ISOLIERUNG, CHARAKTERISIERUNG, MODIFIZIERUNG UND BIOLOGISCHE EVALUIERUNG VON ANTITUMORAKTIVEN TRITERPENSÄUREN

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Für meinen Großvater

und all jene, die mir meine Flügel ließen

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ABKÜRZUNGSVERZEICHNIS

11ß-HSD	11β–Hydroxysteroid-Dehydrogenase
2D-NMR	Zweidimensionale Kernresonanzspektroskopie
5FU	5-Fluoruracil
5LO	Arachidonat-5-Lipoxygeanse
AO	Acridine Orange
AKT	Proteinkinase B, auch PKB
Apt	Adenosintriphosphat
Atg	Autophagozytosegene
B10	28-Acetyl-(3β)-D-glycosylbetulin
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
Bax	Bcl-2-associated X Protein
BH3	Bcl-2 Homologe Domaine 3
Bcl-2	B-Zell lymphoma 2
B-raf	v-Raf murine sarcoma viral oncogene homolog B1
CDDO-Me	Bardoloxone Methyl; 2-Cyano-3,12-dioxooleana-1,9-dien-28-säuremethylester
COSY	Correlated Spectroscopy
COX	Cyclooxygenase
CV	Variationskoeffizient
DC	Dünnschichtchromatographie
DCM	Dichlormethan
DMF	N,N-Dimethylformamid
DNA	Desoxyribonukleinsäure
E2F	Elongationsfaktor 2
EB	Ethidiumbromid
EE	Essigsäureethylester
EC_{50}	Mittlere effektive Konzentration
ER	Endoplasmatisches Retikulum
ERK	Extracellular-signal regulated kinases
et al.	Und andere
FACS	Fluoresence activated cell sorting, Durchflusszytometrie
FSC	Forward Scatter, Vorwärtsstreulicht
G0	Ruhephase
G1	Gap 1 Phase

G2	Gap 2 Phase
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
HMBC	Heteronuclear Multiple Bond Correlation
gHSQC	Heteronuclear Single Quantum Coherence
GP	Glycogenphosphorylase
HIF-1α	Hypoxie-induzierter Faktor 1α
HIV	Humanes Immundefizienz-Virus
HPLC	Hochleistungsflüssigkeitschromatographie
HPLC-MS	Hochleistungsflüssigkeitschromatographie mit Massenspektrometrie-Kopplung
IAP	Apoptose Inhibitor
JNK	C-Jun-N-terminale Kinasen; auch stress-activated phospho-kinases (SAPK)
IC_{50}	Mittlere inhibitorische Konzentration
IR	Infrarot
KBp	Kilobasenpaare
M	Mitose
MB64	4-Oxo-4-phenyl-butyl-Ester der Maslinsäure
MC	Mitotische Katastrophe
MM	Mitochondirenmembran
MOA	Mode-of-Action
MST1	Macrophage stimulating 1
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamidadenindinukleotid
NADP	Nicotinamidadenindinukleotidphosphat
NCI	National Cancer Institut
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Kernresonanzspektroskopie
NOESY	Nuclear Overhauser Enhancement Spectroscopy
NVX-207	3-acetyl-betulinic acid – 2-amino-3-hydroxy-2-hydroxymethyl-propanoate
p21	Auch WAF1, Inhibitor der Cyclin-abhängigen Kinasen
p53	Tumorsupressorgen
PCD	Programmierter Zelltod
PI	Propidiumiodid
PLX4032/RG7204	Vemurafenib

PKC	Proteinkinase C
PTP1B	Tyrosine-protein phosphatase non-receptor type 1
Rock	Rho-activated serine/threonine kinases
ROS	Reaktive Sauerstoffspezies
RR	Rutheniumrot
SAHF	Senescence-Associated Heterochromatin Foci
SAR	Struktur-Aktivitäts-Beziehung
SA-ß-gal	Senescence-associated beta-galactosidase
SB203580	4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1*H*-imidazol-5-yl]pyridine
SD	Standardderivation
SE	Standardfehler
SRB	Sulforhodamin B
SS	Single stranded
SSC	Side scatter
ssNA	Einzelsträngige Nukleinsäuren
THF	Tetrahydrofuran
T.M.	Trockenmasse
TRPV	Transient Receptor Potential Vanilloid
TTAGGGG	Strukturelement der Telomerasen
u.a.	Unter anderem
UV-Vis	Ultraviolettes-Sichtbares Licht
vgl.	vergleiche
WAF1	p21
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
z.B.	Zum Beispiel

EINLEITUNG

In PIERERS Universal Lexikon[1] von 1877 wird für ein dynamisches System, der, durch *„zwei oder mehrere, auf einen Punkt oder ein System wirkenden Kräfte, hervorgebrachte Zustand der Ruhe“* als Gleichgewicht beschrieben. Eine Aktion, Bewegung oder allgemein eine Unruhe tritt dem entsprechend genau dann ein, wenn nicht *„zwei gleiche Kräfte nach gerade entgegengesetzter Richtung wirken“*.[1] Auch wenn die zitierte Definition sich auf statische Systeme, wie das einer Waage, bezieht, kann die nahezu 150 Jahre alte Erklärung in abstrakter Form für jedes physikalische, chemische oder biologische System verwendet werden - aus Kräften werden dann analog Energien, Potentiale oder Faktoren. Wird von detaillierteren Definitionen der Spezialsysteme abgesehen, so kann der beschriebene, statisch-mechanische Prozess der klassischen Waage dennoch als Metapher dienen. Betracht man das *„Elementorgan des thierischen Organismus“*[1], die Zelle, und betrachtet man deren zur Wahl stehende Aktionen, so handelt es sich vereinfacht (Abbildung 1) um ein Vermehren (Proliferation), ein Sterben (z.B. Apoptose) oder ein Pausieren (G_0-Arrest).

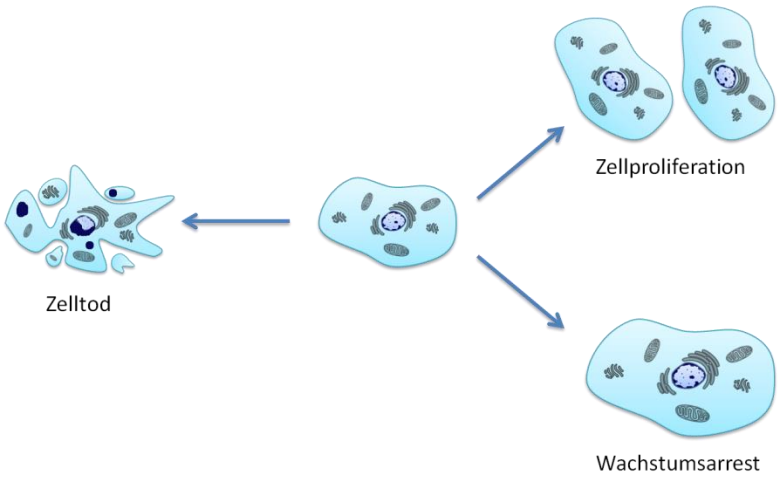


Abbildung 1: *Vereinfachte Darstellung der Reaktionsmöglichkeiten einer Zelle auf intrinsische und extrinsische Einflüsse*

Tierische, wie auch pflanzliche Systeme, bestehen aus einem Konglomerat unzähliger Zellen, sie differenzieren, proliferieren oder sterben kontrolliert durch ein hochreguliertes, im Gleichgewicht stehendes System. Würden z.B. nicht ausreichend genügend Zellen eines Organismus täglich sterben, so würde eine ca. achtzig jährige Person ungefähr zwei Quadratkilometer Haut besitzen[2]. Würden hingegen zu viele Zellen sterben, so wäre der Organismus nicht lebensfähig. Eine stark abstrakte und nicht ganz genaue Form des Zellwachstumsarrestes, könnte in dem Hauptprotagonisten aus Günther Grass´s Roman „Die Blechtrommel“ gesehen werden; im Alter von drei Jahren beschließt dieser im Körper eines Dreijährigen zu verweilen. Unabhängig davon, dass der Autor vermutlich nicht an den G_0-Arrest dachte, kann zu Recht angezweifelt werden, dass ein rein mental ausgesendetes Signal alle Zellen des Körpers in die G_0-Phase versetzen würde.

Vielmehr sind es die unzähligen biochemischen Signale des hochregulierten, genetisch konvertierten Prozesses, welche zwischen dem Zellwachstum und dem Zellsterben entscheiden. Die Proliferation wird über einen Kreis, den Zellwachstumszyklus (Abbildung 2[3]), beschrieben. Innerhalb dieses wird an mehreren Kontrollpunkten überprüft, ob z.B. die energetischen Umweltbedingungen vorteilhaft sind oder die empfindliche DNA-Duplikation fehlerfrei erfolgte. Alternativ können verschiedene Reparaturprogramme oder - in schwerwiegenden Fällen - der Zelltod eingeleitet werden. Eine Dysregulation dieses hochsensiblen Gleichgewichtes stellt für verschiedene Erkrankungen von Mensch, Tier und Pflanze eine Ursache dar. Neurogenerative Krankheiten, wie z.B. Alzheimer können durch eine erhöhte Zelltodinduktion beschrieben werden.[4] Ein zur anderen Seite hin gestörtes System, mit der Folge von ungehinderten Zellwachstum, wird u.a. bei Tumorerkrankungen als eine Hauptursache angesehen.[5]

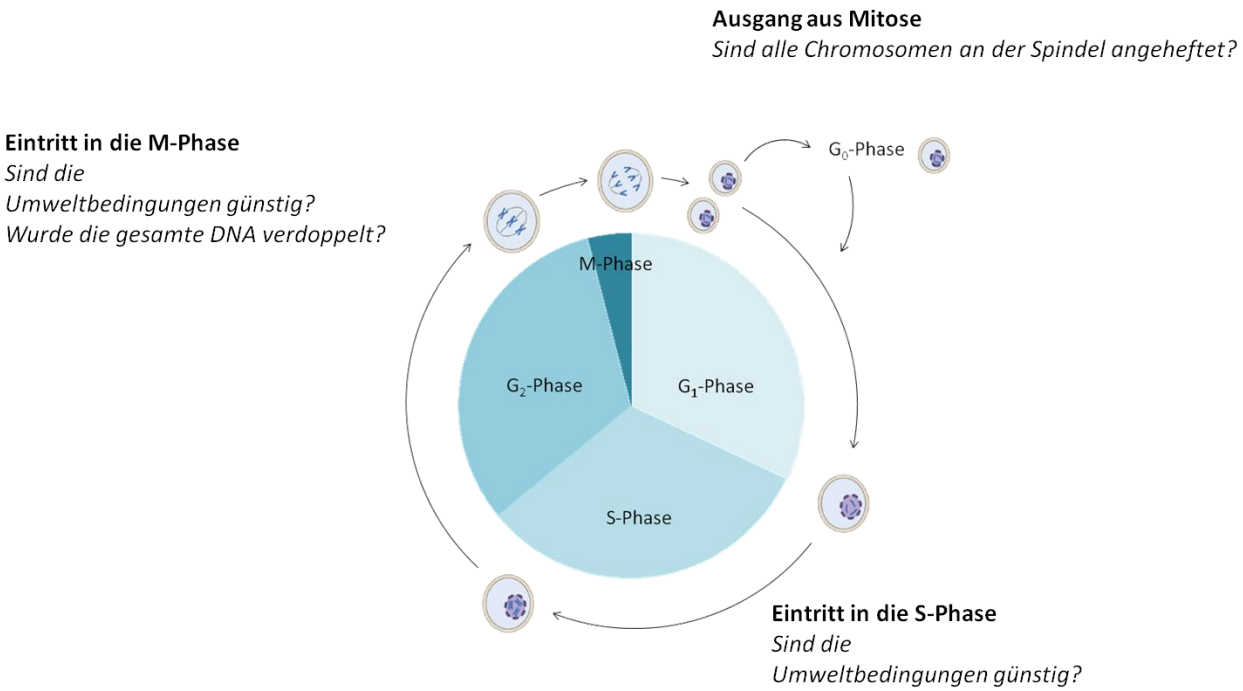


Abbildung 2: *Vereinfachte Darstellung des Zellzyklus nach LÖFFLER[3]*

Gewebeneubildungen, maligne neoplastische Erkrankungen, gehören in der westlichen Welt zu den häufigsten Todesursachen; ca. zwanzigtausend Menschen erlagen im Jahr 2010 in Deutschland an dieser Erkrankung.[6] Durch Mutationen von bestimmten Genen, den sogenannte Protoonkogenen oder den Tumorsupressorgenen, ist das Zellwachstumsgleichgewicht gestört und es kommt zu dem charakteristischen ungehinderten Wachstum von Zellen und damit zur Gewebsneubildung. Bei über 50% aller Tumore wird z.B. festgestellt, dass das Protein p53, welches als Transkriptionsfaktor im

Kontrollsystem des Zellzyklus DNA-Reparaturprogramme oder den Zelltod induzieren kann, geschädigt und somit nicht funktionstüchtig ist.[7]

Klassifiziert wird das neoplastischen Gewebe je nach Dignität, nach Eigenschaften, in gutartige (benigne) oder bösartige (maligne) Neubildungen.[8] Letztere weisen sich u.a. durch eine sehr hohe Proliferationsrate, eine hohe Mutationsrate und die Bildung von Metastasen aus. Eine mögliche Behandlungsform stellt, neben der operativen Entfernung des neoplastischen Gewebes, die mittels chemischer Präparate hervorgerufene gezielte Steuerung bzw. Beeinflussung des Zellwachstums dar.[9] Das hier beschriebene System, bestehend aus gesundem Organismus und malignen Tumor, ist jedoch hochkomplex; es ist in seiner Gesamtheit, seinen internen Wechselwirkungen schwer beschreibar und seine gezielte Beeinflussung kaum möglich. Allein für den Zelltod sind bislang fünf verschiedene, programmierte Wege bekannt.[2] Gleichzeitig ermöglicht die, sich selbst immer wieder weiterentwickelnde, Methodik exaktere Untersuchungen (vergl. bspw. Methods in Cell Biology[10]), deren Ergebnisse wiederum darstellen, dass die unzähligen Subgleichgewichte bzw. Systeme untereinander und zueinander zusätzlich verknüpft sind, was die gezielte, selektive chemotherapeutische Behandlung erschwert.

Trotz aller Hindernisse können die in der klinischen Behandlung eingesetzten Chemotherapeutika prinzipiell gute Erfolge erzielen. So ist z.B. laut einer Statistik des Robert Koch Instituts eine durchschnittlich 61 %ige Überlebenschance bei Krebserkrankungen des Gebärmutterhalses gegeben[11], was aber keine Garantie für die einzelne, betroffene Patientin darstellt. Rezidive, das Wiederauftreten des Tumors, ungewünschte Nebenreaktionen, wie Haarverlust, Schleimhautreduktion und Entzündungen, rechtfertigen und erfordern die weitere Suche nach neuen antitumoraktiven Substanzen bzw. Medikamenten. Die genannten Komplikationen sind oftmals auf unselektive und nicht tumorspezifische Wechselwirkungen zurückzuführen, welche ihren Ursprung u.a. wiederum in der hohen Komplexität und dem subtil entarteten, natürlichen System haben. Die Suche nach einem spezifischen molekularen Target, einem FISCHERschen Schloss, entspricht, wie die Suche nach dem passenden Schlüssel, daher einer Sisyphusarbeit.

Aus wissenschaftstheoretischem Blickwinkel betrachtet, kann ein Lösungsversuch klassisch entweder vom Allgemeinen zum Speziellen, im Sinne eines deduktiven, oder vom Speziellen zum Allgemeinen im Sinne eines induktiven Ansatzes erfolgen (Abbildung 3).[12] Wird dieser theoretische Ansatz auf die Wirkstoffforschung übertragen, kann entweder von einem speziellen Target aus begonnen werden, wie z.B. die DNA-Replikation oder das p53 Protein, und gezielt ein Inhibitor gesucht bzw. modelliert werden. Oder, alternativ von einem allgemeinen Befund, z.B. Wirkstoff X führt zu dem erwünschten Zelltod, ausgegangen und anschließend versucht werden das molekulare Target zu identifizieren. Diese Ketten können unendlich fortgesetzt werden; hat man ausgehend von dem allgemeinen Befund ein Target gefunden, welches durch den Wirkstoff beeinflusst wird, kann induktiv eine neue Hypothese aufgestellt werden, die anschließend wieder deduktiv zu verifizieren ist.

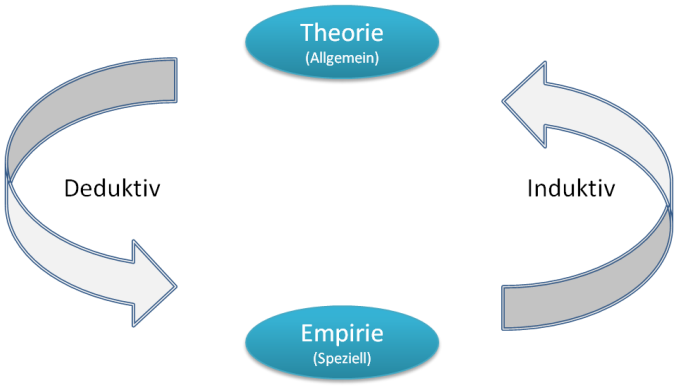


Abbildung 3: *Lösungsstrategien analog zur Erkenntnistheorie*

Erstgenannte, induktive Ansätze werden seit mehreren Jahren intensiv verfolgt. Kinasehemmer können hier als Beispiel dienen und zeigen wie im Falle des Onkogen B-Raf Hemmers Vemurafenib (Zelboraf, PLX4032/RG7204) vielversprechende Resultate.[13] Jedoch schaffen es zu wenige, der auf diesem Wege gefundenen, Wirkstoffe bis zum Medikament.

Historisch betrachtet, verzeichnet der deduktive Weg bisher die meisten Erfolge. Zufällige Beobachtungen, wie die zellproliferations-inhibitorische Wirkung von Senfgasen auf dem Kriegsfeld[14] oder die von entsprechend gleicher Wirkung des Cisplatins (was bei der Untersuchung der Wirkung des Wechselstroms auf Bakterien durch B. ROSENBERG an Platin-Elektroden gefunden wurde[15]) führten, nachdem rückwirkend das Target (bei beiden die DNA-Synthese) charakterisiert werden konnte, gefolgt von anschließenden medizinisch-chemischen modifizierenden, induktiven Ansätzen zu heute weit verbreiteten Antitumormitteln (z.B. Platiblastin und Analoge, Chlorambucil).[16]

Allen gemeinsam ist jedoch die nicht vorhandene oder schwach ausgeprägte Tumorselektivität, da deren Wirkung auf rein kinetischen Faktoren basiert und somit die Selektivität ausschließlich auf die erhöhte Proliferationsrate von Tumorzellen zurückführbar ist. Charakteristische Nebenwirkungen der

Chemotherapie, wie z.B. der Haarausfall, liegen in diesem begründet; alle schnell wachsenden Zellen, zu denen auch Haar und Schleimhautzellen zählen, werden durch die Behandlung getötet. Forschungen, die nun weiter diesen Ansatz verfolgen, scheinen zunächst, mit den Worten von PAUL *et al.* gesprochen[17], kaum Potential zu, Mehrwert für den Patienten generierenden, neuen Behandlungsformen innezuhalten. Ein Problem wiederum, das nicht nur die pharmazeutisch forschende Industrie vor existenzielle Hürden stellt, sondern auch im universitären Rahmen die Erforschung neuer Ansätze erfordert. Neben der molekularbiologischen Suche nach tumorspezifischen Targets, wie z.B. IAPs[18], findet sich auf natur- und wirkstoffchemischer Seite ein Ansatz zur Suche von neuen Hits (Leitstrukturen), welcher zum einen auf das jahrhundertalte Wissen aus der Heilkunde der verschiedensten Kulturen zurückgreift[19] und zum anderen die Natur analog zu LAVOISIER als enormes chemisches Laboratorium ansieht.

Entsprechend inspirierend ist somit der „Chiral Pool“, welcher die Gesamtheit der enantiomerenreinen Naturstoffe umfasst. Die neben Aminosäure, Glykosiden und Flavonen einzuordnenden Kategorien der Alkaloide und Isoprenoide, beinhalten hochkomplexe Strukturen (Abbildung 4), die in der Flora zumeist als Sekundärmetaboliten gebildet werden. Sie spielen weder im Energie- noch im anabolen oder katabolen Stoffwechsel eine Rolle, sind aber, trotz anmutender primärer Irrelevanz, gerade als Signalstoffe, Pathogene oder Membranbildner für den Organismus lebensnotwendig.[20] Zusätzlich sind es gerade jene Substanzen, die in großen pharmakologischen Screens als Hits auffallen, die als Hauptwirkstoffe von, seit Jahrhunderten bekannten, Naturheilmitteln identifiziert werden können und Basis vieler heute zugelassener Präparate bilden.[21]

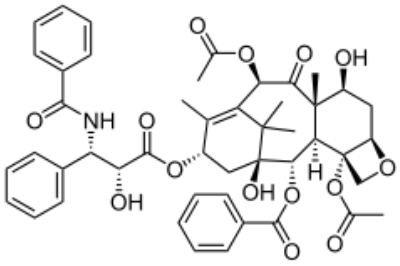


Abbildung 4: *Taxol – ein Vertreter aus der Naturstoffklasse der Triterpene*

Die, zu der Klasse der Isoprenoide zählenden, pentacyclischen Triterpene sind aus sechs Isopreneinheiten aufgebaute Moleküle, die als strukturbildendes Element mindestens vier, zumeist *trans*-verknüpfte Cyclohexanringe, mehrere terminale Methylgruppen und im Speziellen, bedingt durch den biosynthetischen Grundbaustein 2,3-Oxidosqualen, an Position drei eine Hydroxylgruppe besitzen.[22] In Abbildung 5 wird am Beispiel des Oleans der Aufbau graphisch verdeutlicht und zusätzlich die Nomenklatur der Cyclohexanringe sowie die Nummerierung der einzelnen Kohlenstoffatome nach IUPAC gezeigt[23].

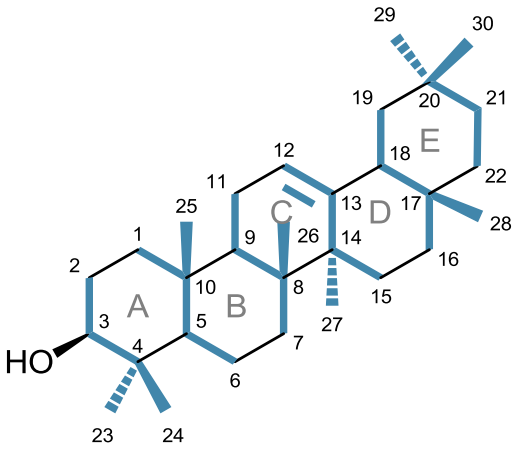


Abbildung 5: *Olean-Grundgerüst mit blaumarkierten Isopreneinheiten. Nummerierung nach IUPAC*

Während unzählige pflanzliche Systeme in der Lage sind z.B. Lupeol in einem Schritt aus dem Grundkörper (2,3-Oxidosqualen, Abbildung 6) enzymatisch zu synthetisieren[24], ist die analoge enantioselektiv verlaufende chemische Synthese laut COREY bis heute eine unfassbar schwere Herausforderung.[25] Selbst eine mehrstufige chemische Synthese war, neben der Beschreibung zur Synthese eines racemischen Gemisches (STORK, 1971[26]), bis 2009 quasi nicht existent.

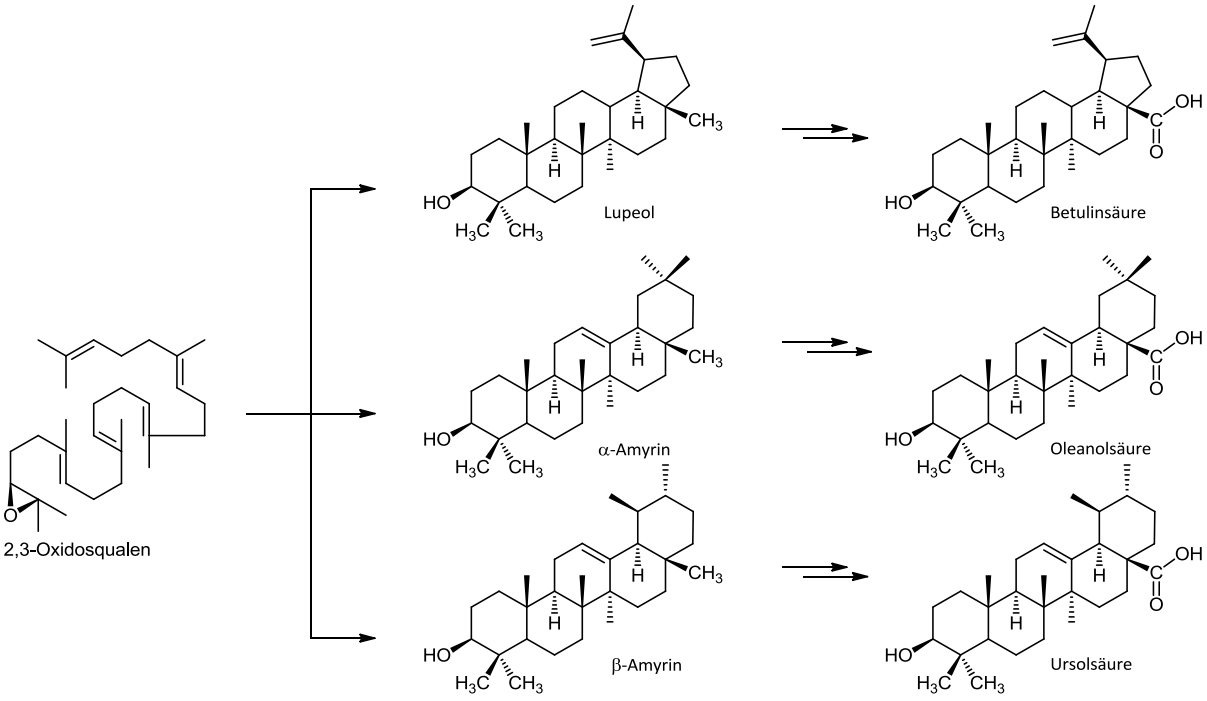
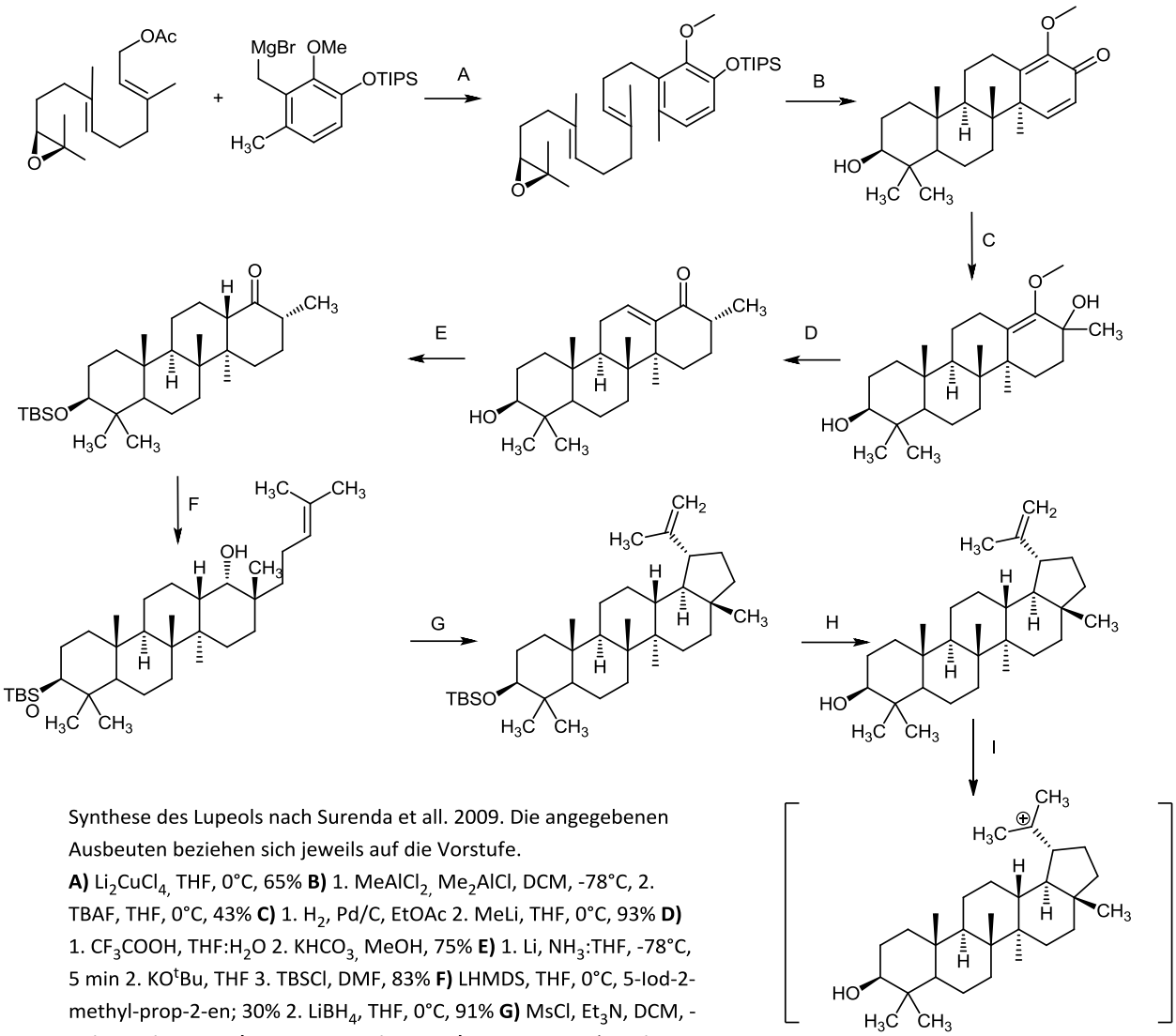


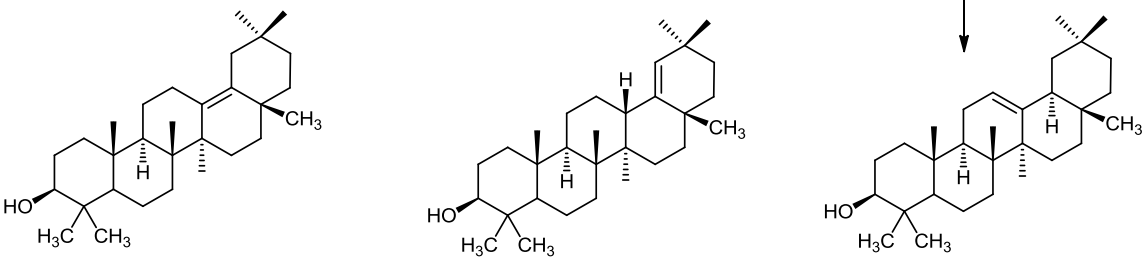
Abbildung 6: *Vereinfachte Darstellung der Biosynthese von Triterpensäuren*

COREY und seinen Mitarbeitern gelang es einhundert Jahre nach der erstmaligen Beschreibung von Lupeol durch COHEN[27] von (*S*)-Epoxy-acetat (E1 vergl. Abb. 7) ausgehend, welches selbst über mehrere Schritte aufgebaut wurde, in vierzehn Stufen das Lupeolgerüst stereoselektiv aufzubauen. Vom Lupeol wiederum konnten SURENDRA und COREY[25] durch eine Trifluormethylsulfonsäure-vermittelte Umlagerung die Bildung von anderen pentacyclischen Triterpene in einem NMR-Experiment nachweisen (vgl. Abbildung 7 unten). Neben diesen Grundkörpern, welche zur Klasse der vom Baccharan abgeleiteten pentacyclischen Triterpene gehören, werden auch das Multiforan, Glutinan, Baueran, Taraxeran, Friedelan und Pachysanan gezählt.[28]

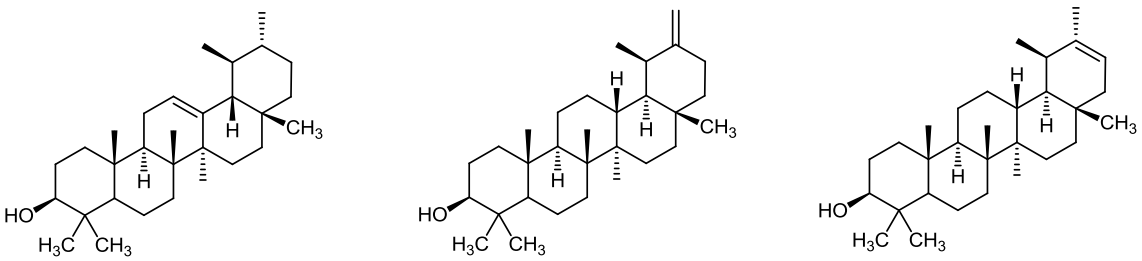
Generell fesselte die Totalsynthese von pentacyclischen Triterpenoiden seit den strukturellen Aufklärungen in der ersten Hälfte des 20. Jahrhunderts, welche vor allem mit dem Namen des Nobelpreisträgers LEOPOLD RUZICKA[29] verbunden sind, die wissenschaftliche Welt. Die synthetische Herausforderung der acht chiralen Zentren ist als Hauptursache für die intensive Forschung zu betrachten.[30] Neben E. J. COREY, einen weiteren Forscher, der wegen seiner Methodenentwicklungen - u.a. auf dem Feld der enantiomerenreinen Synthese von Triterpenoiden[25,31] - mit der größten wissenschaftlichen Auszeichnung geehrt wurde[32], sind vorallem STORK[26], IRELAND[33] und JOHNSON[34] zu würdigen. IRELAND beispielsweise publizierte 1970 eine, von einem bicyclischen System ausgehende, 31ig-stufige Synthese zu einer racemischen Form des Germanicols mit 0.1% Gesamtausbeute.[33] In einem kürzlich erschienen Review sind diese und weitere Totalsynthesen detailreich zusammengefasst.[30] Werden die einzelnen Schritte, die zeitlichen aber auch die finanziellen Aufwendungen zur synthetischen Darstellung dieser Naturstoffe subsummiert betrachet, muss unbestreitbar festgehalten werden, dass diese, trotz Schönheit und Eleganz, ökonomisch irrelevant sind. Dem energieeffizienten natürlichen, enzymatischen System ist bisher keine wirtschaftlich relevante Synthese gegenüberzustellen. Die Gewinnung aus Pflanzenteilen erscheint daher weit sinnvoller; weit über einhundert natürliche Vertreter der pentacyclischen Triterpene konnten auf diesem Weg identifiziert und charakterisiert werden.[35] Durch die bewusst gewählte Extraktion von als Heilmittel bekannten pflanzlichen Materialien, konnte ferner festgestellt werden, dass die besprochenen Strukturen ein ngewöhnlich breites, wenn auch im Speziellen nicht exorbitant signifikantes, Wirkspektrum besitzen.[22]



Synthese des Lupeols nach Surenda et all. 2009. Die angegebenen Ausbeuten beziehen sich jeweils auf die Vorstufe.
A) Li_2CuCl_4, THF, 0°C, 65% **B)** 1. $MeAlCl_2$, Me_2AlCl, DCM, -78°C, 2. TBAF, THF, 0°C, 43% **C)** 1. H_2, Pd/C, EtOAc 2. MeLi, THF, 0°C, 93% **D)** 1. CF_3COOH, THF:H_2O 2. $KHCO_3$, MeOH, 75% **E)** 1. Li, NH_3:THF, -78°C, 5 min 2. KO^tBu, THF 3. TBSCl, DMF, 83% **F)** LHMDS, THF, 0°C, 5-Iod-2-methyl-prop-2-en; 30% 2. $LiBH_4$, THF, 0°C, 91% **G)** MsCl, Et_3N, DCM, -20°C to 0°C, 72% **H)** TBAF, THF, 50°C, 90% **I)** F_3CSO_3H, $CDCl_3$, 23°C



δ - Amyrin | Germanicol | 18-epi-β-Amyrin



α - Amyrin | ψ-Taraxasterol | Taraxasterol

Abbildung 7: *Chemoselektive Synthese von pentacyclischen Triterpenen nach SURENDA und COREY*[25]

Glycyrrhizinsäure konnte beispielsweise als ein aktiver Wirkstoff der, seit über 3000 Jahren als Heilpflanze geschätzten, Süßholzwurzel (*glycyrrhiza glabra*) identifiziert und in die Klasse der Oleane eingeordnet werden.[36] Studien über den Metabolismus der, in Abbildung 8 dargestellten, glykosidischen Verbindung durch den menschlichen Körper wiederum zeigten, dass hauptsächlich das Aglycon, die Glycyrrhetinsäure resorbiert wird, wodurch davon auszugehen ist, dass die freie Säure die pharmazeutisch aktive Komponente ist.[37]

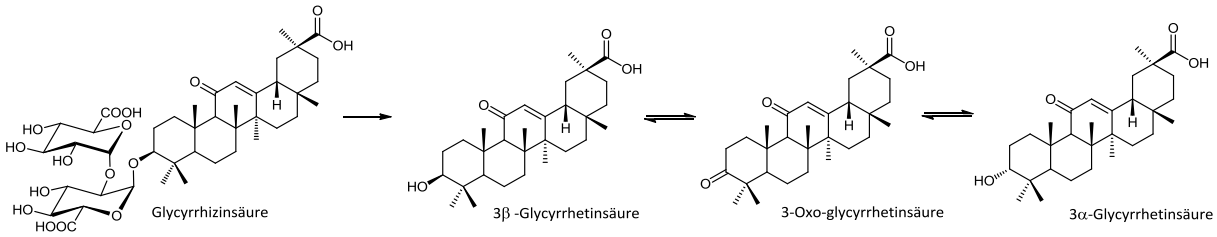


Abbildung 8: *Metabolismus der Glycyrrhezinsäure im Intestinalsystem des Menschen nach AKAO [37b]*

Aber auch andere, indirekte Studien weisen darauf hin, dass Triterpensäuren in verschiedenen, als Heil- oder Nahrungsmittel dienenden, Pflanzen hauptverantwortlich für deren positive Effekte sind. So belegen HE und LIU[38], dass die gesundheitsfördernden Inhaltstoffe von Apfelschalen u.a. den hauptsächlich in der Schale vorkommenden Triterpensäuren, Ursol- und Oleanolsäure, entsprechen. Die erhöhte Lebenserwartung in den mediterranen Ländern kann mit der typischen Ernährung, expliziter betrachtet, mit dem erhöhten Genuss an Oliven und der hauptsächlichen Verwendung von Olivenöl, in Korrelation gesetzt werden. Die damit einhergehende, kontinuierliche orale Aufnahme von geringen Mengen an Oleanol- sowie Maslinsäure wird weitverbreitet als Urasache der erhöhten Lebenserwartung angenommen.[39] Belegt wird letztere These durch Studien, welche beiden Naturstoffen u.a. antivirale, antiinflammatorische und antikanzerogene Wirkung zusprechen.[22] Vor allem durch Arbeiten von JUAN [40], REYES-ZURITA[41] und PARRA[42] ist bekannt, dass u.a. Maslinsäure Apotose-induzierend wirkt. Versteht man darunter den programmierten Zelltod, der bei vielen Tumorzellen bewiesener Weise inhibiert ist und nimmt man die Beobachtung von SÁNCHEZ-GONZÁLEZ[43] hinzu, dass diese Wirkung nur malignes neoplastisches Gewebe betrifft, so hält diese Substanzklasse ein beachtliches antitumorales Potential inne. Bedingt durch eine, ökonomisch gesehene, irrelevante chemische Totalsynthese, bei einer gleichzeitigen genügenden bis sehr guten Extrahierbarkeit aus pflanzlichen Materialen (vgl. Kapitel 3.3 und die darin angeführten Referenzen) sind semisynthetische Modifikationen die Methode der Wahl um Struktur-Wirkungsbeziehungen im Sinne eines deduktiven Ansatzes aufzubauen. Derartige sind für die Gesamtheit der Triterpensäuren, mannigfaltig durchgeführt worden und führten zu vielversprechenden Ergebnissen. Die Überführung von Bardoloxon-Methyl (CDDO-Me)[44] oder NVX-207[45] in klinische Studien unterstreicht nur weiter das

Potential dieser Wirkstoffklasse. Abbildung 8 zeigt die Strukturformeln der Maslinsäure sowie jener Triterpenoide, welche sich in Phase 1 bzw. Phase 2 der Medikamentenzulassung befinden.

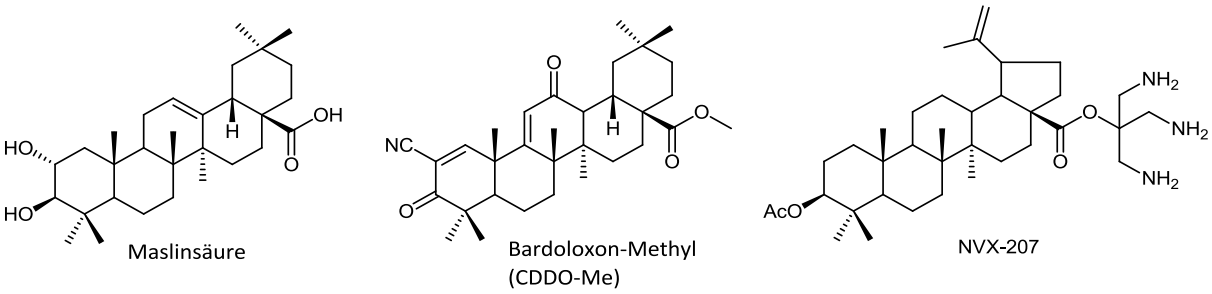


Abbildung 9: *Ausgewählte pharmakologisch aktive Triterpensäuren*

Wenn auch die natürlich vorkommenden Säuren und Analoga zum Teil schon sehr detailreich biochemisch hinsichtlich ihrer Aktivität untersucht wurden sind, muss konstatiert werden, dass es an einem schlüssigen Gesamtbild mangelt. Provokant behauptet, findet sich für nahezu jeden, biochemisch beschriebenen, putativ Zelltod auslösenden Mechanismus ein Indiz (vgl. Kapitel 3.4.1) und dennoch kein eindeutiges, belegtes Target der Triterpensäuren. Basierend auf dieser Tatsache, könnte man vermuten, dass mehrere Targets in der Zelle oder in Zell-Zell-Wechselwirkungsprozessen existieren, welche die unterschiedlichen, nachgewiesenen molekularen Ereignisse hervorrufen.

Folgt man dem Gedanken, dass durch deduktiv aufgebaute Modifikationen, spezifische Target-Drug Interaktionen erkennbar seinen könnten und auf diesem Wege putative Targets beweisbar wären, so muss man feststellen, dass trotz unzähliger Publikationen und Dissertationen eine solche theoretische Studie bisher kaum machbar ist. Unterschiedliche Zytotoxizitätsassay *per se*, fehlende Standardisierungen, marginal publizierte weiterführende biochemische Studien, sowie im Sinne einer SAR eher lückenhafte Datensätze können als Gründe angeführt werden.

ZIELSTELLUNG

Die Isolierung der Maslinsäure und deren anschließende chemische Modifikation soll im Rahmen dieser Arbeit, wie auch die chemische Trennung der konstitutionsisomeren Triterpensäuren (Oleanol- und Ursolsäure), vordergründig sein. Die Eröffnung eines geeigneten Zugangs zu den Ausgangsmolekülen ist essenziell und soll wenn möglich kosten- und zeiteffektiv sein. Ferner ist gerade bei der Trennung das anschließende Ziel, die Synthese von antitumoralen Verbindungen, von hoher Priorität, sodass nebenreaktionsarme Methoden zu potentiellen Leitstrukturen zu präferieren sind.

In einer zweiten Stufe soll eine Struktur-Wirkungsdatenbank aufgebaut werden. Da von der Maslinsäure bisher nur eine geringe Anzahl an Modifikationen literaturbekannt ist und von diesen wiederum nur marginal biologische Daten existent sind, soll der Aufbau möglichst systematisch und rationell erfolgen, um analysieren zu können, welchen Einfluss die funktionellen Gruppen auf die Aktivität ausüben. Anschließend soll der Wirkmechanismus in Abhängigkeit zur chemischen Struktur eruiert werden, um putativ unterschiedliche Mechanismen zu entdecken und aufzuklären. Um unter diesem speziellen Blickwinkel eine erhöhte strukturelle Mannigfaltigkeit zu gewährleisten, sollen zusätzliche semisynthetische Derivate der Tormentillsäure als auch der Glycyrrhetinsäure untersucht werden.

Neben diesen, als wirkungs-theoretisch zu klassifizierenden, Zielen ist die Erhöhung der Aktivität und der Selektivität ein weiteres Hauptziel. Im zunehmenden Maße ist letzterem eine höhere Priorität zu zusprechen. Vorangegangene Studien zeigen evident die prinzipielle Möglichkeit der Aktivitätssteigerung; eine signifikante, erklärbare Selektivitätssteigerung wäre hingegen zweifellos von hohem Wert.

Grundlagebildend hierfür sind die Optimierung der Mess- und Auswertebedingungen zur Bestimmung der EC_{50}-Werte, die theoretische Erarbeitung des biologischen Hintergrundes und die Methodenentwicklung zum Nachweis ebenjener.

THEORETISCHER TEIL

Nach einer kurzen botanischen wie phytochemische Beschreibung ausgewählter Pflanzen, die die Ausgangsstoffe beinhalten, werden in diesem Teil die theoretischen Grundlagen diskutiert, welche für die erfolgreiche Umsetzung der Zielstellung essenziell sind und in den entsprechenden Publikationen nicht ausführlich berücksichtigt werden konnten. Anschließend wird die NMR-spektroskopischen Charakterisierung beleuchtet und das pharmakologische Profil der Substanzklasse im Allgemeinen betrachtet. Um das Ziel der vorliegenden Promotionsarbeit zu erfüllen, ist zusätzlich eine genauere Betrachtung der Tumorerkrankung und der Tumorgenesis unabdingbar. Der einleitend beschriebene Weg zur Behandlung von Tumorerkrankungen zielt auf eine selektive Induktion von letalen Signalen im malignen Gewebe ab. Um dies zu untersuchen sind mehrere theoretische Ebenen zu erarbeiten: von der allgemeineren Betrachtung der bekannten Zelltodarten, über deren Alleinstellungsmerkmale hinweg bis zu den verwendeten Analysenmethoden. Abschließend wird der aktuelle wissenschaftliche Kenntnisstand bezüglich der Antitumorwirkung durch die Maslinsäure zusammengefasst. Die Summe aus diesen und in den Publikationen angeführten, umfassenden, wie auch kritischen, theoretischen Erarbeitungen ermöglichte die Bearbeitung des Themas und führte zu den, dieser Arbeit zugrundeliegenden Publikationen.

3.1. Natürliches Vorkommen und strukturelle Beschreibung

Von den über 4000 verschiedenen, in fast vierzig unterschiedliche Gerüstgruppen unterteilten, isolierten Triterpenen kommen pentacyclische Triterpensäuren in höheren Pflanzen häufig glykosidisch gebunden vor.[47] Der aus dieser Verknüpfung resultierende amphipathische Charakter bestimmt maßgeblich das chemische Verhalten. Wie die, sich aus dem lateinischen Wort für Seife – *Sapo* – ableitende, Fachbezeichnung Saponine impliziert, werden Pflanzenteile mit hohem Saponingehalt, wie z.B. die Nüsse des Waschnussbaumes (*Sapindus mukorossi*) u.a. für ihre Waschkraft geschätzt.[48] Auch pharmakologische Eigenschaften können auf den ambiphilen Charakter zurückgeführt werden, wie z.B. die sekretolytischen (schleimlösenden) der Glycyrrhizinsäure (Abbildung 10).[49] Letztere bildet mit bis zu 20% einen Hauptbestandteil der Wurzel des Süßholzes (*Glycyrrhiza glabra*).

Abbildung 10: *Pflanzentafel des Süßholz* (*Glycyrrhiza glabra*)[46]

Diese mehrjährige, ca. einen Meter hoch wachsende Staude ist im Mittelmeerraum wie auch in Asien weitverbreitet und wird seit mehr als 3000 Jahren als Heilpflanze geschätzt.[36] Die morphologischen Merkmale der zur Klasse der Schmetterlingsblütler gehörenden Pflanze sind in Abbildung 10 zusehen. Neben unpaarig gegliederten Laubblättern sind in der Farbtafel die zehn bis fünfzehn Zentimeter langen, aufrecht aus den Blattachsen entspringenden Blütenstände dargestellt.[50] Auch die als Heilmittel u.a. von ELISABETH VON BINGEN geschätzte[36] Wurzel ist abgebildet. Die pharmakologisch aktive Substanz ist jedoch laut den Metabolismusstudien von AKAO *et al*. [37b] das Aglykon, die Glycyrrhetinsäure (Vgl. Abbildung 8). Diese, zur Klasse der Oleane gehörende, Triterpensäure zeichnet sich, neben der charakteristischen 3β-Hydroxyl-Gruppe, durch eine α,β–ungesättigte Carbonylgruppe in Ring C und durch eine Carbonsäure an Position 30 aus (Abbildung 11). Im Unterschied zu den meisten anderen Triterpensäuren wird die Glycyrrhizinsäure durch eine sehr hohe Süßkraft (ca. 30-50 fach im Vergleich zur Saccharose) gekennzeichnet. Der Süßholzwurzelextrakt findet daher häufig Verwendung als Süßungsmittel und bildet Grundlage der, sowohl als Süßware als auch als Medizin bekannten, Lakritze.[51]

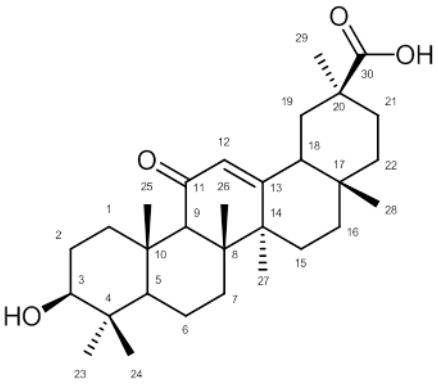


Abbildung 11: *Die Glycyrrhetinsäure*

Andere reich an Triterpensäuren seiende Pflanzen bzw. Pflanzenteile besitzen ein entgegengesetztes Geschmacksprofil.[53] Salbei (*Salvia*), Rosmarin (*Rosmarinus officinalis*) oder z.B. der Blutwurz (*Potentilla erecta*) zeichnen sich gustatorisch eher adstringierend, pelzig, aus. Der letztgenannte gehört zur Klasse der Rosengewächse und ist eine ausdauernde krautige Pflanze, die mäßig saure Böden, wie z.B. die der europäischen Mischwäldern, Heiden oder Magerwiesen, als Lebensraum bevorzugt.[54] Aus dem, von Innen blutroten, im Durchmesser ein bis drei Zentimeter großen Rhizom wächst der mehrästige, beblätterte Stängel bis zu einer Gesamthöhe von ca. fünfzig Zentimetern. (Vgl. Abbildung 12) Die aus vier Kronblättern bestehenden Blüten zeichnen sich durch eine gelbliche Farbe aus und erreichen einen ungefähren Durchmesser von einem Zentimeter. Namensgebend für diese Heilpflanze ist der rötliche Saft, welcher beim Anschneiden der Wurzel austritt.

Abbildung 12: *Pflanzentafel des Blutwurz (Potentilla erecta)*[52]

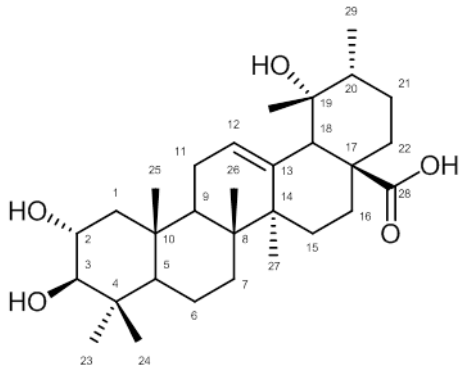


Abbildung 13: *Strukturformel der Tormentillsäure*

Neben Tanninen, die die Verwendung als Gerbstoff erklären, sind es vor allem Flavonoide, Phenole und die Saponine der Tormentillsäure, welche dem Blutwurz sein nützliches Wirkspektrum verleihen.[55] Abgesehen von dem erwähnten Geschmacksprofil wirkt es, wie für die Klasse typisch, antiphlogistisch (entzündungshemmenden). Alkolholische Wurzelextrakte werden daher topisch verwendet um z.B. Entzündungen des Rachenraumes zu mildern.[56] Aus chemisch, struktureller Betrachtung unterscheiden sich die bisher beschriebenen Säuren signifikant. Tormentillsäure (Abbildung 13) gehört zur Klasse der Ursane, Glycyrrhetinsäure hingegen zur Klasse der Oleane. Wie einleitend erwähnt handelt es sich hierbei um Konstitutionsisomere, die, das Grundgerüst betrachtend, ausschließlich im E-Ring durch die Position einer terminalen Methylgruppe zu unterscheiden sind. Im speziellen Fall wird die Tormentillsäure durch eine Carbonsäure an Position 28, eine ungesättigte Bindung im C-Ring und, neben einer Alkoholfunktion an Position 3, durch zwei weitere Hydroxylgruppen an Kohlenstoffatom 2 und 19 gekennzeichnet (Abbildung 13).

Die Tormentillsäure besitzt entsprechend ein α-Amyringerüst und stellt ein von der Ursolsäure ableitbares pentacyclisches Triterpenoid dar. Die, nur an Position 3 eine Hydroxylgruppe tragende, Ursolsäure ist zusammen mit der Oleansäure ein Hauptinhaltstoff des, ebenfalls als Heilpflanze geschätzten, Salbeis.[57] *Salivia officinalis*, der Echte Salbei, wird als Kräutertee zubereitet, gegen übermäßiges Schwitzen (Hyperhidrose), sekretolytisch bei Erkrankungen der Bronchien oder zur Linderung von Problemen des Gastrointestinaltraktes verwendet.[58] Aber auch als Gewürz finden die Blätter der weltweit verbreiteten, zur Klasse der Lippenblütler gehörenden, Sträucher Verwendung.

Die pflanzengattungscharakteristisch geformten, violetten, weißen oder rosafarbenen Blüten blühen von Mai bis Juli in einer bis zu drei Zentimeter langen Krone.[50,53,55] Abbildung 14 zeigt die morphologischen Merkmale der Pflanze.

Abbildung 14: *Farbtafel des Salbeis (Salvia officinalis)*[50]

Die Oleanolsäure kommt ferner mit Ursolsäure gemeinsam in den Schalen von Äpfeln (*Malus*) vor.[59] Von den, zur Gattung der Kernobstgewächse (*Pyrinae*) zählenden,

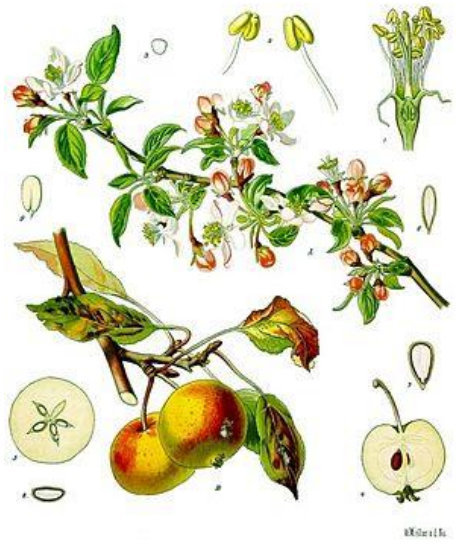


Abbildung 15: *Farbtafel des Apfelbaums (Malus domestica)*[50]

Bäumen sind etwa 42 bis 55 verschiedene Arten bekannt. Die sommergrünen Bäume und Sträucher sind im nördlichen gemäßigten europäischen, asiatischen sowie nordamerikanischen Raum natürlich vorkommend. Der Kulturapfel (*Malus domestica*) ist heute die weitverbreitetste Art und nimmt als Nahrungsmittel eine große Bedeutung ein. Der Laubbaum besitzt wechselständig angeordnete Blätter, von eiförmiger oder elliptischer Form und Rändern, die je nach Art glatt, gesägt oder gelappt sind. Die in den Monaten April und Mai blühenden, aus fünf freien Kornblättern bestehenden, weißen bis roten Blüten sind radiärsymetrisch und flach in einem Durchmesser von ca. zwei bis fünf Zentimeter angeordnet. Aus den Blütenachsen entwickelt sich die essbare Scheinfrucht, der Apfel. Abbildung 14 zeigt die Pflanzentafel der Gattung *malus*. Über phytochemische Studien konnte nachgewiesen werden, dass die, dem Sprichwort *„Ein Apfel am Tag und der Doktor bleibt, wo er mag“* zugrunde liegenden, pharmakologisch wirksamen Stoffe hauptsächlich in den Apfelschalen zu finden sind und, dass den Triterpenoiden, neben den Flavoiden[60], eine nicht ganz unbeachtliche Rolle zuzusprechen sei.[61] Eine aktueller Übersichtsartikel von SZAKIEL *et al.*[62] zeigt, dass die Zusammensetzung der Triterpensäuren in der Schale wiederum von der Apfelsorte abhängig ist. Das triterpenhaltige Extrakt des *Malus pumila* Mill. beispielsweise enthält neben 98% Ursolsäure keine Oleanolsäure, in den Schalen des *Malus domestica* Borkh. –dem Apfel der Sorte Holsteiner Cox- hingegen konnten beide Amyrin-Vertreter nachgewiesen werden.

Die strukturelle Aufklärung der phytochemischen Zusammensetzung mit speziellem Blick auf die pentazyklischen Triterpenoide findet ihren Ursprung im neunzehnten Jahrhundert; Friedelin, Betulin und Ursolsäure wurden bspw. in den Jahren 1807, 1836 und 1854 erstmals beschrieben.[22] Anfang des zwanzigsten Jahrhunderts untersuchte BÄCHLER[63] im Rahmen seiner Dissertation (1927, Basel) die Inhaltsstoffe einer weiteren, zur Familie der rosenartigen zählenden, früchtetragenden Gattung der *Pyrinae.* Der Weißdorn, von dem 200 bis 300 verschiedene Arten bekannt sind, ist vorrangig in Wäldern und Gebüschen der Nordhalbkugel beheimatet. Die, als Sträucher oder Bäume auftretende, Pflanze zeichnet sich durch einen hohen Verzweigungsgrad des dornigen Astwerkes aus (vgl Abbildung 16).[4] Phytochemisch interessant sind die, bis zu zwei Zentimeter groß werdenden, zumeist roten Beeren.[64]

Aus dem Etherextrakt der Früchte isolierte BÄCHLER[63] 1927 ein weißes, amorphes Pulver, dem er die Summenformel $C_{32}H_{52}O_4$ zuordnen konnte und es als Monocarbonsäure identifizierte. Er nannte diese in Analogie zur systematischen Bezeichnung des Weißdorns, *Crataegus oxyacantha*, Crataegussäure. Aufgrund der fehlgeschlagenen Reproduzierbarkeit der Säuretitration sowie der nichtumgesetzten Methylester-Synthese, schlugen H. DIETERLE und O. DOERNER zehn Jahre später eine Laktonstruktur aus der Gruppe der Steroidsapogenine vor, wobei sie die Bezeichnung in Crataeguslakton änderten.[65] T. BERSIN konnte diese Behauptung 1951 widerlegen. In einer ersten Mitteilung[66] veröffentlicht er, dass es sich bei der Crataegussäure um eine Triterpensäure handelt, da die ROSENTHALER Farbreaktion[i] positiv ausfiel, ferner die Säuretitration und die Diazomethan vermittelte Esterbildung gelang. Über die Reaktion mit Tetranitromethan wurde der ungesättigte Charakter nachgewiesen und er postulierte, dass es sich bei der Crataegussäure um die Ursolsäure handelt.[67] Anfang der 50er Jahren publizierten mehrere andere Arbeitsgruppen ihre Erkenntnisse über die chemische Struktur des hochschmelzenden (252 – 254°C) Stoffes. Im Verlaufe der Untersuchung zeigten BERSIN *et al.*[66-67], ULLSPREGER *et al.*[68] sowie TSCHESCHE *et al.*[69], dass es sich um ein Mehrkomponentengemisch handeln muss. Nachdem erst die Ursolsäure als Komponente identifiziert wurde, veröffentlicht BERSIN 1952[67] in einer zweiten Mitteilung Oleanolsäure als weiteren Inhaltstoff. Die Summenformel der Oleanol- wie auch der Ursolsäure, stimmt jedoch nicht mit der von TSCHESCHE 1951 für die Hauptkomponente publizierten, richtigen Summenformel $C_{30}H_{52}O_4$ überein.[69] Erst CAGLIOTTI und CAINELLI[70] gelang 1962 die vollständige Strukturaufklärung. Sie isolierten die Crataegolsäure aus der Schale von Oliven, nannten sie daher auch Maslinsäure, und publizierten, dass es sich um die 2,3-Dihydroxy-Δ^{12}-oleanen-28-säure handele. TSCHESCHE *et al.*[71] bestätigten zwei Jahre später diese Struktur für ihre Crataegolsäure, in die Literatur blieb bis heute jedoch der Name Maslinsäure bestehen.

Abbildung 16: *Farbtafel des gemeinen Weißdorns (Crataegus oxyacantha)*[52]

Mengenmäßig am stärksten vertreten ist Maslinsäure in der *Olea europaea*, genauer in den Schale von Oliven. GUINDA *et al.*[72] beobachteten, dass der Gehalt vom Entwicklungsstand der Oliven abhängig ist. Der Olivenbaum (*Olea europaea*) ist im Gegensatz zu den bisherigen betrachten Bäumen ein immergrüner Vertreter, der vorrangig im Mittelmeerraum verbreitet

[i] Vanillin-Eisessig mit konzentrierter Schwefelsäure

ist. Typische, natürliche Wuchshöhen von zehn bis zwanzig Metern finden sich innerhalb des kommerziellen Anbaus eher selten. Die typisch rissige Borke entsteht mit zunehmendem Alter aus der graugrünen, glatten Rinde junger, eher kantiger Zweige. Die Wurzel entwickelt sich stark abhängig von der Bodenbeschaffenheit entweder senkrecht in die Tiefe oder bildet sich horizontal aus. Die Blätter, welche ebenso einen hohen Triterpenanteil besitzen, sind meist bis zu zehn Zentimetern lang, von länglicher Form und farblich durch Unterschiede in der Vorder- und Rückseite geprägt.[39,55] Während die Blattoberfläche grünlich erscheint, bedingen feine Härchen auf der Unterseite ein leicht silbriges Erscheinungsbild. Die in den Monaten von Ende April bis Anfang Juni blühenden, zwittrigen Blüten sind in zwei bis vier Zentimeter langen Blütenständen angeordnet und besitzen vier Kelchblätter. Von gleicher Anzahl sind die gelblichen Kronblätter, welche mit zweieinhalb bis vier Millimetern um das zwei bis vierfache größer als die Kelchblätter sind. Ferner enthält jede Blüte zwei Fruchtblätter sowie vier Staubblätter. Die in Folge von Wind oder Selbstbestäubung sich entwickelnden Steinfrüchte, sind ellipsoid bis rund und in Abhängigkeit vom Reifezustand grün oder schwarz.[50,53] (Vgl. Abbildung 17)

Abbildung 17: *Farbtafel des Olivenbaums (Olea europaea)*[50]

Um die weitläufige Verbreitung der Triterpensäuren im Pflanzenreich *per se* darzustellen wurden exemplarisch die Pflanzen in Tabelle 1 zusammengefasst, in welchen Maslinsäure nachgewiesen werden konnte. Häufig tritt dieser Naturstoff bei Lippenblütlern, Malven- oder Rosengewächsen auf. Der analytische Nachweis erfolgte vermehrt mittels HPLC-Ms Methoden, sodass über einen effektiven, isolierbaren Gehalt keine stichhaltige Aussage getroffen werden kann.

Lateinischer Name	Englischer Name	Pflanzenteil	Family	Ausbeute (MA)	Lit.
Olea europaea	Olive tree	Schale	Oleaceae	5,7 % (t.m.)	JÄGER[73]
Olea europaea	Olive tree	Trester	Oleaceae	28,2 % (t.m.)	JÄGER[73]
Olea europaea	Olive tree	Öl	Oleaceae	$4.9 \cdot 10^{-3}$ %	ALLOUCHE [74]
Malus domestica	Apple	Trester	Rosaceae	1 % (t.m.)	JÄGER[73]
lagerstroemia speciosa	Giant Crape-myrtle	Blätter	Lythraceae	-	ZONG [75]
Pyrus communis	Peals	Blätter	-	2,8 % (t.m.)	BRIESKORN [76]
Zizyphus jujuba	jujube	Wurzel	Rhamnaceae	-	LEE [77]
Hippophae rhamnoides	Sea-buckthorn	Astrinde		$9 \cdot 10^{-3}$ % as coumaroyl derivate (t.m.)	YANG [78]
Malus pumila	Apple (red delicious)	Schalen		$11.7 \cdot 10^{-3}$% (Aceton)	HE [38]
Dryobalanops aromatica	Borneo Camphor	Harz		$1.2 \cdot 10^{-3}$% as methyl 2,3-diacetyl maslinoate (t.m.)	CHEUNG [79]
***Duchesnea Chrysantha* (heute *potentilla indica*)**	Mock strawberry	Hole plant	Rosaceae	$9.3 \cdot 10^{-3}$ % (t.m.)	KIM [80]
Prunella vulgaris var. lilacina	Common self-heal	Überirdischen Pflanzenteile	lamiaceae	$3.5 \cdot 10^{-4}$ % (t.m.)	LEE [81]
Microcos paniculata	Microcos	Baumstamm	Malvaceae	$4.1 \cdot 10^{-3}$ % (t.m.)	FAN [82]
Vitex cienkowskii Kotschy & Peyritsch	Chastetree or vitex	Rinde des Baumstammes	Lamiaceae	-	DONGMO [83]
Coleus tuberosus	Chinese potato; Coleus tuberosus	Knolle	lamiaceae	1.73 • % (t.m.)	MOOI [84]
Ziziphus jujuba	Jujube; chinese date	Blätter	Rhamnaceae	$4.9 \cdot 10^{-2}$ % (t.m.)	GUO [85]
Z. jujuba var. spinosa	Jujube; chinese date	Blätter	Rhamnaceae	$4.4 \cdot 10^{-1}$ % (t.m.)	GUO [85]
Vaccinium macrocarpon	Large cranberry	Beeren	Ericaceae	$8.1 \cdot 10^{-3}$ % (t.m.)	KIM [86]
Cornus Kousa	Kousa dogwood	Baumstamm	Cornaceae	$15.1 \cdot 10^{-3}$ % (t.m.)	SULTANA [87]
Jacaranda mimosaefolia	Jacaranda	Rinde	Bignoniaceae	0.3 • % (t.m.)	ZAGHLOUL [88]
Viscum liquidambaricolum			Santalaceae	-	YANG [89]
Symplocos lancifolia	Evergreen tree	Blätter	Symplocaceae	$2.8 \cdot 10^{-3}$ % (EE/MeOH)	ACEBEY-CASTELLON [90]
Ficus microcarpa	Chinese Banyan	-	Moraceae	-	LI, Y.W. [91]
Boehmeria nivea	Ramie	Wurzel	Urticaleae	-	XU, Q. [92]
Eriobotrya japonica	loquat	Blätter	Rosaceae	-	LU, H. [93]
Punica granatum L	Pomegranate	Blühten	Lythraceae	$0.5 \cdot 10^{-3}$ % (t.m.)	XIE, Y. [94]
Swertia mileensis	Mile Swertia Herb	Gesamte Pflanze	Gentianaceae	-	LI, X. [95]
Platostoma africanum P. Beauv.,	Akan-Asante	Blattwerk	Lamiaceae	-	ALADEDUNYE, F.A. [96]
Terminalia arjuna	Arjun tree	Rinde	combretaceae	-	SAXENA [97]
Ulmus pumila	Siberian Elm	Wurzel	Ulmaceae	$6.9 \cdot 10^{-3}$ % (t.m.)	WANG, D. [98]
Potentilla chinesis	Chinese cinquefoil	-	Rosaceae	-	LIU, P. [99]
Campsis grandiflora K. Schum.	Chinese trumpet	Blühte	Bignoniaceae	$2.4 \cdot 10^{-2}$ % (t.m.)	KIM, D. [100]
Fructus Rubi	Rubus fruit	Früchte	Rosaceae	-	GUO, Q. [101]
Incarvillea arguta	Incarvillea	Wurzel	Bignoniaceae	$1.0 \cdot 10^{-3}$ % (t.m.)	LUO, Y. [102]
Physocarpus intermedius.	Ninebark	Baumstamm Rinde	Rosaceae	$9.6 \cdot 10^{-4}$ % (t.m.)	KIM, Y.-K. [103]
Luma chequen	White chilean myrtle	Überirdische Part	myrtaceae	-	WACHTER [104]
Geum japonicum	-	Gesamte Pflanze	Rosaceae	$1.2 \cdot 10^{-3}$ % (t.m.)	XU, H. [105]
Hyptis verticillata	John Charles	Gesamte Pflanze	Lamiaceace	-	NOVELO, M. [106]
Agastache rugosa	Korean mint	Wurzel	Lamiaceace	-	ZOU, Z. M. [107]

Tabelle 1: *Verbreitung der Maslinsäure in der Pflanzenwelt. Literatur bis Juni 2011 wurde berücksichtigt.*

3.2. Strukturanalytische Charakterisierung der verschiedenen Triterpensäuren

Die analytische Untersuchung der natürlichen und der synthetisch modifizierten pentazyklischen Triterpene erfolgte unter Zuhilfenahme der Kernresonanz-, Massen-, Schwingungsspektroskopie sowie der Drehwertbestimmungen, UV-Vis-Messungen und verschiedene chromatographischen Analysemethoden (DC und HPLC).

Die Zuordnung der Signale in den Spektren der kernresonanzspektroskopischen Untersuchungen erfolgte unter der Verwendung von 2D-NMR Messungen, dem Inkrementensystem (ACD Labs®) und Literaturdaten (entsprechende Zitate sind in den Veröffentlichungen vermerkt). Die Signale der terminalen Methylgruppen konnten prinzipiell durch ein APT-Experiment von denen der cyclischen Kohlenstoffatome unterschieden werden. Die Aufnahme von gHSQC Spektren ermöglichte ferner die Unterteilung in quaternäre und geminale Signale und die Zuordnung der Kohlenstoffsignale zu den entsprechenden Signalen im Protonen NMR. Von den markanten Signalen der, die Hydroxylgruppen tragenden, Kohlenstoffatomen im A-Ring ausgehend (C2 und C3), konnten die terminalen Methylgruppen über gHMBC-Experimente eindeutig zugeordnet werden.

Exemplarisch soll dies im Folgenden an Hand des, in Kapitel 4.5 und Anlage 9.5 vollständig charakterisierten, Maslinsäuremethylesters beschrieben und durch Abbildung 18 graphisch verdeutlicht werden.

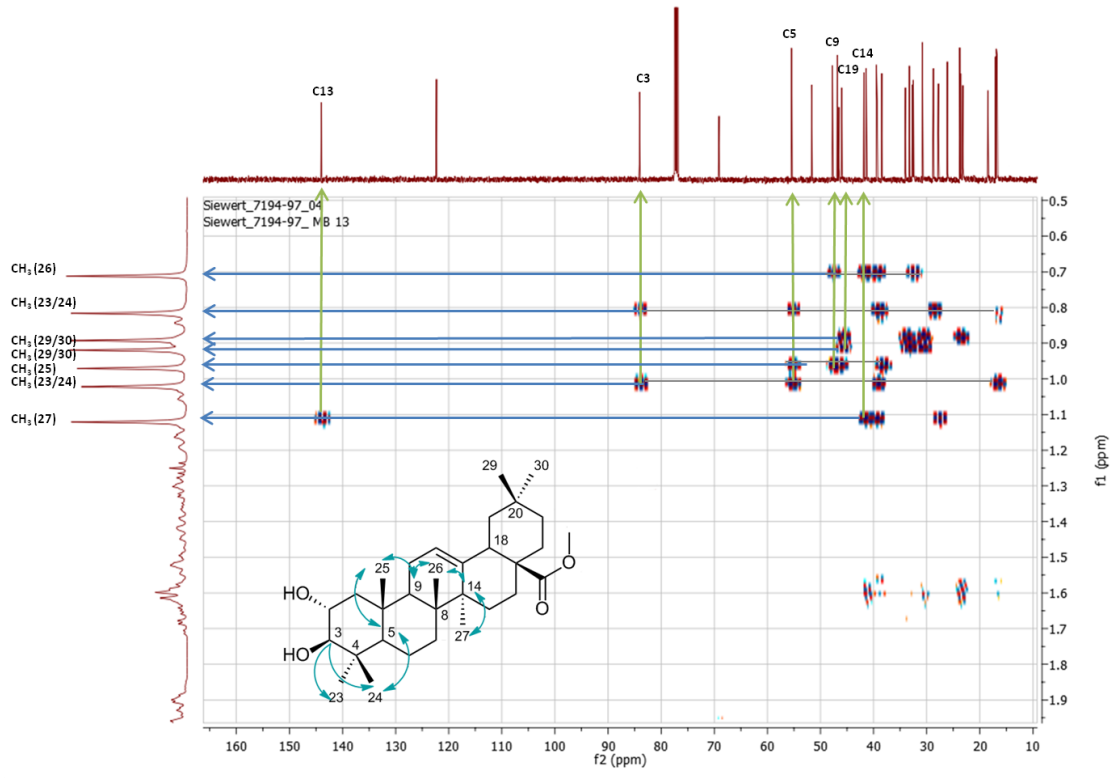


Abbildung 18: *Illustration der terminalen Methylgruppen-Zuordnung via gHMBC-Experiment*

Dem Signal des Kohlenstoffatoms *C*H(3) kann, neben einem Kreuzpeak mit dem Signal des Protons an C2, je einen Kreuzpeak mit den Signalen der Methylgruppen an Position 23 und respektive an Position 24 zugeordnet werden. Für die besonders markanten Signale der axial stehenden Kohlenstoffatome *C*H (5) und *C*H (9) erwartet man im gHMBC je drei Kreuzpeaks; Neben den Signalen für CH_3 (24) und CH_3 (23), einen Kreuzpeak mit CH_3 (25) - im Falle des Kohlenstoffatomsignals *C*H (5). Für C*H* (9) erwartet man, neben einem Kreuzpeak mit CH_3 (25), ein Signal mit den Protonen an C11 und mit denen der terminalen Gruppe CH_3 (26). Von dem so zugeordneten Signal für CH_3 (26) lässt sich das Signal für Position 27 durch einen Kreuzpeak mit *C* (14) bestimmen. Dieses ist, im Gegensatz zu dem, auch mit CH_3 (27) koppelnden, Signal für *C* (8), durch einen zusätzlichen Kreuzpeak mit C*H* (12) und C*H* (18) eindeutig bestimmbar. Letztere zeigen signifikante Kopplungsmuster im Protonen-NMR; Die Kopplungskonstanten in Höhe von ^3J = 13.5 Hz sowie ^3J = 4.8 Hz für das Proton an Position 18 sind charakteristisch für *ß*-Amyrin Gerüste. Die axiale Position des C*H* (18) bedingt dieses Aufspaltungsmuster analog der Karplusbeziehung, welche die Abhängigkeit der Kopplungskonstanten ^3J vom Diederwinkel beschreibt. Für die Klasse der Ursane, in welcher in Nachbarschaft zu C*H* (18) nur ein Proton vorhanden ist, befindet sich bei 2.29 ppm ein Dublett mit einer Kopplungskonstante in Höhe von ^3J = 11.2 Hz, was analog der Karplusbeziehung indirekt die äquatoriale Position der Methylgruppe 29 belegt. Die genaue Zuordnung der Signale für die Positionen 23 und 24 als auch 29 und 30, erfolgte über die bekannte Beobachtung[108], dass in rigiden Cyclohexylsystemen ein dielektrischer Effekt auftritt. In Folge dessen findet man für Protonen entlang der Bindungsachse und im Inneren des Kegels eine negative Abschirmung bzw. positiven Entschirmung des elektrischen Feldes. Im Umkehrschluss werden axialständige Substituenten stärker vom elektrischen Feld abgeschirmt, was zur Folge hat, dass die entsprechenden Signale im Protonen-NMR zu höheren Feld verschoben sind. Den Protonen an Position 24 kann, von den zwei zur Wahl stehenden Signalen (siehe Abbildung 18) auf dieser theoretischen Grundlage, das ins Hochfeld verschobene Signal zugeordnet werden. Analog kann der äquatorial stehenden Methylgruppe am Ring E das ins Tieffeld verschobene Signal zugeordnet werden. Nach der so erfolgten Zuordnung der Signale im ^{1}H NMR können die Kohlenstoffatome eindeutig über ein gHSQC-Experiment den Signalen im ^{13}C NMR zugeordnet werden (vergleiche Abbildung 19). Spezielle Fragen der Raumanordnungen konnten unter zur Hilfenahme von H,H-Cosy Korrelationen sowie durch die Verwendung der Kernoverhauser Korrelation (H,H-Noesy) geklärt werden.

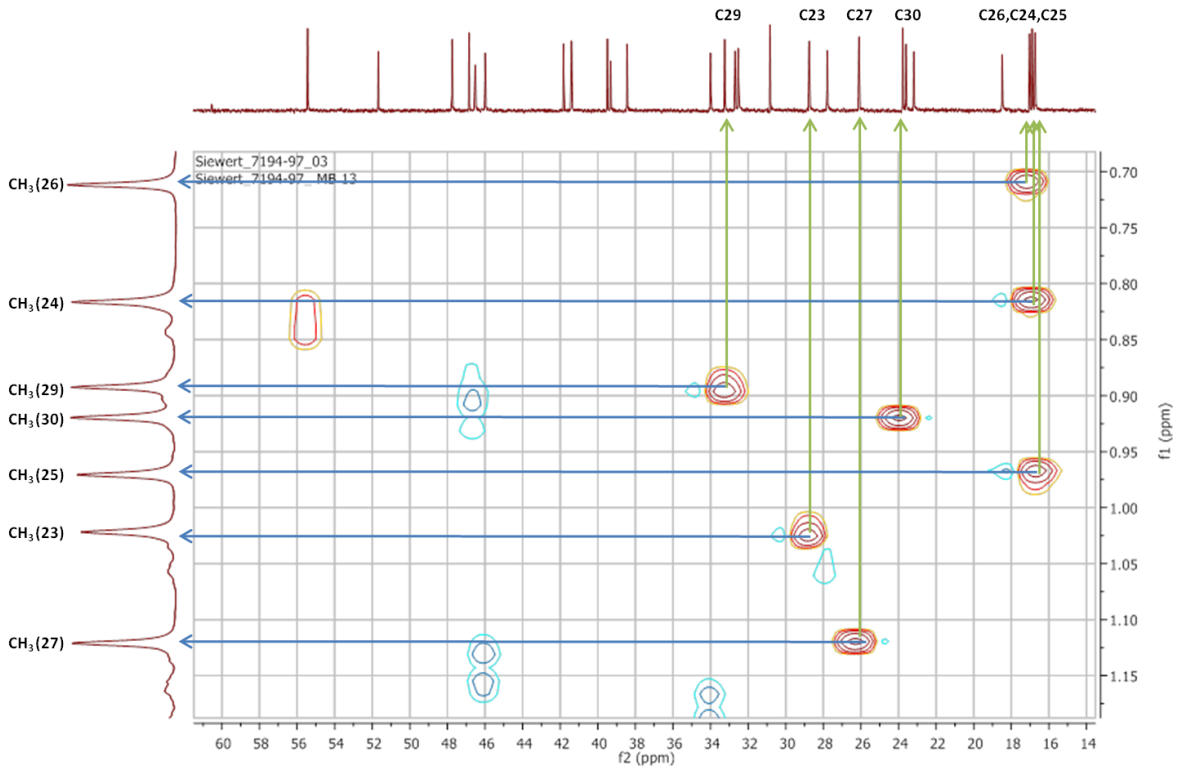


Abbildung 19: *Illustration der Kohlenstoffsignal-Zuordnung der terminalen Methylgruppen*

Mittels IR-Untersuchungen wurden die Molekülschwingungen bestimmt. Charakteristisch für alle Triterpensäuren ist hierbei die Carbonylschwingung bei ca. 1700 cm^{-1}. Im Bereich zwischen 1400 und 800 cm^{-1} finden sich die C-H Valenzschwingen symmetrischer wie auch asymmetrischer Art. Die Veränderungen an den Gerüsten können durch das zusätzliche Auftreten signifikanter IR-Banden bestimmt werden. So führt die Einführung von Acetylgruppen zu einer zusätzlichen Schwingung bei 1200 cm^{-1}; Veränderungen im C-Ringsystem sind ebenso durch zusätzliche oder fehlende Schwingungen verfolgbar (Abbildung 20).

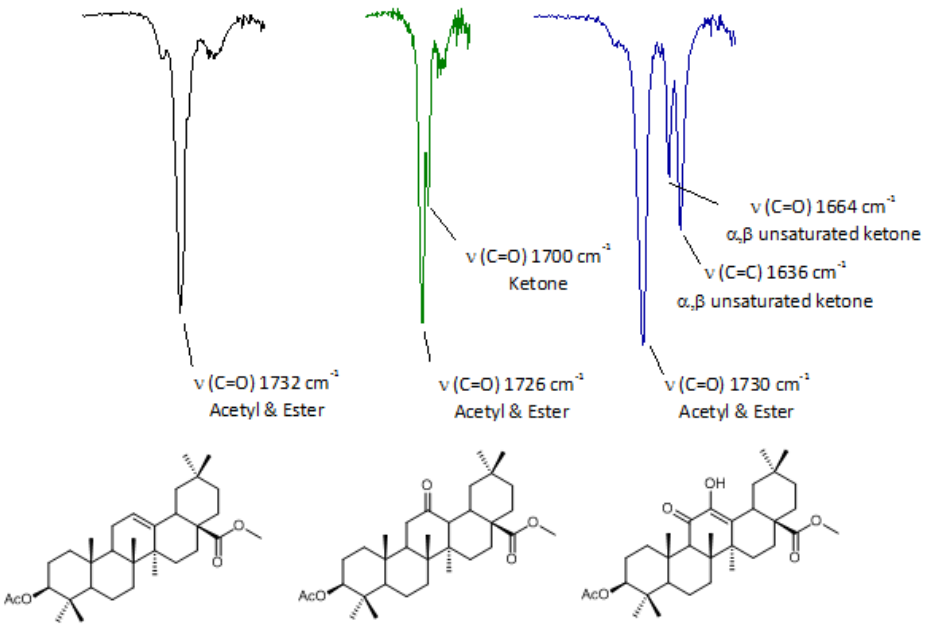


Abbildung 20: *Veränderungen der IR-Absorption in Abhängigkeit von der Modifikation im C-Ring. Die Synthese der abgebildeten Verbindungen wird in P2 beschrieben*

3.3. Pharmakologische Eigenschaften

Die weitläufige Verteilung der, als Sekundärmetaboliten vorkommenden, Triterpene in Heilpflanzen aus den unterschiedlichsten Kulturen lässt ihr enormes pharmakologisches Potential erahnen. Im natürlichen System werden sie hauptsächlich als Membranbildner oder als Pathogene gebildet, was die hohen Stoffkonzentrationen in Schalen, Blättern und Wurzeln erklärt.[109] Die Stoffklasse zeichnet sich, wie in den phytochemischen Betrachtungen schon angedeutet, durch ein mannigfaltiges Wirkspektrum aus, was zur Vermutung führt, dass es sich bei den pentacyclischen Triterpenoiden um Multi-Target aktive Verbindungen handelt.[30] Im Folgenden sollen einige ausgewählte Wirkungen näher betrachtet werden.

3.3.1. Antiphlogtischer Effekt

Im dritten Buch des römischen Arztes PEDANIOS DIOSKURIDES[110] wird die Verwendung der Süßholzwurzel, als Tee zubereitet, gerieben oder gekaut bei Magenbeschwerden und zur schnellen Wundheilung empfohlen. Für die, mit den Überlieferungen aus vielen anderen Kulturen und naturmedizinischen Schriften im Einklang stehenden, pharmakologischen Anwendungen[36], konnten unteranderem durch niederländische Forscher in der Mitte des zwanzigsten Jahrhunderts gezeigt werden, dass der Süßstoff in der Wurzel selbst die aktive Komponente ist. Dieser wiederum wird schon 1821 durch den Kieler Professor für Medizin und Chemie CHRISTOPH PAFF in „Entdeckungen in der Chemie der Materia medica“[111] als Glycyrrhizin bezeichnet und ist im wesentlich auf Arbeiten von ROBIQUET[111] zurückzuführen, der die chemische Komponente sauber extrahieren konnte und Glycion nannte. Die

chemisch-strukturelle Aufklärung gelang TSCHIRCH und CEDERBERG[112] im Jahr 1907. Während des zweiten Weltkrieges konnte der Niederländer Arzt REVERS[113] beobachten, dass die Behandlung mit Stark-Lakritze bei Magengeschwüren innerhalb kurzer Zeit zur Symptomfreiheit führt. Auf diesem Befund aufbauende Untersuchungen zeigten ferner, dass es bei gesunden Menschen in Folge von zu hohem Lakritzgenuss zu Ödembildungen kommt.[114] Im Jahr 1953 konnten CARD *et al.*[115] dann beweisen, dass dieser pharmakologisch unerwartete Effekt der Glycyrrhizin und Glycyrrhetinsäure zuzuschreiben sei. Erst weitere fünfundzwanzig Jahre, nach dieser physiologisch nicht erklärlichen Beobachtung, konnte der Arbeitskreis um EDWARDS *et al.*[116] beweisen, dass Glycyrrhetinsäure, welche nach der oralen Applikation von Glycyrrhizinsäure durch Hydrolyse-Prozesse entsteht, die 11β–Hydroxysteroiddehydrogenase (11β-HSD) inhibiert. Dieses Enzym findet sich hauptsächlich in Mineralcorticoid-sensitiven Gewebe (Leber, Plazenta, Niere)[49] und reguliert den Glycocorticoide-Haushalt durch die Oxidation von Cortisol zu Cortison. Letzteres ist nicht in der Lage mit dem Mineralcorticoid-Rezeptor in Wechselwirkung zutreten und verhindert dadurch eine Cortisol-bedingte Überstimulation des Rezeptors. Durch die Inhibierung von 11β-HSD wird Cortison jedoch nicht mehr gebildet und infolgedessen sammelt sich Cortisol im Gewebe an (vgl. Abbildung 21). Die beschriebenen, pharmakologischen Effekte der Wassereinlagerung und der Antiulcus-Wirkung sind auf die Ansammlung von Cortisol im Gewebe zurückzuführen.

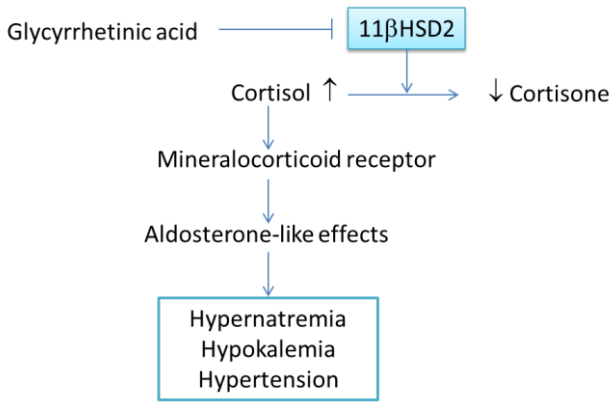


Abbildung 21: *Vereinfachte schematische Darstellung des Wechselspiels zwischen GA und 11βHSD2 nach Sontia et al. [117].*

Die, sich auf die inhibitorische Wechselwirkung mit 11β-HSD gründenden, Eigenschaften konnten auch für andere pentacyclische Triterpene vom α- und β-Amyrintyp nachgewiesen werden.[118] Zusammen mit der putativen Inhibition der im Arachidonsäure-Stoffwechsel involvierten Enzyme Cyclooxygenase (COX) und 5-Lipoxygenase lässt sich der antiphlogtische Effekt dieser Stoffklassen und der, diese Substanzen aufbauenden, Heilpflanzen begründen.[119]

Eine weitere, die antiphlogtische Wirkung untermauernde, Eigenschaft wird in den Arbeiten von ALLOUCHE und Kollegen vorgestellt.[120] Sie zeigten, dass die in Apfelschalen vorkommenden Triterpensäuren sowohl als Radikalfänger agieren können als auch in der Lage sind Kupferionen zu chelatisieren. Im speziellen Fall der Ursolsäure wurde zwar kein wirkungsgebendes Strukturelement in

dieser Abhandlung vorgestellt, Studien an der Glycyrrhetinsäure lassen jedoch den Rückschluss zu, dass die Modifikation des C-Ringes entscheidend ist.[121]

3.3.2. Antidiabetischer Effekt

Die, sich in zwei Haupttypen unterteilende, Stoffwechselstörung Diabetes mellitus lässt sich durch einen erhöhten Blutzuckerspiegel charakterisieren und wird entweder durch genetisch bedingte Prädisposition (Typ 1) oder altersbedingt verursacht (Typ 2).[3] Behandlungsstrategien neben Insulintherapien zeichnen sich durch die gezielte Beeinflussung von Enzymen aus, die am Glucose-Metabolismus beteiligt sind. In den vergangen Jahren konnte mehrfach belegt werden, dass pentacyclische Triterpene durch die Modulation solcher blutzuckersenkend wirken.[122]

Die Inhibierung der 11β-HSD, welche unter 3.3.1 dargestellt wurde, wird zum Beispiel als potentielles therapeutisches Target zur Behandlung von Diabetes Melitus gehandelt. Weitere erklärende Effekte können in der Modulation von Akt/PKB, der Protein-Tyrosin-Phosphatase 1B (PTP1B), dem Insulin Rezeptor, TGR5, dem Glycocorticoid Rezeptor, der α-Amylase sowie α-Glucosidase, GAPDH und der Glycogen Phosphorylase (GP) gesehen werden. Der 2011 erschienene Übersichtsartikel von SHENG und SUN[122] enthält zur thematischen Vertiefung für alle genannten Targets die entsprechenden Literaturzitate sowie eine detailreichere Darstellung bereit.

3.3.3. Antimikrobieller Effekt

Eine weitere vermutete biologische Funktion der, hauptsächlich in den äußeren Epidermalschichten höherer Pflanzen aufzufindenden[109], Stoffe ist von pathogener Natur. Für die antibakterielle Wirkung von Salbeiblättern konnte zum Beispiel gezeigt werden, dass der Triterpensäureanteil mitverantwortlich ist.[123] Für isolierte Triterpensäuren, wie der Oleanolsäure, könnte ferner eine antimikrobielle Wirkung gegen *Staphylococcus aureus* und *Bacillus subtilis* experimentell nachgewiesen werden.[124] HORIUCHI *et al.*[125] zeigten die wachstumsinhibitorische Wirkung gegen über *Enterococcus faecalis* und *Enterococcus faecium*, die ebenso für Ursolsäure nachgewiesen werden konnte. Die nicht vorhandene Wirkung gegenüber gramnegativen Bakterien[125], wie *Enterococcus colie, Staphyloccocus marecescens* und *Pseudomonas aeruginsa* lässt den Rückschluss zu, dass der Membran eine signifikante Rolle einnimmt. Prinzipiell zeigt diese Studie, wie auch eine von CHUNG *et al*.[126], auf, dass die Wirkung zwar existent, jedoch nicht exorbitant ist. Im Gegensatz zu einer, sich putativ auf Membran-destabilisierenden Wirkung begründenden, Aktivität stellen CHUNG *et al.*[126] für die Betulinsäure, Betuninal und α-Amyrin fest, dass 247, 1265 und respektive 179 Gene signifikant durch die Behandlung mit den entsprechenden Naturstoffen reguliert wurden sind, was die Autoren auf ein genetisches Target schließen lässt.

Studien um ZIEGLER *et al.*[127] wiederum bestärken die erstere These; Erythrozyten, welche u.a. mit Betulinsäure beladen wurden, verändern ihre Form[127a] und verhindern durch die Inhibierung der Merozoiten Internalization das parasitäres Wachstum. Auch wenn dies kein stringenter Beweis der membranabhängigen antibakteriellen Wirkung ist, zeigen diese Studien die potentielle Wahrscheinlichkeit dieser MOA, welche ferner durch Untersuchungen von BRONIATWOSKI *et al.*[128] und PRADES *et al.*[129] untermauert wird.

3.3.4. Antiviraler Effekt

Auch antivirale Wirkungen wurden vermehrt für Triterpensäuren beschrieben; erstmals im Jahre 1994 für Betulinsäure.[130] Später konnten OSORIO *et al.*[131] für verschiedene Oleansäurederivate zeigen, dass diese in der Lage sind, die Replikation von HI-Viren post-enteral zu hemmen. Der Arbeitskreis um FLEKHTER[132] zeigt für semi-synthetische Lupananaloge antivirale Aktivitäten und auch für die Maslinsäure ist eine solche Wirkung bekannt.[133] Letztere inhibiert die HIV-1 Protease und interessanterweise zeigt die freie Säure, im Gegensatz zu, mit Dipeptiden modifizierten Derivaten, eine Aktivität. Für die Oleanolsäure und verschiedene Abkömmlinge konnte ein zur Betulinsäure analoger, die Maturation der Viren beeinflussender, Wirkmechanismus nachgewiesen werden. Zusätzlich inhibiert die Behandlung mit Oleanolsäure die Spaltung von p25 zu p24 was zu der Ausbildung von morphologischen Defekten führt, die dafür sorgen, dass die Viren non-infektiös werden.[134]

3.3.5. Antialzheimer Effekt

Die u.a. aus *Origanum majorana L.* isolierbare Ursolsäure zeigte in einer Anti-Demenz Studie eine messbare, gemischte (kompetitive und nicht-kompetitive) Inhibierung der Acetylcholinesterase. Die Autoren um CHUNG *et al.*[135] postulieren einen verzögerten Verlust der kognitiven Fähigkeit durch die therapeutische Behandlung mit Ursolsäure. Auch für andere Amyrine konnte eine solche Aktivität nachgewiesen werden.[58a,136,57b]

3.3.6. Antitumor Effekt

Mit ca. eintausend Einträgen, welche bei der Suche nach den Schlagworten Krebs und den verschiedenen Säuren sowie der anschließenden Subsummierung der Einzelergebnisse, bei PubMed resultieren, wird neben der Relevanz *per se* gleichzeitig die enorme Komplexität des vorhandenen Wissens angedeutet. LOGEMANN und Kollegen[137] veröffentlichten bereits 1960 in Nature eine *in vivo* Studie welche den Titel *„Antileukemic activity of Glycyrrhetinic acid“* trägt. Ferner deutet sich durch diese erste Studie schon die Existenz sehr interessanter Struktur-Wirkungs-Abhängigkeiten an. Während die 18α wie auch die 18β-Glycyrrhetinsäure vielversprechende Aktivitäten aufweisen, verringert sich das Tumorgewicht durch die Behandlung mit einem Methylsulfonsäuresalz des 3-Oxo-glycyrrhetinsäure-dimethylaminoethylesters kaum. Mit der, nicht nur aus wirkstoffforschendem

Blickwinkel heraus betrachten, revolutionären Immortalisierung von Krebszellen (Vgl. 3.4.1.1) und der daraus sich ermöglichenden *in vitro* Testung steigt historisch die Anzahl an Veröffentlichungen, welche belegen, dass es sich bei den antitumor aktiven Komponenten vieler Heilpflanzen um die Triterpensäuren handelt. Über ein halbes Jahrhundert nach der Ersterwähnung im Journal Nature[137] ist das Interesse an antitumor Untersuchungen noch nicht erloschen. Die selektive Induktion eines letalen Effektes, die erwiesene Möglichkeit der Aktivitätsmodulierung *via* chemischer Modifikationen sowie u.a. die gute, zumeist nebenwirkungsarme Verträglichkeit können als Hauptursache angesehen werden. Wissenschaftlich bewiesen ist, dass die meisten Vertreter dieser Naturstoffklasse es vermögen in den Krebszellen eine kontrollierte Form des Zelltodes auszulösen – die Apoptose. Zusätzlich existieren verschiedenste Erkenntnisse, welche verdeutlichen, dass auch ein zellwachstumshemmender Effekt durch die betrachteten Verbindungen ausgelöst werden kann. Um eine der Zielstellung gerecht werdende Betrachtung des Themas zu ermöglichen, ist eine genauere Betrachtung der Krankheitsentstehung sowie der molekularbiologischen Ereignisse, welche zum Zelltod führen, von Nöten.

3.4. Tumorerkrankungen und potentielle Behandlungsstrategien

„Das Geschwultsproblem ist ein Zellenproblem“

THEODOR BOVERI

Der, mit dem revolutionierenden Übergang der Wissenschaft von der deskriptiven zur experimentellen Phase einhergehende, Erkenntniszugewinn der Tumorentstehung ist im Wesentlichen mit den Namen THEODOR BOVERI[138] (1862-1915) und DAVID PAUL VON HANSEMANN[139] (1858-1920) in Korrelation zu setzen.[140] Die Biologen beschäftigten sich während der Jahrhundertwende und zu Beginn des zwanzigsten Jahrhunderts mit der Krebsforschung. HANSEMANN, als Vertreter der deskriptiven Periode anzusehen, war der histologischen Krebsforschung verschrieben und untersuchte u.a. die, in den 80igern des neunzehnten Jahrhunderts entdeckten und beschriebenen, Chromosomen in Krebszellen.[140] Durch Lichtmikroskopie, Einbettungen, Färbeverfahren und anderen technische Neuerungen ermöglicht, begründete HANSEMANN mit MAX BORST die wissenschaftliche Tumordiagnose.[140] Die in den Jahren 1902 und 1910 erschienenen Bücher „Die mikroskopische Diagnose der bösartigen Geschwülste“[141] sowie „Atlas der bösartigen Geschwülste“[142] bezeichnet WUNDERLICH[140] als Höhepunkte der deskriptiven Tumorcharakterisierung. (vergleiche Abbildung 22) Im Gegensatz zu BOVERI war HANSEMANN primär an der formalen Tumorgenese, der Entwicklung von Tumoren, und nicht wie ersterer an der Entstehung, der kausalen Genese, von bösartigen Geschwülsten interessiert.

Als unübertroffener Meister des Experimentes beschreibt HANS SPEMANN (1869-1941) den als Vertreter der neuen, experimentellen Wissenschaftsperiode anzusehenden, BOVERI.[143] Als Zoologe beschäftigte

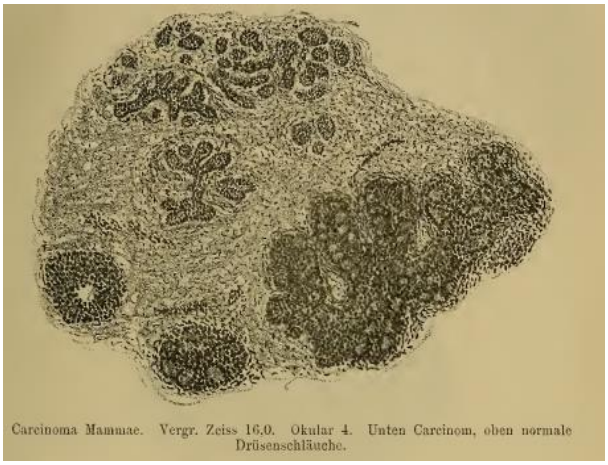


Abbildung 22: *Historische, histologische Abbildung eines Mamma-Karzinoms*[142]

er sich hauptsächlich mit entwicklungsbiologischen Fragen. Die Eier des *Echinus* (Seeigel) und der *Ascaris megalocephala* (Spülwurm, heutige Nomenklatur *Parascaris equorum*) dienten ihm als Modellorganismen. Seine vornehmlich deduktive entwickelte Hypothese, dass der Tumorentstehung permanente Störungen des Gleichgewichts der Chromosomen zugrunde liegen, welche durch abnorme Mitose zustande kommen, leitet sich von einem seiner Hauptwerke[144], dem 1902 erschienen Artikel[144a] „Über Mehrpolige Mitose als Mittel zur Analyse des Zellkerns“ ab.[140,145] Wie viele seiner Thesen konnte diese verifiziert werden; es bedurfte jedoch über fünf Jahrzehnte. Das so generierte Wissen über die Tumorpromotion, -progression und -definition lässt heute VIRCHOW´s Ausspruch - *„Wollte man auch Jemand auf das Blut pressen, dass er sagen sollte, was Geschwülste eigentlich seien, so glaube ich nicht, dass man irgend einen lebenden Menschen finden würde, der in der Lage wäre, dies sagen zu können.“*[146]- der 1897 von HANSEMANN[141] noch validiert werden musste, klarer verneinen. Der 1821 in Pommern geborene Arzt, Archäloge und Politiker beschrieb 1845 erstmalig die maligne Erkrankung des lymphatischen Systems, die Leukämie und überführte mit seinem Theorem *„Omnis cellula e cellula“*[146] im Jahr 1855 die Zytologie in ein neues Zeitalter.[147]

3.4.1. Tumorgenesis

Das ursprüngliche Gewebe, oder expliziter die Zelle mit der ersten Mutation, klassifiziert die verschiedenen malignen Tumorarten in die Hauptarten Karzinome (aus Ephitelzellen), Sarkome (Binde und Stützgewebe) und Leukämien (Blut und Knochenmark) ein.[5] Die Entwicklung der tödlichen, metastierenden Form des Tumors selbst ist ein Prozess, der im Durchschnitt über einen Zeitraum von zwanzig bis vierzig Jahren verläuft. Der lange Entwicklungsprozess verdeutlicht zum einen die ungemeine Komplexität und wird ferner zur Beschreibung in ein Dreistufenmodell untergliedert. Das in der Mitte des zwanzigsten Jahrhunderts entwickelte Modell gilt zwar heute als nicht mehr aktuell, vielmehr geht man von einem non-linearen Mehrstufenmodell aus, es ist aber, durch den Mangel an evidenten, besseren Theorien, allgemeinhin akzeptiert und in Abbildung 23 schematisch dargestellt.[5]

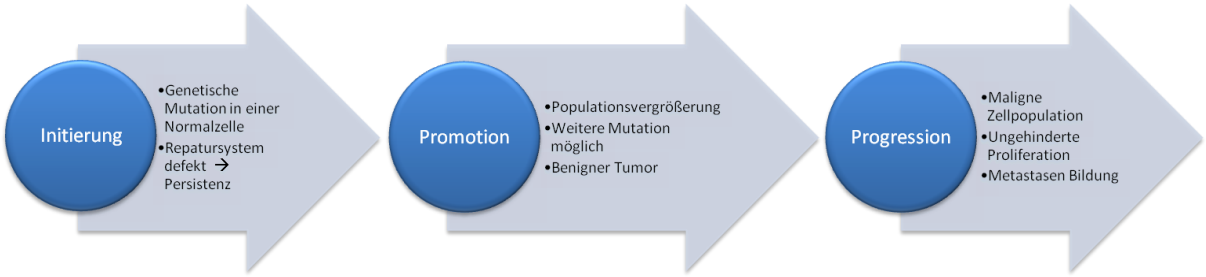


Abbildung 23: *Dreistufenmodell der Tumorgenesis*

Nach der ersten ursprünglichen Mutation einer einzigen Zelle, benötigt es eine gewisse Weile, bis sich aus der einzelnen, mutierten Zelle eine relevante Population gebildet hat (ca. 10^6 Zellen), in einer zweiten Phase kommt ein weiterer Defekt hinzu, der sich aber erst als tumorbedingend bezeichnen lässt, wenn weitere genetische Veränderungen (zwischen vier und sechs) hinzugekommen sind. Beobachtungen, wie z.B. die epigenetischen Veränderungen der Gene, im Konkreten, die Methylierung von Promotorgenen, verkomplizieren die Tumorgenesis.[5] In Abbildung 24, welche dem Übersichtsartikel „The Hallmarks of Cancer" von HANAHAN und WEINBERG[148] entnommen ist, ist dieses komplexe Zusammenspiel dargestellt.

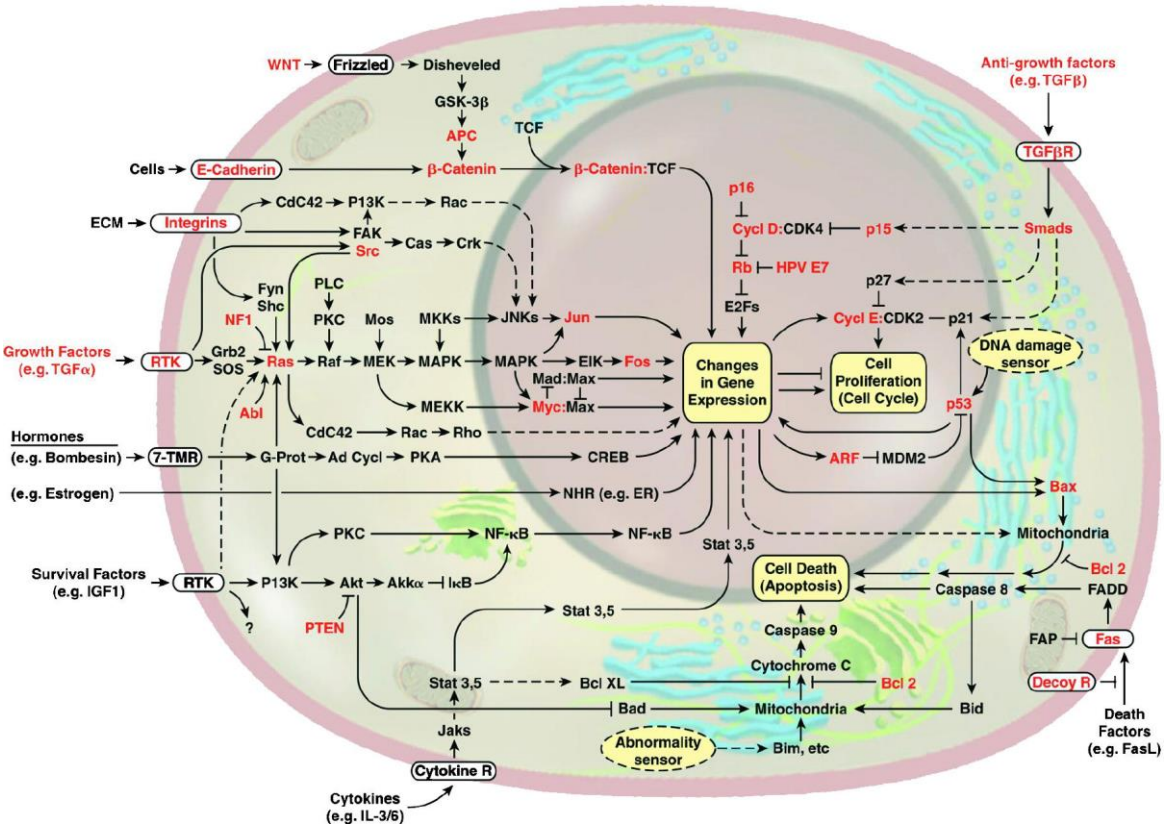


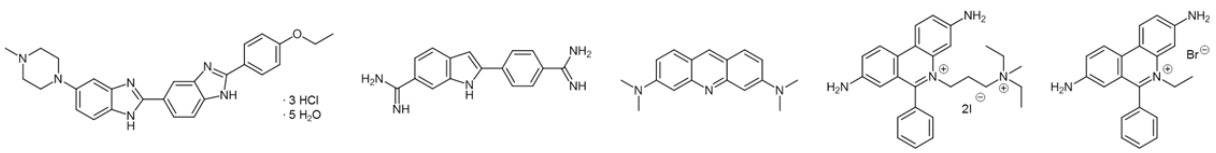
Abbildung 24: *Molekulare Wechselwirkungen, die zur Tumorgenesis führen. Abbildung aus Hanahan und Weinberg[149]. Rot markierte Gene stellen bekannte mutierte Gene in Krebszellen dar.*

3.4.2. Der Zellzyklus

Mutationen, die tumorinitiierend wirken können, treten während des Proliferationsvorganges in der Zelle auf. In Abbildung 2 ist eine schematische Abbildung des Zellzyklus zu sehen; eine schnell wachsende eukaryontische Zelle durchläuft diesen in ca. 24h. Nach durchschnittlich zwölf Stunden erreicht eine proliferierende Zelle den G1-Checkpoint.[150] Reguliert durch Cyclin-abhängige Proteasen wird die Zelle an dieser Stelle in die Synthesephase überführt, wenn eine ausreichende Zellgröße erreicht ist und keine Schädigungen der DNA erkennbar sind. Vermittelt durch reversible Dephosphorylierung und Phosphorylierung, Aktivierung von spezifischen Inhibitoren und die Regulation von subszellulären Lokalisationen erfolgt die Regulation; im speziell betrachteten Fall wird ein [CylcomD/CDK4] Komplex benötigt. Wie in Abbildung 24 ferner dargestellt sind die Proteine $p21^{WAF}$ und p53 indirekte Inhibitoren für die Ausbildung dieses Komplexes und somit Tumorsupressorgene.[5] Eine erhöhte Expression des Gens, wie es z.B. kürzlich bei der Behandlung von Wistar Ratten mit Glycyrrhizinsäure nachgewiesen werden konnte[151], führt zu einem Zellwachstumsarrest und somit zu einer Eindämmung des Tumorwachstums. Zwei in ähnlicher Art und Weise ablaufende Kontrollpunkte sind ferner in der Zelle bekannt, einer nach der G2 Phase und ein weiterer in der M-Phase. Neben der Überführung der Zelle in den G0 Zustand, dem Wachstumsarrest, kann es in Folge von entsprechenden Signalen zur Apoptose oder zu einem Verweilen in der entsprechenden Phase kommen. Ferner ist bekannt, dass bei einer Tumorerkrankung diese Kontrollpunkte inaktiviert sind bzw. deaktiviert wurden, und zum anderen, dass viele Zytostatika Einfluss auf den Zellzyklus haben.

3.4.2.1. Zellzyklusuntersuchungen

Zur Analyse tendenzieller Zellzyklusveränderungen stehen prinzipiell mehrere Methoden zu Verfügung. Die am weitverbreitetsten beruhen auf FACS-Messungen (Fluorescence-activated cell sorting).[152] Nach Vereinzelung und laserunterstützter Zählung der zu untersuchenden Zellpopulation können mittels Emissionsdetektoren Fluoreszenzsignale aufgenommen werden, die selektiv einzelnen Events (Zellen), zugeordnet werden können. Wird eine behandelte Zellpopulation mit einem DNA-Farbstoff versetzt, kann, wenn die Bindung proportional erfolgt, die DNA-Verteilung bestimmt werden. Aus dem Pool der zur Wahl stehenden DNA-Farbstoffe[153] (Tabelle 2) wird Propidiumiodid bevorzugt, da durch Färbungen mit diesem zweifach positiv geladenen, interkalierenden Farbstoff die schärfsten Phasen erzeugt werden und das Vertrauensintervall den minimalsten Wert erreicht.[153]



A B C D E

Abbildung 25: *DNA und RNA Fluoreszenzfarbstoffe*

	Farbstoffbezeichnung	λ_{max}	Farbe	Selektivität bzgl. NA
A	Höchst 33342	461	blau	DNA
B	DAPI	455	blau	DNA
C	Acridine Orange (AO)	520	grün	DNA
D	Propidium Iodid (PI)	615	rot	DNA & RNA
E	Ethidium Bromid (EB)	600	rot	DNA & RNA

Tabelle 2*: Emmissionen und Selektivität der dargestellten DNA-Farbstoffe*

Abbildung 26 zeigt ein typisches, im Rahmen der vorliegenden Arbeit aufgenommenes DNA Histogramm. Theoretische Grundlage zur Auswertung bildet die Tatsache, dass Zellen der G1 bzw. G0-Phase einen einfachen diploiden DNA-Satz besitzen, Zellen der M bzw. G2-Phase einen doppelten, sogenannten tetraploiden Satz.[154] Bindet der Farbstoff proportional, so muss die Signalintensität der G2/M-Phase doppelt so groß sein, wie die der G1/G0-Phase. Events mit einer dazwischenliegenden Signalintensität werden entsprechend der S-Phase zugeordnet, in welcher die DNA aufgebaut wird.

Um eine Färbung mit Propidiumiodid (PI) *per se* zu ermöglichen, muss die Zellpopulation zuvor fixiert und permeabilisiert werden, da der Farbstoff aufgrund seiner zweifach positiven Ladung, nicht zellmembranpermeabel ist.[153] Als Reagenz wurde eine eiskalte, siebzig prozentige ethanolische Lösung verwendet, die gleichzeitig fixierend und permeablilisierend wirkt.[155] Als Nebeneffekt kommt es bei dieser Methode jedoch zu einer verstärkten Konglomeratisierung der Zellen, sodass die Vereinzelung der Zellen während der Messung nicht mehr hundertprozentig gewährleistet werden kann und von dem aufgenommenen Histogramm zuvor die Dupletts entfernt werden müssen, um falsch positive G2/M Populationsanteile zu entfernen. Dies geschieht digital durch eine zweidimensionale Auftragung der Signalhöhe gegen die Signalbreite; direkt proportionale Events entsprechen einzelnen Zellen (Polt B der Abbildung 26). Zur statistischen Auswertung der Verteilung existieren zwei verschiedene Methoden. Erstere, welche das Integral unterhalb des Graphen eines manuell festgelegten Bereiches berechnet, sollte aufgrund der Ungenauigkeit (Abbildung 26 Teil C und D) wenn möglich vermieden werden, da die einzelnen Phasen sich überlappen. Zur korrekteren Analyse des Zellzyklus kann auf ein von RABINOVITCH[154] geschriebenes Programm (MultiCycle™ DNA analysis) zugegriffen werden. Dieses basiert auf einer strikt mathematischen Analyse, welche es vermag die überlappenden Integrale zu trennen. Es beinhaltet die meisten etablierten Modelle, die das Zellzyklushistogramm als Ergebnis mehrerer Gaus-Verteilung betrachten und die bestmögliche theoretische Verteilung berechnen. Zusätzlich können Zellfragmente „debris“ und Hintergrundrauschen, welche das Ergebnis verfälschen, herausgerechnet werden.[156] Das iterativen Verfahren bestimmt über eine Maxima-Suche, kombiniert mit strikten Regeln für die Standardabweichung der einzelnen Integrale und ein nicht zu unterschreitendes Minimum, zunächst das G1 Signal, von diesem ausgehend wird der tetraploide

Peak, der im Mittelwert dem 1.93 – 2.03 fachen des G1 Signal entsprechen sollte, gesucht.[157] Die Synthesephase wird nach einem Model von DEAN UND JETT[158] gefittet. Ferner werden, wie von RABINOVITCH[154] als Ergebnis der DNA Cytometry Consensus Conference empfohlen, mindestens 10.000 Zellen pro Messung gezählt. Um eine reproduzierbare S-Phase zu erhalten, werden Zellfragmente wie auch Aggregate bei der Zählung nicht berücksichtigt. SHANKEY *et. al.*[159] hebt an dieser Stelle auch hervor, dass das Vertrauensintervall von nicht maligen Zellen <8% liegen sollte, für Tumorzellen mag zwar ein CI von >8% durchaus nicht unwahrscheinlich sein, eine gute S-Phasen Kalkulation, zur Aussage über das Tumorwachstum, lässt sich jedoch auch hier besser realisieren mit einem geringen CI (<8%).

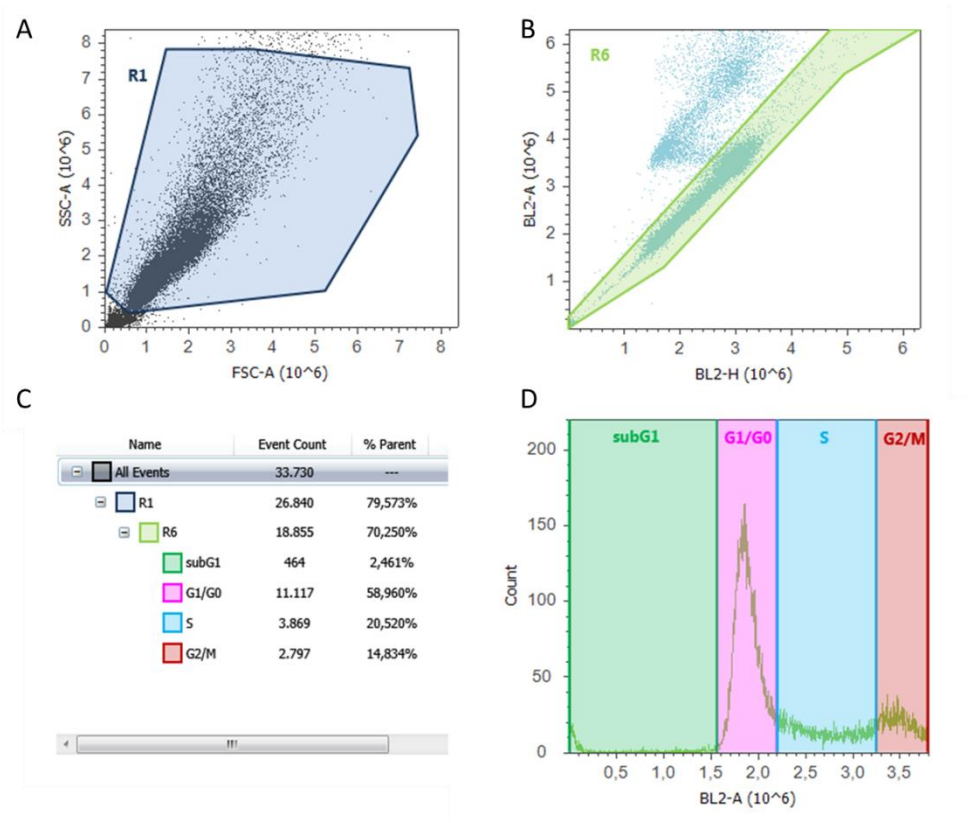


Abbildung 26: *Beispielhafte Darstellung der Zellzyklusuntersuchung via FACS Messung*
***A** Gesammelte gesamt Zellpopulation; R1 stellt die für die Analyse ausgewählten Events dar **B** 2D-Plot der Propidiumiodid Emission; R6 entspricht den einzelnen Zellen, parallel versetzt sind Zell-Dupletts zu sehen. **C** Prozentuale Verteilung der in **D** markierten Bereiche*

Die Veränderung der Normalverteilung als Folge der Behandlung mit zytotoxischen Präparaten ist in Abbildung 27 zusehen.

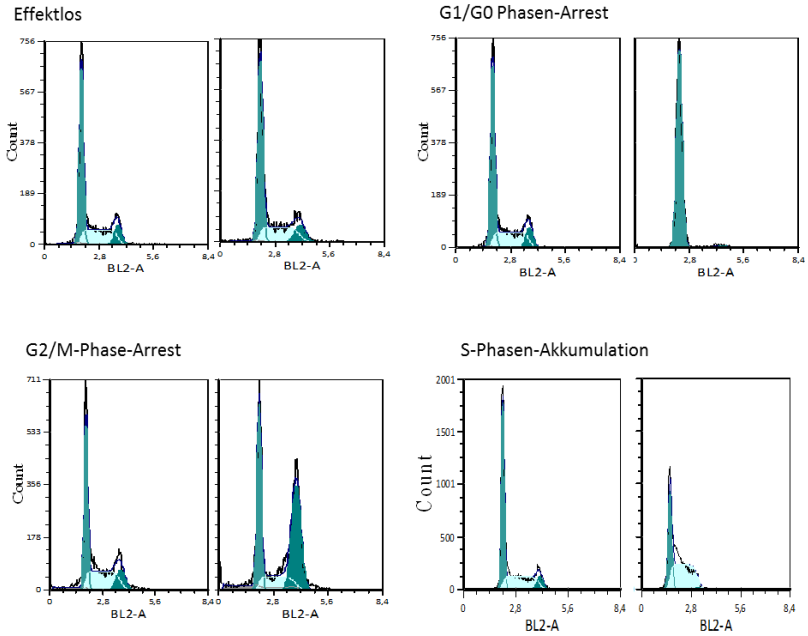


Abbildung 27: *Ausgewählte Histogramme, die die einzelnen Zustände, welche für lebende Zellpopulationen nach einer Behandlung bekannt sind, darstellen.*

Während das jeweils linke Histogramm die Verteilung in der unbehandelten Kontrolle zeigt, ist in dem entsprechend rechtem Graph jene nach der Behandlung einer Zellpopulation zusehen. Über diese Analyse ist es möglich ein erstes Indiz über einen putativen Mechanismus der Wirkung zu erhalten; eine M-Phasen Anreicherung beispielsweise deutet darauf hin, dass der Wirkstoff die Öffnung der Spindel während der Anaphase verhindert.[160] Verstärkt sich die S-Phase hingegen, kann vermutet werden, dass durch einen Eingriff in die DNA-Synthese diese nicht korrekt ausführbar ist.[161] Die Anreichung der M-Phase bleibt in Folge dessen aus und die Zellen verweilen in der S-Phase recht breit verteilt, da es keinen Kontrollpunkt gibt, an dem diese arretiert werden könnten. Durch die Inhibierung von z.B. Cytokinen auf Protein oder Genebene kann alternativ der Übergang von der G1 in die S Phase gehemmt werden, was zu einem G1 Arrest führen würde und in der Zellzyklusanalyse durch eine typische Verteilung (Abbildung 27 links oben) detektierbar wäre. Wie aus Abbildung 24 ersichtlich ist, kann durch Modulation von zellzyklusarrestinduzierenden Proteinen (e.g. p53) parallel über Bid die Caspase Kaskade gestartet werden, welche zum Zelltod führt.

3.4.1. Der Zelltod

Unter dem Begriff werden drei grundlegend verschiedene Formen subsummiert, welche zum Absterben der Zellen führen. Neben der Seneszenz, dem Alterungsprozess, klassifiziert man in traumatisch-nekrotische und programmiert ablaufende Zelltode. Letztere werden wiederum in verschiedene Unterformen unterteilt.[2]

3.4.1.1. Seneszenz

Unter Seneszenz wird der irreversibel induzierte Zellwachstumsarrest verstanden und wurde erstmals für menschliche Fibroblasten, als eine Phase, in welcher sich diese am Ende ihrer replizierenden Lebensspanne befinden, beschrieben. Durch den Verlust der Fähigkeit auf Wachstumsfaktoren zu reagieren sind die Zellen in der G1/G0 Phase des Zellzyklus arretiert.[162] Eine charakteristische, morphologische Veränderung ist das flache, vergrößerte Erscheinungsbild der Zelle und auf molekularer Ebene findet sich häufig eine Induktion der SA-β-gal Aktivität. Signifikante Veränderungen in der Gen-Expression rufen u.a. die Bildung des Seneszenz-assozierten Sekretom hervor, das wiederum schwerwiegende Veränderungen in angrenzenden Zellen und der Mikrostruktur bedingt. Die Bildung des charakteristischen SAHF (senescence-associated heterochromatic foci) erfordert die Hemmung von E2F-abhängigen Promotoren durch pRb was eine stabile Repression von E2F spezifischen Genen zur Folge hat und womöglich die Irreversibilität der Seneszenz begründet.[163]

Hervorgerufen wird die Seneszenz nach aktuellem Stand der Wissenschaft durch die Verkürzung der Chromosomenenden, den Telomeren. Bei jeder Replikation der DNA wird das spezifische, mehrere KBp-große, aus der Sequenz TTAGGG bestehende, Endstrukturelement verkürzt. Nach der Erreichung eines kritischen Minimums kann sich die Zelle nicht mehr teilen, was als Seneszenz auslösend angesehen wird. Die artifizielle Überschreitung dieses Kontrollpunktes gelang mit Telomerasen und bildet Grundlage für die Immortalisierung von Zellen und damit für die Zellkultur.[164] Das Enzym selbst ist in nur in wenigen gesunden Zellen exprimiert (z.B. in Stammzellen), hingegen in bis 95 % der malignen kolorektalen Zellen.[165] Für Ginsenoside konnte durch ZHOU *et al*.[166] nachgewiesen werden, dass diese Triterpene Seneszenz in menschlichen Fibroblasten auslösen.

3.4.1.2. Nekrose

Der auch als „Accidental Cell Death“ beschriebene Zelltod kennzeichnet sich durch das Aufplatzen der Zellmembran, als Folge des signifikanten Anschwellen der Zelle und der Organellen. Auslösende Faktoren sind neben Bakterien und Gifte mechanische Beanspruchungen. Durch das unkontrollierte Austreten des Zellinneren in das umliegende Gewebe kommt es in Folge von nekrotischen Zelltoden zu Entzündungen.[2]

3.4.1.3. Programmierte Zelltod

Nach der erstmaligen, wissenschaftlichen Beschreibung des programmierten Zelltodes durch KARL VOGT im Jahre 1842[167] vermehrte sich die Kenntnis in Folge intensiver Forschungen explosionsartig. Verschiedene Monographien befassen sich ausschließlich mit den verschiedenen Formen, die wohl bedeutendste ist die Apoptose. Neben dieser, welche sich durch die Aktivierung von Caspasen auszeichnet, sind die kontrollierten, zum Tod führenden Prozesse unter Beteiligung von Autophagozytose sowie der Mitotische Katastrophe (MC) anzuführen. Zusätzlich findet sich in der Literatur der Caspase-unabhängige, programmierte Zelltod beschrieben.[2]

3.4.1.3.1. Autophagozytose

Der ubiquitär hoch konservierte eukaryotische Mechanismus wird bei Nährstoffmangel, Absenz von Wachstumsfaktoren oder oxidativen Stress beobachtet und in drei Typen unterteilt. Im Kontext der Antitumor-Forschung sind die Mikro- und die Chaperon-vermittelte Autophagozytose von geringer Relevanz.[2] Der Makroautophagozytose, welche auch als „self-eating mechanism“ beschrieben wird, spricht man dagegen eine hohe Signifikanz als potentiellen anti-tumorös wirkenden Prozess zu. Zentrale Rolle spielen doppelwandige Vesikel, die Autophagosomen, in deren pH-sauren Inneren u.a. Proteine zum Energiegewinn degeneriert werden. Die Typ II-Unterform, die ausschweifende Autophagozytose, führt hierbei – putative unter Beteiligung von apoptotischen oder nekrotischen Mechanismen – [168] zum Zelltod. Die genetisch und molekulare Ebene ist noch nicht vollständig aufgeklärt, als bewiesen gilt jedoch die Involviertheit verschiedener Atg´s (Autophagy related genes).[169] Für B10, einen Tris-Ester der Betulinsäure[170] sowie andere Betulinsäurederivate[171] konnten beispielsweise typische Autophagymerkmale nachgewiesen werden.

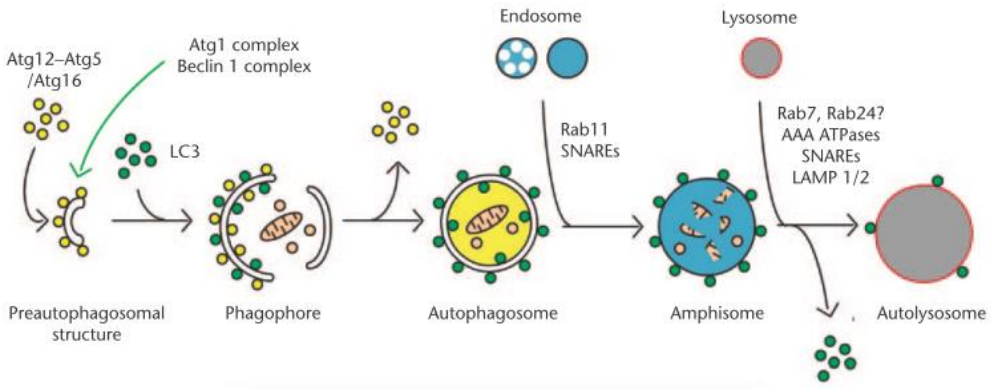


Abbildung 28: *Schematische Darstellung der Autolysosomebildung von MELINO*[2]

3.4.1.3.1.1. Autophagy Nachweis

Neben der Möglichkeit Expressionsveränderung von Autophagozytose relevanten Proteinen über gelelektrophoretische Methoden, wie den Westernplot, zu analysieren, existieren verschiedene, auf pH-Wert sensiblen, fluoreszenzaktiven Farbstoffen beruhende, Protokolle.[172] Der im Rahmen dieser Arbeit u.a. zur Detektion von Autophagy verwendete Fluoreszenzfarbstoff, Acridin Orange (AO), ist eine leicht wasserlösliche Base, die in reinen, wässrigen Lösungen in zwei Hauptformen (als Dimeres oder Monomeres) auftreten kann. Die Monomere-Form besitzt ein Absorptionsmaximum bei 488 nm und fluoresziert bei 530 nm mit einer Halbwertzeit von $1*10^{-9}$ s grün.[173] Die Dimere-Form besitzt ein Absorptionsmaximum bei 465 nm und fluoresziert bei 640 nm mit einer Halbwertszeit von 10^{-8} s rot. Bei mikroskopischen Untersuchungen unterscheidet man zwischen dem A- und B-Typ von AO. Ersterer tritt bei Wechselwirkungen mit doppelsträngigen Nukleinsäuren auf, wenn das Verhältnis von Nukleinbasen zu AO-Molekülen 4:1 beträgt. In der interkalierten Form hat das Monomere AO eine 2 bis 2.5 fach höher Fluoreszenzintensität als im ungebunden Zustand. Prinzipiell sind die Eigenschaften des A-Typs vergleichbar mit denen des Monomeren, als einziger Unterschied lässt sich ein Shift im Absorptionsmaximum (Δ = 15 nm) hervorheben. Der B-Typ von AO tritt bei Wechselwirkungen mit einzelstrangigen Nukleotidsäuren (ssNA) auf, in diesem speziellen Fall bleibt die Fähigkeit Dimere auszubilden erhalten und eine rote Fluoreszenz kann beobachtet werden. In detektierbaren Mengen kommen ssNA in den sauren Organellen der Zelle vor. Lysosomen, Biovesikel mit einem Inneren pH von 4.5-5.0, spalten Proteine und Enzyme in Monomere und spielen somit eine essentielle Rolle im Energiemetabolismus der Zelle. Unter Nährstoffmangelbedingungen fusionieren die Lysosomen zu größeren Autophagosomen (Vgl. Abbildung 28) und der Gesamtanteil dieser Kompartimente in der Zelle nimmt zu, was durch eine vermehrte Anzahl an orange gefärbten Organellen bei der Fluoreszenz-mikroskopischen Untersuchung detektierbar ist.[173]

3.4.1.3.2. Mitotische Katastrophe

In Folge von Hitzestress oder als zelluläre Antwort auf die Behandlung mit verschiedenen Reagenzien wie z.B. 5-FU (5-Fluoruracil), Paclitaxel oder Temozolomid kommt es zu Aneuploidie.[162] Die sich morphologisch hauptsächlich durch anormale Kernmorphologie charakterisierende Mitotische Katastrophe (MC) ist auf eine frühzeitige Überführung der Zelle in die M-Phase und auf den Verlust der Regulation der Zellteilung zurückzuführen.[174] Auf molekularer Ebene korreliert die MC u.a. mit einer Dysfunktion des Proteins p53 und einer Überakkumulation an CyclinB1. Der so induzierte

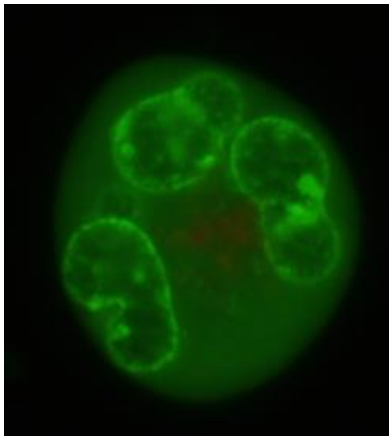


Abbildung 29: *Fluoreszenzmikroskopische Aufnahme einer Zelle mit den Merkmalen der Mitotischen Katastrophe*

Zelltod verläuft zumeist apoptotisch und kann sowohl mikroskopisch (vgl. Abbildung 29) als auch durch Zellzyklusuntersuchungen (vgl. 3.4.2.1) detektiert werden.

3.4.1.3.3. Apoptose

Unter der, sich aus dem altgriechischen Begriff für abfallen - *apopiptein* - ableitenden, Bezeichnung versteht man den programmierten Abbau der Zelle in kleine Membran umschlossen Fragmente (vgl. Abbildung 32) die von Makrophagen oder anliegenden Zellen entzündungsfrei aufgenommen werden. Diese daher erstrebenswerte Form des Zelltodes wird durch eine Kaskade von verschiedenen Cystein-abhängigen Proteasen (Caspasen) beschrieben und kann durch multiple Faktoren ausgelöst werden; vereinfacht unterscheidet man zwischen extrinsisch und intrinsisch initiierenden Signalen, die zur Aktivierung von Initiator-Caspasen führen. Auf beiden Wegen werden die Exekutor-Caspasen C3 und C6 aktiviert, welche die Degeneration der Zelle einleiten. Abbildung 30 verdeutlicht das komplexe System. Erkenntlich ist in dieser Abbildung auch die Existenz eines verstärkten, extrinsischen Mechanismus; Nach initiierender C8 Aktivierung können über verschiedene BH-3-only Proteine die Poren der Mitochondrienmembran geöffnet werden, was eine C9 Aktivierung zur Folge hat und somit verstärkt über die intrinsische Signalkaskade die Exekutor-Caspasen aktiviert werden können.

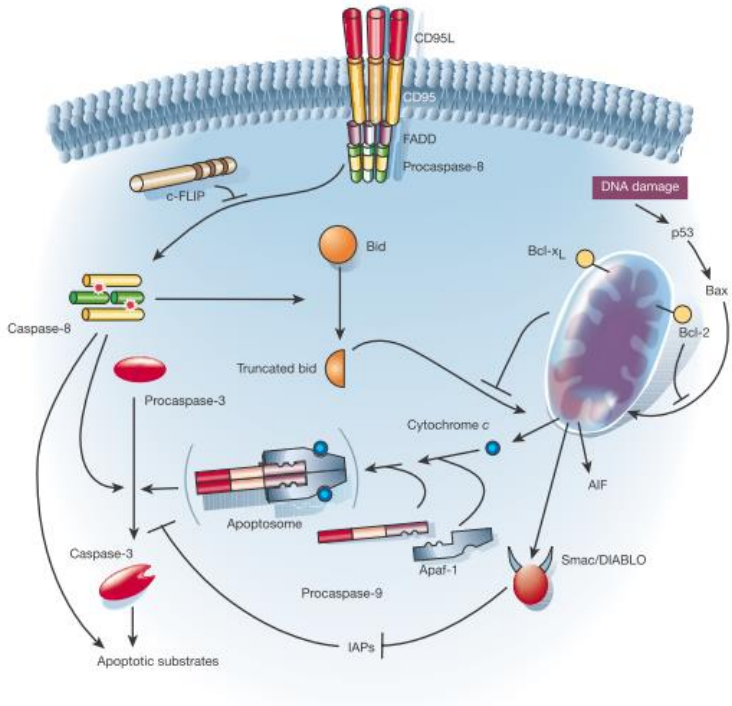


Abbildung 30: *"The road to the ruin" Hengartner (2000)*
Darstellung der extrinsischen, über Caspase – 8 verlaufenden, und der, über durch die Ausbildung des Apoptosoms gekennzeichneten, intrinsischen apoptotischen Signalkaskade

Die typischen, markanten morphologischen Veränderungen, die sich alle indirekt auf die beiden Exekutor-Caspasen C3 und C6 zurückführen lassen, resultieren unabhängig von dem ursprünglichen initiierenden Signal. Im Gegensatz zum nekrotischen Zelltod geht die Apoptose mit einem Zellschrumpfen, Membranaufstülpungen, Nukleinkondensation, DNA-Fragmentierung und vielen mehr einher. Abbildung 31 verdeutlicht eine Auswahl der unterschiedlichen Caspasen Substrate.

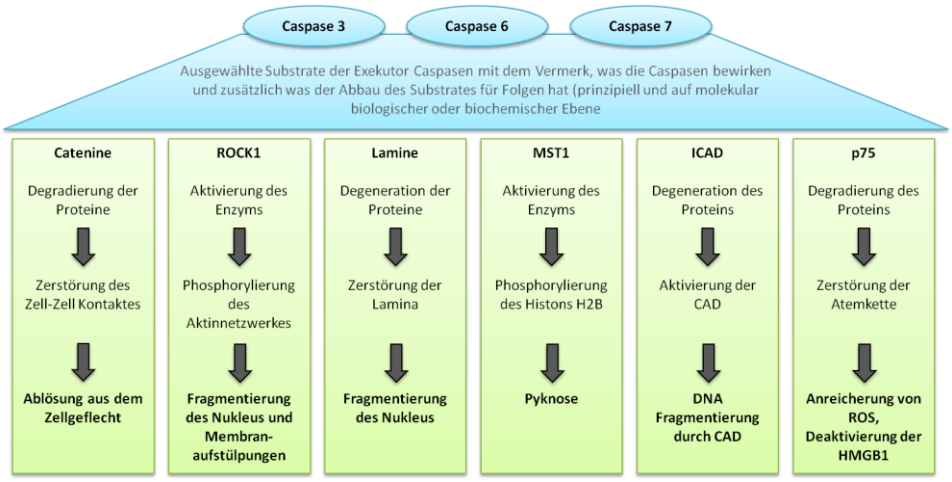


Abbildung 31: *Ausgewählte Konsequenzen der Spaltung bzw. des Abbaus von Caspase-Substraten nach Melino*[2]

3.4.1.3.3.1. Nachweis von Apoptose

Die Anzahl an Nachweismethoden des apoptotischen Zelltodes ist nahezu äquivalent zu den mannigfaltigen morphologischen Merkmalen.[153,169,175] Membranaufstülpungen (Vgl. Abbildung 32) als Folge des Abbaus von u.a. Aktin können mikroskopisch nachgewiesen werden. Verwendet man bei solchen Untersuchungen ein Farbstoffgemisch, kann gleichzeitig die Membranstabilität überprüft werden. Hierbei ist jedoch zu beachten, dass dem ursprünglichen AO/EB-Assay[176], in welchem neben AO das einfach positiv geladene Ethidiumbromid verwendet wird, der AO/PI-Assay vorzuziehen ist. SHAPIRO[153] hebt in seiner Abhandlung über die verschiedenen Fluoreszenzfarbstoffe hervor, dass auch wenn Ethidiumbromid im Allgemeinen als nicht Zellmembranpermeabel angesehen wird, dies nicht der Wahrheit entspricht. Vielmehr kann es die Zellmembran passieren. Es wird zwar anschließend durch Protonenpumpen schnell wieder heraus transportiert aber eine 100%ige Zellimpermeabilität, wie sie ein Farbausschlusstest *per se* erfordert, ist nicht gegeben.[153] Im Rahmen der vorliegenden Arbeit wurde daher das zweifach positive Propidiumiodid verwendet. Abbildung 32 verdeutlicht graphisch zudem die unterschiedlichen morphologischen Eigenschaften zwischen PCD und Nekrose die u.a. durch die Wahl der Fluoreszenzfarbstoffe mikroskopisch untersucht werden können.

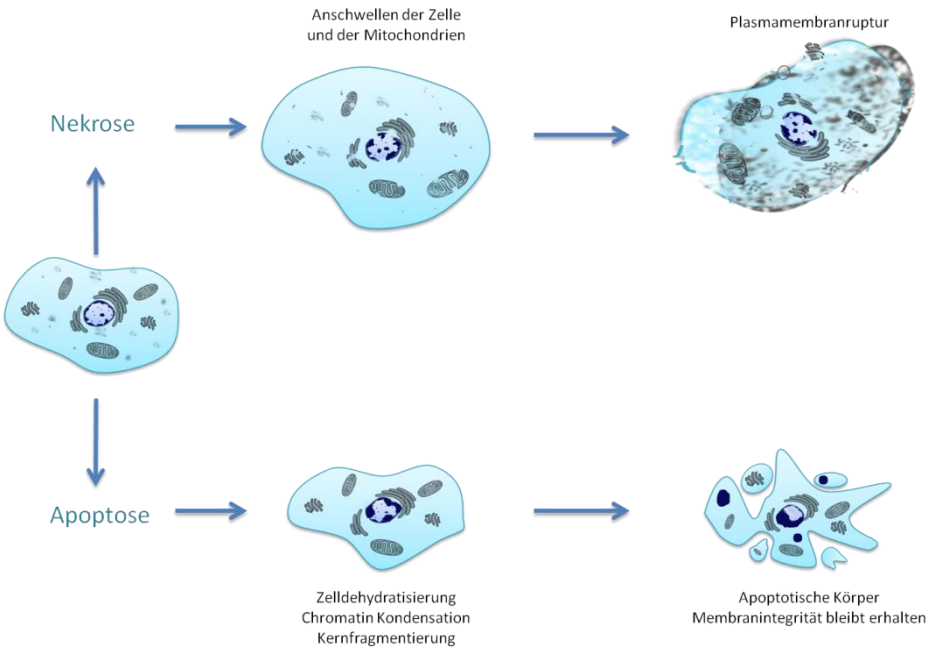


Abbildung 32: *Schematische Illustration der morphologischen und biochemischen Änderungen während der Apoptose (einer Art des PCD) und der Nekrose nach Darzynkiewicz*[177]

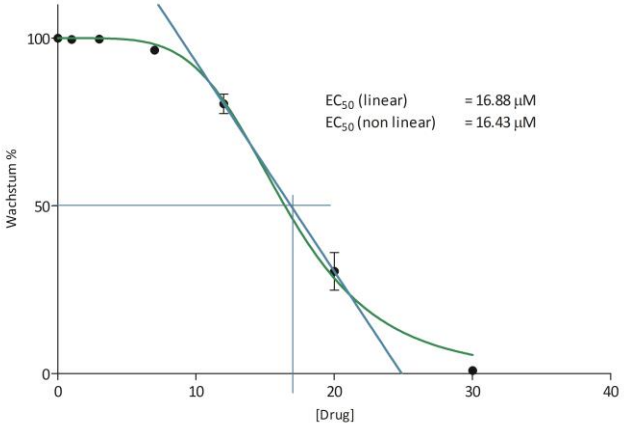
Das typische Zellschrumpfen kann mikroskopisch beobachtet werden, aber auch durch die Änderung in dem FSC-Signal (Forwardscatter-Signal) der FACS-Untersuchungen verfolgt werden.[178] Mittels Annexin V kann die Existenz von Phosphatidylserin auf der äußeren Zelloberfläche entweder qualitativ durch Fluoreszenz-mikroskopische Untersuchungen oder quantitativ durch FACS-vermittelte Analysen bestimmt werden. Die durch ICAD initiierte Fragmentierung der DNA kann entweder elektrophoretisch oder ebenfalls durch eine FACS-Analyse belegt werden.[152] Auf die quantitative Untersuchung der DNA-Fragmentierung *via* FACS-Analyse wurde aufgrund der hohen Fehlerbelastung verzichtet. Neben der problematischen Unterscheidung zwischen Fragmenten (Debris) und kleinen apoptotischen Zellen ist ferner zu berücksichtigen, dass wenn DNA-Stränge tetraploidaler Zellen degeneriert werden, die verkürzten DNA-Stücke zunächst zu einer falsch positiven Anreicherung der S- und G1/G0 Phase führen würden.[177,179] Auch hat der Zellwaschprozess einen wesentlichen Einfluss auf das Analysenergebnis, und es ist zu beachten, dass die Färbedauer wie auch die Farbstoffkonzentration je Zelle einen signifikanten Einfluss auf die Analyse besitzt. Da zum Nachweis für apoptotische Prozesse neben den qualitativen (morphologische Änderung, AO/PI-Assay sowie DNA-Fragmentierung) der Annexin-V Assay einen guten quantitativen Nachweis darstellt, wurde im Rahmen dieser Arbeit darauf verzichtet den DNA-Fragmentierungsassay als quantifizierenden Apoptose-Nachweis zu nutzen.

Mittels FACS-Analytik kann auch die Aktivität der Caspasen bestimmt werden, was gegenüber der auf Kolorimetrie-basierenden Methode den Vorteil hat, dass die Proteine nicht isoliert werden müssen, sondern die Zellpopulation als solches ohne weitere Aufarbeitung analysiert werden kann. Die einzelnen Methoden sind mit Ihren Vor- und Nachteilen in dem publizierten Ergebnisteil diskutiert.

3.4.2. Zytotoxizitätsassay

Um die Aktivität eines potentiellen Wirkstoffes *per se* festzustellen muss dieser zunächst auf seine prinzipielle Fähigkeit die maligne Zellpopulation zu verkleinern untersucht werden. In sogenannten *in vitro* Testungen werden dafür immortalisierte, maligne Zellkulturen für einen definierten Zeitraum mit variierenden Konzentrationen der zu untersuchenden Substanzen versetzt. Zur Detektion der Zellpopulation gibt es prinzipiell zwei grundlegend verschiedene kolorimetrische Methoden. Auf der einen Seite wird der Farbumschlag von Tetrazoliumsalzen durch NAD(P)-abhängige Oxidoreduktasen über eine OD-Messung ermittelt, um die relative Abnahme der Zellpopulation zu bestimmen. Prinzipiell beruht diese Methodik auf der Detektion der vitalen Zellatmung. Als Farbstoffe haben sich neben MTT[180] ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) und XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) sowie MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) etabliert. Der von MOSMANN[180] 1983 entwickelte MTT-Assay war bis zu den frühen 90iger Jahren der am weitesten verbreitete Chemosensitivitätsassay. Fehlende Linearitäten bei einer hohen Zelldichte, Unterschiede zwischen den verschiedenen Zelllinien in der Fähigkeit reduzierend zu wirken und größere Variationen von „intra- und interassay“ Art machten diesen Assay jedoch unattraktiv.[181]

Der vom US National Cancer Institute (NCI)[182] publizierte SRB-Assay basiert auf der proportionalen Bindung eines Rhodamin-Farbstoffes (Sulforhodamin B) an Proteine der Zellmembran. Da diese unabhängig von der Zellvitalität vorhanden sind, muss vor der Färbung eine Abtrennung der toten Zellpopulation erfolgen. Im Falle von adhärent wachsenden Zelllinien, wie sie in der vorliegenden Arbeit ausschließlich verwendet wurden, kann dies recht einfach realisiert werden; tote Zellen lösen sich von der Zellkulturplatte und schwimmen im Medium; durch ein Verwerfen des überstehenden Mediums werden sie entfernt. Da dies ein rein mechanisches Verfahren darstellt, ist hier eine der größten Schwachstellen der Methode zu finden; Falsch negative Ergebnisse können durch die Färbung von Zellfragmenten und Totenzellen hervorgerufen werden, wenn diese nicht hinreichend gut abgetrennt werden. Dennoch ist der SRB-Assay aufgrund seiner höheren Sensitivität und besseren Linearität als Methode der Wahl anzusehen.[181]



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Als direktes Ergebnis des SRB-Assays erhält man die relative Wachstumsrate der Zellpopulation nach 96ig stündiger Inkubation. Diese aufgetragen gegen die Konzentration ergibt eine Dosis-Wirkungs-Beziehung, welche durch einen sigmoidalen Verlauf zu charakterisieren ist (Abbildung 33). Zur statistisch abgesicherten Bestimmung des EC_{50} bzw. des IC_{50} Wertes werden mindestens drei unabhängige Messungen benötigt, wobei jede einzelne als Triplikat aufzunehmen ist, um „technische“ bedingte Abweichungen (Fehler des Messgerätes, Wäagefehler, Pipettierfehler, etc.) zu mitteln. Die drei so gemittelten Wachstumsraten können entweder durch eine lineare Auftragung und die Fällung des Lotes bei entsprechenden 50%igen Wachstum oder über eine non-lineare Regression (blauer Graph) bestimmt werden (vgl. grüne Graphen in Abbildung 33). Wie zu sehen ist, unterscheiden sich die Ergebnisse der Berechnungen nicht, oder nur marginal. Da der „wahre“ EC_{50}-Wert nicht bekannt ist, kann keine absolute Aussage zur Güte der einzelnen Berechnungsmethoden getätigt werden. Alternativ wurde daher ein Datensatz mit 50 verschiedenen, simulierten Datensätzen unter Verwendung der Monte Carlo Simulation generiert. Definiert wurden die Variablen Top, Botton, $logEC_{50}$ und Hill-Slope wie in Abbildung 34 rechts dargestellt. Es gilt als allgemeinhin angenommen, dass die pharmakodynamische Beschreibung eines Wirkstoffes auf ein System diesem Verlauf folgt, wenn das Massenerhaltungsgesetz eingehalten wird.[183] Die Datensätze wurden mit einer relativen, Gauß-Verteilung von 10% erstellt. Für den Fit nach der graphischen, linearen Methode wurden alle Werte bis auf den über und direkt unter 50% Wachstum liegenden entfernt und die erhaltenen Daten jeweils mittels eines non-linearen Ansatzes (y = mx+n) gefittet.

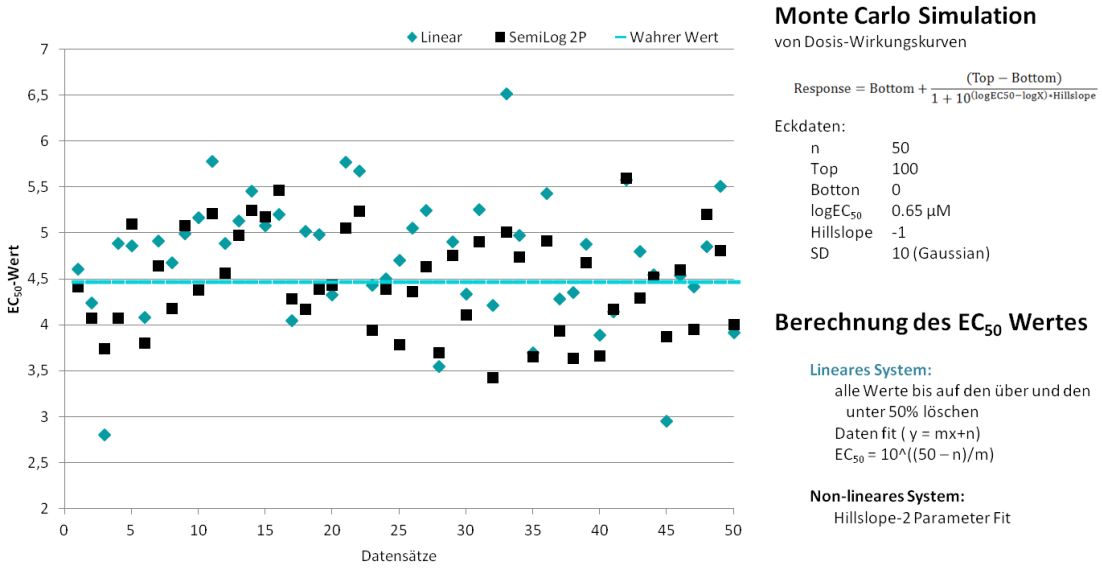


Abbildung 34: *Ergebnisverteilung der EC_{50}-Berechnung simulierter Datensätze*

Die Darstellung (Abbildung 34) belegt die aufgestellte These, dass sich die Ergebnisse nicht signifikant unterscheiden. Die Auswertung mittels einer non-linearen Gleichung ist der linearen dennoch vorzuziehen, da die Evaluation der Güte des berechneten Wertes essenziell für anschließende Dateninterpretationen ist. Auch wenn über eine Mittelung der drei linear bestimmten EC_{50}-Werte eine Aussage zur Verteilung gemacht werden kann, ist diese Standardabweichung kritisch zu betrachten, da der Kurvenverlauf und mögliche Fehler in diesem nicht berücksichtigt werden. Bei der zu bevorzugenden non-linearen Regression werden alle Datenpunkte verwendet. Zu beachten ist, dass die berechneten SE aus der non-linearen Regression nur approximiert sind und somit keine, auf diesen Werten aufbauenden, statistischen Bestimmung durchgeführt werden dürfen.[184] Beeinflussende Effekte auf die SE haben die, sich über die Sum of Square (SS) charakterisierenden, Abweichungen der Kurve, die Anzahl der Datenpunkte, die ausgewählten X-Werte und die Anzahl der zu fittenden Parameter. MOTULSKY und CHRISTOPOULOS[184] zeigen ferner, dass es für die vollständige Angabe des SE notwendig ist, den Logarithmus der Konzentration anstatt die Konzentration als solches zu verwenden. Die Generierung eines aus 5000 einzelnen Datensätzen bestehenden Systems, mit den in Abbildung 35 dargestellten Konstanten und einer Gauß-Verteilung (Standartabweichung 15), belegt diese Aussage: Ist der zu berechnende IC_{50} oder EC_{50} gering, so werden für die zu fittende Größe auch negative Werte berechnet, was unlogisch ist und damit falsche Werte ergibt. Dies im Umkehrschluss führt zu einem nicht verwertbaren Vertrauensintervall, was zusätzlich durch ein Versagen des p-Test angezeigt wird. Aus diesen Gründen sollte eine semi-logarithmische Auftragung verwendet werden. Die Angabe des Fehlerwertes erfolgt dann anschließend im Vertrauensintervall;[184] Angaben mit SE sind an dieser Stelle wiederum nur möglich wenn drei unabhängige EC_{50} Werte nach der Berechnung gemittelt werden.

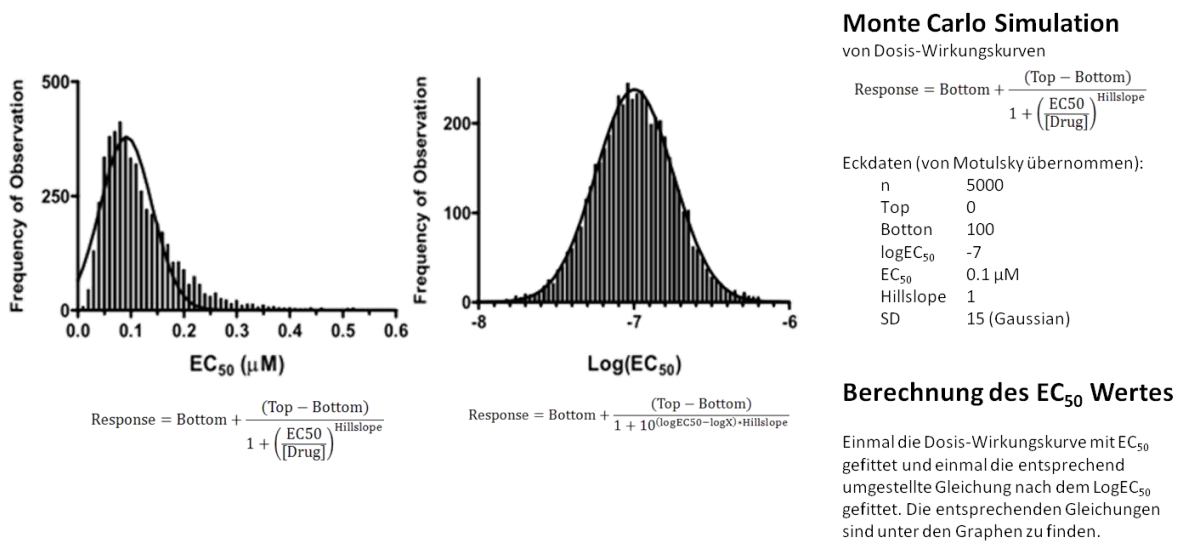


Abbildung 35: *Vergleich zwischen dem logarithmischen und dem nichtlogarithmischen Fit-Verfahren nach Motulsky[184]*

Das Vertrauensintervall, welches als Bereich zwischen $BestFit- t^* \cdot SE = CI = BestFit + t^* \cdot SE$ definiert wird, gibt an wo sich der wahre Kurvenverlauf befinden wird. Die Studentische t- Verteilung (in der Gleichung t^*) ist abhängig von dem Freiheitsgrad (d*f*), welcher durch die Differenz zwischen den Datenpunkten und den zu fittenden Variable definiert wird, und der gewünschten Präzision. Wird durch die Definition zweier, der im speziellen Fall insgesamt vier, zu fittenden Parameter der Freiheitsgrad erhöht, verringert sich daher das Konfidenzintervall. Die Auftragung relativer Wachstumswerte auf der Ordinate legitimiert die Definition der besprochenen Variablen (Bottom = 0, Top = 100) bzw. der Spanne und zusätzlich wird dadurch ein möglicher systematischer Fehler vermieden. Bei der Berechnung des EC_{50}-Wertes wird die obere, wie auch die untere Grenze gefittet und daraus die Spanne gebildet wird, in welcher sich der gewünschte EC_{50} Wert befindet. Der wiederum ist definiert als die Konzentration, bei welcher ein halbmaximaler Effekt beobachtet wird. Sind Datensätze aufgenommen wurden, in welchen die Zellpopulation nicht zu 100% beeinträchtigt wurde sondern bspw. nur zu 30% wird im Umkehrschluss der eigentliche EC_{65}-Wert berechnet (Vgl. Abbildung 36).

log (Konz)	Fall A	Fall B
-1,0	100	100
0,5	80	75
1,0	60	60
1,3	**50**	**50**
1,6	40	40
1,8	20	35
2,0	15	30
3,0	0	30
5,0	0	30

Berechnete Werte via 4P Hill	
EC_{50} (Fall A) =	1,3
EC_{50} (Fall B) =	0,8

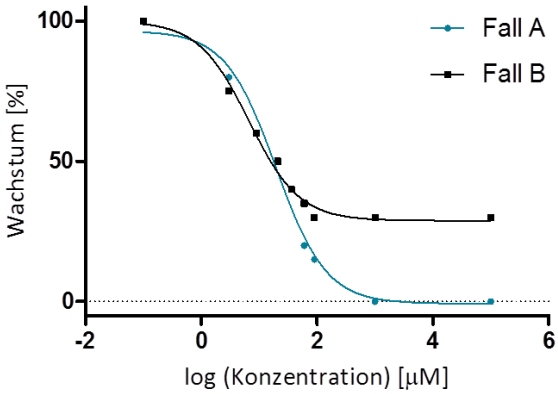


Abbildung 36: *Darstellung zur EC_{50} Berechnung. Zur Verdeutlichung des systematischen Fehlers, welcher bei der vier parametrischen Hill-Berechnung entstehen kann wurden fiktive Daten verwendet.*

Ferner ist die Bezeichnung des berechneten Wertes als EC_{50}-Wert (mittlere effektive Konzentration) der Bezeichnung als IC_{50}–Wert (mittlere inhibitorische Konzentration) vorzuziehen, da aus dem SRB-Assay *per se* keine Aussage über den Wirkmechanismus zu generieren ist. Die Verwendung des Kürzels IC_{50} könnte implizieren, dass ein zellwachstumsinhibitorischer Effekt antagonistisch ausgelöst worden ist, im SRB-Assay als solches wird aber lediglich die Größe der Zellpopulation nach 96h bestimmt und in Relation zu der unbehandelten Kontrolle gesetzt. Ob die gemessen Dezimierung der Populationsgröße auf einen letalen agonistischen oder antagonistischen Effekt zurückgeführt werden kann oder ob die Population unter der Behandlung nicht wachstumsfähig ist, kann nicht bestimmt werden, da weder die tote Population in Betracht gezogen wird, noch die Lebende auf vollständige Vitalität hin überprüft wird. Die Fähigkeit lebendiger Zellen adhärent zuwachsen wird ausschließlich

als differenzierendes Merkmal verwendet, weswegen die Bezeichnung als effektive Konzentration am geeignetsten erscheint.

3.4.3. Selektivität

Die synthetisierten, wie auch extrahierten Verbindungen wurden hinsichtlich verschiedener, humaner Krebszelllinien getestet. In Tabelle 2 sind die Namen der verschiedenen Zelllinien sowie deren Ursprungsgewebe zusammengefasst. Um zu testen, ob die Strukturen selektiv auf malignes neoplastisches Gewebe wirken oder ob sie eher unselektiv auch gesundes Gewebe im Wachstum inhibieren bzw. eine Form des PCD auslösen, wurde im Routine Assay zusätzlich die non-maligne Maus-Fibroblasten Zelllinie NiH 3T3 verwendet. Die 1963 etablierte, immortalisierte Zelllinie kann dazu dienen Tendenzen abzulesen.[185] Es ist jedoch zu beachten, dass es sich zu einem um eine Zelllinie, also eine genetisch veränderte Form handelt und zusätzlich, dass das ursprüngliche Gewebe aus embryonalem Albino-Mäusen gewonnen wurde. Trotz dieser zwei einschränkenden Faktoren ist diese Zelllinie als negativ-Kontrolle sehr vorteilhaft; Sie kann in Kultur analog zu den malignen Zelllinien gehalten und in den Assays als Indikator verwendet werden, was primär ökonomische Vorteile in sich birgt.

Name	Ursprung
518A2	Melanom
8505C	Schilddrüse
A253	Kopf- und Nackenepithel
A549	Lunge
A2780	Eierstöcke
DLD-1	Dickdarm
Liposarcoma	Weichteilgewebe
MCF-7	Brust
HT29	Dickdarm
SW1736	Schilddrüse

Tabelle 3: *Verwendete humane Zelllinien mit Angabe des ursprünglichen Gewebes*

Durch die Verwendung von humanen, primären Fibroblasten (WWW030272) konnte für verschiedene Hits die Selektivität zusätzlich validiert werden. Im Gegensatz zur Zelllinie sind primär Kulturen analog der Hayflick-Grenze nur eine bestimmte Zeitspanne zur Zellteilung fähig und somit ein besseres Modell um Einschätzungen über die Toxizität abgeben zu können. Von zusätzlichem Vorteil ist, dass diese Zellen aus menschlichem Gewebe stammen.

3.4.4. Explizite Betrachtung der Antitumor-Wirkung von Maslinsäure

Im Jahr 1989 wird durch den Arbeitskreis um NUMATA *et al.*[186] erstmalig die Antitumoraktivität der Maslinsäure erwähnt. Diese Säure konnte neben anderen Triterpensäuren aus den Exkrementen des Milne-Edwards Streifenhörnchens (*Trogopterus xanthipes*) isoliert werden. Mittels einer lymphozytischen Leukemie Zellkultur (P-388) wurde der, zuvor durch KOSUGE *et al.*[187] in vivo nachgewiesene, antitumorale Effekt der Chinesischen Medizin Goresishi näher klassifiziert. Es stellte sich dabei heraus, dass u.a. Oleanolsäure und Euscaphsäure einen signifikanten Einfluss innehalten, der Maslinsäure hingegen wurde nur ein sekundärer Einfluss auf das Wirkspektrum des Naturheilmittels zugesprochen.[186] Erst siebzehn Jahre nach dieser Veröffentlichung findet die Antitumor-Aktivität der Maslinsäure wieder in der Literatur Beachtung. Im Jahre 2006 publizieren zwei spanische Arbeitskreise unabhängig voneinander Studien zu den aus Oliven gewonnenen Triterpensäuren Maslinsäure und Oleanolsäure.[40b,41a] Evident durch beide Arbeiten wird, dass in Konzentrationen über 50 µM und nach mindestens 24h in Darmkrebszelllinien morphologische Veränderungen beobachtbar sind, die den typischen Merkmalen der Apoptose entsprechen. In den darauffolgenden Jahren erscheinen die Ergebnisse weitere Studien, die versuchen den Apoptose induzierenden Wirkmechanismus der Maslinsäure aufzuklären. Insgesamt sind sieben wesentliche Arbeiten in dem Zeitraum von 2006 bis 2011 bezüglich der speziellen Fragestellung zu beachten,[40-41,188] von denen sich vier mit einer Darmkrebszelllinie (HT29) beschäftigen.[40a,41] Die primär scheinbare Vergleichbarkeit ist jedoch bei einer expliziten Betrachtung der Messbedingungen nicht gegeben. Unabhängig von unterschiedlich eingesetzten Konzentrationen, unterschiedliche mittlere inhibitorischen Konzentrationen wurden zusätzlich unterschiedliche Zeitpunkte verwendet, was subsummiert einen zweifelsfreien Vergleich negiert.

Unabhängig davon lässt sich allgemeinhin festhalten, dass MA in der Lage ist den apoptotischen Tod auszulösen. In einem signifikanten Maßstab wird dies jedoch erst nach einer längeren Inkubationszeit (min. 48h) beobachtet. Ferner kann als allgemeingültig angenommen werden, dass der Prozess Caspasen-abhängig verläuft. Tabelle 4 fasst die unterschiedlichen Studienergebnisse bzgl. der Darmkrebszelllinie HT29 mit Messbedingungen zusammen. Eindeutig ersichtlich wird bei der Betrachtung, dass die Aktivierung der Effektor-Caspase 3 Zeit- sowie Dosis-abhängig verläuft.

Eine zusätzliche Analyse des Aktivierungsgrades der Initiatorcaspasen durch REYES-ZURITA und Kollegen[41b,c] lässt diese darauf schließen, dass der initiierende Schritt vermeintlich intrinsisch erfolgt. Als weitere Indizien werden die erhöhte messbare Konzentration an Cytochrom c, die erhöhte Expression des proapototischen Faktors Bax sowie der verringerte Anteil an Bcl-2 angeführt.

HT29		[MA]	4h	6h	12h	24h	36h	48h	72h
F. Reyes-Zurita 2009	Caspase 9	IC50 (28.5)	minimal	39 %	~ 0 %	200 %	n.D	n.D	n.D
		IC80 (37.5)	↓	28 %	~ 0 %	300 %	n.D	n.D	n.D
	Caspase 3	IC50 (28.5)	↓	119 %	75 %	50 %	n.D	n.D	n.D
		IC80 (37.5)	↓	220 %	210 %	200 %	n.D	n.D	n.D
	Caspase 8	IC50 (28.5)	~ 0 %	~ 0 %	~ 0 %	~ 0 %	n.D	n.D	n.D
		IC80 (37.5)	~ 0 %	~ 0 %	~ 0 %	~ 0 %	n.D	n.D	n.D
E. Juan, 2008	Caspase 3	150 yM	~ 0 %	~ 0 %	20%	1400%	1400%	1400%	n.D
		250 yM	~ 0 %	~ 0 %	30%	60000%	1500%	1000%	n.D.
E. Juan, 2006	Caspase 3	150 yM	~ 0 %	~ 0 %	activated	400 %	600 %	600 %	n.D.
		250 yM	~ 0 %	~ 0 %	activated	750 %	1400 %	18000 %	n.D.
F. Reyes-Zurita 2011	Caspase 9	61 yM	n.D.	n.D.	n.D.	n.D.	n.D.	380 %	420 %
		76 yM	n.D.	n.D.	n.D.	n.D.	n.D.	480 %	500 %
	Caspase 3	61 yM	n.D.	n.D.	n.D.	n.D.	n.D.	400 %	500 %
		76 yM	n.D.	n.D.	n.D.	n.D.	n.D.	460 %	1100 %
	Caspase 7	61 yM	n.D.	n.D.	n.D.	n.D.	n.D.	200 %	2000 %
		76 yM	n.D.	n.D.	n.D.	n.D.	n.D.	200 %	4500 %
	Caspase 8	61 yM	n.D.	n.D.	n.D.	n.D.	n.D.	160 %	160 %
		76 yM	n.D.	n.D.	n.D.	n.D.	n.D.	220 %	1000 %

Tabelle 4: *Zusammenfassung der gemessen Caspasensktivität. Farblich hervorgerufene Werte sind Diagrammen entnommen. n.D. nicht detektiert*

Werden die Ergebnisse jedoch im Detail kritisch betrachtet, so fällt auf, dass die relative Aktivierung der Caspase 9 zum gleichen Zeitpunkt geringer ausfällt als selbige der Caspase 3. Auch die Cytochrom c Konzentration erreicht erst nach der erhöhten Aktivität von Caspase 3 einen signifikant gestiegenen Wert. Ähnliches ist für die Antagonisten Bcl-2 und Bax zu vermerken, was subsummiert die Interpretation der Daten in Frage stellen lässt, da es eher auf einen ausschließlich Caspasen-abhängig exekutiven Prozess hindeutet und nicht auf einen zusätzlichen Caspasen-induzierten. Alternative Indizien für die Initiierung finden sich u.a. bei JUAN *et al.*[40a]. Sie zeigt die Aktivierung von ROS nach nur 4h und eine Inhibition der antitumoralen Wirkung von Maslinsäure durch Antioxidationen, was diese zur Behauptung eines ROS-vermittelten, Caspase-abhängigen Mechanismus bewegt. Dieser Befund ist jedoch durch die Verwendung einer sehr hohen Konzentration an Maslinsäure (150 µM) ebenfalls kritisch zu hinterfragen. Analoges ist für die Studien von HSUM *et al.*[189] anzumerken; bei über 100 µM Konzentration finden die Wissenschaftler einen halbmaximalen Inhibitionseffekt von COX und eine 70% Inhibition von NFκB durch Maslinsäure.[189]

Zweifelsfrei kristallisiert sich durch diese unterschiedlichen Befunde die Vermutung heraus, dass Maslinsäure in der Lage ist mehrere Mechanismen auszulösen. Hierfür spricht auch der zusätzlich nachgewiesene, induzierte Zellzyklusarrest und die JNK-Aktivierung, welche wiederrum indirekt die Mitochondrienmembran modelliert und somit die Caspasen-Kaskade initiiert.[41b]

Aufschlussreicher erscheint der primäre Widerspruch in der Wirkung, welcher in der Studie von REYES-ZURITA *et al.*[41a] aus dem Jahre 2006 vorgestellt wird. Der Arbeitskreis untersucht in jener neben der p53

mutierten Zelllinie eine p53-Wildtyp Darmkrebskultur (Caco-2). Für letztere führt die Behandlung mit Maslinsäure schon nach sechs Stunden zu signifikanten morphologischen und biochemischen Modulationen, wie z.B. dem frühzeitigen, signifikanten Anstieg an Cytochrom c, Zellschrumpfen und einem Ablösen aus dem Zellgefüge. In ihrer These schlussfolgern die Wissenschaftler, dass Maslinsäure Differenzierungen der Zelle in p53 abhängiger Art und Weise verursacht, respektive die Apoptose.

Wird der Betrachtungsblickwinkel auf die Studien um WU *et al.*[188b] und MARTIN *et al.*[188a] erweitert, welche Gehirnkrebszellen untersuchten, lassen sich interessante Erkenntnisse zur MOA gewinnen. MARTIN *et al.*[188a] zeigen eine sehr schnelle Wirkungsinduktion, welche schon nach dreißig Minuten zu signifikanten morphologischen Veränderungen führt. Diese wiederum werden auf exorbitante Veränderung des zellulären Filaments F-Aktin und des fehlenden Aktin-Netzwerks zurückgeführt. Die zusätzliche Untersuchung des Aktingehaltes zeigte jedoch eine nicht veränderte Gesamtkonzentration an selbigen, was auf einen Inhibierung des Aktinnetzwerkaufbaus hindeutet. Das Ergebnis der Caspase-3-Aktivität weißt nach 6h eine Steigerung um 10% auf. Dieses Ergebnis steht in guter Korrelation zu obigen These, welche die Apoptose auslösende Wirkung der Proteasen dementiert. In der besprochenen Studie wird die Induktion *via* ROS präferiert und durch eine Apoptose-inhibition mittels Co-Behandlung mit Antioxidantien belegt. Die explizite Betrachtung der experimentellen Rahmenbedingungen bedingt jedoch eine Sonderbehandlung dieser Ergebnisse; die Zellen wurden vor der Behandlung mit Maslinsäure für 24h in FKS freien Medium gehalten und anschließend mit 5% Medium und Maslinsäure versetzt. Das fetale Kalbsserum ist reich an diversen Wachstumsfaktoren, essentiellen Proteinen und wird in der Regel in zehn volumenprozentigen Medium zur Haltung von Zelllinien verwendet, wo durch die Wachstumsfaktoren verschiedene Zellmembranrezeptoren stimuliert werden. Diese Signale führen gerade bei Zellkulturlinien zur Proliferation. In Abstinenz solcher wird der G1/G0-Phase schneller induziert, bzw. die Proliferationszeit der Zelllinien erhöht.

Die Studie um WU *et al.*[188b] zeichnet sich vor allem durch die Verwendung von verschiedenen Inhibitoren zur Signifikanzuntersuchung der einzelnen, messbaren Proteingehaltsveränderungen aus. In der Studie selbst zeigen sie zunächst die Dosis-Zeitabhängige Wirkung von Maslinsäure, beobachten typische morphologische Veränderungen und einen Anstieg von intrazellulärem Calcium. Dieses kann entweder im Inneren der Zelle freigesetzt werden (ER) oder u.a. durch TRPV-Kanäle transportiert werden. Durch die Verwendung des intrazellulären Calcium Chelators BAPTA/AM konnte die zytotoxische Wirkung von Maslinsäure inhibiert werden, was mittels MTT und den nicht mehr beobachtbaren morphologischen Änderungen bewiesen wurde. Auch die Caspase 3 Aktivität konnte durch die Bindung der Ionen inhibiert werden, was die Wissenschaftler zu dem Schluss kommen ließ, dass Maslinsäure Apoptose *via* Calciumüberladung induziert und somit dem MAPK-Signalweg entspricht. Die zur Zeit-proportionalen Anstiege an ERK und p38 untermauern diese These. Die

Verwendung des selektiven p38 MAPK Inhibitors SB203580 zeigte ferner, dass die Caspasenaktivität nahezu vollständig blockiert werden kann (nach 24h). Die Abhängigkeit des Faktors zu intrazellulärem Calcium konnte durch die Verwendung von BAPTA belegt werden, der Anstieg der Calciumionenkonzentration hingegen ist unabhängig von p38 MAPK. Um zu testen woher das Calcium kommt wurden weitere Calciumioneninhibitoren verwendet; ein extrazellulärer Chelator und ein unspezifischer TRPV-Kanalinhibitor (RR). Die Ergebnisse beweisen, dass die Calcium-Ionen von außen durch die Kanäle in die Zelle gelangen. Bemerkenswert sind ferner noch zwei Befunde; zum einen der nicht signifikante Einfluss von ERK (die Verwendung eines Inhibitors hat keinen Einfluss auf die Zytotoxizitätswirkung von Maslinsäure, dafür jedoch auf die der Caspasen) und zum anderen, dass der p38 MAPK Inhibitor die Caspasenaktivität inhibiert aber nur zum Teil die Zytotoxizität aufhebt (80% Lebende Zelle nach Behandlung mit der IC50 Konzentration Maslinsäure). Letzteres belegt zusätzlich, dass neben dieser MOA ein weiterer Mechanismus in der Zelle durch Maslinsäure ausgelöst werden kann. Kritisch anzumerken ist noch, dass die Caspasenaktivität nur zu einem Zeitpunkt (24h) bestimmt wird, sodass nicht ausgeschlossen ist, dass die Zelle auch unter der Inhibierung von p38 MAPK einem Caspase-abhängigen Zelltod erfährt. Dieser würde dann analog zu den Erkenntnissen aus der HT29 Zellkultur zu einem sehr späten Zeitpunkt Caspasen vermittelt erfolgen (Vgl. Tabelle; die höchste Caspasenaktivität findet sich erst nach 36h).

Der Vergleich des genetischen Profils zwischen den unterschiedlichen Zelllinien lässt an dieser Stelle ein weiteres Indiz auffinden. Die sehr schnell sterbenden Zelllinien ACC und Caco zeichnen sich gemeinsam dadurch aus, dass sie keine Mutation des p53 Gens besitzen. Bei der Darmzelllinie HT29 hingegen findet man eine p53 Mutation unter gleichzeitiger, nahezu vollständig fehlender transkriptionalen Aktivität gegenüber dem WAF1-Promotor (ca. 1% im prozentualen Vergleich zum Wildtyp). Dies führt dazu, dass das Zellwachstumsregulierende Protein p21 in dieser Zelllinie nicht durch p53 exprimiert wird. Für eine Umfangreiche Listung der p53 Mutationen und eine Sammlung des charakteristischen Genprofils der meisten Zelllinien vergleiche Edlund et al. [190] sowie die dort aufgeführte Zitierung der p53 Datenbank http://p53/free.fr.

Subsummiert lässt sich aus diesen Betrachtungen schließen, dass es zu einer späten Caspaseninduktion durch Maslinsäure in geringen Konzentrationen kommt, der Effekt jedoch auch Konzentrationsabhängig beschleunigbar ist. Ferner lassen sich die, in frühen Zeitphasen gemessen, Caspasen-Aktivitäten nicht linear korrelieren, was den Schluss erzwingt, dass keine Caspasen-induzierte Apoptose vorherrschend ist, sondern der initiierende Einfluss der Proteasenklasse putativ eher effektiv ist. Auch lässt sich aus den Analysen schließen, dass Maslinsäure verstärkt zytostatisch bzw. antimetastatisch oder antitumorgenetisch wirkt. Dieser Rückschluss wird durch Untersuchungen bestätigt, welche nach 2011 publiziert wurden. Der Arbeitskreis um PARK[191] zeigt, dass Maslinsäure

sowohl die basale als auch die endothermal induzierte Migration inhibiert. Ergebnisse von MOOI und Kollegen[192] untermauern diesen pharmakologischen Befund *per se*, im Detail weisen sie aber auf eine verminderte PKC-Konzentration hin. PARK et al.[191] führen die Inhibierung von HIF1α, AKT und ERK als Ursache an und zeigen, dass es zu einer späten mitochondrialen Apoptose mit mitotischem Einfluss kommt.

Erkenntnisse aus den unterschiedlichen Arbeiten sind in Abbildung 37 graphisch veranschaulicht. Für ein signifikantes Target in Form eines zu inhibierenden Enzym gibt es bisher noch keinen Beweis (in der Graphik wird die Aktivierung in diesem Falle durch ein „?“ symbolisiert). Die verstärkte Aktivität des Calciumionen-Kanals[188b] und die zusätzliche Inhibierung der PKC-Aktivierung[192] durch Maslinsäure deuten darauf hin, dass das „Ur-Signal“ der Maslinsäure in der Membran ausgelöst wird.

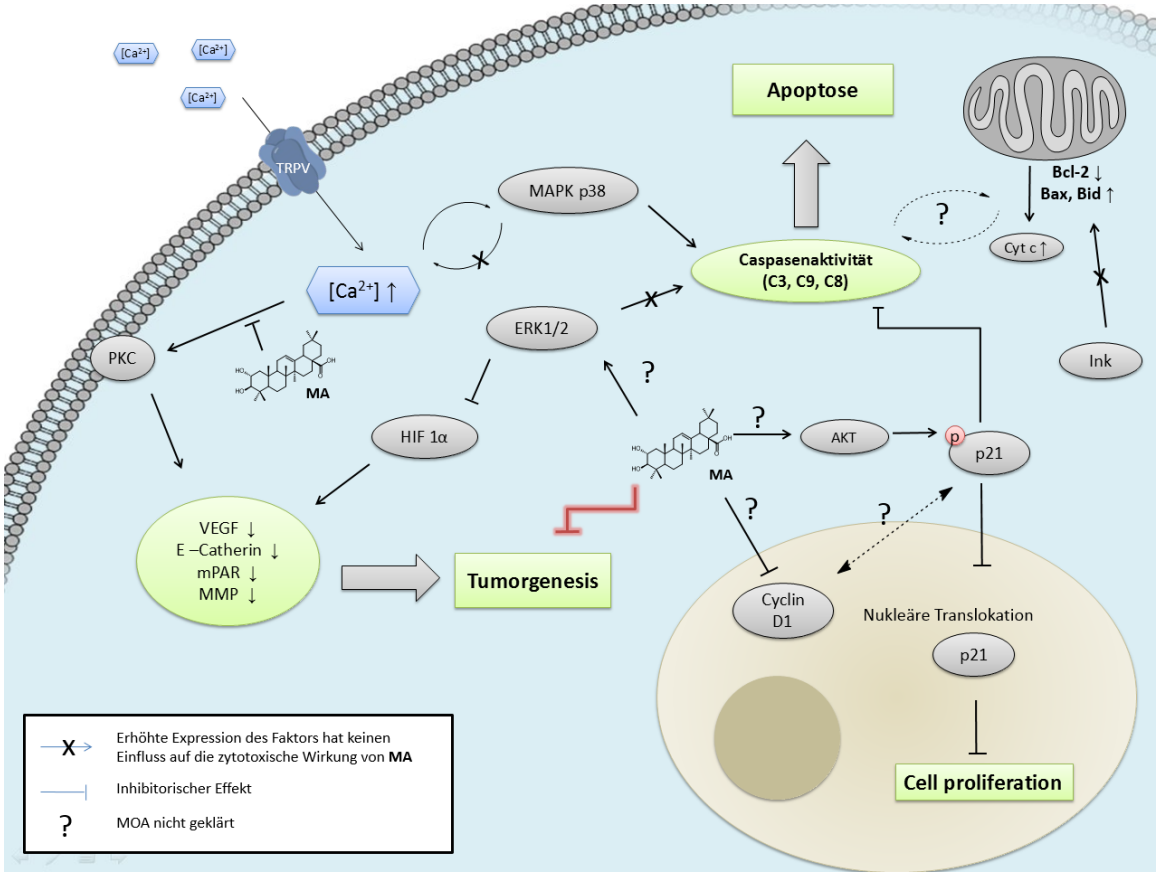


Abbildung 37: *Schematische Zusammenfassung der putativen Wirkung von **MA***

Aktuelle Studien zur Toxikologie der Maslinsäure belegen die non-toxische Wirkung. Weder die einmalige Gabe von 1000 mg/Kg noch die Verabreichung von 50 mg/Kg über einen Zeitraum von 28 Tagen führte zu toxischen Effekten.[193] Körpergewicht, biochemische, wie auch hämatologische Variablen werden durch die Behandlung nicht beeinflusst. Eine Studie von YIN und Kollegen zeigten in einem Mausexperiment die Anreicherung der Säuren in der Leber nach vier bis acht wöchiger Einnahme von 500 mg Maslinsäure.[194]

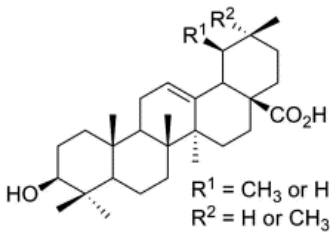
Ergebnisse und Diskussion

Die im Rahmen dieser Dissertation entstandenen Ergebnisse sind in folgenden Publikationen und Manuskripten veröffentlicht bzw. eingereicht.

4.1. A convenient separation of ursolic and oleanolic acid. .

Csuk, R. and B. Siewert *Tetrahedron Lett.* **2011**, *52* (49), 6616-6618.

Graphical abstract:



Abstract:

A convenient route has been developed to separate regioisomeric ursolic and oleanolic acid by treating the mixture with mCPBA or formic acid/hydrogen peroxide.

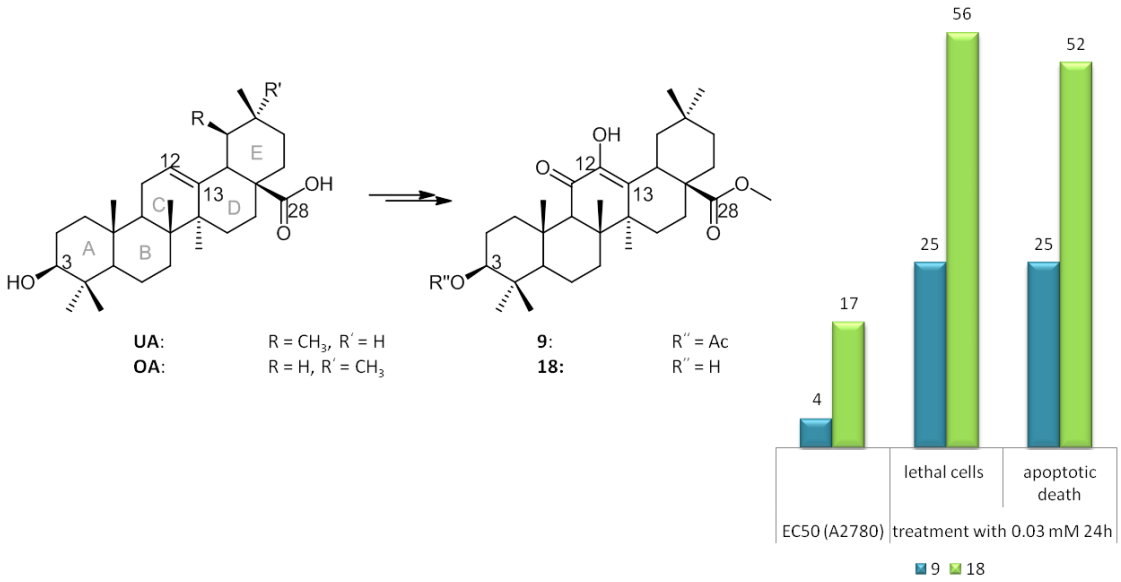
Keywords:

Ursolic acid; Oleanolic acid; Separation

4.2. The chemical and biological potential of C ring modified triterpenoids. .

Bianka Siewert, Jana Wiemann, Alexander Köwitsch, René Csuk, *Eur J Med. Chem.,* **2014**, *72*, 84-101.

Graphical abstract:



Abstract:

A convenient and elegant route has been developed to separate the natural regioisomers triterpenoids ursolic acid (UA) and oleanolic acid (OA) as well as derivatives thereof. Eleven unknown derivatives of OA were designed, synthesized and biologically investigated for their cytotoxicity. Further sixteen compounds were prepared to investigate their biological profile and to correlate all compounds in a SAR study. As a result, C-ring modifications of OA and UA have only a moderate influence onto the anti-cancer activity of the compounds. The presence of this structural element, however, has a significant impact onto the ability to trigger apoptosis in ovarian cancer cells (cell line A2780).

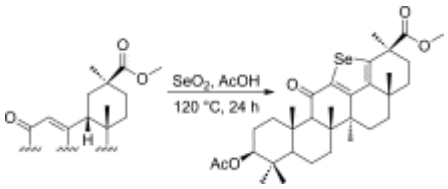
Keywords:

oleanolic acid acid, ursolic acid, triterpenoids, antitumor activity, apoptosis, structure-activity relationships

4.3. A bioassay-driven discovery of an unexpected selenophene and its cytotoxicity. .

Csuk, R.; Siewert, B.; Wiemann, J; *Bioorg. Med. Chem. Lett*, **2013**, *23* (12), 3542-3546

Graphical abstract:



Abstract:

During the reaction of methyl 3beta-acetoxy-glycyrrhetinate (1) with SeO2 significant amounts of a cytotoxic hitherto unprecedented triterpenoic selenophene 3 are formed. This compound stops cell proliferation and acts by apoptosis.

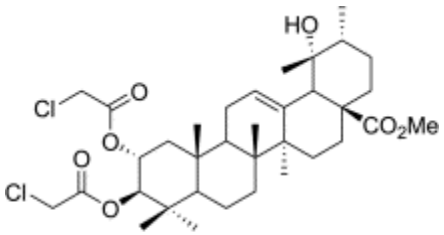
Keywords:

Glycyrrhetinate; SeO_2 oxidation; Selenophene; Cytotoxic; Apoptosis

4.4. Tormentic acid derivatives: Synthesis and apoptotic activity.

Csuk, R., Siewert, B. Dressel, C., Schäfer, R. *Eur. J. Med. Chem.*, **2012**, *56*, 237-245

Graphical abstract:



IC_{50} = 0.8 µmol (A2780; human ovarian carcinoma)

Abstract:

Several derivs. of tormentic acid have been prepd. and tested for their antitumor activity. The dichloroacetate I is an excellent antitumor active agent acting by an apoptose inducing pathway as demonstrated by OA/PI staining, DNA laddering expts. as well as by an annexin V binding assay.

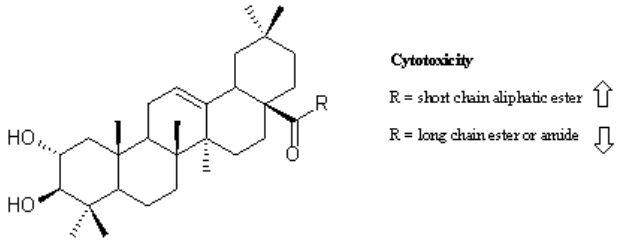
Keywords:

Tormentic acid; Antitumor activity; Apoptosis; Acridine orange/propidium iodide assay; Annexin V; DNA laddering

4.5. Esters and amides of maslinic acid trigger apoptosis in human tumor cells and alter their mode of action with respect to the substitution pattern at C-28. .

Bianka Siewert, Elke Pianowski, René Csuk*, *Eur J Med Chem*, **2013**, *70*, 259-272

Graphical abstract:



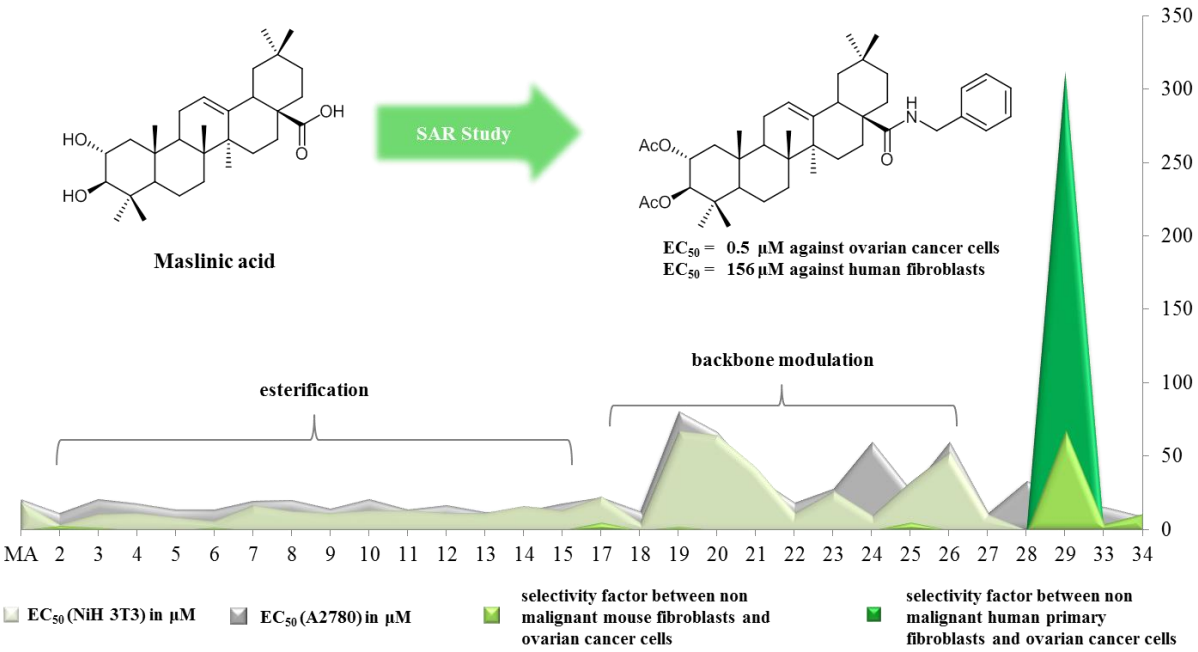
Abstract

Cancer is one of the most commonly diagnosed diseases worldwide; its mortality rate is high, and there is still a demand for the development of antitumor active drugs. Triterpenoic acids show many pharmacological effects, among them antitumor activity. One of these, maslinic acid (1) is of interest because of its antitumor profile. It is not only cytotoxic but also triggers apoptosis in various human tumor cell lines. To improve the cytotoxicity of parent 1 we set out to synthesize new derivatives of it – differing in structure and lipophilicity. These compounds were tested in a sulforhodamine B assay for cytotoxicity, and screened for their ability to induce apoptosis using an acridine orange/propidium iodide assay, DNA laddering and cell cycle experiments. A series of 30 different esters and amides was prepared and screened for their antitumor activity. Esters containing small-chain, lipophilic residues increase the cytotoxicity whereas amides as well long-chain esters led to a decrease in activity. The antitumor activity seems to be independent from the substitution pattern at position C-28 of parent maslinic acid for esters and amides but rather alters their mode of action.

4.6. Towards cytotoxic and selective derivatives of maslinic acid.

Bianka Siewert, Elke Pianowski, Anja Obernauer and René Csuk*, *Bioorg. Med. Chem*, **2014**, *22*, 594-615.

Graphical abstract:



Abstract:

Several novel esters and amides of maslinic acid were prepared and evaluated for their antitumor activity in a panel of human cancer cell lines using a sulforhodamine B (SRB) assay. Some of them showed a noteworthy antitumor activity. The results from annexinV-FITC and caspase-assays as well as from DNA laddering experiments provided evidence for an apoptotic cell death. One of these compounds (15) displayed an extraordinary cytotoxicity for tumor cells but a 300 times lower toxicity for non-malignant primary human fibroblasts. As parent maslinic acid, compound 15 induces a G1/G0 arrest in tumor cells.

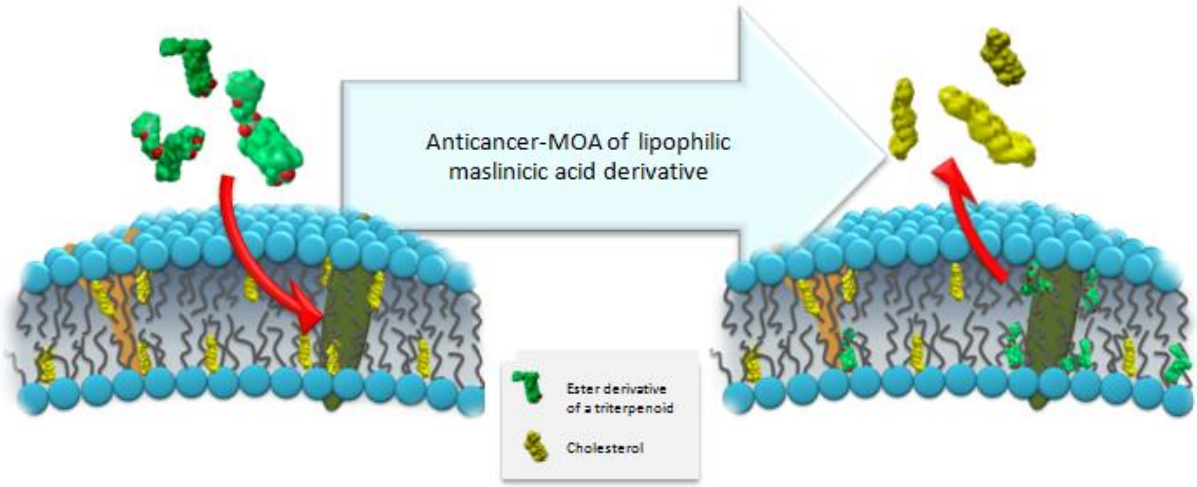
Keywords:

Maslinic acid; esters; cancer; SRB assay; antitumor; DNA laddering; annexin V assay; cell cycle investigations; apoptosis; caspase activity

4.7. Membrane damaging activity of a maslinic acid analogue.

Bianka Siewert, René Csuk*, *Eur. J. Med. Chem.*, **2014**, *74*, 1-6.

Graphical abstract:



Incubation of human ovarian cancer cells A2780 with **1** results in an incorporation of **1** into the cell's membrane followed by an extrusion of cholesterol from the lipid rafts. Crystals consisting of cholesterol and excess **1** are formed at the perimeter of the cell. As a consequence of the alterations of the cell membrane, a volume decrease is initiated that triggers apoptosis, thus extending previous models on apoptosis initiating mechanisms.

Abstract:

Close inspection of human ovarian cancer cells A2780 in the course of an antitumor screening using maslinic acid analogs revealed for one of the compounds, 4-oxa-4-phenyl-butyl 2,3-dihydroxy-olean-12-en-28-oate (**1**) an unusual behavior. During this incubation of the cells with **1** at the perimeter of the cells or close by crystals were formed consisting of cholesterol and excess **1**. Compound **1** is incorporated into the cell's membrane followed by an extrusion of cholesterol from the lipid rafts. As a consequence of the alterations of the cell membrane, a volume decrease is initiated that triggers apoptosis, thus extending previous models on apoptosis initiating mechanisms.

Keywords: maslinic acid, triterpenoids, antitumor activity, apoptosis, membrane modulation, cholesterol crystals

Zusammenfassung und Ausblick

Die Ergebnisse der vorliegenden Arbeit wurden in sieben Publikationen veröffentlicht. Die in der Zielstellung benannte Trennung der Konstitutionsisomere vom α- und β-Amyrin-Grundgerüst wird in der ersten Publikation (Kapitel 4.1) beschrieben und konnte in einer darauffolgenden ausgebaut werden (Kapitel 4.2). Die Verwendung von Selendioxid als Oxidationsmittel führte zu neuartigen, hochaktiven Leitstrukturen und, angewendet auf das System der Glycyrrhetinsäure, zu einer neuartigen, selenhaltigen Spezies (Kapitel 4.3). Am Grundgerüst der Tormentillsäure wurde in Kapitel 4.4 gezeigt, welchen profitablen Einfluss die Einführung von lipophilen Strukturen auf die biologische Aktivität ausübt. In den drei sich anschließenden Publikationen konnten, neben der, zur Aufgabe stehenden, Extraktion von Maslinsäure (Kapitel 4.5), durch den Aufbau einer systematischen Strukturdatenbank vielfältige Mehrwert generierende Erkenntnisse gewonnen werden (Kapitel 4.5 und 4.6). Neben umfangreichen SAR-Zusammenhängen konnte eine immens aktive und selektiv wirkende Verbindung synthetisiert, charakterisiert und umfassend biologisch evaluiert werden. Ferner konnte deduktiv erstmalig ein primäres Target in der Zelle beschrieben und damit der Wirkmechanismus für eine bestimmte Modifikationsklasse ergründet werden (Kapitel 4.7). Im Folgenden sollen nun die Einzelergebnisse genauer betrachtet werden und im globalen Wechselspiel evaluiert werden, was mit dem Aufzeigen zukünftig interessanter Forschungsgebieten einhergeht.

5.1. Chemoselektive Trennung.

Eine neuartige Trennung der natürlichen Kontsitutionsisomere Ursolsäure und Oleanolsäure konnte im Rahmen der vorliegenden Arbeit zugänglich gemacht werden (Vgl. 4.1). Analog zu dem Standardtrennverfahren von Lewis[195] aus dem Jahre 1983 erfolgte die Separation über eine elektrophile Addition an dem ungesättigten System im C-Ring. Die, durch die sterische Hinderung der terminalen Methylgruppe CH_3(19) im Falle der Ursolsäure hervorgerufene, erhöhte Aktivierungsenergie stellt die theoretische Grundlage der Trennung dar.

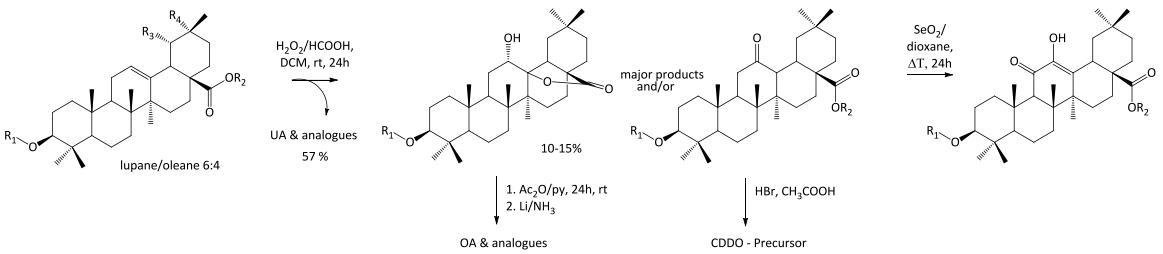


Abbildung 38: *Trennungsstrategie und die sich anschließenden Umsetzungsstrategien*

Bei milden Temperaturen wird durch die Umsetzung mit Persäuren nur das Oleangerüst angegriffen, das α-Amyrin Gerüst hingegen bleibt unverändert. Wie in Kapitel 4.2 dargestellt, können verschiedene Peroxide eingesetzt werden. Zusätzlich konnte gezeigt werden, dass die Trennstrategie bezüglich der zu trennenden Mischungen universal einsetzbar ist, da weitere, häufig als Schutzgruppen eingeführte funktionelle Gruppen nicht stören sind (Vgl. Kapitel 4.2). Die letzte Erkenntnis birgt aus naturstoffchemischer, als auch aus medizinisch-chemischer Sicht, mehrere Vorteile in sich, die im folgendem kurz betrachtet werden sollen. Durch mannigfaltig durchgeführte Extraktionen von, an Triterpensäuren reichen, Pflanzen ist allgemeinhin bekannt, dass die Säuren häufig glykosidisch gebunden vorkommen. Ferner zeigt sich, dass die terpenhaltigen Extrakte vermehrt aus Gemischen von Vertretern unterschiedlicher Triterpenklassen bestehen. Bei der Extraktion erschwert der amphipathische Charakter der Saponine die chromatographische Trennung, sodass eine Verseifung der Reinigung vorzugsweise vorangestellt wird. Auch ein Veresterungsschritt, welcher zusätzlich die Lipophilie erhöht und damit die Reinigung wesentlich vereinfacht, ist nicht ungewöhnlich in den verschiedenen Extraktionsprotokollen. Wird nun der hypothetische Fall betrachtet, dass in Folge solcher Verfahren je ein α-Amyrin und β-Amyrin Vertreter in dem, chromatographisch zu trennenden, Rohextrakt vorliegt, dann wird der generierte, universelle Mehrwert des, in Kapitel 4.1 vorgestellten und in Kapitel 4.2 validierten, Trennverfahrens ersichtlich (Vgl. Abbildung 39). Die kostengünstige und im Vergleich zur Literaturvariante, welche Brom verwendet, weniger gesundheitsschädigende Umsetzung mit Ameisensäure und Wasserstoffperoxid bei Raumtemperatur, könnte den Extraktionsprozess von α-Amyrin Abkömmlingen wesentlich vereinfachen. Zusätzlich kann die gleichzeitige Bildung von geeigneten Vorstufen der β-Amyrine für medizinisch-chemische Studien als Mehrwert angesehen werden.

Denn wie gezeigt werden (Kapitel 4.2) konnte, kann durch die Oxidation von verschiedenen Estergemischen, ein direkter Zugang zu einer vielversprechenden semi-synthetischen Klasse der Triterpensäuren, die Klasse der 12-Oxo-oleane, ermöglicht werden. Ferner zeigte die biologische Evaluierung von verschiedenen Laktonstrukturen, welche sich aus den freien Säuren bilden, sehr interessante SAR und äußerst vielversprechende biologische Verhaltensmuster, wie eine sehr effektive Apoptose-induktion nach 24 h (vgl. Abbildung 40).

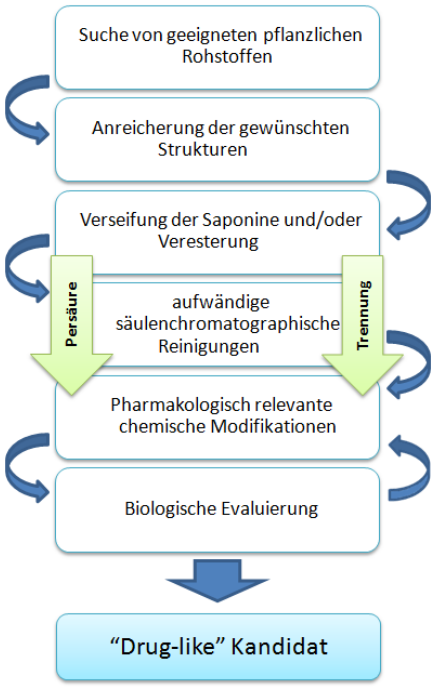


Abbildung 39: *Konzeptionelle Darstellung einer möglichen zusätzlichen Verwendung der Ergebnisse aus 5.1 und 5.2*

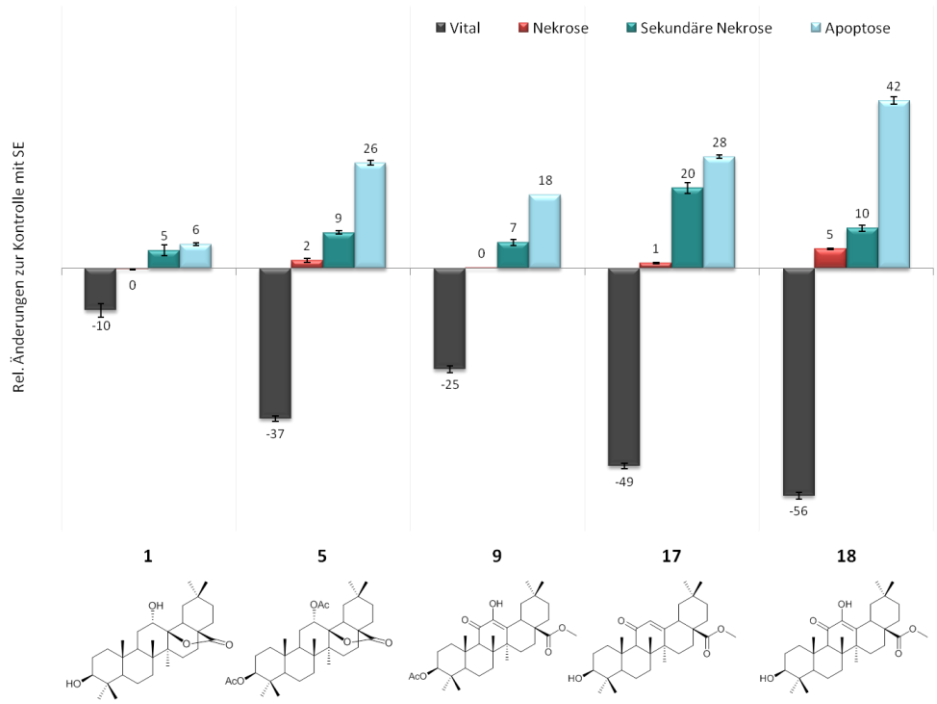


Abbildung 40: *Annexin V-PI Assay.*

Gebärmutterhals-Krebszellen wurden für 24h mit je 30 µM der indizierten Verbindungen behandelt. Das Ergebnis entspricht der relativen Verteilungsveränderung zur unbehandelten Kontrolle

Durch die Wiederentdeckung der Selendioxid-Chemie an β–Amyringerüsten konnte der Zugang zu einer neuartigen 11-Oxo-12-Hydroxy-en Strukturklasse erschlossen werden, welche in Abhängigkeit von den Modifikationen in Ring A und an Position 28 sehr vielversprechende Wirkungen gegenüber Krebszellen zeigt. (vgl. Abbildung 40 und 4.2).

5.2. Chemische und biologische Studien an der Glycyrrhetinsäure und der Tormentillsäure.

Im Rahmen einer betreuten Bachleorarbeit[196], welche sich mit Lakton-Bildungen am C-Ring der Glycyrrhetinsäure beschäftigte, konnte die Bildung einer Selenoorganometallverbindung zunächst NMR-spektroskopisch identifiziert und später massenspektroskopisch nachgewiesen werden. Diese Verbindung stellt für die Klasse der Triterpensäuren im Generellen eine der Ersten ihrer Art dar. Ausschließlich von der Betulonsäure ist ferner eine selenhaltige Verbindung bekannt, von der jedoch keine biologischen Daten existieren.[197] Umfangreichste Studien zu Selendioxid vermittelten Oxidationsreaktion an Triterpengerüsten gehen auf PRADHAN[197-198] in den späten Achtzigern zurück. Zusätzlich konnte der in 4.3 beschriebenen Verbindung und der Klasse als solches erstmalig eine Antitumor-Aktivität nachgewiesen werden und gezeigt werden, dass durch die Einführung von Selen die profitable Apoptose-induzierende Wirkung der Glycyrrhethinsäure erhalten bleibt. Aus SAR-Sicht ist die Struktur für zukünftige Untersuchungen sehr interessant, da durch die Einführung des Selenophen-Strukturelements die 3D-Struktur der Glycyrrhetinsäure modifiziert wurde (Vgl. Abbildung 41)

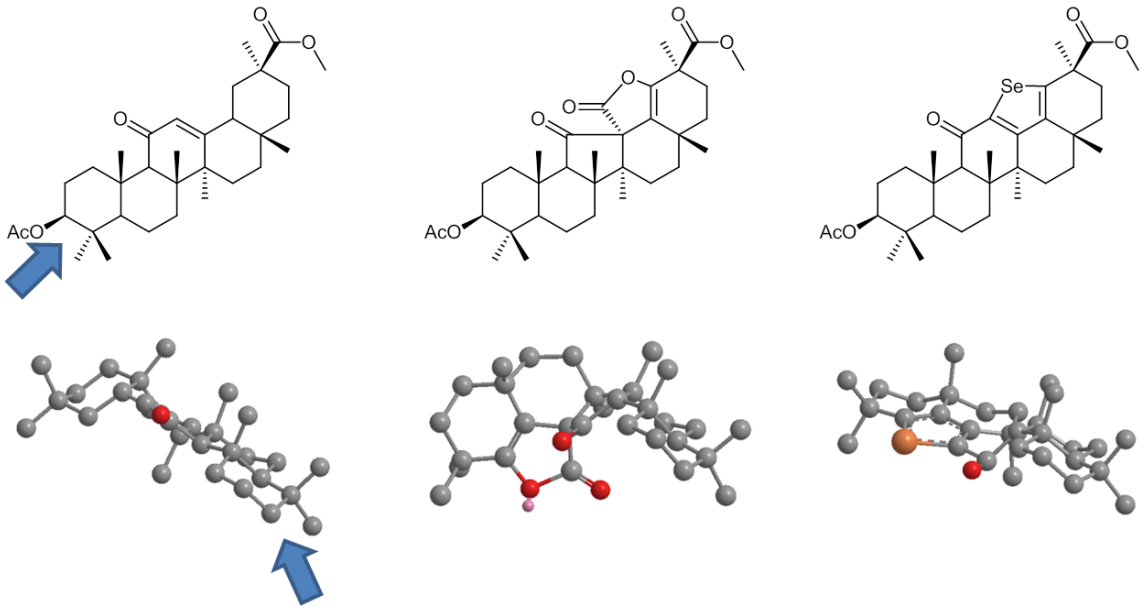


Abbildung 41: *Vergleich der 3D-Struktur der Verbindungen aus P3 (Kapitel 4.3). Zu besseren Übersichtlichkeit sind die Esterfunktion an 3 und 30 in der 3D Struktur unsichtbar. Die Berechnung erfolgte mittels MM2-Berechnung (ChemDraw). Der blaue Pfeil gibt den Blickwinkel an.*

Wie die biologische Evaluierung der, im Rahmen einer Diplomarbeit[199] synthetisierten, Tormentillsäurederivate aus Kapitel 4.4 eindeutig belegt, wäre es zukünftig interessant zu untersuchen, welchen Einfluss der Acetylschutzgruppe im A-Ring und dem Esters an Position 30 zu zuschreiben ist. Für die untersuchten Tormentillderivate konnten gezeigt werden, dass es durch solche Modifikationen zu sehr vielversprechenden Aktivitätssteigerungen kommen (Vgl. Abbildung 42). Weiterführende biologische Studien zeigten außerdem, dass der Apoptose induzierende Effekt der Tormentillsäure auch nach chemisch-modifizierenden Eingriffen erhalten bleibt; ein Befund welcher bis *dato* nicht evident existent war. Die gleichzeitige Verwendung von primären humanen Fibroblasten (WW030272) ermöglichte es im Rahmen dieser Studie ferner die Selektivität der Verbindungen an Hand eines geeigneteren Systems zu untersuchen. Festzuhalten ist, dass Aktivitätsunterschiede zw. malignen und nicht malignen Zelllinien im Bereich des zwei bis drei-Fachen messbar sind. Wie die Ergebnisse des zweiten Hauptschwerpunktes (Kapitel 534.5 bis 4.7) dieser Arbeit zeigen sind Faktoren in Größenordnungen von bis zu zwei Potenzen möglich, womit das Selektivitätsverhalten der untersuchten Tormentillsäurederivate als moderat einzuschätzen ist.

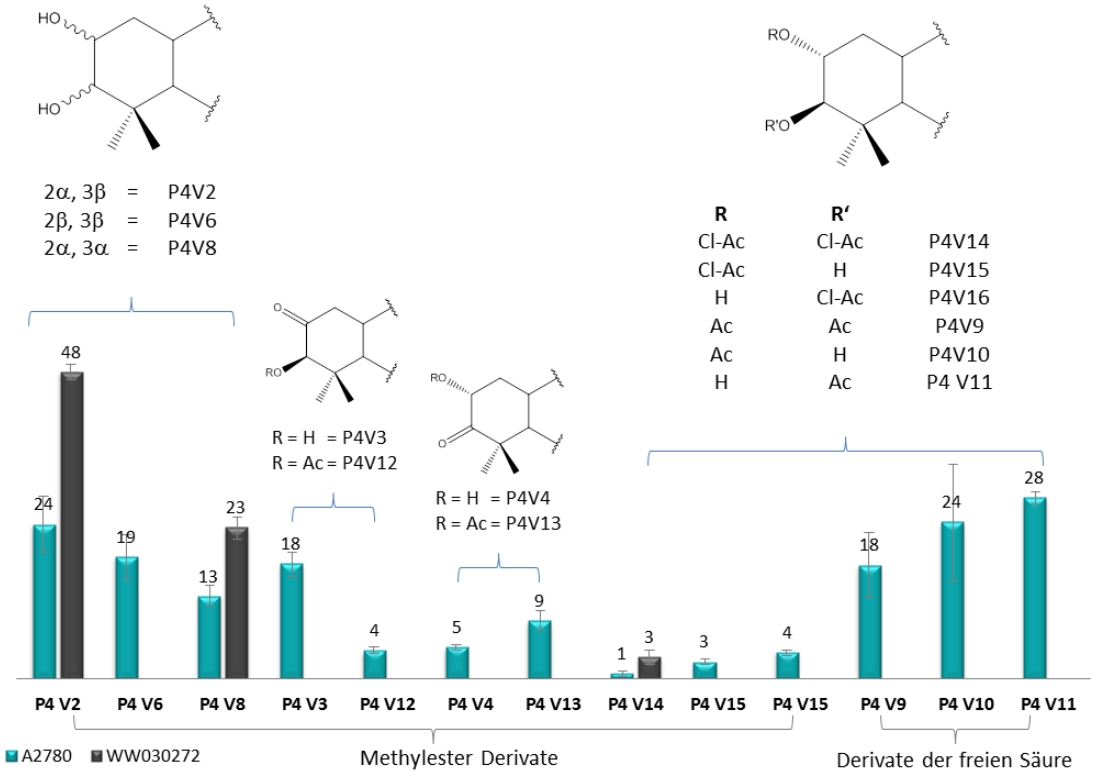


Abbildung 42: *Die Ergebnisse der SAR Untersuchung von Tormentillsäurederivaten bezüglich Gebärmutterhalskrebszellen (A2780) und einer menschlichen, non-malignen primär Fibroblasten-Kultur (WW030272). Die Werte sind in µM mit SE angegeben.*

5.3. SAR-Studien der Maslinsäure.

Maslinsäurederivate, die sich durch eine erhöhte Selektivität auszeichnen, wurden, nach der erfolgreichen Extraktion aus verschiedenen Materialien (Oliven und Oliventrester), über eine systematisch aufgebaute SAR, erschlossen. Die SAR-Studie beschäftigt sich hauptsächlich mit Modifikationen der Säurefunktion an C28, welche z.T. in einer weiteren betreuten Diplomarbeit synthetisiert wurden[200]; die strikte Umsetzung des gespannten Modifikationschemata führte dabei zunächst zu den folgenden Thesen.

Eine Veresterung der Carbonsäurefunktion führt zu einer Aktivitätssteigerung, wenn es sich hierbei um kleine, lipophile Ester handelt. Die Einführung eines ungesättigten Charakters scheint, wie auch die Einführung von zusätzlichen Halogeniden, zu keinen weiteren, signifikanten Wirksteigerungen zuführen. Hiervon lässt sich ableiten, dass der Esterfunktion oder allgemeiner gesprochen, dass dieser Molekülbereich nicht für spezielle Wirkstoff-Enzym-Wechselwirkungen entscheidend ist. Putative nukleophile Angriffe durch das biologischen System scheinen ebenso keinen Einfluss zunehmen. Die Synthese von dreizehn, verschieden substituierten Benzylderivaten zeigt ein zu den aliphatischen Estern analoges Bild; eine Aktivitätssteigerung ist im Vergleich zur Maslinsäure messbar, jedoch entlang der unterschiedlichen Benzylderivate nicht signifikant. Bemerkenswert ist hingegen, dass die

Selektivität zwischen malignen und nicht malignen Gewebe in Abhängigkeit zur Estermodifikation steigerungsfähig ist (Kapitel 4.6).

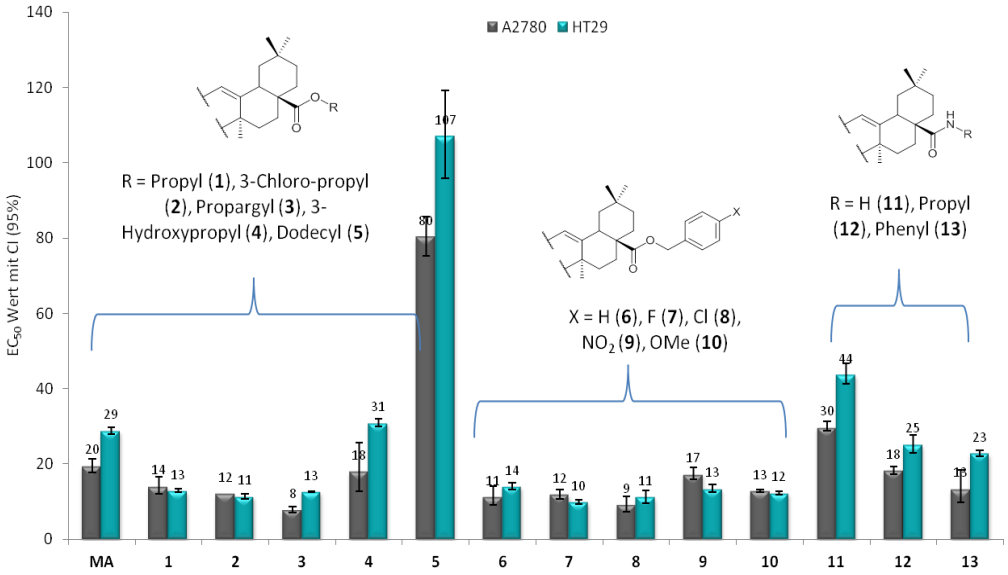


Abbildung 43: *Ergebnisse der SAR für Maslinsäurederivate*

Die Verlängerung der aliphatischen Esterkette führt zu einem gänzlichen Verlust der Aktivität was vermutlich auf die erhöhte Lipophilie zurückführbar ist. Ebenso mit einer Verringerung der Aktivität einhergehend sind jene Esterverbindungen, welche polar protische Endgruppen besitzen (Vgl. Abbildung 43). In Kombination mit dem messbaren Aktivitätsverlust, welcher durch die Amidbildung zu verzeichnen ist, lässt sich darauf schließen, dass die Einführung von Protonendonatorfunktionen an Position 28 für den speziellen Fall, der ansonsten unveränderten Maslinsäure, unvorteilhaft ist. Für analoge Derivate der Oleanolsäure, welche sich nur im A-Ring von der Maslinsäure unterscheiden, wie auch für Modifikationen der Ursolsäure, konnte ein hierzu gegenläufiges Verhalten beobachtet werden. Der bedeutende, Aktivitätstendenzen verändernde Einfluss des A-Ringes konnte im Speziellen auch durch Amidstrukturen der Maslinsäure, gezeigt werden; Diese sind inaktiv solange im A-Ring freie Hydroxylgruppen vorhanden sind, es steigt die Aktivität jedoch nahezu exponentiell durch die Einführung von Acetylschutzgruppen.

Neben dieser Beobachtung, die sich aus dem SRB-Assay schließt, zeigte die fortführende biologische Untersuchung, dass sich die Strukturen im Wirkmechanismus signifikant unterscheiden. Führen Esterstrukturen zu einer schnellen, über einen G1-Arrest verlaufenden, Apoptoseinduktion, kann für die Amide hingegen festgestellt werden, dass sie zytostatisch wirken. Der durchgeführte Caspase-Assay zeigt, dass im Falle des 2,3-diacetylierten Maslinsäurebenzylamids die Caspasen wesentlich später induziert werden. Erst nach längerer Inkubation führt diese Struktur zu apoptotischen Ereignissen. Die sensationelle Selektivität, welche für die 2,3-diacetylierten Amide im Allgemeinen

gefunden werden konnte, lässt das 2,3 diacetylierte Maslinsäurebenzylamid zusammen mit der im nanomolaren Bereich liegenden effektiven halbmaximalen Konzentration bezüglich Gebärmutterhalskrebszellen, im Speziellen zu einem äußerst vielversprechenden anti-Tumorgenesis Kandidaten werden.

Wirkmechanistisch aufklärend sind die Ergebnisse, welche für den 4-Oxo-4-phenyl-butyl Ester (MB64) der Maslinsäure generiert werden konnten. Ausgehend von der zufälligen Beobachtung einer Kristallbildung, einer intensiven Literaturbetrachtung und die Verwertung der, aus ebenjenen gewonnenen, Erkenntnissen konnte durch die Kombination mit mannigfaltigen experimentellen Versuchen, deduktiv folgende These aufgestellt werden. Der Ester MB64, und putative alle analogen Strukturen, wird in die Zellmembran eingebaut, Cholesterol dort verdrängt, die Ionenkanalpumpen dadurch so beeinflusst, dass ein sehr schnelles Zellschrumpfen hervorrufen wird, was wiederum zur Aktivierung von Caspasen führt und schließlich den Zelltod einleitet. (Vgl Abbildung 44)

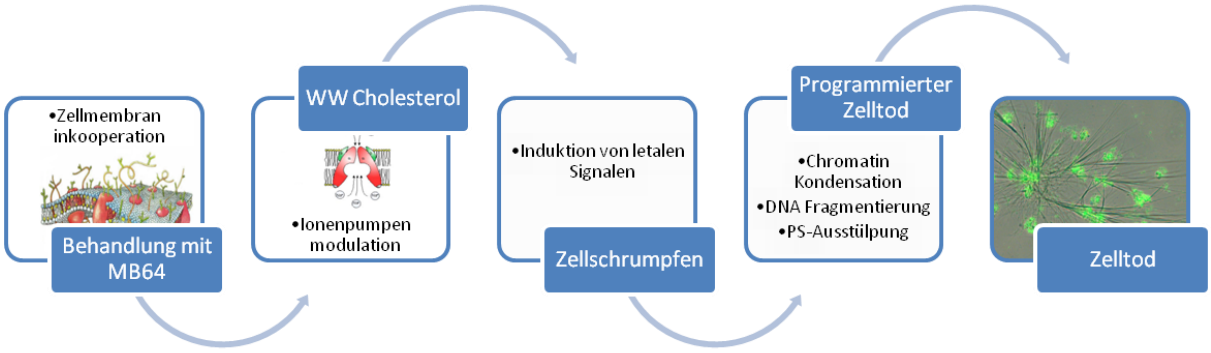


Abbildung 44: *Schematische Darstellung der These zum Wirkmechanismus von MB64*

Mit dieser fundierten These kann erstmalig ein Target für die zytotoxische Wirkung der Triterpene bewiesen werden. Vorangegangene Studien beweisen zwar evident die Modulation verschiedener Proteine, Membranpotentialen und die Aktivierung von mannigfaltigen Enzymen, jedoch sind diese nachgewiesenen potentiellen Targets eher kritisch zu betrachten. Denn werden die unterschiedlichen Substrate der Caspasen betrachtet und die gesamte, molekulare Auswirkung einer solchen Aktivierung, dann könnte zwar bspw. die, als Target von FULDA *et al.*, HOLY *et al.* und LIU *et al.* für Betulinsäure, Dimethylaminopyridine Derivate des Lupans und respektive für Betulin proklamierte, Mitochondrienmembran (MM) immer noch ein Target sein, aber der durch die Wissenschaftler als Beweis hinzugezogene Verlust des MM-Potentials stellt gleichzeitig auch „nur“ eines der sogenannten „Hallmarks of Apoptotic Death“ dar. Die im Rahmen dieser wissenschaftlichen Arbeit vorgestellte These (vgl. Kapitel 4.7) hingegen beweist, durch Kristalle, welche aus der Zellmembran, bei Konzentrationen weit unterhalb der Löslichkeitsgrenze, wachsen und sich aus Maslinsäureester und

Cholesterol zusammensetzen, erstmalig eine direkte Wechselwirkung. Wenn die These zwar durch zusätzliche physikochemische Untersuchungen noch weiter zu verifizieren, auszubauen und eventuell auch zu modifizieren ist, um sie als endgültig darzustellen, ist dennoch der geschaffene Mehrwert unbestreitbar. Durch die als Target definierte Zellmembran kann ferner der Selektivitätsverlust der meisten Ester im Vergleich zu der Klasse der Amide, der eine andere MOA zuzusprechen ist, erklärt werden: Die Zellmembran und putative Cholesteroldrafts bzw. Ionenpumpen sind keine tumorspezifischen Targets.

5.4. Ausblick.

Wie gezeigt werden konnte, führen zwar lipophile, aprotische Veränderungen der Grundgerüste zu signifikanten Aktivitätssteigerungen, unter dem klar definierten Hauptziel der Wirkstoffforschung – hochaktive und selektive Verbindungen zu generieren – sind diese Modifikationen, wie in der vorliegenden Arbeit bewiesen werden konnte, von nur mäßigem Wert. Putativ werden sie alle in die Membran eingebaut, was zu einem Gewebe unspezifischen, apoptotischen Zelltod führt. Die Erkenntnis als solche ist hingegen für andere pharmakologische Forschungsgebiete, wie die die anti-Pathogene und antivirale sehr hilfreich. Membran-Destabilisierungen stellen in diesen Zweigen der Wissenschaft einige der Haupttargets dar.

Subsummiert konnte im Rahmen dieser Arbeit, neben der Erschließung eines neuen, universal anwendbaren Trennverfahrens, der Identifikation und Charakterisierung einer Apoptose auslösenden Selenophen-Verbindung und dem Nachweis von apoptotisch hochaktiven, moderat selektiven Tormetillsäurederivaten, deduktiv gezeigt werden, dass Maslinsäurederivate in Abhängigkeit von ihrer Modifikation unterschiedliche Wirkweisen hervorrufen. Neben der Identifizierung, Charakterisierung und biologischen Evaluierung eines vielversprechenden antitumorgenesis-Kandidaten konnte ein putativer Wirkmechanismus für die letal wirkenden Ester eruiert und grundlegend verifiziert werden.

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ANHANG

Die der Arbeit zugrunde liegenden Publikationen sind im Folgenden zufinden.

1. A convenient separation of ursolic and oleanolic acid A-3
2. The chemical and biological potential of C ring modified triterpenoids. A-6
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   3.1. Addendum: A bioassay-driven discovery of an unexpected selenophene and its cytotoxicity A-45
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A convenient separation of ursolic and oleanolic acid

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ABSTRACT

A convenient route has been developed to separate regioisomeric ursolic and oleanolic acid by treating the mixture with *m*CPBA or formic acid/hydrogen peroxide.

Introduction

The family of the pentacyclic triterpenes includes some important biologically active natural products. Many of them are bactericidal, fungicidal, antiplasmodial, antiviral, spermicidal, analgesic, antiallergic, cardiovascular-active, and some of them possess remarkable cytotoxic and anticancer[1] activities. Recently, ursolic acid (**1**) and oleanolic acid (**2**, Fig. 1) came in the focus of scientific interest. Oleanolic acid was shown to possess hepatoprotective activities[2] and might be of interest for developing antidiabetics[3] whereas **1** is used as an antioxidant[4] and as an antitumor-active[5] agent. Compound **1** induces apoptosis in tumor cells[6] and is claimed[7] for to treat skin cancer.

Although **1** was obtained from the leaves of bearberry (*Arctostaphylos uva-ursi*) by Trommsdorff[8] as early as 1854, its separation from accompanying **2** is challenging because of similarity[9] in structure. Many analytical methods have been elaborated including HPLC,[10–16] TLC,[11,17] MEEC,[18] CD-MEKC,[19–21] CE,[22,23] HP-TLC,[24] and immunochromatography,[25] separation of these two compounds on a preparative scale remains by and large an unresolved problem.

Results and discussion

Lewis' approach from 1983[26] uses the different reactivity of **1** and **2** toward elemental bromine. This synthesis, however, is lengthy and yields drop significantly on scaling-up. Keeping in mind the alkenic bond between carbons C-12 and C-13 in α-amyrin-type triterpenes is less reactive than that in β-amyrins because of steric hindrance by the methyl groups close by, b-amyrin-type triterpenoids are known to react with *m*CPBA and other peracids[27,28] to form inter alia the corresponding 12,13-epoxides.

Thus, treating a 60:40 mixture of **1** and **2** with *m*CPBA or with formic acid/hydrogen peroxide in dry DCM at room temperature for one day (Scheme 1) gave a mixture of unreacted **1**, lactone

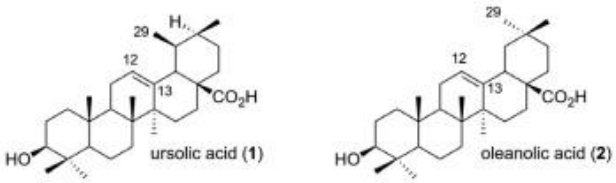

Figure 1. Structure of ursolic acid (**1**) and oleanolic acid (**2**).

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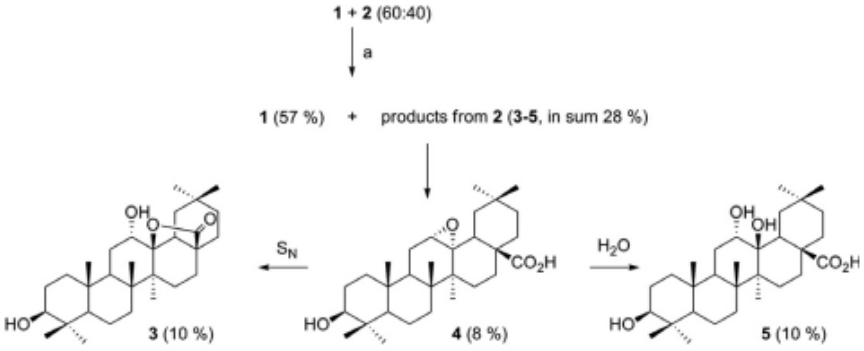

Scheme 1. Separation of ursolic and oleanolic acid. Reagents and conditions: (a) *m*CPBA, DCM, 25 °C, 24 h, or HCOOH/H_2O_2, 25 °C, 24 h followed by chromatography (silica gel, hexane/ethyl acetate 5:3).

3,[29–31] epoxide **4**,[32,33] and dihydroxylated **5**.[34,35] These compounds were easily separated by chromatography.[36,37]

The absolute configuration of HO-C(12) in **3** was deduced from the NOESY spectra showing a cross peak between H-18 and H-12. Compound **3** is formed by a nucleophilic attack of the carboxylic group of epoxide leading to the 12 α-hydroxy lactone **3** whereas nucleophilic ring opening of the epoxide **4** by water leads to the 12,13-dihydroxy derivative **5**.

This synthetic approach can be generalized. Reaction of a mixture of the methyl or benzyl esters with *m*CPBA or formic acid/hydrogen peroxide yielded mixtures containing unreacted ursolic esters and the corresponding reaction products from the oleanolic esters. Again, these mixtures were easily separated by column chromatography.

In summary, our approach allows an easy separation of ursolic acid and oleanolic acid (or of their respective methyl or benzyl esters) on a preparative scale leading to ursolic acid of superior purity as determined by HPLC.[38]

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36. In a typical experiment, to 1 equiv of **1**/**2** (60:40) in DCM, *m*CPBA (2 equiv) [or HCOOH (concd, 2 equiv) /H_2O_2 (30%, 2 equiv)] was added, and the mixture was stirred at 25 °C for 24 h. After washing with an aq soln. of sodium thiosulfate, the solvents were evaporated under diminished pressure. The residue was subjected to chromatography (silica gel, hexane/ethyl acetate, 5:3) to yield the products.
37. *Selected analytical data.* **1**: colorless solid, mp 257–259 °C (lit. 258 °C: White, D. E.; Zampatti, L.S. *J. Chem. Soc.* **1952**, 5040); R_f = 0.64 (hexanes/ethyl acetate 8:2) = 0.64; **3** (usually obtained by ozonolysis of **2**) mp 249–252; decomp. at 297 °C (lit. [31] 247–249) ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.83–3.80 (*dd*, 1H, ^3J = 2.7 Hz, ^3J = 2.7 Hz, *CH* (12)) 3.15 (*dd*, 1H, ^3J = 11.4 Hz, ^3J = 4.4 Hz, *CH* (3)), 2.07 (*ddd*, 1H, ^2J = 13.3 Hz, ^3J = 5.8 Hz, ^3J = 5.8 Hz, CH_2 (16)), 2.01–1.88 (*m*, 3H, *CH* (18) + CH_a (19) + CH_a (11)), 1.86–1.75 (*m*, 2H, CH_b (19) + CH_a (15)), 1.66 (*ddd*, 1H, ^2J = 12.8 Hz, ^3J = 12.8 Hz, ^3J = 3.5 Hz, CH_a (1)), 1.59–1.51 (*m*, 3H, CH_2 (2) + CH_a (22)), 1.53–1.48 (*m*, 1H, *CH* (9)), 1.49–1.44 (*m*, 2H, CH_a (6) + CH_a (7)), 1.42–1.37 (*m*, 2H, CH_b (11) + CH_b (6)) 1.35–1.30 (*m*, 1H, CH_b (21)), 1.24 (*s*, 3H, CH_3 (27)), 1.22–1.15 (*m*, 3H, CH_b (21) + CH_b (7) + CH_b (6)), 1.12–1.09 (*m*, 1H, CH_b (22)), 1.08 (*s*, 3H), 0.92 (*s*, 3H, *CH* (26)), 0.91 (*s*, 3H, *CH* (23)), 0.92–0.87 (*m*, 1H, CH_b (21)), 0.83 (*s*, 3H, *CH* (30)), 0.81 (*s*, 3H, *CH* (29)), 0.71 (*s*, 3H, *CH* (24)), 0.68 (*dd*, 1H, ^3J = 11.8 Hz, ^3J = 1.9 Hz, *CH* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 179.9 (C28, *C*=O), 90.6 (C13, C_{quart}), 78.9 (C3, *CH*), 76.4 (C12, *CH*), 55.2 (C5, *CH*), 51.1 (C18, *CH*), 44.7 (C17, C_{quart}), 44.6 (C9, *CH*), 42.3 (C14, C_{quart}), 42.1 (C8, C_{quart}), 39.4 (C19, CH_2), 38.9 (C1, CH_2), 38.9 (C4, C_{quart}), 36.5 (C10, C_{quart}), 34.2 (C21, CH_2), 34.0 (C7, CH_2), 33.3 (C30, CH_3), 31.6 (C20, C_{quart}), 28.8 (C11, CH_2), 28.1 (C23, CH_3), 28.1 (C15, CH_2), 28.0 (C22, CH_2), 27.5 (C2, CH_2), 23.9 (C29, CH_3), 21.2 (C16, CH_2), 18.6 (C26, CH_3), 18.5 (C27, CH_3), 17.8 (C6, CH_2), 16.3 (C24, CH_3), 15.4 (C25, CH_3) ppm; **4** colorless amorphous solid, R_f = 0.26 (hexane/ethyl acetate 5:3); **5** mp 255 °C; R_f = 0.22 (hexanes/ethyl acetate 5:3); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.67 (*dd*,1H, ^3J = 11.7 Hz, ^3J = 5.8 Hz, *CH* (12)), 3.14 (*dd*, 1H, ^3J = 11.5 Hz, ^3J = 4.7 Hz, *CH* (3)), 2.64 (*dd*, 1H, ^3J = 14.0 Hz, ^3J = 3.0 Hz, *CH* (18)), 2.32–2.19 (*m*, 1H, CH_a (16)), 2.05 (*dd*, 1H, ^2J = 13.2 Hz, ^3J = 5.7 Hz, ^3J = 5.7 Hz, CH_a (15)), 2.01–1.88 (*m*, 3H + CH_a (22) + CH_a (19) + CH_a (1)),

1.73–1.65 (*m*, 2H, CH_2 (2)), 1.64–1.49 (*m*, 2H, CH_a (6) + CH_b (15)), 1.51–1.45 (*m*, 2H, CH_b (6) + CH_a (7), 1.46–1.40 (*m*, 2H, CH_b (19) + $CHHH_a$ (21)), 1.42–1.21 (*m*, 6H, CH_b (21) + CH_b (7) + CH_b (22) + CH_b (16) + CH_2 (11)), 1.22–1.20 (*m*, 1H, *CH* (9)), 1.19 (*s*, 3H, CH_3 (26)), 0.99 (*s*, 3H, CH_3 (27)), 0.92 (*s*, 3H, CH_3 (30)), 0.92 (*s*, 3H, CH_3 (23)), 0.92–0.91 (*m*, 1H, CH_b (1)), 0.84 (*s*, 3H, CH_3 (29)), 0.81 (*s*, 3H, CH_3 (24)), 0.70 (*s*, 3H, CH_3 (25)), 0.60 (*dd*, 1H, ^3J = 11.2 Hz, ^3J = 2.2 Hz, *CH* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 179.5 (C28, C=O), 93.0 (C13, C_{quart}), 78.8 (C3, CH), 67.2 (C12, CH), 55.0 (C5, CH), 48.9 (C9, CH), 44.0 (C17, C_{quart}), 42.5 (C18, CH), 42.4 (C14, C_{quart}), 42.4 (C8, C_{quart}), 38.9 (C1, CH_2), 38.8 (C4, C_{quart}), 37.2 (C19, CH_2), 34.3 (C7, CH_2), 33.4 (C21, CH_2), 33.3 (C30, CH_3), 31.7 (C20, C_{quart}), 29.7 (C11, CH_2), 29.7 (C15, CH_2), 29.1 (C23, CH_3), 29.4 (C2, CH_2), 29.0 (C22, CH_2), 27.3 (C29, CH_3), 23.9 (C16, CH_2), 18.4 (C27, CH_3), 17.7 (C26, CH_3), 16.1 (C24, CH_3), 15.3 (C25, CH_3) ppm

38. For HPLC: Prontosil C-18, MeOH/H_2O 86/6, 1% H_3PO_4.

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Original article

The chemical and biological potential of C ring modified triterpenoids

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ABSTRACT

A convenient and elegant route has been developed to separate the natural regioisomers triterpenoids ursolic acid (**UA**) and oleanolic acid (**OA**) as well as derivatives thereof. Eleven unknown derivatives of **OA** were designed, synthesized, and their cytotoxicity was investigated. Further sixteen compounds were prepared to correlate all compounds in a SAR study. It could be shown that C-ring modifications of **OA** and **UA** have only a moderate influence onto the cytotoxic activity of the compounds but a significant impact onto the ability to trigger apoptosis in ovarian cancer cells (cell line A2780).

1. Introduction

Triterpenoids are promising candidates [1] for the development of new drugs. As products of the secondary metabolism they fulfill a function in nature in their own right. But in terms of a future use for medicinal applications, they are far off of being optimized for this use. For these highly complex molecules carrying several stereogenic centers, however, total syntheses are quite challenging but for larger scale synthesis highly uneconomic and libraries of analogs are difficult to obtain. Thus, semi-synthetic strategies are most rewarding; they shorten the time-to-market period tremendously. As a prerequisite, suitable precursors have to be gained from plant material. However, these precursors occur only in small amounts, and, in addition, they are most often part of complex mixtures. Thus, their isolation and/or separation is difficult, sometimes laborious and uneconomic. For their straightforward isolation, extractive steps have to be combined with selective derivatization reactions. The demand for these compounds has increased since clinical trials for NVX-207 [2] or CDDO-Me [3,4] have already begun.

To guarantee the successful commercial exploitation and the development of analogs, effective isolation methods are mandatory and called for. Recently, we were able to present [5] a convenient separation of regioisomeric triterpenoids, ursolic (**UA**) and oleanolic acid (**OA**) on a larger scale. These compounds (Scheme 1) are widely spread in the plant kingdom and known for their broad pharmaceutical activities, *e.g.*, for noteworthy antiviral, antibacterial and anticancer activities [6–8]. However, both molecules occur very often together in leafs or peels of plants [9]. In continuation of our previous work looking for triterpenoids of improved cytotoxic properties, the development of an easy protocol for their separation was still in the focus of our scientific interest.

Several groups were able to show that the introduction of an oxygen substituent into ring C increased the biological activity of the compounds. Due to their ability to act as "reactive oxygen species (ROS) producer", the presence of α,β-unsaturated systems seems promising. For example, for the well-known compound CDDO (2-cyano-3,12-diooxoolean-1,9-dien-28-oic acid) several pharmaceutical activities, *e.g.*, anti-inflammatory, anti-diabetic nephropathy and cytotoxic activities, have been reported [10,11]. Furthermore, clinical trial using ester derivatives of CDDO have been started, thus increasing the demand for this class of compounds [10,12,13]. CDDO esters showed a fast first-pass metabolism; hence, there is still a necessity for improvement [12]. Recently, Leal et al. [14] presented several promising anticancer active lactones possessing an **UA** backbone.

Many data reported for the biological activity of triterpenoids, however, are hardly comparable, and the impact of modifications of the C-ring onto the cytotoxicity of the compounds still remains unclear. Thus, we became interested in studying the structure–activity relationships (SAR) of some of these derivatives combined with a more detailed inspection of their mode of action (MOA).

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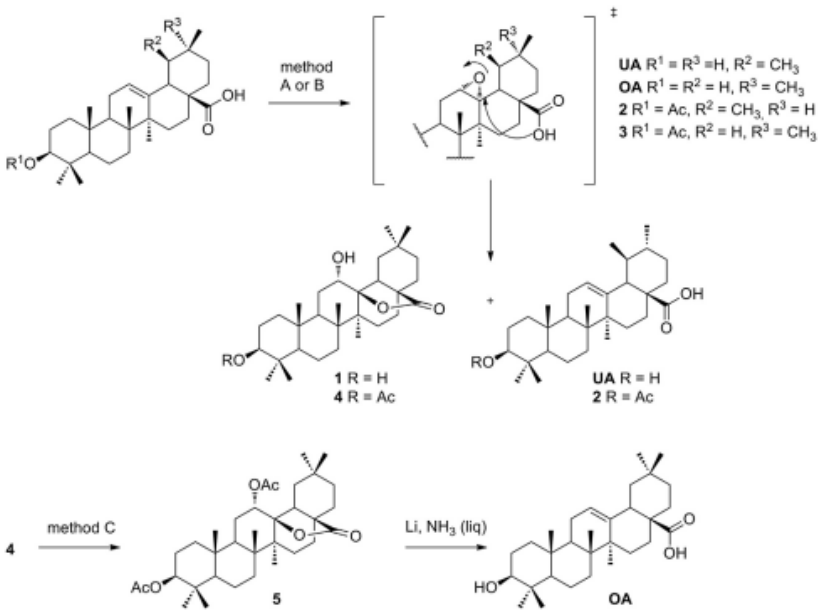


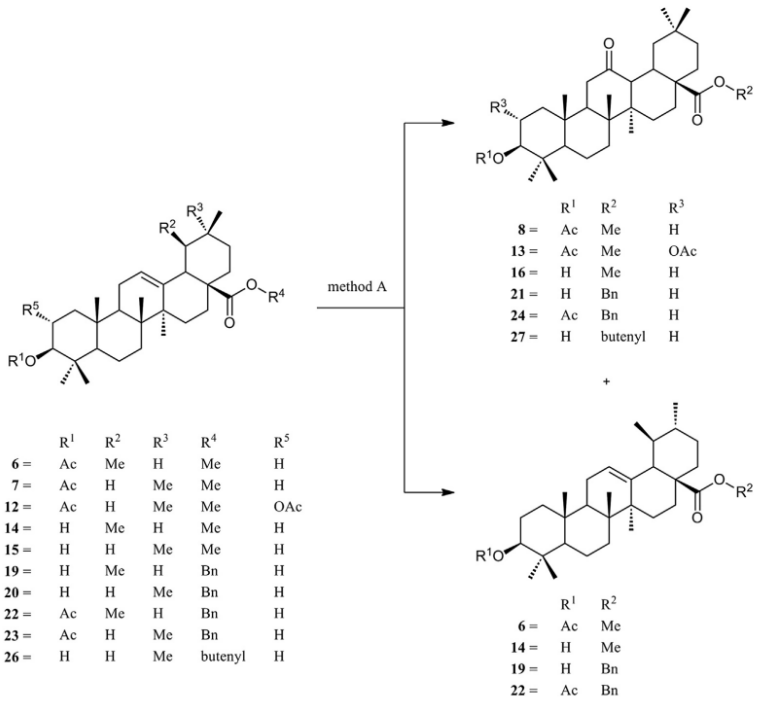
Scheme 1. Separation of a mixture of **OA**/**UA** (and derivatives thereof) and the regeneration of **OA**.

2. Chemistry

Usually the separation of regio- or diastereoisomers can easily be accomplished by chromatography, by recrystallization or by chemo-selective reactions with/without the use of enzymes. Previously, smaller amounts of **UA**/**OA** mixtures have been separated by chromatography [15,16] or by treating these mixtures with HBr [17]. Recently, we presented a new chemo-selective separation of the two constitutional isomers **UA** and **OA** by treating of a **UA**/**OA** mixture with peracids (either performic acid or *m*-chloroperbenzoic acid) [5] under mild conditions. Thus, **OA** is selectively transformed into its corresponding 12-hydroxy-lactone **1** (Scheme 1) while leaving **UA** unchanged. The formation of an epoxide from **UA** is not observed using mild conditions and low temperatures. **UA** is a very valuable starting material [18] for the synthesis of cytotoxic compounds. As early as in 1966, Barton et al. [19] showed that **OA** can be recovered by a reductive elimination (using lithium in ammonia) starting from the 12-acetoxy-lactone **5**. Compound **5** can be prepared either from the 3-hydroxy-lactone **1** by its acetylation with acetic anhydride in pyridine or from the 3-acetoxy-lactone **4**, which was accessed by the separation of a mixture of **2** and **3**. The presence of an additional sharp singulet at $\delta = 2.10$ ppm in the ^{1}H NMR spectrum of **5** indicates – together with an IR vibration band at $\nu = 1766$ cm^{-1} – the formation of **5**. Nevertheless, a low turnover rate of 20% devaluates this separation [19,20]. Consequently, we were looking for a more elegant strategy. Given the potential of C-ring modifications – as exemplified in CDDO-Me – we tried to shorten the synthetic scheme and to optimize the process of extraction and separation.

The treatment of mixtures of ester analogs from **OA**/**UA** mixtures with peracids furnished 12-oxo derivatives from **OA** exclusively but not from **UA**. Thus, **UA** esters remained unchanged under these mild reaction conditions. Moreover, this type of reaction (Scheme 2) could be used for the separation **OA**/**UA** quite universally. Neither the presence of a second double bond in the molecule (as exemplified for **26**) nor the presence of an additional hydroxyl group in ring A (as in **12**) restricted these reactions.

To investigate the influence of C-ring modifications onto the biological activity an oxygen substituent at position 11 (compounds **10** and **17**, Scheme 3) was inserted *via* an α-allylic, chromate-assisted oxidation. For compound **10**, the characteristic IR band for the newly created α,β-unsaturated system was found at $\nu = 1724$ cm^{-1}, in combination with an a signal in the ^{13}C NMR spectrum at $\delta = 200.3$ ppm. Subsequent treatment of **8**, **15** and **24** with selenium dioxide in refluxing 1,4-dioxane resulted in the formation of unknown products. Usually, this type of reaction is known [21–23] to establish a 9,11-ene-12-oxo moiety in steroid-derived structures. However, due to the presence of terminal methyl groups located at positions 26 and 27 in the backbone of the triterpenoids, an elimination of the hydroxyl group at position C-11 is not possible any longer. Hence, a keto group is created instead and rearranged into a thermodynamically more stable enol-system (Scheme 3). For this structural feature, a new signal at $\delta = 142.3$ ppm (C12) is observed in the ^{13}C NMR spectrum. Additional IR vibrations at $\nu = 1652$ and 1614 cm^{-1} confirm the structure [24]. The configuration (Scheme 3) of ring C (compounds **9** and **25**) was ascertained by the shift of the equatorial proton H-1 to higher fields. This shifting (which has already been observed in the past for glycyrrhetinic acid and was also found in compounds **10** and **17**) is caused by an anisotropy tensor of the 11-oxo function [25–27]. Furthermore, cross-peaks in gHMBC NMR experiments between C12 and C18 as well as between C9 and C11 finally confirm this structure which is further ascertained by its ESI-MS spectra ($\Delta m/z = +16$ as compared to the starting material).



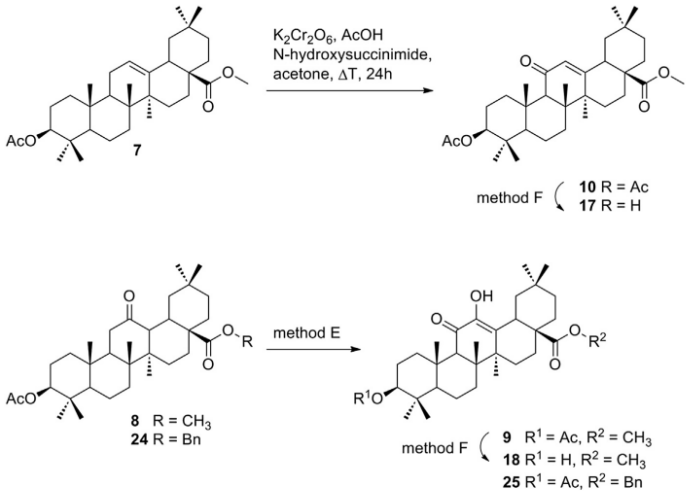
	R^1	R^2	R^3	R^4	R^5
6 =	Ac	Me	H	Me	H
7 =	Ac	H	Me	Me	H
12 =	Ac	H	Me	Me	OAc
14 =	H	Me	H	Me	H
15 =	H	H	Me	Me	H
19 =	H	Me	H	Bn	H
20 =	H	H	Me	Bn	H
22 =	Ac	Me	H	Bn	H
23 =	Ac	H	Me	Bn	H
26 =	H	H	Me	butenyl	H

	R^1	R^2	R^3
8 =	Ac	Me	H
13 =	Ac	Me	OAc
16 =	H	Me	H
21 =	H	Bn	H
24 =	Ac	Bn	H
27 =	H	butenyl	H

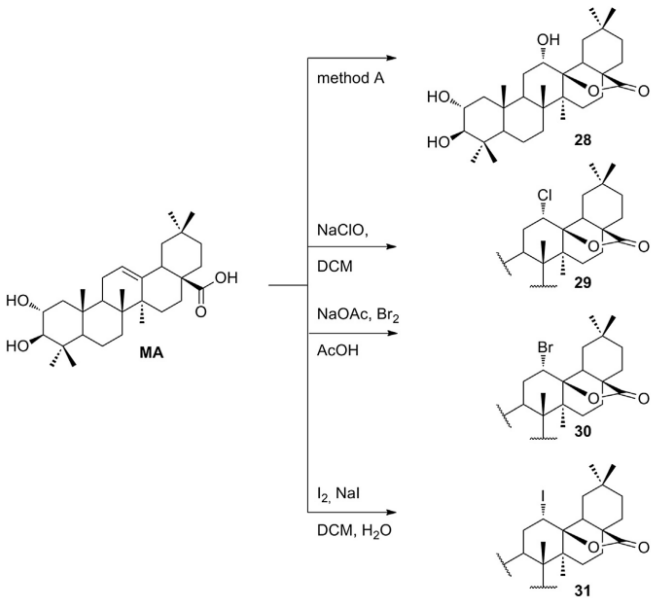
	R^1	R^2
6 =	Ac	Me
14 =	H	Me
19 =	H	Bn
22 =	Ac	Bn

Scheme 2. Separation of mixtures of the **OA**/**UA** ester analogs.

As depicted in Scheme 4, in general, the position of H-12 was assumed to be axial. By comparison with data from literature and by the presence of a cross-peak between H-12 and H-18 in the 2D NMR spectra as well as suiting coupling constants (*e.g.*, $^3J_{H12(ax)-H11(ax)} = 3.9$ Hz, $^3J_{H12(ax)-H11(eq)} = 2.3$ Hz for compound **30**) the configuration of this stereogenic center is confirmed. A characteristic IR absorption at $\nu = 1699$ cm^{-1} was detected for compounds **4**, **5** and **28**–**31** being typical for the presence of a lactone moiety.



Scheme 3. Oxidation of the C-28 ester analogs.



Scheme 4. Lactonization of maslinic acid (**MA**).

3. Biological investigations

Many natural occurring triterpenoids are well-known for their cytotoxic activity with IC_{50} values between 10 and 80 μM [8]. Several of them trigger apoptosis, and **OA** as well as **MA** show promising tumor-to-control selectivity. Screening of the compounds to detect their cytotoxic activity is usually performed either by an MTT [28] or a SRB [29] assay; the first of these uses the mitochondrial dependent reduction of a thiazolium salt whereas the latter is based on the proportional binding of a rhodamine dye to surface membrane proteins. Advantages of Skehan's SRB assay are found in the linearity between cell density and the optical density. The comparison of cytotoxicity measured by different assays (using different theoretical assumptions to describe more or less the same cellular feature) remains critical, and is even more complicated by numerous modifications of the original assay. Hence, we decided to include several already known compounds into this study, and to test all compounds using the same protocol and standardized conditions. Several triterpenoids have been shown to exhibit some selectivity towards cancer cells and some derivatives of **OA** are able to induce apoptosis [8]; hence, we set out to investigate the influence of the C ring onto antitumor activity, their tumor-to-control selectivity as well as onto their ability to trigger apoptosis.

Table 1 and Fig. 1 summarize some of the results. Most of the compounds exhibited a moderate to good IC_{50} value in the SRB tests. As far as the cytotoxic activity of the lactones (**4**, **5**, **28**–**32**) is concerned, the presence of a hydroxyl group seems unfavorable. For the halide-substituted compounds activity increased with the atomic radius or was reciprocally proportional to an increasing electronegativity. The importance of an intact C ring for retaining good biological activity has already been demonstrated for glycyrrhetinic acid derivatives. The mode of action of these compounds, however, has been subject to controversial discussions. Thus, on the one side the presence of an α,β-unsaturated moiety was regarded less important for the ability to produce ROS; a comparison of the cytotoxicity of a 12-oxo-9,11-ene and a 11-oxo-12,13-ene revealed no significant differences between these two compounds [30,31]. Contrary to these findings, activity was claimed to depend crucial on the presence of an intact *Michael* acceptor [31], *e.g.*, the presence of a 12-oxo-9,11-ene moiety. The significance of an intact C ring has also been discussed for the explanation of interactions between tyrosine phosphatase 1B and triterpenoids [32]. In addition, several semicarbamates derived from **1** showed an increased cytotoxicity as compared to parent OA [33]. The introduction of lipophilic moieties into ring A seems to result in better cytotoxicity of these compounds [14].

Our own data make a nucleophilic attack onto position 13 as a key step quite improbable. If an attack at position 13 should be important, one would expect an increasing activity with an increasing positive polarization of C-13 – or at least the activity should be proportional to an increasing electronegativity of the adjacent halogen substituent. Quite on the contrary, the iodolactone is the most active compound of this series. This led to the assumption that either a nucleophilic attack at position 12 or a stereoelectronic hindrance by this substituent must be essential. Our results parallel previous findings of Salvador et al. [33] suggesting a nucleophilic attack.

Comparison of the IC_{50} values of the products resulting from the oxidation of the **OA** esters (Fig. 1) indicate that introducing an extra oxygen seems less important for obtaining good cytotoxicity than variations at *e.g.*, position 3. Cytotoxicity of the **OA** derivatives can be raised by these modifications up to ca. 111 fold [33]. Inspection of the IC_{50} values (listed in Table 1) shows oxidation in ring C by and large insignificant for obtaining good cytotoxic effects. These findings are in perfect agreement with results obtained by Chadalapaka

Table 1
Cytotoxicity of **OA**, **MA** and derivatives (IC_{50} values in μM from SRB assays after 96 h of treatment; the values are averaged from at least three independent experiments performed each in triplicate; confidence interval CI = 95%; the individual errors (positive upper and negative lower values are given; n.D. not detected). Cell lines 518A2, 8505C, A2780, A549, HT29 and MCF are human cancer cell lines, NiH 3T3 cells are a non-malignant mouse fibroblasts, STP is stauroporine.

IC_{50}	518A2		8505C		A2780		A549		HT29		MCF7		NiH 3T3	
OA	>60	0.0	>60	0.0	14.0	1.1	72.3	3.5	38.8	5.4	>60	0.0	76.4	1.5
		0.0		0.0		1.1		1.9		4.8		0.0		1.4
MA	13.7	1.9	17.0	2.1	19.5	1.8	23.4	0.6	28.8	1.0	16.6	0.9	21.4	0.7
		1.7		1.9		1.7		0.6		0.9		0.8		0.7
1	>30	0.0	>30	0.0	>30	0.0	>30	0.0	>30	0.0	>30	0.0	>30	0.0
		0.0		0.0		0.0		0.0		0.0		0.0		0.0
		0.0		0.0		0.0		0.0		0.0		0.0		0.0
4	>90		>90		63.2	2.1	>90		>90		>90		85.7	4.5
						2.5								2.3
5	6.8	0.0	7.0	0.0	5.6	0.3	6.6	0.0	14.1	2.3	18.5	0.3	7.2	0.1
		0.0		0.0		0.3		0.0		2.0		0.3		0.1
7	34.1	1.9	20.5	0.8	8.6	1.3	26.1	3.2	26.7	5.9	16.7	1.0	34.3	4.1
		1.8		0.8		1.1		2.8		4.9		1.0		3.7
8	40.8	5.5	31.1	5.8	>30		>30	0.0	46.8	2.9	>30	0.0	28.2	0.3
		4.8		4.9				0.0		2.8		0.0		0.3
9	7.7	0.1	8.4	0.6	4.0	0.7	8.2	0.4	13.5	2.7	9.4	0.4	12.5	1.3
		0.1		0.5		0.6		0.4		2.2		0.4		1.2
10	24.1	2.0	16.2	4.6	40.0	1.7	66.1	0.0	29.4	6.0	0.0	0.0	33.6	2.6
		1.9		3.6		1.6		0.0		5.0				2.4
11	15.6	1.3	14.7	1.3	17.8	4.3	17.8	0.5	12.8	1.5	16.3	0.9	21.4	0.7
		1.2		1.2		3.4		0.4		1.3		0.8		0.7
12	8.6	1.8	13.3	3.0	6.8	2.3	7.8	2.1	26.3	1.1	>30		30.5	1.6
		1.5		2.5		1.7		1.7		0.7				1.5
13	9.6	0.0	10.3	0.1	11.6	0.9	14.1	0.9	17.6	1.2	23.8	1.9	31.7	0.3
		0.0		0.1		0.8		0.8		1.1		1.8		0.3
14	17.7	0.6	20.6	2.2	15.3	3.1	20.2	0.0	25.4	0.4	22.2	2.1		
		0.6		2.0		2.6		0.0		0.4		1.9		
17	15.6	0.1	13.0	1.8	6.6	1.2	15.1	0.2	18.5	0.3	21.3	0.1	24.8	0.2
		0.1		1.6		1.0		0.2		0.3		0.1		0.2
18	21.2	0.2	23.3	0.5	17.5	1.2	25.6	1.3	15.3	1.4	25.8	1.7	25.8	0.0
		0.2		0.5		1.1		1.2		1.3		1.6		0.0
20	28.0	2.1	29.1	1.5	23.0	1.7	28.0	2.6	28.7	2.5	15.8	0.9	22.4	3.0
		2,0		1,7		1,8		2,4		2,3		0,8		2,7
24	73.2	1.6	41.9	6.5	19.8	5.7	69.1	3.9	>90	0.0	>90	0.0	>90	0.0
		1.6		5.6		4.5		3.7		0.0		0.0		0.0
25	>60		16.4	1.4	8.2	3.0	>60		>60	0.0	>60	0.0	>60	0.0
				0.8		2.2				0.0		0.0		0.0
28	78.4	2.5	>90		39.9	1.0	>90		92.8	0.3	>90		22.1	2.3
		3.8				0.9				0.3				3.9
29	n.D.	0.0	18.0		34.2	3.8	33.1		509.7		n.D.	0.0	>90	
		0.0				3.4						0.0		
30	n.D.	0.0	36.5	2.7	25.9	4.3	29.1	2.2	>90		n.D.	0.0	43.1	8.5
		0.0		2.5		3.7		2.7				0.0		7.1
31	n.D.	0.0	72.1	7.0	31.5	3.8	34.4	3.6	>90		n.D.	0.0	80.2	2.1
		0.0		6.4		3.4		3.2				0.0		1.9
32	n.D.	0.0	22.2	0.4	15.5	0.7	16.4	1.9	46.9	3.2	n.D.	0.0	29.2	4.4
		0.0		0.4		0.7		1.7		3.0		0.0		3.8
STP	0.2	0.0	0.2	0.0	0.2	0.0	0.6	0.0	0.2	0.0	0.4	0.0	0.2	0.0
		0.0		0.0		0.0		0.0		0.0		0.0		0.0

et al. [34]; these authors were able to show that the location of an α,β-unsaturated system ($\Delta^{9,11}$ or $\Delta^{12,13}$) is insignificant for obtaining good cytotoxicity.

However, a comparison of IC_{50} values is not sufficient to describe the biological activity of these compounds. To get a deeper insight, some representative molecules were selected and submitted to several MOA investigation assays applying the same concentration (30 μM) for all compounds. Fig. 2 shows some results from cell cycle investigations.

To evaluate whether the activity (as shown in the SRB assay) is caused by a cytostatic effect or rather by a cytotoxic effect, living ovarian cancer cells were investigated after 24 h of treatment with the compounds. A significant G1/G0 arrest (as previously found for parent maslinic acid, unpublished data) or ursolic acid [14], was not detected at all. A weak G0/G1 arrest (<20%) was observed for compounds **8** and **25**. Compounds **4**, **5** or **7**, however, showed practically no influence onto the cell cycle. The treatment of the cells with compounds **1**, **9** or **24** led to a reduction of the G2/M phase; compounds **1** or **24** were the most active compounds of this series.

Obviously at least compounds **4**, **5** and **7** are cytotoxic, and hence investigations of the dead cells seemed highly interesting. Usually an undesired necrotic cell death (happening most accidentally) is described by a loss of the integrity of the cell membrane [35]. The integrity of the cell membrane can be investigated by a microscopic fluorescence dye-exclusion assay (AO/PI; Fig. 3). In this test, acridine orange (AO) as an un-polar green fluorescence dye permeates the cell membrane and stains dead cells possessing an intact membrane [36]. Thus, cells having died by activating a programmed cell death show green fluorescence. Propidium iodide (PI) [37], however, a double positive charged dye, stains only dead cells with a broken cell membrane; hence, a deep red color is observed.

As depicted in Fig. 4, all dead cells had died due to a programmed process. Several death mechanisms are known [38]; for

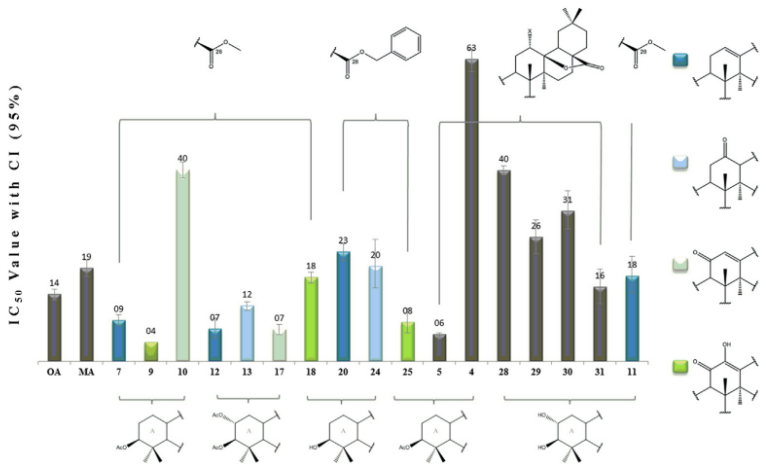


Fig. 1. IC_{50} values (in μM from SRB with a confidence interval (95%) for **OA**, **MA** and derivatives utilizing the human ovarian cancer cell line A2780. The colors of the bars indicate the type of modification at positions C-3 and C-28. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

triterpenoids most commonly apoptosis [8] as well as autophagy [39] was reported.

Microscopic inspection of the dead cells allowed a preliminary evaluation: membrane blebbing and condensed nuclei as well as cell shrinking indicated apoptosis [40]. Staining the cells with AO/PI gave red dots – a sign for at least partial autophagy [41]. Fig. 3 depicts representative microscopic views of the cells, and significant morphologic changes have been marked using different colored arrows. One unique feature of apoptosis is the activation of ICAD [42]; this results in the degradation of DNA into several 178 bp long pieces (Fig. 3). After 24 h of treatment only compounds **5** and **17** showed significant DNA ladders as indicated by electrophoresis and staining of the DNA. In the electrophoretic gels for compounds **1**, **9** and **10** only weak ladders were found.

Membrane integrity and DNA-laddering are typical hallmarks for an apoptotic death; another hallmark of apoptosis is the translocation of phosphatidylserine. This compound is located on the inner side of the cell and translocates to the outer cell membrane during apoptosis thus acting as a signal for macrophages [43]. During apoptosis several executing proteinases, also known as caspases, are activated in a highly controlled manner. During this lethal caspase-cascade process other enzymes get activated and/or essential parts of the cell skeleton are broken down leading to a subsequent exposition of the phosphatidylserine. This process can be measured using an annexin V assay. By double staining and submitting the stained cell population to a FACS measurement the distribution of vital *versus* dead cells can be determined. Fig. 4 shows the result of one of these assays. The experimental data were evaluated by plotting the green fluorescence (belonging to the annexin V-FITC) *versus* the red one (belonging to propidium iodide) and splitting these plots into four quadrants. While PI stained only cells possessing a leak membrane, red labeled cells (upper left) are usually regarded as necrotic cells. Cells carrying both signals (upper right) are assumed to be secondary necrotic; this phenomenon is known to occur for cells held in cell cultures.

As indicated in Fig. 4, the treatment of the cells with **17** or **18** triggered apoptosis after incubating for 24 h in >50% of the cell population. In addition, 3,12-diacetylated **5** as well as compound **9** triggered apoptosis. These results are in excellent agreement to the results from the DNA ladder assays; therein, after treatment of the cells with **5**, **9**, **17** or **18** the typical DNA ladders were found. Furthermore, the ability of **9**, **17** and **18** to trigger apoptosis affirms that the presence of an α,β-unsaturated system in ring C seems necessary for inducing apoptosis very quickly.

The ability to trigger apoptosis is independent from the IC_{50} values; although compound **9** exhibited a lower IC_{50} value in the SRB tests than compound **18**, the latter of which showed a significantly higher ability to trigger apoptosis. The drop down of cytotoxicity found for compounds **24** and **25** might be explained by the low polarity of these compounds. The presence of an increased necrotic population after having treated the cells with **9** might be due to a concentration dependent toxic effect. Hence, **OA** methyl esters possessing a modified ring C are promising compounds possessing the ability to induce programmed cell death very quickly.

4. Conclusion

A convenient method for separating a mixture of regioisomeric triterpenoids **UA**/**OA** was found. This separation technique using per-acids was shown to be quite universal. **UA** could be recovered very easily from the mixtures, and the intermediates were transformed into compounds showing promising antitumor activity. In addition, different MOAs were found for compounds differing only slightly in their structure. As far as the OA derived lactone 1 is concerned, the introduction of an additional hydroxy substituent at position C-2 (as in 28) as well as of a lipophilic moiety at position C-12 (as in 5) decreases the IC50 values significantly. An additional oxygen substituent in ring C increases the cytotoxicity, too. Thus, the highest activity was determined for the previously unknown 11-oxo-12-hydroxy-olean-12-enes **17** and **18**, respectively.

5. Experimental part

5.1. General – chemistry

Reagents were bought from commercial suppliers without any further purification. Melting points were measured with a LEICA hot stage microscope and were not corrected. NMR spectra were recorded on VARIAN Gemini 2000 or Unity 500 spectrometers at

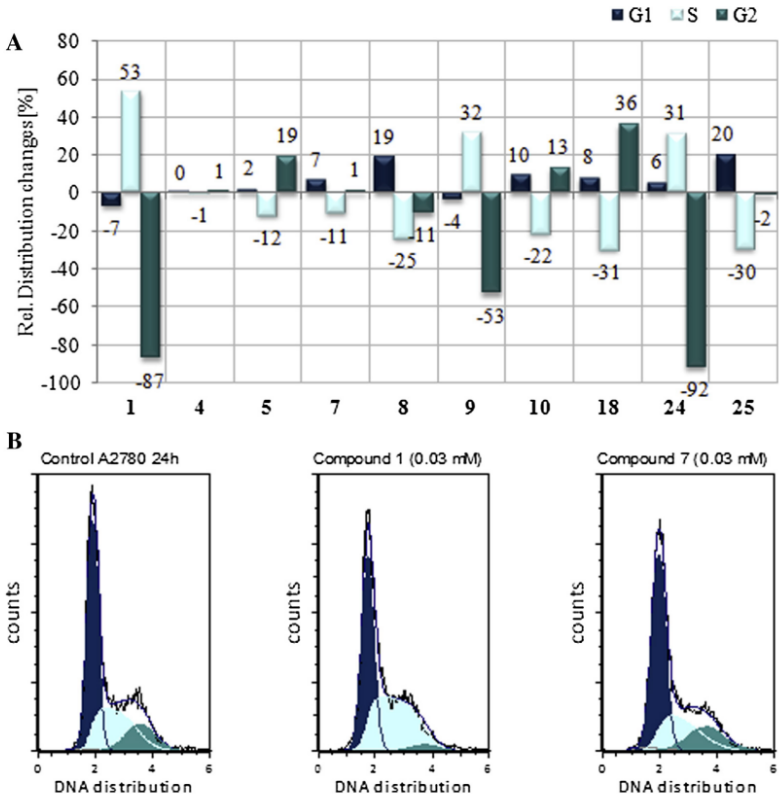


Fig. 2. Cell cycle investigations. Ovarian cancer cells (A2780) were treated for 24 h with a 30 μM solution of compounds **1**, **4**, **5**, **7**–**10**, **18**, **24** or **25**, respectively. The living cells were harvested, fixed, the population was aligned and submitted to a FACS supported measurement. The DNA was dyed with PI after treatment with RNAse. In the upper part of the figure (A) the relative changes to control have been depicted; the lower part (B) shows typical graphs for the cell cycle distribution.

27 °C (chemical shifts δ are given in ppm and J in Hz. Mass spectra were taken on a FINNIGAN MAT TSQ 7000 (electrospray, voltage 4.5 kV, sheath gas nitrogen) instrument. Elemental analyses were measured on a Foss-Heraeus Vario EL unit. IR spectra were recorded on a Perkin Elmer FT-IR spectrometer Spectrum 1000, optical rotations on a Perkin Elmer 341 polarimeter (1 cm micro cell, 25 °C) and UV–vis spectra on a Perkin Elmer unit, Lambda 14. Elemental analysis was performed for all new compounds and correct values (±0.3 for C, H, N) were obtained. TLC was performed on silica gel (Merck 5554, detection with ceriummolybdate spray reagent). Solvents were dried according to usual procedures. The purity of the compounds was checked by HPLC/DAD (Prontosil C18, MeOH/H_2O 95/5, 1% H_3PO_4) and found to be >98% for each compound.

5.2. General procedures

5.2.1. Oxidation with hydrogen peroxide and formic acid (method A)

To a mixture of ursolic acid and oleanolic acid (1 equiv., 2:1) in DCM, formic acid (concd, 2 equiv.)/H_2O_2 (30%, 2 equiv.) was added, and the mixture was stirred at 25 °C for 24 h. After washing with an aq soln. of sodium thiosulfate, and usual work up, the solvents were evaporated under diminished pressure. The residue was subjected to chromatography (silica gel, hexane/ethyl acetate).

5.2.2. Oxidation with mCPBA (method B)

To a mixture of ursolic acid and oleanolic acid (1 equiv., 2:1) in DCM *m*CPBA (1.5 equiv.) was added, and the mixture was stirred at 25 °C for 24 h. After washing with an aq soln. of sodium thiosulfate and usual workup, the solvents were evaporated under diminished pressure. The residue was subjected to chromatography (silica gel, hexane/ethyl acetate).

5.2.3. Acetylation (method C)

Acetylation was performed in dry DCM (100 mL) with acetic anhydride (2 equiv.) and triethylamine (0.3 equiv.) for 12 h at 24 °C and gave, after usual work-up and recrystallization from ethanol, the product.

5.2.4. Esterfication (method D)

The starting material (1 equiv.) was dissolved in dry DMF (5 mL), and finely grounded potassium carbonate (5 equiv.) was added. After 60 min of stirring at room temperature, the alkylbromide (2

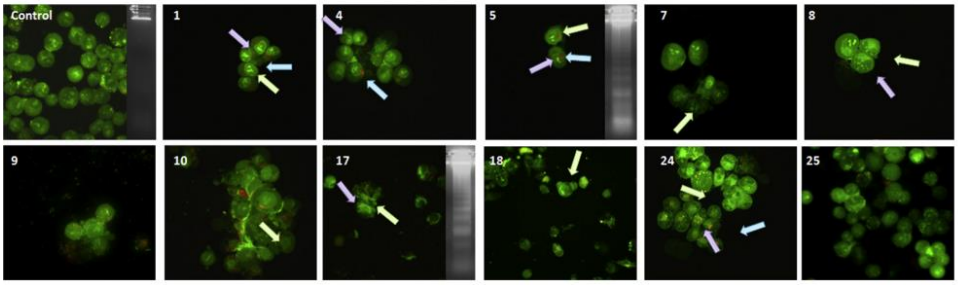


Fig. 3. Dye exclusion (AO/PI) assay and representative DNA laddering experiments. Ovarian cancer cells (A2780) were treated with the compounds **1**, **4**, **5**, **7**–**10**, **17**, **18**, **24** or **25** (30 μM each, 24 h). The floating (dead) cell cells were collected, washed with PBS and submitted to a fluorescence microscopic investigation. Green dyed cells are typical for a programmed, non-necrotic death. Light green colored arrows indicate typical membrane blebbing; light purple arrows indicate the typical membrane blebbing of the nucleus membrane, and the blue arrows point to orange colored dots which could be autophagosomes and indicate an autophagy mediated apoptosis. In addition, the results from the DNA laddering experiment are shown for a control experiment as well as for the experiments using compounds **5** and **17**, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

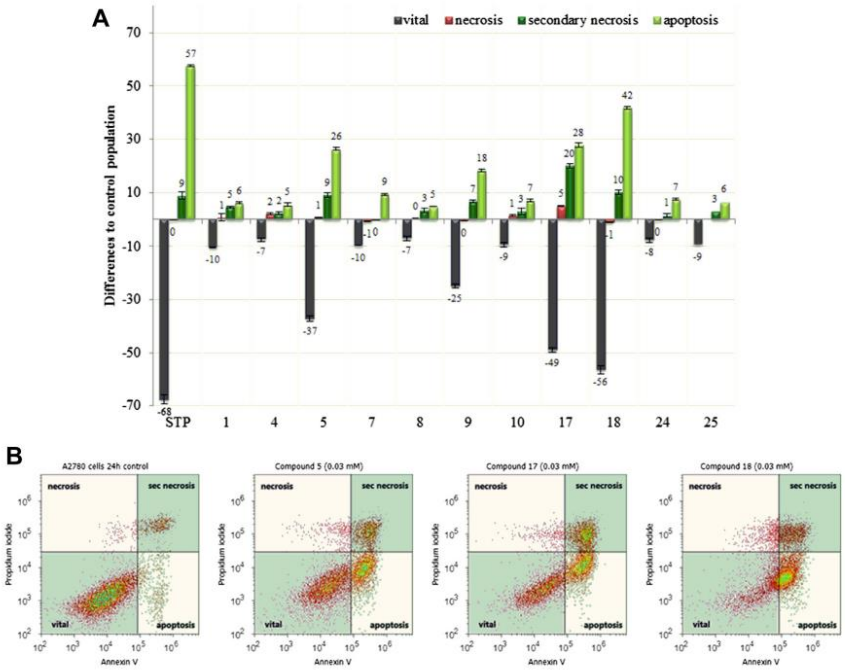


Fig. 4. Annexin V/PI assay: Ovarian cancer cells (A2780) were treated with compounds **1**, **4**, **5**, **7**–**10**, **17**, **18**, **24** or **25** (30 μM each), respectively. After 24 h, all cells were harvested and submitted to a FACS based assay. Diagram A (upper part) shows the relative changes in the distribution compared to control. Measurements were performed as technical replicates. Typical samples of these experiments are depicted in the lower part (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

equiv.) was added, and stirring was continued for an additional 18 h. The mixture was poured into an ice cold solution of aq HCl (3.7%, 50 mL), and the white precipitate was filtered off. Chromatographic purification (silica gel, hexane/ethyl acetate, 7:3) and recrystallization (ethanol) afforded the product.

5.2.5. Oxidation with selenium dioxide (method E)

Equal molar amounts of selenium dioxide and the 12-oxo compound were stirred in refluxing 1,4-dioxane for 24 h. After filtration, an aq solution of sodium thiosulfate was added, and the reaction mixture was extracted with DCM. After following usual

workup, the crude product was subjected to a chromatographic purification followed by recrystallization from ethanol.

5.2.6. Deacetylation (method F)

The acetylated starting material (1 equiv.) was dissolved in methanol. Finally grounded KOH (1.2 equiv.) was added, and the mixture was stirred at room temperature until completed as checked by TLC. The mixture was poured into a cold aq solution of HCl (3.7%), and the precipitate was collected. Usual work up furnished the product.

5.3. Biological material and procedures

5.3.1. Cell lines and culture conditions

The cell lines 518A2, 8505C, A2780, A549, HT29, MCF-7, NiH 3T3 and WW030272 were included in this study. Cultures were maintained as monolayer in RPMI 1640 (PAA Laboratories, Pasching, Germany) supplemented with 10% heat inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

5.3.2. Cytotoxicity assay, dye-exclusion assay, DNA-laddering and apoptosis assay

Instrumentation, cell lines and culture conditions, cytotoxicity assay, dye exclusion tests and DNA fragmentation was performed as previously described [44,45].

5.3.3. Cell cycle investigation

Approximately $1*10^6$ cells (HT29, A2780 or NIH 3T3) were seeded in cell culture flasks (25 cm^2), and the cells were allowed to grow for 24 h. After removing of the used medium, the substance loaded medium was reloaded (or a blank fresh medium as a control). After 24 or 48 h, the living cells were harvested, washed with PBS (with Mg^{2+} and Ca^{2+}) twice and fixed with ethanol (70%, 4 °C, 1 h). After removing of the fixation and permeabilization agent, the cells were washed with PBS buffer (with Mg^{2+} and Ca^{2+}, containing 1% BSA and 0.1% NaN_3, 3 × 1 mL, 1000 rpm) and adjusted to $1*10^5$ million cells. The pellet was gently suspended in staining buffer (PBS buffer containing BSA, RNAase, NaN_3 and PI following the procedure of Darzynkiewicz et al. [41]) and incubated for 30 min at 37 °C. Analyses were performed using the Attune® FACS machine; collecting data from the BL-2A channel. Doublet cells were excluded from the measurements by plotting BL-2A against BL-2H. For each cell cycle distribution 20.000 events were collected. Distribution was calculated by the method of Dean et al. [46].

5.3.4. Annexin V/PI assay

Approx. 500,000 cells (A2780) were seeded in cell culture flasks and were allowed to grow for 1 day. After removing of the medium, the substance loaded medium was added, and the flasks were incubated for about 24 h. All cells were harvested, centrifuged (1200 rpm, 5 min) and washed twice (PBS (w/w). Approx. 100,000 cells were washed with annexin V bounding buffer (BD) and treated with a propidium iodide solution (3 μL, 50 μg/mL) and Annexin V (5 μL, life technologies™) for 15 min at room temperature in the dark. After adding Annexin V bounding buffer (400 μL), the suspension was submitted to a FACS measurement. Calculation was performed as suggested from the supplier (BD Biosciences®).

5.4. Syntheses

*5.4.1. Ursolic acid (**UA**)*

By method **A** or **B**, **UA** was obtained from a mixture of **UA**/**OA** (ca. 2:1); yield: 57%; m.p.: 257–259 °C (lit.: [47]: 258°); R_F = 0.62 (*n*-hexane/ethyl acetate, 5:3); ^{1}H NMR (400 MHz, d_6 – pyridine): δ = 5.45 (*dd*, *J* = 3.5, 3.5 Hz, 1H, C*H* (12)), 3.43 (*dd*, *J* = 9.6, 6.5 Hz, 1H, C*H* (3)), 2.60 (*d*, *J* = 11.2 Hz, 1H, C*H* (18)), 2.29 (*ddd*, *J* = 13.3, 13.3, 4.5 Hz, 1H, CH_a (15)), 2.09 (*ddd*, *J* = 13.3, 13.3, 4.1 Hz, 1H, CH_a (16)), 1.98–1.90 (*m*, 5H, CH_b (16) + CH_2 (7) + CH_2 (11)), 1.88–1.77 (*m*, 2H, CH_2 (2)), 1.62–1.51 (*m*, 4H, C*H* (9) + CH_a (6) + CH_a (22) + CH_b (1)), 1.46–1.39 (*m*, 3H, C*H* (19) + CH_2 (21)), 1.38–1.25 (*m*, 2H, CH_b (6) + CH_b (22)), 1.21 (*s*, 3H, CH_3 (27)), 1.20 (*s*, 3H, CH_3 (23)), 1.23–1.11 (*m*, 1H, CH_b (15)), 1.02 (*s*, 3H, CH_3 (30)), 0.99 (*s*, 3H, CH_3 (25)), 0.97 (*d*, 3H, ^3J = 6.8 Hz, CH_3 (29)), 0.92 (*d*, 3H, ^3J = 6.1 Hz, CH_3 (24)), 0.86 (*s*, 3H, CH_3 (26)), 0.82 (*m*, 1H, C*H* (5)) ppm; ^{13}C NMR (400 MHz, d_6 – pyridine): δ = 180.1 (*C*=O, C28), 139.5 (*C*=CH, C13), 125.9 (*C*H=C, C12), 78.4 (*C*HOH, C3), 56.1 (*C*H, C5), 53.8 (*C*H, C18), 48.3 (*C*H, C9), 48.3 (C_{quart}, C17), 42.7 (C_{quart}, C14), 40.2 (C_{quart}, C8), 39.7 (*C*H, C19), 39.7 (*C*H, C20), 39.6 (C_{quart}, C4), 39.3 (CH_2, C1), 37.7 (CH_2, C22), 37.5 (C_{quart}, C10), 33.8 (CH_2, C7), 31.3 (CH_2, C21), 29.1 (CH_3, C23), 28.9 (CH_2, C15), 28.4 (CH_2, C2), 25.2 (CH_2, C16), 24.2 (CH_3, C27), 23.8 (CH_2, C11), 21.7 (CH_3, C30), 19.0 (CH_2, C6), 17.7 (CH_3, C29), 17.7 (CH_3, C26), 16.8 (CH_3, C24), 15.9 (CH_3, C25) ppm; ESI-MS (MeOH): *m*/*z* = 456.6 (44%, $[M - H]^-$), 501.2 (100%, $[M + HCO_2]^-$), 911.0 (44%, $[2M - H]^-$).

*5.4.2. (3β, 12α) 3,12 Dihydroxy-18β-olean-28-oic acid 28,13-lactone (**1**)*

By using method **A** or **B**, compound **2** was obtained from a mixture of **UA**/**OA** (ca. 2:1); yield: 10%; m.p.: 249–252 °C (lit.: [32]: 247–249 °C); R_F = 0.25 (hexane/ethyl acetate, 5:3); $[\alpha]_D$ = +32.4° (*c* 0.35, $CHCl_3$); IR (KBr): ν = 3668*w*, 3524*s*, 2947*s*, 2867*s*, 1736*s*, 1470*m*, 1396*m*, 1362*m*, 1306*w*, 1255*m*, 1226*m*, 1176*w*, 1146*m*, 1092*w*, 1077*m*, 1060*m*, 1037*m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.83–3.80 (*dd*, *J* = 2.8, 2.8 Hz, 1H, C*H* (12)), 3.15 (*dd*, *J* = 11.4, 4.4 Hz, 1H, C*H* (3)), 2.07 (*ddd*, *J* = 13.3, 5.8, 5.8 Hz, 1H, CH_a (16)), 2.01–1.88 (*m*, 3H, C*H* (18) + CH_a (19) + CH_a (11)), 1.86–1.75 (*m*, 2H, CH_b (19) + CH_a (15)), 1.66 (*ddd*, *J* = 12.8, 12.8, 3.5 Hz, 1H, CH_a (1)), 1.59–1.51 (*m*, 3H, CH_2 (2) + CH_a (22)), 1.53–1.48 (*m*, 1H, C*H* (9)), 1.49–1.44 (*m*, 2H, CH_a (6) + CH_a (7)), 1.42–1.37 (*m*, 2H, CH_b (11) + CH_b (16)), 1.35–1.30 (*m*, 1H, CH_b (21)), 1.24 (*s*, 3H, CH_3 (27)), 1.22–1.15 (*m*, 4H, CH_2 (15) + CH_b (7) + CH_b (6)), 1.12–1.09 (*m*, 1H, CH_b (22)), 1.08 (s, 3H, CH_3 (26)), 0.92 (*s*, 3H, C*H* (23)), 0.91 (*s*, 3H, C*H* (29)), 0.92–0.87 (*m*, 1H, CH_b (21)), 0.83 (*s*, 3H, C*H* (30)), 0.81 (*s*, 3H, C*H* (25)), 0.71 (*s*, 3H, C*H* (24)), 0.68 (*dd*, *J* = 11.8, 1.9 Hz, 1H, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 179.9 (*C*=O, C28), 90.6 (C_{quart}, C13), 78.9 (*C*HOH, C3), 76.4 (CH, C12), 55.2 (*C*H, C5), 51.1 (CH, C18), 44.7 (C_{quart}, C17), 44.6 (*C*H, C9), 42.3 (C_{quart}, C14), 42.1 (C_{quart}, C8), 39.4 (CH_2, C19), 38.9 (CH_2, C1), 38.9 (C_{quart}, C4), 36.5 (C_{quart}, C10), 34.2 (CH_2, C21), 34.0 (CH_2, C7), 33.3 (CH_3, C29), 31.6 (C_{quart}, C20), 28.8 (CH_2, C11), 28.1 (CH_3, C23), 28.1 (CH_2, C15), 28.0 (CH_2, C22), 27.5 (CH_2, C2), 23.9 (CH_3, C30), 21.2 (CH_2, C16), 18.6 (CH_3, C27), 18.5 (CH_3, C26), 17.8 (CH_2, C6), 16.3 (CH_3, C25), 15.4 (CH_3, C24) ppm; MS (ESI, MeOH): *m*/*z* = 473.5 (27%, $[M + H]^+$), 495.6 (15%, $[M + Na]^+$), 527.1 (26%, $[M + Na + MeOH]^+$), 967.3 (100%, $[2M + Na]^+$).

*5.4.3. (3β) 3-Acetoxy-urs-11-en-28-acid (**2**)*

Compound **2** was obtained as a colorless solid using method **A** from a mixture of acetylated **2** and **3.** The latter compounds were obtained either using method **C** or directly from **UA** applying method **C**; yield: 88%; m.p.: 257–259 °C (lit.: 258 °C [47]); $[\alpha]_D$ = +69° (*c* 0.19, $CHCl_3$); R_F = 0.64 (*n*-hexane/ethyl acetate, 8:2); UV–Vis (MeOH): λ_{max} (log ε) = 224 nm (3.71); IR (KBr): ν = 3423*br*, 2947*s*, 1736*s*, 1696*s*, 1464*m*, 1366*m*, 1245*s*, 1180*w*, 1148*w*, 1097*w*, 1076*w*, 1028*m*, 1009 *m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.26 (*m*, 1H, C*H* (12)), 4.48 (*dd*, *J* = 9.4, 7.0 Hz, 1H, C*H* (3)), 2.80 (*dd*, *J* = 3.8, 4.3 Hz, 1H, C*H* (18)), 2.08 (*s*, 3H, Ac), 1.97 (*ddd*, *J* = 13.7, 13.5, 4.0 Hz, 1H, CH_a (16)), 1.90–1.84 (*m*, 2H, CH_2 (11)), 1.76 (*ddd*, *J* = 14.0, 13.8, 4.3 Hz, 1H, CH_a (22)), 1.69 (*ddd*, *J* = 13.8, 13.8, 3.7 Hz, 1H, CH_a (15)),

1.64–1.49 (*m*, 8H, *CH* (9) + CH_a (1) + CH_a (19) + CH_a (6) + CH_b (22) + CH_a (16) + CH_2 (2)), 1.45–1.22 (*m*, 5H, CH_b (6)+ CH_2 (21) + CH_2 (7)), 1.20–1.02 (*m*, 3H, CH_a (19) + CH_b (1) + CH_b (15)), 1.12 (*s*, 3H, CH_3 (27)), 0.93 (*s*, 3H, CH_3 (23)), 0.91 (*s*, 3H, CH_3 (30)), 0.89 (*s*, 3H, CH_3 (29)), 0.86–0.81 (*m*, 1H, *CH* (5)), 0.85 (*s*, 3H, CH_3 (25)), 0.84 (*s*, 3H, CH_3 (26)), 0.74 (*s*, 3H, CH_3 (24)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 184.4 (*C*=O, C28), 171.1 (*C*=O, Ac), 143.5 (*C*=CH, C13), 122.5 (*CH*=C, C12), 80.9 (CHOH, C3), 55.3 (*CH*, C5), 47.5 (CH, C9), 46.5 (C_{quart}, C17), 45.8 (CH_2, C19), 41.5 (C_{quart}, C14), 40.9 (CH, C18), 39.3 (C_{quart}, C8), 38.0 (CH_2, C1), 37.6 (C_{quart}, C4), 36.9 (C_{quart}, C10), 33.7 (CH_2, C21), 33.0 (CH_3, C29), 32.5 (CH_2, C7), 32.4 (CH_2, C22), 30.6 (C_{quart}, C20), 28.0 (CH_3, C23), 27.6 (CH_2, C15), 25.9 (CH_3, C27), 23.6 (CH_3, C30), 23.5 (CH_2, C11), 23.4 (CH_2, C2), 22.8 (CH_2, C16), 21.3 (CH_3, Ac), 18.1 (CH_2, C6), 17.0 (CH_3, C24), 16.6 (CH_3, C26), 15.3 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 497.7 (100%, $[M - H]^-$), 543.3 (50%, $[M + HCO_2^-]^-$).

*5.4.4. (3β) 3-Acetoxy-olean-11-en-28-acid (**3**)*

Compound **3** was obtained as colorless needles applying method **C** starting from **OA**; yield: 95%; m.p.: 257–261 °C, (lit.: 257–259 °C [48]); $[\alpha]_D$ = +65° (*c* 0.33, $CHCl_3$) [lit.: +74° (*c* 1.0; $CHCl_3$ [49])]; R_F = 0.60 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:20:30:4); IR (KBr): ν = 3219*br*, 2942*s*, 1731*s*, 1680*m*, 1466*m*, 1370*m*, 1252*s*, 1179*m*, 1161*m*, 1127*w*, 1026*m*, 1010 *m* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.20 (*dd*, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 4.47 (*dd*, *J* = 8.5, 7.3 Hz, 1H, *CH* (3)), 2.80 (*dd*, *J* = 13.7, 4.1 Hz, 1H, *CH* (18)), 2.02 (*s*, 3H, Ac), 1.95 (*ddd*, *J* = 13.4, 13.4, 3.9 Hz, 1H, CH_a (16)), 1.90–1.77 (*m*, 2H, CH_2 (11)), 1.73 (*ddd*, *J* = 14.9, 14.6, 4.4 Hz, 1H, CH_a (7)), 1.67 (*ddd*, *J* = 13.3, 13.3, 4.1 Hz, 1H, CH_a (15)), 1.54–1.34 (*m*, 8H, CH_a (1) + CH_b (16) + CH_a (19) + *CH* (9) + CH_a (6) + CH_a (22) + CH_2 (2)), 1.34–1.11 (*m*, 5H, CH_b (7) + CH_b (6) + CH_2 (21) + CH_b (22)), 1.10 (*s*, 3H, CH_3 (27)), 1.08–0.93 (*m*, 3H, CH_b (19) + CH_b (15) + CH_b (1)), 0.92 (*s*, 3H, CH_3 (25)), 0.90 (*s*, 3H, CH_3 (30)), 0.88 (*s*, 3H, CH_3 (29)), 0.84 (*s*, 3H, CH_3 (23)), 0.86–0.74 (*m*, 1H, *CH* (5)), 0.83 (*s*, 3H, CH_3 (26)), 0.72 (*s*, 3H, CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 184.3 (*C*=O, C28), 171.0 (*C*=*O*, C31), 143.6 (*C*=CH, C13), 122.5 (*CH*=C, C12), 80.9 (CHOAc, C3), 55.3 (CH, C5), 47.5 (CH, C9), 46.5 (C_{quart}, C17), 45.8 (CH_2, C19), 41.5 (C_{quart}, C14), 40.8 (CH, C18), 39.2 (C_{quart}, C8), 38.0 (CH_2, C1), 37.6 (C_{quart}, C4), 36.9 (C_{quart}, C10), 33.7 (CH_2, C21), 33.0 (CH_3, C29), 32.5 (CH_2, C7), 32.4 (CH_2, C22), 30.6 (C_{quart}, C20), 28.0 (CH_3, C23), 27.6 (CH_2, C15), 26.9 (CH_2, C2), 25.8 (CH_3, C27), 23.6 (CH_3, C30), 23.5 (CH_2, C16), 23.3 (CH_2, C11), 21.2 (CH_3, Ac), 18.1 (CH_2, C6), 17.1 (CH_3, C24), 16.6 (CH_3, C26), 15.3 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 497.5 (100%, $[M - H]^-$), 543.1 (66%, $[M + HCO_2]^-$), 995.1 (64%, $[2M - H]^-$), 1017.5 (16%, $[2M - 2H + Na]^-$).

*5.4.5. (3β, 12α) 3-Acetyl-12-hydroxy-18β-olean-28-oic acid 28,13-lactone (**4**)*

Compound **4** was obtained from a mixture of **2** and **3** or directly from **1** using method **C**; yield: 54.4% (from **1**); m.p.: 295–298 °C (lit.: [50]: 261–263 °C; lit.: [51]: 285–287 °C); R_F = 0.36 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:20:30:4); $[\alpha]_D$ = +44.4° (*c* 0.34, $CHCl_3$), IR (KBr): ν = 3528 *brs*, 3448*vs*, 2948*s*, 1736*vs*, 1468*m*, 1384*s*, 1248*s*, 1060*m*, 1028 *m* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.48 (*dd*, *J* = 10.1, 6.2 Hz, 1H, *CH* (3)), 3.87 (*dd*, *J* = 2.7, 2.7 Hz, 1H, *CH* (12)), 2.13 (*ddd*, *J* = 13.3, 13.3, 5.8 Hz, 1H, *CH* (16)), 2.03 (*s*, 3H, CH_3 (Ac)), 2.06–1.84 (*m*, 4H, *CH* (18) + CH_2 (19) + CH_a (7)), 1.84 (*ddd*, *J* = 13.5, 13.5, 6.1 Hz, 1H, CH_a (15)), 1.76–1.66 (*m*, 1H, CH_a (1)), 1.69–1. 1.52 (m, 6H, *CH* (9) + CH_a (22) + CH_2 (11) + CH_2 (2)), 1.52–1.40 (m, 3H, CH_2 (6) + CH_b (7)), 1.39–1.32 (*m*, 1H, CH_b (22)), 1.30 (*s*, 3H, CH_3 (27)), 1.29–1.22 (*m*, 3H, CH_b (16) + CH_2 (21)), 1.19–1.12 (*m*, 1H, CH_b (15)), 1.14 (*s*, 3H, CH_3 (26)), 1.13–1.09 (*m*, 1H, CH_b (1)), 0.97 (*s*, 3H, CH_3 (30)), 0.89 (*s*, 6H, CH_3 (25) + CH_3 (29)), 0.86 (*s*, 3H, CH_3 (23)), 0.85 (*s*, 3H, CH_3 (24)), 0.85–0.82 (*m*, 1H, *CH* (5)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 180.2 (*C*=O, C28), 171.3 (*C*=O, Ac), 90.8 (C_{quart}, C13), 81.0 (CHOH, C3), 76.3 (CHOH, C12), 55.4 (CH, C5), 51.2 (CH, C18), 44.8 (C_{quart}, C17), 44.6 (CH, C9), 42.4 (C_{quart}, C14), 42.2 (C_{quart}, C8), 39.5 (CH_2, C19), 38.6 (CH_2, C1), 38.0 (C_{quart}, C4), 36.5 (C_{quart}, C10), 34.3 (CH_2, C7), 34.1 (CH_2, C21), 33.4 (CH_3, C30), 31.7 (C_{quart}, C20), 28.9 (CH_2, C22), 28.2 (CH_2, C15), 28.3 (CH_3, C23), 27.6 (CH_2, C11), 24.0 (CH_3, C27), 23.7 (CH_2, C2), 21.4 (CH_3, Ac), 21.4 (CH_2, 16), 18.7 (CH_3, C29), 18.7 (CH_3, C26), 17.8 (CH_2, C6), 16.6 (CH_3, C24), 16.5 (CH_3, C25) ppm; MS (ESI): *m*/*z* = 527.3 (100%, $[M + H]^+$), 549.3 (10%, $[M + Na]^+$), 1053.2 (25%, $[2M + H]^+$), 1075.2 (25%, $[2M + Na]^+$).

*5.4.6. (3β, 12α) 3,12-Diacetoxy-olean-28-oic acid 28,13-lactone (**5**)*

From compound **4** following method **C**, **5** was obtained as fine colorless needles (from ethanol); yield: 76%; m.p.: 236–239 °C (lit.: 241–243 °C [50]); R_F = 0.80 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +60.6° (*c* 0.27, $CHCl_3$); IR (KBr): ν = 3448*brs*, 2938*s*, 1766*s*, 1746*m*, 1724*m*, 1474*m*, 1466*m*, 1458*m*, 1438*m*, 1394*m*, 1376*m*, 1246*m*, 1232*m*, 1044*m*, 1032*m*, 1012 *m* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.7 (*brs*, 1H, *CH* (12)), 4.48 (*dd*, *J* = 9.8, 6.3 Hz, 1H, *CH* (12)), 2.10–2.03 (*m*, 1H, *CH* (16)), 2.10 (*s*, 3H, CH_3 (Ac)), 2.04 (*s*, 3H, CH_3 (Ac)), 1.96–1.86 (*m*, 4H, *CH* (18) + CH_a (19) + CH_a (7) + CH_a (15)), 1.66–1.48 (*m*, 9H, CH_a (6) + CH_a (1) + CH_a (16) + *CH* (9) + CH_a (22) + CH_2 (11) + CH_2 (2)), 1.45–1.28 (*m*, 6H, CH_b (6) + CH_b (19) + CH_b (7) + CH_2 (21) + CH_b (16)), 1.27 (*s*, 3H, CH_3 (27)), 1.21 (*dd*, *J* = 14.5, 8.1 Hz, 1H, CH_b (22)), 1.16 (*s*, 3H, CH_3 (26)), 1.19–1.15 (*m*, 2H, CH_b (1) + CH_b (15)), 0.96 (*s*, 3H, CH_3 (30)), 0.88 (*s*, 3H, CH_3 (25)), 0.87 (*s*, 3H, CH_3 (29)), 0.84 (*s*, 3H, CH_3 (23)), 0.82 (*s*, 3H, CH_3 (24)), 0.91–0.88 (*m*, 1H, *CH* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 179.0 (*C*=O, C28), 171.1 (*C*=O, Ac), 169.4 (*C*=O, Ac), 89.4 (C_{quart}, C13), 80.7 (CHOH, C3), 55.3 (CH, C5), 50.2 (CH, C18), 45.3 (C_{quart}, C17), 44.6 (CH, C9), 42.3 (C_{quart}, C14), 42.2 (C_{quart}, C8), 39.5 (CH_2, C19), 38.4 (CH_2, C1), 37.8 (C_{quart}, C4), 36.4 (C_{quart}, C10), 33.9 (CH_2, C7), 34.1 (CH_2, C21), 33.3 (CH_3, C29), 31.5 (C_{quart}, C20), 27.9 (CH_2, C22), 27.8 (CH_2, C15), 27.4 (CH_3, C23), 25.2 (CH_2, C11), 23.8 (CH_3, C27), 23.5 (CH_2, C2), 21.4 (CH_3, Ac), 21.3 (CH_3, Ac), 21.1 (CH_2, 16), 18.5 (CH_3, C30), 18.4 (CH_3, C26), 17.6 (CH_2, C6), 16.4 (CH_3, C24), 16.3 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 579.3 (100%, $[M + Na]^+$), 1135.3 (100%, $[2M + Na]^+$).

*5.4.7. (3β) Methyl 3-acetoxy-urs-11-en-28-oate (**6**)*

Compound **6** was obtained as colorless needles either by using method **A** from a mixture of the methyl triterpenoates **6** and **7** or directly using method **D** starting from **2**; yield: 91%; m.p.: 243–246 °C (lit.: 243–245 °C [45,52]); R_F = 0.75 (hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +64.7° (*c* 0.30, $CHCl_3$) [lit [45,52].: +61 °C ($CHCl_3$)]; IR (KBr): ν = 3433*br*, 2942*s*, 2868*m*, 1734*s*, 1458*m*, 1385*m*, 1371*m*, 1312*w*, 1244*s*, 1202*m*, 1187*m*, 1167*w*, 1150*w*, 1114*w*, 1073*w*, 1027*m*, 1006*w* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$):δ = 5.23 (*m*, 1H, *CH* (12)), 4.48 (*dd*, *J* = 10.6, 5.5 Hz, 1H, *CH* (3)), 3.59 (*s*, 3H, CH_3), 2.17 (*d*, *J* = 11.3 Hz, 1H, *CH* (18)), 2.03 (*s*, 3H, Ac), 1.98 (*ddd*, *J* = 13.5, 13.4, 4.3 Hz, 1H, CH_a (16)), 1.89 (*dd*, *J* = 8.7, 3.4 Hz, 2H, CH_2 (11)), 1.85 (*ddd*, *J* = 13.6, 13.5, 4.3 Hz, 1H, CH_a (15)), 1.69–1.55 (*m*, 6H, CH_2 (7) + CH_a (1) + CH_b (16) + CH_2 (2)), 1.54–1.43 (*m*, 4H, *CH* (9) + CH_a (6) + CH_a (21) + CH_a (22)), 1.38–1.26 (*m*, 4H, *CH* (19) + CH_b (6) + CH_b (22) + CH_b (21)), 1.10–1.02 (*m*, 2H, CH_b (15) + CH_b (1)), 1.06 (*s*, 3H, CH_3 (27)), 0.99–0.95 (*m*, 1H, *CH* (20)), 0.93 (*s*, 3H, CH_3 (25)), 0.93 (*d*, *J* = 5.0 Hz, 3H, CH_3 (30)), 0.87–0.83 (*m*, 1H, *CH* (5)), 0.86 (*s*, 3H, CH_3 (23)), 0.85 (*d*, *J* = 5.4 Hz, 3H, CH_3 (29)), 0.84 (*s*, 3H, CH_3 (26)), 0.73 (*s*, 3H, CH_3 (24)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.0 (*C*=O, C28), 170.9 (*C*=O, Ac), 138.1 (*C*=CH, C13), 125.4 (*CH*=C, C12), 80.9 (CHOH, C3), 55.3 (*CH*, C5), 52.8 (*CH*, C18), 51.4 (CH_3, C31), 48.0 (C_{quart}, C17), 47.5 (CH, C9), 41.9 (C_{quart}, C14), 39.5 (C_{quart}, C8), 39.0 (CH, C19), 38.8 (CH, C20), 38.3 (CH_2, C1), 37.6 (C_{quart}, C4), 36.8 (CH_2, C21), 36.6 (C_{quart}, C10), 32.9 (CH_2, C7), 30.6 (CH_2, C22), 28.0 (CH_3, C23), 28.0 (CH_2, C15), 24.2 (CH_2, C16), 23.5 (CH_3, C27), 23.5 (CH_2,

C2), 23.3 (*C*H$_2$, C11), 21.3 (*C*H$_3$, Ac), 21.1 (*C*H$_3$, C29), 18.2 (*C*H$_2$, C6), 17.0 (*C*H$_3$, C30), 17.0 (*C*H$_3$, C24), 16.8 (*C*H$_3$, C26), 15.5 (*C*H$_3$, C25) ppm; MS (ESI, MeOH): m/z = 513.2 (40%, [M + H]$^+$), 535.5 (100%, [M + Na]$^+$).

*5.4.8. (3β) Methyl 3-acetoxy-olean-11-en-28-oate (**7**)*

Compound **7** was obtained from **3** as a colorless solid using method **D**; yield: 85%; m.p.: 220–221 °C (lit.: 219–220 °C [53]); [α]$_D$ = +66° (*c* 0.32, CHCl$_3$) [lit.: +69° (CHCl$_3$ [53])]; R_F = 0.70 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:20:30:4); IR (KBr): ν = 3428*br*, 2938*s*, 2861*m*, 1731*s*, 1451*m*, 1364*m*, 1266*m*, 1240*s*, 1162*m*, 1123*w*, 1038*m*, 1022 *m* cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$): δ = 5.28 (*dd*, *J* = 3.5, 3.5 Hz, 1H, C*H* (12)), 4.49 (*dd*, *J* = 8.9, 7.1 Hz, 1H, C*H* (3)), 3.62 (*s*, 3H, C*H*$_3$ (31)), 2.86 (*dd*, *J* = 13.9, 4.1 Hz, 1H, C*H* (18)), 2.04 (*s*, 3H, C*H*$_3$ (Ac)), 1.97 (*ddd*, *J* = 14.3, 5.6, 3.8 Hz, 1H, C*H*$_a$ (16)), 1.92–1.82 (*m*, 2H, C*H*$_a$ (2) + C*H*$_a$ (11)), 1.70 (*dd*, *J* = 13.8, 4.5 Hz, 1H, C*H*$_a$ (19)), 1.67–1.57 (*m*, 6H, C*H*$_a$ (1) + C*H*$_b$ (11) + C*H*$_b$ (2) + C*H*$_b$ (16) + C*H*$_a$ (15) + C*H*$_a$ (7)), 1.57–1.47 (*m*, 2H, C*H*$_a$ (22) + C*H*$_a$ (6)), 1.51 (*dd*, *J* = 10.9, 3.8 Hz, 1H, C*H* (9)), 1.47–1.40 (*m*, 2H, C*H*$_b$ (7) + C*H*$_b$ (6)), 1.34 (*ddd*, *J* = 14.7, 10.5, 5.9 Hz, 1H, C*H*$_a$ (21)), 1.28–1.14 (*m*, 3H, C*H*$_b$ (21) + C*H*$_b$ (19) + C*H*$_b$ (22)), 1.12 (*s*, 3H, C*H*$_3$ (27)), 1.10–0.94 (*m*, 2H, C*H*$_b$ (15) + C*H*$_b$ (1)), 0.93 (*s*, 3H, C*H*$_3$ (30)), 0.92 (*s*, 3H, C*H*$_3$ (25)), 0.90 (*s*, 3H, C*H*$_3$ (29)), 0.86 (*s*, 3H, C*H*$_3$ (23)), 0.85 (*s*, 3H, C*H*$_3$ (24)), 0.79 (*dd*, *J* = 12.9, 7.2 Hz, 1H, C*H* (5)), 0.72 (*s*, 3H, C*H*$_3$ (26)) ppm; ^{13}C NMR (125 MHz, CDCl$_3$): δ = 178.3 (*C*=*O*, C28), 170.9 (*C*=*O*, Ac), 143.8 (*C*=CH, C13), 122.2 (CH=*C*, C12), 80.9 (*C*HOAc, C3), 55.3 (*C*H, C5), 51.5 (*C*H$_3$, C31), 47.6 (*C*H, C9), 46.7 (*C*$_{quart}$, C17), 45.8 (*C*H$_2$, C19), 41.6 (*C*$_{quart}$, C4), 41.3 (*C*H, C18), 41.2 (*C*$_{quart}$, C8), 38.1 (*C*H$_2$, C1), 37.7 (*C*$_{quart}$, C14), 36.9 (*C*$_{quart}$, C10), 33.4 (*C*H$_2$, C21), 33.1 (*C*H$_3$, C29), 32.6 (*C*H$_2$, C7), 32.4 (*C*H$_2$, C22), 30.7 (*C*$_{quart}$, C20), 28.0 (*C*H$_3$, C23), 27.6 (*C*H$_2$, C15), 25.9 (*C*H$_3$, C27), 23.6 (*C*H$_3$, C30), 23.5 (*C*H$_2$, C2), 23.4 (*C*H$_2$, C16), 23.1 (*C*H$_2$, C11), 21.2 (*C*H$_3$, Ac), 18.2 (*C*H$_2$, C6), 16.8 (*C*H$_3$, C26), 16.7 (*C*H$_3$, C24), 15.3 (*C*H$_3$, C25) ppm; MS (ESI, MeOH): m/z = 535.5 (22%, [M + Na]$^+$), 1047.3 (100%, [2M + Na]$^+$).

*5.4.9. (3β) Methyl 3-acetoxy-12-oxo-olean-28-oate (**8**)*

Compound **7** was treated according to method **A** to yield **8**; yield 75%; R_F = 0.5 (*n*-hexane/ethyl acetate, 8:2); m.p.: 189–191 °C (lit.: 194 °C [54]; [α]$_D$ = −13.3° (*c* 0.54, CHCl$_3$; lit.: −12° (*c* 1.4, CHCl$_3$ [54]); IR (KBr): ν = 3426*br*, 2948*s*, 2866*m*, 1728*vs*, 1700*s*, 1466*m*, 1388*m*, 1368*m*, 1244*vs*, 1192*m*, 1162*m*, 1082*w*, 1030*m*, 1004 *m* cm^{-1}; UV–Vis (CHCl$_3$): λ$_{max}$ (log ε) = 278 nm (4.31); ^{1}H NMR (500 MHz, CDCl$_3$): δ = 4.47 (*dd*, *J* = 11.5, 4.9 Hz, 1H, C*H* (3)), 3.67 (*s*, 3H, C*H*$_3$ (31)), 2.81–2.76 (*ddd*, *J* = 13.4, 4.3, 3.5, 1H, C*H* (18)), 2.61 (*d*, *J* = 4.3 Hz, 1H, C*H* (13)), 2.23 (*dd*, *J* = 16.8, 5.1 Hz, 1H, C*H*$_a$ (11)), 2.14 (*dd*, *J* = 16.8, 3.5 Hz, 1H, C*H*$_b$ (11)), 2.04 (*s*, 3H, C*H*$_3$ (Ac)), 1.93 (*ddd*, *J* = 13.5, 3.6, 2.5 Hz, 1H, C*H*$_a$ (19)), 1.89 (*ddd*, *J* = 14.8, 14.8, 4.2 Hz, 1H, C*H*$_a$ (16)), 1.79 (*ddd*, *J* = 13.7, 13.7, 4.6 Hz, 1H, C*H*$_a$ (7)), 1.68–1.58 (*m*, 6H, C*H* (18) + C*H*$_a$ (15) + C*H*$_2$ (2) + C*H*$_a$ (6) + C*H*$_b$ (16)), 1.55 (*ddd*, *J* = 12.9, 7.5, 3.8 Hz, 1H, C*H*$_a$ (1)), 1.49–1.43 (*m*, 3H, C*H*$_b$ (6) + C*H*$_b$ (7) + C*H*$_a$ (22)), 1.36–1.28 (*m*, 2H, C*H*$_b$ (22) + C*H*$_a$ (21)), 1.27–1.17 (*m*, 2H, C*H*$_b$ (21) + C*H*$_b$ (19)), 1.06 (*ddd*, *J* = 13.3, 4.1, 1.8 Hz, 1H, C*H*$_b$ (15)), 1.04–0.98 (*m*, 1H, C*H*$_b$ (1)), 0.97 (*s*, 3H, C*H*$_3$ (30)), 0.96 (*s*, 3H, C*H*$_3$ (26)), 0.93 (*s*, 3H, C*H*$_3$ (27)), 0.90 (*s*, 3H, C*H*$_3$ (29)), 0.87 (*s*, 3H, C*H*$_3$ (25)), 0.86 (*s*, 3H, C*H*$_3$ (23)), 0.85 (*s*, 3H, C*H*$_3$ (24)), 0.84–0.80 (*m*, 1H, C*H* (5)) ppm; ^{13}C NMR (125 MHz, CDCl$_3$): δ = 211.7 (*C*=O, C12), 178.5 (*C*=O, C28), 171.0 (*C*=O, Ac), 80.6 (*C*HOAc, C3), 55.3 (*C*H, C5), 52.0 (*C*H$_3$, C13), 51.9 (*C*H, C31), 49.8 (*C*H, C9), 47.5 (*C*$_{quart}$, C17), 42.0 (*C*$_{quart}$, C14), 41.4 (*C*$_{quart}$, C8), 38.6 (*C*H$_2$, C11), 37.9 (*C*H$_2$, C1), 37.8 (*C*$_{quart}$, C10), 37.0 (*C*$_{quart}$, C4), 36.4 (*C*H$_2$, C19), 34.6 (*C*H$_2$, 21), 33.5 (*C*H$_3$, C29), 33.1 (*C*H$_2$, C22), 32.1 (*C*H, C18), 31.9 (*C*H$_2$, C7), 30.8 (*C*$_{quart}$, C20), 28.1 (*C*H$_3$, C23), 27.7 (*C*H$_2$, C15), 23.6 (*C*H$_2$, C2), 23.3 (*C*H$_3$, C30), 22.9 (*C*H$_2$, C16), 21.4 (*C*H$_3$, Ac), 20.7 (*C*H$_3$, C27), 18.3 (*C*H$_2$, C6), 16.6 (*C*H$_3$, C24), 16.3 (*C*H$_3$, C26), 15.4 (*C*H$_3$, C25) ppm; MS (ESI, MeOH): m/z = 529.1 (20%, [M + H]$^+$), 551.3 (100%, [M + Na]$^+$), 583.2 (17%, [M + Na + MeOH]$^+$).

*5.4.10. (3β) Methyl 3-acetoxy-12-hydroxy-11-oxo-olean-Δ-12,13-en-28-oate (**9**)*

By using method **E** compound **9** was obtained from **8**; yield 83%; R_F = 0.5 (n-hexane/ethyl acetate, 8:2); m.p.: 199–201 °C; [α]$_D$ = +112.7° (*c* 0.34, CHCl$_3$); IR (KBr): ν = 3454*br*, 2948*s*, 2868*m*, 1730*vs*, 1664*m*, 1636*s*, 1466*m*, 1390*m*, 1366*s*, 1306*m*, 1244*vs*, 1202*m*, 1164*m*, 1140*w*, 1120*w*, 1034*m* cm^{-1}; UV–Vis (CHCl$_3$): λ$_{max}$ (log ε) = 280.0 nm (4.51); ^{1}H NMR (500 MHz, CDCl$_3$): δ = 6.21 (*s*, 1H, O*H*), 4.50 (*dd*, *J* = 11.6, 4.9 Hz, 1H, C*H* (3)), 3.71–3.64 (*ddd*, *J* = 9.2, 9.2, 1.6, 1H, C*H* (18)), 3.63 (*s*, 3H, C*H*$_3$ (31)), 2.80 (*ddd*, *J* = 13.6, 13.6, 3.6 Hz, 1H, C*H*$_a$ (1)), 2.46 (*s*, 1H, C*H* (9)), 2.04 (*ddd*, *J* = 13.8, 13.8, 3.0 Hz, 1H, C*H*$_a$ (16)), 2.05 (*s*, 3H, C*H*$_3$ (Ac)), 1.76 (*ddd*, *J* = 13.9, 13.9, 4.4 Hz, 1H, C*H*$_a$ (7)), 1.73–1.67 (*m*, 2H, C*H*$_2$ (2)), 1.67–1.60 (*m*, 2H, C*H*$_b$ (7) + C*H*$_b$ (16)), 1.60–1.55 (*m*, 3H, C*H*$_a$ (21) + C*H*$_a$ (15) + C*H*$_a$ (6)), 1.43–1.36 (*m*, 5H, C*H*$_2$ (19) + C*H*$_a$ (22) + C*H*$_b$ (21) + C*H*$_b$ (6)), 1.36 (*s*, 3H, C*H*$_3$ (27)), 1.29–1.23 (*m*, 1H, C*H*$_b$ (22)), 1.20 (*ddd*, *J* = 14.0, 14.0, 3.4 Hz, 1H, C*H*$_b$ (15)), 1.13 (*s*, 3H, C*H*$_3$ (25)), 1.10 (*ddd*, *J* = 13.3, 13.3, 4.1 Hz, C*H*$_b$ (1)), 1.00 (*s*, 3H, C*H*$_3$ (30)), 0.93 (s, 3H, C*H*$_3$ (29)), 0.92 (*s*, 3H, C*H*$_3$ (26)), 0.87 (*s*, 6H, C*H*$_3$ (23) + C*H*$_3$ (24)), 0.85–0.77 (*m*, 1H, C*H* (5)) ppm; ^{13}C NMR (125 MHz, CDCl$_3$): δ = 195.5 (*C*=O, C11), 178.0 (*C*=O, C28), 171.1 (C=O, Ac), 142.3 (*C*OH=C, C12), 136.9 (*C*=CHOH, C13), 80.6 (*C*HOH, C3), 60.5 (*C*H, C9), 55.2 (*C*H, C5), 52.0 (*C*H$_3$, C31), 46.2 (*C*$_{quart}$, C1), 45.6 (*C*$_{quart}$, C17), 41.7 (*C*$_{quart}$, C8), 40.4 (*C*H$_2$, C19), 38.8 (*C*H$_2$, C1), 38.2 (*C*$_{quart}$, C4), 37.5 (*C*$_{quart}$, C10), 34.2 (*C*H$_2$, C22), 33.4 (*C*H, C18), 33.2 (*C*H$_2$, C21), 33.0 (*C*H$_3$, C29), 32.0 (*C*H$_2$, C7), 30.7 (*C*$_{quart}$, C20), 28.2 (*C*H$_3$, C23), 28.0 (*C*H$_2$, C15), 23.7 (*C*H$_3$, C27), 23.4 (*C*H$_3$, C30), 23.3 (*C*H$_2$, C2), 21.4 (*C*H$_3$, Ac), 19.0 (*C*H$_3$, C26), 17.4 (*C*H$_2$, C6), 16.8 (*C*H$_3$, C24), 16.5 (*C*H$_3$, C25) ppm; MS (ESI, MeOH): m/z = 543.2 (38%, [M + H]$^+$), 565.4.5 (50%, [M + Na]$^+$), 1107.3 (100%, [2M + Na]$^+$).

*5.4.11. (3β) Methyl 3-acetoxy-11-oxo-olean-12-en-28-oate (**10**)*

N-Hydroxysuccinimide (1.2 g, 10.4 mmol) and potassium dichromate (1.1 g, 3.6 mmol) were added to a solution of **7** (0.5 g, 0.94 mmol) in acetone (50 mL) containing glacial acetic acid (5 mL); the mixture was stirred at 40° for 48 h. The reaction was quenched with potassium disulfite solution and after usual work up **10** was obtained as a colorless solid; yield: 65%; m.p.: 216–220 °C (lit.: [55]: 245–247 °C); [α]$_D$ = +82° (*c* 0.14, CHCl$_3$); R_F = 0.62 (hexane/ethyl acetate, 7:3); UV–Vis (MeOH): λ$_{max}$ (log ε) = 269 nm (4.03); IR (KBr): ν = 3339*br*, 2949*s*, 2866*s*, 1724*s*, 1661*s*, 1466*m*, 1387*m*, 1365*w*, 1330*w*, 1304*w*, 1261*m*, 1227*w*, 1209*m*, 1189*m*, 1162*m*, 1125*w*, 1089*w*, 1039*m*, 1013*w* cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$): δ = 5.61 (*s*, 1H, C*H* (12)), 3.61 (*s*, 3H, C*H*$_3$ (31)), 3.20 (*dd*, *J* = 10.8, 5.5 Hz, 1H, C*H* (3)), 2.98 (*dd*, *J* = 13.8, 3.7 Hz, 1H, C*H* (18)), 2.85–2.75 (*m*, 1H, C*H*$_a$ (1)), 2.30 (*s*, 1H, C*H* (9)), 2.02 (*ddd*, *J* = 13.8, 13.8, 4.0 Hz, 1H, C*H*$_a$ (16)), 1.75–1.52 (*m*, 9H, C*H*$_a$ (19) + C*H*$_b$ (16) + C*H*$_2$ (7) + C*H*$_a$ (15) + C*H*$_a$ (22) + C*H*$_2$ (2) + C*H*$_a$ (6)), 1.42–1.13 (*m*, 6H, C*H*$_b$ (19) + C*H*$_b$ (22) + C*H*$_2$ (21) + C*H*$_b$ (15) + C*H*$_b$ (6)), 1.34 (*s*, 3H, C*H*$_3$ (27)), 1.08 (*s*, 3H, C*H*$_3$ (25)), 1.00–0.95 (*m*, 1H, C*H*$_b$ (1)), 0.97 (*s*, 3H, C*H*$_3$ (23)), 0.92 (*s*, 3H, C*H*$_3$ (30)), 0.91 (*s*, 3H, C*H*$_3$ (29)), 0.89 (*s*, 3H, C*H*$_3$ (26)), 0.78 (*s*, 3H, C*H*$_3$ (24)), 0.66 (*brd*, *J* = 11.3 Hz, 1H, C*H* (5)) ppm; ^{13}C NMR (125 MHz, CDCl$_3$): δ = 200.3 (*C*=O, C11), 177.4 (C=O, C28), 168.6 (*C*=CH, C13), 127.9 (CH=*C*, C12), 78.7 (*C*HOH, C3), 61.7 (CH, C9), 55.0 (CH, C5), 51.8 (CH$_3$, C31), 46.2 (C$_{quart}$, C17), 45.0 (C$_{quart}$, C8), 44.2 (*C*H$_2$, C19), 43.4 (C$_{quart}$, C14), 41.5 (CH, C18), 39.1 (*C*H$_2$, C1), 38.8 (C$_{quart}$, C4), 37.2 (C$_{quart}$, C10), 33.7 (*C*H$_2$, C21), 32.9 (*C*H$_2$, C7), 32.8 (*C*H$_3$, C29), 31.6 (*C*H$_2$, C22), 30.6 (C$_{quart}$, C20), 28.1 (*C*H$_3$, C23), 27.7 (*C*H$_2$, C15), 27.3 (*C*H$_2$, C2), 23.5 (*C*H$_3$, C27), 23.4 (*C*H$_3$, C30), 22.9 (*C*H$_2$, C16), 18.9 (*C*H$_3$, C26), 17.4 (*C*H$_2$, C6), 16.1 (*C*H$_3$, C25), 15.5 (*C*H$_3$, C24) ppm; MS (ESI, MeOH): m/z = 485.6 (100%, [M + H]$^+$), 507.5 (35%, [M + Na]$^+$).

*5.4.12. (2α,3β) Methyl 2,3-dihydroxy-olean-12-en-28-oate (**11**)*

Compound **11** [56] was obtained from **MA** (100 mg, 0.21 mmol) using method **D** as fine colorless needles; yield: 88%; m.p. 229–231 °C (214–216 °C [56]); R_F = 0.4 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +60.9° (*c* 0.65, $CHCl_3$); IR (KBr) ν = 3571*br*, 3300*s*, 2947*s*, 1739*s*, 1461*s*, 1386*m*, 1363*m*, 1262*m*, 1229*m*, 1190*s*, 1162*s*, 1124*m*, 1052*s*, 1037*s*, 984*m*, 958*s*, 921*s* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.28 (*dd*, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 3.72–3.65 (*m*, 1H, *CH* (2)), 3.61 (*s*, 3H, CH_3 (31)), 3.00 (*d*, *J* = 9.4 Hz, 1H, *CH* (3)), 2.85 (*dd*, *J* = 13.8, 4.1 Hz, 1H, *CH* (18)), 2.21 (*br*, 2H, *OH*), 1.97 (*dd*, *J* = 10.4, 3.9 Hz, 1H, CH_a (1)), 1.96–1.83 (*m*, 3H, CH_a (16) + CH_2 (11)), 1.68 (*ddd*, *J* = 13.9, 13.9, 4.4 Hz, 1H, CH_a (7)), 1.64–1.56 (*m*, 4H, *CH* (9) + CH_a (19) + CH_a (15) + CH_b (16)), 1.55–1.52 (*m*, 1H, CH_a (6)), 1.50 (*ddd*, *J* = 14.0, 3.4, 3.4 Hz, CH_a (22)), 1.43 (*m*, 1H, CH_b (7)), 1.38 (*ddd*, *J* = 12.4, 12.4, 2.5 Hz, CH_b (6)), 1.29 (*ddd*, *J* = 13.8, 9.6, 2.7 Hz, 1H, CH_a (21)), 1.29–1.27 (*m*, 1H, CH_b (22)), 1.16 (*ddd*, *J* = 14.0, 4.2, 4.2 Hz, 1H, CH_b (21)), 1.15–1.11 (*m*, 1H, CH_b (19)), 1.12 (*s*, 3H, CH_3 (C27)), 1.06–1.02 (*m*, 1H, CH_b (15)), 1.02 (*s*, 3H, CH_3 (C23)), 0.97 (*s*, 3H, CH_3 (C25)), 0.92 (*s*, 3H, CH_3 (C30)), 0.92–0.87 (*m*, 1H, CH_b (1)), 0.89 (*s*, 3H, CH_3 (C29)), 0.84–0.82 (*m*, 1H, *CH* (5)), 0.82 (*s*, 3H, CH_3 (C24)), 0.71 (*s*, 3H, CH_3 (C26)) ppm; ^{13}C NMR (125 Hz, $CDCl_3$): δ = 178.2 (*C*=O, C28), 143.8 (*C*=CH, C13), 122.1 (*CH*=C, C12), 84.0 (*CHOH*, C3), 68.9 (*CHOH*, C2), 55.3 (*CH*, C5), 51.5 (CH_3, C31), 47.6 (*CH*, C9), 46.7 (C_{quart}, C17), 46.3 (CH_2, C1), 45.8 (CH_2, C19), 41.6 (C_{quart}, C14), 41.2 (*CH*, C18), 39.3 (C_{quart}, C8), 39.1 (C_{quart}, C4), 38.3 (C_{quart}, C10), 33.8 (CH_2, C21), 33.1 (CH_3, C29), 32.5 (CH_2, C22), 32.3 (CH_2, C7), 30.7 (C_{quart}, C20), 28.6 (CH_3, C23), 27.6 (CH_2, C15), 25.9 (CH_3, C27), 23.6 (CH_3, C30), 23.4 (CH_2, C16), 23.0 (CH_2, C11), 18.3 (CH_2, C6), 16.9 (CH_3, C26), 16.7 (CH_3, C24), 16.6 (CH_3, C25) ppm; MS (ESI, MeOH, source CID): *m/z* = 487.4 (48.8%, $[M + H]^+$), 504.5 (53.4%, $[M + NH_4]^+$), 509.5 (100%, $[M + Na]^+$), 541.2 (22.0%, $[M + Na + MeOH]^+$), 741.5 (14.6%, $[3M + Na + H]^{2+}$), 749.6 (73.2%, $[3M + K + H]^{2+}$), 992.8 (48.8%, $[4M + K + H]^{2+}$).

*5.4.13. (2α,3β) Methyl 2,3-diacetyl-olean-12-en-28-oate (**12**)*

Obtained from **11** using method **C** as a colorless solid; yield: 92%; m.p.: 173–175 °C (lit.: 165–168 °C [57], 176–178 °C [58]; R_F = 0.48 (n-hexane/ethylacetate, 8:2); $[\alpha]_D$ = +25.1° (*c* 0.37, $CHCl_3$); IR (KBr): ν = 3430*br*, 2972*s*, 2949*s*, 2882*m*, 2866*m*, 1745*vs*, 1722*s*, 1460*m*, 1433*m*, 1393*m*, 1369*m*, 1250*vs*, 1190*m*, 1165*m*, 1042*m*, 1034*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.28 (*dd*, *J* = 3.6, 3.6 Hz, 1H, *CH* (12)), 5.11 (*ddd*, *J* = 11.7, 10.4, 4.8 Hz, 1H, *CH* (2)), 4.73 (*d*, *J* = 10.4 Hz, 1H, *CH* (3)), 3.60 (*s*, 3H, CH_3 (31)), 2.85 (*dd*, *J* = 13.8, 4.4 Hz, 1H, *CH* (18)), 2.04 + 1.97 (*s*, 6H, CH_3 (Ac)), 2.08–1.93 (*m*, 3H, CH_a (1) + CH_a (16) + CH_a (11)), 1.93–1.88 (*m*, 1H, CH_b (11)), 1.73–1.68 (*m*, 1H, CH_a (7)), 1.66–1.58 (*m*, 4H, CH_a (15) + CH_a (19) + CH_b (16) + *CH* (9)), 1.55–1.48 (*m*, 2H, CH_a (6) + CH_b (7)), 1.48–1.38 (*m*, 2H, CH_a (22) + CH_b (6)), 1.35–1.28 (*m*, 2H, CH_b (22) + CH_a (21)), 1.20–1.12 (*m*, 2H, CH_b (21) + CH_b (19)), 1.11 (*s*, 3H, CH_3 (27)), 1.10 (*m*, 2H, CH_b (1) + CH_b (15)), 1.06 (*s*, 3H, CH_3 (23)), 1.00–0.92 (*m*, 1H, *CH* (5)), 0.94 (*s*, 3H, CH_3 (25)), 0.90 (*s*, 3H, CH_3 (30)), 0.89 (*s*, 3H, CH_3 (29)), 0.89 (*s*, 3H, CH_3 (24)), 0.74 (*s*, 3H, CH_3 (26)); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.3 (*C*=O, C28), 170.7 (*C*=O, Ac), 170.3 (*C*=O, Ac), 143.8 (*C*=CH, C13), 122.2 (*CH*=C, C12), 80.6 (*CHOAc*, C3), 70.0 (*CHOAc*, C2), 54.9 (*CH*, C5), 47.6 (*CH*, C9), 46.7 (C_{quart}, C17), 45.9 (CH_2, C19), 43.8 (CH_2, C1), 41.6 (C_{quart}, C14), 41.2 (*CH*, C18), 39.5 (C_{quart}, C4), 39.3 (C_{quart}, C8), 38.3 (C_{quart}, C10), 33.8 (CH_2, C21), 33.0 (CH_3, C29), 32.4 (CH_2, C22), 32.3 (CH_2, C7), 30.6 (C_{quart}, C20), 28.5 (CH_3, C23), 27.6 (CH_2, C15), 25.8 (CH_3, C27), 23.8 (CH_3, C30), 23.4 (CH_2, C11), 23.1 (CH_2, C16), 21.0 (CH_3, Ac), 20.9 (CH_3, Ac), 18.3 (CH_2, C6), 17.7 (CH_3, C26), 16.9 (CH_3, C24), 16.5 (CH_3, C25) ppm; MS (MeOH): *m/z* = 571.1 (41%, $[M + H]^+$), 588.2 (71%, $[M + NH_4]^+$), 593.5 (16%, $[M + Na]^+$, 1163.4 (100%, $[2M + Na]^+$). $C_{35}H_{54}O_6$

*5.4.14. Methyl (2α,3β)2,3–di-O-acetyl-12-oxo-olean-28-oate (**13**)*

Compound **13** obtained as colorless solid from **12** using method **B**; yield: 84%; m.p.: 144–147 °C; R_F = 0.21 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = −46.7° (*c* 0.40, $CHCl_3$); IR (KBr): ν = 3588*w*, 3442*s*, 3430*s*, 2949*m*, 2867*w*, 1742*vs*, 1727*s*, 1700*m*, 1472*m*, 1458*m*, 1440*w*, 1395*w*, 1387*m*, 1369*m*, 1250*vs*, 1192*m*, 1160*m*, 1154*m*, 1119*w*, 1086*w*, 1042*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.06 (*ddd*, *J* = 11.4, 10.4, 4.7 Hz, 1H, *CH* (2)), 4.74 (*d*, *J* = 10.4 Hz, 1H, *CH* (3)), 3.65 (*s*, 3H, CH_3 (31)), 2.80 (*ddd*, *J* = 13.6, 4.0, 4.0 Hz, 1H, *CH* (18)), 2.60 (*d*, *J* = 4.0 Hz, 1H, *CH* (13)), 2.18 (*m*, 1H, CH_a (11)), 2.16 (*m*, 1H, CH_b (11)), 2.05 + 1.98 (*s*, 6H, 2× CH_3 (Ac)), 2.00–1.88 (*m*, 3H, CH_a (19) + CH_a (1) + CH_a (16)), 1.85–1.78 (*m*, 1H, CH_a (7)), 1.79–1.71 (*m*, 1H, *CH* (9)), 1.70–1.60 (*m*, 3H, CH_a (15) + CH_b (16) + CH_a (6)), 1.52–1.42 (*m*, 3H, CH_b (6) + CH_b (7) + CH_a (22)), 1.39–1.28 (*m*, 2H, CH_b (22) + CH_a (21)), 1.24–1.18 (*m*, 1H, CH_b (19) + CH_b (21)), 1.12–1.08 (*m*, 1H, CH_b (15)), 1.06–0.88 (*m*, 1H, CH_b (1)), 1.02 (*s*, 3H, CH_3 (27)), 0.98 (*s*, 3H, CH_3 (25)), 0.97 (*s*, 3H, CH_3 (30)), 0.96 (*m*, 1H, *CH* (5)), 0.94 (*s*, 3H, CH_3 (23)), 0.90 (*s*, 3H, CH_3 (24)), 0.89 (*s*, 3H, CH_3 (29)), 0.89 (*s*, 3H, CH_3 (26)); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 210.6 (*C*=O, C12), 178.0 (*C*=O, C28), 170.4 (*C*=O, Ac), 170.5 (*C*=O, Ac), 80.1 (*CHOAc*, C3), 69.5 (*CHOAc*, C2), 54.8 (*CH*, C5), 51.6 (*CH*, C13), 49.6 (*CH*, C9), 47.4 (C_{quart}, C17), 43.3 (CH_2, C1), 41.7 (C_{quart}, C14), 41.2 (C_{quart}, C8), 39.0 (C_{quart}, C4), 38.5 (CH_2, C11), 38.1 (C_{quart}, C10), 36.4 (CH_2, C19), 34.5 (CH_2, C21), 33.5 (CH_3, C29), 32.8 (CH_2, C7), 32.0 (*CH*, C18), 31.6 (CH_2, C22), 30.8 (C_{quart}, C20), 28.2 (CH_3, C23), 27.7 (CH_2, C15), 23.0 (CH_3, C30), 22.9 (CH_2, C16), 21.0 (CH_3, Ac), 21.1 (CH_3, Ac), 20.4 (CH_3, C27), 18.3 (CH_2, C6), 17.5 (CH_3, C26), 16.5 (CH_3, C24), 16.0 (CH_3, C25) ppm; MS (ESI, MeOH): *m/z* = 587.3 (37%, $[M + H]^+$), 604.4 (26%, $[M + NH_4]^+$), 609.5 (100%, $[M + Na]^+$), 1190.2 (29%, $[2M + NH_4]^+$), 1195.1 (85%, $[2M + Na]^+$). $C_{35}H_{54}O_7$

*5.4.15. (3β) Methyl 3-hydroxy-urs-11-en-28 oate (**14**)*

Compound **14** was obtained from **UA** as fine colorless needles using method **D**; yield: 95%; m.p.: 165–167 °C (lit [59].: 166–168 °C); R_F = 0.47 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +68° (*c* 0.53; $CHCl_3$) [lit.: 49.8° (*c* 1.0; $CHCl_3$) [52]; UV–Vis (MeOH): λ_{max} (log ε) = 224 nm (3.62); IR (KBr): ν = 3374*br*, 2927*s*, 2871*m*, 1728*s*, 1457*m*, 1386*m*, 1378*m*, 1308*w*, 1270*w*, 1232*m*, 1200*m*, 1187*m*, 1168*w*, 1142*m*, 1113*w*, 1093*w*, 1031m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.22 (*m*, 1H, *CH* (12)), 3.59 (*s*, 3H, CH_3 (31)), 3.20 (*dd*, *J* = 11.0, 4.9 Hz, 1H, *CH* (3)), 2.21 (*d*, *J* = 11.3 Hz, 1H, *CH* (18)), 1.98 (*ddd*, *J* = 13.4, 13.4, 4.6 Hz, 1H, CH_a (2)), 1.90 (*dd*, *J* = 8.9, 3.7 Hz, 2H, CH_2 (11)), 1.85 (*ddd*, *J* = 13.8, 13.8, 4.8 Hz, 1H, CH_a (15)), 1.69–1.56 (*m*, 5H, CH_2 (7) + CH_a (1) + CH_a (16) + CH_b (2)), 1.55–1.44 (*m*, 5H, *CH* (9) + CH_a (6) + CH_a (21) + CH_a (22) + CH_b (16)), 1.36–1.24 (*m*, 4H, *CH* (19) + CH_b (6) + CH_b (22) + CH_b (21)), 1.08–1.03 (*m*, 1H, CH_b (15)), 1.06 (*s*, 3H, CH_3 (27)), 1.01–0.95 (*m*, 2H, *CH* (20) + CH_b (1)), 0.97 (*s*, 3H, CH_3 (23)), 0.93 (*d*, *J* = 6.0 Hz, 3H, CH_3 (30)), 0.91 (*s*, 3H, CH_3 (25)), 0.85 (*d*, *J* = 6.4 Hz, 3H, CH_3 (29)), 0.77 (*s*, 3H, CH_3 (24)), 0.73 (*s*, 3H, CH_3 (26)), 0.71 (*brd*, *J* = 10.4 Hz, 1H, *CH* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.9 (*C*=O, C28), 138.1 (*C*=CH, C13), 125.5 (HC=C, C12), 79.0 (*CHOH*, C3), 55.2 (*CH*, C5), 52.9 (*CH*, C18), 51.4 (CH_3, C31), 48.0 (C_{quart}, C17), 47.5 (*CH*, C9), 41.9 (C_{quart}, C14), 39.5 (C_{quart}, C8), 39.0 (*CH*, C19), 38.8 (*CH*, C20), 38.7 (C_{quart}, C4), 38.6 (CH_2, C1), 36.9 (C_{quart}, C10), 36.7 (CH_2, C22), 32.9 (CH_2, C7), 30.6 (CH_2, C21), 28.1 (CH_3, C23), 28.0 (CH_2, C15), 27.2 (CH_2, C16), 24.2 (CH_2, C2), 23.6 (CH_3, C27), 23.3 (CH_2, C11), 21.1 (CH_3, C30), 18.3 (CH_2, C6), 17.0 (CH_3, C29), 16.9 (CH_3, C26), 15.6 (CH_3, C24), 15.4 (CH_3, C25) ppm; MS (ESI, MeOH): *m/z* = 471.2 (80%, $[M + H]^+$), 493.4 (100%, $[M + Na]^+$).

*5.4.16. (3β) Methyl 3-hydroxy-olean-11-en-28 oate (**15**)*

OA (1 equiv.) was treated with iodomethane following method **D.** Compound **16** was obtained as fine colorless needles; yield: 88.3%; m.p.: 198–200 °C (lit [60].: 198–200 °C); R_F = 0.50 (n-

hexane/ethyl acetat, 8:2); $[\alpha]_D$ = +70° (*c* 0.43; $CHCl_3$) [lit [61].: +70° (*c* 1.0; $CHCl_3$)]; IR (KBr): ν = 3334*br*, 2947*s*, 2865*m*, 1725*s*, 1662*w*, 1464*m*, 1386*m*, 1363*m*, 1304*w*, 1263*m*, 1241*m*, 1202*m*, 1190*m*, 1162*m*, 1125*m*, 1094*w*, 1065*w*, 1032*m* cm^{-1}; UV–Vis (MeOH): λ_{max} (log ε) = 223 nm (3.66); ^1H NMR (500 MHz, $CDCl_3$): δ = 5.26 (*brs*, 1H, *CH* (12)), 3.60 (*s*, 3H, CH_3 (31)), 3.19 (*dd*, *J* = 11.0, 4.4 Hz, 1H, *CH* (3)), 2.84 (*dd*, *J* = 13.9, 4.2 Hz, 1H, *CH* (18)), 1.95 (*ddd*, *J* = 14.5, 14.4, 4.1 Hz, 1H, CH_a (16)), 1.88–1.82 (*m*, 2H, CH_2 (11)), 1.67 (*ddd*, *J* = 13.9, 13.9, 4.4 Hz, 1H, CH_a (7)), 1.63–1.47 (*m*, 9H, *CH* (9) + CH_a (1) + CH_a (19) + CH_a (6) + CH_a (15) + CH_b (7) + CH_b (16) + CH_2 (2)), 1.43–1.22 (*m*, 4H, CH_a (21) + CH_2 (22) + CH_b (6)), 1.19–1.12 (*m*, 2H, CH_b (19) + CH_b (21)), 1.10 (*s*, 3H, CH_3 (27)), 1.08–0.98 (*m*, 1H, CH_b (15)), 0.97–0.92 (*m*, 1H, CH_b (1)), 0.96 (*s*, 3H, CH_3 (23)), 0.90 (*s*, 3H, CH_3 (30)), 0.88 (*s*, 3H, CH_3 (29)), 0.87 (*s*, 3H, CH_3 (25)), 0.76 (*s*, 3H, CH_3 (24)), 0.73–0.68 (*m*, 1H, *CH* (5)), 0.70 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.2 (*C*=O, C28), 143.7 (*C*=CH, C13), 122.3 (*CH*=C, C12), 79.0 (C3, CHOH), 55.2 (*CH*, C5), 51.5 (CH_3, C31), 47.6 (CH, C9), 46.7 (C_{quart}, C17), 45.8 (CH_2, C19), 41.6 (C_{quart}, C14), 41.3 (*CH*, C18), 39.2 (C_{quart}, C8), 38.7 (C_{quart}, C4), 38.4 (CH_2, C1), 37.0 (C_{quart}, C10), 33.8 (CH_2, C21), 33.1 (CH_3, C29), 32.6 (CH_2, C7), 32.3 (CH_2, C22), 30.6 (C_{quart}, C20), 28.1 (CH_3, C23), 27.7 (CH_2, C15), 27.1 (CH_2, C2), 25.9 (CH_3, C27), 23.6 (CH_3, C30), 23.4 (CH_2, C11), 23.0 (CH_2, C16), 18.3 (CH_2, C6), 16.8 (CH_3, C26), 15.5 (CH_3, C24), 15.3 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 493.5 (100%, $[M + Na]^+$).

*5.4.17. (3β) Methyl 3-hydroxy-12-oxo-olean-28-oate (**16**)*

Compound **16** was obtained using method **A**; yield: 42%; R_F = 0.20 (hexane/ethyl acetate, 8:2); m.p.: 194–196 °C (lit [62].: 132–133 °C); $[\alpha]_D$ = −26.0° (*c*0.35; $CHCl_3$); IR (KBr): ν = 3492*vs*, 3446*vs*, 3432*s*, 2994*w*, 2970*m*, 2946*m*, 2926*m*, 2860*w*, 1718*m*, 1688*s*, 1654*w*, 1636*w*, 1628*w*, 1470*w*, 1458*w*, 1438*w*, 1240*m*, 1192*w*, 1178*w*, 1162*w*, 1048*w*, 1038*w*, 994*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 3.67 (*s*, 3H, CH_3 (31)), 3.19 (*dd*, *J* = 11.4, 4.4 Hz, 1H, *CH* (3)), 2.81–2.74 (*m*, 1H, *CH* (18)), 2.61 (*d*, *J* = 3.9 Hz, 1H, *CH* (13)), 2.23 (*dd*, *J* = 16.7, 4.8 Hz, 1H, CH_a (11)), 2.12 (*dd*, *J* = 16.1, 13.8 Hz, 1H, CH_b (11)), 1.94 (*m*, 1H, CH_a (19)), 1.87 (*ddd*, *J* = 14.6, 14.6 Hz, 4.1 Hz, 1H, CH_a (16)), 1.79 (*ddd*, *J* = 13.7, 13.7, 4.3 Hz, 1H, CH_a (7)), 1.69–1.56 (*m*, 4H, *CH* (9) + CH_2 (16) + CH_a (2)), 1.54 (*m*, 1H, CH_a (1)), 1.50–1.39 (*m*, 2H, CH_a (22) + CH_a (21)), 1.35–1.24 (*m*, 2H, CH_b (22) + CH_b (21)), 1.20 (*dd*, *J* = 13.3, 13.3 Hz, 1H, CH_b (19)), 1.07 (*d*, 1H, CH_b (2)), 0.98 (*s*, 3H, CH_3 (30)), 0.98–0.95 (*m*, 1H, CH_b (16)), 0.96 (*s*, 3H, CH_3 (27)), 0.95 (*s*, 3H, CH_3 (23)), 0.93 (*s*, 3H, CH_3 (28)), 0.89 (*s*, 3H, CH_3 (29)), 0.84 (*s*, 3H, CH_3 (26)), 0.77 (*s*, 3H, CH_3 (25)), 0.74–0.67 (*m*, 1H, *CH* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 211.7 (*C*=O, C12), 178.2 (*C*=O, C28), 78.6 (*C*HOH, C3), 55.1 (*CH*, C5), 51.8 (*CH*, C13), 51.9 (CH_3, C31), 49.7 (CH, C9), 47.3 (C_{quart}, C17), 41.9 (C_{quart}, C14), 41.2 (C_{quart}, C8), 38.8 (CH_2, C11), 38.5 (CH_2, C1), 37.9 (C_{quart}, C10), 36.9 (C_{quart}, C4), 36.2 (CH_2, C19), 34.4 (CH_2, C1), 33.4 (CH_3, C29), 32.9 (CH_2, C7 + C22), 32.0 (CH_2, C21), 31.8 (*CH*, C18), 30.6 (C_{quart}, C20), 27.9 (CH_3, C29), 27.5 (CH_2, C2), 27.04 (CH_3, C23), 23.1 (CH_3, C27), 22.8 (CH_2, C16), 20.5 (CH_3, C30), 18.3 (CH_2, C6), 16.0 (CH_3, C24), 15.3 (CH_3, C26), 15.2 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 487.5 (54.8%, $[M + H]^+$), 509.6 (79.6%, $[M + Na]^+$), 995.3 (100%, $[2M + Na]^+$).

*5.4.18. (2α, 3β) Methyl 3,12-dihydroxy-11-oxo-olean-12-en-28-oate (**17**)*

Compound **17** was obtained using method **E** starting from compound **16** as colorless crystals; yield: 96%; m.p.: 206–210 °C; R_F = 0.43 (*n*-hexane/ethyl acetate, 7:3); $[\alpha]_D$ = +70° (*c* 0.43; $CHCl_3$); IR (KBr):ν = 3530*s*, 3472*m*, 3008*m*, 2986*s*, 2948*s*, 2928*s*, 2864*s*, 1726*vs*, 1652*m*, 1614*s*, 1470*m*, 1454*m*, 1390*s*, 1366*s*, 1306*m*, 1276*m*, 1262*s*, 1242*m*, 1204*m*, 1192*s*, 1162*m*, 1046*s*, 758*s*, 736 *s* cm^{-1}; UV–Vis ($CHCl_3$): λ_{max} (log ε) = 281.2 nm (4.48); ^1H NMR (500 MHz, $CDCl_3$): δ = 6.22 (*s*, 1H, *OH*), 3.66 (*ddd*, *J* = 9.0, 9.0, 1.8 Hz, 1H, *CH* (18)), 3.62 (*s*, 3H, CH_3 (31)), 3.22 (*dd*, *J* = 10.4, 5.9 Hz, 1H, *CH* (3)), 2.79 (*ddd*, *J* = 13.4, 3.4, 3.4 Hz, 1H, CH_a (1)), 2.44 (*s*, 1H, *CH* (9)), 2.04 (*ddd*, *J* = 13.7, 13.7, 3.9 Hz, 1H, CH_a (16)), 1.76 (*ddd*, *J* = 13.9, 13.9, 4.4 Hz, 1H, CH_a (7)), 1.70 (*brd*, *J* = 13.7 Hz, 1H, CH_b (6)), 1.67–1.52 (*m*, 6H, CH_b (7) + CH_a (21) + CH_2 (11) + CH_a (15) + CH_a (6)), 1.38–1.26 (*m*, 5H, CH_2 (19) + CH_b (21) + CH_a (22) + CH_b (6)), 1.36 (*s*, 3H, CH_3 (27)), 1.26–1.22 (*m*, 1H, CH_b (22)), 1.19 (*ddd*, *J* = 14.0, 3.2, 3.2 Hz, 1H, CH_b (15)), 1.10 (*s*, 3H, CH_3 (25)), 1.04–0.95 (*m*, 1H, CH_b (1)), 0.99 (*s*, 6H, CH_3 (30) + CH_3 (23)), 0.92 (*s*, 3H, CH_3 (29)), 0.91 (*s*, 3H, CH_3 (26)), 0.79 (*s*, 3H, CH_3 (24)), 0.70 (*brd*, *J* = 11.4 Hz, 1H, *CH* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 195.6 (*C*=O, C11), 178.0 (*C*=O, C28), 142.3 (*C*OH=C, C12), 136.8 (*C*=COH, C13), 78.8 (*C*HOH, C3), 60.6 (*CH*, C9), 55.1 (*CH*, C5), 52.0 (CH_3, C31), 46.2 (C_{quart}, C14), 45.6 (C_{quart}, C17), 41.7 (C_{quart}, C8), 40.4 (CH_2, C1), 39.3(CH_2, C19), 39.2 (C_{quart}, C4), 37.6 (C_{quart}, C10), 34.2 (CH_2, C22), 33.4 (*CH*, C18), 33.2 (CH_2, C21), 33.0 (CH_3, C29), 32.0 (CH_2, C7), 30.7 (C_{quart}, C20), 28.2 (CH_3, C23), 28.0 (CH_2, C15), 27.4 (CH_2, C11), 23.5 (CH_3, C27), 23.4 (CH_3, C30), 23.3 (CH_2, C2), 18.9 (CH_3, C26), 17.5 (CH_2, C6), 16.5 (CH_3, C25), 15.7 (CH_3, C24) ppm; MS (ESI): *m*/*z* = 501.3 (100%, $[M + H]^+$), 1023.4 (64%, $[2M + Na]^+$).

*5.4.19. (3β) Methyl 3-hydroxy-11-oxo-olean-12-en-28-oate (**18**)*

To a mixture of **16** (450 mg, 0.88 mmol) in acetone (50 mL) and glacial acetic acid (5 mL) *N*-hydroxysuccinimide (950 mg, 8.25 mmol) and potassium dichromate (780 mg, 2.64 mmol) were added, and the mixture was stirred at 45 °C for 48 h. The mixture was quenched with an aq potassium disulfide solution and washed with an aq. sodium hydrogen carbonate solution. After extracting with DCM, the organic phases were combined, washed, dried and evaporated. Chromatographic purification gave compound **18**; yield 71%; m.p.: 184–188 °C (lit.: [36]: 181–188 °C); R_F = 0.62 (n-hexane/ethyl acetate, 7:3; $[\alpha]_D$ = +82° (*c* 0.14; $CHCl_3$); UV–Vis (MeOH): λ_{max} (log ε) = 269 nm (4.03); IR (KBr): ν = 3339*br*, 2949*s*, 2866*s*, 1724*s*, 1661*s*, 1466*m*, 1387*m*, 1365*w*, 1330*w*, 1304*w*, 1261*m*, 1227*w*, 1209*m*, 1189*m*, 1162*m*, 1125*w*, 1089*w*, 1039*m*, 1013*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.61 (*s*, 1H, *CH* (12)), 3.61 (*s*, 3H, CH_3 (31)), 3.20 (*dd*, *J* = 10.8, 5.5 Hz, 1H, *CH* (3)), 2.98 (*dd*, *J* = 13.8, 3.7 Hz, 1H, *CH* (18)), 2.80 (*d*, *J* = 13.4 Hz, 1H, CH_a (1)), 2.30 (*s*, 1H, *CH* (9)), 2.02 (*ddd*, *J* = 13.8, 13.8, 4.0 Hz, 1H, CH_a (16)), 1.75–1.52 (*m*, 9H, CH_a (19) + CH_b (16) + CH_a (7) + CH_a (15) + CH_2 (22) + CH_2 (2) + CH_a (6)), 1.42–1.13 (*m*, 6H, CH_b (19) + CH_b (7) + CH_2 (21) + CH_b (15) + CH_b (6)), 1.34 (*s*, 3H, CH_3 (27)), 1.08 (*s*, 3H, CH_3 (23)), 1.00–0.95 (*m*, 1H, CH_b (1)), 0.97 (*s*, 3H, CH_3 (30)), 0.92 (*s*, 3H, CH_3 (29)), 0.91 (*s*, 3H, CH_3 (25)), 0.89 (*s*, 3H, CH_3 (24)), 0.78 (*s*, 3H, CH_3 (26)), 0.66 (*brd*, *J* = 11.3 Hz, 1H, *CH* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 200.3 (*C*=O, C11), 177.4 (*C*=O, C28), 168.6 (*C* = CH, C13), 127.9 (CH = C, C12), 78.7 (*C*HOH, C3), 61.7 (*CH*, C9), 55.0 (*CH*, C5), 51.8 (CH_3, C31), 46.2 (C_{quart}, C17), 45.0 (C_{quart}, C8), 44.2 (CH_2, C19), 43.4 (C_{quart}, C14), 41.5 (CH, C18), 39.1 (CH_2, C1), 38.8 (C_{quart}, C4), 37.2 (C_{quart}, C10), 33.7 (CH_2, C21), 32.9 (CH_2, C7), 32.8 (CH_3, C29), 31.6 (CH_2, C22), 30.6 (C_{quart}, C20), 28.1 (CH_3, C23), 27.7 (CH_2, C15), 27.3 (CH_2, C2), 23.5 (CH_3, C27), 23.4 (CH_3, C30), 22.9 (CH_2, C16), 18.9 (CH_3, C26), 17.4 (CH_2, C6), 16.1 (CH_3, C24), 15.5 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 485.6 (100%, $[M + H]^+$), 507.5 (35%, $[M + Na]^+$).

*5.4.20. (3β) Benzyl 3-hydroxy-urs-12-en-28-oate (**19**)*

UA (1 equiv.) was dissolved in DMF and treated with benzylchloride following method **D**. Compound **19** was obtained as a colorless solid; yield: 82%; m.p.: 182 °C (lit.: 180–182 °C [63]); R_F = 0.25 (*n*-hexane/ethyl actetate 8:2); $[\alpha]_D$ = +45.6° (*c* 0.30, $CHCl_3$); IR (KBr): ν = 3518*vs*, 3474*br*, 3266*m*, 3034*w*, 2988*s*, 2970*s*, 2942*s*, 2922*vs*, 2868*s*, 2854*s*, 2836*m*, 2798*w*, 1714*vs*, 1498*w*, 1464*m*, 1452*m*, 1376*m*, 1308*w*, 1288m, 1272*m*, 1248*m*, 1230*m*, 1198*m*, 1180*m*, 1164*m*, 1140*m*, 1114*m*, 1096*w*, 1050*m*, 1028 *m* cm^{-1}; ^1H NMR

(500 MHz, $CDCl_3$): δ = 7.53–7.29 (m, 5H, CH_{aromat}), 5.37 (*br*, 1H, OH), 5.24 (*dd*, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 5.10 (*d*, *J* = 12.5 Hz, 1H, CH_a (31)), 4.98 (*d*, *J* = 12.5 Hz, 1H, CH_b (31)), 3.21 (*dd*, *J* = 11.0, 4.5 Hz, 1H, *CH* (3)), 2.27 (*d*, *J* = 11.3 Hz, 1H, *CH* (18)), 2.01 (*ddd*, *J* = 13.3, 13.3, 4.4 Hz, 1H, CH_a (16)), 1.94–1.84 (*m*, 2H, CH_2 (2)), 1.84–1.75 (*m*, 1H, CH_a (15)), 1.75–1.67 (*m*, 1H, CH_b (16)), 1.67–1.57 (*m*, 5H, CH_2 (22) + CH_a (1), CH_2 (11)), 1.52–1.40 (*m*, 4H, CH_a (6) + CH_a (7) + CH_a (21) + *CH* (9)), 1.41–1.20 (*m*, 5H, *CH* (19) + *CH* (20) + CH_b (7) + CH_b (21) + CH_b (6)), 1.07 (*s*, 3H, CH_3 (27)), 1.02–0.95 (*m*, 2H, CH_b (15) + CH_b (1)), 0.99 (*s*, 3H, CH_3 (23)), 0.94 (*d*, *J* = 6.2 Hz, 3H, CH_3 (30)), 0.89 (*s*, 3H, CH_3 (25)), 0.85 (*d*, *J* = 6.4 Hz, 3H, CH_3 (29)), 0.78 (*s*, 3H, CH_3 (24)), 0.71 (*brd*, *J* = 11.7 Hz, 1H, *CH* (5)), 0.65 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.4 (*C*=O, C28), 138.2 (*C*=CH, C13), 136.5 (C_{aromat}, C32), 128.5 (CH_{aromat}, C33), 128.3 (CH_{aromat}, C34), 128.1 (CH_{aromat}, C35), 125.9 (*CH*=C, C12), 79.2 (*CH*, C3), 66.1 (CH_2, C31), 55.4 (*CH*, C5), 53.0 (*CH*, C18), 48.3 (C_{quart}, C17), 47.7 (*CH*, C9), 42.2 (C_{quart}, C14), 39.7 (C_{quart}, C8), 39.2 (*CH*, C19), 39.0 (*CH*, C20), 38.9 (C_{quart}, C4), 38.8 (CH_2, C1), 37.1 (C_{quart}, C10), 36.8 (CH_2, C22), 33.2 (CH_2, C7), 30.8 (CH_2, C21), 28.3 (CH_3, C23), 28.1 (CH_2, C15), 27.4 (CH_2, C11), 24.4 (CH_2, C16), 23.7 (CH_3, C27), 23.4 (CH_2, C2), 21.3 (CH_3, C30), 18.5 (CH_2, C6), 17.2 (CH_3, C29), 17.1 (CH_3, C26), 15.8 (CH_3, C24), 15.6 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 547.3 (10%, $[M + H]^+$), 569.5 (100%, $[M + Na]^+$), 1115.2 (82%, $2M + Na]^+$).

*5.4.21. (3β) Benzyl 3-hydroxy-olean-12-en-28-oate (**20**)*

OA (1 equiv.) was treated with benzylchloride following method **D**, and **21** was obtained as fine colorless needles; yield: 92%; m.p.: 185–187 °C (lit [64].: 189 °C); $[\alpha]_D$ = +58.4° (*c* 0.45; $CHCl_3$) [lit.: [65]: 50.1° (*c* 2.44; $CHCl_3$)]; R_F = 0.42 (*n*-hexane/ethyl acetate, 8:2); IR (KBr): ν = 3583*s*, 2938*s*, 1726*s*, 1498*w*, 1463*m*, 1386*m*, 1363*w*, 1323*w*, 1305*w*, 1264*w*, 1234*w*, 1201*m*, 1182*m*, 1162*m*, 1129*m*, 1095*w*, 1044*m*, 1016 *m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.37–7.27 (*m*, 5H, CH_{aromat}), 5.29 (*dd*, *J* = 3.6, 3.6 Hz, 1H, *CH* (12)), 5.09 (*d*, *J* = 12.6 Hz, 1H, CH_a (31)), 5.05 (*d*, *J* = 12.5 Hz, 1H, CH_b (31)), 3.20 (*dd*, *J* = 11.3 Hz, 4.5 Hz, 1H, *CH*OH (3)), 2.91 (*dd*, *J* = 13.8, 4.2 Hz, 1H, *CH* (18)), 1.98 (*ddd*, *J* = 13.4, 13.0, 4.1 Hz, 1H, CH_a (16)), 1.85 (*dd*, *J* = 8.9, 3.6 Hz, 1H, CH_2 (11)), 1.76–1.14 (*m*, 16H, *CH* (9) + CH_2 (19) + CH_a (1) + CH_2 (21)+ CH_2 (7) + CH_2 (22) + CH_a (15)+ CH_2 (2)+ CH_b (16)+ CH_2 (6)), 1.13 (*s*, 3H, CH_3 (27)), 1.09–1.00 (*m*, 2H, CH_b (1) + CH_b (15)), 0.98 (*s*, 3H, CH_3 (23)), 0.92 (*s*, 3H, CH_3 (30)), 0.90 (*s*, 3H, CH_3 (29)), 0.88 (*s*, 3H, CH_3 (25)), 0.77 (*s*, 3H, CH_3 (24)), 0.70 (*brd*, *J* = 11.5 Hz, 1H, *CH* (5)), 0.61 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.4 (*C*=O, C28), 143.7 (*C*=CH, C13), 136.4 (C_{quart}, C32), 128.4 (CH_{aromat}, C35), 128.0 (CH_{aromat}, C34), 127.9 (CH_{aromat}, C33), 122.5 (*CH*=C, C12), 80.0 (*CHOH*, C3), 65.9 (CH_2, C31), 55.2 (*CH*, C5), 47.6 (*CH*, C9), 46.7 (C_{quart}, C17), 45.9 (CH_2, C19), 41.7 (C_{quart}, C14), 41.4 (*CH*, C18), 39.3 (C_{quart}, C8), 38.7 (C_{quart}, C4), 38.4 (CH_2, C1), 37.0 (C_{quart}, C10), 33.8 (CH_2, C21), 33.1 (CH_3, C29), 32.7 (CH_2, C7), 32.4 (CH_2, C22), 30.7 (C_{quart}, C20), 28.1 (CH_3, C23), 27.6 (CH_2, C15), 27.2 (CH_2, C2), 25.9 (CH_3, C27), 23.6 (CH_3, C30), 23.4 (CH_2, C11), 23.0 (CH_2, C16), 18.3 (CH_2, C6), 16.9 (CH_3, C26), 15.6 (CH_3, C24), 15.3 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 569.5 (100%, $[M + Na]^+$).

*5.4.22. (3β) Benzyl 3-hydroxy-12-oxo-olean-28-oate (**21**)*

Compound **21** was obtained following method **A** as a colorless solid; yield: 83%; m.p.: 198–201 °C (lit.: 196–198 °C [4]); R_F = 0.74 (silica gel, toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:20); $[\alpha]_D$ = +54.1° (*c* 0.5, $CHCl_3$); UV–Vis ($CHCl_3$): λ_{max} (log ε) = 258.17 (1.35) nm; IR (KBr):ν = 2942*s*, 1728*s*, 1711*s*, 1472*m*, 1456*m*, 1387*w*, 1372*m*, 1240*s*, 1204*m*, 1180*m*, 1161*m*, 1140*m*, 1119*m*, 1086*w*, 1037*m*, 1005*w* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.36–7.29 (*m*, 5H, *CH* (33) + *CH* (34) + *CH* (35)), 5.19 (*d*, *J* = 11.6 Hz, 1H, CH_a (31)), 5.07 (*d*, *J* = 11.6 Hz, 1H, CH_b (31)), 4.45 (*dd*, *J* = 11.3, 5.0 Hz, 1H, *CH* (3)), 2.83 (*ddd*, *J* = 10.2, 3.3, 3.3 Hz, 1H, *CH* (18)), 2.45 (*d*, *J* = 4.2 Hz, 1H, *CH* (13)), 2.18 (*dd*, *J* = 16.7, 16.7, 4.8 Hz, 1H, CH_a (16)), 2.03 (*s*, 3H, CH_3 (37)), 2.04–1.98 (*m*, 1H, CH_a (15)), 1.95–1.90 (m, 1H, CH_a (19)), 1.89–1.80 (m, 2H, CH_a (7) + CH_b (16)), 1.69–1.52 (*m*, 5H, CH_b (15) + CH_a (6) + CH_2 (11) + CH_a (2)), 1.51–1.47 (*m*, 3H, *CH* (9) + CH_a (21) + CH_b (7)), 1.44–1.30 (*m*, 3H, CH_a (1) + CH_b (6) + CH_a (22)), 1.29–1.15 (*m*, 3H, CH_b (1) + CH_b (22) + CH_b (19)), 1.05–0.94 (*m*, 1H, CH_b (2)), 0.99 (*s*, 3H, CH_3 (30)), 0.97 (*s*, 3H, CH_3 (23)), 0.90 (*s*, 6H, CH_3 (27) + CH_3 (29)), 0.87–0.84 (*m*, 1H, CH_b (15)), 0.85 (*s*, 3H, CH_3 (25)), 0.81 (*s*, 3H, CH_3 (24)), 0.79 (*m*, 1H, *CH* (5)), 0.61 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 211.6 (*C*=O, C12), 177.6 (*C*=O, C28), 171.0 (*C*=O, C36), 136.5 (C_{quart}, C32), 128.7 (C_{aromat}, C33), 128.5 (C_{aromat}, C35), 128.3 (C_{aromat}, C34), 80.6 (*CH*, C3), 66.1 (CH_2, C31), 55.3 (*CH*, C5), 52.0 (*CH*, C13), 49.8 (*CH*, C9), 47.4 (C_{quart}, C17), 42.0 (C_{quart}, C14), 41.3 (C_{quart}, C8), 38.6 (C_{quart}, C4), 37.9 (CH_2, C21), 37.8 (CH_2, C11), 36.9 (C_{quart}, C10), 36.4 (CH_2, C19), 34.6 (CH_2, C7), 33.5 (CH_3, C29), 33.0 (CH_2, C22), 32.2 (*CH*, C18), 31.8 (CH_2, C1), 30.8 (C_{quart}, C20), 28.0 (CH_3, C23), 27.5 (CH_2, C15), 23.5 (CH_2, C2), 23.3 (CH_3, C30), 22.9 (CH_2, C16), 21.4 (CH_3, C37), 20.6 (CH_3, C27), 18.3 (CH_2, C6), 16.6 (CH_3, C26), 15.9 (CH_3, C24), 15.3 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 605.1 (56%, $[M + H]^+$), 622.1 (26%, $[M + NH_4]^+$), 585.3 (32%, $[M + Na]^+$), 929.9 (26%, $[3M+2Na]^{2+}$), 1209.2 (26%, $[2M + H]^+$), 1231.3 (100%, $[2M + Na]^+$).

*5.4.23. (3β) Benzyl 3-acetoxy-urs-11-en-28-oate (**22**)*

Compound **22** [66] was obtained from a mixture of **22** and **23** by using method **A**; yield: 56%; R_F = 0.7 (*n*-hexane/ethyl acetate, 85:15); m.p.: 173–174 °C; $[\alpha]_D$ = +45.1° (*c* 0.66, $CHCl_3$); IR (KBr): ν = 3433*w*, 3069*w*, 2926*m*, 2926*s*, 2874*s*, 1727*s*, 1499*w*, 1465*s*, 1388*m*, 1379*s*, 1039*m*, 1283*m*, 1270*m*, 1237*s*, 1197*s*, 1182*s*, 1140*s*, 1110*s*, 1028*s*, 1005*m*, 986*s*, 970*s*, 697*s* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.37–7.27 (*m*, 5H, *CH* (33) + *CH* (35) + *CH* (36)), 5.23 (*dd*, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 5.10 (*d*, *J* = 12.5 Hz, 1H, CH_a (31)), 4.98 (*d*, *J* = 12.5 Hz, 1H, CH_b (31)), 4.49 (*dd*, *J* = 9.7, 6.3 Hz, 1H, *CH* (3)), 2.26 (*d*, *J* = 11.2 Hz, 1H, *CH* (18)), 2.04 (*s*, 3H, CH_3 (37)), 2.00 (*dd*, *J* = 13.2, 13.2, 4.6 Hz, 1H, CH_a (16)), 1.88 (*m*, 2H, CH_a (2)), 1.85 (*ddd*, *J* = 10.5, 10.5, 4.5 Hz, 1H, CH_a (11)), 1.80 (*ddd*, *J* = 13.5, 13.5, 4.6 Hz, 1H, CH_a (15)), 1.69 (*ddd*, 1H, *J* = 10.5, 3.2, 3.2 Hz, CH_b (11)), 1.66–1.60 (*m*, 4H, CH_2 (22) + CH_a (1) + CH_b (2)), 1.52–1.44 (*m*, 4H, *CH* (9) + CH_a (6) + CH_a (21) + CH_b (16)), 1.33–1.30 (*m*, 1H, *CH* (20)), 1.30–1.27 (*m*, 1H, CH_b (21)), 1.28–1.25 (*m*, 1H, CH_b (7)), 1.07 (*s*, 3H, CH_3 (27)), 1.11–1.03 (*m*, 2H, CH_b (1) + CH_b (15)), 0.99 (*dd*, *J* = 11.6, 5.7 Hz, 1H, *CH* (19)), 0.94 (*s*, 3H, CH_3 (30)), 0.91 (*s*, 3H, CH_3 (25)), 0.86 (*s*, 6H, CH_3 (23) + CH_3 (29)), 0.85 (*s*, 3H, CH_3 (24)), 0.87–0.80 (*m*, 1H, *CH* (5)), 0.64 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (500 MHz, $CDCl_3$): δ = 177.2 (*C*=O, C28), 170.9 (*C*=O, C36), 138.1 (*C*=CH, C13), 136.4 (C_{quart}, C32), 128.4 (CH_{aromat}, C34), 128.1 (CH_{aromat}, C33), 127.9 (CH_{aromat}, C35), 125.6 (*CH*=C, C12), 80.9 (*CHOAc*, C3), 65.9 (CH_2, C31), 55.3 (*CH*, C5), 52.9 (*CH*, C18), 48.1 (C_{quart}, C17), 47.5 (*CH*, C9), 42.0 (C_{quart}, C14), 39.5 (C_{quart}, C8), 39.1 (*CH*, C19), 38.8 (C_{quart}, C20), 38.3 (CH_2, C1), 37.7 (C_{quart}, C4), 36.8 (C_{quart}, C10), 36.6 (CH_2, C21), 32.9 (CH_2, C7), 30.6 (CH_2, C22), 28.1 (CH_3, C29), 27.9 (CH_2, C15), 24.2 (CH_2, C16), 23.5 (CH_2, C11), 23.5 (CH_3, C27), 23.2 (CH_2, C2), 21.3 (CH_3, Ac), 21.1 (CH_3, C30), 18.2 (CH_2, C6), 17.0 (CH_3, C26), 17.0 (CH_3, C24), 16.7 (CH_3, C23), 15.5 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 589.2 (30%, $[M + H]^+$), 611.5 (30%, $[M + Na]^+$), 1199.2 (100%, $[2M + Na]^+$).

*5.4.24. (3β) Benzyl 3-acetoxy-olean-11-en-28-oate (**23**)*

Compound **23** [67] **was** obtained using method **D** as a colorless solid; yield: 83%; R_F = 0.7 (*n*-hexane/ethyl acetate, 85:15); m.p.: 228–230 °C; $[\alpha]_D$ = +55.8° (*c* 0.33, $CHCl_3$); IR (KBr): ν = 2940*brs*, 1726*vs*, 1468*w*, 1456*w*, 1388*w*, 1378*w*, 1364*w*, 1264*m*, 1242*s*, 1200*w*, 1178*m*, 1162*m*, 1122*w*, 1030*m*, 1012*m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.37–7.27 (*m*, 5H, CH_{aromat}), 5.28 (*dd*, *J* = 3.5, 3.5 Hz, 1H,

CH (12)), 5.09 (*d*, *J* = 12.6 Hz, 1H, CH_a (31)), 5.04 (*d*, *J* = 12.5 Hz, 1H, CH_b (31)), 4.48 (*m*, 1H, CH (3)), 2.90 (*dd*, *J* = 13.7, 4.1 Hz, 1H, CH (18)), 2.04 (*s*, 3H, CH_3 (Ac)), 1.98 (*ddd*, *J* = 13.5, 13.5, 4.1 Hz, 1H, CH_a (16)), 1.85 (*dd*, *J* = 8.9, 3.5 Hz, 2H, CH_2 (11)), 1.75–1.68 (*ddd*, *J* = 13.8, 13.8, 4.3 Hz, 1H, CH_a (7)), 1.68–1.58 (*m*, 7H, CH_a (19) + CH_a (1) + CH_b (7) + CH_a (15) + CH_2 (2) + CH_b (16)), 1.60–1.48 (*m*, 1H, CH_a (6)), 1.42 (*ddd*, *J* = 12.6, 12.6, 3.4 Hz, 1H, CH_a (21)), 1.38–1.29 (*m*, 2H, CH_b (6) + CH_b (22)), 1.27–1.20 (*ddd*, *J* = 9.7, 3.5, 2.5 Hz, 1H, CH_b (21)), 1.22–1.16 (*ddd*, *J* = 11.7, 4.1, 2.4 Hz, 1H, CH_b (22)), 1.20–1.13 (*ddd*, *J* = 11.5, 4.5, 2.3 Hz, 1H, CH_b (19)), 1.12 (*s*, 3H, CH_3 (27)), 1.08–0.98 (*m*, 2H, CH_b (1) + CH_b (15)), 0.92 (*s*, 3H, CH_3 (30)), 0.90 (*s*, 3H, CH_3 (25)), 0.90 (*s*, 3H, CH_3 (29)), 0.86 (*s*, 3H, CH_3 (23)), 0.85 (*s*, 3H, CH_3 (24)), 0.84–0.78 (*dd*, *J* = 11.8, 1.7 Hz, 1H, CH (5)), 0.61 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.6 (C=O, C28), 171.1 (C=O, Ac), 143.9 (C=CH, C13), 136.6 (C_{aromat}, C32), 128.6 (CH_{aromat}, C34), 128.1 (CH_{aromat}, C33), 128.0 (CH_{aromat}, C35), 122.6 (CH=C, C12), 81.1 (CHOH, C3), 66.1 (CH_2, C31), 55.5 (CH, C5), 47.7 (CH, C9), 46.9 (C_{quart}, C17), 46.0 (CH_2, C19), 41.8 (C_{quart}, C8), 41.6 (CH, C18), 39.5 (C_{quart}, C20), 38.3 (CH_2, C1), 37.8 (C_{quart}, C4), 37.1 (C_{quart}, C14), 34.0 (CH_2, C22), 33.2 (CH_3, C29), 32.8 (CH_2, C21), 32.5 (CH_2, C7), 30.9 (C_{quart}, C10), 28.2 (CH_3, C23), 27.8 (CH_2, C15), 26.0 (CH_3, C27), 23.8 (CH_3, C30), 23.7 (CH_2, C2), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 21.5 (CH_3, Ac), 18.4 (CH_2, C6), 17.0 (CH_3, C26), 16.8 (CH_3, C24), 15.5 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 589.2 (42.9%, $[M + H]^+$), 611.3 (82.5%, $[M + Na]^+$), 1199.4 (100%, $[2M + Na]^+$).

*5.4.25. (3β) Benzyl 3-acetoxy-12-oxo-olean-28-oate (**24**)*

Treatment of **23** following method **A** gave **24** as a colorless solid; yield: 83%; m.p.: 196–198 °C (lit [68].: 196–198 °C); R_F = 0.38 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +5.2° (*c* 0.63, $CHCl_3$); IR (KBr): ν = 3404*w*, 2943*s*, 1728*s*, 1711*s*, 1472*m*, 1456*m*, 1420*w*, 1387*m*, 1366*m*, 1332*w*, 1308*w*, 1241*s*, 1204*m*, 1180*m*, 1161*m*, 1140*m*, 1119*m*, 1086*w*, 1038*w*, 1006*w* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.39–7.27 (*m*, 5H, CH_{aromat}), 5.21 (*d*, *J* = 12.2 Hz, 1H, CH_a (31)), 5.08 (*d*, *J* = 12.2 Hz, 1H, CH_b (31)), 4.47 (*dd*, *J* = 11.5, 4.8 Hz, 1H, CH (3)), 2.85 (*ddd*, *J* = 10.3, 3.2, 3.8 Hz, 1H, CH (18)), 2.46 (*d*, *J* = 4.2 Hz, 1H, CH (13)), 2.19 (*dd*, *J* = 16.6, 4.8 Hz, 1H, CH_a (11)), 2.05 (*s*, 3H, CH_3 (Ac)), 2.02 (*dd*, *J* = 16.5, 3.1 Hz, 1H, CH_b (11)), 1.94 (*ddd*, *J* = 12.8, 2.4, 2.2 Hz, 1H, CH_a (1)), 1.90–1.85 (*m*, 1H, CH_a (16)), 1.88 (*ddd*, *J* = 13.8, 4.4, 4.4 Hz, 1H, CH_a (22)), 1.70–1.64 (*m*, 1H, CH_b (16)), 1.64–1.55 (*m*, 5H, CH (9) + CH_2 (2) + CH_a (21) + CH_a (6)), 1.56–1.48 (*m*, 1H, CH_b (19)), 1.47 (*ddd*, 1H, *J* = 14.0, 3.4, 3.4 Hz, CH_b (7)), 1.41–1.33 (*m*, 3H, CH_b (22) + CH_b (6) + CH_a (15)), 1.33–1.26 (*m*, 3H, CH_b (22) + CH_b (15) + CH_b (1)), 1.06–0.99 (*m*, 1H, CH_b (21)), 1.00 (*s*, 3H, CH_3 (27)), 0.99–0.95 (*m*, 1H, CH_b (19)), 0.91 (*s*, 6H, CH_3 (30) + CH_3 (28)), 0.86 (*s*, 3H, CH_3 (29)), 0.86 (*s*, 3H, CH_3 (24)), 0.83 (*s*, 3H, CH_3 (25)), 0.80 (*dd*, *J* = 11.1, 1.1 Hz, 1H, CH (5)), 0.68 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 211.4 (C=O, C12), 177.4 (C=O, C28), 170.8 (C=O, Ac), 136.3 (C_{aromat}, C32), 128.5 (CH_{aromat}, C34), 128.4 (CH_{aromat}, C33), 128.1 (CH_{aromat}, C35), 80.4 (CHOH, C3), 65.9 (CH_3, C31), 55.1 (CH, C5), 51.8 (CH, C13), 49.6 (CH, C9), 47.2 (C_{quart}, C17), 41.8 (C_{quart}, C14), 41.2 (C_{quart}, C8), 38.4 (CH_2, C11), 37.7 (C_{quart}, C10), 37.6 (CH_2, C19), 36.8 (C_{quart}, C4), 36.2 (CH_2, C1), 34.5 (CH_2, C21), 33.4 (CH_3, C29), 32.9 (CH_2, C7), 32.1 (CH, C18), 31.7 (CH_2, C22), 30.6 (C_{quart}, C20), 27.9 (CH_3, C30), 27.4 (CH_2, C16), 23.4 (CH_2, C2), 23.2 (CH_3, C27), 22.7 (CH_2, C15), 21.2 (CH_3, Ac), 20.5 (CH_3, C23), 18.1 (CH_2, C6), 16.4 (CH_3, C24), 15.7 (CH_3, C26), 15.2 (CH_3, C25) ppm; (ESI, MeOH): *m*/*z* = 605.5 (100%, $[M + H]^+$), 622.4 (54.8%, $[M + NH_4]^+$), 627.5 (32.6%, $[M + Na]^+$), 929.8 (20%, $[3M+2Na]^{2+}$), 1209.2 (27.9%, $[2M + H]^+$), 1231.2 (76.0%, $[2M + Na]^+$).

*5.4.26. (3β) Benzyl 3-acetoxy-11-oxo-12-hydroxy-olean-Δ-12,13-en-28-oate (**25**)*

Obtained from **24** as a colorless solid following method **E**; yield: 75%; m.p.: 237–240 °C; R_F = 0.69 (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:20); $[\alpha]_D$ = +98.9° (*c* 0.34, $CHCl_3$); IR (KBr): ν = 3461*m*, 3033*w*, 2948*s*, 2868*m*, 1720*s*, 1667*m*, 1640*s*, 1498*s*, 1463*m*, 1372*s*, 1322*w*, 1306*m*, 1286*m*, 1263*s*, 1238*m*, 1205*m*, 1182*m*, 1163*s*, 1140*m*, 1118*m*, 1085*m*, 1085*w*, 1070*w*, 1035*s*, 1012*m* cm^{-1}; UV–Vis ($CHCl_3$): λ_{max} (log ε) = 289.93 (4.09) nm; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41–7.27 (*m*, 5H, CH (33) + CH (34) + CH (35)), 6.23 (*s*, 1H, COH=C (12)), 5.12–5.05 (*m*, 2H, CH_2 (31)), 4.50 (*dd*, *J* = 11.5, 4.9 Hz, 1H, CH (3)), 3.75–3.71 (m, 1H, CH (18)), 2.78 (*ddd*, *J* = 13.4, 3.4, 3.4 Hz, 1H, CH_a (1)), 2.42 (*s*, 1H, CH (9)), 2.09–2.00 (m, 1H, CH_a (16)), 2.04 (*s*, 3H, CH_3 (Ac)), 1.80 (*ddd*, *J* = 13.9, 13.9, 4.5 Hz, 1H, CH_a (7)), 1.76–1.71 (*m*, 3H, CH_b (16) + CH_2 (2)), 1.71–1.62 (*m*, 1H, CH_b (7)), 1.58–1.50 (m, 3H, CH_a (6) + CH_a (15) + CH_a (22)), 1.43–1.34 (*m*, 2H, CH_2 (19) + CH_a (21)), 1.34 (*s*, 3H, CH_3 (27)), 1.33–1.24 (*m*, 3H, CH_b (21) + CH_b (6) + CH_b (22)), 1.18–1.13 (*ddd*, *J* = 14.2, 3.3, 3.3 Hz, 1H, CH_b (15)), 1.07 (*s*, 3H, CH_3 (25)), 1.09–1.03 (*m*, 1H, CH_b (1)), 0.99 (*s*, 3H, CH_3 (30)), 0.92 (*s*, 3H, CH_3 (29)), 0.86 (*s*, 6H, CH_3 (23) + CH_3 (24)), 0.82–0.75 (*m*, 1H, CH (5)), 0.69 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 195.4 (C=O, C11), 177.1 (C=O, C28), 171.1 (C=O, CAc), 142.2 (COH=C, C12), 136.7 (C=COH, C13), 136.3 (C_{quart}, C32), 128.6 (C_{aromat}, C34), 128.1 (C_{aromat}, C33), 128.1 (C_{aromat}, C35), 80.6 (CH, C3), 66.2 (CH_2, C31), 60.4 (CH, C9), 55.2 (CH, C5), 46.0 (C_{quart}, C17), 45.6 (C_{quart}, C14), 41.7 (C_{quart}, C8), 40.4 (CH_2, C19), 38.8 (CH_2, C1), 38.2 (C_{quart}, C4), 37.4 (C_{quart}, C10), 34.2 (CH_2, C21), 33.4 (CH, C18), 33.2 (CH_2, C7), 33.0 (CH_3, C29), 32.0 (CH_2, C22), 30.7 (C_{quart}, C20), 28.2 (CH_3, C23), 27.9 (CH_2, C15), 23.6 (CH_2, C2), 23.4 (CH_3, C27), 23.3 (CH_3, C30), 23.2 (CH_2, C16), 21.4 (CH_3, C37), 18.8 (CH_3, C26), 17.4 (CH_2, C6), 16.8 (CH_3, C24), 16.5 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 619.1 (48%, $[M + H]^+$), 641.3 (29%, $[M + Na]^+$), 1259.3 (100%, $[2M + Na]^+$).

*5.4.27. (3β) But-3-enyl 3–hydroxy-olean-12-en-28-oate (**26**)*

Compound **26** was prepared according to method **D** and obtained as colorless, fine needles (recrystallization from ethanol); yield 84%; m.p.: 80–81 °C; R_F = 0.52 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +110.8 (*c* 0.31, $CHCl_3$); IR (KBr): ν = 3442*br*, 2944*m*, 1636*m*, 1620*w*, 1458*s*, 1446*m*, 1432*s*, 1174*w*, 1106*m*, 1032*m*, 1010*m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.79 (*dddd*, *J* = 17.0, 10.2, 6.7, 6.7 Hz, 1H, CH (33)), 5.27 (*dd*, *J* = 3.5 Hz, 1H, CH (12)), 5.10 (*ddd*, *J* = 17.2, 3.2, 1.5 Hz, 1H, CH_a (34)), 5.06 (*ddd*, *J* = 10.3, 3.1, 1.6 Hz, 1H, CH_b (34)), 4.12–4.01 (*m*, 2H, CH_2 (31), *AA* 'XX'-system), 3.21 (*dd*, *J* = 11.4, 4.6 Hz, 1H, CH (3)), 2.86 (*dd*, *J* = 13.9, 4.3 Hz, 1H, CH (18)), 2.39–2.32 (*m*, 2H, CH_2 (32), AA'XX' system), 1.96 (*ddd*, *J* = 11.6, 11.6, 4.5 Hz, 1H, CH_a (16)), 1.89–1.84 (*m*, 2H, CH_2 (11)), 1.69 (*ddd*, *J* = 13.8, 13.8, 4.4 Hz, 1H, CH_a (7)), 1.66–1.50 (*m*, 9H, CH_a (19) + CH (9) + CH_a (1) + CH_b (7) + CH_2 (2) + CH_a (15) + CH_b (16) + CH_a (6)), 1.50–1.45 (*m*, 1H, CH_a (22)), 1.45–1.38 (*m*, 1H, CH_b (6)), 1.38–1.30 (*m*, 1H, CH_a (21)), 1.31–1.25 (*m*, 1H, CH_b (22)), 1.20–1.10 (*m*, 2H, CH_b (19) + CH_b (21)), 1.12 (*s*, 3H, CH_3 (27)), 1.07–1.00 (*m*, 1H, CH_b (15)), 0.98 (*s*, 3H, CH_3 (23)), 0.97–0.92 (*m*, 1H, CH_b (1)), 0.91 (*s*, 3H, CH_3 (30)), 0.90 (*s*, 3H, CH_3 (25)), 0.89 (*s*, 3H, CH_3 (29)), 0.77 (*s*, 3H, CH_3 (24)), 0.73 (*s*, 3H, CH_3 (26)), 0.72 (*dd*, *J* = 9.6, 1.4 Hz, 1H, CH (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.8 (C=O, C28), 143.9 (C=CH, C13), 134.5 (CH=CH_2, C33), 122.5 (CH=C, C12), 117.1 (CH_2=CH, C34), 79.2 (CHOH, C3), 63.4 (CH_2, C1), 55.4 (CH, C5), 47.8 (CH, C9), 46.8 (C_{quart}, C17), 46.1 (CH_2, C19), 41.9 (C_{quart}, C8), 41.4 (CH, C18), 39.5 (C_{quart}, C20), 38.9 (C_{quart}, C14), 38.6 (CH_2, C1), 37.2 (C_{quart}, C4), 34.1 (CH_2, C22), 33.3 (CH_3, C29), 33.3 (CH_2, C32), 32.9 (CH_2, C21), 32.6 (CH_2, C7), 30.9 (C_{quart}, C10), 28.3 (CH_3, C23), 27.8 (CH_2, C15), 27.4 (CH_2, C2), 26.0 (CH_3, C27), 23.7 (CH_3, C30), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.5 (CH_2, C6), 17.2 (CH_3, C26), 15.7 (CH_3, C24), 15.5 (CH_3, C25) ppm; MS (ESI): *m*/*z* = 511.3 (30%, $[M + H]^+$), 1043.4 (100%, $[2M + Na]^+$).

*5.4.28. (3β) But-3-enyl 3–hydroxy-12-oxo-olean-28-oate (**27**)*

Compound was prepared using method **B** followed by chromatography (silica gel, hexane/ethyl acetate, 7:3) and recrystallization from ethanol as a colorless solid; yield 76%; m.p.: 72–73 °C; R_F = 0.36 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = −19.2 (*c* 0.31, $CHCl_3$); IR (KBr): ν = 3440*br*, 2972*s*, 2940*s*, 2862*m*, 2362*m*, 2344*m*, 1700*vs*, 1654*m*, 1646*m*, 1636*m*, 1466*m*, 1458*m*, 1438*m*, 1418*m*, 1388*m*, 1304*m*, 1256*m*, 1242*m*, 1188*m*, 1176*m*, 1088*m*, 1048*m*, 1036*m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.82–5.73 (*m*, 1H, C*H* (33)), 5.11 (*ddd*, *J* = 17.2, 3.2, 1.5 Hz, 1H, CH_a (34)), 5.06 (*ddd*, *J* = 10.2, 1.6, 1.1 Hz, 1H, CH_b (34)), 4.19–4.08 (*m*, 2H, C*H* (31)), 3.20 (*dd*, *J* = 11.4, 4.7 Hz, 1H, C*H* (3)), 2.79 (*dd*, *J* = 10.1, 3.4, 3.4 Hz, 1H, C*H* (18)), 2.62 (*d*, *J* = 4.3 Hz, 1H, C*H* (13)), 2.38 (*brdd*, *J* = 6.6, 6.6 Hz, 2H, CH_2 (32)), 2.23 (*dd*, *J* = 16.7, 5.0 Hz, 1H, CH_a (11)), 2.13 (*dd*, *J* = 16.6, 13.2 Hz, 1H, CH_b (11)), 1.97–1.90 (*m*, 1H, CH_a (19)), 1.91–1.84 (*m*, 1H, CH_a (16)), 1.79 (*ddd*, *J* = 13.8, 13.8, 4.7 Hz, 1H, CH_a (7)), 1.70–1.51 (*m*, 6H, CH_b (16) + CH_a (6) + CH_2 (2) + C*H* (9) + CH_a (1)), 1.48–1.37 (*m*, 3H, CH_b (7) + CH_b (6) + CH_a (22)), 1.36–1.29 (*m*, 2H, CH_a (21) + CH_b (22)), 1.25–1.18 (*m*, 1H, CH_b (19)), 1.11–1.02 (*m*, 1H, CH_b (15)), 0.99 (*s*, 3H, CH_3 (30)), 0.97 (*s*, 3H, CH_3 (23)), 0.97 (*s*, 3H, CH_3 (25)), 0.94 (*s*, 3H, CH_3 (27)), 0.90 (*s*, 3H, CH_3 (29)), 0.92–0.87 (*m*, 1H, CH_b (1)), 0.85 (*s*, 3H, CH_3 (24)), 0.78 (*s*, 3H, CH_3 (26)), 0.74–0.70 (*m*, 1H, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 211.9 (*C*=O, C12), 177.9 (*C*=O, C28), 134.2 (*C*H, C13), 117.4 (CH_2=CH, C34), 78.8 (CHOH, C3), 63.6 (CH_2, C31), 55.3 (*C*H, C5), 52.0 (*C*H, C13) 49.9 (*C*H, C9), 47.5 (C_{quart}, C17), 42.1 (C_{quart}, C14), 41.5 (C_{quart}, C8), 39.0 (CH_2, C11), 38.7 (C_{quart}, C20), 38.1 (CH_2, C1), 37.1 (C_{quart}, C4), 36.4 (CH_2, C19), 34.7 (CH_2, C32), 33.5 (CH_3, C29), 33.3 (CH_2, C21), 33.2 (CH_2, C7), 32.1 (CH, C18), 32.0 (CH_2, C32), 30.8 (C_{quart}, C10), 28.1 (CH_3, C23), 27.7 (CH_2, C15), 27.2 (CH_2, C2), 23.3 (CH_3, C30), 22.9 (CH_3, C21), 20.7 (CH_3, C30), 18.5 (CH_2, C6), 16.4 (CH_3, C24), 15.5 (CH_3, C26), 15.4 (CH_3, C25) ppm; MS (ESI): *m*/*z* = 527.2 (100%, $[M + H]^+$), 544.2 (20%, $[M + NH_4]^+$), 1053.3 (51%, $[2M + Na]^+$), 1075.3 (42%, $[2M + Na]^+$).

*5.4.29. (2α, 3β, 12α) 2,3,12–Trihydroxy-olean-28-oic acid 28,13-lactone (**28**)*

Compound **28** obtained from **MA** following method **A**; yield: 40%; m.p.: 229–231 °C; R_F = 0.21 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D$ = 18.8° (*c* 0.36, $CHCl_3$); IR (KBr): ν = 3442*vs*, 3432*vs*, 2950*s*, 2936*s*, 2866*m*, 1754*s*, 1466*m*, 1456*m*, 1386*m*, 1366*m*, 1252*m*, 1222*m*, 1144*m*, 1134*m*, 1084*m*, 1046*m*, 1032*m*, 974*m*, 942*m*, 732*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 3.89 (*brs*, 1H, C*H* (12)), 3.85–3.68 (*m*, 1H, C*H* (2)), 3.06 (*d*, *J* = 8.6 Hz, 1H, C*H* (3)), 3.15–2.80 (*brs*, 2H, O*H*), 2.15 (*ddd*, *J* = 13.5, 13.5, 5.6 Hz, 1H, CH_a (16)), 2.10–1.91 (*m*, 5H, C*H* (18) + CH_a (1) + CH_a (19) + CH_2 (11)), 1.90–1.79 (*m*, 1H, CH_a (15)), 1.72–1.59 (*m*, 3H, C*H* (9) + CH_2 (7)), 1.58–1.45 (*m*, 4H, CH_a (22) + CH_2 (6) + CH_b (16)), 1.46–1.34 (*m*, 2H, CH_b (22) + CH_a (21)), 1.31 (*s*, 3H, CH_3 (27)), 1.29–1.20 (*m*, 2H, CH_b (19) + CH_b (21)), 1.15–1.09 (*m*, 1H, CH_b (15)), 1.13 (*s*, 3H, CH_3 (26)), 1.03 (*s*, 3H, CH_3 (23)), 0.98 (*s*, 3H, CH_3 (30)), 1.08–0.89 (*m*, 1H, CH_b (1)), 0.94 (*s*, 3H, CH_3 (25)), 0.90 (*s*, 3H, CH_3 (29)), 0.88–0.83 (*m*, 1H, C*H* (5)), 0.82 (*s*, 3H, CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 180.2 (C=O, C28), 90.8 (*C*–O, C13), 84.0 (CHOH, C3), 76.1 (CHOH, C12), 69.2 (CHOH, C2), 55.4 (CH, C5), 51.2 (CH, C18), 46.7 (CH_2, C1), 44.9 (C_{quart}, C17), 44.7 (*C*H, C9), 42.5 (C_{quart}, C14), 42.3 (C_{quart}, C8), 39.5 (CH_2, C19), 39.4 (C_{quart}, C4), 37.9 (C_{quart}, C10), 34.3 (CH_2, C21), 34.0 (CH_2, C7), 33.4 (CH_3, C30), 31.7 (C_{quart}, C20), 29.0 (CH_2, C11), 28.6 (CH_3, C23), 28.1 (CH_2, C15), 27.6 (CH_2, C22), 24.0 (CH_3, C29), 21.3 (CH_2, C16), 18.8 (CH_3, C27), 18.7 (CH_3, C26), 17.9 (CH_2, C6), 17.7 (CH_3, C25), 16.7 (CH_3, C24) ppm; MS (ESI, MeOH): *m*/*z* = 489.2 (60%, $[M + H]^+$), 977.4 (12%, $[2M + H]^+$), 944.4 (100%, $[2M + Na]^+$).

*5.4.30. (2α,3β) 2,3–Diydroxy-12-chloro-olean-28-oic acid 28,13-lactone (**29**)*

MA (0.3 g, 0.6 mmol) was solved in a DCM/water mixture (1:1, 40 mL) and sodium hypochlorite (3 mL; 13% Cl_2) was added. After stirring for 5 h at 25 °C, the mixture was quenched with sodium sulfide and stirred for one additional hour. Usual workup afforded **29** as a colorless solid; yield: 84%; m.p.: 306–309 °C; R_F = 0.40 (*n*-hexane/ethyl acetate, 1:2); $[\alpha]_D$ = +34.5° (*c* 0.5, $CHCl_3$); IR (KBr): ν = 3395*m*, 2950*s*, 1767*s*, 1464*m*, 1395*m*, 1361*m*, 1254*m*, 1216*m*, 1134*m*, 1050*m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 4.16 (*dd*, *J* = 3.9, 2.3 Hz, 1H, C*H* (12)), 3.72 (*ddd*, *J* = 11.5, 9.7, 4.7 Hz, 1H, C*H* (2)), 3.03 (*d*, *J* = 9.6 Hz, 1H, CH (3)), 2.28 (*ddd*, *J* = 15.0, 12.9, 4.0 Hz, 1H, CH_a (11)), 2.18–2.10 (*m*, 2 H, CH_a (19) + CH_a (16)), 2.08–2.05 (*m*, 1H, CH_a (1)), 2.04–1.87 (*m*, 3H, CH_b (19) + C*H* (18) + CH_a (15)), 1.77–1.71 (*m*, 2H, C*H* (9) + CH_b (11)), 1.68–1.59 (*m*, 2H, CH_2 (7)), 1.57–1.49 (*m*, 2H, CH_a (22) + CH_a (6)), 1.47–1.42 (*m*, 1H, CH_b (6)), 1.37 (*s*, 3H, CH_3 (27)), 1.36–1.26 (*m*, 2H, CH_a (21) + CH_b (22)), 1.27–1.10 (*m*, 3H, CH_a (16) + CH_b (21) + CH_b (15)), 1.15 (*s*, 3H, CH_3 (26)), 1.00 (*s*, 3H, CH_3 (23)), 1.03–0.96 (*m*, 1H, CH_b (1)), 0.99 (*s*, 3H, CH_3 (30)), 0.95 (*s*, 3H, CH_3 (25)), 0.89 (*s*, 3H, CH_3 (29)), 0.90–0.84 (*m*, 1H, C*H* (5)), 0.84 (*s*, 3H, CH_3 (24)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 179.2 (*C*=O, C28), 91.7 (C_{quart}, C13), 83.4 (CHOH, C3), 69.1 (CHOH, C2), 64.6 (CHCl, C12), 55.5 (CH, C5), 51.8 (CH, C18), 46.6 (CH_2, C1), 45.1 (C_{quart}, C17), 44.8 (CH, C9), 43.2 (C_{quart}, C14), 42.4 (C_{quart}, C8), 39.6 (CH_2, C19), 39.2 (C_{quart}, C4), 37.9 (C_{quart}, C10), 34.2 (CH_2, C7), 33.7 (CH_2, C21), 33.5 (CH_3, C30), 31.7 (C_{quart}, C20), 29.4 (CH_2, C11), 29.3 (CH_2, C15), 28.4 (CH_3, C23), 27.3 (CH_2, C22), 23.5 (CH_3, C29), 21.2 (CH_2, C16), 20.2 (CH_3, C27), 18.7 (CH_3, C26), 18.1 (CH_3, C25), 17.9 (CH_2, C6), 16.5 (CH_3, C24) ppm; MS (ESI, MeOH): *m*/*z* = 529.5 (85%, $[M + Na]^+$), 561.1 (37%, $[M + Na + MeOH]^+$), 1035.3 (100%, $[2M + Na]^+$).

*5.4.31. (2α,3β) 2,3–Dihydroxy-12-bromo-olean-28-oic acid 28,13-lactone (**30**)*

MA (300 mg, 0.63 mmol) in acetic acid (20 mL, 90%) was treated with sodium acetate (0.5 g, 6.1 mmol) and bromine (50 μL, 1 mmol). After 10 min stirring at room temperature the reaction was quenched with water and an aq. solution of sodium sulfide. The white precipitate were filtered off and washed with water to yield **30** as a colorless solid; yield: 72%; m.p.: 260–262 °C (lit. 261–263 °C [69]); R_F = 0.44 (*n*-hexane/ethyl acetate, 1:2); $[\alpha]_D$ = +33.0° (*c*0.50, $CHCl_3$) [lit. 31.6° (*c* 3.0, $CHCl_3$ [69])]; IR (KBr): ν = 3384*s*, 2950*s*, 1768*s*, 1462*m*, 1394*m*, 1360*m*, 1301*w*, 1246*w*, 1210*m*, 1130*m*, 1051 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 4.26 (*dd*, *J* = 3.7, 2.3, 1H, C*H* (12)), 3.70 (*ddd*, *J* = 11.3, 9.6, 4.6 Hz, 1H, CH_a (2)), 3.05 (*d*, *J* = 9.6 Hz, 1H, C*H* (3)), 2.39 (*ddd*, *J* = 15.0, 12.4, 3.9 Hz, 1H, CH_a (11)), 2.34–2.28 (*m*, 1H, CH_a (19)), 2.18–2.00 (*m*, 2H, CH_a (16) + CH_a (1)), 2.01–1.90 (*m*, 3H, C*H* (18) + CH_b (19) + CH_a (15)), 1.89–1.83 (*m*, 1H, CH_b (11)), 1.82–1.75 (*m*, 1H, C*H* (9)), 1.69–1.52 (*m*, 2H, CH_2 (7)), 1.60–1.50 (*m*, 1H, CH_a (22)), 1.55–1.48 (*m*, 1H, CH_a (6)), 1.47–1.44 (*m*, 1H, CH_b (6)), 1.44 (*s*, 3H, CH_3 (27)), 1.40–1.37 (*m*, 1H, CH_a (21)), 1.33–1.38 (*m*, 1H, CH_b (22)), 1.29–1.20 (*m*, 2H, CH_b (16) + CH_b (21)), 1.17 (*s*, 3H, CH_3 (26)), 1.19–1.10 (*m*, 1H, CH_b (15)), 1.01 (*s*, 3H, CH_3 (23)), 1.03–0.95 (*m*, 1 H, CH_b (1)), 0.99 (*s*, 3H, CH_3 (30)), 0.94 (*s*, 3H, CH_3 (25)), 0.92–0.89 (*m*, 1H, C*H* (5)), 0.89 (*s*, 3H, CH_3 (29)), 0.82 (*s*, 3H, CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.7 (*C*=O, C28), 91.5 (*C*–O, C13), 83.8 (CHOH C3), 69.3 (CHOH, C2), 56.0 (CHBr, C12), 55.5 (CH, C5), 52.1 (CH, C18), 46.1 (CH_2, C1), 45.6 (CH, C9), 45.6 (C_{quart}, C17), 43.7 (C_{quart}, C14), 42.4 (C_{quart}, C8), 39.8 (CH_2, C19), 39.2 (C_{quart}, C4), 37.9 (C_{quart}, C10), 34.3 (CH_2, C7), 33.8 (CH_2, C21), 33.5 (CH_3, C30), 31.7 (C_{quart}, C20), 30.5 (CH_2, C11), 29.3 (CH_2, C15), 28.4 (CH_3, C23), 27.4 (CH_2, C22), 23.6 (CH_3, C29), 21.2 (CH_2, C16), 21.1 (CH_3, C27), 19.0 (CH_3, C26), 18.0 (CH_3, C25), 17.8 (CH_2, C6), 16.8 (CH_3, C24) ppm; MS (ESI, MeOH): *m*/*z* = 551.3 (11%, $[M + H]^+$), 568.3 (19%,

$[M + NH_4]^+$), 573.5 (62%, $[M + Na]^+$), 605.1 (20%, $[M + Na + MeOH]^+$), 1123.1 (100%, $[2M + Na]^+$).

*5.4.32. (2α,3β) O,O′-2,3–Diydroxy-12-iodo-olean-28-oic acid 28,13-lactone (**31**)*

MA (0.3 g, 0.6 mmol)was solved in DCM/water (1:1, 40 mL) and treated with sodium hydrocarbonate (250 mg, 3 mmol), potassium iodide (1 g, 6 mmol) and iodine (500 mg, 2 mmol). After 48 h stirring at 25 °C and work up as described above, compound **31** was obtained as a colorless solid; yield: 46%; m.p.: 230–233 °C; R_F = 0.44 (*n*-hexane/ethyl acetate, 1:2); $[\alpha]_D$ = +69.1° (*c* 0.40, $CHCl_3$); IR (KBr): ν = 3424*s*, 2954*s*, 1775*s*, 1637*w*, 1468*m*, 1386*m*, 1368*w*, 1301*w*, 1210*w*, 1198*w*, 1179*m*, 1130*m*, 1103*w*, 1052*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 4.45 (*dd*, *J* = 4.5, 2.3 Hz, 1H, *CH* (12)), 3.76 (*ddd*, *J* = 11.7, 9.2, 4.5 Hz, 1H, CH_a (2)), 3.07 (*d*, *J* = 9.2 Hz, 1H, *CH* (3)), 2.60–2.51 (*m*, 1H, CH_a (19)), 2.42–2.36 (*m*, 1H, CH_a (11)), 2.14–2.08 (*m*, 1H, CH_a (16)), 2.10–2.06 (*m*, 1H, CH_a (1)), 1.98–1.83 (*m*, 4H, CH_b (11) + CH_b (15) + CH_b (19) + *CH* (18)), 1.79–1.72 (*m*, 1H, *CH* (9)), 1.68–1.40 (*m*, 4H, CH_2 (22) + CH_a (7) + CH_a (6)), 1.50 (*s*, 3H, CH_3 (27)), 1.50–1.40 (*m*, 1H, CH_b (6)), 1.33–1.20 (*m*, 3H, CH_b (7) + CH_a (21) + CH_b (16)), 1.23 (*s*, 3H, CH_3 (26)), 1.21–1.13 (*m*, 2H, CH_b (21) + CH_a (15)), 1.12–1.08 (*m*, 1H, CH_b (1)), 1.04 (*s*, 3H, CH_3 (23)), 0.99 (*s*, 3H, CH_3 (30)), 0.94 (*s*, 3H, CH_3 (25)), 0.95–0.85 (*m*, 1H, *CH* (5)), 0.88 (*s*, 3H, CH_3 (29)), 0.80 (*s*, 3H, CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.3 (*C*=*O*, C28), 91.4 (C_{quart}, C13), 83.6 (*C*HOH, C3), 69.2 (*C*HOH, C2), 55.2 (*C*H, C5), 52.7 (*C*H, C18), 47.1 (*C*H, C9), 45.8 (C_{quart}, C17), 45.9 (CH_2, C1), 44.1 (C_{quart}, C14), 42.6 (C_{quart}, C8), 40.0 (CH_2, C19), 39.4 (C_{quart}, C4), 38.0 (C_{quart}, C10), 34.6 (CH_2, C7), 33.7 (CH_2, C21), 33.6 (CH_3, C30), 33.1 (CH_2, C11), 32.0 (*C*HI, C12), 32.1 (C_{quart}, C20), 29.6 (CH_2, C15), 28.3 (CH_3, C23), 27.6 (CH_2, C22), 23.4 (CH_3, C29), 22.9 (CH_3, C27), 21.5 (CH_2, C16), 19.4 (CH_3, C26), 18.5 (CH_3, C25), 17.6 (CH_2, C6), 16.8 (CH_3, C24) ppm; MS (ESI, MeOH): *m*/*z* = 599.1 (16%, $[M + H]^+$), 621.3 (100%, $[M + Na]^+$), 652.9 (49%, $[M + Na + MeOH]^+$), 1218.9 (87%, $[2M + Na]^+$).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.11.025.

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Supplementary material

The chemical and biological potential of C ring modified triterpenoids.

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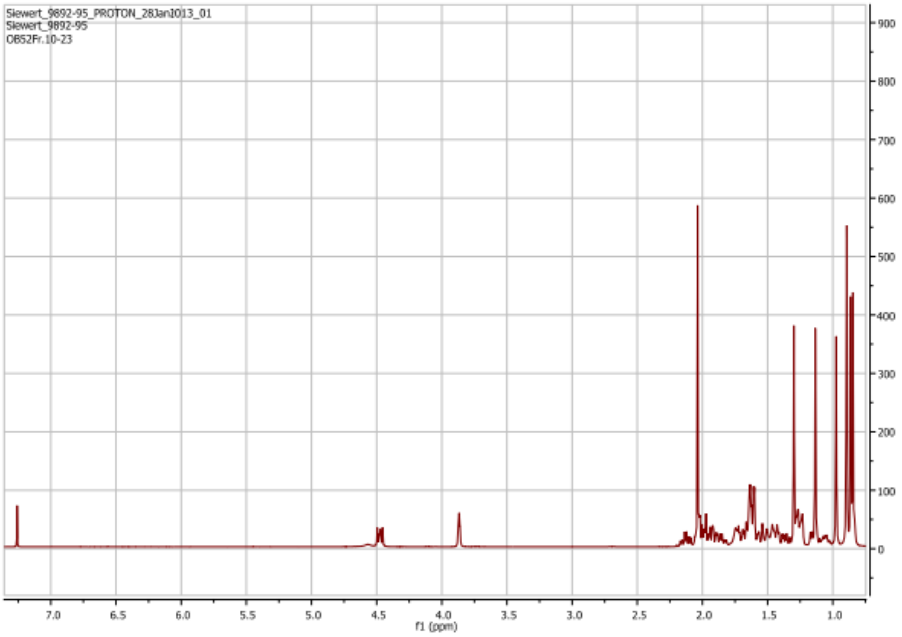
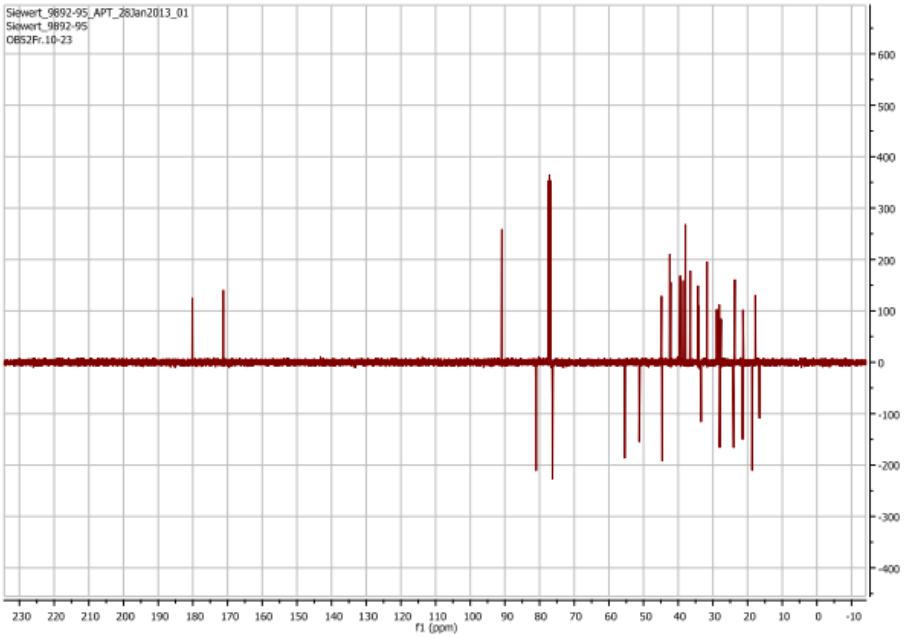
NMR-Spectroscopic results for representative compounds

*(3β, 12α) 3-Acetyl-12-hydroxy-18β-olean-28-oic acid 28,13-lactone (**4**)*

^{1}H NMR



^{13}C-APT NMR



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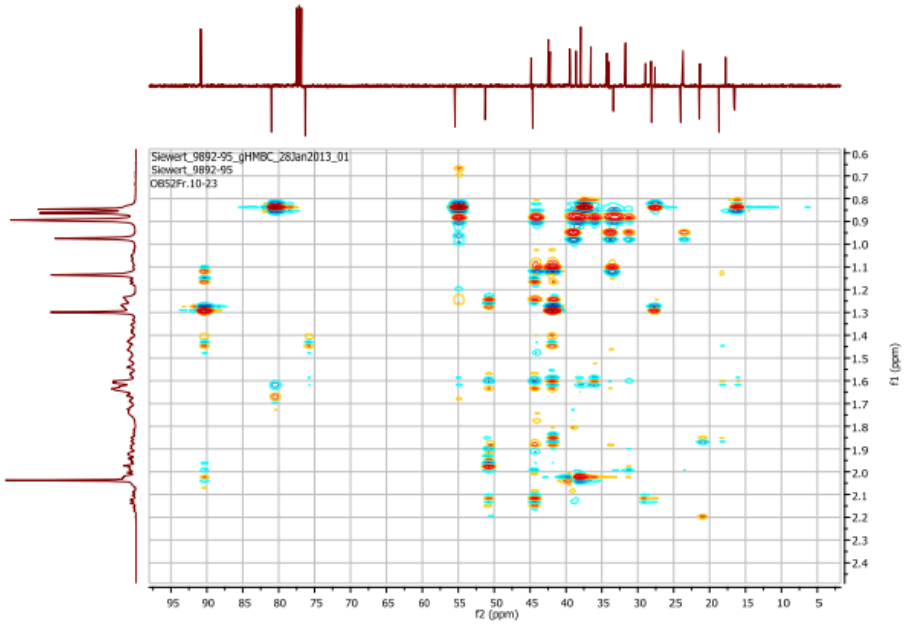

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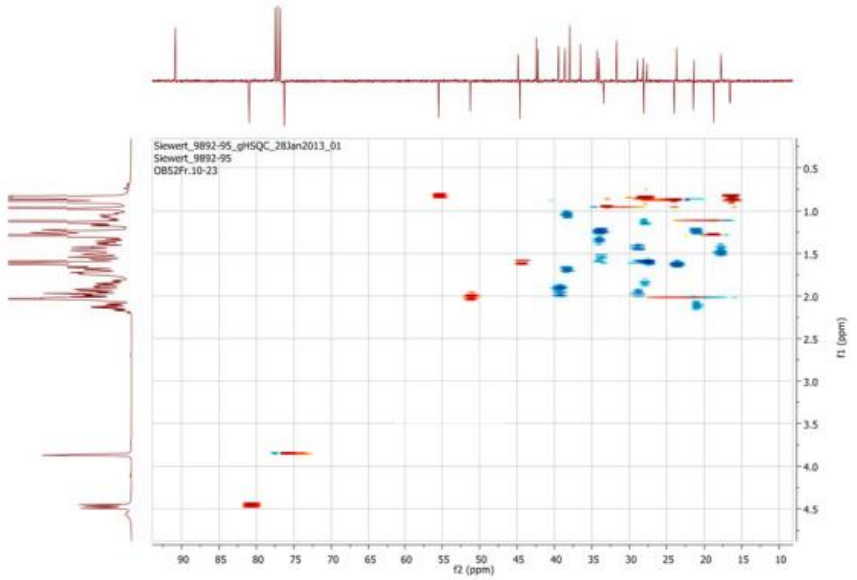
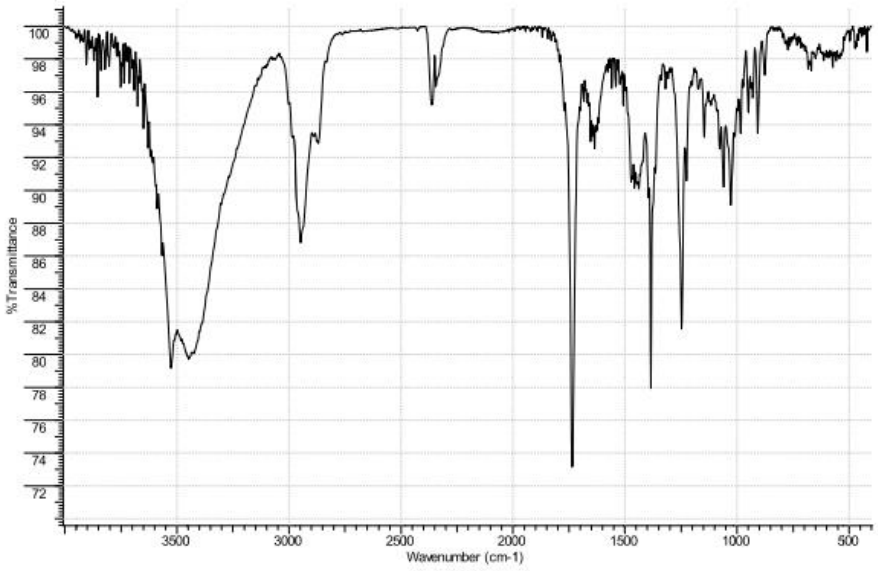
gHSQC



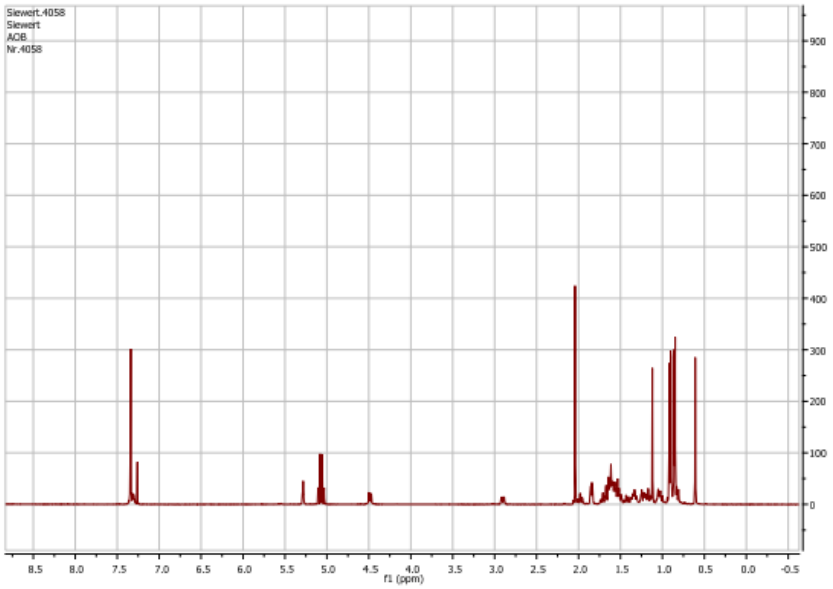
IR-Spectra



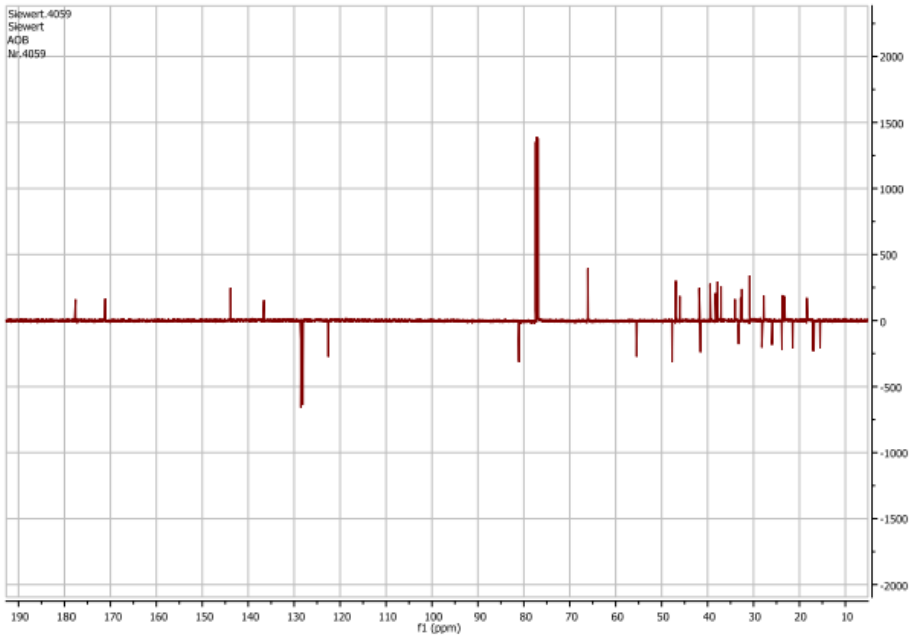
3

(3β) Benzyl 3-acetoxy-olean-11-en-28-oate (23)

^{1}H NMR



^{13}C-APT NMR



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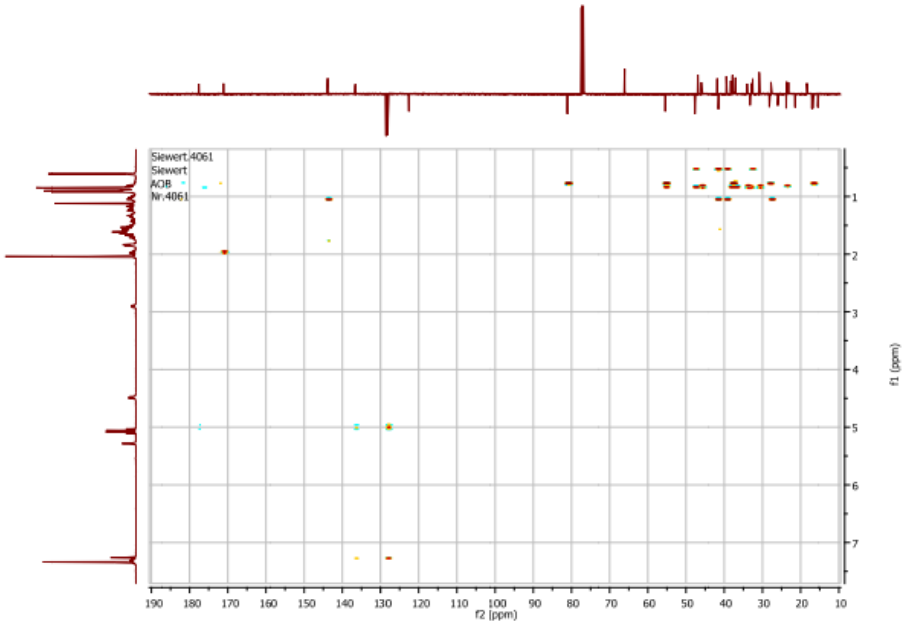
gHMBC



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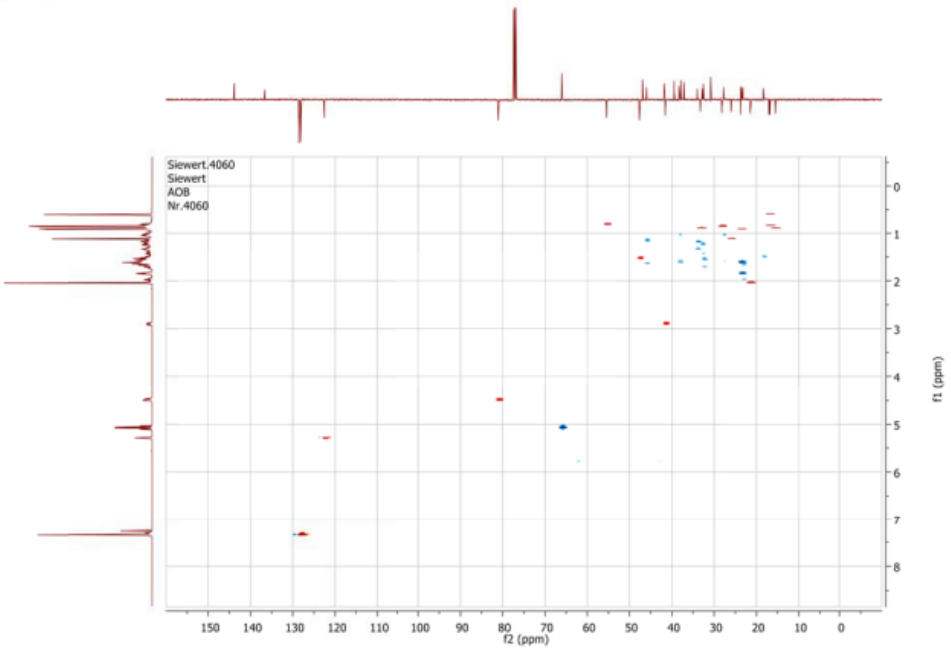

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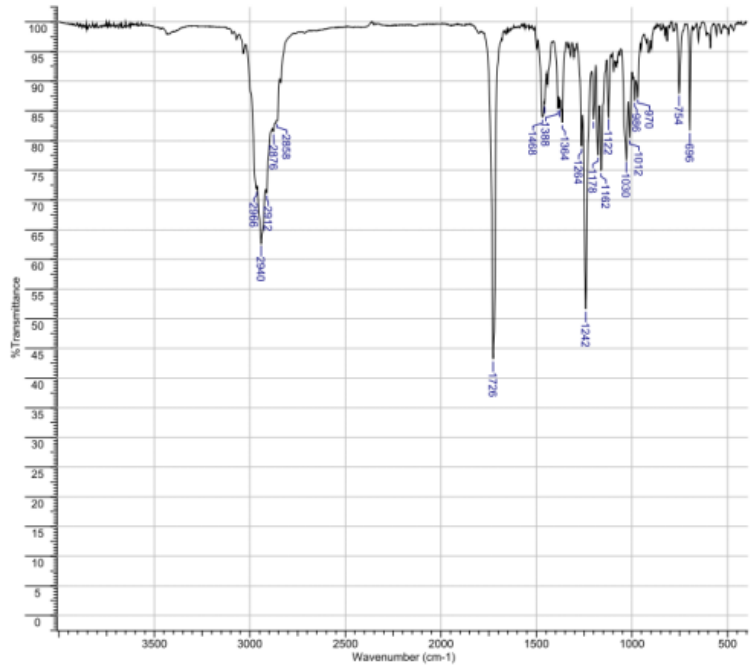
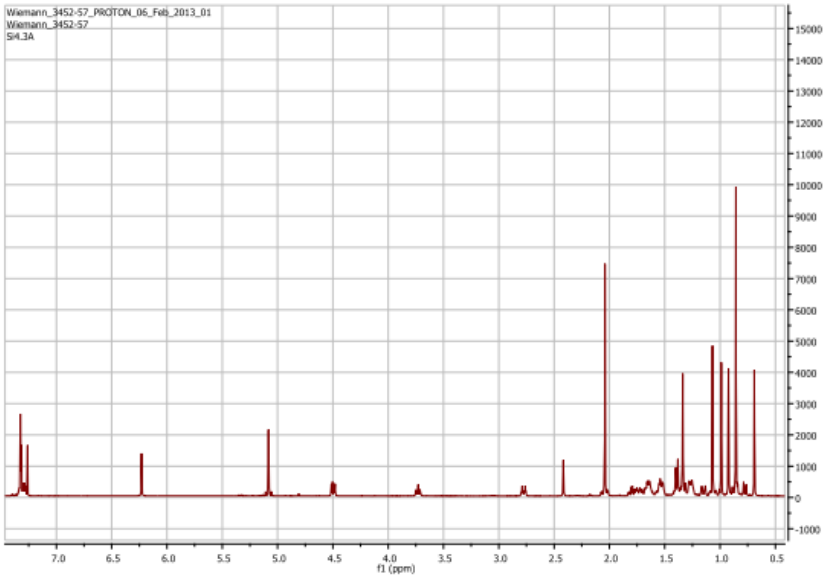
IR-spectra



(3β) Benzyl 3-acetoxy-11-oxo-12hydroxy-olean-Δ-12,13-en-28-oate ***(25)***

^{1}H-NMR



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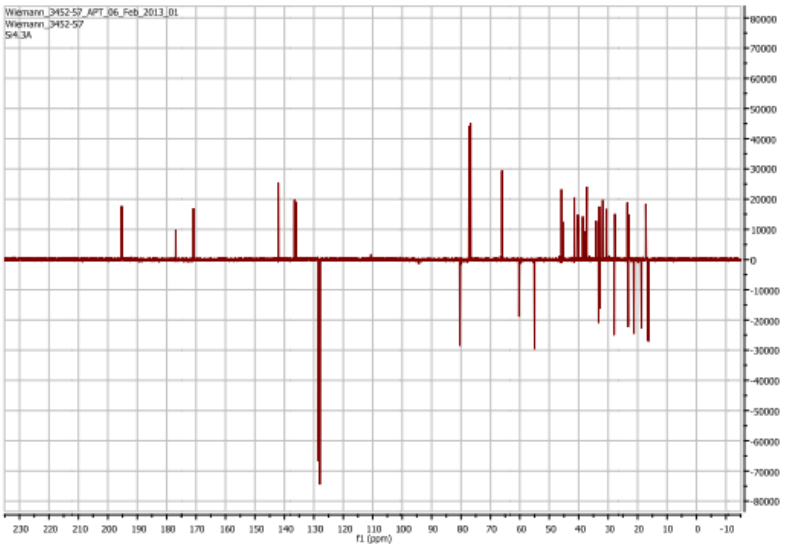
^{13}C-APT-NMR



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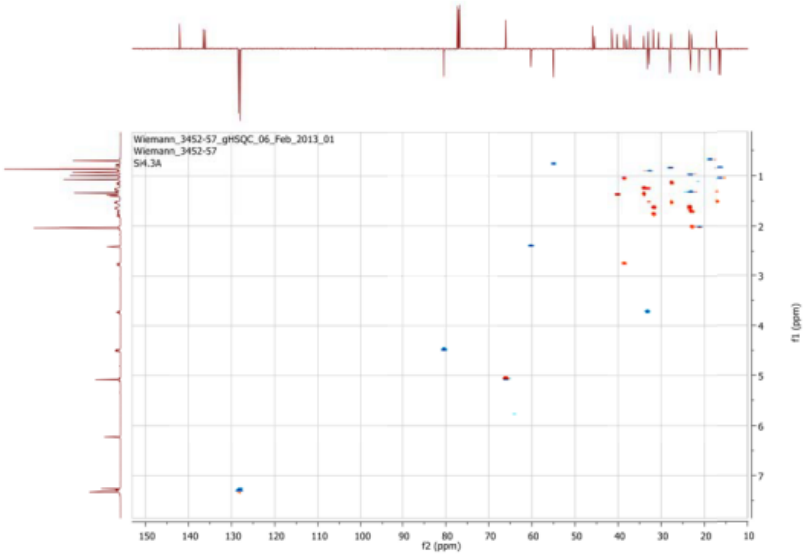

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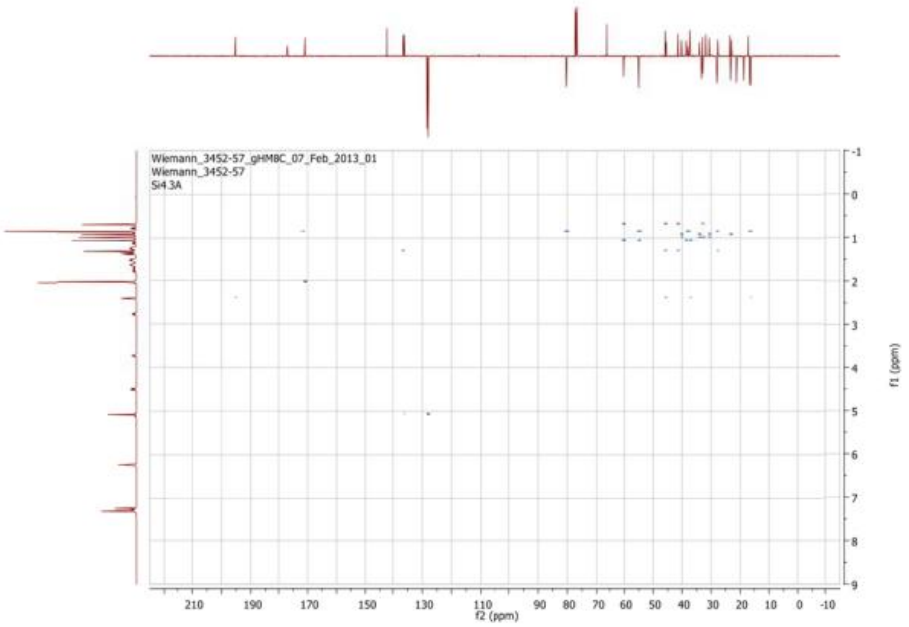
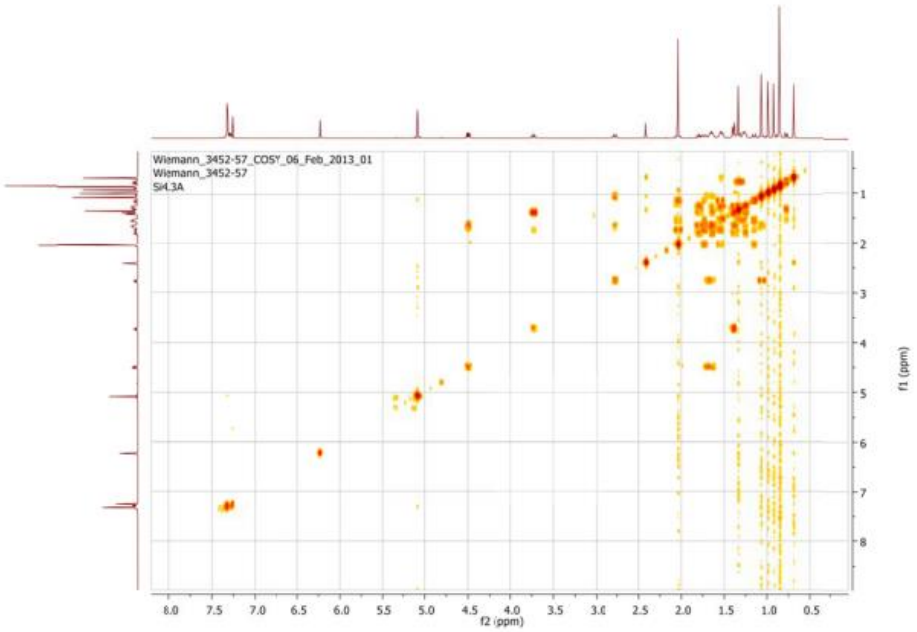
gHMBC

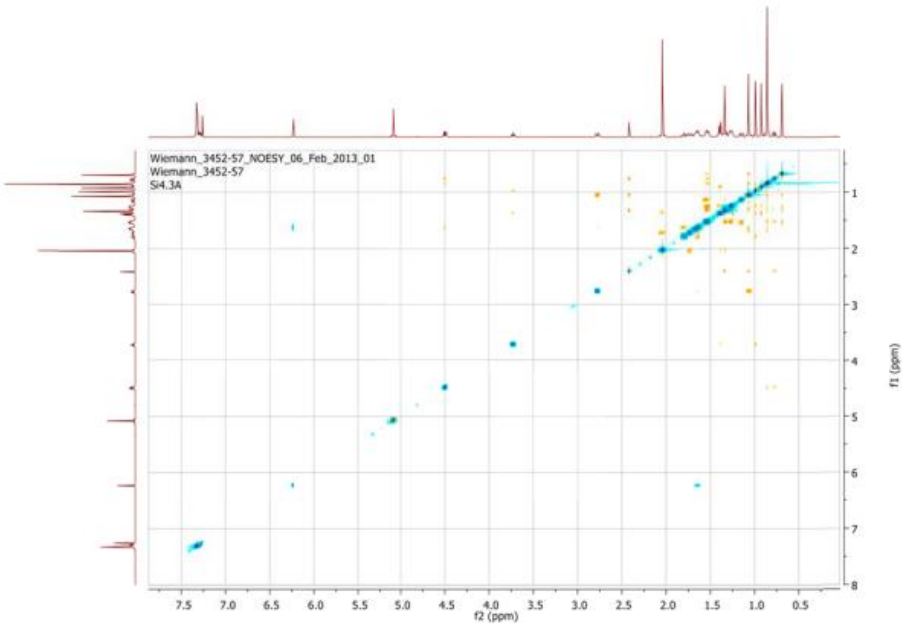


H,H-COSY

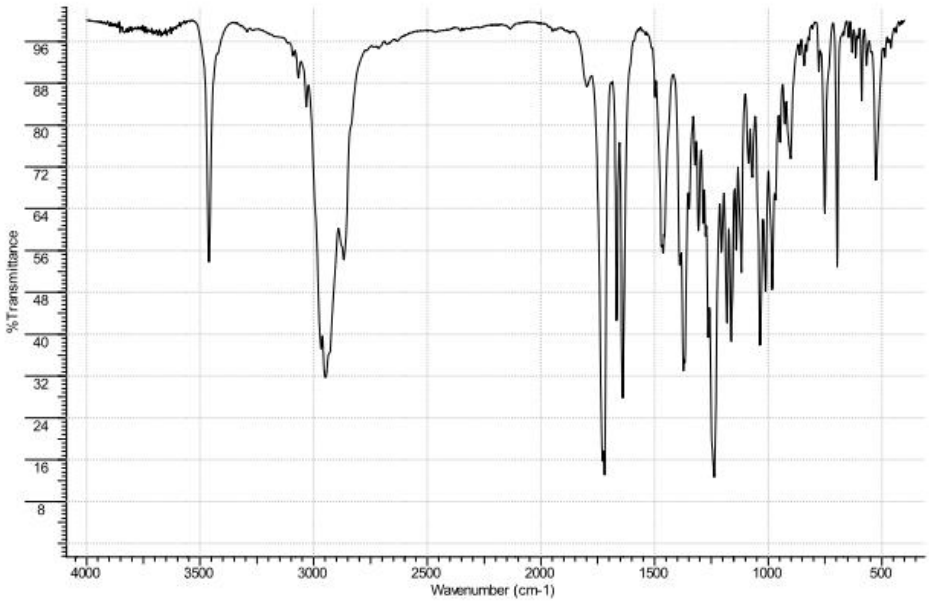


8

H,H-NOESY



IR spectra

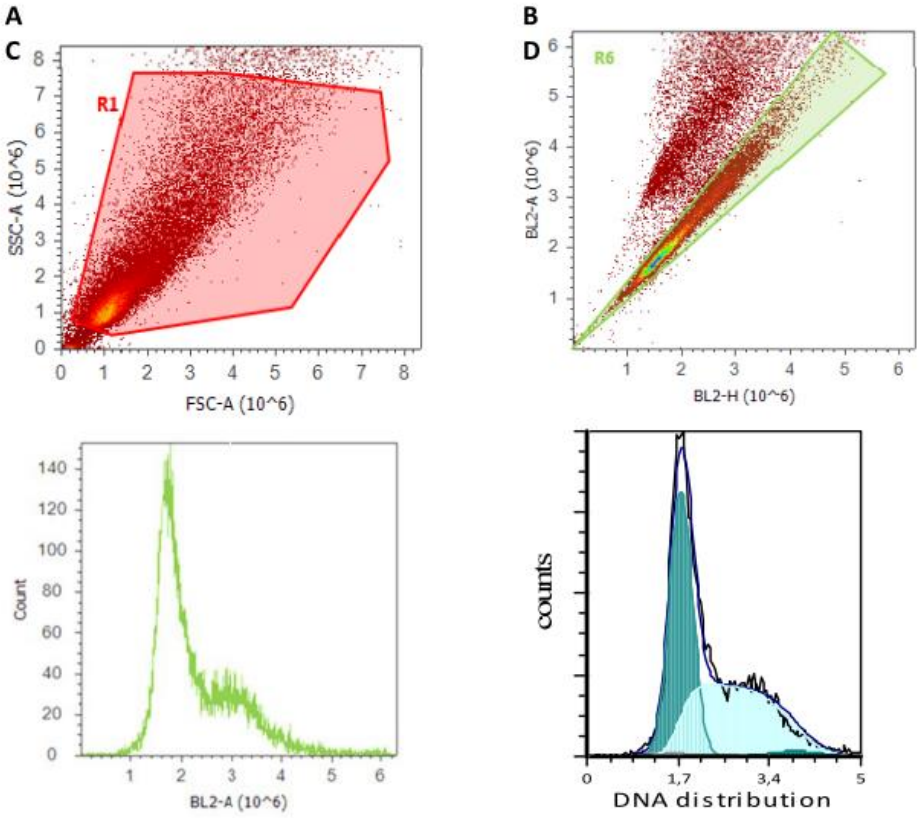


9

Biological Investigations

Cell Cycle

The experiments were performed as described in the experimental part (5.3.3). Below, all graphs (Fig. S3) are depicted in more detail. In addition, for compound **1** the detailed calculated process, starting with the original FACS data is shown (Fig S1). Figure S3 gives detail to the single-cell extraction protocol.



G1 Mean	G1 CV	%G1	G2 Mean	G2 CV	%G2	%S	G2/G1	%Total
1767332,25	12.293	48.672	3780241,00	12.264	3.157	48.171	2.139	100.000

Figure S1_

A: Density plot of the measured cell population. **B**:_The doublets were removed by plotting BL-2H (488 nm, 20 mW, pulse height) against BL-2A ((488 nm, 20 mW, pulse

area) and selecting the diagonal events. When a single-cell signal and a doublet-cell signal show the same height (BL-2H value), the signal area (BL2-A) shows in case of doublet cells the two-fold area under the curve. The single-cell events are represented by the main diagonal, doublet-cell events are represented by the parallel diagonal. Thus, separation is possible by choosing the main diagonal events (green colored gate, R6) see figure S3 **C**: The BL2-A plot of the corrected cell cycle. **D**: The multicycle fit (FCS-Express™ De Novo Software) with CV for G1 and G2.

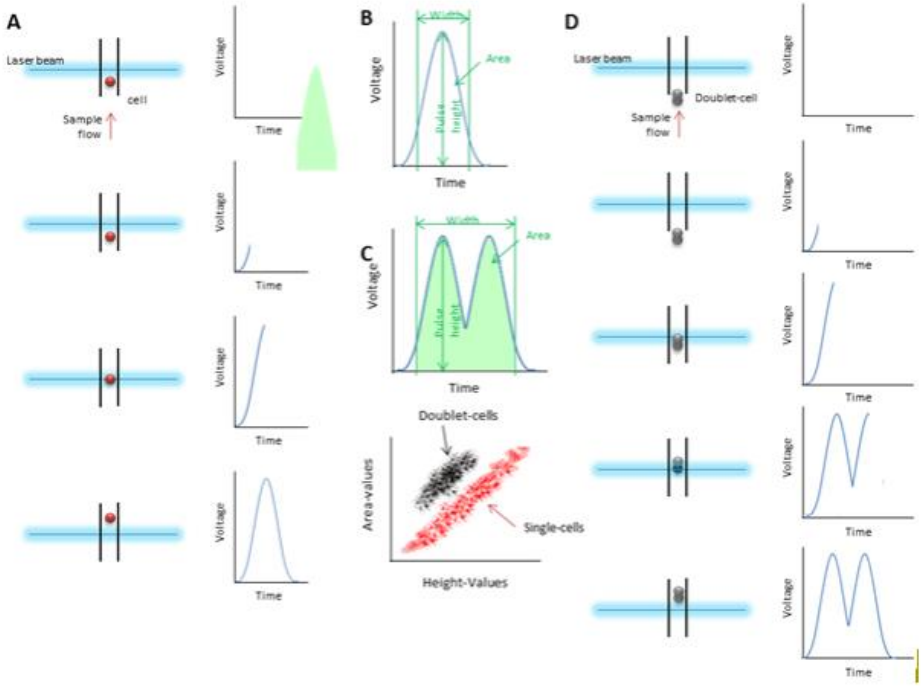
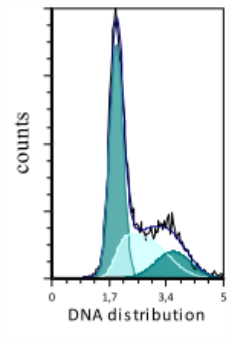


Figure S2: Pulse measurement and event separation

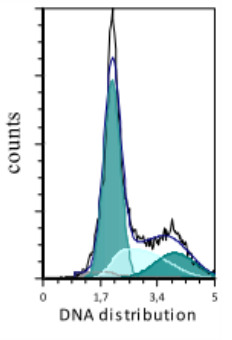
A) The process of normal pulse recording **B**) Pulse measurement in case of a single cell **C**) Pulse measurement in case of a cell-doublet; **D**) Schematic representation of the correlation between A-Pulse measurement and H-pulse measurement of single vs. double-cells; **E**) The process of doublet-cell recording

A



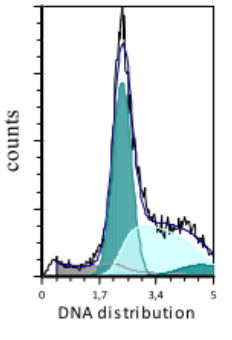
%G1	%G2	%S
51.839	15.721	32.439

B



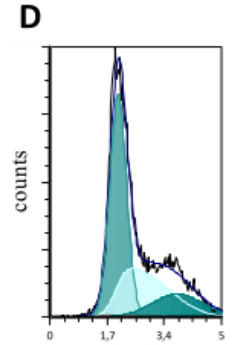
%G1	%G2	%S
56.831	17.835	25.334

C



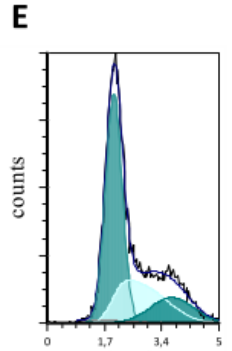
%G1	%G2	%S
49.973	7.345	42.682

D



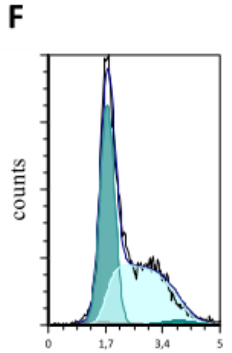
%G1	%G2	%S
51.879	15.860	32.261

E



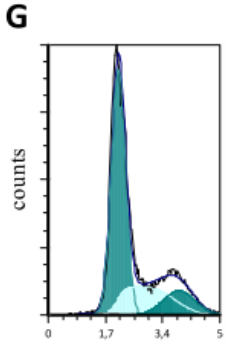
%G1	%G2	%S
55.294	15.901	28.805

F



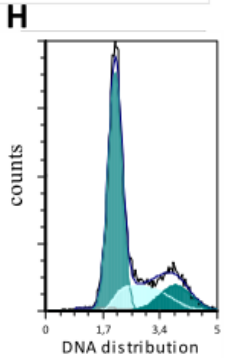
%G1	%G2	%S
48.320	1.975	49.704

G



%G1	%G2	%S
61.565	14.004	24.431

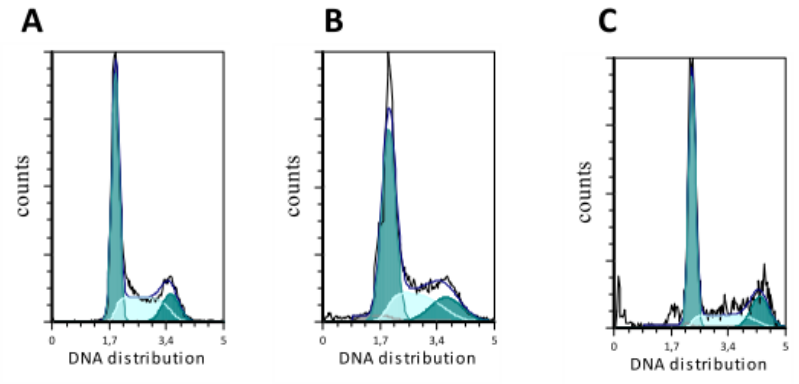
H



%G1	%G2	%S
62.269	15.469	22.262

Figure S2

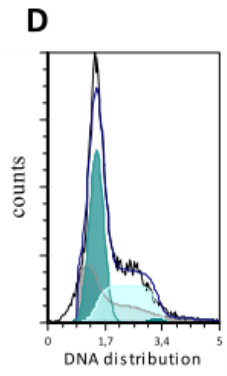
A) control; **B)** compound **10**; **C)** compound **9**; **D)** compound **4**; **E)** compound **7**; **F)** compound **1**; **G**) compound **8**; **H)** compound **25**.



%G1	%G2	%S
54.950	13.912	31.137

%G1	%G2	%S
56.115	16.597	27.288

%G1	%G2	%S
59.438	18.968	21.594



%G1	%G2	%S
58.015	1.057	40.927

Figure S3

A) control; **B)** compound **5**; **C)** compound **18**; **D)** compound **24**.

Annexin V/ PI Assay

The experiments were performed as described in the experimental session (5.3.4). Below (Fig. S4) representative graphs and the extracted values (Tab. S1) for all compounds are given (measured as triplicate). In addition, for compound **17** the detailed measurement process (S5) is shown.

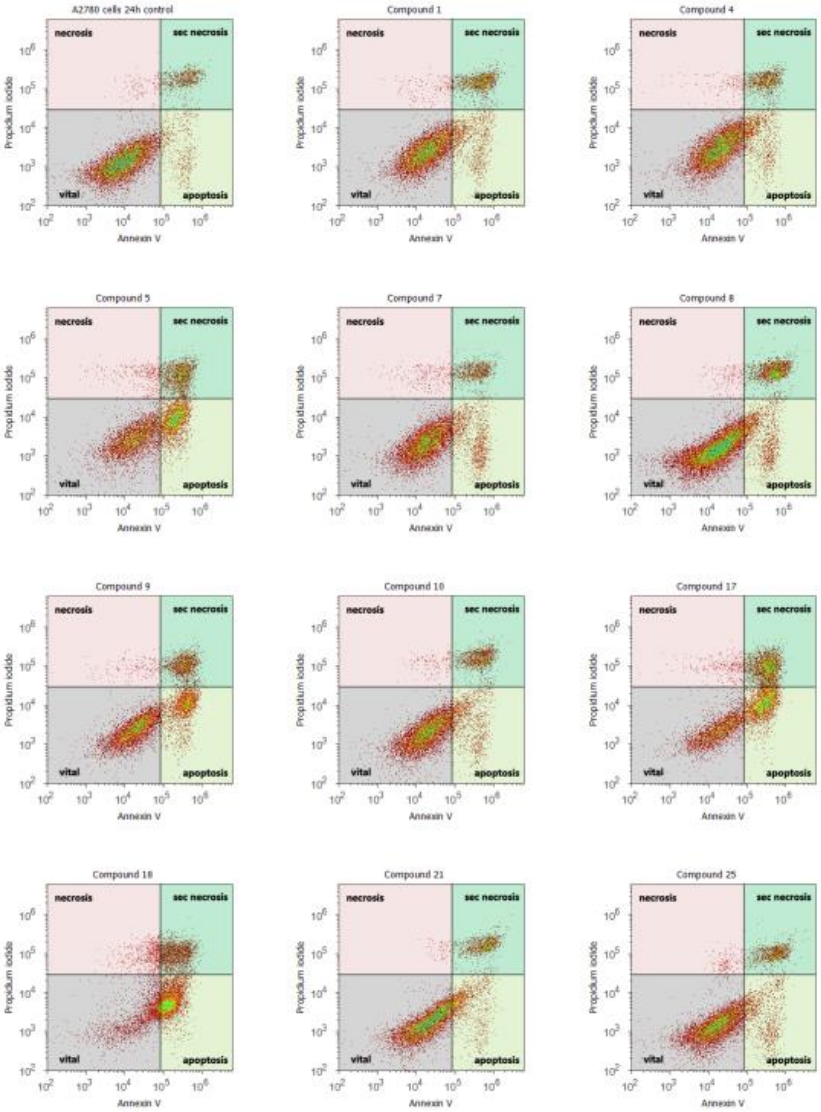


Figure S4: Representative annexin V/PI plots for all investigated compounds.

	nec	Average	STD	sec nec	Average	STD	vital	Average	STD	apop	Average	STD
C M1	1.33	1.78	0.64	7.87	9.71	2.60	84.89	82.15	3.88	5.91	6.37	0.65
C M2	2.23			11.54			79.40			6.83		
9 M1	1.99	2.00	0.01	16.72	16.21	0.77	56.60	57.12	0.79	24.69	24.67	0.03
9 M2	2.01			16.59			56.72			24.68		
9 M3	2.01			15.33			58.03			24.63		
18 M1	6.76	6.65	0.22	19.10	19.74	0.77	26.61	25.72	0.85	47.57	48.08	0.94
18 M2	6.79			20.60			25.65			47.50		
18 M3	6.40			19.52			24.91			49.17		
21 M1	0.68	0.62	0.08	11.98	11.08	0.82	73.00	74.47	1.44	14.34	13.82	0.56
21M2	0.66			10.89			74.55			13.90		
21M3	0.53			10.37			75.87			13.23		
25M1	1.85	1.55	0.31	12.79	12.64	0.56	72.70	72.96	0.85	12.60	12.83	0.24
25M2	1.56			13.11			72.26			13.07		
25M3	1.24			12.02			73.91			12.83		
17M1	3.37	3.11	0.23	28.33	29.68	1.32	33.78	33.08	0.72	34.52	34.13	0.41
17M2	3.00			30.96			32.34			33.70		
17M3	2.96			29.75			33.12			34.17		
10M1	1.42	1.40	0.19	13.12	12.57	0.56	72.21	72.75	0.62	13.25	13.28	0.51
10M2	1.20			12.58			73.43			12.79		
10M3	1.58			12.00			72.61			13.81		
7M1	2.57	2.41	0.15	10.08	9.43	0.94	72.25	72.47	1.06	15.10	15.68	0.55
7M2	2.39			9.87			71.54			16.20		
7M3	2.27			8.35			73.63			15.75		
1M1	1.66	1.56	0.11	14.05	14.23	1.29	72.11	71.74	1.63	12.18	12.47	0.35
1M2	1.59			15.60			69.95			12.86		
1M3	1.44			13.03			73.15			12.38		
4M1	1.54	1.54	0.01	11.61	11.89	0.39	74.79	74.74	0.07	12.06	11.84	0.31
4M2	1.53			12.16			74.69			11.62		
5M1	4.25	3.78	0.45	18.92	18.67	0.44	44.68	44.89	0.68	32.15	32.66	0.64
5M2	3.35			18.93			44.34			33.38		
5M3	3.75			18.16			45.65			32.44		
8M1	0.91	0.96	0.08	12.77	12.96	0.26	75.09	75.08	0.02	11.23	11.00	0.33
8M2	1.02			13.14			75.07			10.77		

Table S1: Uncorrelated values from the annexin V/PI-assay.

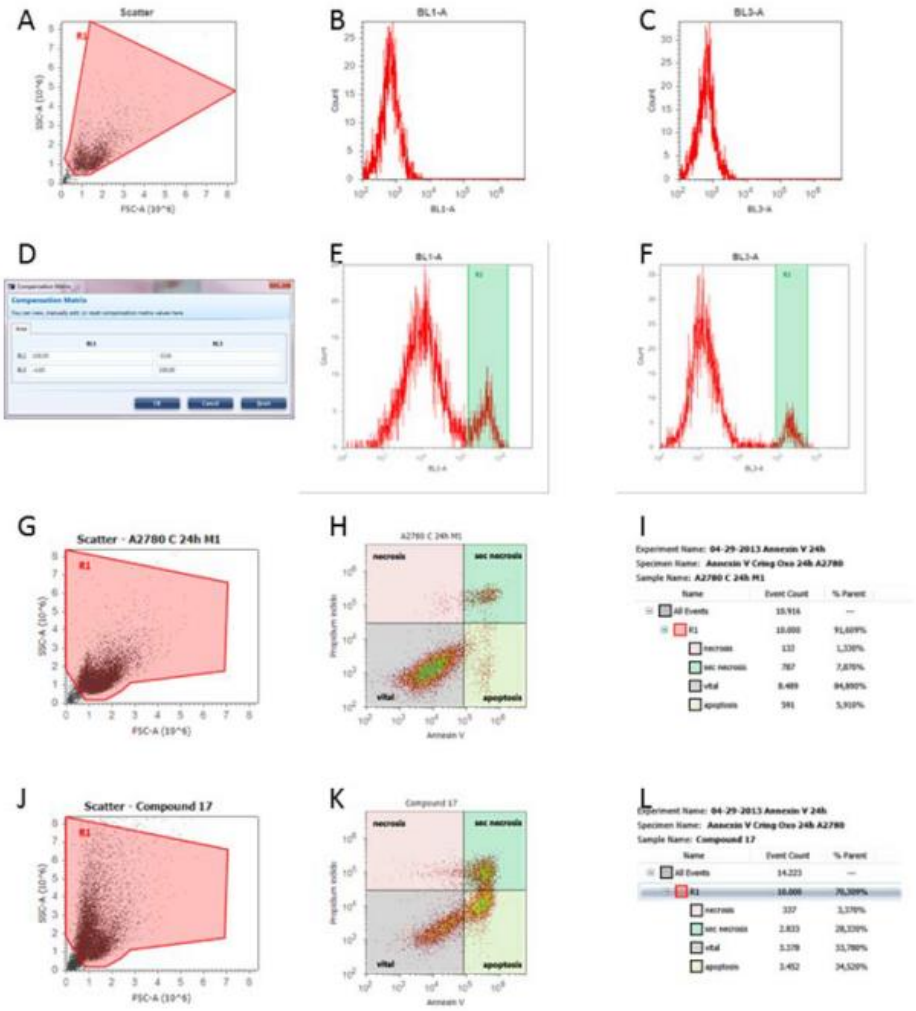


Figure S4: Detailed measurement of the annexin V/PI assay.

A to **F** = compensation:

A) Unstained control population; **B)** BL1-histogram unstained cell population; **C)** BL3-histogram unstained cell population; **D)** compensation matrix; E) BL1-histogram of the annexin V-stained control population; **F)** BL-3 histogram of the PI-stained control population

G to **L** = detailed measurement for the control and for compound **17.**

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A bioassay-driven discovery of an unexpected selenophene and its cytotoxicity

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ABSTRACT

During the reaction of methyl 3β-acetoxy-glycyrrhetinate (**1**) with SeO_2 significant amounts of a cytotoxic hitherto unprecedented triterpenoic selenophene **3** are formed. This compound stops cell proliferation and acts by apoptosis.

Almost seven decades ago Ruzicka and co-workers[1] described the 'oxidation' of methyl 3β-acetoxy-glycyrrhetinate (**1**, Fig. 1) with SeO_2 leading to an unusual product **2** of unknown structure. As an alternative[1,2] to SeO_2 the use of CrO_3 was suggested, but the structure of **2** remained unknown at that time. Several years later, McKean and Spring[3] identified this compound as an α,β-unsaturated lactone, the structure of which was deduced from additional experiments[4,5] including derivatization, combustion analysis and stereochemical considerations. The unusual structure of an anellated enol-lactone is not limited to glycyrrhetinic acid derivatives but was also found for other triterpenoids[6,7] of the oleanane type. Finally, in 2010 the structure of **2** was secured[8] by NMR data and a single crystal X-ray analysis. Hence, the 'oxidation' of **1** introduces two more oxygen atoms, it eliminates four hydrogens, it re-arranges one ring, and it generates an additional unsaturated γ-lactone ring.[8] Although compound **2** has been known for decades, no biological data have been published so far.

During our continuing investigation of antitumor active derivatives of glycyrrhetinic acid derivatives, we became interested in the synthesis and biological evaluation of this type of compound. Thus, reaction of **1** with SeO_2 as described (AcOH, SeO_2, 120 °C, 24 h)[1,8] gave a mixture of many compounds as indicated by TLC. In a bioassay-driven LC fractionation several of these products (often as a mixture of compounds as indicated by ESI-MS) were isolated by preparative LC, and they were tested for their cytotoxic activity using a photometric SRB assay employing human tumor cell lines and mouse fibroblasts NiH 3T3. As a result, two fractions were identified containing cytotoxic compounds: The first bio-active fraction contained lactone **2**, the second fraction consisted of an unknown side-product **3**[9] that was formed in significant amounts (up to 15% isolated yield).

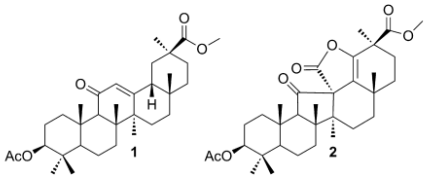

Figure 1. Structure of **1** and 'oxidation product' **2**.

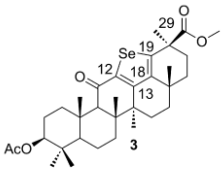

Figure 2. Structure of the unexpected selenophene **3**.

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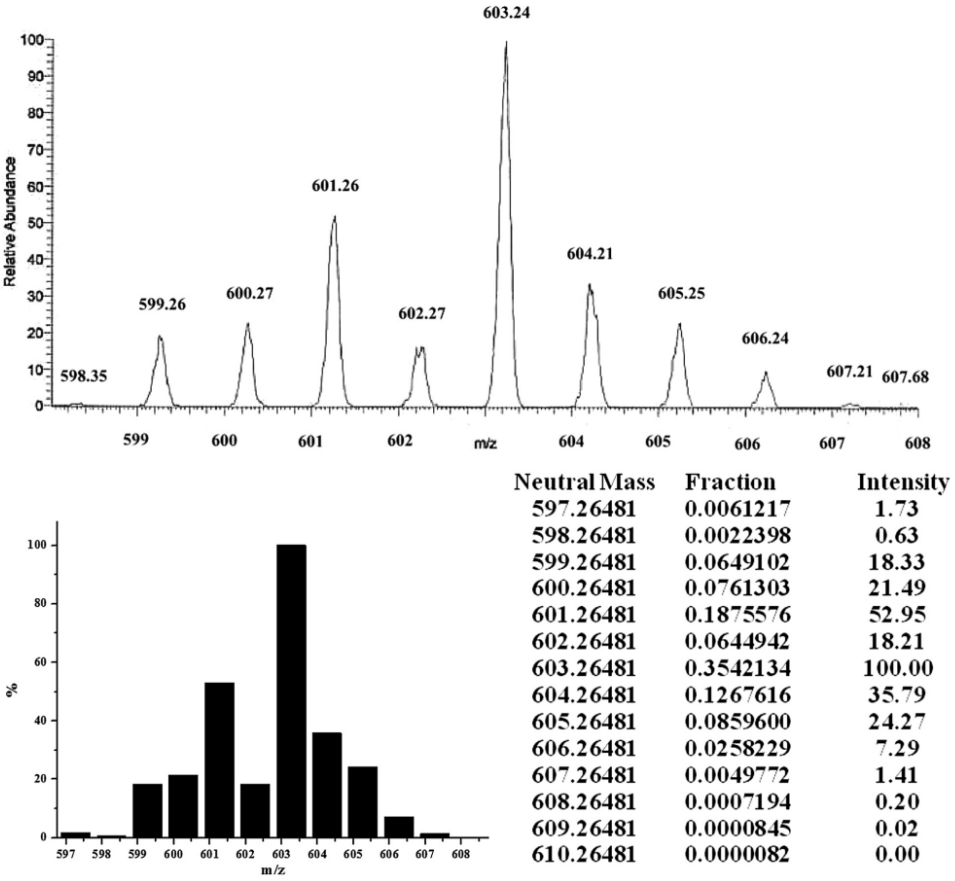

Figure 3. Experimental and calculated isotopic distribution for the quasimolecular ion $[M+H]^+$ of compound **3**.

Close inspection of the ^1H and ^{13}C NMR data of **3** revealed the absence of protons at C-18 and C-19 as well as at C-12 and C-13. The protons $H_3C(29)$ are shifted to lower fields ($\delta = 1.48$ ppm). An additional ^{77}Se NMR spectrum revealed the presence of a signal at $\delta = 592.27$ ppm being typical[10] for a selenophene. In the IR spectrum typical C=Se stretching vibrations[11] were found at $\nu = 684$ and 1469 cm^{-1}.

An ESI-MS experiment showed the presence of a quasimolecular ion $[M+H]^+$ $m/z = 603.3$ with a rather complex pattern (Fig. 2) corresponding to a molecular formula of $C_{33}H_{46}O_5Se$. The calculated isotope distribution pattern for this molecule is also depicted in Figure 3 and matches the result from the experiment.

^{75}Se labeled derivatives have been used as pancreatic imaging agents[12] using γ-ray scintigraphy, and several selenium compounds[13] were discussed in the chemoprevention of colon-cancer representing one of the leading causes[14] for cancer-associated death in the Western world.[15–17] Thus, selenium compounds have been suggested[15–17] as potent novel cytotoxic agents leading to an apoptotic response[18] either by a caspase-dependent process[19] or by their interaction[15] with glutaredoxin proteins.

Recently, the cytotoxicity of selenophenes has been investigated in more detail. Thus, for some benzo[*b*]selenophene derivatives cytotoxic activity on human fibrosarcoma cell has been established,[20] and for compound D-501036 (a bis-(2-selenienyl)-*N*-pyrrole derivative) Chang and co-workers[21] showed, that this compound induces cellular apoptosis through the p53-associated mitochondrial pathway. Also, several selenophenyl-substituted quinolines[22] as well as selenocarbamates[23] exhibited significant cytotoxic activity. Hence, we became interested in the cytotoxic activity of **3** as compared to its parent compound **1** and lactone **2**.

Table 1
Cytotoxicity (IC_{50} values in μmol from SRB assays after 96 h of treatment; averaged results from three independent experiments and using the two-parametric Hill-slope equation using Graphpad Prism 5.04 and JMP7 software for calculations) of **1**–**3** against a panel of selected human tumor cell lines and a nonmalignant mouse fibroblast cell line (NiH 3T3)

Cell line	1	2	3
518A2	26.1 ± 1.6	14.7 ± 0.6	36.7 ± 0.5
A2780	40.5 ± 3.7	19.7 ± 3.3	33.7 ± 0.6
MCF7	51.6 ± 2.6	33.5 ± 1.3	45.6 ± 3.3
8505C	116.4 ± 10.7	73.2 ± 5.5	42.2 ± 2.8
HT29	77.4 ± 7.7	58.7 ± 6.6	67. 2 ± 7.8
A549	42.6 ± 7.3	15.0 ± 0.9	46.9 ± 1.8
NiH 3T3	101.4 ± 7.2	38.1 ± 4.3	46. 1 ± 4.4

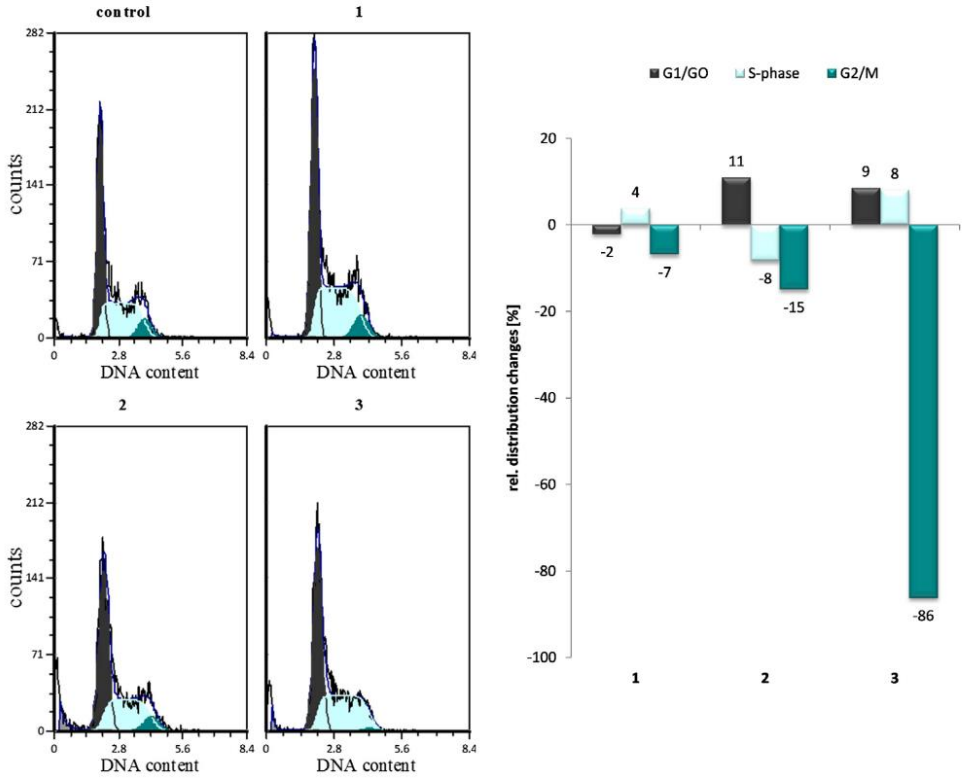

Figure 4. Cell cycle distribution. Melanoma 518A2 cells were incubated with the respective compound for 24 h. After harvesting of all living cells, the cell cycle distribution was measured using a propidium iodide (PI) assay.

Table 1 summarizes the results from the SRB assays[24] using several human tumor cell lines as well as nonmalignant mouse fibroblasts (NiH 3T3). These experiments (Table 1) showed that seleno-**3** is by and large as cytotoxic as parent compound **1** except for human breast adenocarcinoma cells MCF7 and human adenocarcinoma cell HT29. For the human thyroid-carcinoma cells 8505C a 2.8-fold higher cytotoxicity was found. In comparison, lactone **2** shows approximately twice the cytotoxicity than parent **1**.

Thus, comparison of the cytotoxicity of parent **1** with lactone **2** and selenophene **3** indicates that a modified C-ring either leads to an increase in cytotoxicity (as observed for **2**) or leaves the biological activity at least unchanged. To investigate the mode of action for these new glycyrrhetinic acid derivatives, we investigated their influence onto the cell proliferation of 518A2 melanoma cells. As depicted in Figure 4, compound **3** induced a significant stop of cell proliferation. The lactone **2** gave a similar effect after incubation for 24 h whereas parent compound **1** does not affect the cell proliferation rate at all. In summary, seleno-**3** and lactone **2** act rather cytostatic whereas compound **1** is cytotoxic.

Next, we became interested whether these compounds are still able to trigger apoptosis. Typical morphological characteristics of an apoptotic cell death are cell membrane stability, chromatin

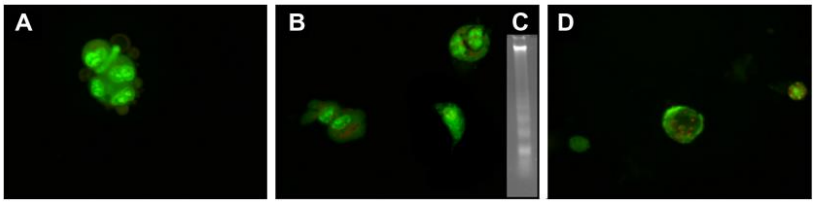

Figure 5. Dye-exclusion assay (A, B, D). Melanoma 518A2 cells were incubated with the respective compound for 24 h. After collecting of the dead cells, these were stained with AO/PI and investigated by fluorescence microscopy; cells treated with compound **1** (50 μM, picture A), **2** (30 μM, picture B) and **3** (50 μM, picture C). DNA-laddering experiment (C): Melanoma 518A2 cells were incubated with **2** (30 μM). After collecting of the dead cells, these were submitted to the DNA-ladder experiment.

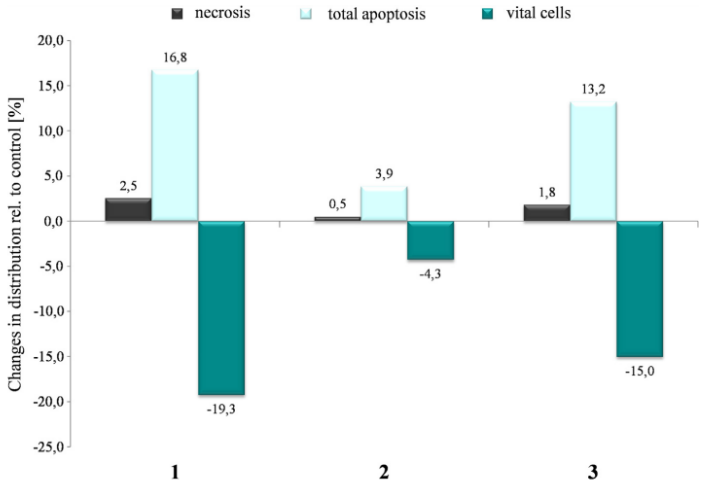

Figure 6. Annexin V assay. Melanoma 518A2 cells were incubated with the respective compound for 6 h. After harvesting of all cells, the apoptotic cells were measured using the Annexin V assay.

condensation as well as cell shrinking. Further, during this programmed cell death the iCAD (inactivated caspase activated DNAse) becomes activated and cuts the DNA into smaller pieces of approx. 178 bp. Caspase mediated ROCK (Rho-activated serine/threonine kinases) activation leads to a degeneration of the actin, cell contraction and membrane blebbing. Also, the exposure of phagocytic signals (e.g. of phosphatidylserine) is a typical hallmark of an apoptotic cell death.

By an AO/PI dye exclusion assay the membrane integrity and other typical morphological characteristics were observed (Fig. 5). In addition, the formation of a typical DNA-ladder was observed for selenophene **3** (cf. Fig. 5C). Combined with the results from the annexin V assay (Fig. 6) an apoptotic cell death becomes evident. Furthermore, for the selenophene **3** an increased ability to induce apoptosis (after an incubation time of 6 h) as compared to lactone **2** was found.

The formation of a selenophene from the reaction of an α,β-unsaturated ketone with SeO_2 is—to the best of our knowledge—unprecedented for triterpenoids. α,β-Unsaturated ketones usually form intramolecular[25] or intermolecular[26] diselenides.

Presumably, selenophene **3** is formed by an SeO_2 mediated allylic oxidation followed by an elimination under acidic conditions[1,5] to a 1,3-diene. This diene[1,5] undergoes a [4+2] cycloaddition with SeO_2 to yield finally compound **3**. Previously, the formation of selenophenes from the reaction of 1,3-dienes with SeO_2 at elevated temperatures has been reported by Lee et al.[27,28] for several verbenone derived dienes.

The biological properties make triterpenoid selenophenes interesting candidates for further biological evaluation.

Acknowledgments

We like to thank Mrs. J. Wiese for skillful experimental help in the analysis of the compounds. Many thanks are due to Dr. D. Ströhl for the NMR spectra and to Dr. R. Kluge for the ESI-MS experiments. The cell lines were kindly provided by Dr. Th. Müller (Dept. of Haematology/Oncology, Universität Halle-Wittenberg). Support by 'Gründerwerkstatt—Biowissenschaften' is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.04.036.

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9. Selected analytical data for **3**: yield: 15%; off-white solid; R_f = 0.67 (SiO_2, toluene/ethyl acetate/heptane/HCOOH, 80:20:30:4); Mp: 199–201 °C; $[\alpha]_D$ +9.23° (*c* 0.27, $CHCl_3$); UV–vis (MeOH): λ_{max} (log ε) = 307.03 (3.87) nm; ^1H NMR (400 MHz, $CDCl_3$): δ = 4.52 (dd, *J* = 11.4, 5.1 Hz, 1H, *CH* (3)), 3.75 (s, 3H, CH_3 (31)), 3.00 (ddd, *J* = 13.6, 7.3, 3.6 Hz, 1H, CH_a (1)), 2.88–2.80 (m, 1H, CH_a (21)), 2.81 (s, 1H, *CH* (9)), 2.07 (dd, *J* = 13.7, 3.0 Hz, 1H, CH_a (15)), 2.03 (s, 3H, CH_3 (33)), 1.83 (ddd, *J* = 14.3, 6.8, 3.4 Hz, 1H, CH_b (21)), 1.74 (ddd, *J* = 14.0, 14.0, 3.7 Hz, 1H, CH_a (16)), 1.70–1.61 (m, 6H, CH_2 (22) + *CH*a (7) + CH_2 (2) + CH_a (6)), 1.60–1.48 (m, 2H, CH_b (7) + CH_b (16)), 1.48 (s, 3H, CH_3 (29)), 1.42 (ddd, *J* = 12.7, 12.7, 3.0 Hz, 1H, CH_b (6)), 1.32 (s, 3H, CH_3(27)), 1.26 (ddd, *J* = 13.8, 6.4, 3.3 Hz, 1H, CH_b (15)), 1.16 (s, 3H, CH_3 (25)), 1.15–1.10 (m, 1H, CH_b (1)), 1.06 (s, 3H, CH_3 (28)), 1.02 (s, 3H, CH_3 (26)), 0.91–0.85 (m, 1H, *CH* (5)), 0.87 (s, 6H, CH_3 (23) + CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 193.9 (C=O, C11), 176.1 (C=O, C30), 171.1 (C=O, C32), 156.7 (C13), 153.9 (C18), 143.2 (C12), 139.1 (C19), 80.7 (C3), 63.7 (C9), 55.8 (C5), 52.8 (C31), 48.6 (C14), 48.2 (C20) 45.4 (C8), 39.0 (C1), 38.4 (C10), 38.3 (C4), 34.8 (C17), 34.4 (C16), 34.1 (C22), 33.5 (C7), 32.3 (C29), 30.4 (C21), 28.3 (C23), 28.1 (C28), 25.4 (C15), 24.3 (C27), 23.8 (C2), 21.5 (C33), 19.2 (C26), 17.4 (C6), 17.0 (C25), 16.8 (C24) ppm; ^{77}Se NMR (95 MHz, $CDCl_3$): δ = 592.27 ppm; MS (ESI, MeOH): *m/z* = 603.3 (100%, $[M+H]^+$), 625.7 (10%, $[M+Na]^+$), 656.5 (15%, $[M+Na+MeOH]^+$), 1227.1 (10%, $[2M+Na]^+$); analysis for $C_{33}H_{46}O_5Se$ (601.68): C, 65.87; H, 7.71; found: C, 65.69; H, 7.82.
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Supplementary material

A bioassay-driven discovery of an unexpected selenophene and its cytotoxicity

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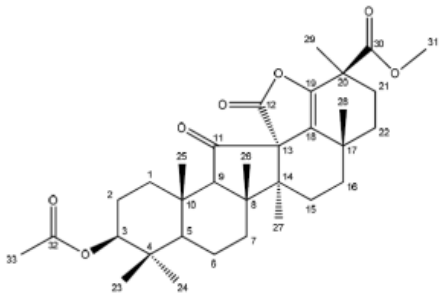
Methyl 3β 3-*O*-acetyl-13-carboxy-19-hydroxy-11-oxo-C-norolean-18-ene-30-oate (2) [125637-17-4]

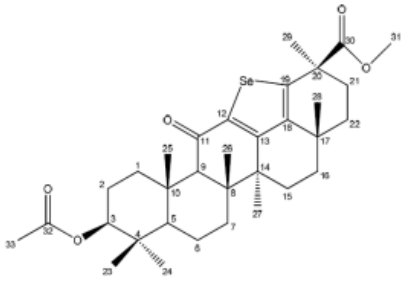


To a solution of **1** (0.8 g, 1.56 mmol) in glacial acetic acid (50 mL) freshly sublimed SeO_2 (0.8 g, 7.45 mmol) was added, and the mixture was heated for 20 h at 120 °C. Another portion of SeO_2 (0.8 g, 7.45 mmol) was added, and heating continued for another 4 h. After cooling to room temperature, the mixture was filtered, water (150 mL) was added, and the mixture was extracted with dichloromethane (4 x 50 mL). After drying (Na_2SO_4), the solvents were removed at diminished pressure, and the residue was subjected to chromatography (SiO_2, hexanes/ethyl acetate, 9:1) to yield **2** (0.4 g, 50%) as an off-white solid;

R_f = 0.52 (SiO_2, toluene/ethyl acetate/heptane/HCOOH 80:20:30:4);mp 285 – 289 °C (lit.: 291 – 292 °C);$[\alpha]_D$ = + 2.99° (*c* 0.5, $CHCl_3$);IR (KBr): ν = 3449*br*, 3024*w*, 2970*m*, 2948*m*, 1778*s*, 1736*s*, 1690*w*, 1449*m*, 1382*m*, 1369*m*, 1248*s*, 1218*m*, 1197*m*, 1153*m*, 1081*m*, 1109*m*, 1081*m*, 1058*m*, 1024*m*, 1010*m*, 980*m* cm^{-1}; UV-vis ($CHCl_3$): λ_{max} (log ε) = 232 (2.22) nm; ^1H NMR (400 MHz, $CDCl_3$):δ = 4.55 (*dd*, *J* = 9.2, 7.3 Hz, 1H, C*H* (3)), 3.72 (*s*, 3H, CH_3(31)), 3.08 (*s*, 1H, C*H* (9)), 2.33 (*ddd*, *J* = 13.9, 13.9, 3.3 Hz, 1H, CH_a (21)), 2.07 (*ddd*, *J* = 14.0, 7.0, 3.5 Hz, 1H, CH_a (1)), 2.04 (s, 3H, CH_3 (33)), 1.87 (*ddd*, *J* = 15.0, 14.9, 3.6 Hz, 1H, CH_a (16)), 1.76 (*ddd*, *J* = 13.9, 6.6, 3.3 Hz, 1H, CH_b (21)), 1.76 – 1.65 (*m*, 4H, CH_a (15) + CH_a (6) + CH_2 (2)), 1.63 – 1.53 (*m*, 4H, CH_2 (7) + CH_a (22) + CH_b (6)), 1.47 (*ddd*, *J* = 13.6, 6.9, 3.4 Hz, 1H, CH_b (22)), 1.42 (*s*, 3H, CH_3 (29)), 1.45 – 1.38 (*m*, 1H, CH_b (16)), 1.32 – 1.18 (*m*, 2H, CH_b (15) + CH_b (1)), 1.18 (*s*, 3H, CH_3 (26)), 1.16 (*s*, 6H, CH_3 (25) + CH_3 (28)), 1.11 (*s*, 3H, CH_3 (27)), 0.96 – 0.90 (*m*, 1H, C*H* (5)), 0.88 (*s*, 6H, CH_3 (23) + CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 203.9 (*C*=O, C11), 174.7 (*C*=O, C30), 174.4 (*C*=O, C12), 170.9 (*C*=O, C32), 151.5 ($C_{quart.}$, C19), 119.2 ($C_{quart.}$, C18), 80.5 (*C*H, C3), 67.8 ($C_{quart.}$, C13), 65.6 (*C*H, C9), 55.3 (*C*H, C5), 52.9 (CH_3, C31), 48.5 ($C_{quart.}$, C14), 43.4 ($C_{quart.}$, C20), 42.4 ($C_{quart.}$, C8), 37.9 ($C_{quart.}$, C4), 36.8 (CH_2, C1), 36.6 (CH_2, C16),

35.8 ($C_{quart.}$, C10), 35.3 (CH_2,C22), 34.1 (CH_2, C7), 32.8 (CH_2, C15), 31.4 (CH_2, C21), 30.8 ($C_{quart.}$, C17), 28.2 (CH_3, C23), 26.9 (CH_3, C28), 26.8 (CH_3, C27), 23.4 (CH_3, C26), 23.3 (CH_2, C2), 21.4 (CH_3, C33), 21.3 (CH_3, C29), 18.9 (CH_2, C6), 17.3 (CH_3, C25), 16.5 (CH_3, C24) ppm; MS (ESI, MeOH): m/z = 555.3 ($[M+H]^+$, 22%), 572.3 ([M+NH_4]+, 35 %), 577.4 ($[M+Na]^+$, 100 %); 1131.3 ($[2M+Na]^+$, 62 %).

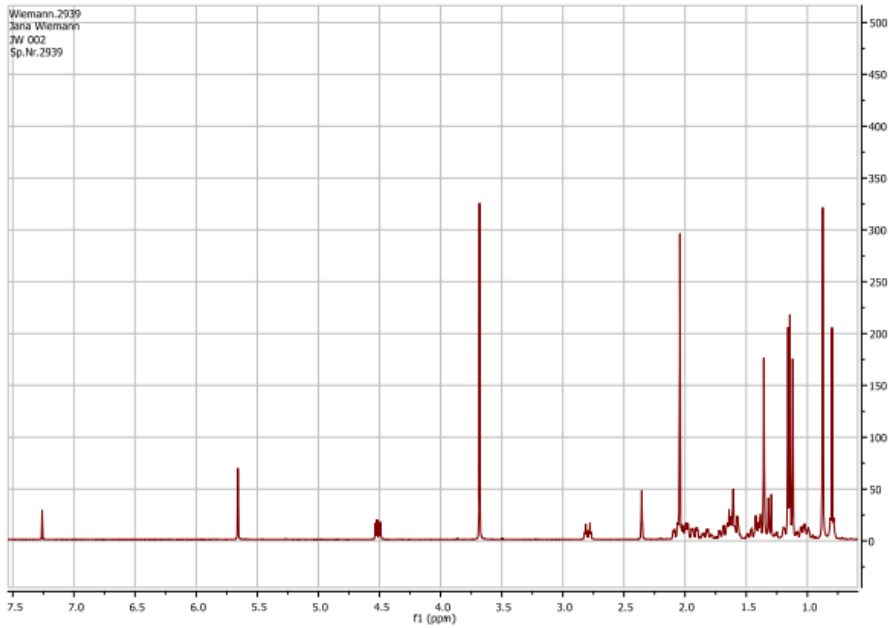
Methyl 3β 3-*O*-acetyl-11-oxo-12,19-selena-olean-12,18-diene-30-oate (3)



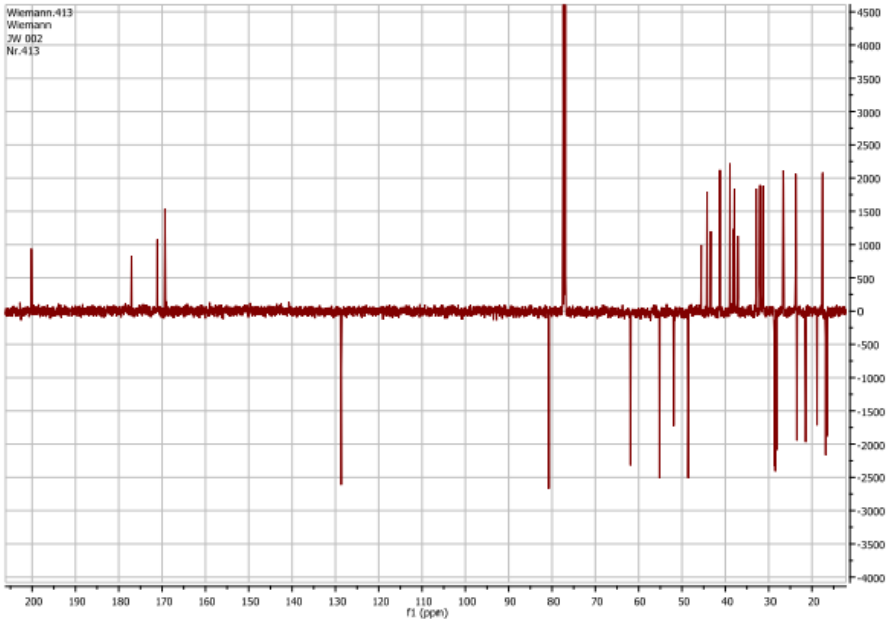
was obtained as an off-white solid (15 %); M.p.: 199 – 201 °C; $[\alpha]_D$ = + 9.23° (c 0.27, $CHCl_3$); UV-vis (MeOH): λ_{max} (log ε) = 307.03 (3.87) nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.52 (*dd*, J = 11.4, 5.1 Hz, 1H, C*H* (3)), 3.75 (*s*, 3H, CH_3 (31)), 3.00 (*ddd*, J = 13.6, 7.3, 3.6 Hz, 1H, CH_a (1)), 2.88 – 2.80 (*m*, 1H, CH_a (21)), 2.81 (*s*,1H, C*H* (9)), 2.07 (*dd*, J = 13.7, 3.0 Hz, 1H, CH_a (15)), 2.03 (*s*, 3H, CH_3 (33)), 1.83 (*ddd*, J =14.3, 6.8, 3.4 Hz, 1H, CH_b (21)), 1.74 (*ddd*, J = 14.0, 14.0, 3.7 Hz, 1H, CH_a (16)), 1.70 – 1.61(*m*, 6H, CH_2 (22) + C*H*a (7) + CH_2 (2) + CH_a (6)), 1.60 – 1.48 (*m*, 2H, CH_b (7) + CH_b (16)),1.48 (*s*, 3H, CH_3 (29)), 1.42 (*ddd*, J = 12.7, 12.7, 3.0 Hz, 1H, CH_b (6)), 1.32 (*s*, 3H, CH_3(27)), 1.26 (*ddd*, J =13.8, 6.4, 3.3 Hz, 1H, CH_b (15)), 1.16 (*s*, 3H, CH_3 (25)), 1.15 – 1.10 (*m*, 1H, CH_b (1)), 1.06 (*s*, 3H, CH_3 (28)), 1.02 (*s*, 3H, CH_3 (26)), 0.91 – 0.85 (*m*, 1H, C*H* (5)), 0.87 (*s*, 6H, CH_3 (23) + CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 193.9 (*C*=O, C11), 176.1 (*C*=O, C30), 171.1 (*C*=O, C32), 156.7 (C13), 153.9 (C18), 143.2 (C12), 139.1 (C19), 80.7 (C3), 63.7 (C9), 55.8 (C5), 52.8 (C31), 48.6 (C14), 48.2 (C20) 45.4 (C8), 39.0 (C1), 38.4 (C10), 38.3 (C4), 34.8 (C17), 34.4 (C16), 34.1 (C22), 33.5 (C7), 32.3 (C29), 30.4 (C21), 28.3 (C23), 28.1 (C28), 25.4 (C15), 24.3 (C27), 23.8 (C2), 21.5 (C33), 19.2 (C26), 17.4 (C6), 17.0 (C25), 16.8 (C24) ppm; ^{77}Se NMR (95 MHz, $CDCl_3$): δ = 592.27 ppm; MS (ESI, MeOH): m/z = 603.3 (100 %, $[M+H]^+$), 625.7 (10 %, $[M+Na]^+$), 656.5 (15 %, [M+Na+MeOH]$^+$), 1227.1 (10 %, $[2M+Na]^+$); analysis for $C_{33}H_{46}O_5Se$ (601.68): C, 65.87; H, 7.71; found: C, 65.69; H, 7.82.

Starting material (1)

^{1}H-NMR

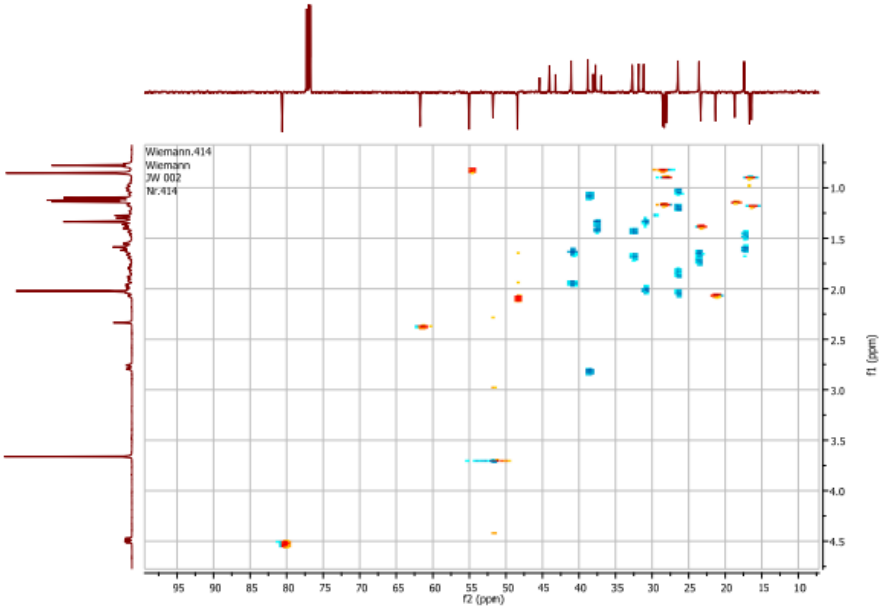


^{13}C NMR (^{13}C- APT)



3

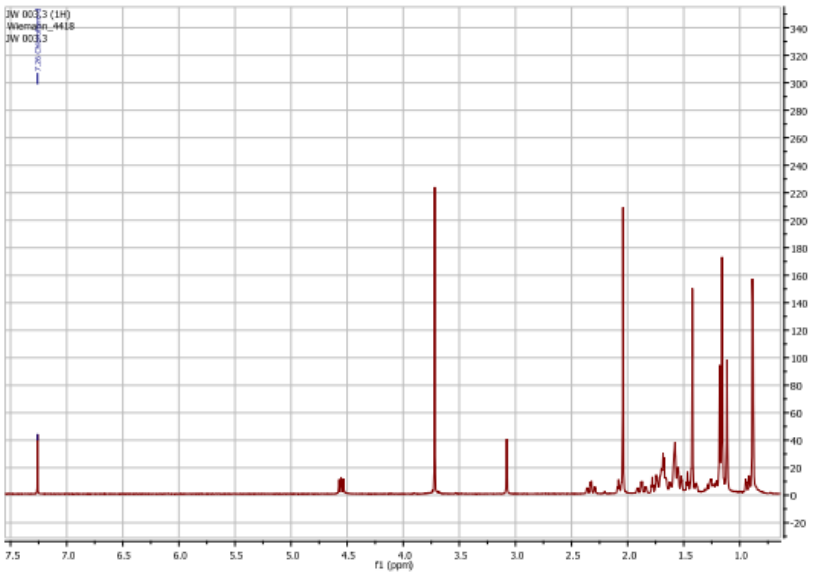
gHSQC



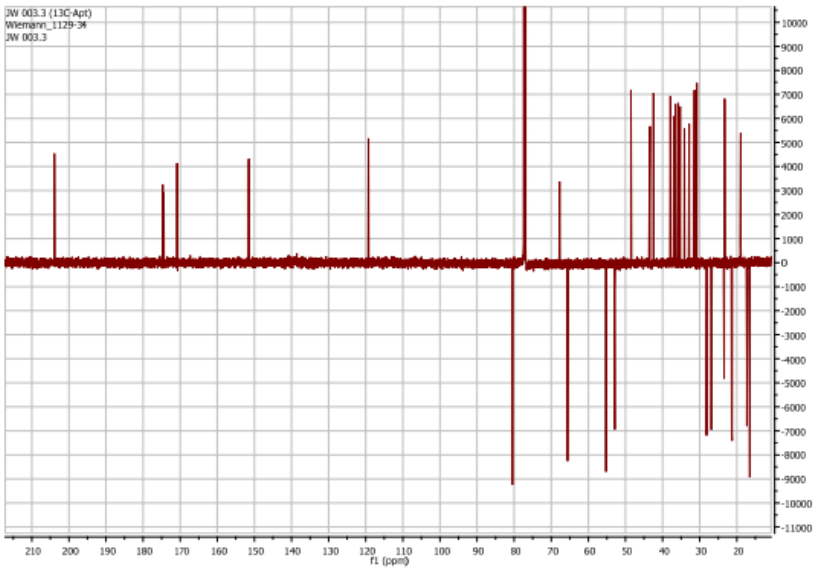
4

Lactone (2)

^{1}H NMR

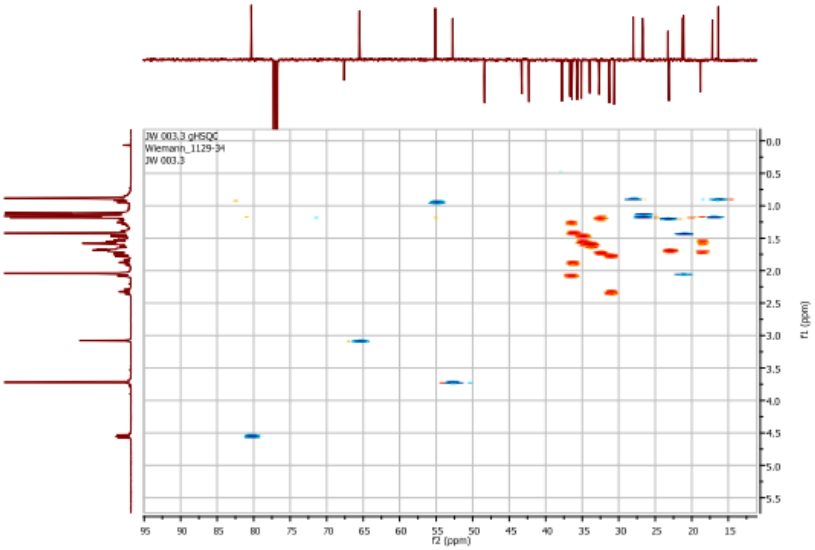


^{13}C NMR (^{13}C-APT)



5

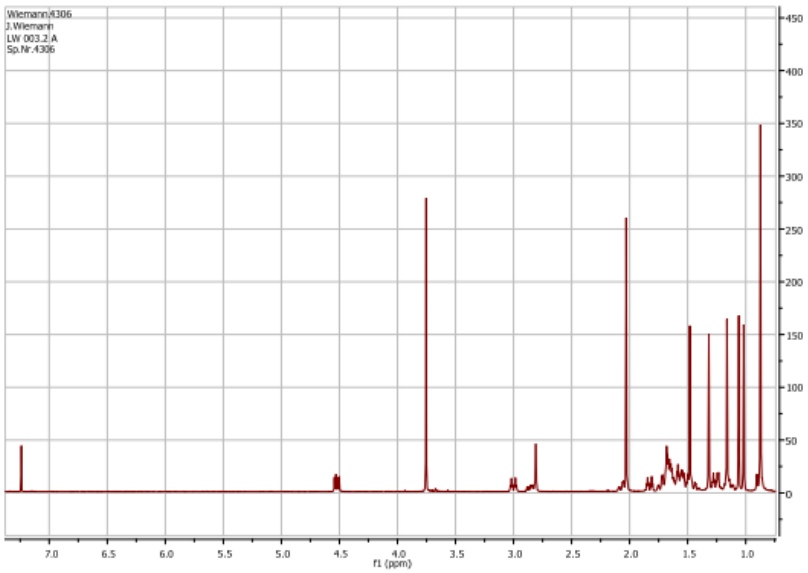
gHSQC



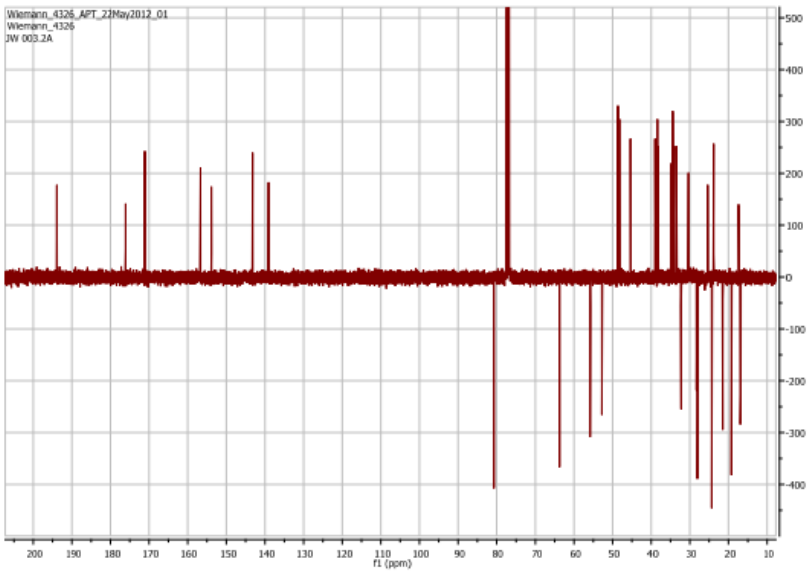
6

Selenophene (3)

^{1}H NMR

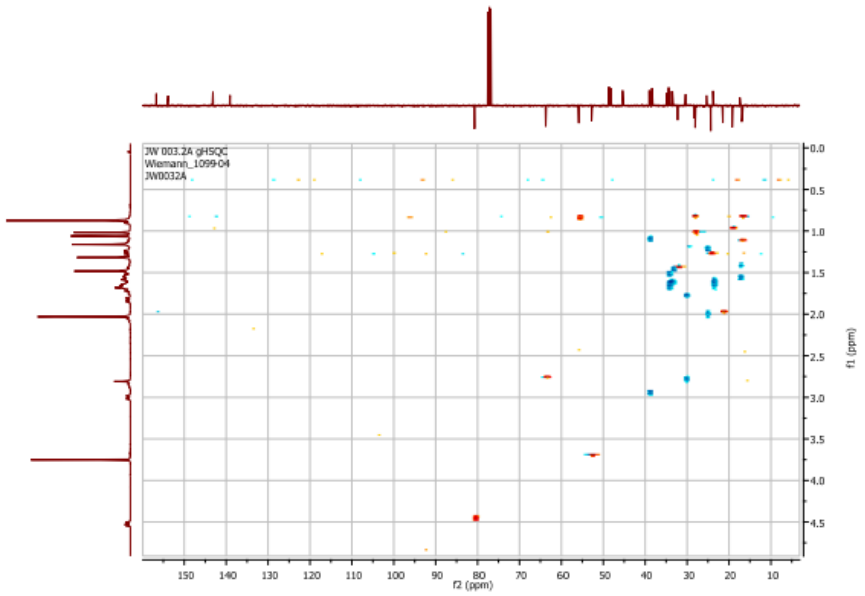


^{13}C NMR (^{13}C-APT)

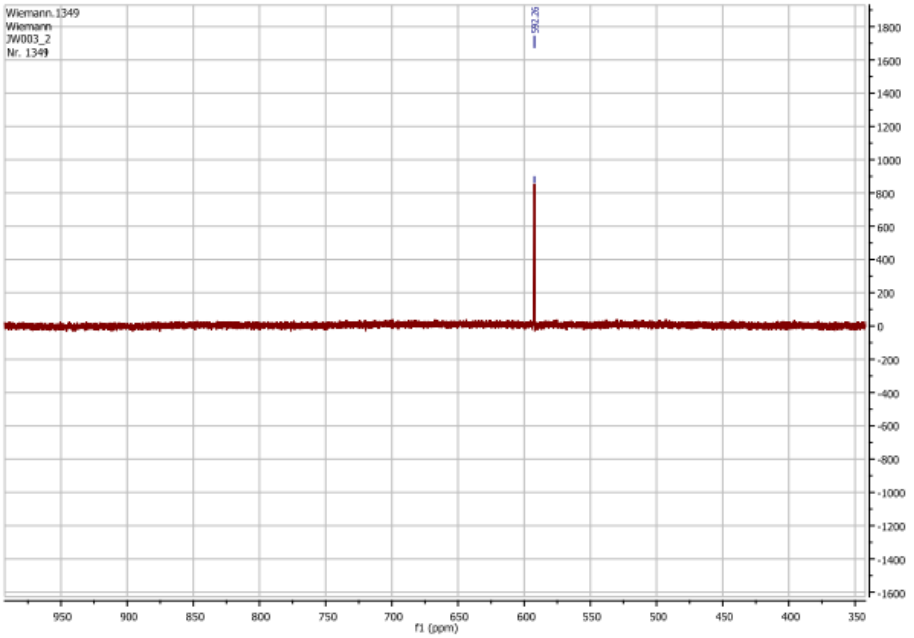


7

gHSQC



^{77}Se NMR:

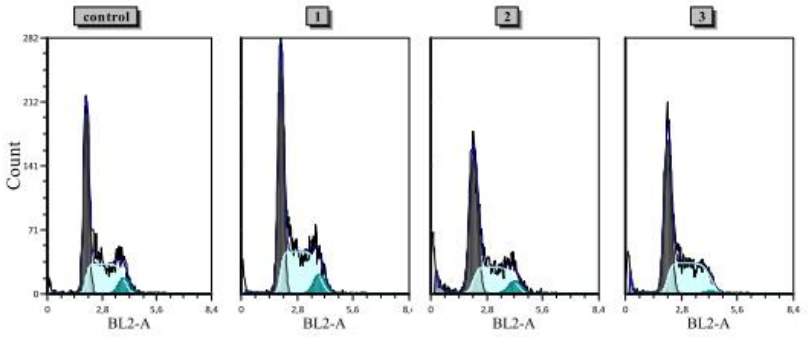


8

Cell Cycle investigations

Approximately $1.2*10^5$ cells (518A2) were seeded in 6-well plates, and the cells were allowed to grow for 24 h. After removing of the used medium, the substance-loaded medium (5 ml) was reloaded (**1** [50µM], **2** [30µM], **3** [50µM] or a blank fresh medium as a control). After 24 h, the living cells were harvested, washed with PBS (with Mg^{2+} and Ca^{2+}) twice and fixed with ethanol (70 %, 4°C, 1h). After removing of the fixation and permeabilization reagent, the cells were washed with PBS buffer (with Mg^{2+} and Ca^{2+}, containing 1% BSA and 0.1% NaN_3, 3 x 1 ml, 1000 rpm) and adjusted to $1*10^5$ million cells. The pellet was gently suspended in staining buffer (PBS buffer containing BSA, RNAse, NaN_3 and PI analog Darzynkiewicz *et al.*) and incubated at 37°C for 30 min. Analyses were performed using an Attune® FACS machine; collecting data from the BL-2A channel. Doublet cells were excluded from the measurements by plotting BL-2A against BL-2H. For each cell cycle distribution 5.000 events were collected. Distribution was calculated by the method of Dean *et al.*.

Result:



%G1	%G2	%S
46.662	8.694	44.643

%G1	%G2	%S
45.600	8.055	46.345

%G1	%G2	%S
51.694	7.402	40.903

%G1	%G2	%S
50.569	1.207	48.224

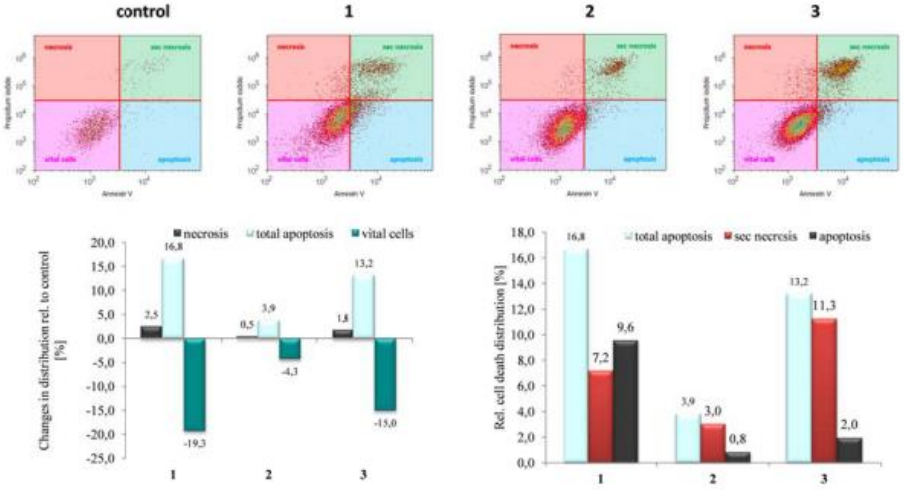
Dye-exclusion assay (AO/PI) with DNA-ladder

The Dye-exclusion assay and the DNA-ladder experiment was preformed like described[1].

Annexin V – Assay

Approximately 1.2*10^5 cells (518A2) were seeded in 6-well plates and the cells were allowed to grow for 24 h. After removing of the used medium, the substance-loaded medium was reloaded (**1** [50µM], **2** [30µM], **3** [50µM] or a blank fresh medium as a control). After 6 h, all cells were collected and washed with PBS (with Mg^{2+} and Ca^{2+}) twice. The cells were washed with annexin-V binding buffer (BD Pharming^{TN}) and adjusted to 1*10^5 cells. AnnexinV-FITC (5 µl, Life Technologies^{TM}) and propidium Iodide (5 µl, 50 µg/ml) and incubated in the dark at room temperature for 15 min. Analyses were performed using an Attune® FACS machine; collecting data from the BL1-A and BL3-A channel after compensation.

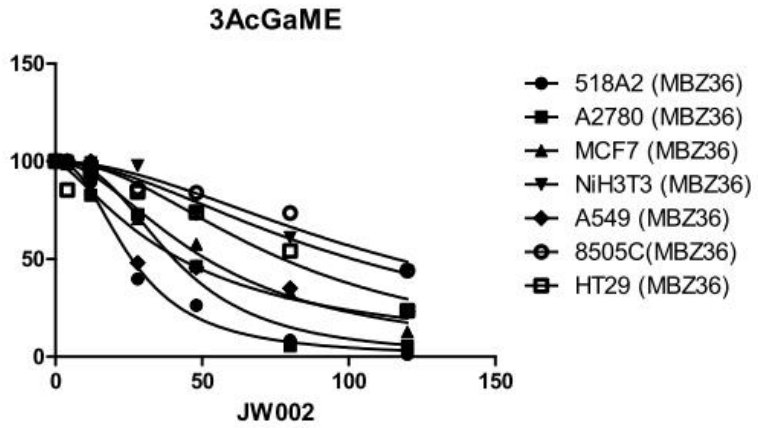
Results:



(1) Csuk, R.; Siewert, B.; Dressel, C.; Schäfer, R. *Eur. J. Med. Chem.* **2012**, *56*, 237-245.

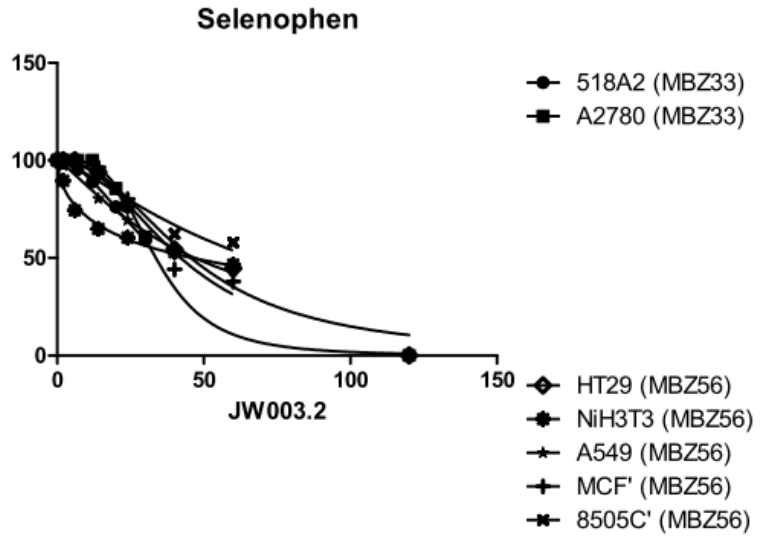
Detailed data from the SRB assay:

Compound **1**:



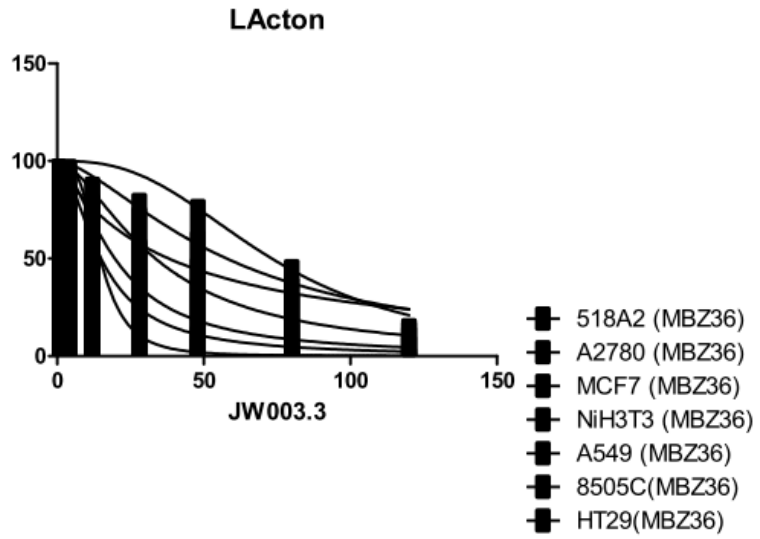
	Nonlin fit Table of results	A 18A2 (MBZ36	B 2780 (MBZ36	C MCF7 (MBZ36	D H3T3 (MBZ36	E 549 (MBZ36	F 8505C(MBZ36	G HT29 (MBZ36)
		Y	Y	Y	Y	Y	Y	Y
1	Inhibition vs. response -- Variable slope (tw							
2	Best-fit values							
3	Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
4	Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
5	IC50	26.06	40.54	51.56	101.4	42.63	116.4	77.60
6	Hillslope	2.240	2.537	1.837	1.788	1.360	1.839	1.997
7	Span	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
8	Std. Error							
9	IC50	1.611	3.676	2.581	7.162	7.280	10.71	7.687
10	Hillslope	0.2707	0.5461	0.1768	0.2683	0.3502	0.3526	0.4501
11	95% Confidence Intervals							
12	IC50	21.92 to 30.2	31.09 to 49.9	44.93 to 58.2	82.97 to 119	23.91 to 61.3	88.89 to 144.	57.84 to 97.3
13	Hillslope	1.544 to 2.93	1.134 to 3.94	1.382 to 2.29	1.098 to 2.47	0.4592 to 2.2	0.9329 to 2.7	0.8399 to 3.1
14	Goodness of Fit							
15	Degrees of Freedom	5	5	5	5	5	5	5
16	R square	0.9913	0.9688	0.9902	0.9705	0.9230	0.9500	0.9301
17	Absolute Sum of Squares	98.74	309.6	69.88	93.97	537.0	121.1	300.0
18	Sy.x	4.444	7.869	3.739	4.335	10.36	4.921	7.746
19	Constraints							
20	Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
21	Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
22	Number of points							
23	Analyzed	7	7	7	7	7	7	7
24								

Compound **3**:



	Nonlin fit Table of results	A 18A2 (MBZ33	B 2780 (MBZ33	C HT29 (MBZ56)	D iH3T3 (MBZ56	E 549 (MBZ56)	F MCF' (MBZ56)	G 505C' (MBZ56
		Y	Y	Y	Y	Y	Y	Y
1	Inhibition vs. response -- Variable slope (tw							
2	Best-fit values							
3	Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
4	Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
5	IC50	36.66	33.67	45.60	46.09	46.89	42.16	67.22
6	Hillslope	1.893	3.645	2.214	0.5966	1.245	2.225	1.361
7	Span	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
8	Std. Error							
9	IC50	0.4891	0.5952	3.252	4.397	1.769	2.759	7.848
10	Hillslope	0.04593	0.2436	0.3556	0.04831	0.07298	0.3680	0.2288
11	95% Confidence Intervals							
12	IC50	35.30 to 38.0	32.22 to 35.1	37.64 to 53.5	34.79 to 57.4	42.34 to 51.4	35.06 to 49.2	47.04 to 87.3
13	Hillslope	1.765 to 2.02	3.049 to 4.24	1.343 to 3.08	0.4724 to 0.7	1.058 to 1.43	1.279 to 3.17	0.7730 to 1.9
14	Goodness of Fit							
15	Degrees of Freedom	4	6	6	5	5	5	5
16	R square	0.9993	0.9991	0.9756	0.9875	0.9947	0.9673	0.9600
17	Absolute Sum of Squares	0.9431	8.141	222.1	28.17	15.95	147.7	82.41
18	Sy.x	0.4856	1.165	6.084	2.373	1.786	5.435	4.060
19	Constraints							
20	Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
21	Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
22	Number of points							
23	Analyzed	6	8	8	7	7	7	7
24								

Compound **2**:



	Nonlin fit Table of results	A 18A2 (MBZ36	B A2780 (MBZ36	C MCF7 (MBZ36	D iH3T3 (MBZ36	E A549 (MBZ36	F 8505C(MBZ36	G HT29(MBZ36)
		Y	Y	Y	Y	Y	Y	Y
1	Inhibition vs. response -- Variable slope (tw							
2	Best-fit values							
3	Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
4	Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
5	IC50	14.71	19.70	33.52	38.08	15.00	73.24	57.82
6	Hillslope	3.290	1.691	1.673	0.9991	1.796	2.687	1.582
7	Span	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
8	Std. Error							
9	IC50	0.5526	3.255	1.332	4.315	0.9215	5.513	6.633
10	Hillslope	0.3673	0.4058	0.1075	0.1388	0.1677	0.5461	0.3282
11	95% Confidence Intervals							
12	IC50	13.29 to 16.1	11.33 to 28.0	30.09 to 36.9	26.98 to 49.1	12.63 to 17.3	59.06 to 87.4	40.77 to 74.8
13	Hillslope	2.345 to 4.23	0.6477 to 2.7	1.396 to 1.94	0.6423 to 1.3	1.365 to 2.22	1.283 to 4.09	0.7378 to 2.4
14	Goodness of Fit							
15	Degrees of Freedom	5	5	5	5	5	5	5
16	R square	0.9966	0.9507	0.9960	0.9696	0.9939	0.9562	0.9410
17	Absolute Sum of Squares	43.90	477.6	34.16	154.5	63.81	258.6	307.0
18	Sy.x	2.963	9.774	2.614	5.559	3.572	7.192	7.836
19	Constraints							
20	Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
21	Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
22	Number of points							
23	Analyzed	7	7	7	7	7	7	7

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Original article

Tormentic acid derivatives: Synthesis and apoptotic activity☆

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Annexin V
DNA laddering

ABSTRACT

Several derivatives of tormentic acid have been prepared and tested for their antitumor activity. The dichloroacetate **14** is an excellent antitumor active agent acting by an apoptose inducing pathway as demonstrated by OA/PI staining, DNA laddering experiments as well as by an annexin V binding assay.

1. Introduction

Cancer is still one of the leading causes of death. The index of cancer cure is often low and its treatment is still a challenge. Cancer cells hold the ability to evade death, and expressing multidrug resistance is an important draw-back in the chemotherapy of cancer.

Natural products have been used to treat diseases for thousands of years. They still play an important role in development of new drugs. Among them, triterpenes represent a class of most significant compounds. They have been shown to possess a broad variety of medicinal properties. In continuation of our previous studies on betuline, betulinic, glycyrrhetinic and boswellic acid derivatives as antitumor active compounds, we became interested in tormentic acid as a lead compound in the synthesis of antitumor active derivatives.

Common tormentil (bloodroot, *Potentilla erecta*), also known as shepherd's knot, is a low, clump-forming plant growing wild all over northern Europe and all over Asia. Extracts prepared from the dried root have been used to treat bleedings and diarrhea (because of its high content in tannins acting as adstringents) or to dye leather red (because of the presence of phlobaphenes).

As early as 1915 tormentol (tormentoside) [1] was isolated as the β-D-glucopyranosyl ester of tormentic acid the structure of which was established in 1966 [2–4]. Tormentic acid, i.e. (2 *R*, 3 *R*, 19 *R*) 2,3,19-trihydroxy-urs-12-en-28-carboxylic acid (**1**), can be extracted [5–17] from various plants, among them *Myrianthus serratus*, *Perilla frutescens*, *Cotoneaster simsonsii*, *Rubus sieboldii* but also from species of *Potentilla*, e.g. *Potentilla anserina*, *Tormentilla tormentilla* or *P. erecta*.

There are ample examples for the antitumor activity of pentacyclic triterpenes; less is known, however, about the biological activity of **1** and even fewer data have been reported for derivatives of **1**. Thus, **1** is able to inhibit *in vitro* platelet aggregation [18], and the influence of **1** on forming atherosclerotic plaques [19] in mice has been investigated. In addition, **TA** reduced vascular smooth muscle cell proliferation [20] and possesses [21,22] some anti-inflammatory activity. Compound **1** reduced also the viability of human gastric cells [13] by an inhibition [13,23,24] of α- and β-DNA polymerases. Only a weak cytotoxic activity has been established [25,26] for different tumor cell lines; some anticancer activity has been found for **1** for lymphocytic leukemua cells [27]. Interesting to note that **1** shows little toxicity [13] to normal cells, and **1** has been suggested [20] to be developed for the treatment of post-angioplasty re-stenosis. Recently, **1** methyl ester (**2**) has been shown [28] to act as a selective, low micromolar inhibitor of 11β-hydroxysteroid dehydrogenase and to display anti-inflammatory effects [29,30].

☆ Dedicated to Prof. Dr. Rainer Beckert, Friedrich-Schiller Universität Jena, on the occasion of his 60th birthday. Ad multos annos!

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2. Results

2.1. Chemistry

Quite recently, we became aware that small structural modifications, e.g. esterification or acylation of a triterpenoid skeleton [31–38], might result in obtaining compounds of improved cytotoxicity. Thus, **1** was used as an easy accessible starting material for the synthesis of "simple" derivatives.

Treatment of **1** (Scheme 1) with MeI/K_2CO_3 gave the methyl ester **2** [4,28,39,40] in almost quantitative yield. TEMPO oxidation [41,42] of **2** yielded the 2-oxo compound **3** [1] in 83% yield; compound **3** is characterized by the presence of a carbonyl signal in its ^{13}C NMR spectra at $\delta = 211.0$ ppm. This oxidation advances in a regioselective way; no oxidation at position C-3 could be noted. The reason for this regioselectivity might be the steric hindrance at position C-3 because of the presence of the two geminal methyl groups at C-4.

Oxidation of **2** using bis(tri-*n*-butyl-tin)oxide [43,44] in the presence of bromine at 0 °C, however, yielded the 3-oxo compound **4** whose carbonyl group can be found in the ^{13}C NMR spectrum at $\delta = 216.6$ ppm. Using a prolonged reaction time and an excess of oxidizing agent gave the 3-oxo-1,12-diene **5** in 57% yield. Reduction of **3** with sodium borohydride proceeded in a stereoselective way and provided the 2-epi compound **6**; compound **6** represents a 2,3-bis epimer to euscaphic ester **8**; the latter is easily obtained from naturally occurring euscapic acid (**7**) by esterification with diazomethane. As an alternative, reduction of **5** under the same conditions gave a 70% yield of **6**. Compound **8** was oxidized in a regioselective manner to afford **4**.

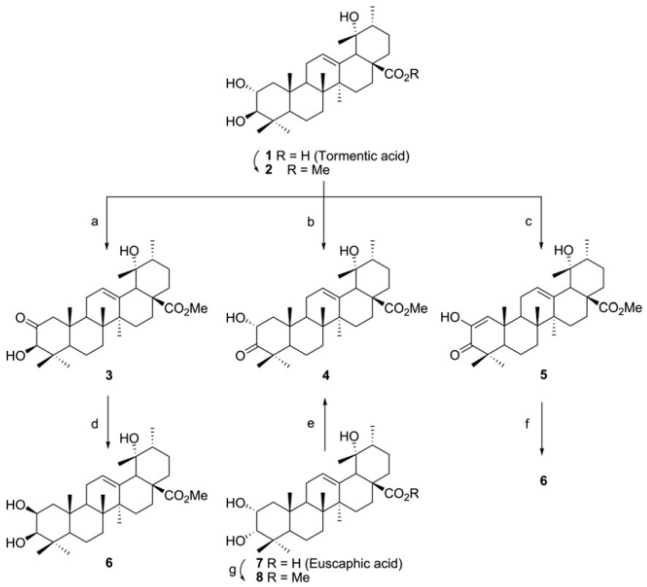
For betulin and betulinic acid, several acylated derivatives showed a higher antitumor activity than their parent compounds [38,45]. Therefore, it seemed of interest to prepare several acylated analogs of **2** and to compare their biological activity with parent **2**.

Acetylation of **1** (Scheme 2) with acetic anhydride in dry pyridine for 24 h yielded 66% of the diacetate **9**. If the reaction was stopped after 3 h, the 2-*O*-acetyl derivative **10** and the 3-*O*-acetyl derivative **11** were isolated in 56% and 21%, respectively. The monoacetates **10** and **11** were previously isolated [25] from *Cecropia lyratiloba*, and shown to be effective inhibiting the viability of a chronic myeloid leukemia blast crisis cell line by inducing apoptosis [2]. Acetylation of **3** under similar conditions provided acetate **12** whereas from compound **4** mono-acetylated **13** was formed. Acylation of **2** with chloroacetyl chloride yielded the 2,3-bis(chloroacetyloxy)-compound **14**, the 2-*O*-chloro acetate **15** and the 3-*O*-chloro acetate **16**.

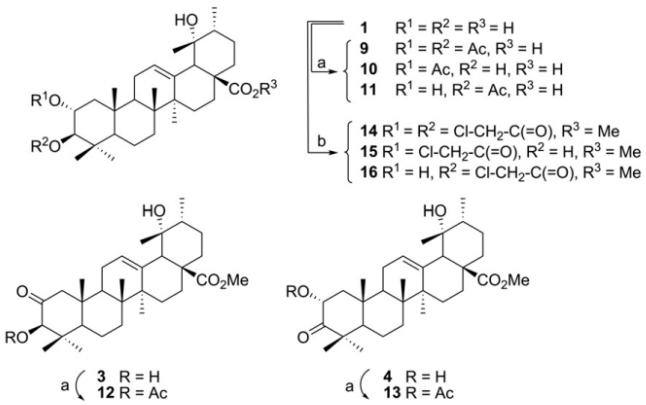
2.2. Biology

Thus, contrary to previous findings with betulinic acid, neither an esterification nor an acetylation resulted in products of significantly increased cytotoxicity (Table 1). Mono- or diacetylated products **9**–**11** showed only moderate cytotoxicity ($IC_{50} > 30$ μmol) for all human tumor cell lines tested. A similar behavior can be found for the mono-acetylated keto compounds **12** [1] and **13** and for the keto compounds **3**, **4** and **5**. An improvement was observed for the mono-chloroacetylated compounds **15** and **16**; a significantly improved cytotoxicity, however, was observed for bis-chloroacetylated **14**.

Cell death can occur [46,47] either necrotic or programmed by a variety of different forms being known for the latter. Apoptosis is characterized [46] *inter alia* by cell shrinking, membrane blebbing, an enhanced activity of caspases, a translocation of phosphatidylserine and DNA fragmentation. Previous work of Rocha et al. [2,25] and Fogo et al. [20] gave evidence for **1** and several alkynic derivatives thereof for acting by inducing apoptosis. To evaluate the ability of our compounds, tormentic acid methyl ester **2** and the



Scheme 1. a) TEMPO, NaOCl, CH_2Cl_2, 8 h, 63%; b) $[(nBu)_3Sn]_2O$, Br_2, 0 °C, 1 min, 62%; c) $[(nBu)_3Sn]_2O$, Br_2, 0 °C, 15 min, 57%; d) $NaBH_4$, MeOH, reflux, 1 h, 82%; e) $[(nBu)_3Sn]_2O$, Br_2, 0 °C, 1 min, 67%; f) $NaBH_4$, MeOH, reflux, 1 h, 70%; g) CH_2N_2, MeOH, 95%.



Scheme 2. a) Ac_2O, pyridine, CH_2Cl_2, 24 °C, 3–24 h, **9**: 66%, **10**: 56%, **11**: 21%, **12**: 66%, **13**: 68%; b) $ClCH_2COCl$, pyridine, CH_2Cl_2, 24 °C, 4 h, **14**: 26%, **15**: 40%, **16**: 11%.

most active compound of this series, bis-chloroacetyl **14** were tested in more detail.

As indicated above, programmed cell death is characterized [47] by different morphological changes. Thus, living cells (518A2) were stained with acridine orange (AO) and investigated by fluorescence microscopy. AO is an uncharged cationic dye; it binds to nucleic acids. AO and nucleic acids form either monomeric complexes with double stranded nucleic acids (exhibiting a green fluorescence) or dimers with single stranded nucleic acids (emitting orange light). Maslinic acid (MA) is well known for its ability to induce apoptosis in cancer cells and was used as a control [22,27,48–57]. A typical condensation of the chromatin as well as blebbing of the nuclear membrane and a shrinking of the cells was observed. Compound **2**, however, shows only a weak ability to induce cell death for 518A2 cells at a concentration of 30 μM. The condensation of chromatin as well as the blebbing of the nuclear membrane indicates a programmed cell death process. The same phenomenon was observed for 8505C human thyroid carcinoma cell cells. Some red dots were seen during microscopy; these were assigned to proteasomes or are due to lysosomal activity [58].

Additional investigations using an AO/PI exclusion dye assay (Fig. 1) showed that the majority of the dead cells still possess an intact cell membrane. While membrane disruption is a characteristic feature of necrosis [leading to deep red light emission from the propidium iodide (PI)], an intact membrane indicates a programmed cell death since PI – as a double charged molecule – cannot enter the cell as long as the cell membrane is intact [59]. All compounds used in this AO/PI assay induced a controlled cell death hence paralleling previous findings [2,25,50,60] for other triterpenoic acids.

To gain a deeper insight, additional experiments were called for. Phosphatidylserine – a label for cell death – switches from the inner to the outer cell membrane during the cascade of apoptosis [47]. Annexin V, a cellular protein of the annexin group, selectively binds to phosphatidylserine. By a combination of the protein with fluorescine isothiocyanate (FITC) a fluorescence active dye is formed. In this assay 8505C cancer cells emitted green light hence having bound annexin V-FITC and thus indicating that these cells died by apoptosis [61]. The same was true for experiments employing 518A2 cancer cells. Fig. 2 depicts the results from the annexin V-FITC/PI stained cells by FACS-analysis.

Another typical hallmark of apoptosis is an exactly determined cutting of the DNA by endonucleases into multiple 180 bp fragments (and multiples thereof) [62,63]. All tested compounds gave the characteristic DNA ladders. To evaluate the cancer-to-control selectivity of some of our compounds, additional experiments

Table 1
Cytotoxicity (IC_{50} in μmol; SRB assay) for tormentic acid (**1**) and compounds **2**–**16** in a panel of various cancer cell lines [518A2 (melanoma), 8505C (anaplastic thyroid), A253 (head), A2780 (ovarian), A549 (lung), DLD1 (colon), MCF7 (mamma)], non malignant mouse fibroblast (NiH 3T3), and human fibroblast primary culture cells (WW030272). Values were obtained from SRB assays after 96 h of treatment; the values are averaged from at least 5 independent experiments (n.d. not determined).

	518A2	8505C	A253	A2780	A549	DLD-1	MCF7	NiH 3T3	WW030272
1	>30	23.4 ± 0.8	>30	>30	31.0 ± 0.1	31.0 ± 0.2	32.3 ± 2.5	>30	47.7 ± 1.1
2	31.3 ± 3.9	42.0 ± 5.2	17.0 ± 2.0	23.9 ± 4.3	n.d.	37.4 ± 1.0	31.3 ± 3.4	15.6 ± 5.0	47.5 ± 1.1
3	>30	>30	n.d.	17.8 ± 1.9	n.d.	>30	28.7 ± 0.5	19.2 ± 0.6	n.d.
4	8.9 ± 0.5	12.9 ± 0.2	6.8 ± 1.8	4.9 ± 0.4	11.8 ± 0.2	18.0 ± 0.1	9.4 ± 0.5	7.7 ± 2.0	n.d.
5	17.2 ± 2.7	25.0 ± 0.9	>30	>30	>30	>30	26.0 ± 5.4	>30	n.d.
6	>30	>30	15.3 ± 2.0	18.9 ± 3.5	>30	>30	22.6 ± 1.0	>30	n.d.
8	27.7 ± 0.2	29.3 ± 2.5	16.4 ± 12.6	12.8 ± 1.5	30.6 ± 0.4	35.8 ± 1.8	17.8 ± 4.5	>30	23.4 ± 1.7
9	>30	>30	27.3 ± 3.5	17.6 ± 4.9	>30	>30	20.4 ± 3.7	32.3 ± 0.1	n.d.
10	>30	>30	>30	24.3 ± 9.0	>30	>30	25.3 ± 2.6	>30	n.d.
11	>30	>30	>30	28.1 ± 0.9	>30	>30	26.2 ± 0.7	>30	n.d.
12	5.6 ± 1.2	7.0 ± 0.2	6.7 ± 1.1	4.4 ± 0.4	7.8 ± 0.4	13.5 ± 0.4	8.2 ± 0.9	6.7 ± 1.1	n.d.
13	28.9 ± 0.7	>30	18.5 ± 0.4	9.0 ± 1.4	31.3 ± 0.4	28.2 ± 0.5	14.8 ± 0.4	25.1 ± 4.6	n.d.
14	1.1 ± 0.2	1.6 ± 0.7	1.6 ± 0.9	0.8 ± 0.4	1.2 ± 0.5	2.2 ± 0.2	1.5 ± 0.8	1.1 ± 0.1	3.4 ± 1.1
15	4.5 ± 0.4	4.6 ± 0.5	4.6 ± 0.5	2.6 ± 0.3	9.7 ± 0.6	3.7 ± 0.4	2.6 ± 0.3	2.1 ± 0.2	n.d.
16	5.5 ± 0.6	7.5 ± 0.8	4.1 ± 0.4	4.1 ± 0.4	10.0 ± 1.0	6.0 ± 0.6	6.9 ± 0.7	4.2 ± 0.4	n.d.

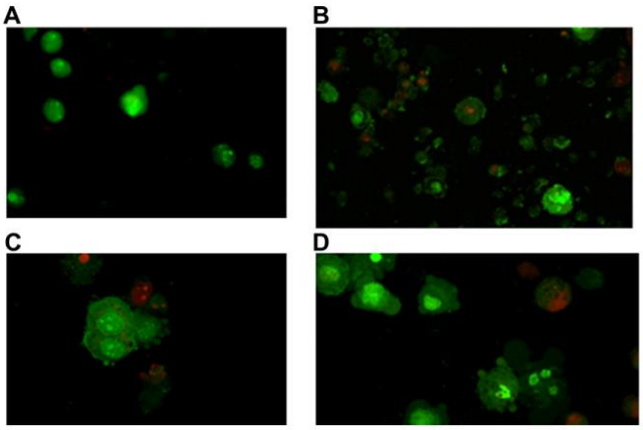


Fig. 1. AO/PI assay of dead 8505C cells. The cells were treated with **MA** (A), tormentic acid (B), **2** (C) and **14** (D); green cells indicate a controlled cell death, deep red cells a necrotic way of exitus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

using human fibroblast primary culture cells WW030272 were used. The results of these experiments are included in the table.

3. Conclusion

In summary, tormentic acid (**1**) as well as its methyl ester **2** are adequate and its dichloroacetate **14** is an excellent antitumor active agent acting by an apoptose inducing pathway as demonstrated by OA/PI staining, DNA laddering experiments as well as by an annexin V binding assay.

4. Experimental

4.1. Biological material

4.1.1. Cell lines and culture conditions

The cell lines 518A2, 8505C, A253, A2780, A549, DLD-1, MCF-7, NiH 3T3 and WW030272 were included in this study. Cultures were maintained as monolayer in RPMI 1640 (PAA Laboratories, Paschings, Germany) supplemented with 10% heat inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

4.1.2. Cytotoxicity assay

The cytotoxicity of the compounds was evaluated using the sulforhodamine-B (SRB) (Sigma–Aldrich) microculture colorimetric assay. In short, exponentially growing cells were seeded into 96-well plates on day 0 at the appropriate cell densities to prevent confluence of the cells during the period of experiment. After 24 h, the cells were treated with serial dilutions of the compounds (0–30 μM) for 96 h. The final concentration of DMSO or DMF solvent never exceeded 0.5%, which was non-toxic to the cells. The percentages of surviving cells relative to untreated controls were determined 96 h after the beginning of drug exposure. After a 96 h treatment, the supernatant medium from the 96 well plates was thrown away and the cells were fixed with 10% TCA. For a thorough fixation, the plates were allowed to rest at 4 °C. After fixation, the cells were washed in a strip washer. The washing was done five times with water using alternate dispensing and aspiration procedures. Afterward the plates were dyed with 100 μl of 0.4% SRB (sulforhodamine B) for about 30 min. The plates were washed

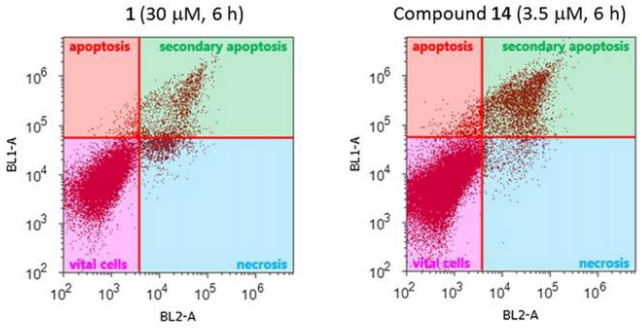


Fig. 2. FACS analysis of 8505C cells after 6 h incubation with **1** (left) or compound **14** (right).

with 1% acetic acid to remove the excess of the dye and allowed to air dry overnight. Tris base solution (100 μl of 10 mM) was added to each well and absorbance was measured at 570 nm (using a 96 well plate reader, Tecan Spectra, Crailsheim, Germany). The IC_{50} was estimated from the dose–response curves.

4.1.3. Morphological investigation of living cells

In an eight-well chamber slide (Sigma–Aldrich) 10.000 cells of the human thyroid cancer cell line 8505C or 10.000 cells of the melanoma cell line 518A2 were seeded. After 24 h of incubation, the medium was removed, and the cells were treated with maslinic acid (MA), tormentic acid (**1**) or tormentic acid methyl ester (**2**) (4 ml, 30 μM). On the next day the supernatant medium was removed, and the cells were washed with PBS (w(o, 1 ml)) and stained with acridine orange (5.10^{-6} mol). Visual inspection was performed using a fluorescence microscope (Zeiss Axioskop).

4.1.4. Apoptosis assay of dead cells by AO/PI dye exclusion test and annexin V-FITC

The death of the cells was analyzed employing an OA/PI assay as well as with annexin V-FITC dye using fluorescence microscopy and human cancer cell lines 518A2 and 8505C, respectively. Approx. $1*10^6$ cells were seeded in cell culture flasks (25 cm^2), and the cells were allowed to grow up to 80%. After removing of the used medium, the substance loaded fresh medium was reloaded (or a blank new medium as a control). After 24–48 h, the supernatant medium was collected and centrifuged (300 g, 4 °C), the pellet was gently suspended in phosphate-buffered saline (PBS, 1 ml) and centrifuged again. The PBS was removed, and the pellet again gently suspended in PBS (100 μl). The analysis of the cells was performed using a fluorescence microscope after having mixed the cell suspension (10 μl) with a solution of AO/PI (10 μl). A green fluorescence indicates apoptosis whereas a red colored cell indicates necrosis.

For the investigations using annexin V-FITC, the cells were washed with annexin V binding buffer after the treatment with PBS, then centrifuged and dyed for 15 min using an annexin V staining buffer. Analyses were performed using a fluorescence microscope; green colored cells indicate cells with phosphatidylserine on the outer cell membrane, a phenomenon that is typical for apoptosis.

4.1.5. DNA laddering experiments

Approximately $1*10^6$ cells (518A2 or 8505C) were seeded in cell culture flasks (25 cm^2), and the cells were allowed to grow up to 80%. After removing of the used medium, the substance loaded medium was reloaded (or a blank fresh medium as a control). After 24–48 h, the supernatant medium was collected and centrifuged (300 g, 4 °C). The pellet was gently suspended in phosphate-buffered saline (PBS 1 ml) and centrifuged again. The PBS was removed and lyses buffer (30 μl, 0 °C, 10 min) was added. The cells were incubated for 2 h (37 °C) after treatment with RNAse (10 μl, 0 °C, 10 min) and for 12 h at 50 °C after having been treated with proteine kinase K (10 μl). The extract was mixed with DNA-ladder dye (10 μl) and analyzed by gel electrophoresis (agarose, 150 mV, 2 h).

4.2. General – chemistry

Reagents were bought from commercial suppliers without any further purification. Melting points were measured with a LEICA hot stage microscope and were not corrected. NMR spectra were recorded on VARIAN Gemini 200, Gemini 2000 or Unity 500 spectrometers at 27 °C with trimethylsilane as an internal standard, δ are given in ppm and J in Hz. Mass spectra were taken on a FINNIGAN MAT TSQ 7000 (electrospray, voltage 4.5 kV, sheath gas nitrogen) instrument. Elemental analyses were measured on a Foss-Heraeus Vario EL unit. IR spectra were recorded on a Perkin–Elmer FT-IR spectrometer Spectrum 1000, optical rotations on a Perkin–Elmer 341 polarimeter (1 cm micro cell, 25 °C) and UV–vis spectra on a Perkin–Elmer unit, Lambda 14. TLC was performed on silica gel (Merck 5554, detection by UV absorption). Solvents were dried according to usual procedures. The purity of the compounds was checked by HPLC/DAD and found to be >98% for each compound.

*4.3. (3 R, 19 R) methyl 3,19-dihydroxy-2-oxo-urs-12-en-28-carboxylate (**3**)*

To a solution of **2** (376 mg, 0.75 mmol) and TEMPO (2 mg, 0.01 mmol) in dichloromethane (20 ml), a solution of KBr (9 mg, 0.075 mmol) and $(n\text{-Bu})_4NBr$ (120 mg, 0.37 mmol), in an aq. solution of $NaHCO_3$ (5%, 3 ml) was added. Under vigorous stirring an aq. solution of NaOCl (1 M, 0.8 ml) was slowly added with 2 h (no further discoloration of the reaction mixture), and stirring was continued for another 6 h. The reaction was quenched by the addition of water (50 ml), and extracted with dichloromethane (4 × 40 ml). The combined organic phases were washed with brine (2 × 30 ml), dried (Na_2SO_4), and the solvent was evaporated. The residue was subjected to chromatography (silica gel, toluene/ethyl acetate/formic acid/*n*-heptane 80:20:3:10) to yield **3** (237 mg, 63%) as a colorless solid; mp 104–106 °C; $[\alpha]_D^{20} = +37.3°$ ($c = 0.47$, $CHCl_3$); R_F = 0.47 (toluene/ethyl acetate/formic acid/*n*-heptane 80:20:3:10); IR (KBr): ν = 3488 br, 2949 s, 1717 s, 1458 m, 1394 m, 1234 m, 1208 m, 1153 m, 1117 m, 1057 m, 1034 m, 970 w, 772 w, 733 w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.28 (dd, $J_{11',12}$ = 3.1, $J_{11'',12}$ = 3.1 Hz, 1 H, H-12), 3.83 (s, 1 H, H-3_{ax}), 3.53 (s, 3 H, H-31), 2.54 (m, 1 H, H-16_{ax}), 2.39 (d, $J_{1eq,1ax}$ = 12.5 Hz, H-1_{eq}), 2.03 (d, $J_{1ax,1eq}$ = 12.5 Hz, 1 H, H-1_{ax}), 1.89 (m, 3 H, H-9, H-11′ and H-11″), 1.67 (m, 1 H, H-22″), 1.66–1.51 (m, 6 H, H-6″, H-7″, H-15_{ax}, H-16_{eq}, H-21″ and H-22′), 1.39 (m, 2 H, H-6′, H-5), 1.37–1.30 (m, 2 H, H-7′, H-20), 1.24 (s, 3 H, H-27), 1.19 (m, 1 H, H-21′), 1.14 (s, 3 H, H-29), 1.13 (s, 3 H, H-23), 0.97 (m, 1 H, H-15_{eq}), 0.87 (d, $J_{20,30}$ = 6.5 Hz, 3 H, H-30), 0.81, 0.63 and 0.62 (each s, 9 H, H-24, H-25, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 211.0 (C2), 178.2 (C28), 138.4 (C13), 128.2 (C12), 82.6 (C3), 73.1 (C19), 54.4 (C5), 53.2 (C18), 53.1 (C1), 51.6 (C31), 47.8 (C17), 47.2 (C9), 45.7 (C4), 43.7 (C10), 41.3 (C14), 41.1 (C20), 40.3 (C8), 37.3 (C22), 32.4 (C7), 29.4 (C23), 28.2 (C15), 27.4 (C29), 25.9 (C21), 25.4 (C16), 24.3 (C27), 23.6 (C11), 21.0 (C6), 18.6, 16.5, 16.1 (C24, C25, C26), 16.2 (C30) ppm; MS (ESI, MeOH): m/z (%) = 501.4 ($[M + H]^+$, 10), 518.4 ($[M + NH_4]^+$, 10), 523.3 ($[M + Na]^+$, 100), 539.3 ($[M + K]^+$, 19); analysis for $C_{31}H_{48}O_5$ (500.71): C, 74.36; H, 9.66; found: C, 74.21; H, 9.82.

*4.4. (2 R, 19 R) methyl 2,19-dihydroxy-3-oxo-urs-12-en-28-carboxylate (**4**)*

From **2**: To a solution of **2** (500 mg, 0.99 mmol) in dry chloroform (20 ml) at 0 °C $[(n\text{-Bu})_3Sn]_2O$ (0.5 ml, 0.99 mmol) and bromine (51 μl, 0.99 mmol) were added. After stirring for 1 min, NEt_3 (0.10 ml) was added, the solvents were removed under diminished pressure, and the residue was subjected to chromatography (silica gel, toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10) to afford **4** (310 mg, 62%) as a colorless solid.

From **8**: Analogous synthesis starting from **8** (673 mg, 0.99 mmol) gave **4** (452 mg, 67%) as a colorless solid; mp 114–117 °C; $[\alpha]_D^{20} = +31.3°$ (c = 0.42, $CHCl_3$); R_F = 0.46 (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10); IR (KBr): ν = 3483 br, 2935 s, 1720 s, 1458 m, 1390 m, 1263 m, 1232 m, 1207 m, 1192 m, 1153 s, 1094 m, 1056 m, 961 w, 866 w, 771 w cm^{-1}; ^1H NMR

(500 MHz, $CDCl_3$): $\delta = 5.32$ (dd, 1 H, $J_{12,\,11'} = 3.5$, $J_{12,\,11''} = 3.5$ Hz, H-12), 4.51 (dd, 1 H, $J_{2ax,\,1ax} = 12.5$, $J_{2ax,\,1eq} = 6.5$ Hz, 1 H, H-2_{ax}), 3.58 (s, 3 H, H-31), 2.57 (s, 1 H, H-18), 2.51 (m, 1 H, H-16_{ax}), 2.40 (dd, $J_{1eq,\,1ax} = 12.5$, $J_{1eq,\,2ax} = 6.5$ Hz, 1 H, H-1_{eq}), 2.04–2.01 (m, 2 H, H-11′, H-11″), 1.72–1.28 (m, 12 H, H-6′, H-6″, H-7′, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-20, H-21′, H-21″, H-22′, H-22″), 1.25 (s, 3 H, H-25), 1.22 (s, 3 H, H-27), 1.20 (s, 3 H, H-29), 1.16 (s, 3 H, H-24), 1.16–1.13 (m, 2 H, H-1_{ax}, H-5), 1.11 (s, 3 H, H-23), 1.01 (m, 1 H, H-15_{eq}), 0.93 (d, $J_{30,\,20} = 7.0$, 3 H, H-30), 0.73 (s, 3 H, H-26) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 216.6$ (C3), 178.3 (C28), 138.3 (C13), 128.5 (C12), 73.1 (C19), 69.1 (C2), 57.6 (C5), 53.1 (C18), 51.6 (C31), 49.5 (C1), 47.8, 47.7 (C4, C17), 46.9 (C9), 41.2 (C14), 41.1 (C20), 40.0 (C8), 37.6 (C10), 37.3 (C22), 32.4 (C7), 28.2 (C15), 27.4 (C29), 25.9 (C21), 25.4 (C16), 24.7 (C23), 24.4 (C27), 23.8 (C11), 21.6 (C24), 19.2 (C6), 16.8 (C26), 16.1 (C30), 15.9 (C25) ppm; MS (ESI, MeOH): m/z (%) = 501.4 ($[M + H]^+$, 116), 518.4 ($[M + NH_4]^+$, 10), 523.3 ($[M + Na]^+$, 100), 539.3 ($[M + K]^+$, 17); analysis for $C_{31}H_{48}O_5$ (500.71): C, 74.36; H, 9.66; found: C, 74.25; H, 9.81.

4.5. (19 R) methyl 2,19-dihydroxyursa-3-oxo-1,12-dien-28-carboxylate (**5**)

Following the procedure as described above (15 min reaction time), from **2** (214 mg, 0.43 mmol), $[(n\text{-Bu})_3Sn]_2O$ (0.44 ml, 0.86 mmol) and bromine (44 µl, 86 mmol), compound **5** (121 mg, 57%) was obtained as a white solid; mp 115–118 °C; $[\alpha]_D^{20} = +62.8°$ ($c = 0.47$, $CHCl_3$); $R_F = 0.65$ (toluene/ethyl acetate/formic acid/*n*-heptane 80:20:3:10); IR (KBr): $\nu = 3436$ br, 2933 s, 2876 s, 1725 s, 1669 s, 1648 m, 1458 m, 1404 m, 1383 s, 1238 s, 1208 s, 1152 s, 1091 m, 1054 m, 1034 m, 970 w, 930 w, 865 w, 786 w, 772 w, 753 w, 538 w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 6.34$ (s, 1 H, H-1), 5.93 (s, 1 H, OH), 5.40 (dd, 1 H, $J_{12,\,11'} = 3.5$, $J_{12,\,11''} = 3.5$ Hz, H-12), 3.61 (s, 3 H, H-31), 2.62 (s, 1 H, H-18), 2.52 (m, 1 H, H-16_{ax}), 2.21 (dd, 1 H, $J_{11'',\,9} = 6.6$, $J_{11'',\,12} = 3.5$ Hz, H-11″), 2.13 (dd, 1 H, $J_{11',\,9} = 11.2$, $J_{11',\,12} = 3.5$ Hz, H-11′), 1.96 (dd, 1 H, $J_{9,\,11'} = 11.2$, $J_{9,\,11''} = 6.6$ Hz, H-9), 1.76–1.52 (m, 9 H, H-5, H-6′, H-6″, H-7″, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.43–1.37 (m, 2 H, H-7′, H-20), 1.26 (s, 3 H, H-27), 1.24 (m, 1 H, H-21′), 1.22, 1.21, 1.12 (each s, 12 H, H-23, H-24, H-25, H-29), 1.04 (m, 1 H, H-15_{eq}), 0.94 (d, 3 H, $J_{30,\,20} = 7.0$ Hz, H-30), 0.77 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 201.1$ (C3), 178.3 (C28), 143.6 (C2), 138.6 (C13), 128.4 (C12), 128.1 (C1), 73.1 (C19), 53.8 (C5), 53.3 (C18), 51.6 (C31), 47.9 (C17), 43.9 (C4), 42.6 (C9), 41.6 (C10), 42.0 (C20), 40.6 (C14), 38.4 (C8), 37.3 (C22), 32.6 (C7), 28.2 (C15), 27.4 (C29), 27.1 (C23), 26.0 (C21), 25.4 (C16), 24.5 (C27), 23.6 (C11), 21.8, 19.4 (C24, C25), 18.7 (C6), 17.1 (C26) 16.0 (C30) ppm; MS (ESI, MeOH): m/z (%) = 499.7 ($[M + H]^+$, 13), 521.5 ($[M + Na]^+$, 100), 537.4 ($[M + K]^+$, 75), 553.2 ($[M + MeOH]^+$, 98), 569.3 ($[M + K + MeOH]^+$, 34); analysis for $C_{31}H_{46}O_5$ (498.69): C, 74.66; H, 9.29; found: C, 74.53; H, 9.38.

4.6. (2 S, 3 R, 19 R) methyl 2,3,19-trihydroxyurs-12-en-28-carboxylate (**6**)

From **3**: To a solution of $NaBH_4$ (53 mg, 1.41 mmol) in MeOH (2 ml), a solution of **3** (235 mg, 0.47 mmol) was added drop-wise and heated under reflux for 1 h. After quenching with an aqueous solution of NH_4Cl (satd., 5 ml), dilution with water (20 ml), the mixture was extracted with ethyl acetate (3 × 20 ml), the combined extracts were washed (2 × 20 ml) and dried (Na_2SO_4). The solvent was removed and the residue subjected to chromatography (silica gel, *n*-pentane/ethyl acetate, 2:1) to afford **6** (193 mg, 82%) as a colorless solid.

From **5**: In analogous manner from **5** (80 mg, 0.16 mmol) compound **6** (56 mg, 70%) was obtained as a colorless solid; mp 106–107 °C; $[\alpha]_D^{20} = +50.7°$ ($c = 0.6$, $CHCl_3$) (lit.: [3] 16.3°); $R_F = 0.26$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10); IR (KBr): $\nu = 3510$ br, 2929 s, 1721 s, 1648 w, 1458 m, 1380 m, 1368 m, 1322 m, 1262 m, 1229 m, 1208 m, 1152 s, 1114 m, 1095 m, 1050 m, 1030 m, 1000 m, 973 w, 932 w, 900 w, 867 w, 806 w, 787 w, 772 w, 706 w, 684 w, 654 w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.35$ (dd, 1 H, $J_{12,\,11'} = 3.4$, $J_{12,\,11''} = 3.4$ Hz, H-12), 4.07 (ddd, 1 H, $J_{2eq,\,3ax} = 4.0$, $J_{2eq,\,1ax} = 3.6$, $J_{2eq,\,1eq} = 2.8$ Hz, H-2_{eq}), 3.58 (s, 3 H, H-31), 3.20 (d, 1 H, $J_{3ax,\,2eq} = 4.0$ Hz, H-3_{ax}), 2.58 (s, 1 H, H-18), 2.48 (m, 1 H, H-16_{ax}), 2.08 (dd, 1 H, $J_{1eq,\,1ax} = 14.5$, $J_{1eq,\,2eq} = 2.8$ Hz, H-1_{eq}), 2.02–1.99 (m, 2 H, H-11′, H-11″), 1.72–1.47 (m, 9 H, H-6′, H-6″, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.39 (m, 1 H, H-20), 1.29 (m, 1 H, H-21′), 1.26 (m, 1 H, H-7′), 1.23 (s, 3 H, H-27), 1.21 (s, 3 H, H-25), 1.19 (s, 3 H, H-29), 1.14 (dd, 1 H, $J_{1ax,\,1eq} = 14.5$, $J_{1ax,\,2eq} = 3.6$ Hz, H-1_{ax}), 1.01 (m, 1 H, H-15_{eq}), 0.99 and 0.98 (each s, 6 H, H-23, H-24), 0.92 (d, 3 H, $J_{30,\,20} = 6.6$ Hz, H-30), 0.81 (m, 1 H, H-5), 0.68 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$, 125 MHz): $\delta = 178.3$ (C28), 138.1 (C13), 129.3 (C12), 78.5 (C3), 73.2 (C19), 71.1 (C2), 55.1 (C5), 53.2 (C18), 51.6 (C31), 47.9 (C17), 47.6 (C9), 44.0 (C1), 41.3 (C14), 41.1 (C20), 40.0 (C8), 38.1 (C4), 37.4 (C22), 36.7 (C10), 32.7 (C7), 29.7 (C23), 28.1 (C15), 27.4 (C29), 26.0 (C21), 25.5 (C16), 24.6 (C27), 23.7 (C11), 18.2 (C6), 17.3 (C24), 16.6 (C26), 16.2 (C25), 16.1 (C30) ppm; MS (ESI, MeOH): m/z (%) = 525.5 ($[M + Na]^+$, 100), 556.9 ($[M + MeOH]^+$, 21); analysis for $C_{31}H_{50}O_5$ (502.73): C, 74.06; H, 10.02; found: C, 73.96; H, 10.14.

4.7. Euscaphic acid methyl ester (**8**)

From the esterification of euscaphic acid (**7**) with diazomethane; mp: 120–122 °C (lit.: [40] 130–132 °C; [64] 122–124 °C; [65] 140 °C); $[\alpha]_D^{20} = +31.6°$ ($c = 0.46$, $CHCl_3$) (lit: +31° [66]); $R_F = 0.19$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10); MS (ESI, MeOH): m/z (%) = 503.4 ($[M + H]^+$, 10), 520.3 ($[M + NH_4]^+$, 6), 525.5 ($[M + Na]^+$, 100), 556.9 ($[M + MeOH]^+$, 46).

4.8. (2 R, 3 R, 19 R) 2,3-Bis(acetyloxy)-19-hydroxyurs-12-en-28-carboxylic acid (**9**)

Acetylation of **1** (150 mg, 0.31 mmol) in dry pyridine (6 ml) with acetic anhydride for 12 h at 24 °C yielded after usual work-up and re-crystallization from toluene **9** as a colorless solid; mp 178–180 °C (lit.: [55] 186–189 °C); $[\alpha]_D^{20} = +5.8°$ ($c = 0.51$, $CHCl_3$) (lit.: +12° [25]; +6° [67]); $R_F = 0.45$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10); IR (KBr): $\nu = 3433$ br, 2937 s, 1743 s, 1456 m, 1369 s, 1252 s, 1154 m, 1109 w, 1033 m, 965 m, 932 w, 866 w, 759 w, 642 w, 598 w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.31$ (dd, 1 H, $J_{12,\,11'} = 3.2$, $J_{12,\,11''} = 3.2$ Hz, H-12), 5.08 (ddd, 1 H, $J_{2ax,\,1ax} = 11.1$, $J_{2ax,\,3ax} = 10.3$, $J_{2ax,\,1eq} = 4.7$ Hz, H-2_{ax}), 4.73 (d, 1 H, $J_{3ax,\,2ax} = 10.3$ Hz, H-3_{ax}), 2.52 (m, 2 H, H-18, H-16_{ax}), 2.03 (s, 3 H, H-32 or H-34), 2.02 (m, 1 H, H-1_{eq}), 1.96 (m, 5 H, H-32 or H-34 and H-11′, H-11″), 1.79–1.47 (m, 8 H, H-6″, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.42–1.35 (m, 2 H, H-6′, H-20), 1.32–1.27 (m, 2 H, H-7′, H-21′), 1.23 (s, 3 H, H-27), 1.18 (s, 3 H, H-29), 1.09 (m, 1 H, H-1_{ax}), 1.04 (s, 3 H, H-25), 0.99 (m, 1 H, H-15_{eq}), 0.96 (m, 1 H, H-5), 0.93 (d, 3 H, $J_{30,\,20} = 6.6$ Hz, H-30), 0.88 (s, 6 H, H-23, H-24), 0.70 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHZ, $CDCl_3$): $\delta = 184.2$ (C28), 170.8, 170.6 (C31, C33), 138.0 (C13), 128.8 (C12), 80.6 (C3), 73.0 (C19), 70.0 (C2), 54.7 (C5), 52.8 (C18), 47.7 (C17), 47.1 (C9), 43.9 (C1), 41.1 (C14), 41.0 (C20), 39.9 (C8), 39.3 (C4), 38.1 (C10), 37.4 (C22), 32.4 (C7), 28.4 (C23), 28.1 (C15), 27.3 (C29), 25.9 (C21), 25.2 (C16), 24.4 (C27), 23.7 (C11), 22.0, 20.9 (C32, C34), 18.2 (C6), 17.6 (C24), 16.9 (C26), 16.3 (C25), 16.1 (C30) ppm; MS (ESI, MeOH): m/z (%) = 571.4 ($[M - H]^-$, 100), 616.9 ($[M + HCO_2]^-$, 27); analysis for $C_{34}H_{52}O_7$ (572.77): C, 71.30; H, 9.15; found: C, 71.18; H, 9.23.

*4.9. (2 R, 3 R, 19 R) 2-acetyloxy-3,19-dihydroxyurs-12-en-28-carboxylic acid (**10**) and (2 R, 3 R, 19 R) 3-acetyloxy-2,19-dihydroxyurs-12-en-28-carboxylic acid (**11**)*

Acetylation of **1** (300 mg, 0.61 mmol) in dichloromethane (30 ml) containing dry pyridine (2 ml) with acetic anhydride (1 ml) for 3 h at 24 °C followed by usual aqueous work-up and chromatography (silica gel, *n*-pentane/ethyl acetate/ethanol, 17:10:1) afforded **10** (182 mg, 56%) and **11** (67 mg, 21%).

Data for **10**: colorless solid; mp 171–174 °C; $[\alpha]_D^{20} = +4.7°$ ($c = 0.51$, $CHCl_3$); $R_F = 0.23$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10); IR (KBr): $\nu = 3510$ br, 2937 s, 1724 s, 1457 m, 1369 m, 1255 s, 1155 m, 1095 m, 1031 m, 961 m, 933 w, 865 w, 766 w, 660 w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.30$ (dd, 1 H, $J_{12,\ 11'} = 3.2$, $J_{12,\ 11''} = 3.2$ Hz, H-12), 4.92 (ddd, 1 H, $J_{2ax,\ 1ax} = 10.9$, $J_{2ax,\ 3ax} = 10.0$, $J_{2ax,\ 1eq} = 4.4$ Hz, H-2_{ax}), 3.18 (d, 1 H, $J_{3ax,\ 2ax} = 10.0$ Hz, H-3_{ax}), 2.51 (s, 1 H, H-18), 2.45 (m, 1 H, H-16_{ax}), 2.04 (s, 3 H, H-33), 1.99 (m, 1 H, H-1_{eq}), 1.99–1.94 (m, 2 H, H-11′, H-11″), 1.78–1.45 (m, 8 H, H-6″, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.40–1.26 (m, 3 H, H-7′, H-20, H-21′), 1.22 (s, 3 H, H-27), 1.17 (s, 3 H, H-29), 1.03, 1.01 (each s, 6 H, H-23, H-25), 1.00 (m, 1 H, H-15_{eq}), 0.96 (m, 1 H, H-1_{ax}), 0.92 (d, 3 H, $J_{30,\ 20} = 6.6$ Hz, H-30), 0.86 (m, 1 H, H-5), 0.83 (s, 3 H, H-24), 0.69 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 184.1$ (C28), 171.6 (C31), 138.0 (C13), 129.0 (C12), 80.8 (C3), 73.3 (C2), 73.1 (C19), 55.0 (C5), 52.8 (C18), 47.7 (C17), 47.1 (C9), 43.7 (C1), 41.1 (C14), 41.0 (C20), 40.0 (C8), 39.7 (C4), 38.3 (C10), 37.4 (C22), 32.5 (C7), 28.5 (C23), 28.1 (C15), 27.3 (C29), 25.9 (C21), 25.3 (C16), 24.5 (C27), 23.7 (C11), 21.3 (C32), 18.3 (C6), 17.0, 16.6, 16.3 (C24, C25, C26), 16.1 (C30) ppm; MS (ESI, MeOH): *m/z* (%) = 529.7 ($[M - H]^-$, 100), 575.3 ($[M + HCO_2]^-$, 13); analysis for $C_{32}H_{50}O_6$ (530.74): C, 72.42; H, 9.50; found: C, 72.36; H, 9.58.

Data for **11**: mp 190–192 °C; $[\alpha]_D^{20} = +0.99°$ ($c = 0.41$, MeOH); $R_F = 0.20$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10); (KBr): $\nu = 3576$ m, 3432 br, 2930 s, 1737 s, 1689 s, 1461 m, 1369 s, 1253 s, 1158 m, 1104 m, 1049 m, 1031 m, 1005 m, 959 m, 934 w, 907 w, 868 w, 769 w, 650 w, 562 w cm^{-1}; ^1H NMR (500 MHz, CD_3OD): $\delta = 5.29$ (dd, 1 H, $J_{12,\ 11'} = 3.3$, $J_{12,\ 11''} = 3.3$ Hz, H-12), 4.51 (d, 1 H, $J_{3ax,\ 2ax} = 9.9$ Hz, H-3_{ax}), 3.76 (ddd, 1 H, $J_{2ax,\ 1ax} = 10.9$, $J_{2ax,\ 3ax} = 9.9$, $J_{2ax,\ 1eq} = 4.4$ Hz, H-2_{ax}), 2.58 (ddd, 1 H, $J_{16ax,\ 16eq} = 14.3$, $J_{16ax,\ 15ax} = 12.8$, $J_{16ax,\ 15eq} = 4.3$ Hz, H-16_{ax}), 2.50 (s, 1 H, H-18), 2.09 (s, 3 H, H-32), 2.04–1.97 (m, 3 H, H-1_{eq}, H-11′, H-11″), 1.83–1.41 (m, 9 H, H-6″, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-20, H-21″, H-22′, H-22″), 1.35 (s, 3 H, H-27), 1.33–1.30 (m, 3 H, H-6′, H-7′, H-21′), 1.19 (s, 3 H, H-29), 1.03 (s, 3 H, H-23), 1.01–0.96 (m, 3 H, H-1_{ax}, H-5, H-15_{eq}), 0.93 (d, 3 H, $J_{30,\ 20} = 6.7$ Hz, H-30), 0.88, 0.87 (each s, 6 H, H-24, H-25), 0.80 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHz, CD_3OD): $\delta = 180.8$ (C28), 171.9 (C31), 138.7 (C13), 127.7 (C12), 84.4 (C3), 72.1 (C19), 66.1 (C2), 55.0 (C5), 53.6 (C18), 48.1 (C17), 47.1 (C9), 46.9 (C1), 41.7 (C20), 41.2 (C14), 39.7 (C8), 38.9 (C4), 37.7 (C10), 37.6 (C22), 32.6 (C7), 28.1 (C15), 27.7 (C24), 25.9 (C21), 25.6 (C29), 25.2 (C16), 23.4 (C27), 23.3 (C11), 19.7 (C32), 18.1 (C6), 16.7 (C25), 16.0 (C26), 15.6 (C23), 15.2 (C30) ppm; MS (ESI, MeOH): *m/z* (%) = 529.7 ($[M - H]^-$, 100), 575.3 ($[M + HCO_2]^-$, 15); analysis for $C_{32}H_{50}O_6$ (530.74): C, 72.41; H, 9.50; found: C, 72.31; H, 9.66.

*4.10. (3 R, 19 R) methyl 3-acetyloxy-19-hydroxy-2-oxo-urs-12-en-28-carboxylate (**12**)*

To a solution of **3** (100 mg, 0.20 mmol) in dry pyridine (4 ml) acetic anhydride (8 ml) was slowly added and stirring at 24 °C was continued for 24 h. The reaction mixture was poured into ice-cold water, and the precipitate was filtered off. Re-crystallization from methanol yielded **12** (72 mg, 66%) as a colorless solid; mp 218–220 °C; $[\alpha]_D^{20} = +72.2°$ ($c = 0.67$, $CHCl_3$); $R_F = 0.56$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10); IR (KBr): $\nu = 3511$ br, 2935 s, 1721 s, 1458 m, 1396 m, 1371 m, 1292 m 1236 s, 1151 m, 1096 w, 1053 m, 1034 m, 1009 m, 969 w, 930 w, 866 w, 772 w, 691 w, 499 w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.28$ (dd, 1 H, $J_{12,\ 11'} = 2.5$, $J_{12,\ 11''} = 2.5$ Hz, H-12), 4.88 (s, 1 H, H-3_{ax}), 3.53 (s, 3 H, H-31), 2.54 (s, 1 H, H-18), 2.46 (m, 1 H, H-16_{ax}), 2.34 (d, 1 H, $J_{1eq,\ 1ax} = 12.2$ Hz, H-1_{eq}), 2.12 (d, 1 H, $J_{1ax,\ 1eq} = 12.2$ Hz, H-1_{ax}), 2.11 (s, 3 H, H-33), 1.88–1.85 (m, 3 H, H-9, H-11′, H-11″), 1.68–1.51 (m, 7 H, H-6′, H-6″, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.46 (m, 1 H, H-5), 1.37–1.30 (m, 3 H, H-7′, H-7″, H-20), 1.24 (s, 3 H, H-27), 1.21 (m, 1 H, H-21′), 1.15 (s, 3 H, H-29), 1.04 (s, 3 H, H-23), 0.99 (m, 1 H, H-15_{eq}), 0.88 (d, 3 H, $J_{30,\ 20} = 6.7$ Hz, H-30), 0.84, 0.79, 0.62 (each s, 9 H, H-24, H-25, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 204.2$ (C2), 178.2 (C28), 170.5 (C32), 138.3 (C13), 128.2 (C12), 84.1 (C3), 73.1 (C19), 55.2 (C5), 53.9 (C1), 53.2 (C18), 51.6 (C31), 47.9 (C17), 47.1 (C9), 43.6, 43.1 (C4, C10), 41.3 (C14), 41.1 (C20), 40.3 (C8), 37.3 (C22), 32.4 (C7), 29.0 (C23), 28.2 (C15), 27.4 (C29), 26.0 (C21), 25.4 (C16), 24.3 (C27), 23.6 (C11), 20.6 (C33) 18.6 (C6), 17.5, 16.1, 15.9 (C24, C25, C26), 16.2 (C30); MS (ESI, MeOH): *m/z* (%) = 543.4 ($[M + H]^+$, 39), 560.6 ($[M + NH_4]^+$, 38), 565.5 ($[M + Na]^+$, 100), 581.4 ($[M + K]^+$, 18), 597.0 ($[M + Na + MeOH]^+$, 25); analysis for $C_{33}H_{50}O_6$ (542.75): C, 73.03; H, 9.29; found: C, 72.87; H, 9.41.

*4.11. (2 R, 19 R) methyl 2-acetyloxy-19-hydroxy-3-oxo-urs-12-en-28-carboxylate (**13**)*

Following the procedure given for **12**, from **4** (100 mg, 0.20 mmol), pyridine (4 ml) and acetic anhydride (8 ml) **13** (74 mg, 68%) was obtained as a colorless solid; mp 101–104 °C; $[\alpha]_D^{20} = +39.3°$ ($c = 0.46$, $CHCl_3$); $R_F = 0.61$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80:20:3:10); IR KBr: $\nu = 3545$ br, 2936 s, 2878 m, 1724 s, 1458 m, 1371 s, 1235 s, 1152 s, 1093 m, 1032 m, 1011 m, 960 m, 931 w, 906 w, 866 w, 804 w, 772 w, 704 w, 655 w, 601 w, 485 w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.59$ (dd, 1 H, $J_{2ax,\ 1ax} = 13.3$ Hz, $J_{2ax,\ 1eq} = 6.5$ Hz, H-2_{ax}), 5.33 (dd, 1 H, $J_{12,\ 11'} = 3.3$, $J_{12,\ 11''} = 3.3$ Hz, H-12), 3.59 (s, 3 H, H-31), 2.58 (s, 1 H, H-18), 2.49 (m, 1 H, H-16_{ax}), 2.19 (dd, 1 H, $J_{1eq,\ 1ax} = 12.5$ Hz, $J_{1eq,\ 2ax} = 6.5$ Hz, H-1_{eq}), 2.12 (s, 3 H, H-33), 2.02 (m, 2 H, H-11′, H-11″), 1.73–1.50 (m, 8 H, H-6″, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.41–1.32 (m, 5 H, H-1_{ax}, H-6′, H-7′, H-20, H-21′), 1.27 (s, 3 H, H-25), 1.22 (s, 3 H, H-27), 1.18 (s, 3 H, H-29), 1.15 (m, 1 H, H-5), 1.13 (s, 3 H, H-24), 1.10 (s, 3 H, H-23), 1.02 (m, 1 H, H-15_{eq}), 0.92 (d, 3 H, $J_{30,\ 20} = 6.7$ Hz, H-30), 0.73 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 209.3$ (C3), 178.3 (C28), 170.2 (C32), 138.5 (C13), 128.3 (C12), 73.1 (C19), 71.7 (C2) 57.0 (C5), 53.2 (C18), 51.6 (C31), 48.7 (C4), 47.8 (C17), 46.9 (C9), 45.6 (C1), 41.3 (C20), 41.1 (C14), 40.0 (C8), 37.8 (C10), 37.3 (C22), 32.4 (C7), 28.2 (C15), 27.4 (C29), 25.9 (C16), 25.4 (C21), 24.8 (C23), 24.4 (C27), 23.8 (C11), 21.3 (C24), 20.7 (C33), 19.2 (C6), 16.8 (C26), 16.0 (C30), 15.8 (C25) ppm; MS (ESI, MeOH): *m/z* (%) = 543.3 ($[M + H]^+$ 34), 560.4 ($[M + NH_4]^+$, 42), 565.5 ($[M + Na]^+$, 100), 581.4 ($[M + K]^+$, 43), 597.1 ($[M + Na + MeOH]^+$, 27); analysis for $C_{33}H_{50}O_6$ (542.75): C, 73.02; H, 9.29; found: C, 72.88; H, 9.31.

*4.12. (2 R, 3 R, 19 R) methyl 2,3-bis(chloroacetyloxy)-19-hydroxy urs-12-en-28-carboxylate (**14**), (2 R, 3 R, 19 R) methyl 2-chloro acetyloxy-3,19-dihydroxyurs-12-en-28-carboxylate (**15**), and (2 R, 3 R, 19 R) methyl 3-chloroacetyloxy-2,19-dihydroxyurs-12-en-28-carboxylate (**16**)*

Compound **2** (1.03 g, 2.05 mmol) was acylated at 24 °C for 4 h with chloroacetyl chloride (282 mg, 2.5 mmol) and pyridine (0.2 ml) in dry dichloromethane (30 ml). After usual aqueous work-up and chromatography (silica gel, toluene/ethyl acetate/formic acid/*n*-heptane, 80/20/3/10), compounds **14** (341 mg, 26%), **15** (463 mg, 40%) and **16** (124 mg, 11%) were obtained.

Data for **14**: colorless solid; mp 92–93 °C; $[\alpha]_D^{20} = -8.11°$ ($c = 0.39$, $CHCl_3$); $R_F = 0.77$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80/20/3/10); IR (KBr): $\nu = 3568$ br, 2948 s, 2878 m, 1736 s, 1457 m, 1412 m, 1397 m, 1370 m, 1310 s, 1262 s, 1168 s, 1071 w, 1023 m, 1001 m, 968 m, 928 m, 865 w, 791 m, 772 w, 696 w, 656 w, 587 w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.32$ (dd, 1 H, $J_{12,\ 11'} = 3.5$, $J_{12,\ 11''} = 3.5$ Hz, H-12), 5.17 (ddd, 1 H, $J_{2ax,\ 1ax} = 11.4$, $J_{2ax,\ 3ax} = 10.3$, $J_{2ax,\ 1eq} = 4.7$ Hz, 1 H, H-2_{ax}), 4.84 (d, 1 H, $J_{3ax,\ 2ax} = 10.3$ Hz, H-3_{ax}), 4.02, 3.93 (each s, 4 H, H-33, H-35), 3.58 (s, 3 H, H-31), 2.57 (s, 1 H, H-18), 2.49 (m, 1 H, H-16_{ax}), 2.07 (dd, 1 H, $J_{1eq,\ 1ax} = 12.3$, $J_{1eq,\ 2ax} = 4.7$, H-1_{eq}), 1.98–1.95 (m, H, H-11′, H-11″), 1.73–1.51 (m, 8 H, H-6″, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.45–1.26 (m, 4 H, H-6′, H-7′, H-20, H-21′), 1.23 (s, 3 H, H-27), 1.18 (s, 3 H, H-29), 1.13 (m, 1 H, H-1_{ax}), 1.05 (s, 3 H, H-25), 1.02–0.98 (m, 2 H, H-5, H-15_{eq}), 0.92 (d, 3 H, $J_{30,\ 20} = 6.4$, H-30), 0.92 (s, 6 H, H-23, H-24), 0.67 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 178.2$ (C28), 167.2, 166.9 (C32, C34), 138.3 (C13), 128.4 (C12), 82.3 (C3), 73.1 (C19), 72.2 (C2), 54.7 (C5), 53.1 (C18), 51.6 (C31), 47.8 (C17), 47.1 (C9), 43.6 (C1), 41.2 (C14), 41.1 (C20), 40.8 (C33, C35), 39.9 (C8), 39.6 (C4), 38.2 (C10), 37.3 (C22), 32.5 (C7), 28.3 (C23), 28.1 (C15), 27.4 (C29), 26.0 (C21), 25.4 (C16), 24.4 (C27), 23.7 (C11), 18.2 (C6), 17.5 (C24), 16.6 (C26), 16.3 (C25), 16.1 (C30) ppm; MS (ESI, MeOH): m/z (%) = 672.3 ($[M + NH_4]^+$, 14), 677.4 ($[M + Na]^+$, 100); analysis for $C_{35}H_{52}Cl_2O_7$ (655.69): C, 64.11; H, 7.99; found: C, 64.00; H, 8.09.

Data for **15**: colorless solid; mp 93–95 °C; $[\alpha]_D^{20} = -3.42°$ ($c = 0.50$, $CHCl_3$); $R_F = 0.46$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80/20/3/10); IR (KBr): $\nu = 3528$ s, 2947 s, 2877 s, 1727 s, 1457 m, 1380 m, 1310 s, 1263 m, 1231 m, 1192 s, 1168 s, 1074 m, 1046 m, 1033 m, 1015 m, 964 m, 865 w, 791 w, 772 w, 661 w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) $\delta = 5.34$ (dd, 1 H, $J_{12,\ 11'} = 3.5$, $J_{12,\ 11''} = 3.5$ Hz, H-12), 5.03 (ddd, 1 H, $J_{2ax,\ 1ax} = 11.5$, $J_{2ax,\ 3ax} = 10.0$, $J_{2ax,\ 1eq} = 4.5$ Hz, H-2_{ax}), 4.06 (s, 2 H, H-33), 3.60 (s, 3 H, H-31), 3.25 (d, 1 H, $J_{3ax,\ 2ax} = 10.0$ Hz, 1 H, H-3_{ax}), 2.59 (s, 1 H, H-18), 2.50 (m, 1 H, H-16_{ax}), 2.03 (dd, 1 H, $J_{1eq,\ 1ax} = 12.2$, $J_{1eq,\ 2ax} = 4.5$ Hz, H-1_{eq}), 1.99–1.96 (m, 2 H, H-11′, H-11″), 1.74–1.48 (m, 8 H, H-6″, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.45–1.39 (m, 2 H, H-6′, H-20), 1.33 (m, 1 H, H-7′), 1.31 (m, 1 H, H-21′), 1.25 (s, 3 H, H-27), 1.19 (s, 3 H, H-29), 1.06, 1.03 (each s, 6 H, H-23, H-25), 1.02 (m, 1 H, H-1_{ax}), 0.99 (m, 1 H, H-15_{eq}), 0.93 (d, 3 H, $J_{30,\ 20} = 6.7$ Hz, H-30), 0.89 (m, 1 H, H-5), 0.87 (s, 3 H, H-24), 0.68 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 178.3$ (C28), 167.5 (C32), 138.2 (C13), 128.7 (C12), 80.5 (C3), 75.6 (C2), 73.1 (C19), 55.0 (C5), 53.2 (C18), 51.6 (C31), 47.9 (C17), 47.1 (C9), 43.4 (C1), 41.2 (C14), 41.1 (C20, C33), 39.9 (C4, C8), 38.3 (C10), 37.3 (C22), 32.6 (C7), 28.5 (C23), 28.1 (C15), 27.4 (C29), 26.0 (C21), 25.4 (C16), 24.5 (C27), 23.7 (C11), 18.4 (C6), 16.6, 16.2 (C24, C25, C26), 16.1 (C30) ppm; MS (ESI, MeOH): m/z (%) = 601.4 ($[M + Na]^+$, 100); analysis for $C_{33}H_{51}ClO_6$ (579.21): C, 68.43; H, 8.88; found: C, 68.32; H, 9.03.

Data for **16**: colorless solid; mp 108–113 °C; $[\alpha]_D^{20} = +13.82°$ ($c = 0.47$, $CHCl_3$); $R_F = 0.33$ (toluene/ethyl acetate/formic acid/*n*-heptane, 80/20/3/10); IR (KBr): $\nu = 3528$ br, 2946 s, 2878 s, 1725 s, 1669 m, 1456 m, 1380 m, 1311 s, 1263 s, 1230 s, 1192 s, 1152 s, 1095 m, 1043 m, 1016 m, 988 m, 969 m, 929 m, 866 w, 788 w, 772 w, 698 w, 659 w, 465 w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.34$ (dd, 1 H, $J_{12,\ 11'} = 3.5$, $J_{12,\ 11''} = 3.5$ Hz, H-12), 4.58 (d, 1 H, $J_{3ax,\ 2ax} = 10.0$ Hz, H-3_{ax}), 4.14 (s, 2 H, H-33), 3.82 (ddd, 1 H, $J_{2ax,\ 1ax} = 11.5$, $J_{2ax,\ 3ax} = 10.0$, $J_{2ax,\ 1eq} = 4.4$ Hz, H-2_{ax}), 3.59 (s, 3 H, H-31), 2.59 (s, 1 H, H-18), 2.51 (m, 1 H, H-16_{ax}), 2.07 (dd, 1 H, $J_{1eq,\ 1ax} = 12.6$, $J_{1eq,\ 2ax} = 4.4$, 1 H, H-1_{eq}), 2.02–1.98 (m, 2 H, H-11′, H-11″), 1.74–1.48 (m, 8 H, H-6″, H-7″, H-9, H-15_{ax}, H-16_{eq}, H-21″, H-22′, H-22″), 1.43–1.38 (m, 2 H, H-6′, H-20), 1.33 (m, 1 H, H-7′), 1.26 (s, 3 H, H-27), 1.24 (m, 1 H, H-21′), 1.21 (s, 3 H, H-29), 1.06–1.01 (m, 3 H, H-1_{ax}, H-5, H-15_{ax}), 0.99, 0.91, 0.89 (each s, 9 H, H 23, H-24, H-25), 0.93 (d, 3 H, $J_{30,\ 20} = 6.7$ Hz, H-30), 0.68 (s, 3 H, H-26) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 178.3$ (C28), 168.3 (C32), 138.2 (C13), 128.7 (C12), 87.1 (C3), 73.2 (C19), 67.3 (C2), 55.0 (C5), 53.2 (C18), 51.6 (C31), 47.8 (C17), 47.6 (C1), 47.1 (C9), 41.2 (C14), 41.1 (C20, C33), 39.9 (C8), 39.4 (C4), 38.1 (C10), 37.3 (C22), 32.5 (C7), 28.5 (C23), 28.1 (C15), 27.3 (C29), 25.9 (C21), 25.4 (C16), 24.4 (C27), 23.7 (C11), 18.3 (C6), 17.5, 16.6, 16.4 (C24, C25, C26), 16.1 (C30) ppm; MS (ESI, MeOH): m/z (%) = 601.4 ($[M + Na]^+$, 100); analysis for $C_{33}H_{51}ClO_6$ (579.21): C, 68.43; H, 8.88; found: C, 68.27; H, 8.99.

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Original article

Esters and amides of maslinic acid trigger apoptosis in human tumor cells and alter their mode of action with respect to the substitution pattern at C-28

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ABSTRACT

Cancer is one of the most commonly diagnosed diseases worldwide; its mortality rate is high, and there is still a demand for the development of antitumor active drugs. Triterpenoic acids show many pharmacological effects, among them antitumor activity. One of these, maslinic acid-1 is of interest because of its antitumor profile. It is not only cytotoxic but also triggers apoptosis in various human tumor cell lines. To improve the cytotoxicity of parent **1** we set out to synthesize a series of esters and amides differing in structure and lipophilicity. These compounds were tested in a sulforhodamine B assay for cytotoxicity, and screened for their ability to induce apoptosis using an acridine orange/propidium iodide assay, DNA laddering and cell cycle experiments. Esters containing small-chain, lipophilic residues increased the cytotoxicity whereas amides as well long-chain esters led to a decrease in activity. The antitumor activity seems to be independent from the substitution pattern at position C-28 for esters and amides but alters their mode of action.

1. Introduction

Cancer is one of the most commonly diagnosed diseases worldwide. Recent research results [1–5] correlate the low cancer mortality rate in Mediterranean countries to a diet rich in olives. The triterpenoic compounds maslinic acid (**1**, Scheme 1) and oleanolic acid seem to be the main antitumor active ingredients of olives (*Olea europea* L.) [1,3,6–8]. Thus, for **1** an inhibitory activity for glycogen phosphorylases [9,10], protein tyrosine phosphatase 1B [11] as well as an anti-HIV-1 activity [12] were reported. Both triterpenoic acids can be extracted in high amounts from waste [13] of the olive oil production ("olive pomace"), hence underlining an economical interest connected with the investigation of the pharmaceutical effects attributed to these natural products.

Consequently, several groups investigated the effects of maslinic acid onto cancer cell lines [1,6,8,14–16]. Among others, Reyes et al. [8,14,15] could demonstrate that **1** is able to induce cell death by apoptosis in the colon cancer cell lines HT29 and in Caco-2. In 2007, **1** was reported to be cytotoxic for the breast cancer cell line MCF7 [17], and quite recently these initial findings were confirmed by several groups [3,18–21]. Furthermore, compound **1** exhibits [22] some selectivity between malignant and non-malignant cell lines, and selective parasitostatic activities of **1** were established in mice [23,24].

Structural modifications of triterpenoids have a high impact onto their biological activity [25]. As compared to other triterpenoids, for maslinic acid only a limited number of derivatives is known, and even less of them have been investigated with respect to their antitumor activity. Recent studies [26] gave evidence for an apoptotic effect of **1**. To the best of our knowledge, however, only one report [26] has been published showing a correlation between the ability to induce apoptosis as a function of structural modifications – but unfortunately no IC_{50} values have been reported. In that study, Parra et al. [26] demonstrated that the ability for inducing apoptosis increases by "simple" modifications of **1**: Thus, especially amide and benzyl maslinoates induce apoptosis in >90% of the tumor cells – thus making these compounds interesting candidates for further development. For betulinic acid several structure–activity relationships have been deduced but almost nothing is known for compound **1** and derivatives thereof [27–29]. Hence, we became interested in a more systematic investigation on maslinic acid derivatives: Several esters and amides of **1** were prepared, characterized and their cytotoxicity (IC_{50} values from photometric sulforhodamine B protein (SRB) assays) were determined.

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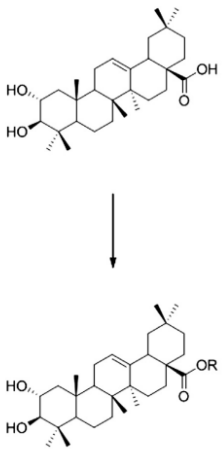


R =		
H	Maslinic acid	**1**
CH_3	Methyl	**2**
CH_2CH_3	Ethyl	**3**
CH_2CH_2OH	2-Hydroxyl-ethyl	**4**
CH_2CH_2Cl	2-Chloro-ethyl	**5**
$CH_2CH_2CH_3$	Propyl	**6**
$CH_2CH=CH_2$	Prop-2-enyl	**7**
$CH_2C\equiv CH$	Propargyl	**8**
$CH_2CH_2CH_2OH$	3-Hydroxy-propyl	**9**
$CH_2CH_2CH_2Cl$	3-Chloro-propyl	**10**
$CH_2CH_2CH_2Br$	3-Bromo-propyl	**11**
$CH_2CH_2CH_2I$	3-Iodo-propyl	**12**
$CH_2CH_2CH_2CH_3$	Butyl	**13**
$CH_2CH_2CH=CH_2$	But-3-enyl	**14**
$CH_2CH_2C\equiv CH$	But-3-inyl	**15**
$CH_2CH_2CH_2CH_2Cl$	4-Chloro-butyl	**16**
$CH_2CH_2CH_2CH_2Br$	4-Bromo-butyl	**17**
(*Z*)-$CH_2CH=CHCH_2Cl$	(Z)-4-Chloro-but-2-enyl	**18**
$CH_2CH_2C\equiv CCl$	4-Chloro-but-2-inyl	**19**
$CH_2(CH_2)_4CH_3$	Hexyl	**20**
$CH_2(CH_2)_{10}CH_3$	Dodecyl	**21**
$CH_2(CH_2)_{16}CH_3$	Octadecyl	**22**
CH_2Cy	Cyclohexylmethyl	**23**

Scheme 1. Synthesis of the esters **2**–**23** from maslinic acid **1**: using DMF, K_2CO_3, 25 °C, 30 min followed by the addition of Hal-R, 25 °C, 12 h.

2. Chemistry

Maslinic acid-1, a triterpenoic acid, carries three different functional groups: two hydroxyl groups in ring A, a double bond in ring C and a carboxylic group (C-28). Antitumor screening of derivatives of betulinic acid, oleanolic or ursolic acid revealed the presence of a C=O moiety to be essential for obtaining good antitumor activity [25–29].

Firstly, we prepared several esters at position C-28. This carboxyl group is remarkably unreactive, and, Fischer esterification protocols failed. The esters were obtained by treating **1** in *N*,*N*-dimethylformamide (DMF) or acetone with alkyl bromides in the presence of freshly grounded potassium carbonate. An aqueous work-up using 5% cold aqueous hydrochloric acid facilitated the isolation of the products by destroying some byproducts [30]. Thus, following this general procedure, aliphatic esters (Scheme 1) were synthesized in good to excellent yields (75–99%), and esters containing side chains of different lengths (**2**,**3**,**6**,**13**,**20**–**21**) as well as halogenated (**5**, **10**–**12**, **16**–**19**) and hydroxylated (*e.g.* **4**) esters were obtained.

The structures of the esters **2**–**23** were confirmed by extensive analysis. Thus, the mass spectrometric analysis revealed for all esters the presence of a characteristic methyl oleanoate decomposition signal $m/z = 203$ [31,32]. Also, the elimination of two molecules of water followed by a subsequent splitting off of two molecules of alcohol was observed ($m/z = 409$). All ^{13}C NMR signals for the triterpenoic skeleton in this series of compounds were similar with exception of the signal for C-28 (and the additional signals for the alcohol part of the ester). For C-28 a shift to higher field for the esters was observed as compared to parent **1** ($\delta = 180.2$ ppm for **1** to $\delta = 177.8 \pm 0.47$ ppm for the esters). In addition, for the alkynic derivatives **8** and **15** in the IR spectra the typical bands for alkynes were found at $\nu = 3314$ and 3296 cm^{-1}, respectively.

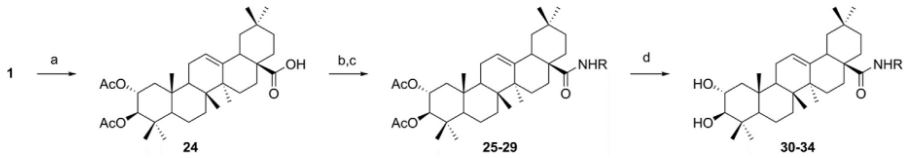
Consecutively, a series of homologous amides was synthesized (Scheme 2) to investigate the influence of a nitrogen substituent. Starting from **1**, diacetylation furnished a diacetate **24** whose treatment with thionylchloride followed by the reaction with amines yielded diacetylated amides **25**–**29** whose de-acetylation with potassium carbonate in MeOH gave amides **30**–**34** in 61–82% isolated yield.

3. Biology

Photometric SRB assays [33] were performed, the results of which are compiled in Table 1. Thus, IC_{50} values (Table 1) were determined for six different human cancer cell lines and non-malignant mouse fibroblasts (NIH 3T3).

By and large, esterification of parent **1** results in an increase of cytotoxicity. As shown for three different human cancer cell lines [A2780 (ovarian carcinoma), HT29 (colon adenocarcinoma) and MCF7 (breast adenocarcinoma)] short alkyl esters of **1** up to a maximum length of six carbons possess a higher cytotoxicity than parent **1** (Fig. 1). Activity is lost, however, for long chain esters (*e.g.* **20**–**22**).

The allyl ester of 2-cyano-3,12-dioxooleana-1,9(11)dien-28-acid (CDDO) has previously been shown to possess a five-fold higher inhibitory activity for the production of nitric oxide NO in mouse macrophages than a homologous butyl ester, whereas a significant drop in activity was determined for the corresponding CDDO ethyl ester [34]. Recently, we were able to show for several derivatives of glycyrrhetinic acid that unsaturated esters exhibit higher cytotoxicity towards several human tumor cell lines as compared to their saturated analogs. However, comparison of the IC_{50} values from the SRB tests using the estrogen receptor positive breast cancer cell line MCF7 showed no significant differences in the cytotoxicity for the allyl (**7**), propargyl (**8**) or but-3-enyl (**14**) ester as compared to parent **1**. A drop in activity was observed for the alkynyl ester **15**.



Scheme 2. a) Ac_2O, pyridine, TEA b) DCM/THF, $SOCl_2$, 0 °C → 25 °C, 30 min c) DCM, RNH_2, 25 °C, 12 h d) K_2CO_3, MeOH, 25 °C, 8 h; R′ = H (**25**), ethyl (**26**), propyl-27, propenyl (**28**), propargyl-29; R = H (**30**), ethyl (**31**), propyl (**32**), propenyl (**33**), propargyl (**34**).

Following these observations we assumed that the improvements in cytotoxicity following the introduction of an ester moiety might be explained by differences in the transport of the compounds through the cell membrane. In addition, the overall lipophilicity of the C-28 ester seems to be more important for activity than individual modifications at this functional group. Thus, the IC_{50} values of several halogenated analogs of the propyl **6** as well as of the butyl ester **13** are similar; these substituents exhibited no strong impact onto the cytotoxicity of the compounds. By and large, these halogenated analogs showed similar cytotoxicity as their non-halogenated analogs. Interestingly, introducing additional polar groups (as exemplified in the 2-hydroxyethyl ester **4**) resulted in a complete loss of activity in melanoma cells (518A2), thyroid carcinoma (8505C) and lung cancer (A549) cells. The same is true for the butinyl analog **19**. Promising results could be seen for the 4-chloro-butyl compound **16** whereas a drop of activity was observed for the chlorobutinyl derivative **19**.

Several amides of **1** [26] are able to induce apoptosis; this parallels previous findings for CDDO derivatives [34]. Contrary to the results for the esters, introduction of a lipophilic moiety resulted in a significant loss of cytotoxicity for the amides. The propyl derivative **32** was shown to be significantly more potent than the ethyl derivative **31**. Introducing moieties containing C═C double bonds led to a decrease in activity. It seems that esters exhibit a 2–3 fold higher cytotoxicity than the amides. These findings may be the result either of different transport mechanisms of the compounds through the membrane or of a different integration into the cell membrane [35,36]. The effects of several analogs of betulinic acid onto the membrane of erythrocytes has been studied several years ago [37]. Compounds possessing a hydrogen bond donor group (for example COOH, $CONH_2$ or CHOH) at position C-28 acted echinocytogenic, whereas esters, aldehydes and lupeol resulted in the formation of stomatocytes. The latter were formed when the incorporation took place predominantly into the inner leaflet. The amides **30**–**34**, parent **1** as well as inactive **4** and **9** carry a hydrogen bond donor group whereas all of the active compounds of our study are devoid of this function.

The amide **30** exhibited IC_{50} values comparable to those of **1** employing the malignant human tumor cell lines A549 (human lung carcinoma) and HT29. This compound showed a significantly higher cytotoxicity towards tumor cell lines than towards the non-malignant mouse fibroblasts. Furthermore, the introduction of a saturated, bulky cyclohexylmethyl residue (as in **23**) led to promising results; for example for the ovarian cancer cell line an IC_{50} value of 3.6 μM was observed.

The IC_{50} values from the SRB assays show the cytotoxicity of a compound but these tests don't indicate whether the cell death occurs *via* apoptosis or necrosis. To investigate the compounds for their ability to induce apoptosis or for a stop of cell growing, additional cell cycle investigations (after incubation periods of 24 and 48 h) were performed.

Treatment of a cell population with a specific and proportionally DNA-binding fluorescence dye allows measuring the distribution of the cell cycle by FACS-analysis [38]. These experiments revealed (Fig. 2) that the compounds induced a significant G1/G0 arrest in the living, adherent growing cells after 24 h. After 48 h, a further increase of cells in the G1/G0 phase with a total reduction of cells in the S as well as the G2/M phase was observed. Hence, these analogs of **1** are potent cell proliferation stopping agents.

Controlled dying of cells is characterized by an intact cell membrane until the middle phases of the death process [39–42]. The combination of a cell impermeable dye (*e.g.* propidium iodide, PI) and a permeable dye (*e.g.* acridine orange, AO) [42] in a fluorescence microscopy based assay allows quantitative information concerning the different modes of cell death. In this AO/PI assay the cells were treated with maslinic acid derivatives possessing a three carbon chain in the ester or amide moiety, and a green fluorescence (Fig. 3) was observed; hence a controlled cell death had been induced. A hallmark of apoptosis is the activation of caspase-activated DNAse (CAD) by caspase 3 [43]. As a result, DNA is cut into several smaller pieces of 178 bps length. Using gel

Table 1
Cytotoxicity (IC_{50} values in microM, from SRB assays after 96 h of treatment; the values are averaged from at least 3 independent experiments; confidence interval = 95%, individual positive (upper value) and negative (lower value) errors are given in the supplementary part; n.D. not detected) for maslinic acid derivatives **1**–**23**, **30**–**34** and betulinic acid (**BA**, as a standard) using a panel of various cancer cell lines (518A2, 8505C, A2780, A549, HT29 and MCF7) and non-malignant mouse fibroblasts NIH 3T3.

	518A2	8505c	A2780	A549	HT29	MCF7	NIH 3T3
BA	11.9	6.7	11.0	14.9	n.d.	14.8	10.0
1	13.7	17	19.5	23.4	28.8	24.4	16.6
2	15.6	14.7	17.3	17.8	12.8	16.3	21.4
3	11.1	12.2	12.6	13.7	12	8.8	12.1
4	32.5	>30	13.4	32.5	24.1	24	>30
5	16.9	18.9	10.2	19	14.9	17.8	14
6	13.9	13.8	14.1	13.2	12.8	13.5	13.4
7	14.2	12.4	n.d	14.2	14.7	15.1	15.1
8	20.4	14.9	7.8	17.3	12.6	14.3	18.1
9	31.6	37.3	18.1	30.3	30.9	36	32.6
10	12.4	11.6	12.1	14	11.4	13.8	17.3
11	19.4	22.2	n.d.	20.6	16.4	21.1	21.6
12	12.8	13.5	11.7	13.8	14.3	13.4	12.3
13	12.2	13.7	17.7	17.5	11.5	13.1	16
14	13.3	7.1	8	12.8	10.1	12.7	14.1
15	22.7	26.3	10.1	18.6	9.8	19.7	26.1
16	8.4	8.5	6.5	12.2	8.9	8.6	12.9
17	13.1	13.2	5.9	12.7	n.D	12.8	12.9
18	27.9	29.1	22.5	27.4	20.5	23.4	33.1
19	12.4	9.2	8.5	9.9	8.5	7.7	12.6
20	12.4	7.2	13	14.4	13.3	13	14.1
21	111	141.3	80.3	135.6	107	67.7	>120
22	38.5	>90	93.8	104.1	75.7	123.4	56.9
23	8	5.9	3.6	10.5	9.3	8.2	11.9
30	41.2	61.3	29.7	>60	43.7	81.1	47.8
31	42.1	45.2	27.4	37.3	37.7	29.1	14.2
32	29.1	28.1	18.3	23.4	25.2	13.9	17.6
33	39.3	42.5	25.8	37	37.6	28.2	24.8
34	24.5	27.7	21.9	29.8	29.5	24.6	26.1

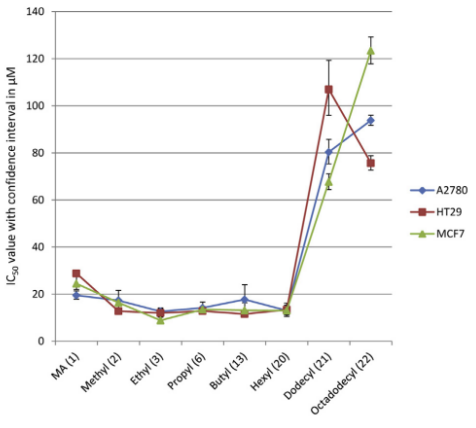


Fig. 1. Comparison of IC_{50} values (from SRB assays after 96 h of incubation with several human tumor cell lines, confidence interval = 95%) for compounds **1**–**3**, **6**, **13**, **20**–**22**.

electrophoresis and staining, the typical "DNA-ladders" are found. The results from these DNA-laddering experiments are depicted in Fig. 3, too. These assays, however, do not allow quantifications. Hence, an annexin-V/PI assay was performed [44]. In these experiments, the whole cell population was treated with fluorescence labeled phosphatidyl-serine specific binding protein annexin-V and PI. Quantitative FACS analysis allowed to distinguish vital from necrotic or apoptotic or secondary necrotic cells. As a result, most of the dead cells showed secondary necrosis after having been incubated for 6 h. Hence, the outer leaf of the membrane carries the phosphatidyl-serine (as expected for an apoptosis), and the integrity of the membrane is lost (as typical for late apoptotic events). These results suggest that either apoptosis is induced slowly or the integrity of the cell membrane might be altered upon incubation of the cells with the compounds.

To investigate whether the apoptotic process is caspase-dependent an extra colorimetric assay [42] was applied. The cell lysate collected from treated as well as untreated cell populations was reacted with *p*-nitroaniline-containing tetra-peptides. The changes in the optical density (Fig. 4) were measured after 12 h of incubation. In this experiment, a high concentration of an aspartate specific cutting caspase correlates with a high optical density. For **1** a low optical density was found after an incubation period of 12 h. For compounds **1** and **8** caspases 3, 8 and 9 were shown to be activated.

Triterpenoic acids are known to intercalate into isolated membranes [35,36,38]. These intercalation processes take place either in the upper or deeper fatty acid regions of the cell membrane depending on the log *P* value of the intercalants. Hence, the entering of PI into cells might occur *via* gaps in the membrane being formed by an intercalation between compounds and the membrane.

4. Summary

Starting from industrial waste a series of different esters and amides of maslinic acid was synthesized. Biological screening of these compounds showed for esters containing small-chain, lipophilic residues an increase in cytotoxicity whereas for amides as well as for long-chain esters a decrease was observed. Results from a dye exclusion assay, from DNA laddering experiments, from a caspase assay as well from annexin-V binding studies indicate that the cytotoxicity seems to be independent from the substitution pattern at position C-28 for esters and amides; modifications at this position determine their mode of action. Of special interest is ethyl ester **3** due to its pronounced cytotoxicity for HT29 cells whereas compounds **8** and **14** exhibited good cytotoxicity against the human ovarian cancer cell line A2780.

5. Experimental

5.1. General

Reagents were bought from commercial suppliers without any further purification. Melting points were measured with a LEICA hot stage microscope and were not corrected, NMR spectra were recorded using the Varian spectrometers Gemini 2000 or Unity 500 (δ given in ppm, *J* in Hz, internal Me_4Si), IR spectra (film or KBr pellet) on a Perkin–Elmer FT-IR spectrometer Spectrum 1000. MS spectra were taken on a Finnigan MAT TSQ 7000 (electrospray, voltage 4.5 kV, sheath gas nitrogen) instrument. Optical rotations were taken on a Perkin–Elmer 341 polarimeter (1 cm micro cell, 20 °C) and UV–vis spectra on a Perkin–Elmer unit, Lambda 14. Elemental analyses were measured on a Foss–Heraeus Vario EL unit and correct elemental analyses were obtained for all compounds (C, H, and N where appropriate, max. deviation from theoretical value < 0.3%). TLC was performed on silica gel (Merck 5554, detection by UV absorption). The solvents were dried according to usual procedures. The purity of the compounds was >95% as checked by HPLC. Atoms were numbered according to usual IUPAC numbering for triterpenoids. The assignments of the NMR signals attribution was realized using a combination of several 2D NMR techniques (H, H-COSY, H, H-NOESY, gHMBC and gHSQC). Carbon shifts for all compounds are summarized in Tables 2–4.

5.2. Biological assays

5.2.1. SRB-assay

SRB-Assays were prepared according to ref [33]. The cell lines 518A2, 8505C, A549, A2780, HT29, MCF7 and NIH 3T3 were amplified with a starting cell number of 2000, 2500, 1500, 1000, 2000, 2000 and 2500, respectively and counted with an automatic cell counter (Invitrogene™ countess®). IC_{50} values were calculated from semi logarithmic dose response curves by non-linear regression applying a two parametrical Hill-slope equation [45]. Values are given with a confidence interval CI = 95% (cf. Supplementary material).

5.2.2. Acridine orange/propidium iodide dye exclusion assay

Approximately 500,000 cells (HT29) were seeded in cell culture flasks and were allowed to grow for 1 day. After removing of the medium, the substance loaded medium was added, and the flasks were incubated for about 24–48 h. The supernatant medium was collected and centrifuged; the cell pellet was suspended in PBS and centrifuged again. Equal amounts of a PI/AO solution (1:1, 100 µg/ml) and a suspension of the cell pellet in PBS (w/o Ca^{2+} and Mg^{2+}) were softly mixed. Visual analysis was performed under a fluorescence microscope (Axioskop II (Carl Zeiss, Jena). While green fluorescence shows apoptosis, a deep colored nucleus indicates necrotic cells; weak orange dots mark lysosomes [39,40].

5.2.3. Cell cycle investigations

Approximately 1,000,000 cells (HT29) were seeded in cell culture flasks and were allowed to grow for 1 day. After removing of the medium, the substance loaded medium was added, and the flasks were incubated for about 24–48 h. The living cells were collected by centrifugation (1200 rpm, 5 min) and washed twice (PBS, w/o). After careful fixation with ice cold ethanol (70%, min. 2

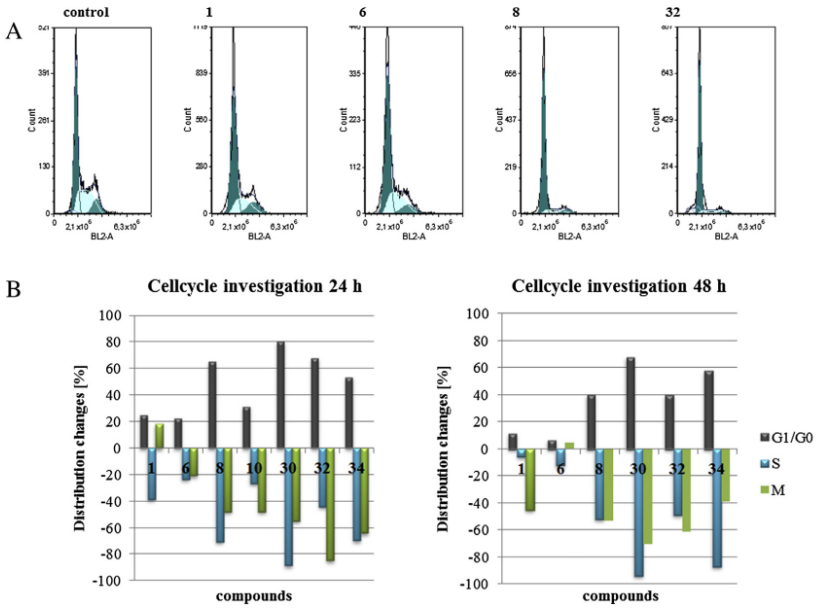


Fig. 2. Upper part: Cell cycle distribution after 24 h treatment of HT29 cells with maslinic acid **1** or derivatives **6**, **8** and **32**. Lower part: Relative changes in the cell cycle of HT29 colon cancer cells after treatment for 24 h and 48 h respectively with MA (**1**, 30 μM), propyl maslinoate (**6**, 15 μM), propargyl (**8**, 30 μM), 3-chloropropyl (**10**, 15 μM), maslinic amide (**30**, 60 μM), propyl amide (**32**, 60 μM) and propargyl amide (**34**, 60 μM).

h), the cells were centrifuged (1200 rpm, 5 min) again, washed twice with staining buffer (PBS (w/w), fetal bovine, sodium azide) and treated with RNAse (100 μL, 100 μg/mL, 30 min) at 37 °C. Then, at room temperature PI (1 mg/ml, 30 min) was added in the dark. Analysis was performed with using an AB™ Attune FACS machine and calculated with FCS express using the method described by Dean [46].

5.2.4. Annexin V/PI assay

Approximately 1,000,000 cells (HT29) were seeded in cell culture flasks and were allowed to grow for 1 day. After removing of the medium, the substance loaded medium was added, and the flasks were incubated for about 6 h. All cells were harvested, centrifuged (1200 rpm, 5 min) and washed twice (PBS, w/w). Approximately 100,000 cells were washed with annexin V bounding buffer (BD) and treated in the dark with propidium iodide (5 μL, life technologies™) for 15 min at room temperature. After adding of the annexin V bounding buffer (400 μL), the suspension was subjected to a FACS measurement. Calculation of the data was performed as described in the suppliers (BD Biosciences®) manual.

5.2.5. General procedure for the synthesis of the esters (method A)

Compound **1** (1 equiv) was dissolved in dry DMF (5 mL), and finely grounded potassium carbonate (5 equiv) was added. After stirring for 1 h at room temperature, the alkyl bromide (2 equiv) was added, and stirring was continued for another 18 h. The mixture was poured into an ice cold solution of aq. hydrochloric acid (5%, 50 mL), and the white precipitate was filtered off. Its chromatographic purification (silica gel, *n*-hexane/ethyl acetate, 7:3) and recrystallization (ethanol) yielded the product in an analytically pure form.

5.2.6. General procedure for the synthesis of the diacetylated amides (method B)

To a solution of **24** (1 equiv) in dry DCM/THF (50 mL, 1:1, v/v), thionyl chloride (1.1 equiv) and triethylamine (1.3 equiv) were added at 0 °C, stirring at 0 °C was continued for 20 min. After stirring at 25 °C for several hours (until TLC showed completion of the reaction), the solvents were removed under reduced pressure, the residue was dissolved in DCM, and the corresponding amine (1.3 equiv) and triethylamine (1.3 equiv) were added. After completion of the reaction (as checked by TLC), the mixture was poured into ice water, the precipitate was filtered off, washed with cold water and purified by chromatography (silica gel, *n*-hexane/ethyl acetate).

5.2.7. General procedure for deacylation (method C)

The diacetylated amides were dissolved in methanol, and KOH (1.2 equiv) was added; the mixture was stirred at room temperature until TLC showed completion of the reaction. Usual workup followed by chromatography (silica gel, *n*-hexane/ethyl acetate mixtures) yielded the amides.

*5.2.7.1. Maslinic acid (**1**).* Maslinic acid was isolated from olive pomace as reported in Refs. [47]; after recrystallization from ethyl acetate **1** was obtained as a colorless solid; m.p. 263–267 °C, (lit.: 266–269 °C [9]); R_F = 0.22 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D = +55°$ (*c* 0.42, $CHCl_3$) (lit.: $[\alpha]_D = +60°$ (*c* 0.1, $CHCl_3$) [48]). MS (ESI, MeOH): *m*/*z* = 471.5 (43%, $[M - H]^-$), 517.0 (100%, $[M + HCO_2]^-$), 943.1 (62%, $[2M - H]^-$).

*5.2.7.2. Methyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**2**).* Obtained from **1** (100 mg, 0.21 mmol) by method **A** as fine colorless needles; yield: 88%; m.p. 229–231 °C (lit.: 214–216 °C [49]); $R_F = 0.4$ (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D = 60.9°$ (*c* 6.5, $CHCl_3$); IR

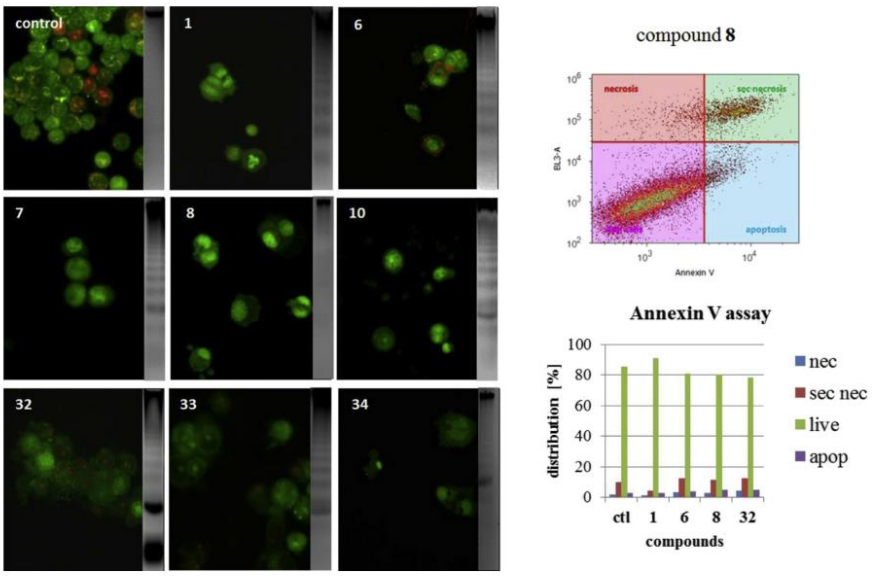


Fig. 3. Left part: Dye exclusion assay and DNA-ladder (HT29, colon cancer cell line): Cells were treated with **1** (30 μM), **6** (15 μM), **7** (30 μM), **8** (30 μM), **10** (15 μM), **32** (60 μM), **33** (60 μM), **34** (60 μM). Right part: Annexin V assay (HT29, colon cancer cells): Cells were treated for 6 h with **1** (30 μM), **6** (15 μM), **8** (30 μM) and **32** (60 μM).

(KBr) ν = 3571, 3300, 2947, 1739, 1461, 1386, 1363, 1262, 1229, 1190, 1162, 1124, 1052, 1037, 984, 958, 921 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.28 (dd, J = 3.4, 3.4 Hz, 1H, H-12), 3.72–3.65 (m, 1H, H-2), 3.61 (s, 3H, CH_3-31), 3.00 (d, J = 9.4 Hz, 1H, H-3), 2.85 (dd, J = 13.8, 4.1 Hz, 1H, H-18), 2.21 (br, 2H, O*H*), 1.97 (dd, J = 10.4, 3.9 Hz, 1H, H_a-1), 1.96–1.83 (m, 3H, H_a-16 + $H_{a,b}$-11), 1.68 (ddd, J = 13.9, 13.9, 4.4 Hz, 1H, H_a-7), 1.64–1.56 (m, 4H, H-9 + H_a-19 + H_a-15 + H_b-16), 1.55–1.52 (m, 1H, H_a-6), 1.50 (ddd, J = 14.0, 3.4, 3.4 Hz, H_a-22), 1.43 (m, 1H, H_b-7), 1.38 (ddd, J = 12.4, 12.4, 2.5 Hz, H_b-6), 1.29 (ddd, J = 13.8, 9.6, 2.7 Hz, 1H, H_a-21), 1.29–1.27 (m, 1H, H_b-22), 1.16 (ddd, J = 14.0, 4.2, 4.2 Hz, 1H, H_b-21), 1.15–1.11 (m, 1H, H_b-19), 1.12 (s, 3H, CH_3–C27), 1.06–1.02 (m, 1H, H_b-15), 1.02 (s, 3H, CH_3-23), 0.97 (s, 3H, CH_3-25), 0.92 (s, 3H, CH_3-30), 0.92–0.87 (m, 1H, H_b-1), 0.89 (s, 3H, CH_3-29), 0.84–0.82 (m, 1H, H-5), 0.82 (s, 3H, CH_3-24), 0.71 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 487.4 (48.8%, $[M + H]^+$), 504.5 (53.4%, $[M + NH_4]^+$), 509.5 (100%, $[M + Na]^+$), 541.2 (22.0%, $[M + Na + MeOH]^+$), 741.5 (14.6%, $[3M + Na + H]^{2+}$), 749.6 (73.2%, $[3M + K + H]^{2+}$), 992.8 (48.8%, $[4M + K + H]^{2+}$).

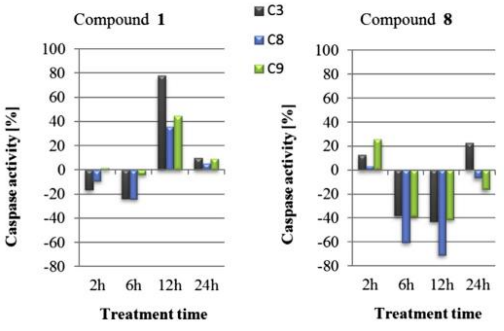


Fig. 4. Relative activity of caspases 3, 8 and 9 after treatment of HT29 colon-cancer cells with 30 μM of **MA**(**1**) or propargyl maslinoate (**8**) for 6, 12 and 24 h, respectively.

*5.2.7.3. Ethyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**3**).* Obtained from **1** by method **A** as a colorless solid; yield: 86%; m.p. 223–224 °C; R_F = 0.4 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +201.5° (*c* 6.2, $CHCl_3$); IR (KBr) ν = 3609, 3363, 2948, 1723, 1463, 1387, 1365, 1320, 1302, 1258, 1180, 1161, 1126, 1098, 1081, 1050, 1037 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.26 (dd, J = 3.4, 3.4 Hz, 1H, H-12), 4.06 (m, 2H, CH_2-31), 3.67 (m, 1H, H-2), 2.98 (d, J = 9.3 Hz, 1H, H-3), 2.84 (dd, J = 13.8, 4.1 Hz, 1H, H-18), 2.10–2.00 (br, 2H, O*H*), 1.95 (dd, 1H, J = 14.8, 3.2 Hz, H_a-1), 1.95–1.82 (m, 3H, H_a-16 + $H_{a,b}$-11), 1.68 (ddd, J = 13.7, 13.7, 4.3 Hz, 1H, H_a-7), 1.64–1.54 (m, 4H, H-9 + H_a-19 + H_a-15 + H_b-16), 1.54–1.47 (m, 1H, H_a-6), 1.50–1.43 (m, 1H, H_b-7), 1.44–1.40 (m, 1H, H_a-22), 1.38–1.33 (m, 1H, H_b-6), 1.32–1.24 (m, 2H, H_a-21 + H_b-22), 1.24–1.15 (m, 1H, H_b-21), 1.20 (t, J = 7.1 Hz, 3H, CH_3-32), 1.16–1.10 (m, 1H, H_b-19), 1.11 (s, 3H, CH_3-27), 1.06–1.00 (m, 1H, H_b-15), 1.01 (s, 3H, CH_3-23), 0.96 (s, 3H, CH_3-25), 0.90 (s, 3H, CH_3-30), 0.92–0.86 (m, 1H, H_b-1), 0.88 (s, 3H, CH_3-29), 0.84–0.82 (m, 1H, H-5), 0.80 (s, 3H, CH_3-24), 0.72 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 523.6 (100%, $[M + Na]^+$), 555.1 (19.5%, $[M + Na + MeOH]^+$), 770.5 (75%, $[3M + K + H]^{2+}$), 1020.8 (38.7%, $[4M + K + H]^{2+}$), 1024.2 (36.4%, $[4M+2Na + H]^{2+}$)).

*5.2.7.4. 2-Hydroxyethyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**4**).* Obtained from **1** by method **A** as a colorless solid; yield: 84%; m.p. 224–226 °C; R_F = 0.08 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = 57.1° (*c* 3.5, $CHCl_3$); IR (KBr) ν = 3422, 2948, 2866, 1706, 1048, 1458, 1034, 1176, 1718, 754,1262, 1162, 1388, 1080, 1364 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.30 (dd, J = 3.4, 3.4 Hz, 1H, H-12), 4.19 (ddd, J = 11.7, 5.8, 3.8 Hz, 1H, H_a-31), 4.13 (ddd, J = 11.8, 5.4, 3.6 Hz, 1H, CH_b-31), 3.79 (dd, J = 5.3, 3.8 Hz, 2H, CH_2-32), 3.68 (ddd, J = 10.7, 10.7, 3.6 Hz, 1H, H-2), 2.99 (d, J = 9.3 Hz, 1H, H-3), 2.87 (dd,

Table 2
^{13}C NMR shifts (100 MHz, $CDCl_3$) for compounds **1**–**12**.

	1	2	3	4	5	6	7	8	9	10	11	12
C-1	46.6	46.3	46.3	46.5	47.0	46.5	46.6	46.3	48.4	46.5	46.5	46.5
C-2	68.6	68.9	68.9	69.1	69.1	69.1	69.1	68.9	69.5	69.1	69.0	69.1
C-3	83.8	84.0	84.0	84.0	84.2	84.1	84.1	84.0	84.5	84.1	84.0	84.1
C-4	39.8	39.1	39.1	39.3	39.3	39.3	39.3	39.1	40.5	39.3	39.3	39.3
C-5	55.9	55.3	55.3	55.4	55.5	55.4	55.4	55.3	55.7	55.4	55.4	55.4
C-6	18.8	18.3	18.3	18.5	18.5	18.5	18.5	18.3	19.5	18.5	18.5	18.5
C-7	33.2	32.5	32.3	32.7	32.6	32.6	32.4	32.4	33.5	32.6	32.6	32.6
C-8	39.8	39.3	39.3	39.5	39.6	39.6	39.6	39.4	40.7	39.6	39.5	39.6
C-9	48.1	47.6	47.6	47.7	47.8	47.7	47.8	47.6	48.6	47.7	47.7	47.8
C-10	38.5	38.3	38.3	38.4	38.5	38.4	38.5	38.3	39.3	38.5	38.4	38.5
C-11	23.9	23.0	23.0	23.8	23.8	23.1	23.8	23.0	24.6	23.8	23.6	23.7
C-12	122.4	122.1	122.1	122.3	122.6	122.3	122.6	122.2	123.7	122.5	122.4	122.4
C-13	144.8	143.8	143.8	144.3	143.7	144.0	143.6	143.8	145.1	144.0	143.9	143.9
C-14	42.4	41.6	41.6	42.0	42.0	41.9	41.9	41.7	42.9	41.5	41.5	41.5
C-15	28.3	27.6	27.6	27.7	27.8	27.7	27.8	27.6	28.7	27.8	27.7	27.8
C-16	23.7	23.4	23.4	23.2	26.7	23.6	23.1	23.4	24.0	23.2	23.1	23.2
C-17	47.7	46.7	46.7	47.1	47.0	46.8	46.9	46.7	47.1	47.0	46.9	47.0
C-18	42.0	41.2	41.2	41.6	41.4	41.4	41.4	41.3	42.8	41.9	41.9	41.9
C-19	46.4	45.8	45.8	45.9	46.0	46.0	46.0	45.8	48.1	45.9	45.9	45.9
C-20	30.9	3.7	30.7	30.8	30.9	30.9	30.8	30.7	31.6	30.9	30.8	30.9
C-21	34.2	33.8	33.8	34.0	33.2	34.1	34.0	33.9	33.7	34.0	34.0	34.0
C-22	33.2	32.3	32.5	32.6	32.6	32.8	32.8	32.6	34.8	31.9	32.8	32.8
C-23	29.3	28.6	28.6	28.8	28.8	28.8	28.8	28.6	29.3	28.8	28.4	28.4
C-24	17.6	16.7	16.7	17.2	17.2	16.9	17.3	16.7	17.8	17.3	16.9	16.9
C-25	17.4	16.5	16.6	16.8	16.8	16.8	16.9	16.6	17.1	16.8	16.7	16.8
C-26	16.8	16.9	16.9	16.9	16.9	17.2	16.8	17.0	17.4	16.9	17.2	17.4
C-27	26.2	25.9	25.9	26.0	26.0	26.0	26.0	25.9	26.4	26.1	26.0	26.0
C-28	180.2	178.2	178.2	178.3	177.6	177.9	176.8	177.3	179.5	177.6	177.7	177.7
C-29	33.3	33.1	33.1	33.2	33.3	33.2	33.1	33.2	33.5	33.2	33.2	33.1
C-30	23.8	23.6	23.6	23.6	23.8	23.6	23.6	23.7	23.9	23.7	23.8	23.6
C-31		51.5	51.5	66.2	64.0	66.0	51.8	64.8	62.6	62.0	61.0	63.8
C-32				61.8	41.7	22.2	78.3	132.5	32.7	31.9	31.8	32.6
C-33						10.8	74.5	177.7	59.6	29.7	41.4	1.9

J = 13.6, 3.9 Hz, 1H, H-18), 2.16 (brs, 3H, O*H*), 1.97 (ddd, J = 13.9, 13.9, 4.3 Hz, 1H, H_a-16), 1.96–1.93 (m, 1H, H_a-1), 1.93–1.87 (m, 2H, $H_{a,b}$-11), 1.72 (ddd, J = 13.8, 13.8, 4.4 Hz, 1H, H_a-7), 1.65–1.59 (m, 4H, H-9 + H_a-15 + H_a-19 + H_b-16), 1.53 (m, 2H, H_a-6 + H_b-7), 1.44 (ddd, J = 10.1, 10.1, 2.7 Hz, 1H, H_a-22), 1.39–1.27 (m, 3H, H_b-6 + CH_2-21), 1.22–1.14 (m, 2H, H_b-22 + H_b-19), 1.14 (s, 3H, CH_3-27), 1.10–1.05 (m, 1H, H_b-15), 1.02 (s, 3H, CH_3-23), 0.97 (s, 3H, CH_3-25), 0.92 (s, 3H, CH_{3-30}), 0.90 (s, 3H, CH_3-29), 0.85–0.81 (m, 1H, H-5), 0.81 (s, 3H, CH_3-24), 0.74 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 539.6 (100%, $[M + Na]^+$), 1055.2 (36%, $[2M + Na]^+$).

*5.2.7.5. 2-Chloroethyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**5**).* Obtained from **1** by method **A** as a colorless solid; yield: 56%; m.p. 169–170 °C; R_F = 0.37 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +34° (*c* 1.3, $CHCl_3$); IR (KBr): ν = 3430, 2944, 2926, 2852, 1730, 1634, 1462, 1434, 1384, 1366, 1302, 1262, 1238, 1196, 1174, 1160, 1124, 1108, 1094, 1082, 1050, 1034 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.32 (dd, J = 3.4, 3.4 Hz, 1H, H-12), 4.31 (ddd, J = 11.6, 5.7, 5.7 Hz, 1H, H_a-31), 4.24 (ddd, J = 11.6, 5.6, 5.6 Hz, 1H, CH_b-31), 3.68 (ddd, J = 10.2, 9.4, 4.7 Hz, 1H, H-2), 3.66 (dd, J = 5.7, 5.7 Hz, 2H, CH_2-32), 3.01 (d, J = 9.4 Hz, 1H, H-3), 2.88 (dd, J = 14.1, 4.4 Hz, 1H, H-18), 2.03–1.88 (m, 4H, H_a-1 + H_a-16 + $H_{a,b}$-11), 1.74 (dd, J = 13.7, 13.7, 4.2 Hz, 1H, H_a-7), 1.68–1.50 (m, 6H, H-9 + H_a-15 + H_a-19 + H_b-16 + H_a-6 + H_b-7), 1.49–1.44 (m, 1H, H_a-22), 1.43–1.20 (m, 5H, CH_2 (21) + H_b-6 + H_b-22 + H_b-19), 1.23–1.15 (m, 1H, H_b-15), 1.14 (s, 3H, CH_3-27), 1.07 (m, 1H, H_b-1), 1.03 (s, 3H, CH_3-23), 0.98 (s, 3H, CH_3-25), 0.93 (s, 3H, CH_3-30), 0.91 (s, 3H, CH_3-29, 0.84 (m, 1H, H-5), 0.83 (s, 3H, CH_3-24), 0.74 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 557.3 (100%, $[M + Na]^+$), 1091.3 (90%, $[2M + Na]^+$).

*5.2.7.6. Propyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**6**).* Obtained from **1** by method **A** as a colorless solid; yield: 78%; m.p. 167–169 °C; R_F = 0.52 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +53.8° (*c* 3.0, $CHCl_3$); IR (KBr): ν = 3384, 2946, 1721, 1462, 1386, 1364, 1262, 1162, 1125, 1052, 1032, 958 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.28 (dd, J = 3.3, 3.3 Hz, 1H, H-12), 4.05–3.88 (m, 2H, CH_2-31), 3.68 (ddd, J = 11.1, 11.1, 4.4 Hz, 1H, H-2), 2.99 (d, J = 9.5 Hz, 1H, H-3), 2.88 (dd, J = 13.8, 3.9 Hz, 1H, H-18), 2.21 (brs, 2H, O*H*), 1.97–1.83 (m, 4H, CH_a-1 + H_a-16 + $H_{a,b}$-11), 1.71 (dd, J = 13.5, 13.5, 4.3 Hz, 1H, H_a-7), 1.71–1.55 (m, 6H, H-9 + H_a-19 + H_a-15 + H_b-16 + CH_2-32), 1.52–1.48 (m, 2H, H_a-6 + H_b-7), 1.42 (ddd, J = 12.2, 12.2, 2.7 Hz, 1H, H_a-22), 1.38–1.34 (m, 1H, H_b-6), 1.32–1.24 (m, 2H, H_a-21 + H_b-22), 1.17–1.13 (m, 2H, H_b-19 + H_b-21), 1.13 (s, 3H, CH_3-27), 1.10–1.01 (m, 1H, H_b-15), 1.02 (s, 3H, CH_3-23), 0.97 (s, 3H, CH_3-25), 0.95 (t, J = 7.4 Hz, CH_3 (33)), 0.92 (s, 3H, CH_3-30), 0.92–0.86 (m, 1H, H_b-1), 0.90 (s, 3H, CH_3-29), 0.86 (m, 1H, H-5), 0.82 (s, 3H, CH_3-24), 0.73 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 537.6 (100%, $[M + Na]^+$), 792.0 (61%, $[3M + K + H]^{2+}$), 1049.3 (49%, $[4M + K + H]^2$).

*5.2.7.7. 2-Propen-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**7**).* Obtained from **1** by method **A** as a colorless solid; yield: 90%; m.p. 203–205 °C; R_F = 0.49 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +55.4° (*c* 3.6, $CHCl_3$); IR (KBr): ν = 3406, 2947, 1727, 1648, 1464, 1386, 1363, 1261, 1199, 1178, 1159, 1123, 1051, 1034 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.87 (ddd, 1H, J = 22.7, 10.8, 5.6 Hz, C*H*-32), 5.32–5.25 (m, 1H, CH_a (33)), 5.27 (m, 1H, H-12), 5.18 (dd, J = 10.4, 1.2 Hz, 1H, CH_b (33)), 4.55–4.44 (m, 2H, CH_2-31), 3.65 (ddd, J = 11.1, 9.7, 4.5 Hz, 1H, H-2), 2.98 (d, J = 9.5 Hz, 1H, H-3), 2.86 (dd, J = 13.8, 4.1 Hz, 1H, H-18), 2.03 (br, 2H, O*H*), 2.00–1.84 (m, 4H, $H_{a,b}$-11 + H_a-16 + H_a-1), 1.69 (ddd, J = 13.7, 13.7, 4.3 Hz, 1H, H_a-7), 1.66–1.46 (m, 4H, H_b-16 + H_a-15 + H_a-19 + H-9), 1.55–1.49 (m, 2H, H_b-7 + H_a-6), 1.44 (ddd, J = 15.4, 15.4, 3.8 Hz, 1H, H_a-22), 1.37 (ddd, J = 12.6, 12.6, 2.1 Hz, 1H, H_b-6), 1.33 (m, 1H, H_a-21), 1.28 (m, 1H, H_b-

Table 3
^{13}C NMR shifts (100 MHz, $CDCl_3$) for compounds **13**-**22**.

	13	14	15	16	17	18	19	20	21	22
C-1	46.4	46.4	46.5	45.9	45.8	46.5	46.6	46.5	46.5	46.6
C-2	68.9	68.9	69.1	69.0	68.9	69.1	69.0	69.1	69.1	69.1
C-3	84.0	83.9	84.1	84.0	83.9	84.1	84.1	84.1	84.1	84.1
C-4	39.2	39.2	39.3	39.4	39.2	39.3	39.3	39.3	39.3	39.3
C-5	55.3	55.3	55.4	55.3	55.3	55.4	55.4	55.4	55.4	55.4
C-6	18.3	18.3	18.5	18.4	18.3	18.5	18.5	18.5	18.5	18.5
C-7	32.7	32.4	32.6	32.5	32.3	32.5	32.8	32.6	32.6	32.6
C-8	39.4	39.4	39.6	39.2	41.2	39.6	39.6	39.6	39.6	39.6
C-9	47.5	47.6	47.8	47.6	47.6	47.7	47.7	47.8	47.8	47.7
C-10	38.2	38.3	38.5	38.3	38.3	38.4	38.4	38.4	38.5	38.5
C-11	23.5	22.9	23.7	23.0	23.0	23.6	23.6	23.1	23.6	23.6
C-12	122.3	122.2	122.4	122.3	122.3	122.4	122.6	122.3	122.3	122.3
C-13	144.0	143.8	143.8	143.9	143.8	143.8	143.6	144.0	144.1	144.0
C-14	41.7	41.7	41.9	41.8	41.7	41.9	41.9	41.9	41.9	41.9
C-15	27.6	27.6	27.8	27.6	27.6	27.8	27.8	27.7	27.8	27.8
C-16	23.0	23.4	23.1	23.5	23.5	23.2	23.2	23.6	23.1	23.1
C-17	46.6	46.6	46.9	46.7	46.7	46.9	46.9	46.8	46.8	46.8
C-18	41.3	41.2	41.4	41.3	39.4	41.4	41.4	41.4	41.0	41.4
C-19	45.8	45.8	46.0	46.4	46.4	46.0	46.0	46.0	46.0	46.0
C-20	30.7	30.7	30.9	30.7	30.9	30.8	30.8	30.9	30.9	30.9
C-21	33.9	33.9	34.0	33.9	33.8	34.0	34.0	34.1	34.1	34.1
C-22	32.6	32.6	32.8	32.6	32.6	32.8	32.4	32.8	32.8	32.8
C-23	28.8	28.1	28.3	28.3	28.3	28.8	28.8	28.8	28.8	29.4
C-24	16.8	16.7	17.2	17.1	17.1	17.3	17.2	16.9	17.2	17.2
C-25	16.6	16.6	16.8	16.6	16.6	16.8	16.8	16.7	16.8	16.7
C-26	17.0	17.0	16.9	16.7	16.7	16.9	16.9	17.2	16.9	16.9
C-27	25.9	25.9	26.0	25.9	25.9	26.0	26.0	26.0	26.0	26.0
C-28	177.9	177.6	177.6	177.7	177.7	177.5	177.0	177.9	177.9	177.9
C-29	33.1	33.1	33.2	33.2	33.3	33.2	33.2	33.3	32.1	33.3
C-30	23.6	23.6	23.6	23.6	23.6	23.8	23.8	23.8	29.4	23.8
C-31	64.0	63.3	62.1	63.3	63.2	59.2	51.8	64.5	64.4	66.0
C-32	30.8	33.1	19.1	26.1	27.3	128.4	80.9	28.8	28.8	29.4
C-33	19.2	134.3	69.8	29.4	29.5	129.9	81.3	25.9	26.2	26.2
C-34	13.7	117.0	28.8	44.7	33.0	39.0	30.6	31.6	29.5	
C-36								14.0		
Chain									29.8	29.8
C-42									14.3	
C-35 + C-45										29.5
C-46										32.1
C-48										14.3

22), 1.22–1.07 (m, 2H, H_b-21 + H_b-19), 1.13 (s, 3H, CH_3-27), 1.05 (m, 1H, H_b-15), 1.02 (s, 3H, CH_3-23), 0.97 (s, 3H, CH_3-25), 0.92 (s, 3H, CH_3-30), 0.90 (s, 3H, CH_3-29), 0.91–0.80 (m, 1H, H_b-1), 0.81 (s, 3H, CH_3-24), 0.81–0.79 (m, 1H, H-5), 0.72 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 495.5 (7.3%, $[M + H]^+$), 530.4 (6.1%, $[M + NH_4]^+$), 535.5 (100%, $[M + Na]^+$), 788.5 (36.6%, $[3M + K + H]^{2+}$), 1047.2 (48.8%, $[2M + Na]^+$).

*5.2.7.8. 2-Propyn-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**8**).* Obtained from **1** by method **A** as a colorless solid; yield: 78%; m.p. 225–227 °C (lit.: 233–234 °C [50]); R_F = 0.23 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +58.6° (*c* 3.0, $CHCl_3$); IR (KBr): ν = 3568, 3406, 3310, 3296, 2946, 2862, 1736, 1466, 1442, 1386, 1364, 1260, 1228, 1196, 1176, 1156, 1118, 1066, 1052, 1034 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.31 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 4.68 (dd, J = 15.6, 2.4 Hz, 1H, CH_a-31), 4.57 (dd, J = 15.6, 2.5 Hz, 1H, CH_b-31), 3.69 (ddd, J = 11.2, 9.6, 4.5 Hz, 1H, H-2), 3.00 (d, J = 9.5 Hz, 1H, H-3), 2.87 (dd, J = 13.7, 3.9 Hz, 1H, H-18), 2.41 (dd, J = 2.5, 2.5 Hz, 1H, *CH* (33)), 2.10 (brs, 2H, O*H*), 2.02 (ddd, J = 12.6, 12.6, 3.5 Hz, 1H, H_a-16), 1.95 (m, 1H, H_a-1), 1.94–1.87 (*m*, 2H, $H_{a,b}$-11), 1.70 (ddd, J = 13.7, 13.7, 4.3 Hz, 1H, H_a-7), 1.68–1.49 (m, 6H, H_b-16 + H_b-19 + H_a-15 + H-9 + H_b-7 + H_a-6), 1.49–1.39 (m, 1H, H_a-22), 1.39–1.32 (m, 2H, H_b-6 + H_a-21), 1.29 (m, 1H, H_b-22), 1.24–1.17 (m, 2H, H_b-21 + H_b-19), 1.14 (s, 3H, CH_3-27), 1.07 (ddd, J = 9.5, 9.5, 5.3 Hz, 1H, H_b-1), 1.03 (s, 3H, CH_3-23), 0.98 (s, 3H, CH_3-25), 0.93 (s, 3H, CH_3-30), 0.90 (s, 3H, CH_3-29), 0.88–0.78 (m, 1H, H-5), 0.82 (s, 3H, CH_3-24), 0.76 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 533.6 (63%, $[M + Na]^+$), 785.4 (46%, $[3M + K + H]^{2+}$), 1040.8 (100%, $[4M + K + H]^+$).

*5.2.7.9. 3-Hydroxy-prop-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**9**).* Obtained from **1** by method **A** as a colorless solid; yield: 80%; m.p. 119–121 °C; R_F = 0.17 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +51.7° (*c* 1.4, $CHCl_3$); IR (KBr): ν = 3446, 2948, 2878, 2864, 1724, 1706, 1646, 1636, 1458, 1384, 1364, 1262, 1178, 1162, 1050 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OH): δ = 5.33 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 4.19 (ddd, J = 12.7, 6.3, 6.3 Hz, 1H, CH_a-31), 4.15 (ddd, J = 12.7, 6.3, 6.3 Hz, CH_b-31), 3.68 (ddd, J = 6.5, 1.4 Hz, 2H, CH_2 (33)), 3.72–3.64 (m, 1H, H-2), 2.96 (d, J = 9.6 Hz, 1H, H-3), 2.95 (dd, J = 13.8, 4.5 Hz, 1H, H-18), 2.10 (ddd, J = 12.8, 12.8, 3.5 Hz, 1H, H_a-16), 2.03–1.96 (m, 3H, CH_a-1 + $H_{a,b}$-11), 1.95–1.85 (m, 2H, CH_2-32), 1.82–1.67 (m, 2H, H_a-19 + H-7), 1.70–1.60 (m, 3H, H-9 + H_a-15 + H_b-16), 1.63–1.38 (m, 5H, CH_2-6 + H_b-7 + H_a-22 + H_a-21), 1.38–1.33 (m, 1H, H_b-22), 1.25–1.20 (m, 1H, H_b-21), 1.22 (s, 3H, CH_3-27), 1.21–1.08 (m, 2H, H_b-15 + H_b-19), 1.07 (s, 3H, CH_3-23), 1.06 (s, 3H, CH_3-25), 1.00 (s, 3H, CH_3-30), 0.97 (s, 3H, CH_3-29), 0.95–0.88 (m, 2H, H-5 + H_b-1), 0.87 (s, 3H, CH_3-24), 0.82 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 531.1 (8%, $[M + H]^+$), 553.3 (92%, $[M + Na]^+$), 815.9 (70%, $[3M + K + H]^{2+}$), 1083.3 (100%, $[2M + Na]^+$).

*5.2.7.10. 3-Chloro-prop-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**10**).* Obtained from **1** by method **A** as a colorless solid; yield

Table 4
^{13}C NMR shifts (100 MHz, $CDCl_3$) for compounds **23**-**34**.

	23	24	25	26	27	28	29	30	31	32	33	34
C-1	46.6	44.0	44.1	44.1	44.1	44.1	44.1	46.5	48.1	48.1	45.5	48.1
C-2	69.1	70.2	70.1	70.1	70.0	70.1	70.1	69.0	69.5	69.5	69.0	69.5
C-3	84.1	80.8	80.7	80.7	80.7	80.7	80.7	84.7	84.5	84.4	84.1	85.5
C-4	39.3	39.5	39.4	39.5	39.5	39.5	39.5	39.3	40.5	40.5	39.3	40.5
C-5	55.5	55.0	55.0	55.0	55.0	55.0	55.0	55.4	56.6	56.6	55.3	56.7
C-6	18.5	18.3	18.4	18.3	18.3	18.4	18.3	18.5	19.5	19.5	18.4	19.5
C-7	32.8	32.5	32.7	32.6	32.7	32.7	32.4	32.6	34.3	34.4	32.6	34.1
C-8	39.6	39.5	39.5	39.6	39.6	39.6	39.6	39.4	40.7	40.7	39.6	40.8
C-9	47.8	47.7	47.6	47.6	47.6	47.6	47.6	47.7	48.6	48.8	47.7	48.4
C-10	38.5	38.3	38.3	38.2	38.2	38.2	38.2	38.4	39.2	39.2	38.3	39.3
C-11	23.6	23.6	23.9	23.9	23.9	24.0	24.0	24.0	24.6	24.0	23.9	24.7
C-12	122.2	122.4	122.6	122.3	122.3	122.6	122.9	122.8	123.8	123.8	122.8	123.9
C-13	144.0	143.8	145.1	145.4	145.4	145.3	145.0	145.1	145.5	145.5	145.2	145.3
C-14	41.9	41.0	42.3	42.3	42.3	42.1	42.2	42.3	43.0	43.0	42.1	42.9
C-15	27.8	27.8	27.4	27.4	27.4	27.4	27.3	27.5	28.5	28.5	27.4	28.4
C-16	23.1	23.0	23.8	23.7	23.7	23.7	23.8	23.7	24.0	23.6	23.7	24.0
C-17	46.9	46.7	46.6	46.3	46.4	46.5	46.5	46.5	47.4	47.5	46.5	47.5
C-18	41.5	41.7	42.7	42.4	42.4	42.3	42.3	42.7	42.6	42.6	42.4	42.5
C-19	46.0	46.0	46.8	47.0	47.0	46.9	46.8	46.8	47.7	47.7	46.9	47.6
C-20	30.9	30.8	30.9	30.9	30.9	30.9	30.9	30.9	31.6	31.6	30.9	31.6
C-21	34.1	33.9	34.2	34.3	34.3	34.3	34.2	34.1	35.1	35.1	34.3	35.1
C-22	32.7	32.5	32.4	32.3	32.3	32.3	32.3	32.5	33.8	33.8	32.4	33.8
C-23	28.8	28.6	28.6	28.6	28.6	28.6	28.5	28.8	29.3	29.3	28.7	29.3
C-24	16.9	17.3	17.2	17.0	17.0	17.0	17.1	16.9	17.4	17.5	16.9	17.4
C-25	16.8	16.6	16.6	16.6	16.6	16.6	16.6	16.8	17.1	17.1	16.8	17.1
C-26	17.3	17.8	17.8	17.8	17.8	17.8	17.7	17.3	17.9	17.9	17.1	18.1
C-27	26.1	26.0	25.9	25.9	25.9	25.9	25.9	25.9	26.5	26.5	25.9	26.5
C-28	177.9	183.8	181.2	178.1	178.1	178.1	178.0	181.4	180.2	180.2	178.2	180.0
C-29	33.3	33.2	33.1	33.2	33.1	33.1	33.1	33.1	33.5	33.6	33.1	33.5
C-30	23.8	23.7	23.7	23.7	23.7	23.7	23.8	23.7	24.0	24.0	23.7	24.0
C-31	69.6			34.5	41.3	42.3	29.5		35.6	42.5	42.3	29.7
C-32	32.3			14.8	22.8	134.6	71.8		14.9	24.6	134.5	81.0
C-33	26.6				11.6	116.4	70.0			11.9	116.5	71.8
C-34	25.9											
C-35	30.0											
Ac		21.1	21.1	21.1	21.1	21.1	21.0					
Ac		21.3	21.3	21.3	21.3	21.3	21.3					
AcC = O		107.7	170.7	170.7	170.7	170.7	170.7					
AcC = O		171.0	171.0	171.0	171.0	171.0	171.0					

84%; m.p. 146–147 °C; R_F = 0.3 (*n*-hexane/ethyl acetate, 5:3); $[\alpha]_D$ = +41.2° (*c* 2.1, $CHCl_3$); IR (KBr): ν = 3440, 2948, 2864, 1726, 1654, 1648, 1636, 1628, 1618, 1458, 1438, 1386, 1260, 1176, 1162, 1050, 1034 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.27 (dd, *J* = 3.3, 3.3 Hz, 1H, H-12), 4.17–4.13 (m, 2H, CH_2-31), 3.67 (ddd, *J* = 11.1, 9.5, 4.4 Hz, 1H, H-2), 3.60 (t, *J* = 6.4 Hz, 2H, CH_2 (33)), 2.98 (d, *J* = 9.5 Hz, 1H, H-3), 2.85 (dd, *J* = 13.8, 4.0 Hz, 1H, H-18), 2.49 (brs, 2H, O*H*), 2.09–2.00 (m, 2H, CH_2-32), 1.98–1.87 (m, 4H, $H_{a,b}$-11 + CH_a-1 + H_a-16), 1.70 (ddd, *J* = 13.9, 13.9, 4.3 Hz, 1H, H_a-7), 1.66–1.56 (m, 4H, H_a-19 + H-9 + H_b-16 + H_a-15), 1.55–1.48 (m, 1H, H_a-6), 1.43–1.35 (m, 3H, H_b-6 + H_a-22 + H_b-7), 1.36–1.32 (m, 2H, H_a-21 + H_b-22), 1.22–1.16 (m, 2H, H_b-21 + H_b-19), 1.12 (s, 3H, CH_3-27), 1.08–1.02 (m, 1H, H_b-15), 1.01 (s, 3H, CH_3-23), 0.96 (s, 3H, CH_3-25), 0.91 (s, 3H, CH_3-30), 0.89 (s, 3H, CH_3-29), 0.91–0.84 (m, 2H, H_b-1 + H-5), 0.81 (s, 3H, CH_3-23), 0.72 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): *m*/*z* = 549.1 (11%, $[M + H]^+$), 571.4 (100%, $[M + Na]^+$), 844.6 (10%, $[3M + Na]^{2+}$), 1119.5 (67%, $[2M + Na]^+$).

*5.2.7.11. 3-Bromo-prop-1-yl (2α, 3β)-2,3-dihydroxy-olean-12-en-28-oate (**11**).* Obtained from **1** by method **A** as a colorless solid; yield: 92%; m.p. 117–119 °C; R_F = 0.30 (*n*-hexane/ethyl acetate, 5:3); $[\alpha]_D$ = +44.4° (*c* 4.0, $CHCl_3$); IR (KBr): ν = 3442, 2948, 2864, 1726, 1628, 1458, 1386, 1364, 1302, 1260, 1176, 1162, 1124, 1050, 1034 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.29 (brs, 1H, H-12), 4.17 (dt, *J* = 11.1, 6.9 Hz, 1H, CH_a-31), 4.15 (dt, *J* = 11.1, 6.9 Hz, 1H, CH_b-31), 3.70 (ddd, *J* = 11.0, 9.5, 4.2 Hz, 1H, H-2), 3.46 (t, *J* = 6.6 Hz, 2H, CH_2 (33)), 3.01 (d, *J* = 9.4 Hz, 1H, H-3), 2.86 (dd, *J* = 13.8, 4.0 Hz, 1H, H-18), 2.17 (dtt, *J* = 12.5, 6.3, 6.3 Hz, 2H, CH_2-32), 1.98 (m, 2H, CH_a-1 + H_a-16), 1.85–1.79 (m, 2H, $H_{a,b}$-11), 1.70 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 1H, H_a-7), 1.68–1.59 (m, 4H, H-9 + H_a-19 + H_a-15 + H_b-16), 1.58–1.53 (m, 1H, H_a-6), 1.45 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 1H, H_a-22), 1.41–1.35 (m, 1H, H_b-7), 1.38 (ddd, *J* = 12.5, 12.5, 2.5 Hz, 1H, H_b-6), 1.37–1.28 (m, 2H, H_b-22 + H_a-21), 1.20–1.15 (m, 2H, H_b-1 + H_b-21), 1.13 (s, 3H, CH_3-27), 1.10–1.04 (m, 1H, H_b-15), 1.03 (s, 3H, CH_3-23), 0.99 (s, 3H, CH_3-25), 0.93–0.89 (m, 1H, H_b-1), 0.92 (s, 3H, CH_3-30), 0.90 (s, 3H, CH_3-29), 0.79–0.75 (m, 1H, H-5), 0.75 (s, 3H, CH_3-24), 0.74 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): *m*/*z* = 615.7 (75%, $[^{79}M + Na]^+$), 617.5 (100%, $[^{81}M + Na]^+$), 1209.1 (60%, $[2^{79}M + Na]^+$), 1211.1 (38%, $[2^{81}M + Na]^+$).

*5.2.7.12. 3-Iodo-prop-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**12**).* Obtained from **1** by method **A** as a colorless solid; yield: 92%; m.p. 145–147 °C; R_F = 0.29 (*n*-hexane/ethyl acetate, 5:3); $[\alpha]_D$ = +47.3° (*c* 3.1, $CHCl_3$); IR (KBr): ν = 3424, 2948, 1726, 2864, 1162, 1034, 1050, 1460, 1170, 1384, 1260, 1630, 1124, 1364, 1302 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.28 (dd, *J* = 3.3, 3.3 Hz, 1H, H-12), 4.14–4.10 (m, 2H, CH_2-31), 3.69 (ddd, *J* = 11.3, 9.7, 4.5 Hz, 1H, H-2), 3.23 (ddd, *J* = 6.8, 6.7, 1.0 Hz, 2H, CH_2 (33)), 3.00 (d, *J* = 9.5 Hz, 1H, H-3), 2.85 (dd, *J* = 13.8, 4.2 Hz, 1H, H-18), 2.20–2.01 (br, 2H, O*H*), 2.15–2.07 (m, 2H, CH_2-32), 2.00–1.93 (m, 2H, CH_a-1 + H_a-16), 1.95–1.83 (m, 2H, $H_{a,b}$-11), 1.71 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 1H, H_a-7), 1.64–1.58 (m, 4H, H-9 + H_a-19 + H_a-15 + H_b-16), 1.60–1.55 (m, 1H, H_a-6), 1.53–1.40 (m, 3H, H_b-6 + H_b-7 + H_a-22), 1.39–1.30 (m, 2H, H_b-22 + H_a-21), 1.22–1.14 (m, 2H, H_b-21 + H_b-

19), 1.13 (s, 3H, CH_3-27), 1.09–1.03 (m, 1H, H_b-15), 1.03 (s, 3H, CH_3-23), 0.98 (s, 3H, CH_3-25), 0.92 (s, 3H, CH_3-30), 0.90 (s, 3H, CH_3-29), 0.92–0.85 (m, 2H, H-5 + H_b-1), 0.82 (s, 3H, CH_3-24), 0.75 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 641.1 (20%, $[M + H]^+$), 658.1 (10%, $[M + NH_4]^+$), 663.3 (62%, $[M + Na]^+$), 1303.2 (100%, $[2M + Na]^+$).

*5.2.7.13. Butyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**13**).* Obtained from **1** by method **A** as a colorless solid; yield: 95%; m.p. 149–152 °C; R_F = 0.51 (*n*-hexane/ethyl acetate 1:1); $[\alpha]_D$ = +51.4° (*c* 4.0, $CHCl_3$); IR (KBr): ν = 3368, 2950, 2870, 2864, 1720, 1460, 1434, 1386, 1380, 1364, 1304, 1260, 1242, 1176, 1164, 1064, 1052, 1032 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.27 (dd, J = 3.4, 3.4 Hz, 1H, H-12), 4.00 (m, 2H, CH_2-31), 3.67 (ddd, J = 11.0, 9.5, 4.3 Hz, 1H, H-2), 2.99 (d, J = 9.5 Hz, 1H, H-3), 2.94 (br, 2H, O*H*), 2.86 (dd, J = 14.0, 4.2 Hz, 1H, H-18), 1.96 (dd, 1H, J = 12.6, 3.6 Hz, H_a-1), 1.96–1.83 (m, 3H, $H_{a,b}$-11 + H_a-16), 1.69 (ddd, J = 13.8, 13.8, 4.3 Hz, 1H, H_a-7), 1.64–1.54 (m, 3H, H_a-19 + H_a-15 + CH_b (11)), 1.54–1.47 (m, 3H, H-9 + CH_2-32), 1.52–1.47 (m, 1H, H_a-6), 1.45–1.40 (m, 1H, H_b-7), 1.43–1.38 (m, 1H, H_a-22), 1.38–1.33 (m, 1H, H_b-6), 1.37 (m, 2H, CH_2 (33)), 1.35–1.31 (m, 1H, H_a-21), 1.32–1.24 (m, 1H, H_b-22), 1.20–1.13 (m, 2H, H_b-21 + H_b-19), 1.12 (s, 3H, CH_3-27), 1.05–1.00 (m, 1H, H_b-15), 1.01 (s, 3H, CH_3-23), 0.96 (s, 3H, CH_3-25), 0.90 (t, J = 7.4 Hz, 3H, CH_3 (36)), 0.91 (s, 3H, CH_3-30), 0.93–0.86 (m, 1H, H_b-1), 0.88 (s, 3H, CH_3-29), 0.83–0.77 (m, 1H, H-5), 0.80 (s, 3H, CH_3-24), 0.72 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 551.6 (100%, $[M + Na]^+$), 812.5 (20%, $[3M + K + H]^{2+}$), 1079.3 (52.4%, $[2M + Na]^+$).

*5.2.7.14. 3-Buten-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**14**).* Obtained from **1** by method **A** as a colorless solid; yield: 87%; m.p. 149–152 °C; R_F = 0.56 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D$ = +55.4° (*c* 3.2, $CHCl_3$); IR (KBr): ν = 3392, 2946, 2864, 1720, 1642, 1460, 1434, 1386, 1364, 1260, 1242, 1176, 1162, 1052, 1032, 992 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.78 (ddd, J = 17.0, 10.3, 6.7 Hz, 1H, C*H* (33)), 5.27 (dd, J = 3.4, 3.4 Hz, 1H, H-12), 5.10 (dd, J = 17.2, 1.5 Hz, 1H, CH_a (34)), 5.06 (dd, J = 10.2, 1.2 Hz, 1H, CH_b (34)), 4.07 (ddd, J = 13.2, 10.8, 5.4 Hz, 2H, CH_2-31), 3.69 (ddd, J = 11.3, 9.7, 4.5 Hz, 1H, H-2), 3.00 (d, J = 9.5 Hz, 1H, H-3), 2.86 (dd, J = 13.8, 4.1 Hz, 1H, H-18), 2.47 (br, 2H, O*H*), 2.35 (ddd, J = 6.6, 6.6, 6.6 Hz, 2H, CH_2-32), 1.99–1.91 (m, 4H, $H_{a,b}$-11 + H_a-16 + H_a-1), 1.69 (ddd, J = 13.8, 13.8, 4.3 Hz, 1H, H_a-7), 1.66–1.46 (m, 4H, H_b-16 + H_a-15 + H_a-19 + H-9), 1.56–1.47 (m, 2H, H_b-7 + H_a-6), 1.47 (dd, J = 17.3, 3.4 Hz, 1H, H_a-22), 1.43–1.38 (m, 1H, H_b-6), 1.38–1.27 (m, 2H, H_a-21 + H_b-22), 1.20–1.14 (m, 2H, H_b-21 + H_b-19), 1.12 (s, 3H, CH_3-27), 1.07–1.03 (m, 1H, H_b-15), 1.02 (s, 3H, CH_3-23), 0.97 (s, 3H, CH_3-25), 0.91 (s, 3H, CH_3-30), 0.89 (s, 3H, CH_3-29), 0.91–0.80 (m, 1H, H_b-1), 0.82 (s, 3H, CH_3-24), 0.85–0.79 (m, 1H, H-5), 0.72 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 549.6 (83.0%, $[M + Na]^+$), 580.9 (9.8%, $[M + Na + MeOH]^+$), 801.6 (12.2%, $[3M + Na + H]^{2+}$), 810.0 (34.1%, $[3M + K + H]^{2+}$), 1075.3 (100%, $[2M + Na]^+$).

*5.2.7.15. 3-Butyn-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**15**).* Obtained from **1** by method **A** as a colorless solid; yield: 89%; m.p. 168–169 °C; R_F = 0.22 (*n*-hexane/ethyl acetate 1:1); $[\alpha]_D$ = +48.3° (*c* 3.7, $CHCl_3$); IR (KBr): ν = 3422, 2942, 2864, 1724, 1636, 1466, 1458, 1386, 1364, 1304, 1260, 1228, 1198, 1178, 1160, 1124, 1088, 1050, 1040 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.30 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 4.19–4.05 (m, 2H, CH_2-31), 3.72 (dd, J = 14.0, 7.4 Hz, 2H, CH_2-32), 3.68 (ddd, J = 11.3, 9.6, 4.5 Hz, 1H, H-2), 3.00 (d, J = 9.5 Hz, 1H, H-3), 2.88 (dd, J = 13.8, 4.1 Hz, 1H, H-18), 2.50 (dt, J = 6.8, 2.7 Hz, 2H, CH_2-32), 2.01–1.95 (m, 2H, H_a-1 + H_a-16), 1.95–1.88 (m, 2H, $H_{a,b}$-11), 1.71 (ddd, J = 13.8, 13.8, 4.4 Hz, 1H, H_a-7), 1.71–1.50 (m, 6H, H_a-19 + H-9 + H_a-15 + H_b-16 + H_a-6 + H_b-7), 1.47–1.35 (m, 2H, H_b-6 + H_a-22), 1.39–1.26 (m, 2H, H_a-21 + H_b-22), 1.21–1.15 (m, 2H, H_b-21 + H_b-19), 1.13 (s, 3H, CH_3-27), 1.11–1.00 (m, 1H, H_b-15), 1.03 (s, 3H, CH_3-23), 0.98 (s, 3H, CH_3-25), 0.93 (s, 3H, CH_3-30), 0.90 (s, 3H, CH_3-29), 0.91–0.81 (m, 2H, H_b-1 + H-5), 0.82 (s, 3H, CH_3-24), 0.74 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): m/z = 525.2 (25%, $[M + H]^+$), 547.4 (81%, $[M + Na]^+$), 806.7 (84%, $[3M + K + H]^{2+}$), 1071.4 (100%, $[2M + Na]^+$).

*5.2.7.16. 4-Chloro-but-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**16**).* Obtained from **1** by method **A** as a colorless solid; yield: 72%; m.p. 134–136 °C; R_F = 0.41 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D$ = +49.0° (*c* 3.3, $CHCl_3$); IR (KBr): ν = 3396, 2945, 1723, 1462, 1387, 1261, 1161, 1124, 1049 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.22 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 3.98 (t, J = 6.2 Hz, 2H, CH_2-31), 3.62 (ddd, J = 11.0, 9.8, 4.6 Hz, 1H, H-2), 3.49 (t, J = 6.5 Hz, 2H, CH_2 (34)), 2.98 (d, J = 9.4 Hz, 1H, H-3), 2.80 (dd, J = 13.5, 3.9 Hz, 1H, H-18), 1.91 (dd, J = 12.5, 4.4 Hz, 1H, H_a-1), 1.89–1.81 (m, 3H, H_a-16 + $H_{a,b}$-11), 1.80–1.75 (m, 2H, CH_2 (33)), 1.74–1.67 (m, 2H, CH_2-32), 1.64 (ddd, J = 13.9, 13.9, 4.4 Hz, 1H, H_a-7), 1.61–1.44 (m, 6H, H_a-19 + CH_2 -9 + CH_b-11 + H_b-7 + H_b-16 + H_a-15 + H_a-22), 1.38 (ddd, J = 14.0, 11.1, 3.4 Hz, 1H, H_a-21), 1.30 (ddd, J = 11.3, 9.6, 2.9 Hz, 1H, H_a-21), 1.27–1.22 (m, 1H, H_b-21), 1.16–1.02 (m, 2H, H_b-21 + H_b-19), 1.07 (s, 3H, CH_3-27), 1.03–0.97 (m, 1H, H_b-15), 0.96 (s, 3H, CH_3-23), 0.91 (s, 3H, CH_3-25), 0.86 (s, 3H, CH_3-30), 0.84 (s, 3H, CH_3-29), 0.83–0.77 (m, 2H, H_b-1 + H-5), 0.76 (s, 3H, CH_3-24), 0.66 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): m/z = 585.5 (21%, $[M + Na]^+$), 1147.2 (100%, $[2M + Na]^+$).

*5.2.7.17. 4-Bromo-but-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**17**).* Obtained from **1** by method **A** as a colorless solid; yield: 55%; m.p. 151–155 °C; R_F = 0.25 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D$ = +45.0° (*c* 4.1, $CHCl_3$); IR (KBr): ν = 3385, 2947, 1725, 1464, 1386, 1260, 1178, 1161, 1051, 1035 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.26 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 4.02 (t, J = 6.2 Hz, 2H, CH_2-31), 3.66 (ddd, J = 11.2, 9.6, 4.5 Hz, 1H, H-2), 3.40 (t, J = 6.7 Hz, 2H, CH_2 (34)), 2.97 (d, J = 9.5 Hz, 1H, H-3), 2.84 (dd, J = 12.9, 3.2 Hz, 1H, H-18), 2.03–1.95 (m, 1H, H_a-1), 1.95–1.86 (m, 5H, CH_2 (33) + H_a-16 + $H_{a,b}$-11), 1.79–1.72 (m, 2H, CH_2-32), 1.72–1.69 (m, 2H, H-7 + H-19)), 1.63–1.48 (m, 5H, H-9 + H_b-16 + H_a-15 + H_a-6 + H_b-7), 1.48–1.33 (m, 2H, H_a-22 + H_b-6), 1.32–1.25 (m, 2H, H_b-22 + H_a-21), 1.20–1.09 (m, 2H, H_b-19 + H_b-21), 1.11 (s, 3H, CH_3-27), 1.07–1.02 (m, 1H, H_b-15), 1.00 (s, 3H, CH_3-23), 0.96 (s, 3H, CH_3-25), 0.90 (s, 3H, CH_3-30), 0.88 (s, 3H, CH_3-29), 0.83–0.77 (m, 2H, H_b-1 + H-5), 0.80 (s, 3H, CH_3-24), 0.70 (s, 3H, CH_{3-26}) ppm; MS (ESI, MeOH): m/z = 629.3 (15%, $[M + Na]^+$), 1237.1 (100%, $[2M + Na]^+$).

*5.2.7.18. (2Z) 4-Chlorobut-2-en-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**18**).* Obtained from **1** by method **A** as a colorless solid; yield: 78%; m.p. 148–149 °C; R_F = 0.27 (*n*-hexane/ethyl acetate, 5:3); $[\alpha]_D$ = +46.9° (*c* 3.2, $CHCl_3$); IR (KBr): ν = 3422, 2948, 2864, 1726, 1636, 1460, 1386, 1364, 1260, 1230, 1196, 1176, 1160, 1122, 1050, 1034 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.82 (dddd, J = 10.8, 7.7, 7.7, 1.3 Hz, 1H, C*H* (33)), 5.71 (ddd, J = 10.8, 6.8, 6.8 Hz, C*H*-32), 5.29 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 4.66 (ddd, J = 13.1, 6.9, 1.2 Hz, 1H, CH_a-31), 4.59 (ddd, J = 13.1, 6.7, 1.2 Hz, 1H, CH_b-31), 4.15 (ddd, J = 11.9, 7.7, 0.9 Hz, 1H, CH_a (34)), 4.25 (ddd, J = 12.0, 7.7, 0.9 Hz, 1H, CH_b (34)), 3.69 (ddd, J = 11.4, 9.5, 4.5 Hz, 1H, H-2), 3.0 (d, J = 9.5 Hz, 1H, H-3), 2.85 (dd, J = 13.7, 3.7 Hz, 1H, H-18), 2.11 (brs, 2H, O*H*), 2.03–1.87 (m, 4H, H_a-16 + CH_a-1 + $H_{a,b}$-11), 1.70 (dd, J = 13.7, 13.7, 4.4 Hz, 1H, H_a-7), 1.68–1.50 (m, 6H, H-9 + H_a-19 + H_a-15 + H_b-16 + H_a-6 + H_b-7), 1.47–1.40 (m, 2H, H_b-6 + H_a-21), 1.39–1.27 (m, 2H, H_b-22 + H_b-21), 1.23–1.15 (m, 2H, H_b-21 + H_b-19), 1.13 (s, 3H, CH_3-27), 1.09–1.01 (m, 1H, H_b-15), 1.02 (s, 3H, CH_3-23), 0.98 (s, 3H, CH_3-25), 0.92 (s, 3H, CH_3-30), 0.90 (s, 3H, CH_3-29), 0.89–0.84 (m, 2H, H-5 + H_b-1), 0.82 (s, 3H, CH_3-24), 0.75 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH, source CID): m/z = 583.3 (100%, $[M + Na]^+$), 584.3

(41%, $[M + Na]^+$), 585.3 (41%, $[M + Na]^+$), 861.9 (6%, $[3M + K + H]^{2+}$), 1038.9 (32%, $[4M + K + H]^{2+}$).

*5.2.7.19. 4-Chloro-but-3-yn-1-yl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**19**).* Obtained from **1** by method **A** as a colorless solid; yield: 72%; m.p. 184–185 °C; $R_F = 0.36$ (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D = +53.6°$ (*c* 3.1, $CHCl_3$); IR (KBr): ν = 3430, 2944, 2862, 1734, 1466, 1458, 1388, 1364, 1262, 1174, 1158, 1118, 1052, 1034 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.30 (dd, *J* = 3.3, 3.3 Hz, 1H, H-12), 4.71 (ddd, *J* = 15.7, 1.9, 1.9 Hz, 1H, C*H*$_a$-31), 4.64 (ddd, *J* = 15.7, 1.9, 1.9 Hz, 1H, C*H*$_b$-31), 4.14 (dd, *J* = 1.9 Hz, 2H, C*H*$_2$ (34)), 3.70 (ddd, *J* = 11.2, 9.5, 4.4 Hz, 1H, H-2), 2.99 (d, *J* = 9.5 Hz, 1H, H-3), 2.86 (dd, *J* = 13.7, 3.7 Hz, 1H, H-18), 2.14 (brs, 2H, O*H*), 2.01 (ddd, *J* = 13.3, 13.2, 3.3 Hz, 1H, H$_a$-16), 2.00–1.87 (m, 3H, C*H*$_a$-1 + H$_{a,b}$-11), 1.71 (ddd, *J* = 13.8, 13.8, 4.2 Hz, 1H, H$_a$-7), 1.68–1.50 (m, 6H, H-9 + H$_a$-19 + H$_a$-15 + H$_b$-16 + H$_a$-6 + H$_b$-7), 1.47–1.40 (m, 2H, H$_b$-6 + H$_a$-21), 1.39–1.27 (m, 2H, H$_b$-22 + H$_a$-21), 1.23–1.15 (m, 2H, H$_b$-21 + H$_b$-19), 1.13 (s, 3H, C*H*$_3$-27), 1.06 (ddd, *J* = 14.6, 3.9, 3.9 Hz, 1H, H$_b$-15), 1.02 (s, 3H, C*H*$_3$-23), 0.98 (s, 3H, C*H*$_3$-25), 0.92 (s, 3H, C*H*$_3$-30), 0.90 (s, 3H, C*H*$_3$-29), 0.89–0.84 (m, 2H, H-5 + H$_b$-1), 0.82 (s, 3H, C*H*$_3$-24), 0.75 (s, 3H, C*H*$_3$-26) ppm; MS (ESI, MeOH, source CID): *m/z* = 581.5 (100%, $[M + Na]^+$), 858.4 (24%, $[3M + K + H]^{2+}$), 1038.9 (54%, $[4M + K + H]^{2+}$).

*5.2.7.20. Hexyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**20**).* Obtained from **1** by method **A** as a colorless solid; yield: 81%; m.p. 161–163 °C; R_F = 0.24 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D = +53.2°$ (*c* 6.2, $CHCl_3$); IR (KBr): ν = 3418, 2950, 2861, 1725, 1466, 1386, 1364, 1302, 1262, 1200, 1178, 1161, 1124, 1051, 1034, 996 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.27 (dd, *J* = 3.5, 3.5 Hz, 1H, H-12), 4.00 (dt, *J* = 6.5, 3.0 Hz, 2H, C*H*$_a$-31), 3.68 (ddd, *J* = 11.2, 9.5, 4.4 Hz, 1H, H-2), 2.99 (d, *J* = 9.5 Hz, 1H, H-3), 2.87 (dd, *J* = 13.8, 4.1 Hz, 1H, H-18), 2.25 (brs, 1H, O*H*), 2.21 (brs, 1H, O*H*), 2.00–1.87 (m, 4H, C*H*$_a$-1 + H$_a$-16 + H$_{a,b}$-11), 1.70 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 1H, H-7), 1.68–1.55 (m, 6H, *H-9* + H$_a$-19 + C*H*$_2$-32 + H$_a$-15 + H$_b$-16), 1.58–1.50 (m, 2H, H$_b$-7 + H$_a$-6), 1.41–1.35 (m, 1H, H$_b$-6), 1.34–1.26 (m, 8H, H$_a$-21 + C*H*$_2$-35 + C*H*$_2$-33 + C*H*$_2$-34 + H$_a$-22), 1.16–1.08 (m, 2H, H$_b$-21 + H$_b$-19), 1.12 (s, 3H, C*H*$_3$-27), 1.08–1.00 (m, 1H, H$_b$-15), 1.02 (s, 3H, C*H*$_3$-23), 0.97 (s, 3H, C*H*$_3$-25), 0.92 (s, 3H, C*H*$_3$-30), 0.90 (s, 3H, C*H*$_3$-29), 0.92–0.85 (m, 1H, H$_b$-1), 0.89 (t, *J* = 6.8 Hz, 3H, C*H*$_3$-36), 0.85–0.79 (m, 1H, H-5), 0.82 (s, 3H, C*H*$_3$-24), 0.73 (s, 3H, C*H*$_3$-26) ppm; MS (ESI, MeOH, source CID): *m/z* = 557.3 (14.6%, $[M + H]^+$), 579.6 (100.0%, $[M + Na]^+$), 607.5 (34.1%, $[M + NH_4$+MeOH + $H]^+$), 855.0 (26.9%, $[3M + K + H]^{2+}$), 1135.3 (85.3%, $[2M + Na]^+$).

*5.2.7.21. Dodecyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**21**).* Obtained from **1** by method **A** as a colorless solid; yield: 99%; M.p. 141–142 °C; R_F = 0.5 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D = +45.7°$ (*c* 2.9, $CHCl_3$); IR (KBr): ν = 3326, 2926, 2854, 1726, 1704, 1466, 1460, 1438, 1386, 1382, 1362, 1262, 1200, 1180, 1160, 1124, 1052, 1034 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.28 (dd, *J* = 3.4, 3.4 Hz, 1H, H-12), 4.06–3.94 (m, 2H, C*H*$_2$-31), 3.68 (ddd, *J* = 10.8, 9.5, 4.1 Hz, 1H, H-2), 3.00 (d, *J* = 9.4 Hz, 1H, H-3), 2.87 (dd, *J* = 13.7, 4.1 Hz, 1H, H-18), 2.20–2.10 (br, 2H, O*H*), 2.98 (dd, *J* = 12.2, 4.0 Hz, 1H, H$_a$-1), 1.97–1.88 (m, 3H, H$_a$-16 + H$_{a,b}$-11), 1.71 (ddd, *J* = 13.8, 13.8, 4.4 Hz, H$_a$-7), 1.66–1.57 (m, 6H, H-9 + H$_b$-16 + H$_a$-15 + C*H*$_2$ (32) + H$_a$-19), 1.54–1.48 (m, 1H, H$_a$-6), 1.47–1.42 (m, 2H, H$_b$-7 + H$_a$-22), 1.37–1.33 (m, 1H, H$_b$-6), 1.33–1.20 (m, 15H, H$_a$-21 + H$_b$-16 + H$_b$-22 + C*H*$_2$-42 + C*H*$_2$-40) + C*H*$_2$-35 + C*H*$_2$-36 + C*H*$_2$-37 + C*H*$_2$-38), 1.21–1.15 (m, 1H, H$_b$-21), 1.17–1.11 (m, 1H, H$_b$-19), 1.13 (s, 3H, C*H*$_3$-27), 1.08–1.04 (m, 1H, H$_b$-15), 1.03 (s, 3H, C*H*$_3$-23), 0.97 (s, 3H, C*H*$_3$-25), 0.92 (s, 3H, C*H*$_3$-30), 0.90 (s, 3H, C*H*$_3$-29), 0.91–0.85 (m, 1H, H$_b$-1), 0.86–0.82 (m, 1H, H-5), 0.82 (s, 3H, C*H*$_3$-24), 0.73 (s, 6H, C*H*$_3$ (42) + C*H*$_3$-26) ppm; MS (ESI, MeOH, source CID): *m/z* = 641.7 (20%, $[M + H]^+$), 663.7 (100%, $[M + Na]^+$), 1303.3 (50%, $[2M + Na]^+$).

*5.2.7.22. Octadecyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**22**).* Obtained from **1** by method **A** as a colorless solid; yield: 82%; m.p. 119–120 °C; R_F = 0.5 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D = 37.2°$ (*c* 3.4, $CHCl_3$); IR (KBr): ν = 3572, 3314, 3150, 2922, 2852, 1726, 1468, 1386, 1362, 1262, 1228, 1200, 1180, 1160, 1124, 1052, 1036 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.28 (dd, *J* = 3.3, 3.3 Hz, 1H, H-12), 4.06–3.93 (m, 2H, C*H*$_2$-31), 3.72–3.64 (m, 1H, H-2), 3.00 (d, *J* = 9.3 Hz, 1H, H-3), 2.87 (dd, *J* = 13.8, 4.1 Hz, 1H, H-18), 2.14 (brs, 2H, O*H*), 2.00–1.87 (m, 4H, H$_{a,b}$-11 + H$_a$-16 + H$_a$-1), 1.69 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 1H, H$_a$-7), 1.65–1.50 (m, 6H, H-9 + H$_a$-19 + H$_a$-15 + C*H*$_2$-32 + H$_b$-16), 1.50–1.43 (m, 1H, H$_a$-6), 1.42–1.30 (m, 3H, H$_b$-7 + H$_a$-22 + H$_b$-6), 1.37–1.22 (m, 27H, H$_a$-21 + C*H*$_2$-33 + C*H*$_2$-47 + H$_b$-22 + C*H*$_2$-46 + C*H*$_2$-(36–44)), 1.22–1.11 (m, 2H, H$_b$-19 + H$_b$-21), 1.13 (s, 3H, C*H*$_3$-27), 1.08–1.00 (m, 1H, H$_b$-15), 1.02 (s, 3H, C*H*$_3$-23), 0.97 (s, 3H, C*H*$_3$-25), 0.91 (s, 3H, C*H*$_3$-30), 0.90 (s, 3H, C*H*$_3$-29), 0.88 (t, *J* = 7.0 Hz, 3H, C*H*$_3$-48), 0.86–0.75 (m, 2H, H-5 + H$_b$-1), 0.82 (s, 3H, C*H*$_3$-23), 0.73 (s, 3H, C*H*$_3$-26) ppm; MS (ESI, MeOH, source CID): *m/z* = 725.5 (36%, $[M + H]^+$), 747.6 (100%, $[M + Na]^+$), 1107.2 (32%, $[3M + K + H]^{2+}$), 1471.5 (85%, $[2M + Na]^+$).

*5.2.7.23. (Cyclohexyl)-methyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**23**).* Obtained from **1** by method **A** as a colorless solid; yield: 74%; m.p. 198–200 °C; R_F = 0.43 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D = +46°$ (*c* 3.2, $CHCl_3$); IR (KBr): ν = 3424, 2928, 1725, 1615, 1451, 1385, 1261, 1161, 1051, 1034 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.21 (dd, *J* = 3.5, 3.5 Hz, 1H, H-12), 3.78–3.70 (m, 2H, C*H*$_2$-31), 3.62 (ddd, *J* = 11.1, 11.0, 4.5 Hz, 1H, H-2), 2.93 (d, 1H, *J* = 9.5 Hz, H-3), 2.81 (dd, *J* = 13.8, 4.2 Hz, 1H, H-18), 1.91 (dd, *J* = 12.5, 4.7 Hz, 1H, H$_a$-1), 1.89–1.81 (m, 3H, H$_a$-16 + H$_{a,b}$-11), 1.69–1.50 (m, 10H, C*H*-32 + C*H*$_a$-33 + C*H*$_a$-34 + C*H*$_2$-35 + H$_a$-7 + H$_a$-19 + H$_b$-16 + H$_a$-15 + H-9), 1.50–1.35 (m, 4H, H$_a$-22 + C*H*$_2$-6 + H-7), 1.30 (ddd, *J* = 14.6, 10.8, 3.2 Hz, 1H, H$_a$-21), 1.25–1.07 (m, 3H, H$_b$-21 + H$_b$-22 + H$_b$-34 + H$_b$-33), 1.12 (dd, *J* = 14.9, 4.8 Hz, 1H, H$_b$-19), 1.06 (s, 3H, C*H*$_3$-27), 0.96 (s, 3H, C*H*$_3$-23), 1.01–0.89 (m, 2H, H$_b$-1 + H$_b$-15), 0.91 (s, 3H, C*H*$_3$-25), 0.86 (s, 3H, C*H*$_3$-30), 0.83 (s, 3H, C*H*$_3$-29), 0.82–0.77 (m, 1H, H-5), 0.76 (s, 3H, C*H*$_3$-24), 0.66 (s, 3H, C*H*$_3$-26) ppm; MS (ESI, MeOH): *m/z* = 569.5 (18%, $[2M + H]^+$), 1159.3 (100%, $[2M + Na]^+$).

*5.2.7.24. (2α, 3β) 2,3-Di-O-acetyl-maslinic acid (**24**).* Acetylation of **1** (1.2 g; 2.54 mmol) in dry dichloromethane (50 mL) with acetic anhydride (0.96 mL, 10.16 mmol) and triethylamine (0.96 mL, 10.16 mmol) for 12 h at 24 °C followed by aqueous work-up and recrystallization from ethanol gave **24** as a colorless solid; yield: 88%; m.p. 170–173 °C, (lit.: 175–180 °C [51]); $[\alpha]_D = +30°$ (*c* 8.3, $CHCl_3$); R_F = 0.61 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); IR (KBr): ν = 2949, 1748, 1694, 1464, 1369, 1252, 1183, 1156, 1045 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.18 (dd, *J* = 3.4, 3.4 Hz, 1H, H-12), 5.01 (ddd, *J* = 11.4, 9.8, 4.7 Hz, 1H, H-2), 4.66 (d, *J* = 10.4 Hz, 1H, H-3), 2.73 (dd, *J* = 13.8, 4.1 Hz, 1H, H-18), 1.96 (s, 3H, C*H*$_3$-32), 1.92 (dd, *J* = 11.3 Hz, 4.3 Hz, 1H, H$_a$-1), 1.91–1.90 (m, 1H, C*H*$_a$ (11)), 1.89 (s, 3H, C*H*$_3$ (33)), 1.84 (ddd, *J* = 12.5, 9.9, 3.5 Hz, 1H, H$_a$-16), 1.80–1.72 (m, 2H, H$_b$-16 + C*H*$_b$ (11)), 1.68 (ddd, 1H, *J* = 14.1, 10.8, 4.5 Hz, H$_a$-7), 1.65–1.62 (m, 1H, H$_a$-15), 1.60 (dd, *J* = 11.8, 4.1 Hz, 1H, H-9), 1.56–1.48 (m, 2H, H$_b$-7 + H$_a$-19), 1.48–1.45 (m, 1H, H$_a$-6), 1.41–1.32 (m, 2H, H$_a$-22 + H$_b$-6), 1.27 (ddd, *J* = 11.7, 11.7, 1.9 Hz, 1H, H$_a$-21), 1.19–1.09 (m, 2H, H$_b$-21 + H$_b$-22), 1.06 (ddd, *J* = 13.9, 4.5, 2.0 Hz, 1H, H$_b$-19), 1.03 (s, 3H, C*H*$_{3-27}$), 1.01–0.86 (m, 4H, C*H*$_2$-15 + H$_b$-1 + H-5), 0.96 (s, 3H, C*H*$_3$-23), 0.84 (s, 3H, C*H*$_3$-25), 0.83 (s, 3H, C*H*$_3$-30), 0.82 (s, 3H, C*H*$_3$-29), 0.81 (s, 3H, C*H*$_3$-24), 0.65 (s, 3H, C*H*$_3$-26) ppm; MS (ESI, MeOH): *m/z* = 557.4 (49%, $[M + H]^+$),

574.5 (100%, $[M + NH_4]^+$), 579.5 (51%, $[M + Na]^+$), 1135.2 (100%, $[2M + Na]^+$).

*5.2.7.25. (2α, 3β) 2,3-Dihydroxy-28-oxo-olean-12-en-28-amide (**25**).* Obtained from **24** by method **B** as a colorless solid; yield: 61%; m.p. 292–295 °C, (lit.: 160–162 °C [25]); R_F = 0.21 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +52° (*c* 3.7, $CHCl_3$), (lit.: +60°, *c* 1.0, $CHCl_3$/MeOH 2:1 [25]); IR (KBr): ν = 3456, 2946, 1660, 1596, 1463, 1364, 1191, 1050 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.81 (brs, 2H, NH_2), 5.31 (dd, *J* = 3.5, 3.5 Hz, 1H, H-12), 3.63 (ddd, *J* = 11.3, 9.9, 4.5 Hz, 1H, H-2), 2.94 (d, *J* = 9.5 Hz, 1H, H-3), 2.46 (dd, *J* = 13.1, 3.9 Hz, 1H, H-18), 2.02–1.92 (m, 2H, H_a-16 + H_a-1), 1.90–1.81 (m, 2H, $H_{a,b}$-11), 1.80–1.71 (m, 2H, H_a-19 + H_a-7), 1.65–1.52 (m, 5H, H-9 + H_b-7 + H_a-15 + H_b-16 + H_a-6), 1.52–1.30 (m, 3H, H_b-6 + H_a-21 + H_a-22), 1.24–1.12 (m, 5H, H_b-22 + H_b-19 + H_b-21), 1.11 (s, 3H, CH_3-27), 1.07–0.98 (m, 1H, H_b-15), 0.97 (s, 3H, CH_3-30), 0.92 (s, 3H, CH_3-23), 0.85–0.77 (m, 1H, H_b-1), 0.85 (s, 3H, CH_3-29), 0.85 (s, 3H, CH_3-24), 0.84–0.82 (m, 1H, H-5), 0.76 (s, 3H, CH_3-25), 0.76 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): *m*/*z* = 943.5 (22%, $[2M + H]^+$), 965.4 (100%, $[2M + Na]^+$).

*5.2.7.26. Ethyl (2α, 3β) 2,3-di-O-acetyl-28-oxo-olean-12-en-28-amide (**26**).* Obtained from **24** by method **B** as a colorless solid; yield: 81%; m.p. 146–149 °C; R_F = 0.41 (toluene/ethyl acetate/*n*-heptane/HCOOH, 80:25:30:4); $[\alpha]_D$ = +16° (*c* 3.1, $CHCl_3$); IR (KBr): ν = 3432, 2947, 1745, 1662, 1517, 1368, 1252, 1044 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.82 (dd, *J* = 5.1, 5.1 Hz, 1H, N*H*), 5.36 (dd, *J* = 3.4, 3.4 Hz, 1H, H-12), 5.09 (ddd, *J* = 11.4, 11.4, 4.6 Hz, 1H, H-2), 4.74 (d, *J* = 10.3 Hz, 1H, H-3), 3.38–3.30 (m, 1H, CH_a-31), 3.19–3.03 (m, 1H, CH_{2-31}), 2.50 (dd, *J* = 12.8, 3.4 Hz, 1H, H-18), 2.05 (s, 3H, CH_3 (Ac)), 2.02 (ddd, *J* = 12.5, 12.5, 4.7 Hz, 1H, H_a-16), 1.98 (s, 3H, CH_3 (Ac)), 1.96–1.87 (m, 3H, $H_{a,b}$-11 + H_a-1), 1.75 (m, 1H, *J* = 13.4, 13.4 Hz, H_a-19), 1.70–1.61 (m, 3H, H-9 + H_b-16 + H_a-7), 1.60–1.47 (m, 4H, H_b-7 + H_a-22 + H_a-15 + H_a-6), 1.44 (ddd, *J* = 11.9, 11.9, 2.3 Hz, 1H, H_b-6), 1.38–1.25 (m, 2H, H_b-22 + H_a-21), 1.22–1.16 (m, 1H, H_b-21), 1.17–1.08 (m, 1H, H_b-19), 1.15 (s, 3H, CH_3-27), 1.10 (t, *J* = 7.3 Hz, CH_3-32), 1.06 (s, 3H, CH_3-25), 1.05–0.98 (m, 2H, H_b-15 + H_b-1), 0.90 (m, 13H, H-5 + CH_3-23 + CH_3-30 + CH_3-24 + CH_3-29), 0.77 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): *m*/*z* = 584.5 (100%, $[M + H]^+$), 606.4 (34%, $[M + Na]^+$), 1166.9 (14%, $[2M + H]^+$), 1189.2 (48%, $[2M + Na]^+$).

*5.2.7.27. Propyl (2α, 3β) 2,3-di-O-acetyl-28-oxo-olean-12-en-28-amide (**27**).* Obtained from **24** by method **B** as an off-white solid; yield: 61%; m.p. 129–130 °C; R_F = 0.54 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +21° (*c* 2.6, $CHCl_3$); IR (KBr): ν = 3432, 2948, 1744, 1661, 1517, 1368, 1251, 1043 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.88 (dd, *J* = 5.9, 4.9 Hz, 1H, N*H*), 5.36 (dd, *J* = 3.4, 3.4 Hz, 1H, H-12), 5.09 (ddd, *J* = 11.4, 10.3, 4.6 Hz, 1H, H-2), 4.75 (d, *J* = 10.3 Hz, 1H, H-3), 3.34 (dt, *J* = 13.6, 7.1 Hz, 1H, CH_a-31), 2.95 (dt, *J* = 13.6, 7.2 Hz, 1H, CH_b-31), 2.51 (dd, *J* = 13.4, 3.3 Hz, 1H, H-18), 2.05 (s, 3H, CH_3 (Ac)), 2.02 (ddd, *J* = 12.5, 12.5, 4.8 Hz, 1H, H_a-1), 1.98 (s, 3H, CH_3 (Ac)), 1.91–1.86 (m, 3H, H_a-16 + $H_{a,b}$-11), 1.77 (dd, *J* = 13.4, 13.4 Hz, H_a-19), 1.74–1.59 (m, 3H, H-9 + H_a-7 + H_b-16), 1.55–1.36 (m, 7H, H_a-22 + H_b-7 + H_a-15 + CH_2 (32) + CH_2-6), 1.36–1.24 (m, 2H, H_a-21 + H_b-22), 1.24–1.16 (m, 1H, C (19)), 1.15 (s, 3H, CH_3 (17)), 1.11–1.00 (m, 2H, H_b-1 + H_b-15), 1.06 (s, 3H, CH_3-25), 1.00–0.94 (m, 1H, H-5), 0.93–0.89 (m, 12H, CH_3-23 + CH_3-30 + CH_3-24 + CH_3-29), 0.77 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): *m*/*z* = 598.4 (100%, $[M + H]^+$), 620.5 (39%, $[M + Na]^+$), 1195.0 (28%, $[2M + H]^+$), 1217.3 (68%, $[2M + Na]^+$).

*5.2.7.28. 2-Propen-1-yl (2α, 3β) 2,3-di-O-acetyl-28-oxo-olean-12-en-28-amide (**28**).* Obtained from **24** by method **B** as a colorless solid; yield: 65%; m.p. 158–159 °C; R_F = 0.48 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +11° (*c* 4.2, $CHCl_3$); IR (KBr): ν = 3428, 2948, 1744, 1662, 1517, 1368, 1252, 1043 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.97–5.91 (dd, *J* = 5.3, 5.2 Hz, 1H, N*H*), 5.88–5.78 (m, 2H, CH_2-31), 5.37 (dd, *J* = 3.3, 3.3 Hz, 1H, *H-12*), 5.16 (dd, *J* = 17.2, 1.4 Hz, 1H, H_a-33), 5.12 (dd, *J* = 10.3, 1.3 Hz, H_b-33), 5.09 (dd, *J* = 11.2, 10.9, 4.5 Hz, 1H, H-2), 4.75 (d, *J* = 10.3 Hz, 1H, H-3), 4.01 (dddd, *J* = 11.9, 6.0, 5.9, 1.2 Hz, 1H, CH_a-32), 3.61 (ddd, *J* = 15.6, 5.8, 4.5 Hz, 1H, H_b-32), 2.54 (dd, *J* = 12.8, 3.4 Hz, 1H, H-18), 2.06–2.00 (m, 1H, H_a-1), 2.05 (s, 3H, CH_3 (Ac)), 2.03 (ddd, *J* = 12.4, 12.4, 4.7 Hz, 1H, H_a-16), 1.98 (s, 3H, CH_3 (Ac)), 1.96–1.82 (m, 2H, $H_{a,b}$-11), 1.77 (dd, *J* = 13.3 Hz, 1H, H_a-19), 1.74–1.69 (m, 1H, H_a-7), 1.69–1.61 (m, 2H, H-9 + H_b-16), 1.61–1.41 (m, 5H, H_a-22 + H_b-7 + H_a-15 + CH_2-6), 1.41–1.24 (m, 3H, H_b-22 + CH_2-21), 1.24–1.16 (m, 1H, H_b-19), 1.15 (s, 3H, CH_3-27), 1.09–0.96 (m, 2H, H_b-1 + H_b-15), 1.05 (s, 3H, CH_3-25), 0.96 (dd, *J* = 11.9, 1.5 Hz, 1H, H-5), 0.90 (s, 12H, CH_3-23 + CH_3-30 + CH_3-24 + CH_3-29), 0.75 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): *m*/*z* = 596.5 (100%, $[M + H]^+$), 618.5 (36%, $[M + Na]^+$), 1191.0 (28%, $[2M + H]^+$), 1213.2 (74%, $[2M + Na]^+$).

*5.2.7.29. 2-Propyn-1-yl (2α, 3β) 2,3-di-O-acetyl-28-oxo-olean-12-en-28-amide (**29**).* Obtained from **24** by method **B** as a colorless solid; yield: 56%; m.p. 226–227 °C; R_F = 0.51 (toluene/ethyl acetate/n-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +1° (*c* 3.8, $CHCl_3$); IR (KBr): ν = 3432, 3250, 2947, 2362, 1744, 1654, 1508, 1369, 1251, 1044 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 6.04 (dd, *J* = 4.8 Hz, 1H, N*H*), 5.40 (dd, *J* = 3.4, 3.4 Hz, 1H, H-12), 5.08 (ddd, *J* = 11.4, 10.7, 4.6 Hz, 1H, H-2), 4.74 (d, *J* = 10.3 Hz, 1H, H-3), 4.03 (ddd, *J* = 17.6, 5.4, 2.6 Hz, 1H, CH_a-31), 3.90 (ddd, *J* = 17.6, 4.4, 2.6 Hz, 1H, H_b-31), 2.53 (dd, *J* = 12.9, 3.4 Hz, 1H, H-18), 2.20 (dd, *J* = 2.6 Hz, 1H, C≡*CH* (33)), 2.05 (s, 3H, CH_3 (Ac)), 2.02 (ddd, *J* = 12.4, 12.4, 4.7 Hz, 1H, H_a-16), 1.97 (s, 3H, CH_3 (Ac)), 1.99–1.84 (m, 3H, $H_{a,b}$-11 + H_a-1), 1.75 (dd, *J* = 13.3, 13.3 Hz, 1H, H_a-19), 1.72–1.67 (m, 1H, H_a-7), 1.66–1.51 (m, 4H, H-9 + H_a-22 + H_b-16 + H_a-6), 1.51–1.41 (m, 2H, H_a-15 + H_b-7), 1.41–1.26 (m, 3H, H_b-6 + H_a-21 + H_b-22), 1.22–1.16 (m, 1H, H_b-21), 1.15 (s, 3H, CH_3-27), 1.14–1.00 (m, 1H, H_b-19), 1.06 (s, 3H, CH_3-25), 1.08–0.99 (m, 2H, H_b-1 + H_b-15), 0.99–0.94 (m, 1H, H-5), 0.90 (s, 12H, CH_3 (23) + CH_3 (30) + CH_3 (24) + CH_3-29), 0.79 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): *m*/*z* = 594.5 (91%, $[M + H]^+$), 616.5 (39%, $[M + Na]^+$), 1186.9 (24%, $[2M + H]^+$), 1209.2 (100%, $[2M + Na]^+$).

*5.2.7.30. (2α, 3β) 2,3-Dihydroxy-28-oxo-olean-12-en-28-amide (**30**).* Obtained from **25** by method **C** as a colorless solid; yield: 61%; m.p. 292–295 °C (lit.: 160–162 °C [26]); R_F = 0.21 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +52° (*c* 3.7, $CHCl_3$), (lit.: +60°, *c* 1.0, $CHCl_3$/MeOH 2:1 [26]); IR (KBr): ν = 3456, 2946, 1660, 1596, 1463, 1364, 1191, 1050 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.81 (brs, 2H, NH_2), 5.31 (dd, *J* = 3.5, 3.5 Hz, 1H, H-12), 3.63 (ddd, *J* = 11.3, 9.9, 4.5 Hz, 1H, H-2), 2.94 (d, *J* = 9.5 Hz, 1H, H-3), 2.46 (dd, *J* = 13.1, 3.9 Hz, 1H, H-18), 2.02–1.92 (m, 2H, H_a-16 + H_a-1), 1.90–1.81 (m, 2H, $H_{a,b}$-11), 1.80–1.71 (m, 2H, H_a-19 + H_a-7), 1.65–1.52 (m, 5H, H-9 + H_b-7 + H_a-15 + H_b-16 + H_a-6), 1.52–1.30 (m, 3H, H_b-6 + H_a-21 + H_a-22), 1.24–1.12 (m, 5H, H_b-22 + H_b-19 + H_b-21), 1.11 (s, 3H, CH_3-27), 1.07–0.98 (m, 1H, H_b-15), 0.97 (s, 3H, CH_3-30), 0.92 (s, 3H, CH_3-23), 0.85–0.77 (m, 1H, H_b-1), 0.85 (s, 3H, CH_3-29), 0.85 (s, 3H, CH_3-24), 0.84–0.82 (m, 1H, H-5), 0.76 (s, 3H, CH_3-25), 0.76 (s, 3H, CH_{3-26}) ppm; MS (ESI, MeOH): *m*/*z* = 943.5 (22%, $[2M + H]^+$), 965.4 (100%, $[2M + Na]^+$).

*5.2.7.31. Ethyl (2α, 3β) 2,3-dihydroxy-28-oxo-olean-12-en-28-amide (**31**).* Obtained from **26** by method **C** as a colorless solid; yield: 82%; m.p. 170–173 °C; $[\alpha]_D$ = +33° (3.0, $CHCl_3$); R_F = 0.15 (toluene/ethyl acetate/n-heptane/formic acid, 80:25:30:4); IR (KBr): ν = 3422, 2945, 1637, 1525, 1384, 1259, 1050, 1031 cm^{-1}; ^1H NMR (500 MHz, CD_3OD): δ = 7.25 (dd, *J* = 5.5, 5.5 Hz, 1H, NH), 5.40 (dd, *J* = 3.4,

3.4 Hz, 1H, H-12), 3.66 (ddd, J = 11.4, 9.8, 4.5 Hz, 1H, H-2), 3.30–3.20 (m, 1H, CH_a-31), 3.20–3.12 (m, 1H, CH_b-31), 2.95 (dd, J = 9.6 Hz, 1H, H-3), 2.83 (dd, J = 13.7, 4.1 Hz, 1H, H-18), 2.10 (ddd, J = 13.8, 13.8, 4.0 Hz, 1H, H_a-16), 1.99 (m, 3H, CH_a-1 + $H_{a,b}$-11), 1.82 (dd, J = 13.5, 13.5 Hz, 1H, H_a-19), 1.70–1.52 (m, 7H, H-9 + H_a-15 + H_b-16 + H_a-6 + CH_2-7 + H_a-22), 1.49 (ddd, J = 11.4, 11.4, 3.3 Hz, 1H, H_a-6), 1.43 (ddd, J = 13.9, 13.9, 4.1 Hz, H_a-21), 1.35 (m, 1H, H_b-22), 1.24–1.19 (m, 2H, H_b-19 + H_b-21), 1.21 (s, 3H, CH_3-27), 1.11 (t, J = 7.2 Hz, 3H, CH_3-32), 1.10–1.01 (m, 1H, H_b-15), 1.05 (s, 3H, CH_3-23), 1.05 (s, 3H, CH_3-25), 0.99 (s, 3H, CH_3-30), 0.95 (s, 3H, CH_3-29), 0.97–0.90 (m, 1H, H_b-1), 0.88 (dd, J = 11.3, 0.7 Hz, 1H, H-5), 0.85 (s, 3H, CH_3-24), 0.83 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): m/z = 500.6 (88%, $[M + H]^+$), 522.7 (20%, $[M + Na]^+$), 999.3 (22%, $[2M + H]^+$), 1021.3 (100%, $[2M + Na]^+$).

*5.2.7.32. Propyl (2α, 3β) 2,3-dihydroxy-28-oxo-olean-12-en-28-amide (**32**).* Obtained from **27** by method **C** as a colorless solid; yield: 80%; m.p. 152–154 °C; R_F = 0.19 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +47° (*c* 4.4, $CHCl_3$); IR (KBr): ν = 3417, 2945, 1639, 1525, 1463, 1384, 1266, 1186, 1050 cm^{-1}; ^1H NMR (500 MHz, CD_3OD): δ = 5.40 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 3.65 (ddd, J = 11.3, 9.8, 4.5 Hz, 1H, H-2), 3.24–3.16 (m, 1H, CH_a-31), 3.09–3.02 (m, 1H, CH_b-31), 2.94 (d, J = 9.6 Hz, 1H, H-3), 2.83 (dd, J = 13.3, 3.8 Hz, 1H, H-18), 2.10 (ddd, J = 13.1, 13.1, 4.4 Hz, 1H, H_a-16), 2.03–1.94 (m, 3H, CH_a-1 + $H_{a,b}$-11), 1.82 (dd, J = 13.5 Hz, 1H, H_a-19), 1.73–1.50 (m, 9H, H-9 + CH_2-7 + H_a-22 + H_a-15 + CH_2 (32) + H_b-16 + H_a-6), 1.49 (ddd J = 11.8, 11.8, 3.5 Hz, 1H, H_b-6), 1.43 (ddd, J = 13.5, 13.5, 3.9 Hz, 1H, H_a-22), 1.37–1.30 (m, 1H, H_b-21), 1.22 (s, 3H, CH_3-27), 1.25–1.17 (m, 2H, H_b-21 + H_b-19), 1.11–1.03 (m, 1H, H_b-15), 1.05 (s, 3H, CH_3-23), 1.04 (s, 3H, CH_3-25), 0.99 (s, 3H, CH_3-30), 0.95 (s, 3H, CH_3-29), 0.94 (t, J = 7.4 Hz, 3H, CH_3 (33)), 1.00–0.87 (m, 1H, H_b-15), 0.88–0.82 (m, 2H, H-5 + H_b-1), 0.85 (s, 3H, CH_3-24), 0.83 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): m/z = 514.7 (54%, $[M + H]^+$), 1027.3 (28%, $[2M + H]^+$), 1049.4 (100%, $[2M + Na]^+$).

*5.2.7.33. N-Prop-2-en-1-yl (2α, 3β) 2,3-dihydroxy-28-oxo-olean-12-en-28-amide (**33**).* Obtained from **28** by method **C** as white solid; yield: 62%; m.p. 152–154 °C; R_F = 0.12 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +42° (*c* 3.6, $CHCl_3$); IR (KBr): ν = 3527, 3416, 2945, 1637, 1540, 1469, 1386, 1259, 1050, 1031 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.97 (dd, J = 5.0, 5.2 Hz, 1H, N*H*), 5.81 (dddd, J = 16.2, 10.6, 5.8, 5.8 Hz, 1H, *CH*-32), 5.38 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 5.16 (dd, J = 17.3, 3.1, 1.5 Hz, 1H, CH_a (33)), 5.12 (ddd, J = 10.3, 2.7, 1.4 Hz, 1H, CH_b (33)), 4.05–3.97 (m, 1H, CH_a-31), 3.69 (ddd, J = 11.1, 11.1, 4.5 Hz, 1H, H-2), 3.64–3.57 (m, 1H, CH_b-31), 3.00 (d, J = 9.3 Hz, 1H, H-3), 2.54 (dd, J = 13.0, 3.6 Hz, 1H, H-18), 2.18 (brs, 2H, O*H*), 2.00 (ddd, J = 12.6, 12.6, 3.9 Hz, 1H, H_a-16), 1.98–1.91 (m, 3H, CH_a-1 + $H_{a,b}$-11), 1.76 (dd, J = 13.5, 13.5 Hz, 1H, H_a-19), 1.73 (ddd, J = 14.0, 3.6, 3.6 Hz, 1H, H_a-7), 1.68–1.52 (m, 3H, H_b-16 + H_b-7 + H-9), 1.53–1.48 (m, 1H, H_a-6), 1.48–1.42 (m, 1H, H_a-15), 1.42–1.32 (m, 2H, H_b-6 + H_a-22), 1.29–1.17 (m, 4H, CH_2 (21) + H_b-22 + H_b-19), 1.16 (s, 3H, CH_3-27), 1.03 (s, 3H, CH_3-23), 1.02–0.95 (m, 1H, H_b-15), 0.98 (s, 3H, CH_3-25), 0.94–0.87 (m, 1H, H_b-1), 0.91 (s, 6H, CH_3 (30) + CH_3-29), 0.89–0.81 (m, 1H, H-5), 0.83 (s, 3H, CH_3-24), 0.75 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): m/z = 512.5 (65%, $[M + H]^+$), 534.5 (15%, $[M + Na]^+$), 1023.3 (18%, $[2M + H]^+$), 1045.5 (100%, $[2M + Na]^+$).

*5.2.7.34. N-Prop-2-yn-1-yl (2α, 3β)-2,3-dihydroxy-28-oxo-olean-12-en-28-amide (**34**).* Obtained from **29** by method **C** as a bright yellow solid; yield: 62%; m.p. 181–183 °C; R_F = 0.22 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +34° (*c* 0.35, $CHCl_3$); IR (KBr): ν = 3418, 2946, 2360, 1646, 1515, 1464, 1387, 1256, 1186, 1050 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): δ = 5.40 (dd, J = 3.5, 3.5 Hz, 1H, H-12), 4.0 (dd, J = 17.3, 2.5 Hz, 1H, CH_a-31), 3.91 (dd, J = 17.3, 2.5 Hz, 1H, CH_b-31), 3.66 (ddd, J = 11.3, 9.7, 4.5 Hz, 1H, H-2), 2.95 (d, J = 9.6 Hz, 1H, H-3), 2.84 (dd, J = 13.2, 3.5 Hz, 1H, H-18), 2.54 (d, J = 2.5 Hz, 1H, *CH* (33)), 2.14 (ddd, J = 13.3, 13.3, 3.5 Hz, 1H, H_a-16), 2.06–1.92 (m, 3H, $H_{a,b}$-11 + H_a-1), 1.81 (dd, J = 13.5, 13.5 Hz, 1H, H_a-19), 1.77–1.39 (m, 9H, H-9 + H_a-15 + H_b-16 + CH_2 -6 + CH_2-7 + H_a-22 + H_a-21), 1.37–1.25 (m, 2H, H_b-21 + H_b-22), 1.22 (s, 3H, CH_3-27), 1.29–1.16 (m, 1H, H_b-19), 1.14–1.06 (m, 1H, H_b-15), 1.05 (s, 3H, CH_3-23), 1.05 (s, 3H, CH_3-25), 0.99 (s, 6H, CH_3 (30) + CH_3-29), 0.95 (s, 3H, CH_3-23), 0.91 (s, 3H, CH_3-24), 0.89–0.81 (m, 2H, H_b-1 + H-5), 0.85 (s, 3H, CH_3-26) ppm; MS (ESI, MeOH): m/z = 510.6 (40%, $[M + H]^+$), 1019.2 (10%, $[2M + H]^+$), 1041.3 (100%, $[2M + Na]^+$).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.10.016.

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Supplementary material

Esters and amides of maslinic acid trigger apoptosis in human tumor cells and alter their mode of action with respect to the substitution pattern at C-28.

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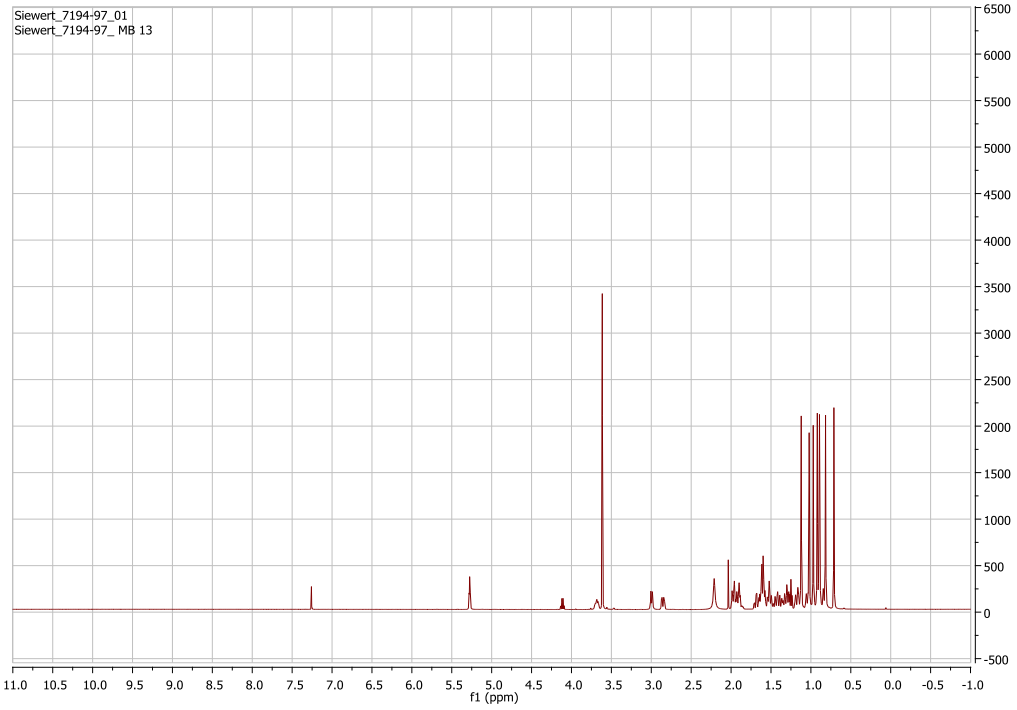
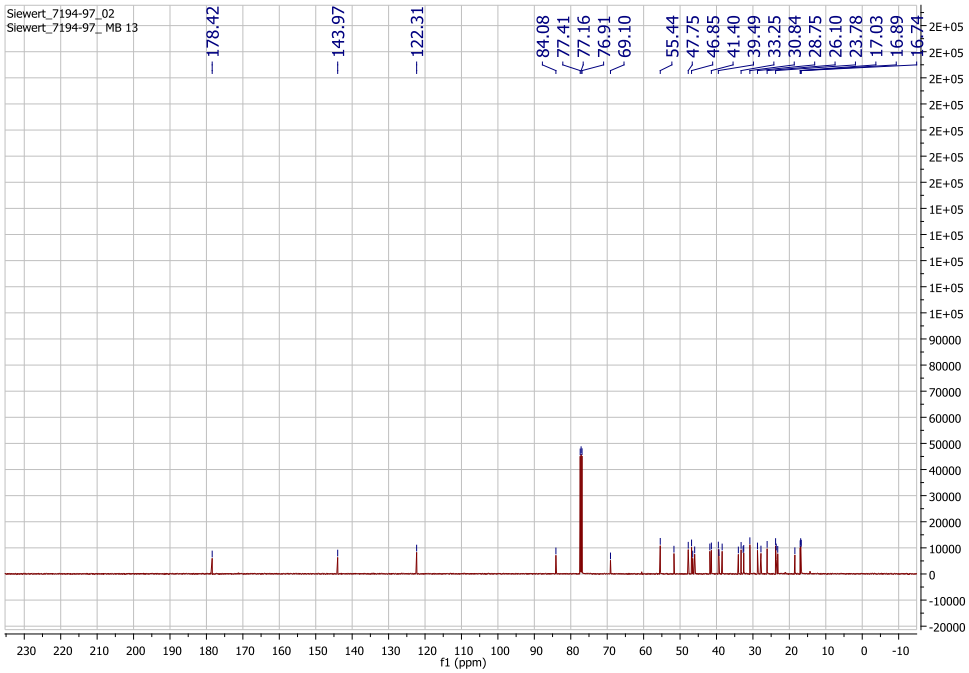
1. Analytical data for Methyl (2α, 3β) 2,3 dihydroxy-olean-12-en-28-oate (**2**)

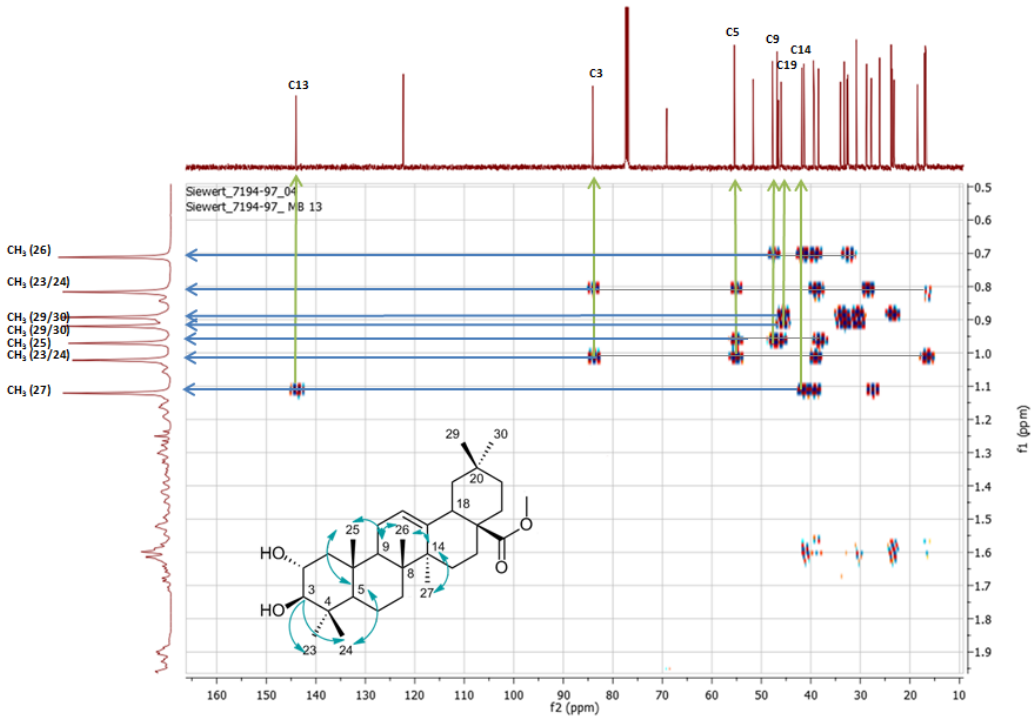
1.1. ^{1}H NMR



1.2. ^{13}C NMR



1.3. gHMBC



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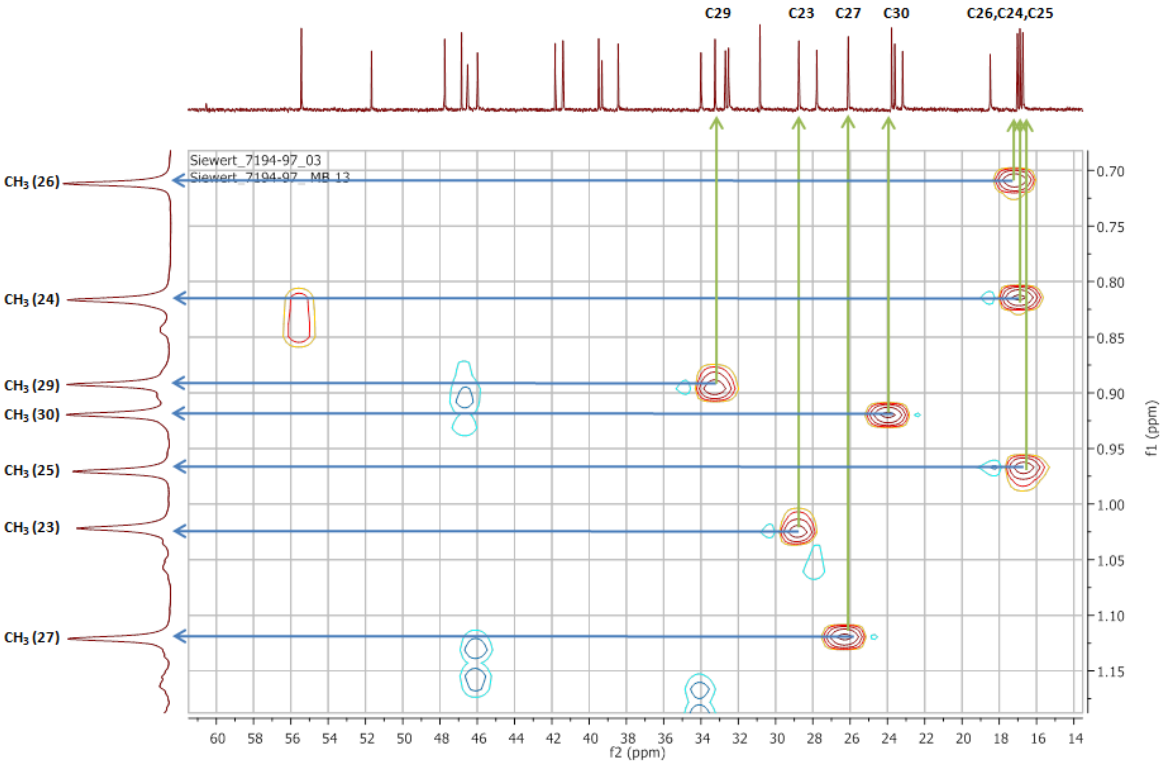
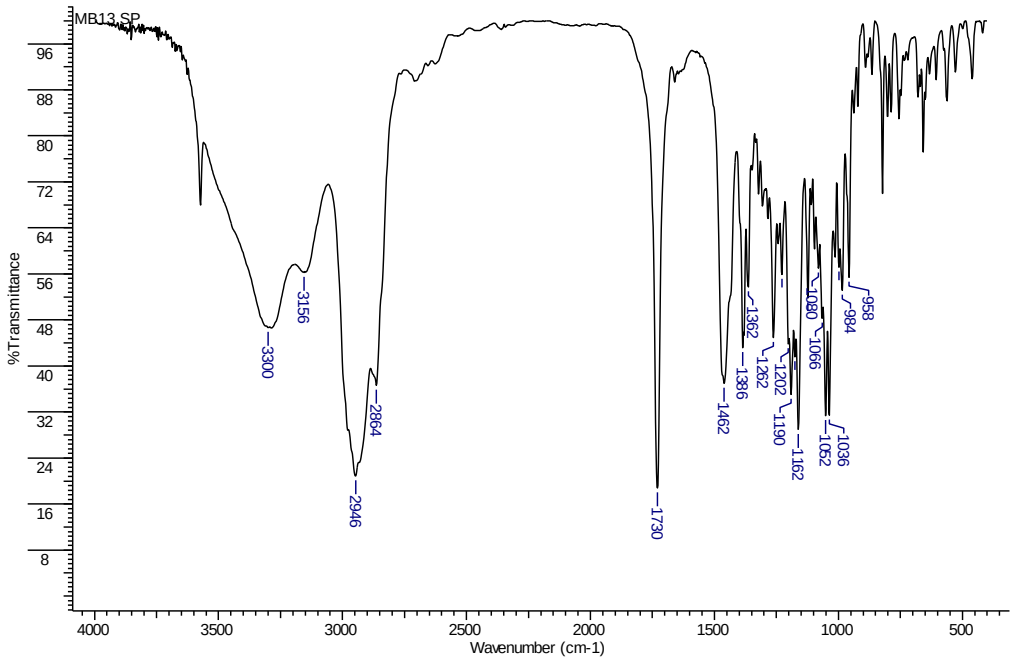
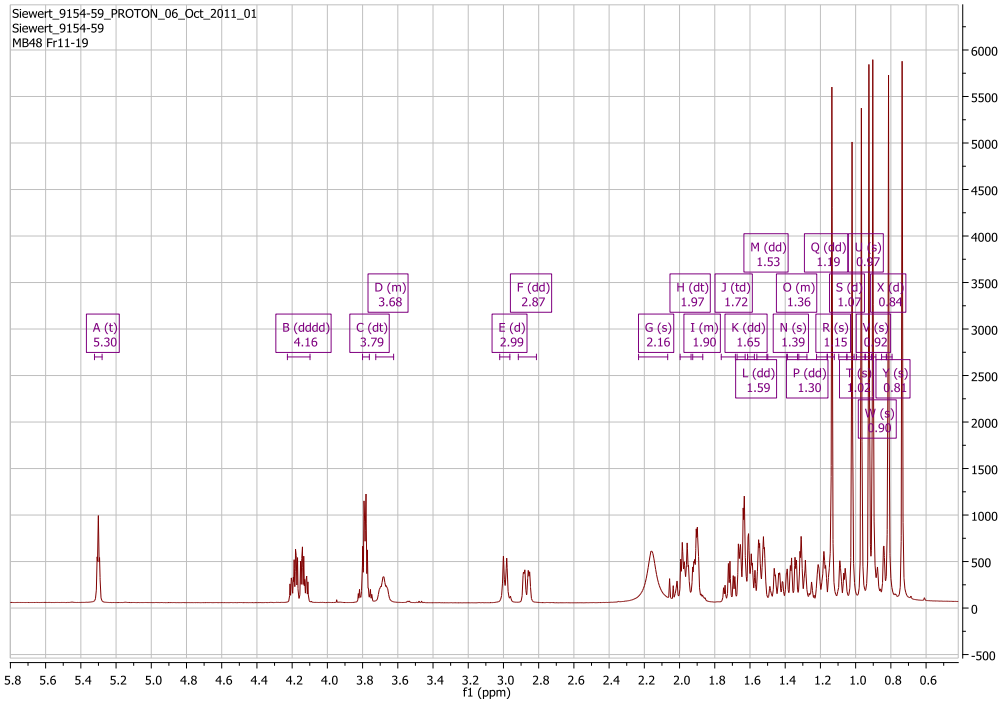


1.5. IR (KBr)

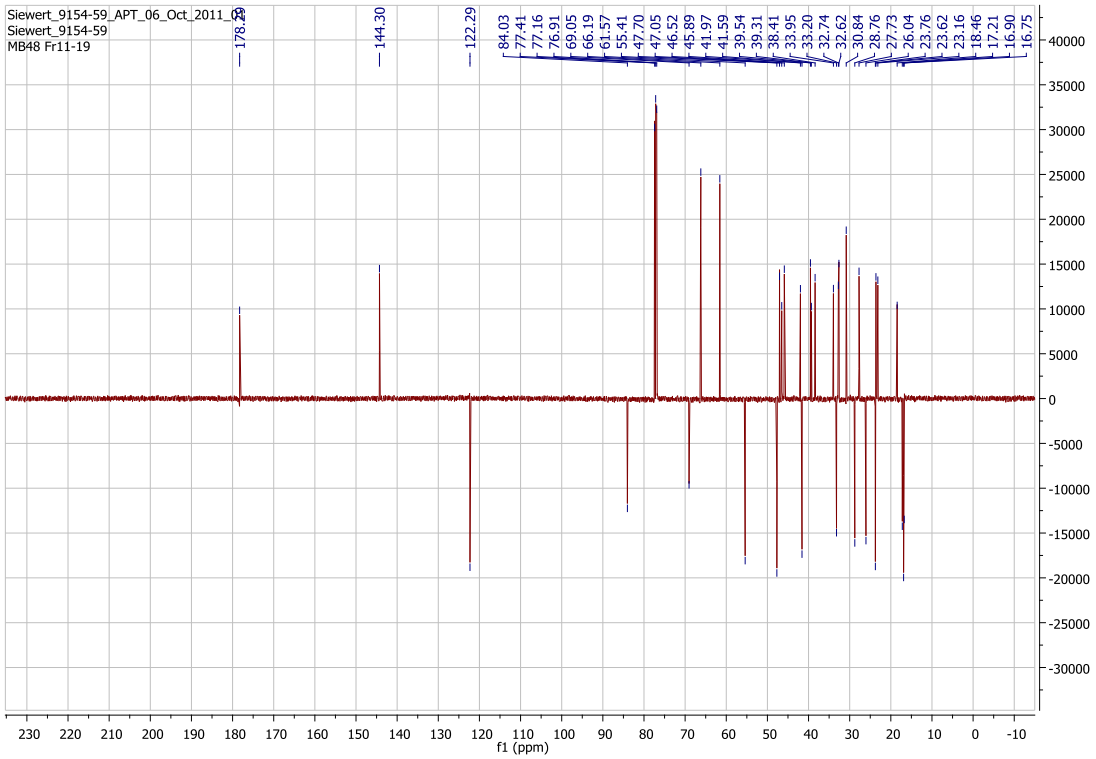


2. Analytical data for 2-hydroxyethyl (2α, 3β) 2,3-dihydroxy-olean-12-en-28-oate (**4**)

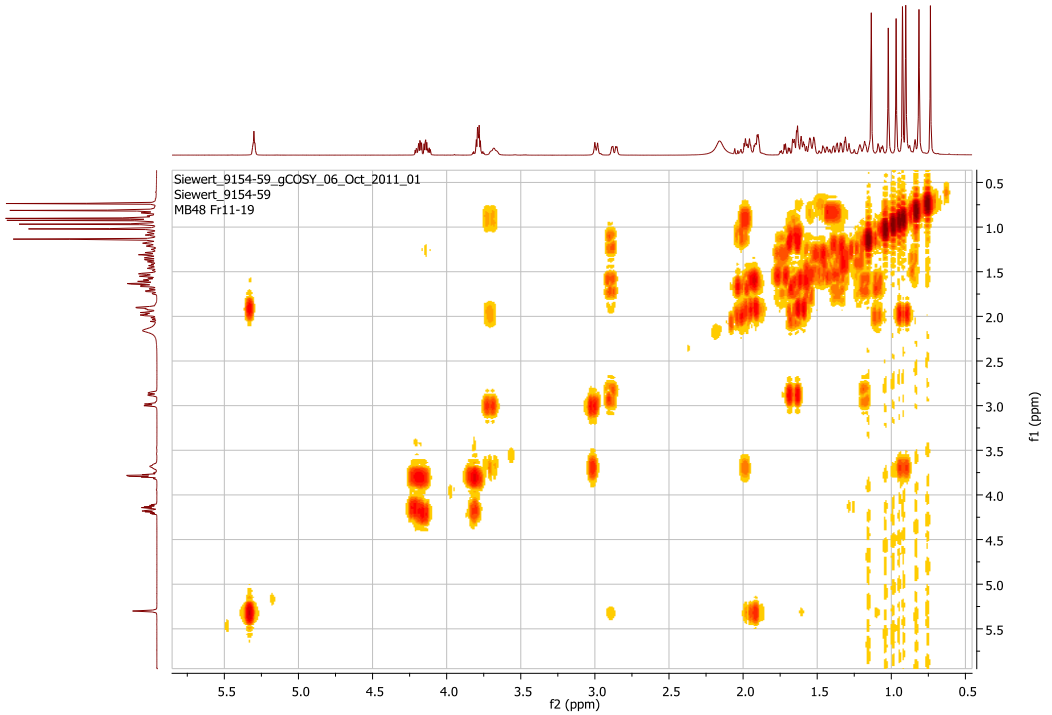
2.1. ^{1}H NMR



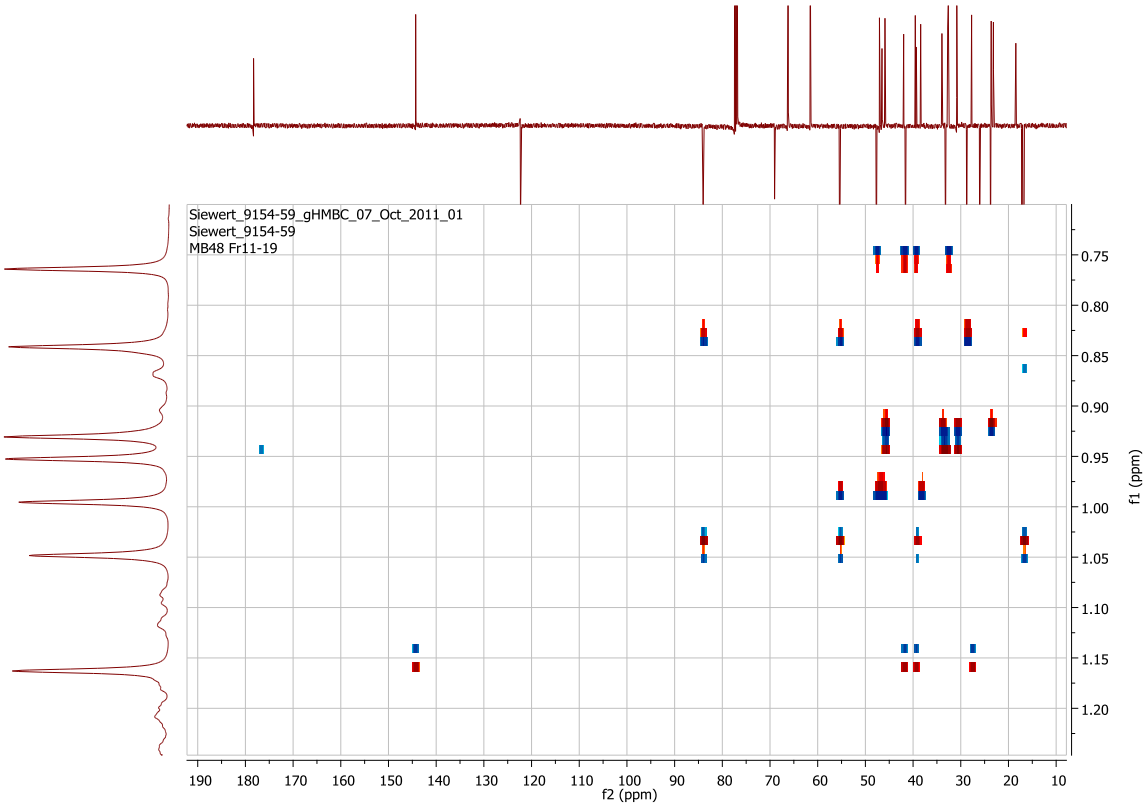
2.2. ^{13}C NMR-APT



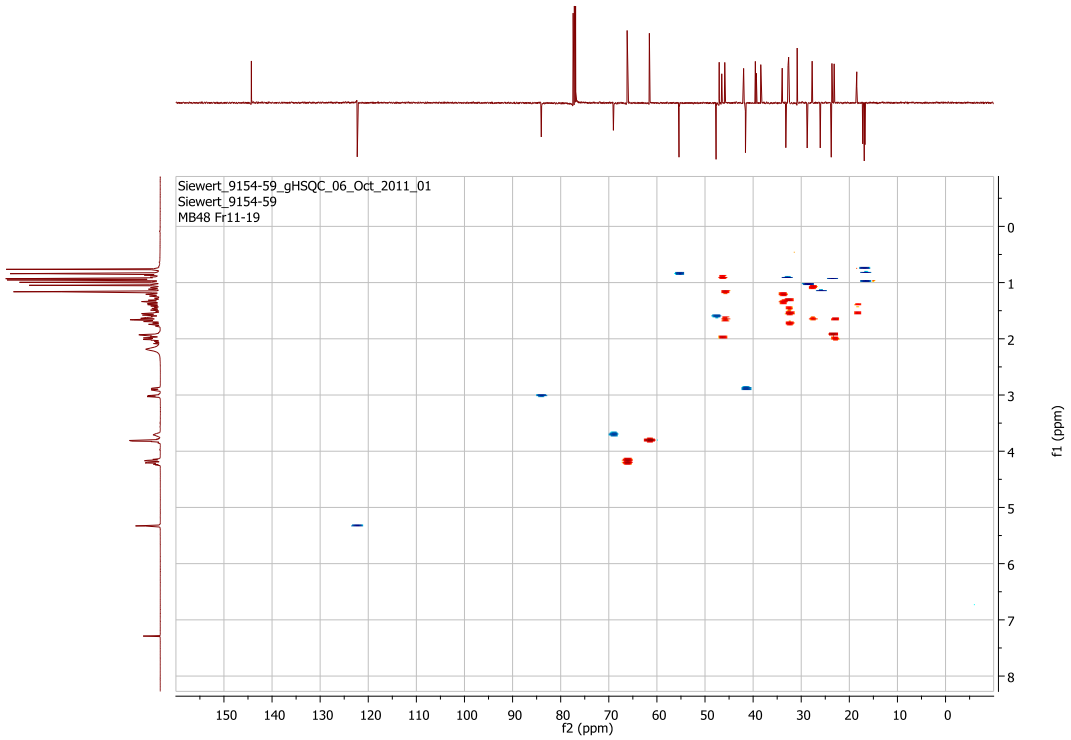
2.3. H,H-Cosy



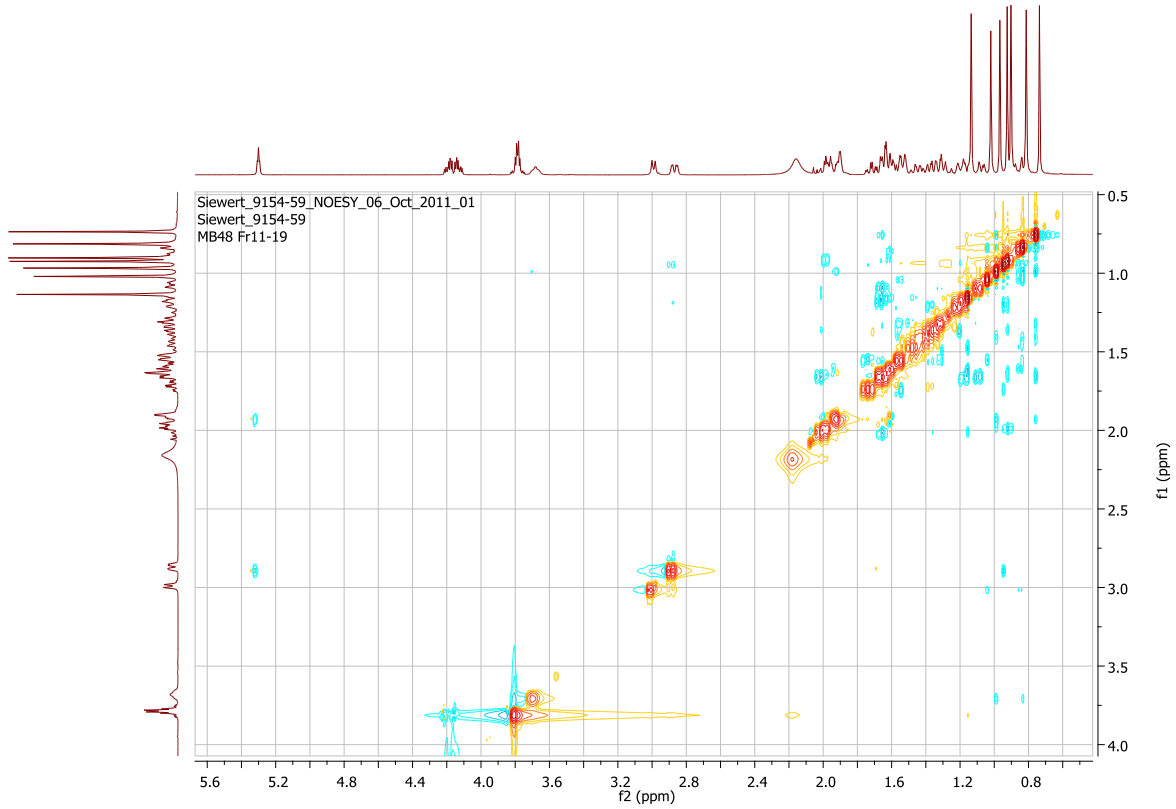
2.4. gHMBC



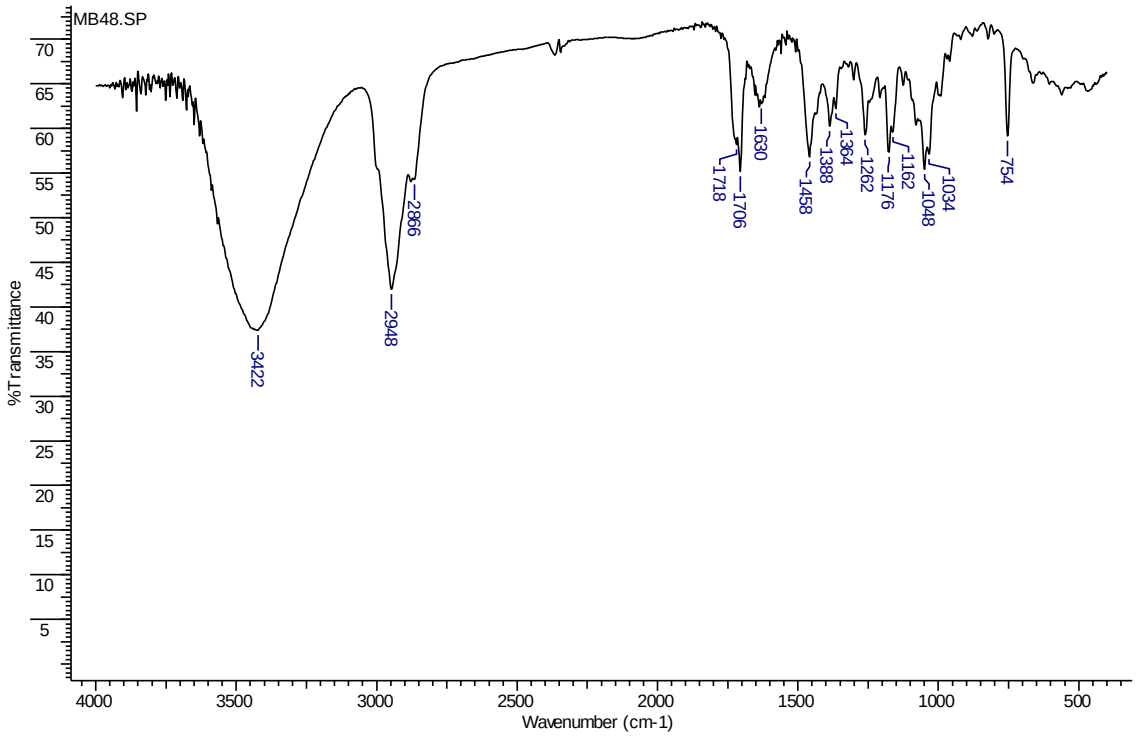
2.5. gHSQC



2.6. H,H-Noesy

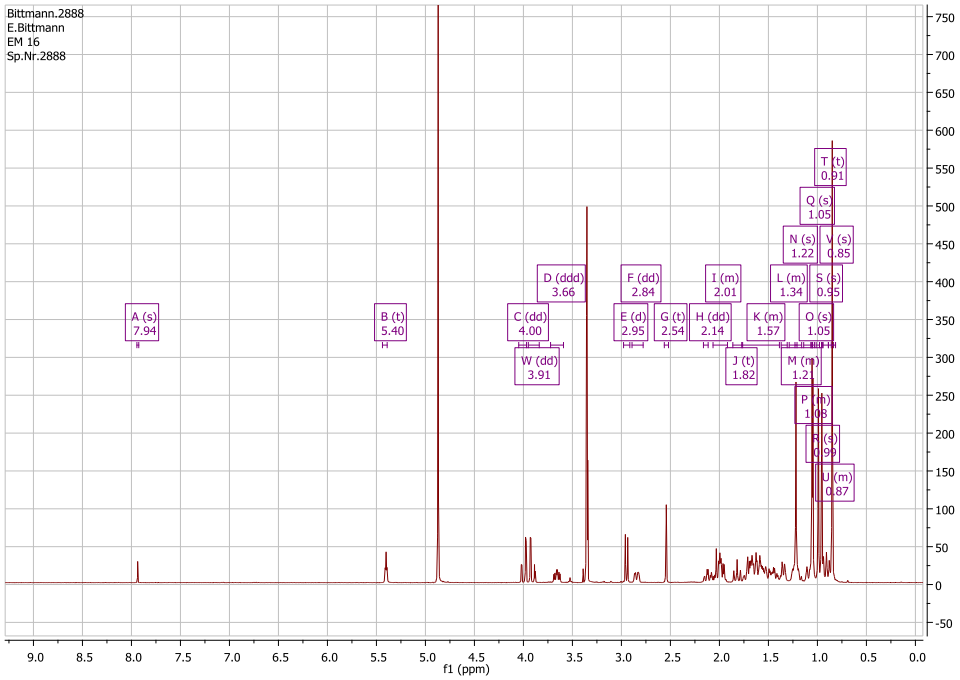


2.7. IR (KBr)

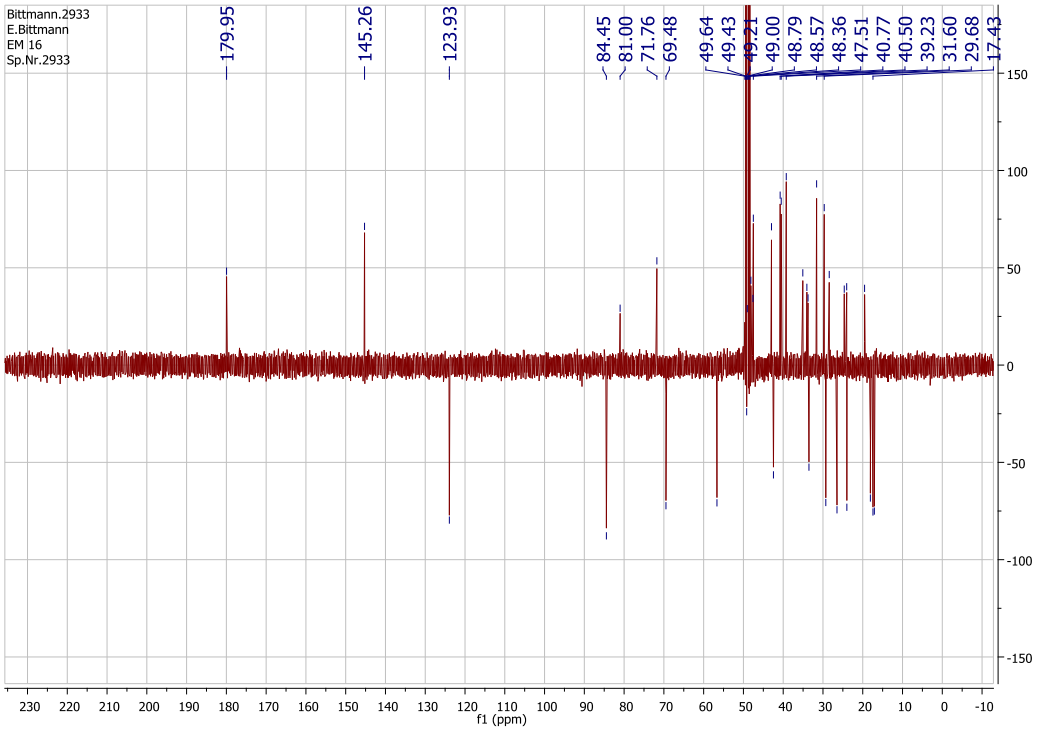


3. Analytical data for N-propyl-2-yn-1-yl (2α, 3β) 2,3-dihydroxy-28-oxo-olean-12-en-28-anide (**34**)

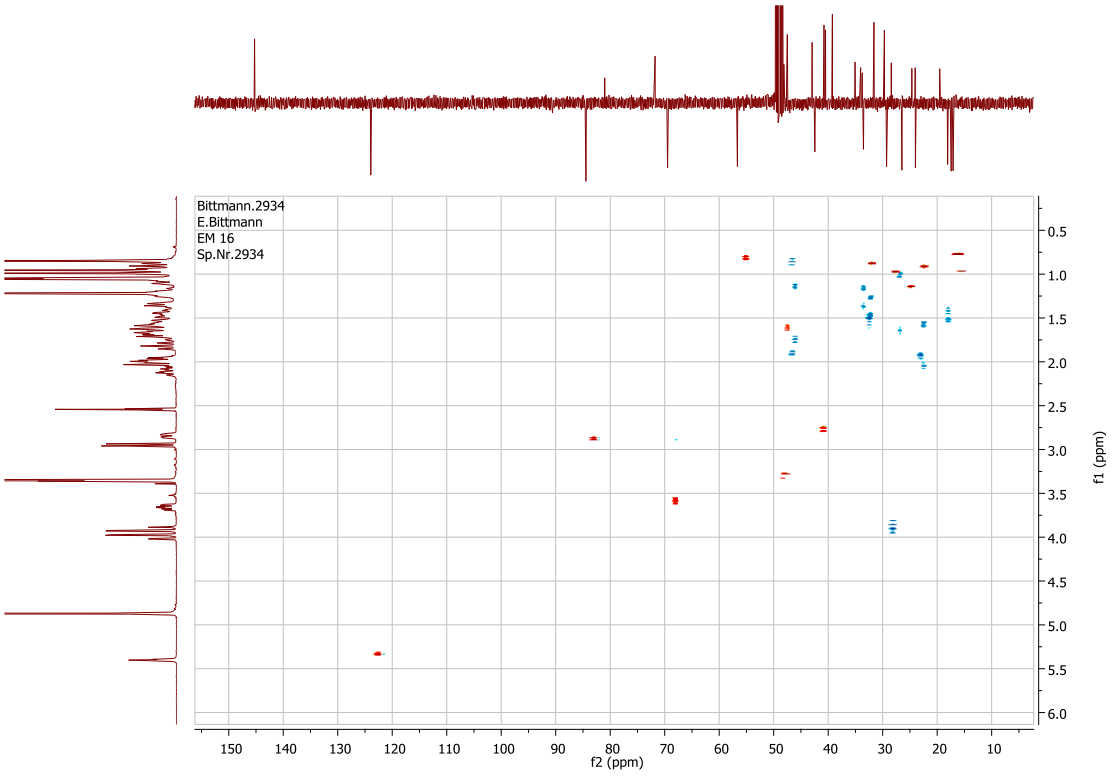
3.1. ^{1}H NMR



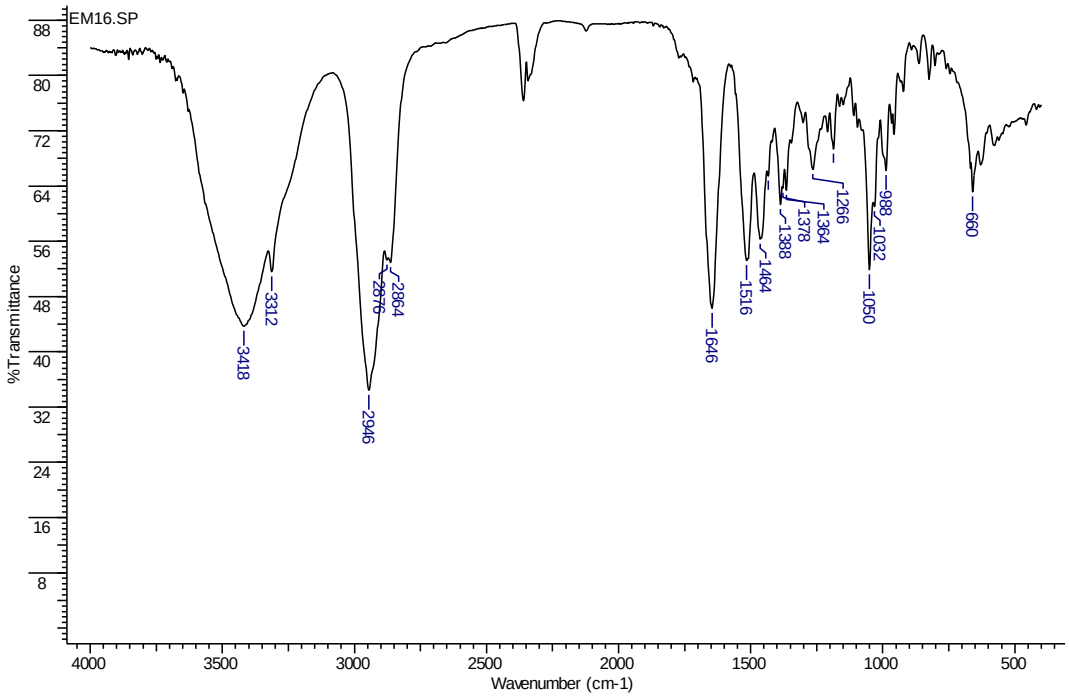
3.2. ^{13}C NMR APT



3.3. gHSQC



3.4. IR (KBr)


EM16.SP
%Transmittance
Wavenumber (cm-1)
3418
3312
2946
2876
2864
1646
1516
1464
1388
1378
1364
1266
1050
1032
988
660

4. Table with IC_{50} values and Confidence Interval (CI = 95 %)

IC_{50}	518A2		8505C		A2780		A549		HT29		MCF7		NIH 3T3	
1	13.7	1.9 1.7	17.0	2.1 1.9	19.5	1.8 1.7	23.4	0.6 0.6	28.8	1.0 0.9	24.4	2.8 2.5	16.6	0.5 0.5
2	15.6	1.3 1.2	14.7	1.3 1.2	17.3	4.3 3.4	17.8	0.5 0.4	12.8	1.5 1.3	16.3	0.9 0.8	21.4	0.7 0.7
3	11.1	2.1 1.8	12.2	0.4 0.4	12.6	1.1 1.0	13.7	1.4 1.3	12.0	2.1 1.8	8.8	0.2 0.2	12.1	1.9 1.7
4	32.5	0.6 0.6	>30		13.4	4.8 3.6	32.5	1.7 1.6	24.1	1.8 1.7	24.0	2.9 2.6	>30	
5	16.9	1.9 1.7	18.9	2.4 2.2	10.2	1.4 1.2	19.0	3.9 3.2	14.9	0.4 0.4	17.8	1.3 1.2	14.0	3.6 2.8
6	13.9	1.0 1.0	13.8	2.5 2.1	14.1	2.4 2.1	13.2	1.5 1.7	12.8	0.5 0.4	13.5	1.3 1.2	13.4	2.8 2.3
7	14.2	1.9 1.7	12.4	0.5 0.5	n.D		14.2	1.9 1.7	14.7	2.9 2.4	15.1	0.8 0.8	15.1	1.6 1.5
8	20.4	1.3 1.2	14.9	1.1 1.0	7.8	0.9 0.8	17.3	0.4 0.4	12.6	0.1 0.1	14.3	1.1 1.0	18.1	1.6 1.5
9	31.6	2.3 2.1	37.3	1.3 1.2	18.1	7.6 5.3	30.3	3.1 2.8	30.9	1.0 1.0	36.0	0.6 0.6	32.6	7.0 5.7
10	12.4	0.4 0.4	11.6	0.2 0.5	12.1	0.0 0.0	14.0	0.1 0.1	11.4	0.7 0.7	13.8	0.9 0.9	17.3	0.4 1.0
11	19.4	1.8 1.7	22.2	1.0 0.9	n.D.		20.6	0.9 0.8	16.4	0.7 0.7	21.1	1.0 0.9	21.6	1.9 1.8
12	12.8	1.3 1.2	13.5	0.0 0.0	11.7	0.4 0.4	13.8	1.6 1.4	14.3	1.4 0.8	13.4	4.3 3.2	12.3	0.9 1.2
13	12.2	1.1 1.0	13.7	2.8 2.3	17.7	6.2 4.6	17.5	2.2 2.0	11.5	1.2 1.1	13.1	3.2 2.6	16.0	1.7 1.5
14	13.3	4.5 3.4	7.1	0.4 0.4	8.0	0.8 0.8	12.8	1.3 0.8	10.1	2.6 2.1	12.7	1.1 1.0	14.1	1.3 1.6
15	22.7	0.5 0.5	26.3	0.4 0.4	10.1	2.7 2.1	18.6	0.1 0.1	9.8	0.9 0.8	19.7	0.6 0.6	26.1	0.0 0.0
16	8.4	0.8 0.8	8.5	1.6 1.3	6.5	1.7 1.4	12.2	0.9 0.8	8.9	1.9 1.6	8.6	3.4 2.4	12.9	2.8 2.3
17	13.1	5.8 4.0	13.2	2.0 1.8	5.9	1.9 1.4	12.7	1.3 1.1	n.D		12.8	0.9 0.8	12.9	2.3 1.9
18	27.9	0.8 1.0	29.1	2.2 1.8	22.5	0.5 0.5	27.4	0.0 0.0	20.5	0.4 0.3	23.4	0.5 0.5	33.1	1.5 1.6
19	12.4	1.2 0.9	9.2	0.6 0.6	8.5	1.0 0.9	9.9	0.4 0.4	8.5	0.1 0.1	7.7	0.5 0.5	12.6	1.0 0.7
20	12.4	1.2 1.1	7.2	0.7 0.7	13.0	2.3 1.9	14.4	0.5 0.5	13.3	1.9 1.7	13.0	3.2 2.5	14.1	1.5 1.3
21	111.0	0.7 0.7	141.3	13.0 11.9	80.3	5.4 5.0	135.6	10.0 9.3	107.0	12.3 11.1	67.7	3.4 3.3	>120	
22	38.5	1.7 1.7	>90		93.8	2.2 2.1	104.1	0.8 0.8	75.7	3.1 3.0	123.4	5.8 5.6	56.9	1.8 1.7
23	8.0	1.4 1.2	5.9	1.3 1.0	3.6	1.5 1.1	10.5	1.9 1.6	9.3	3.8 2.7	8.2	2.8 2.1	11.9	5.0 3.5
30	41.2	2.5 1.6	61.3	6.0 3.2	29.7	14.7 9.8	>60		43.7	3.0 2.4	81.1	4.1 3.2	47.8	6.5 2.7
31	42.1	2.8 2.6	45.2	3.2 3.0	27.4	2.1 1.9	37.3	0.5 0.5	37.7	1.4 1.4	29.1	4.2 3.7	14.2	3.8 1.8
32	29.1	2.7 2.5	28.1	3.3 3.0	18.3	1.1 1.0	23.4	2.5 2.3	25.2	2.5 2.3	13.9	1.9 1.7	17.6	3.3 2.8
33	39.3	15.6 11.2	42.5	1.1 1.1	25.8	1.1 1.1	37.0	1.9 1.8	37.6	2.1 2.0	28.2	3.4 3.1	24.8	1.3 2.6
34	24.5	0.8 0.8	27.7	4.0 3.5	21.9	4.2 3.5	29.8	3.8 3.4	29.5	1.6 1.5	24.6	0.5 0.5	26.1	1.8 1.9

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Towards cytotoxic and selective derivatives of maslinic acid

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Caspase activity

ABSTRACT

Several novel esters and amides of maslinic acid were prepared. Their evaluation for cytotoxic activity with a panel of human cancer cell lines using a sulforhodamine B (SRB) assay revealed for some of them a noteworthy activity. The results from annexinV-FITC and caspase-assays as well as from DNA laddering experiments provided evidence for an apoptotic cell death. A diacetylated benzylamide (15) induced a G1/G0 arrest in tumor cells. It also displayed an extraordinary cytotoxicity against human ovarian cancer cells but a 300 times lower toxicity for non-malignant primary human fibroblasts.

1. Introduction

In 2009/2010 nearly three million tons of olive oil have been produced worldwide, and approximately ten million tons of olives are harvested each year.[1] Per ton of processed olives approximately 800 kg of residues, the olive pomace also known as 'alperujo' are obtained.[2] For natural product chemists this 'waste' (often used as an additive for animal feed) looks like a gold mine for the isolation of natural products. The olive pomace contains high amounts of oleuropein, tyrosol, hydroxytyrosol and the triterpenoid maslinic acid (**MA**, Fig. 1).[3] This oleane type triterpenic acid shows some promising biological properties, such as antiviral,[4–6] antioxidant[7–9] and an anticancer activity.[10–14] Improvements in activities are usually obtained by chemical modifications,[15] and thus we set out synthesizing a series of systematically modified maslinic acid derivatives trying to improve its cytotoxicity.

A SAR study using several aliphatic esters and amides at C-28 revealed that the presence of a proton donor group at position C-28 seems unfavorable for obtaining good cytotoxicity. The presence of a lipophilic residue, however, improved the anticancer activity of the compounds while still retaining their ability to trigger apoptosis. As outlined in Figure 1, bulky ester residues seem to be able to interact quite well with an up to now still unknown intracellular target/receptor. Thus, we became interested in the synthesis and biological evaluation of some substituted benzylic esters of **MA**. All new compounds were evaluated by a photometric sulforhodamin B assay (SRB) for their cytotoxic activity. For some of these compounds annexin V/propidium iodide assays, DNA-fragmentation, caspase assay as well as cell cycle investigations were performed.

2. Synthesis and evaluation of various esters of maslinic acid

Maslinic acid (**MA**) was extracted from olive pomace or from edible green or black olives. Although **MA** has been isolated from many different sources, the residue from the olive oil production seems to be an ideal source for obtaining **MA** by extraction. Unfortunately, this pomace is readily available only in countries producing olive oil.[16] It is only seasonally available, and its transport out of the producing countries is laborious (the pomace has to be kept cool during transport to avoid/slow down fermentation processes) and hence expensive. The isolation of **MA** from edible olives (to be purchased in the nearest supermarket) seems to be a feasible alternative. **MA** was transformed into the corresponding esters by its reaction with alkyl bromides in the presence of finely grounded K_2CO_3 in dry DMF (Fig. 2, Table 1). By this procedure esters **1**–**10** (and chain-extended analogs **12** and **13**) were prepared in good yields. Hydrogenation of **12** gave **13**. These esters showed in their respective IR spectra the typical signals expected for this class of

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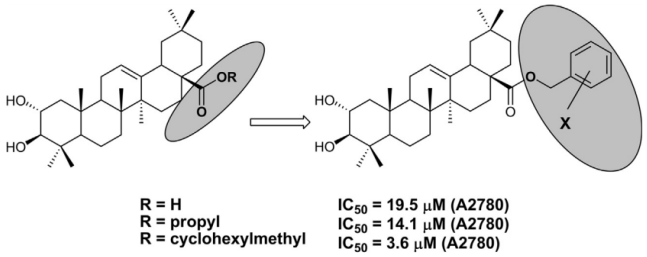


Figure 1. Structure of maslinic acid (MA) and some of its cytotoxic esters.

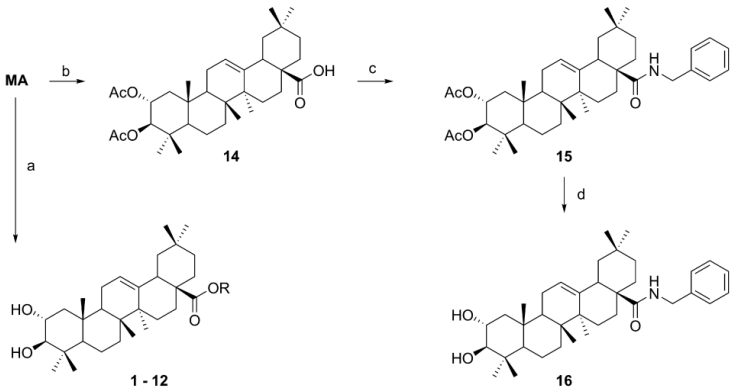


Figure 2. Synthesis of compounds 1–16: (a) K_2CO_3, R-Br, DMF, 25 °C, 24 h; (b) Ac_2O, NEt_3, DCM, 25 °C, 24 h, 88%; (c) $SOCl_2$, NEt_3, DCM/THF (1:1), then $BnNH_2$, DCM, 25 °C, 24 h, 82%; (d) MeOH/KOH, 25 °C, 24 h, 66%.

compounds, and in the ^{1}H NMR spectra the signals of the aromatic protons were detected between δ = 7.20 and 7.50 ppm. For example, for compound **5**, the signals of the aromatic protons were found at δ = 7.47 and 7.21 ppm, each as a doublet exhibiting a coupling constant of J = 8.3 Hz. In addition, compound **3** is characterized in its ^{19}F NMR spectrum by the presence of a signal at δ = −114.18 ppm showing a $J_{H,F}$ = 8.6 Hz to the adjacent and a $^4J_{H,F}$ = 5.4 Hz to the protons in *meta*-position. Diacetyled **MA** (**14**) was obtained by acetylation of **MA**. The benzyl amide **16** was prepared from **14** using a Schotten-Baumann route followed by a deprotection of intermediate **15** (Fig. 2).

Compounds **1**–**13**, **16** and **MA** (as a reference) were submitted to SRB assays.[17] The IC_{50} values were calculated from dose response curves applying a non-linear regression using the two parametric Hills-slope equation. The results of these assays are compiled in Table 1.

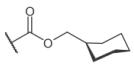
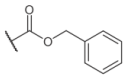
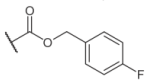
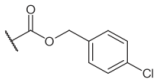
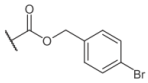
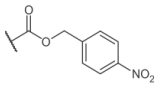
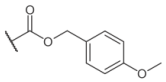
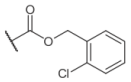
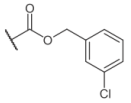
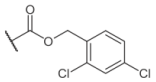
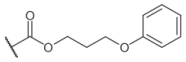
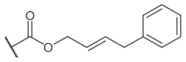
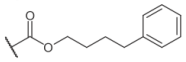
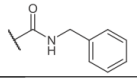
The results from the SRB assay for **MA** and compounds **1**–**13** and **16** using the human ovarian carcinoma cell line A2780 are presented in more detail in Figure 3. Almost all of the compounds showed an increased cytotoxic activity as compared to parent compound **MA**.

The cytotoxicity of compounds **2**–**13** and **16** is similar to that of the cyclohexylmethylester (**1**, Table 1 and Fig. 2). For A2780 cells (Fig. 3) the activity of the *para* substituted compounds increases with the atomic radius of the halogen substituent. In addition, the *para*-bromobenzyl derivative **5** shows a quite promising selectivity in cytotoxicity discriminating between the cancer cell lines and non-malignant mouse fibroblasts NiH 3T3 with a ratio $IC_{50\ [NiH3\ 3T3]}/IC_{50\ [A2780]}$ = SI = ca. 3. The amide **16** and the methoxy substituted benzyl ester **7** exhibit SI values of similar magnitude. Except for a substitution in *para*-position, other substitution patterns seem to lower cytotoxicity. Also the presence of an *ortho* substituent led to a significant lower selectivity between malignant and non-malignant cells, and chain-elongation (as exemplified in compounds **12** and **13**) resulted in a loss in selectivity.

To sum up, these modifications yield some small increase in cytotoxicity and a low but significant improvement for the selectivity of the compounds.

Next, modifications of ring A (concerning the number of hydroxyl groups) and of ring E (α-amyrin or β-amyrin skeleton) came in our focus of our investigations. Whereas **MA** holds two hydroxyl groups, oleanolic acid (**OA**, Fig. 4) and ursolic acid (**UA**) carries only one. As previously shown for glycyrrhetinic acid (**GA**), a 20-fold increase in cytotoxicity can be gained by converting **GA** into its corresponding benzyl ester.[18] Thus, the structure–activity relationships of benzyl oleanoates (**17**–**20**) and of substituted benzyl esters (**24**–**27**) of ursolic acid (**UA**) were investigated in more detail. Compounds **17**–**20** and **24**–**27** were obtained by esterification of **OA** and **UA**, respectively. Acetylation of **OA** or **UA** (Fig. 4) gave acetates **21** and **28**; reaction of these compounds with thionyl chloride/triethylamine and benzylamine furnished amides **22** and **29**. Their deacetylation (methanolic KOH) gave amides **23** and **30**,

Table 1
Cytotoxicity of MA and compounds 1–13 and 16 (IC_{50} values in μM from SRB assays; independent experiments were at least performed in triplicate; standard errors are given) employing human tumor cell lines (518A2, 8505C, A2780, A549, HT29 and MCF7) and non-malignant mouse fibroblasts NiH 3T3

	R	518A2	8505C	A2780	A549	HT29	MCF7	NiH3T3
MA	H	13.7 ± 0.9	17.0 ± 1.0	19.5 ± 0.8	23.4 ± 0.5	28.8 ± 0.5	37.2 ± 1.2	21.1 ± 0.2
1		8.0 ± 0.6	5.9 ± 0.5	3.6 ± 0.6	10.5 ± 0.8	9.3 ± 1.5	8.2 ± 1.1	11.9 ± 1.9
2		19.4 ± 0.8	16.9 ± 0.9	11.1 ± 0.6	20.2 ± 0.1	15.2 ± 0.2	17.2 ± 0.5	19.6 ± 0.5
3		11.0 ± 1.1	12.2 ± 1.1	11.9 ± 0.6	12.3 ± 1.0	9.9 ± 0.3	13.8 ± 1.2	14.6 ± 0.7
4		12.0 ± 0.8	15.3 ± 0.6	11.3 ± 0.8	18.7 ± 1.6	13.1 ± 0.7	14.5 ± 0.5	15.6 ± 0.7
5		9.4 ± 0.1	11.1 ± 0.2	6.6 ± 0.4	13.1 ± 1.0	10.2 ± 0.1	10.7 ± 0.1	20.1 ± 1.2
6		11.0 ± 0.1	15.8 ± 0.6	17.3 ± 1.2	13.2 ± 0.2	13.4 ± 0.5	17.8 ± 1.0	20.5 ± 0.1
7		12.7 ± 0.3	14.2 ± 0.2	12.8 ± 0.1	21.8 ± 0.7	12.3 ± 0.2	14.8 ± 0.1	14.7 ± 0.3
8		20.6 ± 2.0	21.2 ± 2.0	12.1 ± 1.2	21.9 ± 1.3	18.1 ± 0.1	21.1 ± 0.3	21.4 ± 2.1
9		5.5 ± 0.4	7.9 ± 0.2	8.8 ± 0.4	12.5 ± 0.3	13.4 ± 0.1	14.2 ± 0.1	14.4 ± 1.1
10		13.1 ± 1.1	14.7 ± 0.5	13.9 ± 1.3	17.4 ± 1.0	16.3 ± 0.5	18.8 ± 1.0	17.3 ± 0.5
11		12.6 ± 1.2	13.3 ± 0.4	10.6 ± 0.5	13.7 ± 1.3	12.5 ± 0.6	13.6 ± 1.3	12.7 ± 1.1
12		15.2 ± 0.5	15.3 ± 0.8	11.5 ± 0.7	15.7 ± 0.3	15.6 ± 0.2	14.5 ± 0.4	12.8 ± 1.0
13		12.3 ± 0.1	12.8 ± 0.9	13.1 ± 0.1	13.6 ± 0.1	14.3 ± 0.1	14.4 ± 0.5	16.5 ± 1.5
16		21.4 ± 1.2	24.0 ± 1.9	13.4 ± 1.3	24.0 ± 1.2	22.8 ± 0.4	20.2 ± 1.1	18.5 ± 0.9

respectively. These compounds were tested in SRB assays and to our surprise, the introduction of a lipophilic ester led for these compounds to a complete loss of activity (Table 2 and Fig. 5).

As a consequence of these unexpected results, we assume that either the presence of a second proton donating group at position 2 or its bulkiness seems to be crucial for obtaining an improved cytotoxic activity. For betulinic acid, a substitution at position 2 was shown to increase cytotoxicity,[19] and oleanolic acid derivatives carrying substituents at position 2 (e.g., bardoxolone or CDDO and analogs)[20] showed remarkable cytotoxicity paralleling the cytotoxic behavior of some derivatives of glycyrrhetinic acid.[21–23]

Acetylation of the hydroxyl groups in ring A increases cytotoxicity as previously shown for asiatic,[24] corosolic[25] as well as for tormentic acid.[26] Recently, Sarek et al.[27] demonstrated that the

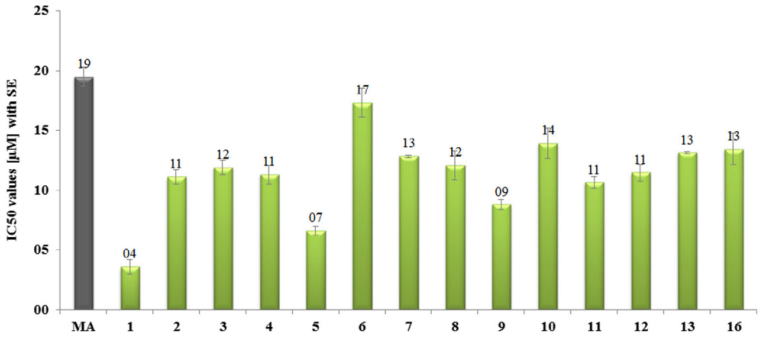


Figure 3. Cytotoxicity (IC_{50} values in µM with standard error (SE), from SRB assays) for MA, esters 1–13 and amide 16 using the human ovarian cancer cell line A2780.

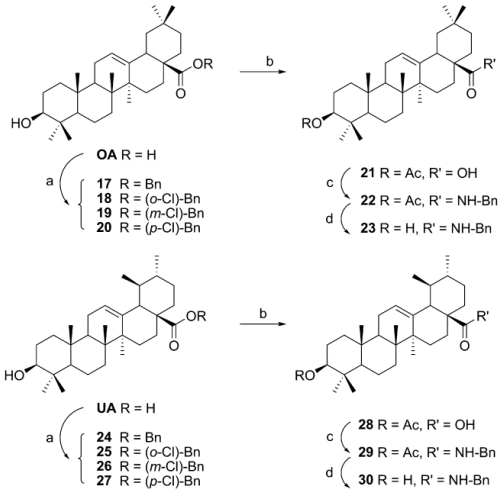


Figure 4. Synthesis of OA and UA derivatives: (a) DMF, K_2CO_3, BnBr (or chloro-substituted BnBr), 24 °C, 24 h, 55–92%; (b) Ac_2O/pyridine, NEt_3/DMAP, 24 °C, 12 h, 88% (for OA), 90% (for UA); (c) $SOCl_2$/NEt_3, then $BnNH_2$, NEt_3, 24 °C, 24 h, 61% (for OA), 86% (for UA); (d) KOH/MeOH, 24 °C, 24 h, 80% (for OA), 85.7% (for UA).

biological activity of acetylated 18-lupene derivatives is strongly influenced by the configuration and substitution pattern of rings D and E. Similar results have been obtained by Kvasnica et al.[28] for derivatives of betulinic acid and by Lu et al.[29] for amino acid conjugates of triterpenoids. Acetylated derivatives of **MA** (as well as for tormentic acid derivatives[26]) retain their ability to trigger apoptosis. Thus, compounds **2**, **4** and **12** were treated with acetyl chloride and NEt_3 in pyridine, and the corresponding diacetates **31**, **32** and **33** were obtained. These diacetates are characterized in the ^1H NMR spectra by the presence of additional sharp singulet signals ad $\delta = 2.05$ and 1.97 ppm, as well as by additional strong absorption bands ($\nu = 1254$ and 1232 cm^{-1}) in the IR spectra. The cytotoxicity data (SRB assays) of these compounds are compiled in Table 3.

Whereas for **31** and **33** cytotoxicity is significantly reduced, compound **15** exhibits strong cytotoxicity for all human tumor cells lines; for human ovarian carcinoma cells (A2780) cytotoxicity even in a nanomolar concentration was observed. A high IC_{50} value, however, was also measured for the non-malignant mouse fibroblasts NiH 3T3 (Fig. 6).

As a consequence, **OA** amides **34**–**37** were prepared in analogy to the preparation of **15** (Fig. 2) and screened for cytotoxic activity (Table 3). All of these compounds exhibited remarkable cytotoxicity especially against the human ovarian cancer cell line A2780. One might speculate that a defined but yet unknown receptor or a special transportation system seems responsible for this pronounced activity. In addition, cytotoxicity of **15** (Fig. 7) was evaluated for several additional human tumor cell lines (DLD1, Lipo and SW1736) as well as against human primary fibroblasts (WW030272).

Whereas **15** is tremendously toxic for all cancer cell lines (especially against the human ovarian cancer cell line A2780 and against melanoma 518A2), it shows a rather low cytotoxicity for the human fibroblasts (Fig. 7).

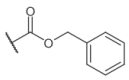
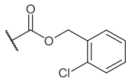
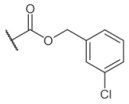
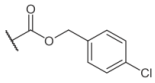
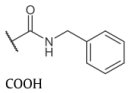
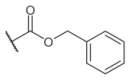
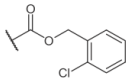
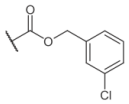
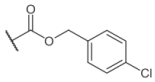
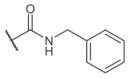
3. Biological evaluation

Cancer diseases are characterized by an uncontrolled proliferation of cells, and approximately 50% of sporadic human cancers carry a P53 gene mutation.[30] This so-called 'guardian of the genome' controls cell growing and induces a G1/G0 arrest for cells possessing genomic modifications. The growing of malignant tissue, however, strongly depends on the inability of the cells to induce apoptosis.[30,31]

In chemotherapeutic approaches for the treatment of cancer either a selective stop of cell proliferation or a controlled cell death has to be induced. Apoptosis, a program of induced cell death, is most often triggered by an activation of several cysteine-dependent aspartate-directed proteases—the caspase cascade. The effector caspases 3, 6 and 7 process several substrates,[30] among them catenins, ROCK1, ICAD or p75. As a consequence morphological changes occur, such as detachment of the cells, nuclear fragmentation and membrane blebbing, DNA fragmentation or the accumulation of ROS.[30,31]

Reyes et al.[10] proposed for **MA** a mitochondrial apoptotic mechanism (a JNK and p53 mediated pathway). In HT29 cells **MA** induces the activation of caspase 3 after an incubation time of 72 h. In this cell line, caspase 3 is already activated after 24 or 48 h. Furthermore, from investigations by Wu et al.[14] it became evident that a $[Ca^{2+}]$ dependent release of cytochrome *c* takes place. For pancreatic cancer cell lines, Li et al.[13] showed that **MA** regulates the transcription factor NF-κB. An inhibition of NF-κB in Raji cells was reported by Hsum et al.[32] Recently, Mooi et al.[6] discussed the inhibition of a protein kinase C (PKC) by **MA** in Raji

Table 2
Cytotoxicity of OA, UA, 17–20, 23–27 and 30 (IC_{50} values from SRB assays in μM together with standard error (SE), n.D. not detected) employing human tumor cell lines and non-malignant mouse fibroblasts NiH 3T3

		518A2	8505C	A2780	A549	HT29	MCF7	NIH 3T3
OA	COOH	>60	0	14.0 ± 2.3	72.3 ± 1.5	38.8 ± 3.1	>60	76.4 ± 0.7
17		28.0 ± 2.7	27.7 ± 0.6	22.9 ± 2.0	27.1 ± 2.7	28.7 ± 3.4	15.8 ± 1.5	22.4 ± 2.6
18		79.6 ± 0.7	91.0 ± 2.2	66.9 ± 1.8	86.2 ± 1.2	139.6 ± 5.2	82.5 ± 0.5	80.4 ± 4.1
19		82.0 ± 0.8	91.2 ± 1.0	64.4 ± 0.8	74.6 ± 1.3	n.D.	76.6 ± 0.2	66.0 ± 2.5
20		61.9 ± 1.5	58.3 ± 1.8	42.6 ± 1.9	49.9 ± 1.6	159.5 ± 5.9	57.6 ± 0.5	36.0 ± 2.0
23		27.3 ± 1.4	15.5 ± 1.5	5.5 ± 0.2	16.2 ± 0.4	9.8 ± 0.6	15.5 ± 1.2	13.4 ± 0.6
UA	COOH	14.7 ± 0.1	13.5 ± 0.7	11.7 ± 0.6	15.5 ± 1.3	10.6 ± 0.3	12.7 ± 0.1	18.7 ± 1.6
24		51.5 ± 1.4	43.7 ± 1.9	26.8 ± 0.9	43.0 ± 0.3	40.4 ± 3.6	32.5 ± 2.6	45.9 ± 4.4
25		>60	>60	32.9 ± 2.9	>60	>60	57.8 ± 5.6	>60
26		>120	>120	>120	>120	>120	>120	>120
27		>60	>60	52.6 ± 1.3	>60	>60	78.6 ± 2.3	>60
30		45.5 ± 4.0	45.1 ± 4.4	10.4 ± 0.1	31.7 ± 1.3	20.9 ± 0.9	n.D	26.9 ± 1.6

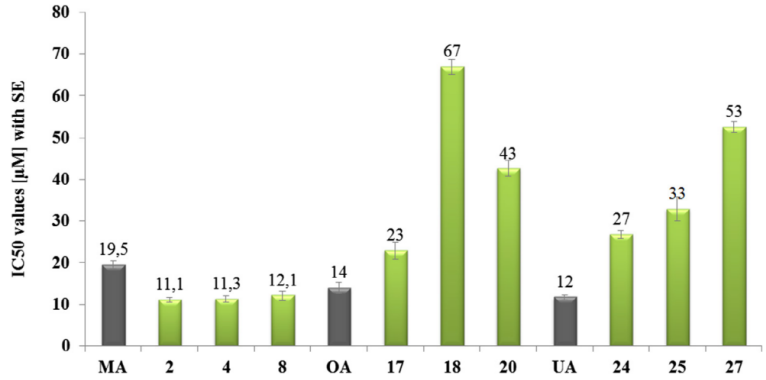
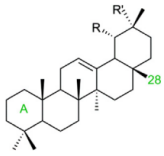
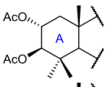
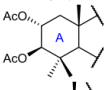
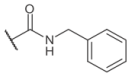
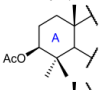
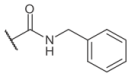
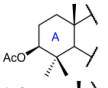
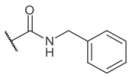
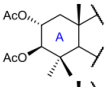
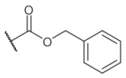
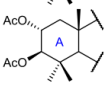
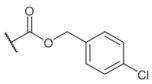
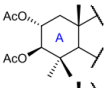
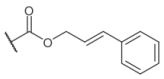
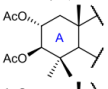
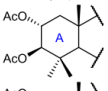
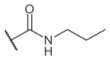
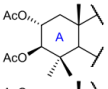
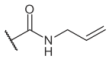
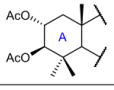
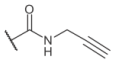


Figure 5. Cytotoxicity (from SRB, in μM) of several benzyl esters of MA (2, 4 and 8), OA (17, 18 and 20) as well as of UA (24, 25 and 27) with standard errors using the human ovarian cancer cell line A2780.

Table 3
Cytotoxicity (IC_{50} in μM from SRB assays; standard error (SE); n.d. not detected) for compounds 14, 15, 22, 29, 31–37



MA and OA: R = H, R' = CH_3
UA: R = CH_3, R' = H

IC_{50}	Ring A	Back-bone	Position 28	518A2	8505C	A2780	A549	HT29	MCF7	NiH 3T3
14		MA	H	n.d.	21.3 ± 1.3	10.5 ± 0.9	18.9 ± 1.5	25.5 ± 2.2	16.6 ± 1.5	12.2 ± 0.8
15		MA		1.5 ± 0.2	13.6 ± 0.4	0.5 ± 0.1	2.5 ± 0.1	4.7 ± 0.2	7.7 ± 0.5	33.8 ± 3.0
22		OA		9.0 ± 0.8	9.6 ± 0.7	4.3 ± 0.3	5.4 ± 0.4	27.3 ± 0.8	8.3 ± 0.6	17.7 ± 1.5
29		UA		25.3 ± 2.0	22.0 ± 2.2	17.8 ± 1.7	43.3 ± 2.0	98.7 ± 3.7	87.4 ± 5.5	>120
31		MA		41.5 ± 1.7	106.5 ± 5.5	20.5 ± 1.7	64.6 ± 5.5	>120	116.3 ± 10.1	103.7 ± 2.1
32		MA		>60	>60	>60	>60	>60	>60	> 60
33		MA		116.5 ± 2.4	>120	28.7 ± 2.5	54.9 ± 4.0	83.5 ± 3.7	56.8 ± 4.2	70.1 ± 3.4
34		MA		2.5 ± 0.3	12.9 ± 1.0	1.4 ± 0.0	5.3 ± 0.1	5.7 ± 0.4	7.8 ± 0.7	15.8 ± 1.6
35		MA		6.7 ± 0.5	7.1 ± 0.4	6.3 ± 0.5	7.2 ± 0.5	5.7 ± 0.3	6.6 ± 0.6	9.4 ± 0.8
36		MA		4.8 ± 0.1	4.4 ± 0.3	1.3 ± 0.0	7.3 ± 0.3	4.5 ± 0.3	4.2 ± 0.2	10.4 ± 0.7
37		MA		6.1 ± 0.6	6.5 ± 0.7	4.0 ± 0.4	6.7 ± 0.5	3.8 ± 0.4	6.2 ± 0.6	6.9 ± 0.7

cells following an **MA**-induced inhibition of NF-κB. It was assumed, that the inhibition of PKC activity may be explained by a previous inhibition of NF-κB. As a possible mechanism for the inhibition of NF-κB an interference of the PKC membrane translocation by **MA** was suggested. To sum up, all of these results show **MA** able to induce apoptosis but still leaving the primary target(s) of **MA** yet unknown.

Two compounds (**3** and **15**) were selected for investigating their action onto the cell cyle, and to distinguish whether they act cytotoxic or cytostatic. Thus, vital cells of A2780, NiH 3T3 and HT29 were treated with the compounds and submitted to a flow-cytometric DNA distribution assay following the procedure of Darzynkiewicz et al.[33,34] As depicted in Figure 8A and B in tumor cell lines (HT29 and A2780) a significant induction of a G1/G0 arrest was observed after having treated the cells with **15** (2 μM) for 48 h; this parallels previous findings for **MA**.[11] Compound **3** (25 μM, 24 h), however, displays a weak G1/G0 arrest in A2780 cells as well as a decrease in the S-phase. Furthermore, an increase of the G2/M phase was observed in A2780 cells. Thus, compound **3** is cytotoxic whereas **15** acts cytostatic.

Investigation of the dead cells by a dye exclusion test (AO/PI) revealed that controlled cell death has been induced (as seen by the

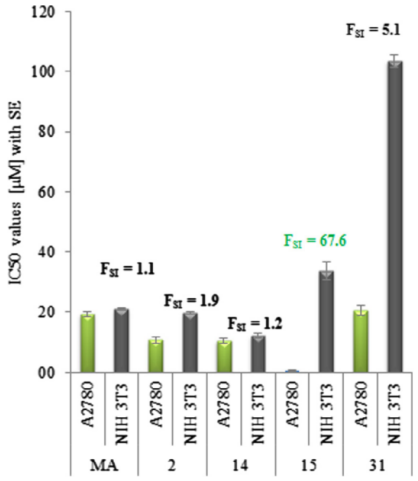


Figure 6. Selectivity of MA and compounds 2, 14, 15 and 31 (tumor cell lines vs non-malignant mouse fibroblasts NiH 3T3) of cytotoxicity (IC_{50} in µM from SRB with standard error (SE)); selectivity is defined as the quotient of IC_{50} values according to $IC_{50\ [NiH3\ 3T3]}/IC_{50\ [tumor\ cell\ line]}$.

presence of green fluorescent cells in this assay; cells possessing ruptures in the membrane—as assumed for a necrotic death—will be stained in deep red; Fig. 9A). In a DNA-laddering assay well separated 178 bp fragments were detected (Fig. 9B). This is an additional indicator for the occurrence of an apoptotic cell death.[39] During apoptosis ICAD will be activated resulting in the defined fragmentation and degeneration of DNA.[30,31]

An extra caspase assay revealed esters **2** and **3** to trigger caspase-dependent death. Crucial analysis of the data showed *p*-fluorobenzyl substituted **3** to activate the effector caspase 3 quicker than the unsubstituted ester. After an incubation period of 48 h, for both compounds caspase 8 (extrinsinic pathway) showed a higher activity (approximately 30%) than caspase 9 (intrinsic pathway). This implicates that the esters trigger a death signal probably resulting from their interaction with the membrane: Activated caspase 8, however, mediates a mitochondrial damage via bid[30] by a modulation of the mitochondrial membrane and in consequence activates caspase 9. In addition, activated caspases activate each other (potentiation loop[35]). This explains the high activity of caspase 9 and a decreasing difference for the activation of caspases 8 and 9 after 72 h. For compound **15** (after an incubation of 48 h) no increased activity of caspases was found at all. After a prolonged period of 72 h, however, and using a concentration of **15** 10 times IC_{50}, a small but significant activation was measured. Both caspases 8 and 9 exhibited activity of similar magnitude but their activity remained lower than that of the effector caspase 3.

From these results we assume that neither an interaction of **15** with a 'death receptor' nor an interaction with the mitochondria seems responsible for the initiation of the caspase cascade. These results support our findings from the investigation of the cell cycle that compound **3** acts cytotoxic whereas **15** is cytostatic.

Thus, to ascertain whether and to what extend the **MA** analogs induce apoptosis, **MA**, **2**, **3** and **15** were submitted to an extra annexin V/PI assay (48 h and 72 h, Fig. 10). Figure 10 depicts the results of this assay using the ovarian tumor cell line A2780. Treatment of the tumor cells with esters **2** or **3** increased the extent of apoptose (approximately 150% compared to control). After an additional period of 24 h for both cell populations, phosphatidyl serine was detected to be located on the membrane.

Treatment of the cells with compound **15** (2 µM) showed only a small increase of annexin positive cells (approximately 28%). Apoptosis increases in a time- and concentration-dependent manner; high concentrations (5 µM) of **15**, however, increased necrosis

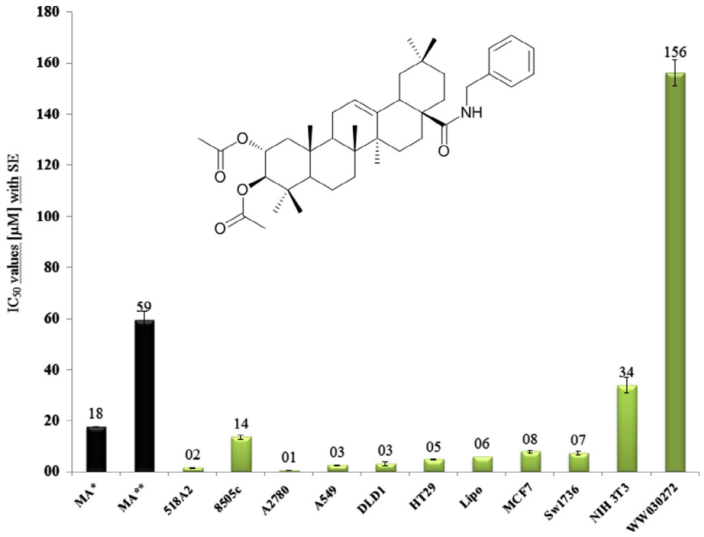


Figure 7. Comparison of cytotoxicity of compound 15 (IC_{50} in µM from SRB assays with SE) for various human tumor cell lines, non-malignant mouse fibroblasts NiH 3T3 as well as for primary human fibroblasts WW030272. MA* corresponds to an averaged IC_{50} value (IC_{50} values from all tumor cell lines), and MA** (IC_{50} for MA employing fibroblasts WW030272).

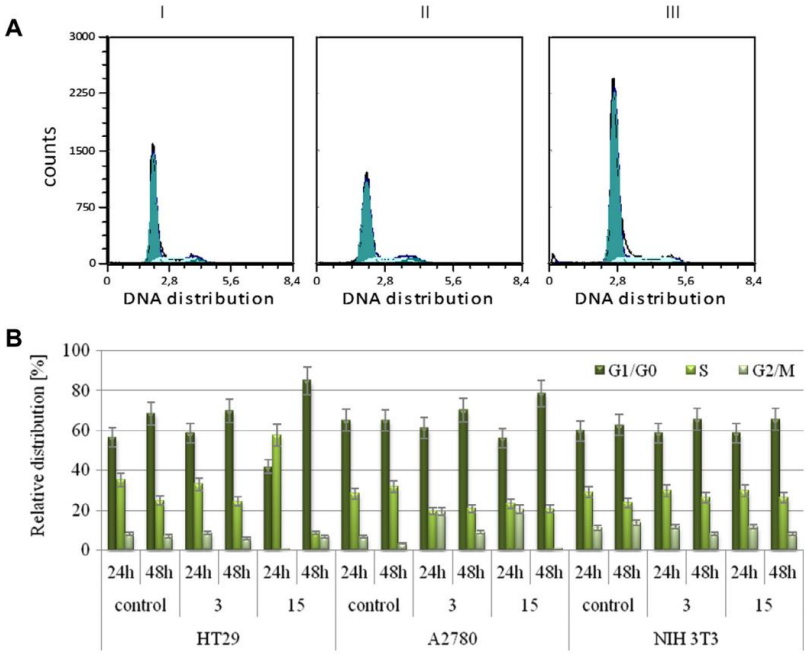


Figure 8. Upper part (A): Representative histograms from the cell cycle investigations. Histogram I shows the control distribution (A2780 cells, 48 h). In histogram II A2780 cells have been treated with 3 (25 μM, after 48 h), in histogram III the cells were treated with 15 (25 μM, after 48 h). In the lower part (B): the cell cycle distribution of living cells is shown resulting from two independent experiments (SE <10%).

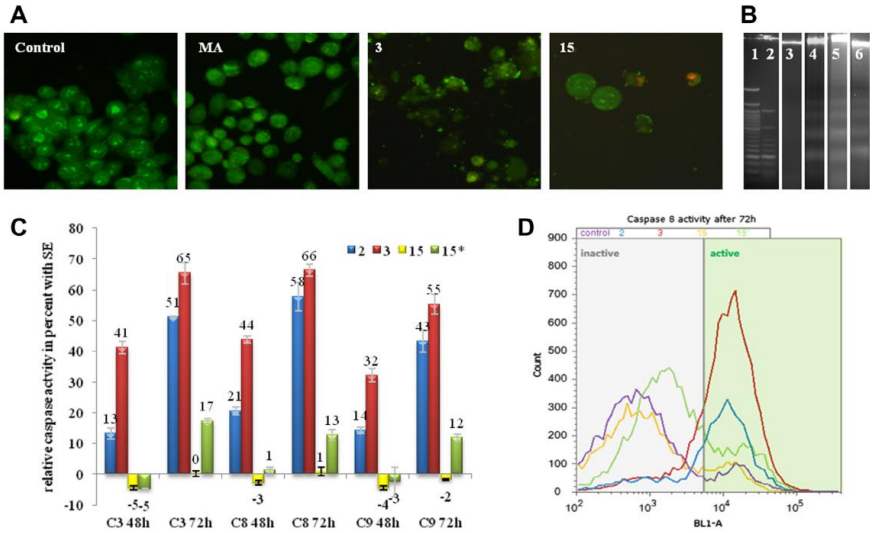


Figure 9. Upper part (A) shows the fluorescence microscopic investigation of dead A2780 cells after 24 h. (B) DNA ladders (A2780, 48–50 h) are shown. B-1 1 kbp, B-2 100 bp, B-3 control, B-4 MA (30 μM), B-5 compound 3 (25 μM), B-6 compound 15 (5 μM). Cells were treated with MA (30 μM), 3 (25 μM) or 15 (2 μM). Lower part (C). FACS assisted measurement of the caspase activity in ovarian cancer cells after having been treated with 2 (30 μM), 3 (25 μM), 15 (2 μM), 15* (5 μM). Shown is the relative activation in percent; values result from three technical replicates and are given with SE. Part (D) shows a sample graph for comparison.

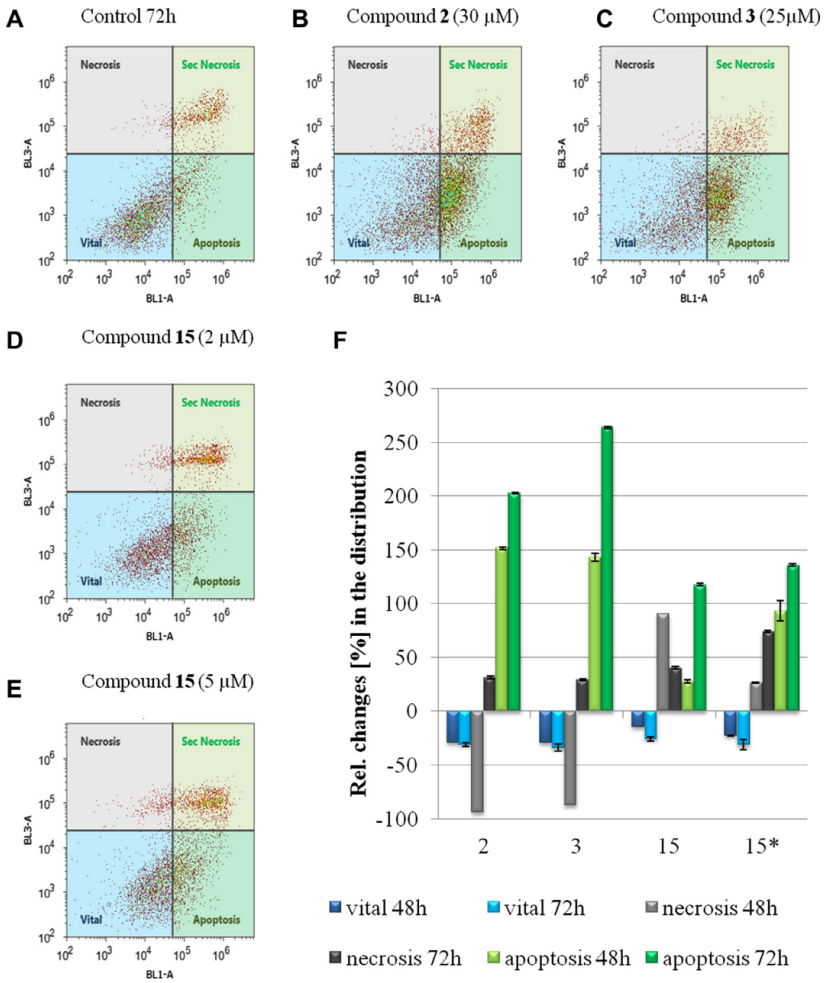


Figure 10. Annexin V assay: Ovarian cancer cells (A2780) were treated for 48 and 72 h with 2 (30 µM), 3 (25 µM), 15 (2 µM) and 15* (5 µM). Pictures A–E show representative samples after 72 h treatment: A control, B compound 2 (30 µM), C compound 3 (25 µM), D compound 15 (2 µM), E compound 15* (5 µM). Diagram F shows the relative changes in the distribution compared to the control.

(75%). This may either be due to a toxic effect or due to a PARP mediated initiation of caspases followed by necrosis.[30] For HL-60 cells, Uto et al.[36] provided experimental evidence of an activation of PARP after having been treated with corosolic acid (a constitutional isomer of **MA**).

4. Conclusions

From the results of these assays we assume that maslinic acid and compound **15** act in a similar mode. Both compounds induce G1/G0 arrest and are acting cytostatic rather than cytotoxic. Compound **3**, however, led to a very fast induction of cell death. From the close inspection of the morphological differences between cells treated with **MA**, **2**, **3** or **15** for 24 h (only weak changes are found in cells treated with **MA** or **15**, whereas cells treated with **3** show significant cell shrinking) an interaction of the compounds with the cell membrane cannot be ruled out. The cells show an altered morphology, and the induced G1/G0 arrest (following an increased G2/M phase) as well as a high number of late-apoptotic cells (found even very early) ascertain this assumption. Ziegler et al.[37] as well as Prades et al.[38] investigated the interaction of triterpenic acids with phosphatidylcholines of the cell membrane, and a correlation between the functional group at C-28 and an incorporation into the membrane was found. For compounds possessing a functional group at C-28 being capable of acting as hydrogen bond donors (COOH or $CONH_2$) incorporation into the outer leaflet membrane was revealed. For ester analogs or for compounds being unable to act as a hydrogen bond donor incorporation into the

inner leaflet of the membrane bilayer seems probable.[37] Furthermore, Prades et al.[38] assumed for triterpenic acids that they are able perturbing membrane domains being rich in cholesterol.

5. Summary

In summary, the cytotoxic activity of maslinic acid is increased by an esterification at position 28 employing lipophilic residues. Furthermore, introducing a benzylic substituent does not result in significant improvement of the cytotoxicity but seems necessary for obtaining selectivity for tumor cells. The presence of a hydroxyl group at position 2 seems necessary for obtaining good cytotoxic effects, and the selectivity in cytotoxicity between tumor and non-tumor cells is obtained by the introduction of two acetyl groups at positions 2 and 3. From the comparison of the cytotoxic activity of compounds **14**–**16** we deduce that the presence of the acetyl groups seems mandatory for obtaining selectivity whereas the nitrogen substituent at position C-28 is essential for obtaining good activity at all. Extra assays demonstrated that these derivatives of maslinic acid induce apoptosis. Incorporation of tumor cells with **15** for 48 h resulted in a G1/G0 arrest; it shows a rather low IC_{50} = 0.5 μM for A2780 ovarian cancer cells and is approximately 300 times more toxic to cancer cells than to primary human fibroblasts. This makes compound **15** a very promising candidate for further screening and evaluation.

6. Experimental

6.1. Biological material

6.1.1. Cell lines and culture conditions

The cell lines 518A2 (human melanoma cells), 8505C (human thyroid cancer cells), A2780 (human ovarian cancer cells), A549 (human lung carcinoma cells), DLD1 (human colorectal adenocarcinoma cells), Lipo (human liposarcoma), HT29 (human colorectal adenocarcinoma cells), MCF-7 (human breast cancer cells), NiH 3T3 (mouse embryonic fibroblast cells), SW1736 (human anaplastic thyroid carcinoma cells) and WW030272 (primary human fibroblast cells) were included in this study. Cultures were maintained as monolayer in RPMI 1640 (PAA Laboratories, Pasching, Germany) supplemented with 10% heat inactivated fetal bovineserum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

6.1.2. Cytotoxicity assay

The cytotoxicity of the compounds was evaluated using the sulforhodamine-B (Kiton-Red S, ABCR) microculture colorimetric assay. In short, exponentially growing cells were seeded into 96-well plates on day 0 at the appropriate cell densities (518A2 [1000 cells], 8505C [2500 cells], A549 [2500 cells], A2780 [1000 cells], DLD-1 [2000 cells], HT29 [2000 cells], Lipo [2000 cells], NIH 3T3 [3000 cells], MCF7 [2000 cells], SW1736 [2500 cells], WW030272 [3000 cells]) to prevent confluence of the cells during the period of experiment. The cells were counted using an Attune® FACS machine. After 24 h, the cells were treated with serial dilutions of the compounds (0, 1, 2, 3, 4, 6, 7, 9, 12, 14, 20, 21, 24, 28, 30, 36, 40, 48, 60, 80, 90, 120 μM) for 96 h. The final concentration of DMSO or DMF solvent never exceeded 0.5%, which was nontoxic to the cells. The percentages of surviving cells relative to untreated controls were determined 96 h after the beginning of drug exposure. After a 96 h treatment, the supernatant medium from the 96 well plates was discarded, and the cells were fixed with 10% TCA. For a thorough fixation, the plates were allowed to rest at 4 °C (min. 2 h). After fixation, the cells were washed in a strip washer. The washing was done five times with water using alternate dispensing and aspiration procedures. Afterward the plates were dyed with 100 mL of 0.4% SRB (sulforhodamine B) for about 30 min. The plates were washed with 1% acetic acid to remove the excess of the dye and allowed to air dry overnight. Tris base solution (100 mL of 10 mM) was added to each well and absorbance was measured at λ = 570 nm (using a 96 well plate reader, Tecan Spectra, Crailsheim, Germany. The IC_{50} values were determined using three technical replicates each in triplicate applying a non-linear 4P Hills-slope equation (software Prism5 and SAS JMP; variables top and bottom were set to 100 and 0, respectively).

6.1.3. Dye exclusion assay, DNA laddering and apoptosis assay

Instrumentation, dye exclusion tests and DNA fragmentation were performed as previously described.[26]

6.1.4. Cell cycle investigations

Approximately 1×10^6 cells (HT29, A2780 or NIH 3T3) were seeded in cell culture flasks (25 cm^2), and the cells were allowed to grow for 24 h. After removing of the used medium, the substance loaded medium was reloaded (or a blank fresh medium as a control). After 24 or 48 h, the living cells were harvested, washed twice with PBS (with Mg^{2+} and Ca^{2+}) and fixed with ethanol (70%, 4 °C, 1 h). After removing of the fixation and the permeabilization agents, the cells were washed with PBS buffer (with Mg^{2+} and Ca^{2+}, containing 1% BSA and 0.1% NaN_3, 3 × 1 mL, 1000 rpm) and adjusted to 1×10^5 million cells. The pellet was gently suspended in staining buffer (PBS buffer containing BSA, RNAase, NaN_3 and PI analog Darzynkiewicz et al.[33]) and incubated for 30 min at 37 °C. Analyses were performed using the Attune® FACS machine; collecting data from the BL-2A channel. Doublet cells were excluded from the measurements by plotting BL-2A against BL-2H. For each cell cycle distribution 20.000 events were collected. Distribution was calculated with FCS Express (De Novo™).

6.1.5. Annexin V/PI assay

Approximately 500,000 cells (A2780) were seeded in cell culture flasks and were allowed to grow for 1 day. After removing of the medium, the substance loaded medium was added, and the flasks were incubated for 48 and 72 h. All cells were harvested, centrifuged (1200 rpm, 5 min) and washed twice (PBS (w/w)). Approximately 100,000 cells were washed with Annexin V bounding buffer (BD) and treated with propidium iodide solution (3 μL, 50 μg/mL) and Annexin V (5 μL, life technologies™) at room temperature for 15 min in the dark. After adding Annexin V bounding buffer (400 μL) the suspension was submitted to a FACS measurement. Calculation was performed as suggested by the supplier (BD Biosiences®).

6.1.6. Caspase activity assay

Approximately 500,000 cells (A2780) were seeded in cell culture flasks and were allowed to grow for 1 day. After removing of the medium, the substance loaded medium was added, and the flasks were incubated for 48 and 72 h. All cells were harvested, centrifuged (1200 rpm, 5 min) and washed twice (PBS (w/w)). Approximately 300,000 cells were treated with Caspase substrates (Promokine, Germany) and incubated at 37 °C and 5% CO_2 in the dark for 50 min. The cell suspension was submitted to FACS assistant measurements after washing with caspase buffer (twice, Promokine, Germany). Each point was measured as a technical triplicate.

6.2. General chemistry

Reagents were bought from commercial suppliers without any further purification. Melting points were measured with a LEICA

hot stage microscope and were not corrected. NMR spectra were recorded on VARIAN Gemini 2000 or Unity 500 spectrometers at 27 °C (chemical shifts δ are given in ppm and J in Hz. Mass spectra were taken on a FINNIGAN MAT TSQ 7000 (electrospray, voltage 4.5 kV, sheath gas nitrogen) instrument. Elemental analyses were measured on a Foss-Heraeus Vario EL unit. IR spectra were recorded on a Perkin Elmer FT-IR spectrometer Spectrum 1000, optical rotations on a Perkin Elmer 341 polarimeter (1 cm micro cell, 25 °C), and UV–vis spectra on a Perkin Elmer unit, Lambda 14. TLC was performed on silica gel (Merck 5554, detection with ceriummolybdate spray reagent). Solvents were dried according to usual procedures. The purity of the compounds was checked by HPLC/DAD (Prontosil C18, MeOH/H_2O 95/5, 1% H_3PO_4) and found to be >98% for each compound.

6.2.1. General procedure for the synthesis of the esters (method A)

Maslinic acid (**MA**, 1 equiv) was dissolved in dry DMF (5 mL), and finely grounded potassium carbonate (5 equiv) was added. After 60 min of stirring at room temperature, the corresponding alkyl or benzyl bromide (2 equiv) was added, and stirring was continued for 18 h. The mixture was poured into an ice cold aq. HCl (5%, 50 mL), and the white precipitate was filtered off. Chromatographic purification (silica gel, hexane/ethyl acetate, 7:3) and recrystallization (ethanol) afforded the product.

6.2.2. Isolation of maslinic acid (MA)

Edible, green, pitted olives (6 kg in saline solution) were crushed, dried (110 °C, 24 h) and extracted with methanol (5 × 2 L for 4 days each). Usual work-up of the extract, followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 1:1) and re-crystallization (ethyl acetate) gave **MA** (5.65 g, 0.36% of dry mass).

As an alternative, black, pitted olives (2 kg) were used and **MA** (1.49 g, 0.36% of dry mass) was obtained.

Olive pomace (4 kg) was dried for 24 h at 110 °C. Methanolic extraction of the solid (1.22 kg)[39] with methanol (4 × 2 L, 4 days each) followed by usual work-up and chromatography gave **MA** (3.07 g, 0.25% of dry mass); mp: 263–267 °C (lit.: 266–269 °C[50]); R_f = 0.22 (hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +55° (*c* 0.42, $CHCl_3$) (lit.: $[\alpha]_D$ = +60° (*c* 0.1, $CHCl_3$)[40]).

6.2.3. Derivatives

6.2.3.1. (Cyclohexyl) methyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (1). Obtained from **MA** by method **A** as a colorless solid; yield: 74%; mp: 198–200 °C; R_f = 0.43 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D$ = +46° (*c* 0.32, $CHCl_3$); IR (KBr): ν = 3424br, 2928s, 1725m, 1615m, 1451m, 1385w, 1261m, 1161m, 1051m, 1034m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.21 (dd, J = 3.5, 3.5 Hz, 1H, C*H* (12)), 3.78–3.70 (m, 2H, CH_2 (31)), 3.62 (ddd, J = 11.1, 11.0, 4.5 Hz, 1H, C*H* (2)), 2.93 (d, 1H, J = 9.5 Hz, C*H* (3)), 2.81 (dd, J = 13.8, 4.2 Hz, 1H, C*H* (18)), 1.91 (dd, J = 12.5, 4.7 Hz, 1H, CH_a (1)), 1.89–1.81 (m, 3H, CH_a (16) + CH_2 (11)), 1.69–1.50 (m, 10H, C*H* (32) + CH_a (33) + CH_a (34) + CH_2 (35) + CH_a (7) + CH_a (19) + CH_b (16) + CH_a (15) + C*H* (9)), 1.50–1.31 (m, 4H, CH_a (22) + CH_b (7) + CH_2 (6)), 1.30 (ddd, J = 14.6, 10.8, 3.2 Hz, 1H, CH_a (21)), 1.25–1.07 (m, 3H, CH_b (21) + CH_b (22) + CH_b (34) + CH_b (33)), 1.12 (dd, J = 14.9, 4.8 Hz, 1H, CH_b (19)), 1.06 (s, 3H, CH_3 (27)), 0.96 (s, 3H, CH_3 (23)), 1.01–0.89 (m, 3H, CH_b (1) + CH_b (15)), 0.91 (s, 3H, CH_3 (25)), 0.86 (s, 3H, CH_3 (30)), 0.83 (s, 3H, CH_3 (29)), 0.82–0.77 (m, 1H, C*H* (5)), 0.76 (s, 3H, CH_3 (24)), 0.66 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.9 (*C*=O, C28), 144.0 (*C*=CH, C13), 122.4 (*CH*=C, C12), 84.1 (CHOH, C3), 69.6 (CH_2, C31), 69.1 (CHOH, C2), 55.5 (CH, C5), 47.8 (CH, C9), 46.9 (C_{quart}, C17), 46.6 (CH_2, C1), 46.0 (CH_2, C19), 41.9 (C_{quart}, C14), 41.5 (CH, C18), 39.6 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.5 (C_{quart}, C10), 37.3 (*CH*, C32), 34.1 (CH_2, C21), 33.3 (CH_3, C29), 32.8 (CH_2, C7), 32.7 (CH_2, C22), 30.9 (C_{quart}, C20), 29.9 (CH_2, C30), 28.8 (CH_3, C23) 27.8 (CH_2, C15), 26.6 (CH_2, C33), 26.1 (CH_3, C27), 25.9 (CH_2, C34), 23.8 (CH_3, C30), 23.6 (CH_2, C11), 23.1 (CH_2, C16), 18.5 (CH_2, C6), 17.3 (CH_3, C26), 16.9 (CH_3, C24), 16.8 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 569.5 (18%, $[2M+H]^+$), 1159.3 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{60}O_4$ (568.87): C 78.12, H 10.63; found: C 78.01, H 10.87.

6.2.3.2. Benzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (2). Obtained from **MA** by method **A** as a colorless solid; yield: 76%; mp: 163–164 °C (lit.: 164 °C[41]); R_f = 0.68 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D$ = +40.2 (*c* 0.43, $CHCl_3$) (lit.: $[\alpha]_D$ = +12 (*c* 1, $CHCl_3$),[42]); UV–vis (MeOH): λ_{max} (logε) = 234 (4.17); IR (KBr): ν = 3572br, 3318s, 2946s, 1728s, 1663m, 1498m, 1456s, 1386m, 1365m, 1303m, 1261m, 1228m, 1197m, 1176s, 1158s, 1121m, 1051s, 1034s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.23–7.21 (m, 5H, C*H* (33) + C*H* (34) + C*H* (35)), 5.22 (dd, J = 3.5, 3.5 Hz, 1H, C*H* (12)), 5.00 (d, J = 12.6 Hz, 1H, CH_a (31)), 4.89 (d, J = 12.6 Hz, 1H, CH_a (31)), 3.62 (ddd, J = 11.3, 9.6, 4.4 Hz, 1H, C*H* (2)), 2.93 (d, J = 9.5 Hz, 1H, C*H* (3)), 2.84 (dd, J = 13.7, 3.9 Hz, 1H, C*H* (18)), 1.95–1.87 (m, 2H, CH_a (16) + CH_a (1)), 1.81 (m, 2H, CH_2 (11), 1.65 (ddd, J = 13.7, 13.7, 4.6 Hz, 1H, CH_a (7)), 1.62–1.57 (m, 1H, CH_b (16)), 1.57 (dd, J = 13.6, 13.6 Hz, 1H, CH_a (19)), 1.55–1.42 (m, 4H, CH_a (15) + CH_b (7) + CH_a (6) + C*H* (9)), 1.40 -1.35 (m, 1H, CH_a (22)), 1.35–1.25 (m, 2H, CH_b (6) + CH_a (21)), 1.26–1.22 (m, 1H, CH_b (21)), 1.20–1.15 (m, 1H, CH_b (22)), 1.14–1.05 (m, 2H, CH_b (1) + CH_b (19)), 1.05 (s, 3H, CH_3 (27)), 0.96–0.93 (m, 1H, CH_b (15)), 0.95 (s, 3H, CH_3 (23)), 0.88 (s, 3H, CH_3 (25)), 0.85 (s, 3H, CH_3 (30)), 0.83 (s, 3H, CH_3 (29)), 0.85–0.80 (m, 1H, C*H* (5)), 0.75 (s, 3H, CH_3 (24)), 0.53 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (500 MHz, $CDCl_3$): δ = 177.4 (*C*=O, C28), 143.7 (*C*=CH, C13), 136.4 (*C*=CH, C32), 128.4 (*CH*=CH, C33), 127.9 (*CH*=CH, C34), 127.9 (CH=CH, C35), 122.3 (*CH*=C, C12), 83.9 (CH, C3), 68.9 (CH, C2), 65.9 (CH_2, C31), 55.3 (CH, C5), 47.6 (CH, C9), 46.7 (C_{quart}, C17), 46.4 (CH_2, C1), 45.8 (CH_2, C19), 41.7 (C_{quart}, C14), 41.3 (CH, C18), 39.3 (C_{quart}, C8), 39.1 (C_{quart}, C4), 38.3 (C_{quart}, C10), 33.8 (CH_2, C21), 33.1 (CH_3, C30), 32.6 (CH_2, C22), 32.3 (CH_2, C7), 30.7 (C_{quart}, C20), 28.7 (CH_3, C23), 27.6 (CH_2, C15), 25.9 (CH_3, C27), 23.6 (CH_3, C29), 23.4 (CH_2, C16), 23.0 (CH_2, C11), 18.3 (CH_2, C6), 16.9 (CH_3, C26), 16.7 (CH_3, C24), 16.6 (CH_3, C25) ppm; MS (ESI, source CID): m/z = 563.4 (10%, $[M+H]^+$), 585.6 (20%, $[M+Na]^+$), 1147.3 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{54}O_4$ (562.82): C 78.96, H 9.67; found: C 78.33, H 9.84.

6.2.3.3. *p*-Fluorobenzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (3). Obtained from **MA** by method **A** as a colorless solid; yield: 95%; mp: 144 °C; R_f = 0.52 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D$ = +45.8° (*c* 0.31, $CHCl_3$); IR (KBr) ν = 3438 br s, 2948vs, 2864m, 1724s, 1636w, 1622w, 1608m, 1514s, 1462m, 1386m, 1366m, 1260m, *1228s*, 1176m, 1154s,1122m, 1050s, 1034s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.31 (dd, J = 8.6 Hz, $^4J_{H,F}$ = 5.4 Hz, 2H, C*H* (33)), 7.02 (dd, J = 8.6, 8.7 Hz, 2H, C*H* (34)), 5.27 (dd, J = 3.5 Hz, 3.5 Hz, 1H, C*H* (12)), 5.05 (d, J = 12.4 Hz, 1H, CH_a (31)), 5.00 (d, J = 12.4 Hz, 1H, CH_b (31)), 3.68 (ddd, J = 11.2, 9.5 Hz, 4.5 Hz, 1H, C*H* (2)), 2.98 (d, J = 9.5 Hz, 1H, C*H* (3)), 2.88 (dd, J = 13.7, 4.0 Hz, 1H, C*H* (18)), 2.30 (br s, 2H, O*H*), 2.00–1.95 (m, 1H, CH_a (16)), 1.97 (dd, J = 12.5, 4.3 Hz, 1H, CH_a (1)), 1.87 (dd, J = 8.8 Hz, 3.5 Hz, 2H, CH_2 (11)), 1.68 (dd, J = 12.6, 12.6, 4.7 Hz, 1H, CH_a (22)), 1.68–1.61 (m, 2H, CH_a (19) + CH_b (16)), 1.60–1.50 (m, 2H, C*H* (9) + CH_a (15)), 1.49–1.42 (m, 2H, CH_b (22) + CH_a (6)), 1.40–1.35 (m, 1H, CH_a (7)), 1.36 - 1.31 (m, 1H, CH_b (16)), 1.32–1.28 (m, 1H, CH_a (21)), 1.23 (ddd, J = 12.5, 2.6, 2.6 Hz, 1H, CH_b (7)), 1.20–1.15 (m, 1H, CH_b (21)), 1.18 -1.11 (m, 1H, CH_b (19)), 1.11 (s, 3H, CH_3 (27), 1.06–1.00 (m, 1H, CH_b (15)), 1.02 (s, 3H, CH_3 (23)), 0.95 (s, 3H, CH_3 (25)), 0.95–0.85 (m, 1H, CH_b (1)), 0.91

(s, 3H, CH_3 (30), 0.89 (s, 3H, CH_3 (29)), 0.85–0.79 (m, 1H, CH (5)), 0.81 (s, 3H, CH_3 (24)), 0.56 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (500 MHz, $CDCl_3$): δ = 177.5 (C=O, C28), 162.6 (C_{aromat}F, d, J = 246.4 Hz, C35), 143.8 (C=CH, C13), 132.4 (C_{aromat}, d, J = 3.2 Hz, C32), 130.2 (CH_{aromat}, d, J = 8.2 Hz, C33), 122.4 (CH=C, C12), 115.4 (CH_{aromat}, d, J = 24.8 Hz, C34), 84.0 (CHOH, C3), 69.0 (CHOH, C2), 65.4 (CH_2, C31), 55.4 (CH, C5), 47.7 (CH, C9), 46.9 (C_{quart}, C17), 46.8 (CH_2, C1), 46.5 (CH_2, C19), 41.8 (C_{quart}, C14), 41.5 (CH, C18), 39.5 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C29), 32.7 (CH_2, C7), 31.9 (CH_2, C22), 30.8 (C_{quart}, C20), 28.8 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.8 (CH_3, C30), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.5 (CH_2, C6), 17.0 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; ^{19}F NMR (375 MHz, $CDCl_3$): δ = −114.18 (tt, 1F, ^3J = 8.6 Hz, ^4J = 5.4 Hz, CF (35)) ppm; MS (ESI, source CID): m/z = 603.6 (100%, $[M+Na]^+$), 1183.1 (41%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}FO_4$ (580.81): C 76.51, H 9.20; found: C 76.43, H 9.45.

6.2.3.4. *p*-Chlorobenzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (4). Obtained from **MA** by method A as a colorless solid; yield: 73%; mp: 105–107 °C; R_f = 0.5 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +34.5° (*c* 0.44, $CHCl_3$); IR (KBr): ν = 3438br s, 2948vs, 2864m, 1724s, 1636w, 1622w, 1608m, 1514s, 1462m, 1386m, 1366m, 1260m, 1228s, 1176m, 1154s, 1122m, 1050s, 1034s cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.35–7.26 (m, 4H, CH_{aromat}), 5.27 (dd, J = 3.3, 3.3 Hz, 1H, CH (12)), 5.06 (d, J = 12.6 Hz, 1H, CH_a (31)), 4.99 (d, J = 12.6 Hz, 1H, CH_b (31)), 3.68 (ddd, J = 11.2, 9.6, 4.4 Hz, 1H, CH (2)), 2.99 (d, J = 9.5 Hz, 1H, CH (3)), 2.88 (dd, J = 13.6, 3.6 Hz, 1H, CH (18)), 2.36–2.06 (m, 2H, OH), 2.01–1.93 (m, 2H, CH_a (1) + CH_a (16)), 1.87 (dd, J = 8.8, 3.3 Hz, 2H, CH_2 (11)), 1.70 (ddd, J = 10.1, 10.1, 5.0 Hz, 1H, CH (7)), 1.63–1.45 (m, 6H, CH (9) + CH_a (19) + CH_b (16) + CH_a (15) + CH_b (7) + CH_a (6)), 1.40–1.25 (m, 3H, CH_b (6) + CH_a (22) + CH_a (21)), 1.30–1.18 (m, 2H, CH_b (22) + CH_b (21)), 1.17–1.12 (m, 1H, CH_b (19)), 1.11 (s, 3H, CH_3 (27)), 1.05–1.00 (m, 1H, CH_b (15)), 1.02 (s, 3H, CH_3 (23)), 0.95 (s, 3H, CH_3 (25)), 0.95–0.85 (m, 1H, CH_b (1)), 0.92 (s, 3H, CH_3 (30)), 0.90 (s, 3H, CH_3 (29)), 0.85–0.79 (m, 1H, CH (5)), 0.82 (s, 3H, CH_3 (24)), 0.54 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.5 (C=O, C28), 143.8 (C=CH, C13), 135.0 (C_{aromat}, C35), 134.0 (C_{aromat}, C32), 129.7 (CH_{aromat}, C34), 128.7 (CH_{aromat}, C33), 122.5 (CH_{aromat}, C12), 84.1 (CHOH, C3), 69.1 (CHOH, C2), 65.3 (CHH_2, C31), 55.4 (CH, C5), 47.7 (CH, C9), 46.8 (C_{quart}, C17), 46.5 (CH_2, C1), 45.9 (CH_2, C19), 41.8 (C_{quart}, C14), 41.5 (CH, C18), 39.5 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C10, C_{quart}), 34.0 (CH_2, C21), 33.2 (CH_2, C7), 32.7 (CH_2, C22), 32.5 (CH_3, C29), 30.8 (C_{quart}, C20), 28.8 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.8 (CH_3, C30), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.4 (CH_2, C6), 17.0 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; ESI (Ms, MeOH, source CID): m/z = 619.5 (100%, $[M+H]^+$), 915.4 (24%, $[3\,M+K+H]^{2+}$), 1213.3 (58%, $[4\,M+K+H]^{2+}$); analysis calculated for $C_{37}H_{53}ClO_4$ (597.27): C 70.40, H 8.94; found: C 74.26, H 9.02.

6.2.3.5. *p*-Bromobenzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (5). Obtained from **MA** by method **A** as a colorless solid; yield: 83%; mp: 102–104 °C; R_f = 0.3 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +36.8° (*c* 0.20, $CHCl_3$); IR (KBr) ν = 3356br, 2948vs, 2878s, 2862s, 1726vs, 1654w, 1596w, 1490s, 1460s, 1434m, 1386s, 1378m, 1366s, 1346m, 1318m, 1302m, 1260s, 1240s, 1210m, 1194m, 1160vs, 1122s, 1094m, 1070s, 1050s, 1032s, 1012s, 994s, 756s cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.47 (d, J = 8.3 Hz, 2H, CH_{aromat} (33) + CH_{aromat} (37)), 7.21 (d, J = 8.3 Hz, 2H, CH_{aromat} (34) + CH_{aromat} (36)), 5.27 (br s, 1H, CH (12)), 5.04 (d, J = 12.6 Hz, 1H, CH_a (31)), 4.97 (d, J = 12.6 Hz, 1H, CH_b (31)), 3.69 (br s, 1H, CH (2)), 2.99 (d, J = 9.2 Hz, 1H, CH (3)), 2.88 (dd, J = 13.6, 3.5 Hz, 1H, CH (18)), 2.22–2.05 (m, 2H, OH), 2.01–1.91 (m, 2H, CH_a (1) + CH_a (16)), 1.87 (dd, J = 8.6, 3.0 Hz, 2H, CH_2 (11)), 1.75–1.67 (ddd, J = 13.7, 13.7, 4.2 Hz, 1H, CH_a (7)), 1.67–1.47 (m, 6H, CH (9) + CH_a (19) + CH_b (16) + CH_a (15) + CH_b (7) + CH_a (6)), 1.48–1.32 (m, 3H, CH_b (6) + CH_a (22) + CH_a (21)), 1.27–1.20 (m, 2H, CH_b (22) + CH_b (21)), 1.18–1.10 (m, 1H, CH_b (19)), 1.11 (s, 3H, CH_3 (27)), 1.02 (s, 3H, CH_3 (23)), 1.04–0.98 (m, 1H, CH_b (15)), 0.95 (s, 3H, CH_3 (25)), 0.95–0.85 (m, 1H, CH_b (1)), 0.91 (s, 3H, CH_3 (30)), 0.90 (s, 3H, CH_3 (29)), 0.90–0.80 (m, 1H, CH (5)), 0.82 (s, 3H, CH_3 (24)), 0.54 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.5 (C=O, C28), 143.8 (C=CH, C13), 135.5 (C_{aromat}, C35), 131.7 (CH_{aromat}, C33), 130.0 (CH_{aromat}, C34), 122.5 (CH, C12), 122.2 (C_{aromat}, C32), 84.1 (CHOH, C3), 69.1 (CHOH, C2), 65.3 (CH_2, C31), 55.4 (CH, C5), 47.7 (CH, C9), 46.9 (C_{quart}, C17), 46.5 (CH_2, C1), 45.0 (CH_2, C19), 41.9 (C_{quart}, C14), 41.5 (CH, C18), 39.5 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_2, C7), 32.7 (CH_2, C22), 32.5 (CH_3, C30), 30.8 (C_{quart}, C20), 28.8 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.8 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.4 (CH_2, C6), 17.0 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; MS (ESI, MeOH, source CID): m/z = 641.3 (66%, $[^{79}M+H]^+$), 663.1 (46.7%, $[^{81}M+H]^+$), 663.3 (50%, $[^{79}M+Na]^+$), 665.5 (53%, $[^{81}M+Na]^+$), 1304.9 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}BrO_4$ (641.72): C 69.25, H 8.32; found: C 69.17, H 8.49.

6.2.3.6. *p*-Nitrobenzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (6). Obtained from **MA** by method **A** as a colorless solid; yield: 82%; mp: 120 °C; R_f = 0.33 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +33.0° (*c* 0.37, $CHCl_3$); UV-Vis (MeOH): λ_{max} (log ε) = 269 (3.94); IR (KBr): ν = 3568br, 3386s, 3364m, 2946vs, 2878s, 2864s, 1728s, 1608m, 1524vs, 1460s, 1434m, 1386m, 1378m, 1364m, 1346vs, 1318m, 1302m, 1260m, 1240m, 1210m, 1194m, 1160s, 1122m, 1110m, 1094m, 1082m, 1050s, 1034s, 1014m cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.20 (d, J = 8.6 Hz, 2H, CH (34)), 7.50 (d, J = 8.4 Hz, 2H, CH (33)), 5.30 (dd, J = 3.3, 3.3 Hz, 1H, CH (12)), 5.14 (d, J = 13.5 Hz, 1H, CH_a (31)), 5.10 (d, J = 13.5 Hz, 1H, CH_b (31)), 3.68 (ddd, J = 10.8, 9.4, 4.2 Hz, 1H, CH (2)), 2.99 (d, J = 9.4 Hz, 1H, CH (3)), 2.89 (dd, J = 13.6, 3.8 Hz, 1H, CH (18)), 1.98 (ddd, J = 14.0, 12.8, 4.2 Hz, 1H, CH_a (16)), 1.95 (dd, J = 12.8, 4.2 Hz, 1H, CH_a (1)), 1.86 (dd, J = 7.4, 3.3 Hz, 2H, CH_2 (11)), 1.72 (ddd, J = 13.7, 13.8, 4.3 Hz, 1H, CH_a (7)), 1.68–1.61 (m, 2H, CH_b (16) + CH_a (19)), 1.61–1.53 (m, 3H, CH_b (7) + CH_a (22) + CH (9)), 1.52–1.48 (m, 1H, CH_a (6)), 1.47–1.44 (m, 1H, CH_a (15)), 1.38–1.28 (m, 2H, CH_b (6) + CH_a (21)), 1.26–1.17 (m, 2H, CH_b (21) + CH_b (22)), 1.18–1.12 (m, 1H, CH_b (19)), 1.12 (s, 3H, CH_3 (27)), 1.09–1.01 (ddd, 1H, J = 13.5, 3.5, 3.0 Hz, CH_b (15)), 1.01 (s, 3H, CH_3 (23)), 0.95–0.90 (m, 1H, CH_b (1)), 0.92 (s, 6H, CH_3 (30) + CH_3 (25)), 0.91–0.85 (m, 1H, CH (5)), 0.90 (s, 3H, CH_3 (29)), 0.80 (s, 3H, CH_3 (24)), 0.54 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.3 (C=O, C28), 147.6 (CNO_2, C35), 143.6 (C=CH, C13), 143.7 (C_{aromat}, C32), 128.6 (CH_{aromat}, C33), 123.8 (CH_{aromat}, C34), 122.6 (CH=C, C12), 84.0 (CHOH, C3), 69.0 (CHOH, C2), 64.7 (CH_2, C31), 55.4 (CH, C5), 47.6 (CH, C9), 47.0 (C_{quart}, C17), 46.5 (CH_2, C1), 45.9 (CH_2, C19), 41.9 (C_{quart}, C14), 41.5 (CH, C18), 39.5 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 33.9 (CH_2, C21), 33.2 (CH_3, C30), 32.7 (CH_2, C22), 32.6 (CH_2, C7), 30.8 (C_{quart}, C20), 28.7 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.7 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.4 (CH_2, C6), 17.0 (CH_3, C26), 16.9 (CH_3, C24), 16.6 (CH_3, C25) ppm; MS (ESI, MeOH, source CID): m/z = 630.5 (100%, $[M+Na]^+$), 1237.1 (95%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}NO_6$ (607.82): C 73.11, H 8.79, N 2.30; found: C 73.02, H 8.91, N 2.17.

6.2.3.7. *p*-Methoxybenzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (7). Obtained from **MA** by method **A** as a colorless solid; yield: 80%; mp: 178–179 °C; R_f = 0.21 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +46° (*c* 0.32, $CHCl_3$); IR (KBr): ν = 3313br, 2943ss, 1728s, 1615m, 1515s, 1465m, 1385m, 1248s, 1158s, 1120m,

1034s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.31–7.27 (m, 2H, C*H* (33)), 6.92–6.86 (m, 2H, C*H* (34)), 5.30 (dd, *J* = 3.5, 3.5 Hz, 1H, C*H* (12)), 5.05 (d, *J* = 12.1 Hz, 1H, CH_a (31)), 5.00 (d, *J* = 12.1 Hz, 1H, CH_b (31)), 3.83 (s, 3H, CH_3 (36)), 3.71 (ddd, *J* = 11.1, 9.8, 4.5 Hz, 1H, C*H* (2)), 3.02 (d, *J* = 9.5 Hz, 1H, C*H* (3)), 2.91 (dd, *J* = 13.8, 4.0 Hz, 1H, C*H* (18)), 2.00 (dd, *J* = 12.7, 4.4 Hz, 1H, CH_a (1)), 1.97–1.86 (m, 3H, CH_a (16) + CH_2 (11)), 1.73 (ddd, *J* = 13.9, 13.9, 4.2 Hz, 1H, CH_a (7)), 1.69–1.51 (m, 6H, CH_a (19) + CH_a (15) + CH_b (16) + CH_a (6) + CH_a (22) + C*H* (9)), 1.50–1.41 (m, 2H, CH_b (7) + CH_b (6)), 1.33 (ddd, *J* = 14.2, 10.4, 8.1 Hz, 1H, CH_a (21)), 1.24–1.15 (m, 3H, CH_b (22) + CH_b (19) + CH_b (21)), 1.14 (s, 3H, CH_3 (27)), 1.05 (s, 3H, CH_3 (23)), 0.98 (s, 3H, CH_3 (25)), 1.04–0.86 (m, 2H, CH_b (1) + CH_b (15)), 0.94 (s, 3H, CH_3 (30)), 0.92 (s, 3H, CH_3 (29)), 0.91–0.85 (m, 1H, C*H* (5)), 0.85 (s, 3H, CH_3 (24)), 0.62 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.5 (C28, *C*=O), 159.4 (C35, *C*OCH_3), 143.8 (C13, *C*=CH), 129.8 (C33, CH_{aromat}), 128.6 (C32, $C_{quart.}$), 122.3 (C12, *C*H=C), 113.8 (C34, CH_{aromat}), 84.0 (C3, *C*HOH), 69.0 (C2, *C*HOH), 65.7 (C31, CH_2), 55.3 (C36, CH_3), 47.6 (C5, *C*H), 46.7 (C17, C_{quart}), 46.4 (C1, CH_2), 46.4 (C9, *C*H), 45.9 (C19, CH_2), 41.7 (C14, C_{quart}), 41.4 (C18, *C*H), 39.3 (C8, C_{quart}), 39.1 (C4, C_{quart}), 38.3 (C10, C_{quart}), 33.8 (C21, CH_2), 33.1 (C29, CH_3), 32.6 (C7, CH_2), 32.3 (C22, CH_2), 30.7 (C20, C_{quart}), 28.6 (C23, CH_3), 27.6 (C15, CH_2), 25.9 (C27, CH_3), 23.7 (C30, CH_3), 23.4 (C16, CH_2), 23.0 (C11, CH_2), 18.3 (C6, CH_2), 16.9 (C26, CH_3), 16.7 (C24, CH_3), 16.6 (C25, CH_3) ppm; MS (ESI, MeOH): *m*/*z* = 615.5 (44%, $[M+Na]^+$), 1207.1 (100%, $[2M+Na]^+$); analysis calculated for $C_{38}H_{56}O_5$ (592.84): C 76.99, H 9.52; found: C 76.87, H 9.65.

6.2.3.8. *o*-Chlorobenzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (8). Obtained from **MA** by method **A** as a colorless solid; yield: 82%; mp: 113 °C; R_f = 0.4 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +35.1° (*c* 0.32, $CHCl_3$); IR (KBr): *ν* = 3374s, 2946vs, 2878s, 2864s, 1726s, 1462s, 1454s, 1386m, 1366m, 1302m, 1260s, 1234m, 1210m, 1194m, 1160s, 1124m, 1094m, 1082m, 1052s, 1032s, 1012m, 752s cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.43 (dd, *J* = 5.5, 3.9 Hz, 1H, C*H* (37)), 7.40–7.34 (ddd, *J* = 6.1, 3.6, 3.6 Hz, 1H, C*H* (35)), 7.27–7.22 (m, 2H, C*H* (34) + C*H* (36)), 5.29 (dd, *J* = 3.3, 3.3 Hz, 1H, C*H* (12)), 5.18 (d, *J* = 13.1 Hz, 1H, CH_a (31)), 5.14 (d, *J* = 13.1 Hz, 1H, CH_b (31)), 3.69 (ddd, *J* = 10.5, 9.6, 3.9 Hz, 1H, C*H* (2)), 3.00 (d, *J* = 9.4 Hz, 1H, C*H* (3)), 2.92 (dd, *J* = 13.7, 3.9 Hz, 1H, C*H* (18)), 2.05–1.93 (m, 1H, CH_a (16)), 1.96 (dd, *J* = 12.0, 3.8 Hz, 1H, CH_a (1)), 1.87 (dd, *J* = 8.8, 3.3 Hz, 2H, CH_2 (11)), 1.74 (ddd, *J* = 13.7, 13.7, 4.3 Hz, 1H, CH_a (7)), 1.72–1.67 (m, 1H, CH_b (16)), 1.67–1.60 (m, 2H, CH_a (19) + CH_a (15)), 1.61–1.53 (m, 2H, C*H* (9) + CH_a (22)), 1.55–1.48 (m, 1H, CH_a (6)), 1.47–1.40 (m, 1H, CH_b (7)), 1.39–1.28 (m, 2H, CH_b (6) + CH_a (21)), 1.28–1.20 (m, 2H, CH_b (22) + CH_b (21)), 1.18–1.13 (m, 1H, CH_b (19)), 1.13 (s, 3H, CH_3 (27)), 1.08–1.02 (m, 1H, CH_b (15)), 1.06 (s, 3H, CH_3 (23), 0.94 (s, 3H, CH_3 (25)), 0.95–0.86 (m, 1H, CH_b (1)), 0.93 (s, 3H, CH_3 (30)), 0.89 (s, 3H, CH_3 (29)), 0.84–0.80 (m, 1H, C*H* (5)), 0.81 (s, 3H, CH_3 (24)), 0.58 (s,s, 3H, CH_3 (26)) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.5 (*C*=O, C28), 143.8 (*C*=CH, C13), 134.2 (C_{aromat}, C13), 133.9 (CCl_{aromat}, C33), 130.2 (CH_{aromat}, C35), 129.6 (CH_{aromat}, C37), 129.4 (CH_{aromat}, C34), 126.9 (CH_{aromat}, C36), 122.5 (*C*H=C, C12), 84.0 (*C*HOH, C3), 69.06 (*C*HOH, C2), 63.6 (CH_2, C31), 55.4 (*C*H, C5), 47.7 (*C*H, C9), 47.0 (C_{quart}, C17), 46.5 (CH_2, C1), 46.0 (CH_2, C19), 41.8 (C_{quart}, C14), 41.5 (*C*H, C18), 39.5 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_2, C7), 32.7 (CH_2, C22), 32.5 (CH_3, C30), 30.8 (C_{quart}, C20), 28.7 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.7 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.5 (CH_2, C6), 17.0 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; MS (ESI, MeOH, source CID): *m*/*z* = 619.7 (100%, $[M+Na]^+$), 1215.2 (95%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}ClO_4$ (597.37): C 74.40, H 8.94; found: C 74.11, H 9.10.

6.2.3.9. *m*-Chlorobenzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (9). Obtained from **MA** by method **A** as a colorless solid; yield: 73%; mp: 110 °C; R_f = 0.37 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +41.5° (*c* 0.32, $CHCl_3$); IR (KBr): *ν* = 3438br, 2948vs, 2864m, 1724s, 1636w, 1622w, 1608m, 1514s, 1462m, 1386m, 1366m, 1260m, 1228s, 1176m, 1154s, 1122m, 1050s, 1034s cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.33 (br s, 1H, C*H* (33)), 7.30–7.25 (m, 1H, C*H* (36)), 7.26–7.23 (m, 1H, C*H* (35)), 7.21–7.18 (m, 1H, C*H* (37)), 5.29 (dd, *J* = 3.4, 3.4 Hz, 1H, C*H* (12)), 5.06 (d, *J* = 12.7 Hz, 1H, CH_a (31)), 4.98 (d, *J* = 12.7 Hz, 1H, CH_b (31)), 3.73–3.63 (m, 1H, C*H* (2)), 2.98 (d, *J* = 9.0 Hz, 1H, C*H* (3)), 2.89 (dd, *J* = 13.7, 3.9 Hz, 1H, CH (18)), 2.07 (br s, 2H, O*H*), 2.03–1.92 (m, 1H, CH_a (1)), 1.96 (dd, *J* = 13.1, 13.1, 3.7 Hz, 1H, CH_a (16)), 1.88 (br s, 2H, CH_2 (11)), 1.76–1.65 (ddd, *J* = 13.7, 13.7, 4.3 Hz, 1H, CH_a (7)), 1.61–1.57 (m, 1H, CH_b (16)), 1.63–1.57 (m, 1H, CH_a (19)), 1.57–1.53 (m, 1H, CH_a (15)), 1.55–1.47 (m, 2H, C*H* (9) + CH_a (22)), 1.50–1.48 (m, 1H, CH_a (6)), 1.46–1.38 (m, 1H, CH_b (7)), 1.38–1.28 (m, 2H, CH_b (6) + CH_a (21)), 1.27 (m, 1H, CH_b (22)), 1.22–1.18 (m, 1H, CH_b (21)), 1.20–1.11 (m, 1H, CH_b (19)), 1.11 (s, 3H, CH_3 (27)), 1.05–0.98 (m, 1H, CH_b (15)), 1.01 (s, 3H, CH_3 (23)), 0.94 (s, 3H, CH_3 (25)), 0.95–0.86 (m, 1H, CH_b (1)), 0.91 (s, 3H, CH_3 (30)), 0.89 (s, 3H, CH_3 (29)), 0.86–0.82 (m, 1H, C*H* (5)), 0.80 (s, 3H, CH_3 (24)), 0.55 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.5 (*C*=O, C28), 143.8 (*C*=CH, C13), 138.5 (C_{aromat}, C32), 134.5 (CCl_{aromat}, C34), 129.8 (CH_{aromat}, C36), 128.2 (CH_{aromat}, C35), 128.2 (CH_{aromat}, C33), 126.2 (CH_{aromat}, C37), 122.6 (*C*H=C, C12), 84.1 (*C*HOH, C3), 69.1 (*C*HOH, C2), 65.2 (CH_2, C31), 55.4 (*C*H, C5), 47.7 (*C*H, C9), 46.9 (C_{quart}, C17), 46.5 (CH_2, C1), 46.0 (CH_2, C19), 41.9 (C_{quart}, C14), 41.5 (*C*H, C18), 39.5 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C29), 32.7 (CH_2, C22), 32.6 (CH_2, C7), 30.8 (C_{quart}, C20), 28.8 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.8 (CH_3, C30), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.5 (CH_2, C6), 17.0 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; MS (ESI, MeOH, source CID): *m*/*z* = 619.5 (100%, $[M+Na]^+$), 1215.1 (95%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}ClO_4$ (597.27): C 74.40, H 8.94; found: C 74.29, H 9.09.

6.2.3.10. 2,4-Dichlorobenzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (10). Obtained from **MA** by method **A** as a colorless solid; yield: 65%; mp: 122 °C; R_f = 0.21 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +36.0° (*c* 0.31, $CHCl_3$); IR (KBr): *ν* = 3440br, 3424vs, 2946vs, 2878m, 2864m, 1728s, 1474s, 1464s, 1386m, 1366m, 1260m, 1240m, 1234m, 1208m, 1160s, 1122m, 1104m, 1082m, 1052s, 1032s cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.40 (d, *J* = 2.2 Hz, 1H, C*H* (34)), 7.37 (d, *J* = 8.4 Hz, 1H, C*H* (37)), 7.23 (dd, *J* = 8.2, 2.0 Hz, 1H, C*H* (36)), 5.27 (br s, 1H, C*H* (12)), 5.15 (d, *J* = 13.1 Hz, 1H, CH_a (31)), 5.08 (d, *J* = 13.1 Hz, 1H, CH_b (31)), 3.69 (br s, 1H, C*H* (2)), 3.00 (d, *J* = 9.4 Hz, 1H, C*H* (3)), 2.89 (dd, *J* = 13.7, 3.7 Hz, 1H, C*H* (18)), 1.98 (ddd, *J* = 13.3, 13.3 Hz, 4.3 Hz, 1H, CH_a (16)), 1.98–1.92 (m, 1H, CH_a (1)), 1.86 (dd, *J* = 10.1, 3.3 Hz, 2H, CH_2 (11)), 1.72 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 2H, CH_a (7)), 1.67–1.61 (m, 1H, CH_b (16)), 1.65 (dd, *J* = 14.1, 14.1 Hz, 1H, CH_a (19)), 1.63–1.55 (m, 2H, CH_a (15) + C*H* (9)), 1.57–1.51 (m, 2H, CH_a (6) + CH_a (22)), 1.47–1.40 (m, 1H, CH_b (7)), 1.38–1.28 (m, 2H, CH_b (6) + CH_a (21)), 1.28–1.20 (m, 2H, CH_b (22) + CH_b (21)), 1.18–1.13 (m, 1H, CH_b (19)), 1.14 (s, 3H, CH_3 (27)), 1.05–1.00 (m, 1H, CH_b (15)), 1.02 (s, 3H, CH_3 (23)), 0.94 (s, 3H, CH_3 (25)), 0.95–0.86 (m, 1H, CH_b (1)), 0.93 (s, 3H, CH_3 (30)), 0.90 (s, 3H, CH_3 (29)), 0.84–0.80 (m, 1H, C*H* (5)), 0.82 (s, 3H, CH_3 (24)), 0.53 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.4 (*C*=O, C28), 143.7 (*C*=CH, C13), 134.8 (C_{aromat}, C35), 134.7 (C_{aromat}, C33), 132.8 (C_{aromat}, C32), 131.3 (CH_{aromat}, C37), 129.5 (CH_{aromat}, C34), 127.2 (CH_{aromat}, C36), 122.6 (*C*H=C, C12), 84.1 (*C*HOH, C3), 69.1 (*C*HOH, C2), 63.0 (CH_2, C31), 55.4 (*C*H, C5), 47.7 (*C*H, C9), 47.0

(C_{quart}, C17), 46.5 (CH_2, C1), 46.0 (CH_2, C19), 41.9 (C_{quart}, C14), 41.5 (*C*H, C18), 39.5 (C_{quart}, C8), 38.6 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_2, C7), 32.7 (CH_2, C22), 32.6 (CH_3, C30), 30.8 (C_{quart}, C20), 28.6 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.7 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.5 (CH_2, C6), 17.0 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; MS (ESI, MeOH, source CID): m/z = 653.3 (100%, $[M+Na]^+$), 1284.9 (78%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{52}Cl_2O_4$ (631.71): C 70.35, H 8.30; found: C 70.27, H 8.41.

6.2.3.11. (4-Oxa-4-phenyl)-butyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (11). Obtained from **MA** and 3-phenoxypropyl bromide by method **A** as a colorless solid; yield: 77%; mp: 166–170 °C; R_f = 0.4 (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D$ = +45.8° (*c* 0.33, $CHCl_3$); IR (KBr): ν = 3424vs, 2946vs, 2930vs, 2878m, 2864m, 1726vs, 1602m, 1498m, 1470s, 1458m, 1384s, 1364m, 1242s, 1202m, 1180m, 1172m, 1162s, 1124m, 1080m, 1052s, 1034m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.30–7.24 (m, 2H, CH_{aromat}), 6.94 (dd, *J* = 7.3, 7.3 Hz, 1H, CH_{aromat}), 6.88 (d, *J* = 8.0 Hz, 2H, CH_{aromat}), 5.25 (dd, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 4.27–4.14 (m, 2H, CH_2 (33)), 4.03 (ddd, *J* = 6.1, 6.1, 1.5 Hz, 2H, CH_2 (31)), 3.68 (ddd, *J* = 11.5, 9.7, 4.4 Hz, 1H, *CH* (2)), 2.99 (d, *J* = 9.5 Hz, 1H, *CH* (3)), 2.87 (dd, *J* = 13.8, 3.8 Hz, 1H, *CH* (18)), 2.18 (br s, 2H, O*H*), 2.15–2.06 (m, 2H, CH_2 (32)), 2.00–1.86 (m, 2H, CH_a (16) + CH_a (1)), 1.83 (dd, *J* = 8.8, 3.4 Hz, 2H, CH_2 (11)), 1.75–1.66 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 1H, CH_a (7)), 1.67–1.58 (m, 3H, CH_a (19) + CH_a (15) + CH_b (16)), 1.57 (m, 1H, *CH* (9)), 1.54–1.45 (m, 2H, CH_a (22) + CH_a (6)), 1.43–1.35 (m, 1H, CH_b (7)), 1.33–1.23 (m, 2H, CH_a (21) + CH_b (6)), 1.22–1.14 (m, 3H, CH_b (19) + CH_b (21) + CH_b (22)), 1.11 (s, 3H, CH_3 (27)), 1.02 (s, 3H, CH_3 (23)), 1.05–0.95 (m, 1H, CH_b (15)), 0.92 (s, 3H, CH_3 (25)), 0.90 (s, 3H, CH_3 (30)), 0.89 (s, 3H, CH_3 (29)), 0.88–0.80 (m, 1H, CH_b (1)), 0.81 (s, 3H, CH_3 (24)), 0.80 (m, 1H, *CH* (5)), 0.66 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 Hz, $CDCl_3$): δ = 177.8 (*C*=O, C28), 158.9 (C_{aromat}, C34), 144.0 (*C*=CH, C13), 129.6 (CH_{aromat}, C35), 122.4 (*CH*=C, C12), 120.9 (CH_{aromat}, C36), 114.5 (CH_{aromat}, C37), 84.1 (*C*HOH, C3), 69.1 (CHOH, C2), 64.3 (CH_2, C31), 61.1 (CH_2, C33), 55.4 (*C*H, C5), 47.7 (*C*H, C9), 46.9 (C_{quart}, C17), 46.5 (CH_2, C1), 46.0 (CH_2, C19), 41.9 (C_{quart}, C14), 41.4 (*C*H, C18), 39.5 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C30), 32.7 (CH_2, C7), 32.6 (CH_2, C22), 30.9 (C_{quart}, C20), 28.8 (CH_2, C32), 28.8 (CH_3, C23), 27.7 (CH_2, C15), 26.1 (CH_3, C27), 23.8 (CH_3, C29), 23.6 (CH_2, C11), 23.1 (CH_2, C16), 18.5 (CH_2, C6), 17.1 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; MS (ESI, MeOH, source CID): m/z = 607.3 (20%, $[M+H]^+$), 629.3 (100%, $[M+Na]^+$), 929.3 (60%, $[3\,M+K+H]^{2+}$); analysis calculated for $C_{39}H_{58}O_5$ (606.87): C 77.19, H 9.63; found: C 76.89, H 9.74.

6.2.3.12. *(E)* (3-Phenyl)-prop-2-enyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (12). Obtained from **MA** by method **A** as a colorless solid; yield: 75%; mp: 186–188 °C; R_f = 0.3 (*n*-hexane/ethyl acetate, 5:3); $[\alpha]_D$ = +45.7° (*c* 0.32, $CHCl_3$); UV–vis ($CHCl_3$): 254 (4.22), 293 (2.98), 283 (3.20) nm; IR (KBr): ν = 3574m, 3422m, 2944vs, 2864s, 1724vs, 1464m, 1452m, 1386m, 1364m, 1260m, 1200m, 1178s, 1160s, 1122m, 1082m, 1066m, 1052s, 1034s, 1010m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): ν = 7.40–7.23 (m, 5H, CH_{aromat}), 6.64 (d, *J* = 15.9 Hz, 1H, *CH* (33)), 6.26 (ddd, *J* = 15.9, 6.3, 6.3 Hz, 1H, *CH* (32)), 5.31 (dd, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 4.71 (ddd, *J* = 12.9, 6.2, 1.2 Hz, 1H, CH_a (31)), 4.67 (ddd, *J* = 13.1, 6.2, 1.2 Hz, 1H, CH_b (31)), 3.68 (ddd, *J* = 11.0, 9.6, 4.6 Hz, 1H, *CH* (2)), 2.99 (d, *J* = 9.4 Hz, 1H, *CH* (3)), 2.91 (dd, *J* = 13.8, 4.2 Hz, 1H, *CH* (18)), 2.12 (br s, 1H, O*H*), 2.04 (br s, 1H, OH), 2.03–1.93 (m, 2H, CH_a (16) + CH_a (1)), 1.93–1.87 (m, 2H, CH_2 (11)), 1.73 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 1H, CH_a (7)), 1.73–1.60 (m, 2H, CH_a (15) + CH_b (16)), 1.64 (dd, *J* = 13.4, 13.4 Hz, 1H, CH_a (19)), 1.59–1.53 (m, 1H, CH_a (22)), 1.53–1.47 (m, 1H, CH_a (6)), 1.45–1.40 (m, 1H, CH_b (22)), 1.39–1.30 (m, 2H, CH_b (6) + CH_a (21)), 1.30–1.25 (m, 1H, CH_b (7)), 1.24–1.14 (m, 2H, CH_b (21) + CH_b (19)), 1.13 (s, 3H, CH_3 (27)), 1.06 (m, 1H, CH_b (15)), 1.02 (s, 3H, CH_3 (23)), 0.94 (s, 3H, CH_3 (25)), 0.92 (s, 3H, CH_3 (30)), 0.89 (s, 3H, CH_3 (29)), 0.85 (m, 2H, CH_b (1) + *CH* (5)), 0.81 (s, 3H, CH_3 (24)), 0.72 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (500 MHz, $CDCl_3$): δ = 177.6 (*C*=O, C28), 144.0 (*C*=CH, C13), 136.6 (C_{aromat}, C35), 133.8 (*CH*=CH, C33), 128.7 (CH_{aromat}, C37), 128.1 (CH_{aromat}, C36), 126.7 (CH_{aromat}, C38), 123.8 (*CH*=CH, C32), 122.4 (CH=C, C12), 84.1 (CHOH, C3), 69.1 (*C*HOH, C2), 64.8 (CH_2, C31), 55.4 (*C*H, C5), 47.8 (CH, C9), 46.9 (C_{quart}, C17), 46.5 (CH_2, C1), 46.0 (CH_2, C19), 41.9 (C_{quart}, C14), 41.5 (*C*H, C18), 39.6 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.0 (CH_2, C21), 33.3 (CH_3, C30), 32.8 (CH_2, C7), 32.6 (CH_2, C22), 30.9 (C_{quart}, C20), 28.8 (CH_3, C23), 27.8 (CH_2, C15), 26.1 (CH_3, C27), 23.8 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.5 (CH_2, C6), 17.3 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 589.3 (16%, $[M+H]^+$), 611.3 (26%, $[M+Na]^+$), 1199.4 (100%, $[2M+Na]^+$); analysis calculated for $C_{39}H_{56}O_4$ (588.86): C 79.95, H 9.59; found: C 79.67, H 9.68.

6.2.3.13. (3-Phenyl)-propyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-oate (13). Hydrogenation (6 bar) of **12** (80 mg, 0.14 mmol) in $CHCl_3$/MeOH (2:1, 5 mL) in the presence of palladium catalyst (10% on carbon) followed by usual work-up gave **13** (70 mg, 85%) as a colorless solid; mp: 169–170 °C; R_f = 0.2 (*n*-hexane/ethyl acetate, 6:4); $[\alpha]_D$ = +41.7° (*c* 0.36, $CHCl_3$); IR (KBr): ν = 3422vs, 2942s, 2926s, 2862m, 1734s, 1726s, 1720s, 1654m, 1474s, 1458s, 1386m, 1364m, 1262m, 1228m, 1200m, 1178m, 1160s, 1124m, 1052m, 1034s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.29 (dd, *J* = 7.4 Hz, 2H, *CH* (37)), 7.20 (d, *J* = 7.3 Hz, 1H, *CH* (38)), 7.17 (dd, *J* = 7.0 Hz, 2H, *CH* (36)), 5.31 (dd, *J* = 3.6 Hz, 1H, *CH* (12)), 4.04 (*t*, *J* = 6.3 Hz, 2H, CH_2 (31)), 3.73–3.66 (ddd, *J* = 11.2, 9.7, 4.4 Hz, 1H, *CH* (2)), 3.02 (d, *J* = 9.5 Hz, 1H, *CH* (3)), 2.90 (dd, *J* = 13.8, 4.5 Hz, 1H, *CH* (18)), 2.70 (*t*, *J* = 7.7 Hz, 2H, CH_2 (33)), 2.52 (br s, 2H, O*H*), 2.02–1.88 (m, 6H, CH_a (1) + CH_2 (32) + CH_2 (11) + CH_a (16)), 1.72 (ddd, *J* = 13.9, 13.9, 4.5 Hz, 1H, *CH* (7)), 1.68–1.57 (m, 4H, *CH* (9) + CH_a (19) + CH_a (15) + CH_b (16)), 1.54–1.48 (m, 2H, CH_a (6) + CH_b (7)), 1.43–1.36 (m, 2H, CH_b (6) + CH_a (22)), 1.36–1.28 (m, 2H, CH_a (21) + CH_b (22)), 1.23–1.17 (m, 1H, CH_b (21)), 1.16–1.12 (m, 1H, CH_b (19)), 1.14 (s, 3H, CH_3 (27)), 1.09–1.05 (m, 1H, CH_b (15)), 1.03 (s, 3H, CH_3 (23)), 0.97 (s, 3H, CH_3 (25)), 0.94 (s, 3H, CH_3 (30)), 0.91 (s, 3H, CH_3 (29)), 0.93–0.88 (m, 1H, CH_b (1)), 0.86–0.81 (m, 1H, *CH* (5)), 0.83 (s, 3H, CH_3 (24)), 0.72 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.8 (*C*=O, C28), 144.0 (*C*=CH, C13), 141.4 (C_{aromat}, C35), 128.6 (CH_{aromat}, C37), 128.5 (CH_{aromat}, C36), 126.1 (CH_{aromat}, C38), 122.3 (*CH*=C, C12), 84.1 (CHOH, C3), 69.1 (*C*HOH, C2), 63.5 (CH_2, C31), 55.4 (*C*H, C5), 47.7 (*C*H, C9), 46.9 (C_{quart}, C17), 46.5 (CH_2, C1), 46.0 (CH_2, C19), 41.9 (C_{quart}, C14), 41.5 (*C*H, C18), 39.6 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.1 (CH_2, C21), 33.3 (CH_3, C30), 32.8 (CH_2, C7), 32.7 (CH_2, C22), 32.4 (CH_2, C33), 30.9 (C_{quart}, C20), 30.4 (CH_2, C32), 28.8 (CH_3, C23), 27.8 (CH_2, C15), 26.1 (CH_3, C27), 23.8 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 18.5 (CH_2, C6), 17.2 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 591.1 (100%, $[M+H]^+$), 1203.3 (80%, $[2M+Na]^+$); analysis calculated for $C_{39}H_{58}O_4$ (590.87): C 79.28, H 9.89; found: C 79.03, H 10.04.

6.2.3.14. (2α,3β) 2,3-Diacetoxy-olean-12-en-28-acid (14). Acetylation of **MA** (1.2 g, 2.54 mmol) in dry DCM (50 mL) with acetic anhydride (0.96 mL, 10.16 mmol) and NEt_3 (0.96 mL, 10.16 mmol) for 12 h at 24 °C yielded, after usual work-up and recrystallization from ethanol, **14** as a colorless solid; yield: 88%; mp: 170–173 °C (lit.: 136–138 °C [43]; 232-234 °C[44]); $[\alpha]_D$ = +30° (*c* 0.83, $CHCl_3$); R_f = 0.61 (toluene/ ethyl acetate/ *n*-heptane/formic acid, 80:25:30:4); IR (KBr): ν = 2949s, 1748s, 1694s,

1464m, 1369s, 1252s, 1183w, 1156m, 1045m, 960w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.18 (dd, J = 3.4, 3.4 Hz, 1H, C*H* (12)), 5.01 (ddd, J = 11.4, 9.8, 4.7 Hz, 1H, C*H* (2)), 4.66 (d, J = 10.4 Hz, 1H, C*H* (3)), 2.73 (dd, J = 13.8, 4.1 Hz, 1H, C*H* (18)), 1.96 (s, 3H, CH_3 (Ac)), 1.92 (dd, J = 11.3 Hz, 4.3 Hz, 1H, CH_a (1)), 1.91–1.90 (m, 1H, CH_a (11)), 1.89 (s, 3H, CH_3 (Ac)), 1.84 (ddd, J = 12.5, 9.9, 3.5 Hz, 1H, CH_a (16)), 1.80–1.72 (m, 2H, CH_b (16) + CH_b (11)), 1.68 (ddd, 1H, J = 14.1, 10.8, 4.5 Hz, CH_a (7)), 1.65–1.62 (m, 1H, CH_a (15)), 1.60 (dd, J = 11.8, 4.1 Hz, 1H, C*H* (9)), 1.56–1.48 (m, 2H, CH_b (7) + CH_a (19)), 1.48–1.45 (m, 1H, CH_a (6)), 1.41–1.32 (m, 2H, CH_a (22) + CH_b (6)), 1.27 (ddd, J = 11.7, 11.7, 1.9 Hz, 1H, CH_a (21)), 1.19–1.09 (m, 2H, CH_b (21) + CH_b (22)), 1.06 (ddd, J = 13.9, 4.5, 2.0 Hz, 1H, CH_b (19)), 1.03 (s, 3H, CH_3 (27)), 1.01–0.86 (m, 4H, CH_2 (15) + CH_b (1) + C*H* (5)), 0.96 (s, 3H, CH_3 (23)), 0.84 (s, 3H, CH_3 (25)), 0.83 (s, 3H, CH_3 (30)), 0.82 (s, 3H, CH_3 (29)), 0.81 (s, 3H, CH_3 (24)), 0.65 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 183.6 (*C*=O, C28), 170.8 (*C*=O, Ac), 170.6 (*C*=O, Ac), 144.7 (*C*=CH, C13), 122.2 (*CH*=C, C12), 80.6 (*CH*OAc, C3), 70.0 (*CH*OAc, C2), 54.8 (*CH*, C5), 47.5 (*CH*, C9), 46.5 (C_{quart}, C17), 45.8 (CH_2, C19), 43.8 (CH_2, C1), 41.6 (C_{quart}, C14), 40.9 (*CH*, C18), 39.4 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.2 (C_{quart}, C10), 33.8 (CH_2, C21), 33.0 (CH_3, C29), 32.4 (CH_2, C22), 31.6 (CH_2, C7), 30.7 (C_{quart}, C20), 28.4 (CH_3, C27), 27.6 (CH_2, C15), 25.9 (CH_3, C30), 23.6 (CH_3, C23), 23.4 (CH_2, C16), 22.8 (CH_2, C11), 21.1 (CH_3, C33), 20.9 (CH_3, C32), 18.2 (CH_2, C6), 17.6 (CH_3, C24), 17.1 (CH_3, C25), 16.4 (CH_3, C26) ppm; MS (ESI, MeOH): m/z = 557.4 (49%, $[M+H]^+$), 574.5 (100%, $[M+NH_4]^+$), 579.5 (51%, $[M+Na]^+$), 1135.2 (100%, $[2M+Na]^+$); analysis calculated for $C_{34x}H_{52}O_6$ (566.77): C 73.34, H 9.41; found: C 73.21, H 9.56.

6.2.3.15. Benzyl (2α,3β) 2,3-diacetoxy-olean-12-en-28-amide (15). Compound **14** (200 mg, 0.36 mmol) was dissolved in dry DCM and THF (1:1); thionyl chloride and TEA (3 drops) were added at 0 °C. After 20 min, the solution was allowed to warm up to room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in DCM and benzylamine (2 mL, 18.4 mmol) and TEA (3 drops) were added. After completion of the reaction (TLC), the mixture was poured into ice water, the precipitate was filtered off, washed with water and subjected to chromatography (silica gel, *n*-hexane/ethyl acetate). After recrystallization from methanol compound **15** was obtained as a colorless solid; yield: 82%; mp: 143–145 °C; R_f = 0.7 (*n*-hexane/ethyl acetate 7:3); $[\alpha]_D$ = −7° (*c* 0.32, $CHCl_3$); IR (KBr): ν = 3426br, 2948s, 1742ss, 1663m, 1509m, 1455m, 1368m, 1253s, 1044m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.28–7.16 (m, 5H, C*H* (37) + C*H* (38) + C*H* (39)), 6.08 (dd, J = 5.1, 5.1 Hz, 1H, N*H*), 5.22 (dd, J = 3.4, 3.4 Hz, 1H, C*H* (12)), 5.01 (ddd, J = 11.3, 11.1, 4.3 Hz, 1H, C*H* (2)), 4.67 (d, J = 10.4 Hz, 1H, C*H* (3)), 4.54 (dd, J = 14.7, 6.3 Hz, 1H, CH_a (35)), 4.08 (dd, J = 14.7, 4.4, 1H, CH_b (35)), 2.47 (dd, J = 13.0, 3.8 Hz, 1H, C*H* (18)), 1.98 (s, 3H, CH_3 (32)), 2.00–1.89 (m, 2H, CH_2 (11)), 1.93 (dd, J = 8.1, 4.4 Hz, 1H, CH_a (1)), 1.91 (s, 3H, CH_3 (33)), 1.86–1.78 (m, 1H, CH_a (22)), 1.76 (dd, J = 7.4, 3.1 Hz, 1H, CH_a (19)), 1.73–1.59 (m, 4H, CH_2 (7) + CH_a (15) + CH_b (22)), 1.57 (dd, J = 12.0, 7.6 Hz, 1H, C*H* (9)), 1.54–1.45 (m, 2H, CH_a (6) + CH_a (16)), 1.39 (ddd, J = 9.9, 9.4, 2.5 Hz, 1H, CH_b (6)), 1.34–1.26 (m, 1H, CH_b (16)), 1.26–1.18 (m, 2H, CH_2 (21)), 1.18–1.09 (m, 1H, CH_b (19)), 1.07 (s, 3H, CH_3 (27)), 1.01–0.96 (m, 2H, CH_b (1) + CH_b (15)), 0.94 (s, 3H, CH_3 (23)), 0.91–0.86 (m, 1H, C*H* (5)), 0.84 (s, 3H, CH_3 (25)), 0.83 (s, 3H, CH_3 (30)), 0.83 (s, 3H, CH_3 (29)), 0.82 (s, 3H, CH_3 (24)), 0.59 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.9 (*C*=O, C28), 170.8 (*C*=O, Ac), 170.5 (*C*=O, Ac), 145.0 (*C*=CH, C13), 138.4 (C_{quart}, C32), 128.7 (CH_{aromat}, C34), 127.8 (CH_{aromat}, C33), 127.4 (CH_{aromat}, C35), 122.4 (*CH*=C, C12), 80.5 (*CH*OAc, C3), 70.0 (*CH*OAc, C2), 54.8 (*CH*, C5), 47.4 (*CH*, C9), 46.6 (CH_2, C19), 46.3 (C_{quart}, C17), 43.9 (CH_2, C31), 43.6 (CH_2, C1), 42.3 (*CH*, C18), 42.1 (C_{quart}, C14), 39.4 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.0 (C_{quart}, C10), 34.1 (CH_2, C21), 33.0 (CH_3, C29), 32.6 (CH_2, C7), 32.2 (CH_2, C16), 30.7 (C_{quart}, C20), 28.3 (CH_3, C23), 27.3 (CH_2, C15), 25.7 (CH_3, C27), 23.7 (CH_2, C22), 23.6 (CH_3, C30), 23.5 (CH_2, C11), 21.1 (CH_3, Ac), 20.9 (CH_3, Ac), 18.1 (CH_2, C6), 17.6 (CH_3, C26), 16.9 (CH_3, C24), 16.4 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 646.5 (100%, $[M+H]^+$), 668.5 (52%, $[M+Na]^+$), 1291.1 (31%, $[2M+H]^+$), 1313.3 (92%, $[2M+Na]^+$); analysis calculated for $C_{41}H_{59}NO_5$ (645.91): C 76.24, H 9.21, N 2.17; found: C 76.11, H 9.39, N 2.08.

6.2.3.16. Benzyl (2α,3β) 2,3-dihydroxy-olean-12-en-28-amide (16). Compound **15** (100 mg, 0.18 mmol) was dissolved in methanol and methanolic KOH (1.2 equiv) was added; the mixture was stirred at room temperature. After completion of the reaction (TLC), the mixture was poured into ice cold aq. HCl (3.7%). The precipitate was collected, and after work-up as described above, **16** was obtained as a colorless solid; yield: 66%; mp: 148–151 °C; R_f = 0.16 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +29° (*c* 0.29, $CHCl_3$); IR (KBr): ν = 3422br, 2945s, 1643m, 1518m, 1455m, 1387m, 1264w, 1050m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.28–7.16 (m, 5H, C*H* (33) + C*H* (34) + C*H* (35)), 6.11 (dd, J = 5.1, 5.1 Hz, 1H, N*H*), 5.24 (dd, J = 3.3, 3.3 Hz, 1H, C*H* (12)), 4.56 (dd, J = 14.7, 6.4 Hz, 1H, CH_a (31)), 4.06 (dd, J = 14.7, 4.3, 1H, CH_b (31)), 3.61 (ddd, J = 11.1, 9.5, 4.4 Hz, 1H, C*H* (2)), 2.93 (d, J = 9.5 Hz, 1H, C*H* (3)), 2.47 (dd, J = 12.8, 3.5 Hz, 1H, C*H* (18)), 2.10–2.00 (br s, 2H, O*H*), 1.94 (ddd, J = 13.7, 13.7, 3.8 Hz, 1H, CH_a (16)), 1.87 (ddd, J = 12.2, 4.4 Hz, 1H, CH_a (1)), 1.83–1.71 (m, 2H, CH_2 (11)), 1.71–1.45 (m, 7H, CH_a (19) + CH_2 (7) + CH_a (6) + CH_b (16) + C*H* (9) + CH_a (15)), 1.43–1.07 (m, 6H, CH_b (6) + CH_2 (21) + CH_2 (22) + CH_b (19)), 1.08 (s, 3H, CH_3 (27)), 1.04–0.97 (m, 1H, CH_b (15)), 0.96 (s, 3H, CH_3 (23)), 0.87 (s, 3H, CH_3 (25)), 0.84 (s, 3H, CH_3 (30)), 0.84 (s, 3H, CH_3 (29)), 0.84–0.79 (m, 1H, CH_b (1)), 0.78–0.74 (m, 1H, C*H* (5)), 0.76 (s, 3H, CH_3 (24)), 0.60 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.0 (*C*=O, C28), 145.0 (*C*=CH, C13), 138.4 (C_{quart}, C32), 128.7 (CH_{aromat}, C34), 127.8 (CH_{aromat}, C33), 127.4 (CH_{aromat}, C35), 122.7 (*CH*=C, C12), 84.0 (*CH*OH, C3), 68.9 (*CH*OH, C2), 55.2 (*CH*, C5), 47.5 (*CH*, C9), 46.7 (CH_2, C19), 46.4 (CH_2, C1), 46.3 (CH_2, C31), 43.6 (C_{quart}, C17), 42.3 (*CH*, C18), 42.1 (C_{quart}, C14), 39.4 (C_{quart}, C8), 39.2 (C_{quart}, C4), 38.2 (C_{quart}, C10), 34.1 (CH_2, C21), 33.0 (CH_3, C30), 32.6 (CH_2, C7), 32.2 (CH_2, C22), 30.8 (C_{quart}, C20), 28.6 (CH_3, C23), 27.3 (CH_2, C15), 25.8 (CH_3, C27), 23.8 (CH_2, C16), 23.6 (CH_3, C29), 23.5 (CH_2, C11), 18.3 (CH_2, C6), 17.0 (CH_3, C26), 16.7 (CH_3, C24), 16.6 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 562.5 (42%, $[M+H]^+$), 1123.2 (32%, $[2M+H]^+$), 1145.4 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{55x}NO_3$ (561.84): C 78.09, H 9.87, N 2.49; found: C 78.91, H 9.99, N 2.35.

6.2.3.17. Benzyl (3β) 3-hydroxy-olean-12-en-28-oate (17). Obtained from **OA** (3.0 g, 6.57 mmol) following method **A** as a colorless solid; yield: 92%; mp: 185–187 °C (lit.: 189 °C[41]); $[\alpha]_D$ = +58.4° (*c* 0.45; $CHCl_3$); R_f = 0.42 (*n*-hexane/ethyl acetate, 8:2); IR (KBr): ν = 3583s, 2938s, 1726s, 1498w, 1463m, 1386m, 1363w, 1323w, 1305w, 1264w, 1234w, 1201m, 1182m, 1162m, 1129m, 1095w, 1044m, 1016m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.37–7.27 (m, 5H, CH_{aromat}), 5.29 (dd, J = 3.6, 3.6 Hz, 1H, C*H* (12)), 5.09 (d, J = 12.6 Hz, 1H, CH_a (31)), 5.05 (d, J = 12.5 Hz, 1H, CH_b (31)), 3.20 (dd, J = 11.3 Hz, 4.5 Hz, 1H, *CH*OH (3)), 2.91 (dd, J = 13.8, 4.2 Hz, 1H, C*H* (18)), 1.98 (ddd, J = 13.4, 13.0, 4.1 Hz, 1H, CH_a (16)), 1.85 (dd, J = 8.9, 3.6 Hz, 1H, CH_2 (11)), 1.76–1.14 (m, 16H, C*H* (9) + CH_2 (19) + CH_a (1) + CH_2 (21) + CH_2 (7) + CH_2 (22) + CH_a (15) + CH_2 (2) + CH_b (16) + CH_2 (6)), 1.13 (s, 3H, CH_3 (27)), 1.09–1.00 (m, 2H, CH_b (1) + CH_b (15)), 0.98 (s, 3H, CH_3 (23)), 0.92 (s, 3H, CH_3 (30)), 0.90 (s, 3H, CH_3 (29)), 0.88 (s, 3H, CH_3 (25)), 0.77 (s, 3H, CH_3 (24)), 0.70 (brd, J = 11.5 Hz, 1H, C*H* (5)), 0.61 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$):

δ = 177.4 (*C*=O, C28), 143.7 (*C*=CH, C13), 136.4 (C_{quart}, C32), 128.4 (CH_{aromat}, C35), 128.0 (CH_{aromat}, C34), 127.9 (CH_{aromat}, C33), 122.5 (*CH*=C, C12), 80.0 (*CH*OH, C3), 65.9 (CH_2, C31), 55.2 (*CH*, C5), 47.6 (*CH*, C9), 46.7 (C_{quart}, C17), 45.9 (CH_2, C19), 41.7 (C_{quart}, C14), 41.4 (*CH*, C18), 39.3 (C_{quart}, C8), 38.7 (C_{quart}, C4), 38.4 (CH_2, C1), 37.0 (C_{quart}, C10), 33.8 (CH_2, C21), 33.1 (CH_3, C29), 32.7 (CH_2, C7), 32.4 (CH_2, C22), 30.7 (C_{quart}, C20), 28.1 (CH_3, C23), 27.6 (CH_2, C15), 27.2 (CH_2, C2), 25.9 (CH_3, C27), 23.6 (CH_3, C30), 23.4 (CH_2, C11), 23.0 (CH_2, C16), 18.3 (CH_2, C6), 16.9 (CH_3, C26), 15.6 (CH_3, C24), 15.3 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 569.5 (100%, $[M+Na]^+$); analysis calculated for $C_{37}H_{54}O_3$ (546.82): C 81.27, H 9.95; found: C 81.04, H 10.11.

6.2.3.18. *o*-Chlorobenzyl (3β) 3-hydroxy-olean-12-en-28-oate (18). Obtained from **OA** by method **A** as a colorless solid; yield: 65%; mp: 175 °C; R_f = 0.28 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +55.1° (*c* 0.4, $CHCl_3$); IR (KBr): *v* = 3590s, 3440s, 2938s, 2926s, 2864m, 2838m, 1728vs, 1474m, 1454m, 1442m, 1384s, 1364m, 1260w, 1234w, 1200m, 1182m, 1162s, 1130m, 1054m, 1042m, 1030m, 1018m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.43–7.22 (m, 4H, *CH* (34) + *CH* (35) + *CH* (36) + *CH* (37)), 5.28 (dd, *J* = 3.6, 3.6 Hz, 1H, *CH* (12)), 5.19 (d, *J* = 13.1 Hz, 1H, CH_a (31)), 5.14 (d, *J* = 13.1 Hz, 1H, CH_b (31)), 3.22 (dd, *J* = 11.0, 4.7 Hz, 1H, *CH* (3)), 2.91 (dd, *J* = 13.8, 4.2 Hz, 1H, *CH* (18)), 1.98 (ddd, *J* = 12.4, 12.4, 4.1, 1H, CH_a (16)), 1.84 (dd, *J* = 8.9, 3.6 Hz, 2H, CH_2 (11)), 1.70 (dd, *J* = 13.9, 13.9, 4.5 Hz, 1H, CH_a (7)), 1.67–1.58 (m, 6H, CH_b (16) + CH_a (19) + CH_a (1) + CH_2 (2) + CH_a (15)), 1.55–1.46 (m, 3H, *CH* (9) + CH_a (22) + CH_a (6)), 1.45–1.29 (m, 3H, CH_a (21) + CH_b (7) + CH_b (6)), 1.29–1.14 (m, 3H, CH_b (19) + CH_b (21) + CH_b (22)), 1.12 (m, 3H, CH_3 (27)), 1.08–1.01 (m, 1H, CH_b (15)), 0.98 (s, 3H, CH_3 (23)), 0.93 (s, 3H, CH_3 (30)), 0.90 (s, 3H, CH_3 (29)), 0.87 (s, 3H, CH_3 (25)), 0.77 (s, 3H, CH_3 (24)), 0.74 (dd, *J* = 11.6, 1.8 Hz, 1H, *CH* (5)), 0.58 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.4 (*C*=O, C28), 143.7 (*C*=CH, C13), 134.2 (C_{aromat}, C32), 133.9 ($C_{aromat}Cl$, C33), 130.2 (CH_{aromat}, C36), 129.6 ($CH_{aromat,}$ C35), 129.4 ($CH_{aromat,}$ C34), 126.8 ($CH_{aromat,}$ C37), 122.8 (*CH*=C, C12), 79.1 (*CH*OH, C3), 63.6 (CH_2, C31), 55.4 (*CH*, C5), 47.8 (*CH*, C9), 47.1 (C_{quart}, C17), 46.0 (CH_2, C19), 41.8 (C_{quart}, C14), 41.5 (*CH*, C18), 39.3 (C_{quart}, C8), 38.9 (C_{quart}, C4), 38.6 (CH_2, C1), 37.1 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C29), 32.6 (CH_2, C7), 30.8 (C_{quart}, C20), 28.3 (CH_3, C23), 27.8 (CH_2, C15), 27.3 (CH_2, C2), 26.0 (CH_3, C27), 23.8 (CH_3, C30), 23.2 (CH_2, C11), 18.5 (CH_2, C6), 17.0 (CH_3, C26), 15.5 (CH_3, C24), 15.4 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 619.3 (16%, $[M+K]^+$), 603.7 (100%, $[M+Na]^+$), 598.5 (32%, $[M+NH_4]^+$), 581.3 (49%, $[M+H]^+$); analysis calculated for $C_{37}H_{53}ClO_3$ (571.27): C 76.45, H 9.19; found: C 76.34, H 9.31.

6.2.3.19. *m*-Chlorobenzyl (3β) 3-hydroxy-olean-12-en-28-oate (19). Obtained from **OA** by method **A** as a colorless solid; yield: 80%; mp: 175 °C; R_f = 0.28 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +53.3° (*c* 0.32, $CHCl_3$); IR (KBr): *v* = 3546s, 3446vs, 2964s, 2946vs, 2936s, 2912s, 2862m, 1714s, 1628m, 1470m, 1384s, 1364m, 1262m, 1168m, 1158m, 1124m, 1032m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.33 (s, 1H, *CH* (33)), 7.28–7.23 (m, 2H, *CH* (35) + *CH* (36)), 7.21–7.17 (m, 2H, *CH* (37)), 5.28 (dd, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 5.07 (d, *J* = 12.8 Hz, 1H, CH_a (31)), 4.96 (d, *J* = 12.8 Hz, 1H, CH_b (31)), 3.19 (dd, *J* = 11.4, 4.5 Hz, 1H, *CH* (3)), 2.88 (dd, *J* = 13.8, 4.0 Hz, 1H, *CH* (18)), 1.97 (ddd, *J* = 13.6, 13.6, 4.1 Hz, 1H, CH_a (16)), 1.85 (dd, *J* = 9.5, 3.3 Hz, 2H, CH_2 (11)), 1.70 (ddd, *J* = 13.9, 13.9, 4.5 Hz, 1H, CH_a (7)), 1.67–1.58 (m, 6H, CH_b (16) + CH_a (19) + CH_a (1) + CH_2 (2) + CH_a (15)), 1.55–1.46 (m, 3H, *CH* (9) + CH_a (22) + CH_a (6)), 1.45–1.29 (m, 3H, CH_a (21) + CH_b (7) + CH_b (6)), 1.29–1.14 (m, 3H, CH_b (19) + CH_b (21) + CH_b (22)), 1.11 (m, 3H, CH_3 (27)), 1.06–1.00 (m, 1H, CH_b (15)), 0.96 (m, 3H, CH_3 (23)), 0.98–0.94 (m, 1H, CH_b (1)), 0.91 (m, 3H, CH_3 (30)), 0.88 (s, 3H, CH_3 (29)), 0.86 (m, 3H, CH_3 (25)), 0.77 (s, 3H, CH_3 (24)), 0.69 (dd, *J* = 11.8, 1.8 Hz, 1H, *CH* (5)), 0.56 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.5 (*C*=O, C28), 143.8 (*C*=CH, C13), 138.6 (CCl_{aromat}, C34), 134.5 (C_{aromat}, C32), 129.8 (CH_{aromat}, C33), 128.2 (CH_{aromat}, C36), 128.2 (CH_{aromat}, C35), 126.2 (CH_{aromat}, C37), 122.8 (*CH*=C, C12), 79.2 (*CH*OH, C2), 65.2 (CH_2, C31), 55.4 (*CH*, C5), 47.8 (*CH*, C9), 47.0 (C_{quart}, C7), 46.0 (CH_2, C19), 41.8 (C_{quart}, C14), 41.5 (*CH*, C18), 39.5 (C_{quart}, C8), 38.9 (C_{quart}, C4), 38.6 (C1, CH_2), 37.2 (C_{quart}, C10), 34.0 (C21, CH_2), 33.2 (CH_3, C29), 32.9 (CH_2, C7), 32.6 (CH_2, C22), 30.8 (C_{quart}, C20), 28.3 (CH_3, C23), 27.7 (CH_2, C15), 27.4 (CH_2, C2), 26.0 (CH_3, C27), 23.8 (CH_3, C30), 23.5 (CH_2, C16), 23.3 (CH_2, C11), 18.5 (CH_2, C6), 17.0 (CH_3, C26), 15.7 (CH_3, C24), 15.4 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 581.3 (59%, $[M+H]^+$), 603.3 (100%, $[M+Na]^+$; analysis calculated for $C_{37}H_{53}ClO_3$ (581.27): C 76.45, H 9.19; found: C 76.36, H 9.32.

6.2.3.20. *p*-Chlorobenzyl (3β) 3-hydroxy-olean-12-en-28-oate (20). Obtained from **OA** by method **A** as a colorless solid; yield: 82%; mp: 172 °C; R_f = 0.3 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +52.1° (*c* 0.33, $CHCl_3$); IR (KBr): *v* = 3538s, 2974s, 2946vs, 2922vs, 2910s, 2876s, 2864s, 2852s, 1714vs, 1494s, 1466s, 1452m, 1380m, 1358m, 1304m, 1262m, 1234m, 1208m, 1200m, 1178s, 1160s, 1136m, 1124m, 1092m, 1050m, 1032m, 1012s cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.31 (d, *J* = 8.1 Hz, 2H, *CH* (34) + *CH* (36)), 7.28 (d, *J* = 8.0 Hz, 2H, *CH* (33) + *CH* (37)), 5.28 (dd, *J* = 3.3, 3.3. Hz, 1H, *CH* (12)), 5.07 (d, *J* = 12.6 Hz, 1H, CH_a (31)), 5.00 (d, *J* = 12.6 Hz, 1H, CH_b (31)), 3.21 (dd, *J* = 10.9, 4.6 Hz, 1H, *CH* (3)), 2.89 (dd, *J* = 13.7, 3.9 Hz, 1H, *CH* (18)), 2.05–1.93 (ddd, 13.3, 13.3, 3.7 Hz, 1H, CH_a (16)), 1.85 (dd, *J* = 8.8, 3.3 Hz, 2H, CH_2 (11)), 1.70 (ddd, *J* = 13.7, 13.7, 4.4 Hz, 1H, CH_a (7)), 1.67–1.58 (m, 6H, CH_b (16) + CH_a (19) + CH_a (1) + CH_2 (2) + CH_a (15)), 1.55–1.46 (m, 3H, *CH* (9) + CH_a (22) + CH_a (6)), 1.45–1.29 (m, 3H, CH_a (21) + CH_b (7) + CH_b (6)), 1.29–1.14 (m, 3H, CH_b (19) + CH_b (21) + CH_b (22)), 1.13 (s, 3H, CH_3 (27)), 1.08–1.03 (m, 1H, CH_b (15)), 0.99 (s, 3H, CH_3 (23)), 0.98–0.94 (m, 1H, CH_b (1)), 0.93 (m, 3H, CH_3 (30)), 0.91 (s, 3H, CH_3 (29)), 0.89 (s, 3H, CH_3 (25)), 0.79 (s, 3H, CH_3 (24)), 0.72 (dd, *J* = 11.7, 1.9 Hz, 1H, *CH* (5)), 0.57 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.5 (*C*=O, C28), 143.8 (*C*=CH, C13), 135.1 (C_{aromat}, C32), 134.0 ($C_{aromat}Cl$, C35), 129.7 (CH_{aromat}, C33 + C37), 128.7 (CH_{aromat}, C34 + C36), 122.7 (*CH*=C, C12), 79.1 (*CH*OH, C3), 65.3 (CH_2, C31), 55.4 (*CH*, C5), 47.7 (*CH*, C9), 46.9 (C_{quart}, C7), 46.0 (CH_2, C19), 41.8 (C_{quart}, C14), 41.5 (*CH*, C18), 39.4 (C_{quart}, C8), 38.9 (C_{quart}, C4), 38.6 (CH_2, C1), 37.1 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C29), 32.9 (CH_2, C7), 32.5 (CH_2, C22), 30.8 (C_{quart}, C20), 28.3 (CH_3, C23), 27.7 (CH_2, C15), 27.3 (CH_2, C2), 26.0 (CH_3, C27), 23.8 (CH_3, C30), 23.5 (CH_2, C16), 23.2 (CH_2, C11), 18.4 (CH_2, C6), 17.0 (CH_3, C26), 15.7 (CH_3, C24), 15.4 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 581.2 (19%, $[M+H]^+$), 596.8 (12%, $[M+NH_4]^+$), 603.5 (12%, $[M+Na]^+$), 620.9 (13%, $[M+K]^+$), 1183.0 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}ClO_3$ (546.82): C 81.27, H 9.95; found: C 81.02, H 10.14.

6.2.3.21. (3α) 3-Acetoxy-olean-12-en-28-acid (21). Acetylation of **OA** (5 g; 10.95 mmol) in dry pyridine (100 mL) with acetic anhydride (2.6 mL 27.4 mmol), NEt_3 (3 mL, 21.7 mmol) and catalytic amounts of DMAP for 12 h at 24 °C yielded, after usual work-up and recrystallization from ethanol, **21** as a colorless solid; yield: 88%; mp: 259–261 °C (lit.: 260–261 °C[45]); $[\alpha]_D$ = +65° (*c* 0.33, $CHCl_3$) (lit.: +74° (*c* 1.0, $CHCl_3$)[45]); R_f = 0.60 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:20:30:4); IR (KBr): *v* = 3219br, 2942s, 1731s, 1680m, 1466m, 1370m, 1252s, 1179m, 1161m, 1127w, 1026m, 1010m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.20 (dd, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 4.47 (dd, *J* = 8.5, 7.3 Hz, 1H, *CH* (3)), 2.80 (dd, *J* = 13.7, 4.1 Hz, 1H, *CH* (18)), 2.02 (s, 3H, Ac), 1.95 (ddd, *J* = 13.4, 13.4, 3.9 Hz, 1H, CH_a (16)), 1.90–1.77 (m, 2H, CH_2 (11)), 1.73 (ddd, *J* = 14.9, 14.6, 4.4 Hz, 1H CH_a (7)), 1.67 (ddd, *J* = 13.3, 13.3, 4.1 Hz, 1H, CH_a (15)), 1.54–1.34 (m, 8H, CH_a (1) + CH_b

(16) + CH_a (19) + CH (9) + CH_a (6) + CH_a (7) + CH_2 (2)), 1.34–1.11 (m, 5H, CH_b (7) + CH_b (6) + CH_2 (21) + CH_a (22)), 1.10 (s, 3H, CH_3 (27)), 1.08–0.93 (m, 3H, CH_b (19) + CH_b (15) + CH_b (1)), 0.92 (s, 3H, CH_3 (25)), 0.90 (s, 3H, CH_3 (30)), 0.88 (s, 3H, CH_3 (29)), 0.84 (s, 3H, CH_3 (23)), 0.86–0.74 (m, 1H, CH (5)), 0.83 (s, 3H, CH_3 (26)), 0.72 (s, 3H, CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 184.3 (*C*=O, C28), 171.0 (*C*=O, Ac), 143.6 (*C*=CH, C13), 122.5 (*CH*=C, C12), 80.9 (*CHOAc*, C3), 55.3 (*CH*, C5), 47.5 (*CH*, C9), 46.5 (C_{quart}, C17), 45.8 (CH_2, C19), 41.5 (C_{quart}, C14), 40.8 (*CH*, C18), 39.2 (C_{quart}, C8), 38.0 (CH_2, C1), 37.6 (C_{quart}, C4), 36.9 (C_{quart}, C10), 33.7 (CH_2, C21), 33.0 (CH_3, C29), 32.5 (CH_2, C7), 32.4 (CH_2, C22), 30.6 (C_{quart}, C20), 28.0 (CH_3, C23), 27.6 (CH_2, C15), 26.9 (CH_2, C2), 25.8 (CH_3, C27), 23.6 (CH_3, C30), 23.5 (CH_2, C16), 23.3 (CH_2, C11), 21.2 (CH_3, Ac), 18.1 (CH_2, C6), 17.1 (CH_3, C24), 16.6 (CH_3, C26), 15.3 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 497.5 (100%, $[M-H]^-$), 543.1 (66%, $[M+HCO_2]^-$), 995.1 (64%, $[2M-H]^-$), 1017.5 (16%, $[2M-2H+Na]^-$); analysis calculated for $C_{32}H_{50}O_4$ (498.74): C 77.06, H 10.10; found: C 76.84, H 10.29.

6.2.3.22. Benzyl (3β) 3-acetoxy-olean-12-en-28-amide (22). Compound **21** (200 mg, 0.36 mmol) was dissolved in dry DCM and THF (1:1) and treated with thionyl chloride and TEA (3 drops) at 0 °C. After 20 min, the solution was allowed to warm up to room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in DCM and benzyl amine (2 mL, 18.4 mmol) and TEA (3 drops) were added. After completion of the reaction (as indicated by TLC), the mixture was worked up as described above. Chromatography (silica gel, *n*-hexane/ethyl acetate) followed by recrystallization from methanol gave **22** as a colorless solid; yield: 61%; mp: 254–259 °C; $[\alpha]_D$ = +29° (*c* 0.56, $CHCl_3$); R_f = 0.57 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:20:30:4); IR (KBr): *ν* = 3388br, 2946s, 2872m, 1719ss, 1648s, 1511m, 1471m, 1386m, 1258s, 1211m, 1027m cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.29–7.16 (m, 5H, *CH* (35) + *CH* (36) + *CH* (37)), 6.12 (dd, *J* = 5.0, 5.0 Hz, 1H, *NH*), 5.23 (dd, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 4.57 (dd, *J* = 14.6, 6.2 Hz, 1H, CH_a (33)), 4.41 (dd, *J* = 8.6, 7.4 Hz, 1H, *CH* (3)), 4.06 (dd, *J* = 14.6, 3.9, 1H, CH_b (33)), 2.47 (dd, *J* = 12.8, 4.0 Hz, 1H, *CH* (18)), 1.98 (s, 3H, CH_3 (32)), 1.96–1.80 (m, 1H, CH_a (11)), 1.79–1.65 (m, 2H, CH_a (2) + CH_a (7)), 1.71 (dd, *J* = 13.2, 13.2 Hz, 1H, CH_a (19)), 1.65–1.42 (m, 8H, CH_b (2) + CH_2 (15) + CH_b (7) + CH_a (22) + CH_a (6) + CH_b (11) + CH_a (1)), 1.46 (dd, *J* = 11.3, 5.1 Hz, 1H, *CH* (9)), 1.39 (ddd, *J* = 14.7, 11.9, 8.3 Hz, 1H, CH_a (16)), 1.25–1.10 (m, 5H, CH_b (6) + CH_2 (21) + CH_b (16) + CH_b (19)), 1.08 (s, 3H, CH_3 (30)), 1.06–0.87 (m, 2H, CH_b (22) + CH_b (1)), 0.85–0.74 (m, 1H, *CH* (5)), 0.84 (s, 3H, CH_3 (29)), 0.84 (s, 3H, CH_3 (23)), 0.83 (s, 3H, CH_3 (25)), 0.80 (s, 3H, CH_3 (27)), 0.79 (s, 3H, CH_3 (26)), 0.60 (s, 3H, CH_3 (24)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.0 (*C*=O, C28), 171.0 (*C*=O, Ac), 145.0 (*C*=CH, C13), 138.4 (C_{quart}, C34), 128.6 (CH_{aromat}, C36), 127.7 (CH_{aromat}, C35), 127.3 (CH_{aromat}, C37), 122.8 (*CH*=C, C12), 80.8 (*CHOAc* C3), 55.2 (*CH*, C5), 47.4 (*CH*, C9), 46.6 (C_{quart}, C17), 46.4 (CH_2, C19), 43.6 (CH_2, C33), 42.3 (*CH*, C18), 42.0 (C_{quart}, C14), 39.4 (C_{quart}, C8), 38.1 (CH_2, C1), 37.7 (C_{quart}, C4), 36.8 (C_{quart}, C10), 34.1 (CH_2, C21), 33.0 (CH_3, C29), 32.6 (CH_2, C7), 32.3 (CH_2, C16), 30.7 (C_{quart}, C20), 28.0 (CH_3, C27), 27.3 (CH_2, C22), 25.7 (CH_3, C30), 23.8 (CH_2, C11), 23.6 (CH_3, C23), 23.5 (CH_2, C2), 23.4 (CH_2, C15), 21.3 (CH_3, C32), 18.1 (CH_2, C6), 16.9 (CH_3, C24), 16.6 (CH_3, C26), 15.4 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 588.5 (64%, $[M+H]^+$), 610.6 (40%, $[M+Na]^+$), 1175.3 (50%, $[2M+H]^+$), 1197.4 (100%, $[2M+Na]^+$); analysis calculated for $C_{39}H_{57}NO_3$ (587.87): C 79.68, H 9.77, N 2.38; found: C 79.51, H 9.91, N 2.24.

6.2.3.23. Benzyl (3β) 3-hydroxy-olean-12-en-28-amide (23). Compound **22** (100 mg, 0.18 mmol) was dissolved in methanol. KOH (1.2 equiv) was added, and the mixture stirred at room temperature. After completion of the reaction (TLC) and work-up as described above, **23** was obtained as a colorless solid; yield: 80%; mp: 247–249 °C; $[\alpha]_D$ = +30.4° (*c* 0.56, $CHCl_3$); R_f = 0.37 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:20:30:4); IR (KBr): *ν* = 3444m, 3426m, 2972s, 2946vs, 2926s, 2876m, 2850m, 1644vs, 1516s, 1482m, 1470m, 1454m, 1378m, 1364m, 1030m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.36–7.19 (m, 5H, CH_{aromat}), 6.20 (dd, *J* = 5.9, 4.8 Hz, 1H, *NH*), 5.30 (dd, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 4.62 (dd, *J* = 14.7, 6.4 Hz, 1H, CH_a (31)), 4.13 (dd, *J* = 14.7, 4.3 Hz, 1H, CH_b (31)), 3.19 (dd, *J* = 10.9, 4.7 Hz, 1H, *CH* (3)), 2.53 (dd, *J* = 12.9, 3.9 Hz, 1H, *CH* (18)), 1.98 (ddd, *J* = 13.7, 13.7, 3.8 Hz, 1H, CH_a (16)), 1.89–1.81 (m, 2H, CH_2 (11)), 1.79–1.73 (m, 2H, CH_a (19) + CH_a (7)), 1.69–1.61 (m, 2H, CH_b (16) + CH_b (7)), 1.61–1.49 (m, 5H, CH_a (6) + CH_2 (2) + CH_a (1) + *CH* (9)), 1.45 (ddd, *J* = 12.6, 12.6, 3.6 Hz, 1H, CH_a (22)), 1.40 (ddd, *J* = 13.3, 13.3, 3.3 Hz, 1H, CH_a (21)), 1.38–1.32 (m, 1H, CH_b (6)), 12.8–1.21 (m, 2H, CH_b (21) + CH_b (22)), 1.17–1.09 (m, 1H, CH_b (19)), 1.15 (s, 3H, CH_3 (27)), 1.04 (ddd, *J* = 13.9, 3.0, 3.0, 1H, CH_b (15)), 0.98 (s, 3H, CH_3 (23)), 0.98–0.90 (m, 1H, CH_b (1)), 0.90 (s, 6H, CH_3 (30) + CH_3 (29)), 0.86 (s, 3H, CH_3 (25)), 0.77 (s, 3H, CH_3 (24)), 0.72–0.68 (m, 1H, *CH* (5)), 0.66 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.2 (*C*=O, C28), 144.9 (C_{aromat}, C33), 138.5 (*C*=CH, C13), 128.7 (*CH*, C33), 127.9 (CH_{aromat}, C34), 127.4 (CH_{aromat}, C35), 123.0 (*CH*=C, C12), 79.0 (*CHOH*, C3), 55.3 (*CH*, C5), 47.6 (*CH*, C9), 46.8 (C_{quart}, C17), 46.5 (CH_2, C19), 43.7 (CH_2, C31), 42.5 (*CH*, C18), 42.2 (C_{quart}, C14), 39.5 (C_{quart}, C8), 38.9 (C_{quart}, C4), 38.6 (CH_2, C1), 37.0 (C_{quart}, C10), 34.3 (CH_2, C21), 33.1 (CH_3, C29), 32.7 (CH_2, C7), 32.5 (CH_2, C22), 30.8 (C_{quart}, C20), 28.2 (CH_3, C23), 27.5 (CH_2, C15), 27.3 (CH_2, C2), 25.6 (CH_3, C27), 24.0 (CH_2, C16), 23.7 (CH_3, C30), 23.6 (CH_2, C11), 18.4 (CH_2, C6), 17.0 (CH_3, C26), 15.7 (CH_3, C24), 15.4 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 546.6 (44%, $[M+H]^+$), 568.4 (10%, $[M+Na]^+$), 1091.3 (31%, $[2M+H]^+$), 1134.4 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{55}NO_2$ (545.83): C 81.42, H 10.15, N 2.57; found: C 81.34, H 10.29, N 2.35.

6.2.3.24. Benzyl (3β) 3-hydroxy-urs-12-en-28-oate (24). Obtained from **UA** by method **A** as a colorless solid; yield: 73%; mp: 182 °C (lit.: 180–182 °C[46]); R_f = 0.25 (*n*-hexane/ethyl acetetate, 8:2); $[\alpha]_D$ = +45.6° (*c* 0.30, $CHCl_3$); IR (KBr): *ν* = 3518vs, 3474br, 3266m, 3034w, 2988s, 2970s, 2942s, 2922vs, 2868s, 2854s, 2836m, 2798w, 1714vs, 1498w, 1464m, 1452m, 1376m, 1308w, 1288 m, 1272m, 1248m, 1230m, 1198m, 1180m, 1164m, 1140m, 1114m, 1096w, 1050m, 1028m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.53–7.29 (m, 5H, CH_{aromat}), 5.37 (br, 1H, OH), 5.24 (dd, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 5.10 (d, *J* = 12.5 Hz, 1H, CH_a (31)), 4.98 (d, *J* = 12.5 Hz, 1H, CH_b (31)), 3.21 (dd, *J* = 11.0, 4.5 Hz, 1H, *CH* (3)), 2.27 (d, *J* = 11.3 Hz, 1H, *CH* (18)), 2.01 (ddd, *J* = 13.3, 13.3, 4.4 Hz, 1H, CH_a (16)), 1.94–1.84 (m, 2H, CH_2 (2)), 1.84–1.75 (m, 1H, CH_a (15)), 1.75–1.67 (m, 1H, CH_b (16)), 1.67–1.57 (m, 5H, CH_2 (22) + CH_a (1), CH_2 (11)), 1.52–1.40 (m, 4H, CH_a (6) + CH_a (7) + CH_a (21) + *CH* (9)), 1.41–1.20 (m, 5H, *CH* (19) + *CH* (20) + CH_b (7) + CH_b (21) + CH_b (6)), 1.07 (s, 3H, CH_3 (27)), 1.02–0.95 (m, 2H, CH_b (15) + CH_b (1)), 0.99 (s, 3H, CH_3 (23)), 0.94 (d, *J* = 6.2 Hz, 3H, CH_3 (30)), 0.89 (s, 3H, CH_3 (25)), 0.85 (d, *J* = 6.4 Hz, 3H, CH_3 (29)), 0.78 (s, 3H, CH_3 (24)), 0.71 (brd, *J* = 11.7 Hz, 1H, *CH* (5)), 0.65 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.4 (*C*=O, C28), 138.2 (*C*=CH, C13), 136.5 (C_{aromat}, C32), 128.5 (CH_{aromat}, C33), 128.3 (CH_{aromat}, C34), 128.1 (CH_{aromat}, C35), 125.9 (*CH*=C, C12), 79.2 (*CH*, C3), 66.1 (CH_2, C31), 55.4 (*CH*, C5), 53.0 (*CH*, C18), 48.3 (C_{quart}, C17), 47.7 (*CH*, C9), 42.2 (C_{quart}, C14), 39.7 (C_{quart}, C8), 39.2 (*CH*, C19), 39.0 (*CH*, C20), 38.9 (C_{quart}, C4), 38.8 (CH_2, C1), 37.1 (C_{quart}, C10), 36.8 (CH_2, C22), 33.2 (CH_2, C7), 30.8 (CH_2, C21), 28.3 (CH_3, C23), 28.1 (CH_2, C15), 27.4 (CH_2, C11), 24.4 (CH_2, C16), 23.7 (CH_3, C27), 23.4 (CH_2, C2), 21.3 (CH_3, C30), 18.5 (CH_2, C6), 17.2 (CH_3, C29), 17.1 (CH_3, C26), 15.8 (CH_3, C24), 15.6 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 547.3 (10%, $[M+H]^+$),

569.5 (100%, $[M+Na]^+$), 1115.2 (82%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{54}O_3$ (546.82): C 81.27, H 9.95; found: C 81.00, H 10.14.

6.2.3.25. *o*-Chlorobenzyl (3β) 3-hydroxy-urs-12-en-28-oate (25). Obtained from **UA** by method **A** as a colorless solid; yield: 61%; mp: 140 °C; R_f = 0.25 (*n*-hexane/ethyl actetate, 8:2); $[\alpha]_D$ = +42.4° (*c* 0.64, $CHCl_3$); IR (KBr): *ν* = 3568s, 3534s, 3446br, 2972s, 2944s, 2922vs, 2868s, 2850s, 1718vs, 1458m, 1446m, 1376m, 1272m, 1248m, 1230m, 1202m, 1180m, 1164m, 1140m, 1114m, 1052m, 1044m, 1032m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): *δ* = 7.51–7.26 (m, 4H, CH_{aromat}), 5.26 (d, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 5.22 (d, *J* = 13.0 Hz, 1H, CH_a (31)), 5.11 (d, *J* = 13.0 Hz, 1H, CH_b (31)), 3.23 (dd, *J* = 11.1, 4.7 Hz, 1H, *CH* (3)), 2.29 (brd, *J* = 11.2, 1H, *CH* (18)), 2.03 (ddd, *J* = 13.1, 13.1, 4.4 Hz, 1H, CH_a (16)), 1.93–1.80 (m, 2H, CH_2 (2)), 1.75 (m, 1H, CH_a (15)), 1.70–1.56 (m, 6H, CH_b (16) + CH_2 (22) + CH_a (1) + CH_2 (11)), 1.54–1.46 (m, 4H, CH_a (6) + CH_a (7) + CH_a (21) + *CH* (9)), 1.44–1.22 (m, 5H, *CH* (19) + *CH* (20) + CH_b (7) + CH_b (21) + CH_b (6)), 1.09 (s, 3H, CH_3 (27)), 1.05–1.00 (m, 1H, CH_b (15)), 1.00–0.95 (m, 1H, CH_b (1)), 1.00 (s, 3H, CH_3 (23)), 0.96 (d, *J* = 6.3 Hz, 3H, CH_3 (30)), 0.90 (s, 3H, CH_3 (25)), 0.88 (d, *J* = 6.5 Hz, 3H, CH_3 (29)), 0.79 (s, 3H, CH_3 (24)), 0.72 (brd, *J* = 11.7 Hz, 1H, *CH* (5)), 0.63 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): *δ* = 177.3 (*C*=O, C28), 138.1 (*C*=CH, C13), 134.2 (C_{aromat}, C32), 134.0 (CCl_{aromat}, C33), 130.3 (CH_{aromat}, C36), 129.6 (CH_{aromat}, C35), 129.4 (CH_{aromat}, C34), 126.6 (CH_{aromat}, C37), 126.0 (*CH*=C, C12), 79.2 (*CHOH*, C3), 63.6 (CH_2, C31), 55.4 (*CH*, C5), 53.1 (*CH*, C18), 48.5 (C_{quart}, C17), 47.7 (*CH*, C9), 42.2 (C_{quart}, C14), 39.7 (C_{quart}, C8), 39.3 (*CH*, C19), 39.0 (*CH*, C20), 38.9 (C_{quart}, C4), 38.8 (CH_2, C1), 37.1 (C_{quart}, C10), 36.9 (CH_2, C22), 33.2 (CH_2, C7), 30.8 (CH_2, C21), 28.3 (CH_3, C23), 28.1 (CH_2, C15), 27.4 (CH_2, C11), 24.4 (CH_2, C16), 23.7 (CH_3, C27), 23.4 (CH_2, C2), 21.3 (CH_3, C30), 18.5 (CH_2, C6), 17.1 (CH_3, C29), 17.1 (CH_3, C26), 15.8 (CH_3, C24), 15.6 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 581.3 (10%, $[M+H]^+$), 603.5 (100%, $[M+Na]^+$), 1183.1 (30%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}ClO_3$ (581.27): C 76.45, H 9.19; found: C 76.30, H 9.31.

6.2.3.26. *m*-Chlorobenzyl (3β) 3-hydroxy-urs-12-en-28-oate (26). Obtained from **UA** by method **A** as a colorless solid; yield: 55%; mp: 181 °C; R_f = 0.25 (*n*-hexane/ethyl actetate, 8:2); $[\alpha]_D$ = +41.5° (*c* 0.43, $CHCl_3$); IR (KBr): *ν* = 3528s, 2990s, 2972s, 2950s, 2920vs, 2870s, 2850s, 1716vs, 1600w, 1576w, 1466m, 1446m, 1438s, 1402w, 1378m, 1358m, 1310m, 1288m, 1272m, 1248m, 1230m, 1218m, 1196m, 1180s, 1162m, 1138m, 1112m, 1094m, 1078m, 1050m, 1028m, 1010m, 996s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): *δ* = 7.51–7.26 (m, 4H, CH_{aromat}), 5.21 (dd, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 5.08 (d, *J* = 12.7 Hz, 1H, CH_a (31)), 4.88 (d, *J* = 12.7 Hz, 1H, CH_b (31)), 3.18 (dd, *J* = 11.1, 4.9 Hz, 1H, *CH* (3)), 2.23 (d, *J* = 11.3 Hz, 1H, *CH* (18)), 1.99 (ddd, *J* = 12.8, 12.8, 5.0 Hz, 1H, CH_a (16)), 1.89–1.72 (m, 3H, CH_2 (2) + CH_a (15)), 1.72–1. 51 (m, 6H, CH_b (16) + CH_2 (22) + CH_a (1) + CH_2 (11)), 1.44–1.22 (m, 5H, *CH* (19) + *CH* (20) + CH_b (7) + CH_b (21) + CH_b (6)), 1.09 (s, 3H, CH_3 (27)), 1.05–1.00 (m, 1H, CH_b (15)), 1.00–0.95 (m, 1H, CH_b (1)), 1.00 (s, 3H, CH_3 (23)), 0.96 (d, *J* = 6.3 Hz, 3H, CH_3 (30)), 0.90 (s, 3H, CH_3 (25)), 0.88 (d, *J* = 6.5 Hz, 3H, CH_3 (29)), 0.79 (s, 3H, CH_3 (24)), 0.72 (brd, *J* = 11.7 Hz, 1H, *CH* (5)), 0.63 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): *δ* = 177.3 (*C*=O, C28), 138.5 (CCl_{aromat}, C34), 138.2 (*C*=CH, C13), 134.5 (C_{aromat}, C32), 129.8 (CH_{aromat}, C35), 128.4 (CH_{aromat}, C36), 128.2 (CH_{aromat}, C33), 126.4 (*CH*=C, C12), 126.0 (CH_{aromat}, C37), 79.2 (*CHOH*, C3), 65.3 (CH_2, C31), 55.4 (*CH*, C5), 53.0 (*CH*, C18), 48.3 (C_{quart}, C17), 47.7 (*CH*, C9), 42.2 (C_{quart}, C14), 39.7 (C_{quart}, C8), 39.3 (*CH*, C19), 39.0 (*CH*, C20), 38.9 (C_{quart}, C8), 38.8 (CH_2, C1), 37.1 (C_{quart}, C10), 36.8 (CH_2, C22), 33.2 (CH_2, C7), 30.8 (CH_2, C21), 28.3 (CH_3, C23), 28.1 (CH_2, C15), 27.4 (CH_2, C11), 24.4 (CH_2, C16), 23.7 (CH_3, C27), 23.4 (CH_2, C2), 21.3 (CH_3, C30), 18.5 (CH_2, C6), 17.1 (CH_3, C29), 17.1 (CH_3, C26), 15.8 (CH_3, C24), 15.6 (CH_3, C25) ppm; MS (ESI, MeOH): *m*/*z* = 581.3 (5%, $[M+H]^+$), 603.5 (20%, $[M+Na]^+$), 1183.1 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}ClO_3$ (581.27): C 76.45, H 9.19; found: C 76.29, H 9.29.

6.2.3.27. *p*-Chlorobenzyl (3β) 3-hydroxy-urs-12-en-28-oate (27). Obtained from **UA** by method **A** as a colorless solid; yield: 86%; mp: 169 °C; R_f = 0.30 (*n*-hexane/ethyl acetate, 8:2); $[\alpha]_D$ = +42.2° (*c* 0.63, $CHCl_3$); IR (KBr): *ν* = 3518s, 2982vs, 2970*v*, 2940vs, 2924vs, 2868s, 2840s, 1714vs, 1494s, 1466s, 1446s, 1402m, 1388m, 1378s, 1358m, 1308m, 1290m, 1272m, 1248m, 1232s, 1206s, 1198s, 1182s, 1174s, 1166s, 1138s, 1114s, 1094s, 1076m, 1050s, 1030s, 1012s, 996s, 810s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): *δ* = 7.31 (ddd, *J* = 8.4, 2.2, 2.2 Hz, 2H, *CH* (34) + *CH* (36)), 7.28–7.26 (ddd, *J* = 8.5, 2.2, 2.2 Hz, 2H, *CH* (33) + *CH* (37)), 5.21 (dd, *J* = 3.7, 3.7 Hz, 1H, *CH* (12)), 5.07 (d, *J* = 12.5 Hz, 1H, CH_a (31)), 4.92 (d, *J* = 12.6 Hz, 1H, CH_b (31)), 3.20 (dd, *J* = 11.0, 4.9 Hz, 1H, *CH* (3)), 2.24 (dd, *J* = 11.2, 1.4 Hz, 1H, *CH* (18)), 2.00 (ddd, *J* = 13.2, 13.2, 4.6 Hz, 1H, CH_a (16)), 1.89 (ddd, *J* = 18.4, 6.4, 4.4 Hz, 1H, CH_a (2)), 1.81 (ddd, *J* = 18.4, 11.2, 3.1 Hz, 1H, CH_b (2)), 1.77 (ddd, *J* = 12.9, 12.9, 4.0 Hz, 1H, CH_a (15)), 1.72–1.69 (m, 1H, CH_b (16)), 1.69–1.58 (m, 5H, CH_2 (22) + CH_a (1) + CH_2 (11)), 1.54–1.40 (m, 4H, CH_a (6) + CH_a (7) + CH_a (21) + *CH* (9)), 1.37–1.24 (m, 5H, *CH* (19) + *CH* (20) + CH_b (7) + CH_b (21) + CH_b (6)), 1.06 (s, 3H, CH_3 (27)), 1.05–1.00 (m, 1H, CH_b (15)), 1.00–0.95 (m, 1H, CH_b (1)), 0.98 (s, 3H, CH_3 (23)), 0.93 (d, *J* = 6.2 Hz, 3H, CH_3 (30)), 0.89 (s, 3H, CH_3 (25)), 0.85 (d, *J* = 6.5 Hz, 3H, CH_3 (29)), 0.78 (s, 3H, CH_3 (24)), 0.70 (dd, *J* = 11.9, 1.3 Hz, 1H, *CH* (5)), 0.59 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): *δ* = 177.3 (*C*=O, C28), 138.2 (*C*=CH, C13), 135.0 (C_{aromat}, C32), 134.0 (CCl_{aromat}, C35), 129.8 (CH_{aromat}, C33), 128.7 (CH_{aromat}, C34), 125.8 (*CH*=C, C12), 79.2 (*CHOH*, C3), 65.3 (CH_2, C31), 55.4 (*CH*, C5), 53.1 (*CH*, C18), 48.3 (C_{quart}, C17), 47.7 (*CH*, C9), 42.2 (C_{quart}, C14), 39.7 (C_{quart}, C8), 39.2 (*CH*, C19), 39.0 (*CH*, C20), 38.9 (C_{quart}, C4), 38.8 (CH_2, C1), 37.1 (C_{quart}, C10), 36.8 (CH_2, C22), 33.2 (CH_2, C7), 30.8 (CH_2, C21), 28.3 (CH_3, C23), 28.1 (CH_2, C15), 27.4 (CH_2, C11), 24.4 (CH_2, C16), 23.7 (CH_3, C27), 23.4 (CH_2, C2), 21.3 (CH_3, C30), 18.4 (CH_2, C6), 17.1 (CH_3, C29), 17.1 (CH_3, C26), 15.8 (CH_3, C24), 15.6 (CH_3, C25) ppm; ESI-MS (MeOH): *m*/*z* = 603.5 (100%, $[M+Na]^+$), 1182.9 (40%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{53}ClO_3$ (581.27): C 76.45, H 9.29; found: C 76.31, H 9.28.

6.2.3.28. (3β) 3-Acetoxy-urs-12-en-28-acid (28). Acetylation of **UA** (5 g; 10.95 mmol) in dry pyridine (100 mL) with acetic anhydride (2.6 mL, 27.4 mmol), NEt_3 (3 mL, 217 mmol) and catalytic amounts of DMAP for 12 h at 24 °C followed by usual work-up and recrystallization from ethanol, **25** as a colorless solid; yield: 90%; mp: 273–277 °C (lit.: 267–268 °C[47]); $[\alpha]_D$ = +69° (*c* 0.19; $CHCl_3$) (lit.: $[\alpha]_D$ = +71.2° (*c* 1.0; $CHCl_3$)[48]); R_f = 0.64 (hexane/ethyl acetate, 8:2); UV-Vis (MeOH): λ_{max} (log *ε*) = 224 nm (3.71); IR (KBr): *ν* = 3423br, 2947s, 1736s, 1696s, 1464m, 1366m, 1245s, 1180w, 1148w, 1097w, 1076w, 1028m, 1009m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): *δ* = 5.26 (m, 1H, *CH* (12)), 4.48 (dd, *J* = 9.4, 7.0 Hz, 1H, *CH* (3)), 2.80 (dd, *J* = 3.8, 4.3 Hz, 1H, *CH* (18)), 2.08 (s, 3H, Ac), 1.97 (ddd, *J* = 13.7, 13.5, 4.0 Hz, 1H, CH_a (16)), 1.90–1.84 (m, 2H, CH_2 (11)), 1.76 (ddd, *J* = 14.0, 13.8, 4.3 Hz, CH_a (22)), 1.69 (ddd, *J* = 13.8, 13.8, 3.7 Hz, 1H, CH_a (15)), 1.64–1.49 (m, 8H, *CH* (9) + CH_a (1) + CH_a (19) + CH_a (6) + CH_a (22) + CH_a (16) + CH_2 (2)), 1.45–1.22 (m, 5H, CH_b (6) + CH_2 (21) + CH_2 (7)), 1.20–1.02 (m, 3H, CH_a (19) + CH_b (1) + CH_b (15)), 1.12 (s, 3H, CH_3 (27)), 0.93 (s, 3H, CH_3 (25)), 0.91 (s, 3H, CH_3 (30)), 0.89 (s, 3H, CH_3 (29)), 0.86-0.81 (m, 1H, *CH* (5)), 0.85 (s, 3H, CH_3 (23)), 0.84 (s, 3H, CH_3 (26)), 0.74 (s, 3H, CH_3 (24)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): *δ* = 184.4 (*C*=O, C28), 171.1 (*C*=O, Ac), 143.5 (*C*=CH, C13), 122.5 (*CH*=C, C12), 80.9 (*CHOH*, C3), 55.3 (*CH*, C5), 47.5 (*CH*, C9), 46.5 (C_{quart},

C17), 45.8 (CH_2, C19), 41.5 (C_{quart}, C14), 40.9 (CH, C18), 39.3 (C_{quart}, C8), 38.0 (CH_2, C1), 37.6 (C_{quart}, C4), 36.9 (C_{quart}, C10), 33.7 (CH_2, C21), 33.0 (CH_3, C29), 32.5 (CH_2, C7), 32.4 (CH_2, C22), 30.6 (C_{quart}, C20), 28.0 (CH_3, C23), 27.6 (CH_2, C15), 25.9 (CH_3, C27), 23.6 (CH_3, C30), 23.5 (CH_2, C11), 23.4 (CH_2, C2), 22.8 (CH_2, C16), 21.3 (CH_3, Ac), 18.1 (CH_2, C6), 17.0 (CH_3, C24), 16.6 (CH_3, C26), 15.3 (CH_3, 25) ppm; MS (ESI, MeOH): m/z = 497.7 (100% $[M\text{-}H]^-$), 543.3 (50% $[M+HCO_2^-]^-$); analysis calculated for $C_{32}H_{50}O_4$ (498.74): C 77.06, H 10.10; found: C 76.87, H 10.19.

6.2.3.29. Benzyl (3β) 3-acetoxy-urs-12-en-28-amide (29). Compound **28** (200 mg, 0.36 mmol) was dissolved in dry DCM and THF (1:1) and treated with thionyl chloride and TEA (3 drops) at 0 °C. After 20 min, the solution was allowed to warm up to room temperature. The solvent was removed under reduced pressure, the residue was dissolved in DCM and benzyl amine (2 mL, 18.4 mmol) and TEA (3 drops) were added. After completion of the reaction (TLC) followed by work up as described above and chromatography (silica gel, *n*-hexane/ethyl acetate) followed by recrystallization from methanol, **29** obtained as a slightly yellowish solid; yield: 86%; mp: 259–265 °C (lit.: 197–198 °C[49]); R_f = 0.42 (toluene/ ethyl acetate/ *n*-heptane/ formic acid, 80:20:30:4);$[\alpha]_D$ = +18.6° (*c* 0.33, $CHCl_3$); IR (KBr): ν=3373br, 2926s, 2872m, 1735s, 1644m, 1520m, 1454m, 1370m, 1246s, 1079w, 1028m cm^{-1}; UV-Vis ($CHCl_3$): λ_{max} (log ε) = 243.7 (3.15) nm, 240.1 (2.58) nm, 241.0 (2.32) nm, 257.7 (2.90) nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.37–7.23 (m, 5H, *CH* (35) + CH (36) + *CH* (37)), 6.16 (dd, *J* = 5.1, 5.1 Hz, 1H, N*H*), 5.23 (dd, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 4.56 (dd, *J* = 14.5, 6.1 Hz, 1H, CH_a (33)), 4.50 (dd, *J* = 8.9, 7.0 Hz, 1H, *CH* (3)), 4.17 (dd, *J* = 14.6, 4.4 Hz, 1H, CH_b (33)), 2.05 (s, 3H, Ac), 1.98 (dd, *J* = 13.7, 4.2 Hz, 1H, CH_a (16)), 1.95–1.86 (m, 2H, CH_a (1) + CH_a (11)), 1.85–1.70 (m, 3H, *CH* (18) + CH_b (16) +, CH_b (11)), 1.70–1.57 (m, 4H, CH_a (22) + CH_a (15) + CH_a (2) + CH_a (6)), 1.56–1.43 (m, 5H, CH_a (9) + CH_b (1) + CH_a (21) + CH_a (7) + CH_b (6)), 1.42–1.35 (m, 1H, *CH* (19)), 1.34–1.24 (m, 2H, CH_b (21) + CH_b (7)), 1.14–1.02 (m, 3H, CH_b (22) + CH_b (15) + CH_b (2)), 1.09 (s, 3H, CH_3 (27)), 0.96 (s, 3H, CH_3 (30)), 0.92 (s, 3H, CH_3 (25)) 0.88–0.85 (m, 1H, *CH* (20)), 0.88 (s, 3H, CH_3 (23)), 0.87 (s, 3H, CH_3 (26)), 0.85 (s, 3H, CH_3 (24)), 0.84–0.80 (m, 1H, *CH* (5)), 0.72 (s, 3H, CH_3 (29)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.0 (*C*=O, C28), 171.1 (*C*=O, Ac), 140.0 (C_{quart}, C32), 138.5 (*C*=CH, C13), 128.8 (*CH*, C34), 128.5 (*CH*, C33), 128.0 (*CH*, C35), 125.8 (*CH*=C, C12), 81.0 (*CH*OAc, C3), 55.4 (*CH*, C5), 54.1 (*CH*, C18), 47.9 (C_{quart}, C17), 47.6 (*CH*, C9), 43.8 (CH_2, C31), 42.6 (C_{quart}, C14), 39.9 (*CH*, C19), 39.7 (C_{quart}, C8), 39.2 (*CH*, C20), 38.4 (CH_2, C1), 37.8 (C_{quart}, C4), 37.4 (CH_2, C22), 36.9 (C_{quart}, C10), 32.9 (CH_2, C21), 31.0 (CH_2, C7), 28.18 (CH_2, C15), 28.0 (CH_3, C23), 25.0 (CH_2, C16), 23.7 (CH_2, C11), 23.4 (CH_3, C27), 23.4 (CH_2, C2), 21.4 (CH_3, Ac), 21.3 (CH_3, C30), 18.3 (CH_2, C6), 17.4 (CH_3, C29), 17.1 (CH_3, C26), 16.8 (CH_3, C24), 15.6 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 588.3 (100%, $[M+H]^+$), 610.3 (22%, $[M+Na]^+$), 1175.2 (32%, $[2M+H]^+$), 1198.4 (65%, [2M+Na; analysis calculated for $C_{39}H_{57}NO_3$ (587.87): C 79.68, H 9.77, N 2.39; found: C 79.51, H 9.82, N 2.11.

6.2.3.30. Benzyl (3β) 3-hydroxy-urs-12-en-28-amide (30). Compound **29** (100 mg, 0.18 mmol) was dissolved in methanol, and KOH (1.2 equiv) was added; the mixture was stirred at room temperature until complete (TLC). Work up as described above gave **30** as a colorless solid; yield: 85.7%; mp: 272–275 °C (lit.: 267–268 °C[49]); R_f = 0.32 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:20:30:4); $[\alpha]_D$ = +41.9° (*c* 0.39, $CHCl_3$); IR (KBr): ν = 3422m, 2970s, 2923s, 2868m, 1647s, 1517s, 1480w, 1452m, 1378w, 1362m, 1293w, 1140w, 1049w, 1028w cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N): δ = 7.81 (dd, *J* = 5.7, 5.7 Hz, 1H, N*H*), 7.51 (d, *J* = 7.5 Hz, 2H, *CH* (33)), 7.39 (dd, *J* = 7.5, 7.5 Hz, 2H, *CH* (34)), 7.30 (dd, *J* = 7.3, 7.3 Hz, 1H, *CH* (35)), 5.46 (dd, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 5.15 (s, 1H, O*H*), 4.75 (dd, *J* = 14.8, 5.7 Hz, 1H, CH_a (31)), 4.66 (dd, *J* = 14.8, 5.8 Hz, 1H, CH_b (31)), 3.49 (dd, *J* = 10.7, 5.4 Hz, 1H, *CH* (3)), 2.46 (d, *J* = 10.8 Hz, 1H, *CH* (18)), 2.28–1.94 (m, 5H, CH_a (1) + CH_a (16) + CH_2 (11) + CH_a (15)), 1.94–1.80 (m, 3H, CH_b (1) + CH_2 (2)), 1.68–1.50 (m, 8H, *CH* (9) + *CH* (19) + CH_2 (22) + CH_a (7) + CH_a (21) + CH_b (15) + CH_a (6)), 1.50–1.31 (m, 3H, CH_b (7) + CH_b (21) + CH_b (6)), 1.28 (s, 3H, CH_3 (23)), 1.23 (s, 3H, CH_3 (27)), 1.18–1.11 (m, 1H, CH_b (16)), 1.08 (s, 3H, CH_3 (24)), 1.06–1.00 (m, 1H, *CH* (20)), 0.99 (s, 3H, CH_3 (25)), 0.99 (d, *J* = 4.5 Hz, 3H, CH_3 (30)), 0.96 (d, *J* = 5.2 Hz, 3H, CH_3 (29)), 0.95 (s, 3H, CH_3 (26)), 0.91–0.86 (m, 1H, *CH* (5)) ppm; ^{13}C NMR (100 MHz, C_5D_5N): δ = 177.8 (*C*=O, C28), 141.1 (C_{quart}, C32), 140.1 (*C*=CH, C13), 129.2 (CH_{aromat}, C34), 128.7 (CH_{aromat}, C33), 127.7 (CH_{aromat}, C35), 126.4 (*CH*=C, C12), 78.6 (*CH*OH, C3), 56.3 (*CH*, C5), 54.1 (*CH*, C18), 48.5 (*CH*, C9), 48.3 (C_{quart}, C17), 44.0 (CH_2, C31), 43.0 (C_{quart}, C14), 40.5 (C_{quart}, C8), 40.3 (*CH*, C19), 39.9 (CH_2, C22), 39.8 (*CH*, C20), 39.6 (C_{quart}, C4), 38.6 (CH_2, C1), 37.8 (C_{quart}, C10), 34.0 (CH_2, C7), 31.7 (CH_2, C21), 29.3 (CH_3, C23), 28.8 (CH_2, C16), 28.6 (CH_2, C2), 25.6 (CH_2, C11), 24.3 (CH_3, C27), 24.1 (CH_2, C15), 21.8 (CH_3, C30), 19.3 (CH_2, C6), 18.0 (CH_3, C26 + C30) 17.1 (CH_3, C24), 16.2 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 546.3 (94%, $[M+H]^+$), 568.3 (27%, $[M+Na]^+$), 1113.5 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{55}NO_2$ (545.84): C 81.32, H 10.16, N 2.57; found: C 81.31, H 10.30, N 2.41.

6.2.3.31. Benzyl (2α,3β) 2,3-Di-acetoxy-olean-12-en-28-oate (31). Obtained as a colorless solid from **14** following method **A**; yield: 82%; R_f = 0.78 (*n*-hexane/ethyl acetetate, 5:3); mp: 104–106 °C; IR (KBr): ν = 3446br, 2972m, 2948s, 2864m, 1742vs, 1626w, 1456m, 1436m, 1384m, 1368m, 1254s, 1232s, 1160m, 1044m, 1032m cm^{-1}; $[\alpha]_D$ = +17.5° (*c* 0.18, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.36–7.28 (m, 4H, CH_{aromat}), 5.27 (dd, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 5.10 (s, *J* = 12.5 Hz, 1H, CH_a (31)), 5.13–5.08 (m, 1H, *CH* (2)), 5.04 (d, *J* = 12.5 Hz, 1H, CH_b (32)), 4.73 (d, *J* = 10.3 Hz, 1H, *CH* (3)), 2.90 (dd, *J* = 13.8, 4.1 Hz, 1H, *CH* (18)), 2.05 (s, 3H, CH_3 (Ac)), 2.03–1.98 (m, 2H, CH_a (16) + CH_a (1)), 1.97 (s, 3H, CH_3 (Ac)), 1.99–1.87 (m, 2H, CH_2 (11)), 1.84 (ddd, *J* = 10.6, 10.6, 4.9 Hz, 1H, CH_a (7)), 1.83–1.78 (m, 1H, CH_b (16)), 1.72 (dd, *J* = 13.8, 13.8 Hz, 1H, CH_a (19)), 1.66–1.60 (m, 2H, *CH* (9) + CH_a (15)), 1.59–1.49 (m, 2H, CH_a (22) + CH_a (6), 1.48–1.39 (m, 1H, CH_b (22)), 1.39–1.36 (m, 1H, CH_b (6)), 1.36–1.28 (m, 1H, CH_a (21)), 1.27–1.22 (m, 1H, CH_b (7)), 1.21–1.17 (m, 1H, CH_b (21)), 1.16–1.10 (m, 1H, CH_b (19)), 1.11 (s, 3H, CH_3 (27)), 1.10–1.0 (m, 2H, CH_b (1) + CH_b (15)), 1.02 (s, 3H, CH_3 (23)), 0.97–0.93 (m, 1H, *CH* (5)), 0.92 (s, 3H, CH_3 (25)), 0.90 (s, 6H, CH_3 (29) + CH_3 (30)), 0.89 (s, 3H, CH_3 (24)), 0.58 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.5 (*C*=O, C28), 171.0 (*C*=O, Ac), 170.7 (*C*=O, Ac), 143.9 (*C*=CH, C13), 136.5 (C_{aromat}, C32), 128.5 (CH_{aromat}, C34 + C36), 128.1 (CH_{aromat}, C33 + C37), 128.1 (CH_{aromat}, C35), 122.2 (CH=C, C12), 80.8 (*CH*OH, C3), 70.2 (*CH*OH, C2), 66.1 (CH_2, C31), 55.0 (*CH*, C5), 47.7 (*CH*, C9), 46.9 (C_{quart}, C17), 46.0 (CH_2, C19), 44.0 (CH_2, C1), 41.8 (C_{quart}, C14), 41.5 (*CH*, C18), 39.5 (C_{quart}, C8), 38.3 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C30), 32.6 (CH_2, C7), 32.5 (CH_2, C22), 30.8 (C_{quart}, C20), 28.6 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.8 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 21.3 (CH_3, C41), 21.0 (CH_3, C39), 18.4 (CH_2, C6), 17.8 (CH_3, C26), 17.0 (CH_3, C24), 16.6 (CH_3, C25) ppm; ESI-MS (MeOH): m/z = 647.1 (30%, $[M+H]^+$), 664.1 (48%, $[M+NH_4]^+$), 989.5 (36%, $[3\,M+K+H]^{2+}$), 1315.3 (100%, $[2M+Na]^+$); analysis calculated for $C_{41}H_{58}O_6$ (646.90): C 76.12, H 9.04; found: C 76.02, H 9.17.

6.2.3.32. *p*-Chlorobenzyl (2α,3β) 2,3-di-acetoxy-olean-12-en-28-oate (32). Obtained as a colorless solid from **14** following method **A**; yield: = 74%; R_f = 0.85 (*n*-hexane/ethyl acetetate, 5:3); mp: 166–168 °C; IR (KBr): ν = 3432w, 2948s, 2878m, 2866m,

1742vs, 1650*v*w, 1636w, 1600w, 1494m, 1462m, 1434w, 1394m, 1368s, 1318w, 1302w, 1254vs, 1234s, 1172m, 1158m, 1122m, 1092m, 1042s, 1032s cm^{-1}; $[\alpha]_D$ = +12.5° (*c* 0.11, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ = 7.31 (ddd, *J* = 8.5, 2.2, 2.2 Hz, 2H, CH_{aromat}), 7.27 (ddd, *J* = 8.4, 2.2, 2.2 Hz, 2H, CH_{aromat}), 5.26 (dd, *J* = 3.4, 3.4 Hz, *CH* (12)), 5.9 (dd, *J* = 11.7, 10.4, 4.8 Hz, 1H, *CH* (2)), 5.06 (d, *J* = 12.5 Hz, 1H, CH_a (31)), 4.99 (d, *J* = 12.5 Hz, 1H, CH_b (31)), 4.73 (d, *J* = 10.5 Hz, *CH* (3)), 2.88 (dd, *J* = 13.8, 4.1 Hz, 1H, *CH* (18)), 2.05 (s, 3H, CH_3 (Ac)), 2.01 (m, 2H, CH_a (16) + CH_a (1)), 1.98 (s, 3H, CH_3 (Ac)), 1.88–1.82 (m, 2H, CH_2 (11)), 1.72–1.66 (ddd, *J* = 13.9, 13.9, 4.3 Hz, 1H, CH_a (7)), 1.67–1.61 (m, 2H, CH_a (19) + CH_b (16)), 1.60–1.50 (m, 4H, *CH* (9) + CH_a (21) + CH_a (15) + CH_a (6)), 1.48–1.26 (m, 3H, CH_b (21) + CH_b (6) + CH_a (22)), 1.25–1.16 (m, 2H, CH_b (7) + CH_b (22)), 1.13–1.10 (m, 1H, CH_b (19)), 1.11 (s, 3H, CH_3 (27)), 1.03–0.97 (m, 2H, CH_b (1) + CH_b (15)), 1.03 (s, 3H, CH_3 (23)), 0.97–0.93 (m, 1H, *CH* (5)), 0.91 (s, 3H, CH_3 (25)), 0.90 (s, 3H, CH_3 (30)), 0.90 (s, 3H, CH_3 (29)), 0.89 (s, 3H, CH_3 (24)), 0.54 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.4 (*C*=O, C28), 171.0 (*C*=O, C38), 170.7 (*C*=O, C40), 143.9 (*C*=CH, C13), 135.0 (C_{aromat}, C32), 134.1 (CCl_{aromat}, C35), 129.7 (CH_{aromat}, C33), 128.8 (CH_{aromat}, C34), 122.3 (*C*H=C, C12), 80.8 (*C*HOH, C3), 70.2 (*C*HOH, C2), 65.3 (CH_2, C31), 55.0 (*C*H, C5), 47.7 (*C*H, C9), 46.9 (C_{quart}, C17), 45.9 (CH_2, C19), 44.1 (CH_2, C1), 41.9 (C_{quart}, C14), 41.5 (*C*H, C18), 39.5 (C_{quart}, C8), 39.5 (C_{quart}, C4), 38.3 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C30), 32.6 (CH_2, C7), 32.5 (CH_2, C22), 30.9 (C_{quart}, C20), 28.6 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.8 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 21.3 (CH_3, C41), 21.1 (CH_3, C39), 18.3 (CH_2, C6), 17.8 (CH_3, C26), 16.9 (CH_3, C24), 16.6 (CH_3, C25) ppm; ESI-MS (MeOH, source CID): *m*/*z* = 703.3 (100%, $[M+Na]^+$), 1041.1 (48%, $[M+NH_4]^+$), 1041.4 (42%, $[3M+K+H]^{2+}$), 1383.3 (100%, $[2M+Na]^+$); analysis calculated for $C_{41}H_{57}ClO_6$ (681.34): C 72.27, H 8.43; found: C 71.99, H 8.57.

6.2.3.33. *(E)* (3-Phenyl)-prop-2-enyl (2α,3β) 2,3-diacetoxy-olean-12-en-28-oate (33). Obtained as a colorless solid from **14** following method **A**; yield: 77%; R_f = 0.88 (*n*-hexane/ethyl acetate, 7:3); mp: 138-142 °C; $[\alpha]_D$ = +16.2° (*c* 0.39, $CHCl_3$); IR (KBr): *v* = 3448m, 2968m, 2948s, 2882m, 2864m, 1750vs, 1714s, 1458m, 1432w, 1366m, 1254vs, 1208m, 1164m, 1060w, 1044m, 1030m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.40–7.22 (m, 5H, CH (32) + C*H* (33) + *CH* (34)), 6.64 (d, *J* = 15.9 Hz, 1H, *CH* (33)), 6.25 (ddd, *J* = 15.9, 6.3, 6.3 Hz, 1H, *CH* (32)), 5.30 (dd, *J* = 3.5, 3.5 Hz, 1H, *CH* (12)), 5.09 (ddd, *J* = 11.1, 11.1, 4.6 Hz, 1H, *CH* (2)), 4.73 (d, *J* = 10.4 Hz, 1H, *CH* (3)), 4.71 (ddd, *J* = 13.0, 6.4, 1.4 Hz, H, CH_a (31)), 4.66 (ddd, *J* = 13.0, 6.4, 1.4, 1H, CH_b (31)), 2.90 (dd, *J* = 13.8, 4.3 Hz, 1H, *CH* (18)), 2.05 (s, 3H, CH_3 (Ac)), 2.03–1.94 (m, 2H, CH_a (16) + CH_a (1)), 1.97 (s, 3H, CH_3 (Ac)), 1.92–1.83 (m, 2H, CH_2 (11)), 1.73 (ddd, *J* = 13.8, 13.8, 4.4 Hz, 1H, CH_a (7)), 1.69–1.52 (m, 6H, CH_a (19) + CH_b (16) + CH_a (6) + CH_a (21) + CH_a (15) + *CH* (9)), 1.47–1.26 (m, 3H, CH_b (21) + CH_b (6) + CH_a (22)), 1.24–1.15 (m, 2H, CH_b (22) + CH_b (7)), 1.13–1.10 (m, 1H, CH_b (19)), 1.12 (s, 3H, CH_3 (27)), 1.10–1.00 (m, 2H, CH_b (1) + CH_b (15)), 1.00 (s, 3H, CH_3 (23)), 0.97–0.93 (m, 1H, *CH* (5)), 0.93 (s, 3H, CH_3 (25)), 0.90 (s, 3H, CH_3 (30)), 0.89 (s, 3H, CH_3 (29)), 0.89 (s, 3H, CH_3 (24)), 0.71 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.5 (*C*=O, C28), 171.0 (*C*=O, Ac), 170.6 (*C*=O, Ac), 144.0 (*C*=CH, C13), 136.5 (C_{aromat}, C34), 133.9 (*CH*=CH, C32), 128.7 (CH_{aromat} C35), 128.1 (CH_{aromat}, C37), 126.7 (CH_{aromat}, C36), 123.8 (CH=CH, C33), 122.2 (CH=C, C12), 80.8 (CHOH, C3), 70.2 (*C*HOH, C2), 64.8 (CH_2, C31), 55.0 (*C*H, C5), 47.7 (*C*H, C18), 46.9 (C_{quart}, C17), 46.0 (CH_2, C19), 44.0 (CH_2, C1), 41.9 (C_{quart}, C14), 41.5 (*C*H, C18), 39.6 (C_{quart}, C8), 39.5 (C_{quart}, C4), 38.3 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C30), 32.6 (CH_2, C7), 32.6 (CH_2, C22), 30.9 (C_{quart}, C20), 28.6 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.8 (CH_3, C29), 23.6 (CH_2, C11), 23.2 (CH_2, C16), 21.3 (CH_3, Ac), 21.0 (*C*H$_3$, Ac), 18.3 (*C*H$_2$, C6), 17.8 (*C*H$_3$, C26), 17.1 (*C*H$_3$, C24), 16.5 (*C*H$_3$, C25) ppm; MS (ESI, MeOH): *m*/*z* = 695.3 (100%, $[M+H]^+$); analysis calculated for $C_{43}H_{60}O_6$ (672.93): C 76.75, H 8.99; found: C 76.51, H 9.14.

6.2.3.34. Ethyl (2α,3β) 2,3-di-acteoxy-olean-12-en-28-amide (34). Compound **34** was obtained as a colorless solid (81%) from **14** (150 mg, 0.27 mmol) following the procedure given for **29**; mp 146–149 °C; R_f = 0.41 (toluene/ethyl acetate/*n*-heptane/HCOOH, 80:25:30:4); $[\alpha]_D$ = +16° (*c* 3.1, $CHCl_3$); IR (KBr): *v* = 3432br, 2947s, 1745s, 1662m, 1517m, 1368s, 1252s, 1044s cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.82 (dd, *J* = 5.1, 5.1 Hz, 1H, N*H*), 5.36 (dd, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 5.09 (ddd, *J* = 11.4, 11.4, 4.6 Hz, 1H, *CH* (2)), 4.74 (d, *J* = 10.3 Hz, 1H, *CH* (3)), 3.38 - 3.30 (m, 1H, CH_a (31)), 3.19–3.03 (m, 1H, CH_2 (31)), 2.50 (dd, *J* = 12.8, 3.4 Hz, 1H, *CH* (18)), 2.05 (s, 3H, CH_3 (Ac)), 2.02 (ddd, *J* = 12.5, 12.5, 4.7 Hz, 1H, CH_a (16)), 1.98 (s, 3H, CH_3 (Ac)), 1.96–1.87 (m, 3H, CH_2 (11) + CH_a (1)), 1.75 (m, 1H, *J* = 13.4, 13.4 Hz, CH_a (19)), 1.70–1.61 (m, 3H, *CH* (9) + CH_b (16) + CH_a (7)), 1.60–1.47 (m, 4H, CH_b (7) + CH_a (22) + CH_a (15) + CH_a (6)), 1.44 (ddd, *J* = 11.9, 11.9, 2.3 Hz, 1H, CH_b (6)), 1.38–1.25 (m, 2H, CH_b (22) + CH_a (21)), 1.22–1.16 (m, 1H, CH_b (21)), 1.17–1.08 (m, 1H, CH_b (19)), 1.15 (s, 3H, CH_3 (27)), 1.10 (*t*, *J* = 7.3 Hz, CH_3 (32)), 1.06 (s, 3H, CH_3 (25)), 1.05–0.98 (m, 2H, CH_b (15) + CH_b (1)), 0.90 (m, 13H, *CH* (5) + CH_3 (23) + CH_3 (30) + CH_3 (24) + CH_3 (29)), 0.77 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.1 (*C*=O, C28), 171.0 (*C*=O, Ac), 170.7 (*C*=O, Ac), 145.4 (*C*=CH, C13), 122.3 (*C*H=C, C12), 80.7 (*C*HOH, C3), 70.1 (*C*HOH, C2), 55.0 (*C*H, C5), 47.6 (*C*H, C9), 47.0 (CH_2, C19), 46.3 (C_{quart}, C17), 44.1 (CH_2, C1), 42.4 (*C*H, C18). 42.3 (C_{quart}, C14), 39.6 (C_{quart}, C8), 39.5 (C_{quart}, C4), 38.2 (C_{quart}, C10), 34.5 (CH_2, C31), 34.3 (CH_2, C31), 33.2 (CH_3, C29), 32.6 (CH_2, C7), 32.3 (CH_2, C22), 30.9 (C_{quart}, C20), 28.6 (CH_3, C23), 27.4 (CH_2, C15), 25.9 (CH_3, C27), 23.9 (CH_2, C11), 23.7 (CH_2, C16). 23.7 (CH_3, C30), 21.3 (CH_3, Ac), 21.1 (CH_3, Ac), 18.3 (CH_2, C6). 17.8 (*C*H$_3$, C26), 17.0 (*C*H$_3$, C24), 16.6 (*C*H$_3$, C25), 14.8 (*C*H$_3$, C32) ppm;; MS (ESI, MeOH): *m*/*z* = 584.5 (100%, $[M+H]^+$), 606.4 (34%, $[M+Na]^+$), 1166.9 (14%, $[2M+H]^+$), 1189.2 (48%, $[2M+Na]^+$); analysis calculated for $C_{36}H_{57}NO_5$ (583.84): C 74.06, H 9.84, N 2.40; found: C 73.81, H 9.92; N 2.35.

6.2.3.35. Propyl (2α,3β) 2,3-di-acetoxy-olean-12-en-28-amide (35). Compound **35** was obtained from **14** (150 mg, 0.27 mmol) following the procedure given for **29** as a colorless solid (61%); mp 129–130 °C; R_f = 0.54 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +21° (*c* 2.6, $CHCl_3$); IR (KBr): *v* = 3432br, 2948s, 1744ss, 1661m, 1517m, 1368s, 1251ss, 1043s cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.88 (dd, *J* = 5.9, 4.9 Hz, 1H, N*H*), 5.36 (dd, *J* = 3.4, 3.4 Hz, 1H, *CH* (12)), 5.09 (ddd, *J* = 11.4, 10.3, 4.6 Hz, 1H, *CH* (2)), 4.75 (d, *J* = 10.3 Hz, 1H, *CH* (3)), 3.34 (d*t*, *J* = 13.6, 7.1 Hz, 1H, CH_a (31)), 2.95 (d*t*, *J* = 13.6, 7.2 Hz, 1H, CH_b (31)), 2.51 (dd, *J* = 13.4, 3.3 Hz, 1H, *CH* (18)), 2.05 (s, 3H, CH_3 (Ac)), 2.02 (ddd, *J* = 12.5, 12.5, 4.8 Hz, 1H, CH_a (1)), 1.98 (s, 3H, CH_3 (Ac)), 1.91–1.86 (m, 3H, CH_a (16) + CH_2 (11)), 1.77 (dd, *J* = 13.4, 13.4 Hz, CH_a (19)), 1.74–1.59 (m, 3H, *CH* (9) + CH_a (7) + CH_b (16)), 1.55–1.36 (m, 7H, CH_a (22) + CH_b (7) + CH_a (15) + CH_2 (32) + CH_2 (6)), 1.36–1.24 (m, 2H, CH_a (21) + CH_b (22)), 1.24–1.16 (m, 1H, CH_b (19)), 1.15 (s, 3H, CH_3 (17)), 1.11–1.00 (m, 2H, CH_b (1) + CH_b (15)), 1.06 (s, 3H, CH_3 (25)), 1.00–0.94 (m, 1H, *CH* (5)), 0.93–0.89 (m, 12H, CH_3 (23) + CH_3 (30) + CH_3 (24) + CH_3 (29)), 0.77 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.1 (*C*=O, C28), 171.0 (*C*=O, Ac), 170.7 (*C*=O, Ac), 154.4 (*C*13, *C*=CH), 122.3 (*CH*=C, C12), 80.7 (CHOH, C3), 70.0 (*C*HOH, C2), 55.0 (CH, C5), 47.6 (CH, C9), 46.4 (C_{quart}, C17), 44.1 (CH_2, C1), 42.4 (*C*H, C18), 42.3 (C_{quart}, C14), 41.3 (CH_2, C31), 39.6 (C_{quart}, C8), 39.5 (C_{quart}, C4), 38.2 (C_{quart}, C10), 34.3 (CH_2, C21), 33.1 (CH_3, C29), 32.7 (CH_2, C7), 32.3 (CH_2, C22), 30.9 (C_{quart}, C20), 28.6 (CH_3, C23), 27.4 (CH_2, C15) 25.9 (CH_3, 27), 23.9 (CH_2, C11), 23.7 (CH_2,

C16), 23.7 (CH_3, C30), 22.8 (CH_2, C32), 21.3 (CH_3, Ac), 21.1 (CH_3, Ac), 18.3 (CH_2, C6), 17.8 (CH_3, C26), 17.0 (CH_3, C24), 16.6 (CH_3, C25), 11.6 (CH_3, C33) ppm; MS (ESI, MeOH): m/z = 598.4 (100%, $[M+H]^+$), 620.5 (39%, $[M+Na]^+$), 1195.0 (28%, $[2M+H]^+$), 1217.3 (68%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{59}NO_5$ (597.87): C 74.33, H 9.95, N 2.34; found: C 74.19, H 10.03; N 2.21.

6.2.3.36. 3-Propen-1-yl (2α,3β) 2,3-di-acetoxy-olean-12-en-28-amide (36). Compound **36** was obtained from **14** (200 mg, 0.36 mmol) and allyl amine following the procedure given for **29** as a colorless solid (65%); mp 158–159 °C; R_f = 0.48 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +11° (*c* 4.2, $CHCl_3$); IR (KBr): ν = 3428br, 2948s, 1744ss, 1662m, 1517m, 1368s, 1252ss, 1043s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.97–5.91 (dd, *J* = 5.3, 5.2 Hz, 1H, N*H*), 5.88–5.78 (m, 2H, CH_2 (31)), 5.37 (dd, *J* = 3.3, 3.3 Hz, 1H, C*H* (12)), 5.16 (dd, *J* = 17.2, 1.4 Hz, 1H, CH_a (33)), 5.12 (dd, *J* = 10.3, 1.3 Hz, CH_b (33)), 5.09 (dd, *J* = 11.2, 10.9, 4.5 Hz, 1H, C*H* (2)), 4.75 (d, *J* = 10.3 Hz, 1H, C*H* (3)), 4.01 (dddd, *J* = 11.9, 6.0, 5.9, 1.2 Hz, 1H, CH_a (32)), 3.61 (ddd, *J* = 15.6, 5.8, 4.5 Hz, 1H, CH_b (32)), 2.54 (dd, *J* = 12.8, 3.4 Hz, 1H, C*H* (18)), 2.06–2.00 (m, 1H, CH_a (1)), 2.05 (s, 3H, CH_3 (Ac)), 2.03 (ddd, *J* = 12.4, 12.4, 4.7 Hz, 1H, CH_a (16)), 1.98 (s, 3H, CH_3 (Ac)), 1.96–1.82 (m, 2H, CH_2 (11)), 1.77 (dd, *J* = 13.3 Hz, 1H, CH_a (19)), 1.74–1.69 (m, 1H, CH_a (7)), 1.69–1.61 (m, 2H, C*H* (9) + CH_b (16)), 1.61–1.41 (m, 5H, CH_a (22) + CH_b (7) + CH_a (15) + CH_2 (6)), 1.41–1.24 (m, 3H, CH_b (22) + CH_2 (21)), 1.24–1.16 (m, 1H, CH_b (19)), 1.15 (s, 3H, CH_3 (27)), 1.09–0.96 (m, 2H, CH_b (1) + CH_b (15)), 1.05 (s, 3H, CH_3 (25)), 0.96 (dd, *J* = 11.9, 1.5 Hz, 1H, C*H* (5)), 0.90 (s, 12H, CH_3 (23) + CH_3 (30) + CH_3 (24) + CH_3 (29)), 0.75 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.1 (*C*=O, C28), 171.0 (*C*=O, Ac), 170.7 (*C*=O, Ac), 145.3 (*C*=CH, C13), 134.6 (*C*H=CH_2, C32), 122.6 (*C*H=C, C12), 116.4 (CH_2=CH, C33), 80.7 (*C*HOH, C3), 70.1 (*C*HOH, C2), 55.0 (*C*H, C5), 47.6 (CH, C9), 46.9 (CH_2, C19), 46.5 (C_{quart}, C17), 44.1 (CH_2, C1), 42.3 (*C*H, C18), 42.3 (CH_2, C31), 42.1 (C_{quart}, C14), 39.6 (C_{quart}, C8), 39.5 (C_{quart}, C4), 38.2 (C_{quart}, C10), 34.3 (Ch_2, C21), 33.1 (CH_3, C29), 32.7 (CH_2, C7). 32.3 (CH_2, C22), 30.9 (C_{quart}, C20), 28.6 (CH_3, C23), 27.4 (CH_2, C15), 25.9 (CH_3, C27), 24.0 (CH_2, C11), 23.7 (CH_2, C16), 23.7 (CH_3, C30), 21.3 (CH_3, Ac), 21.1 (CH_3, Ac), 18.4 (CH_2, C16), 17.8 (CH_3, C26), 17.0 (CH_3, C24), 16.6 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 596.5 (100%, $[M+H]^+$), 618.5 (36%, $[M+Na]^+$), 1191.0 (28%, $[2M+H]^+$), 1213.2 (74%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{57}NO_5$ (595.85): C 74.58, H 9.64, N 2.35; found: C 74.47, H 9.82; N 2.17.

6.2.3.37. 3-Propyn-1-yl (2α,3β) 2,3-di-acetoxy-olean-12-en-28-amide (37). Compound **37** was obtained from **14** (200 mg, 0.36 mmol) as described for **29**; yield: 56%; mp 226–227 °C; R_f = 0.51 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:25:30:4); $[\alpha]_D$ = +1° (*c* 3.8, $CHCl_3$); IR (KBr): ν = 3432br, 3250w, 2947s, 2362w, 1744s, 1654m, 1508m, 1369s, 1251ss, 1044s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 6.04 (dd, *J* = 4.8 Hz, 1H, N*H*), 5.40 (dd, *J* = 3.4, 3.4 Hz, 1H, C*H* (12)), 5.08 (ddd, *J* = 11.4, 10.7, 4.6 Hz, 1H, C*H* (2)), 4.74 (d, *J* = 10.3 Hz, 1H, C*H* (3)), 4.03 (ddd, *J* = 17.6, 5.4, 2.6 Hz, 1H, CH_a (31)), 3.90 (ddd, *J* = 17.6, 4.4, 2.6 Hz, 1H, CH_b (31)), 2.53 (dd, *J* = 12.9, 3.4 Hz, 1H, C*H* (18)), 2.20 (dd, *J* = 2.6 Hz, 1H, C≡C*H* (33)), 2.05 (s, 3H, CH_3 (Ac)), 2.02 (ddd, *J* = 12.4, 12.4, 4.7 Hz, 1H, CH_a (16)), 1.97 (s, 3H, CH_3 (Ac)), 1.99–1.84 (m, 3H, CH_2 (11) + CH_a (1)), 1.75 (dd, *J* = 13.3, 13.3 Hz, 1H, CH_a (19)), 1.72–1.67 (m, 1H, CH_a (7)), 1.66–1.51 (m, 4H, C*H* (9) + CH_a (22) + CH_b (16) + CH_a (6)), 1.51–1.41 (m, 2H, CH_a (15) + CH_b (7)), 1.41–1.26 (m, 3H, CH_b (6) + CH_a (21) + CH_b (22)), 1.22–1.16 (m, 1H, CH_b (21)), 1.15 (s, 3H, CH_3 (27)), 1.14–1.00 (m, 1H, CH_b (19)), 1.06 (s, 3H, CH_3 (25)), 1.08–0.99 (m, 2H, CH_b (1) + CH_b (15)), 0.99–0.94 (m, 1H, C*H* (5)), 0.90 (s, 12H, CH_3 (23) + CH_3 (30) + CH_3 (24) + CH_3 (29)), 0.79 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.0 (*C*=O, C28), 171.0 (*C*=O, Ac), 170.7 (*C*=O, Ac), 145.0 (*C*=CH, C13), 122.9 (CH=C, C12), 80.7 (*C*HOH, C3), 71.8 (CH_2, C31), 71.8 (*C*≡CH, C32), 70.1 (*C*HOH, C2), 70.0 (CH≡*C*, C33), 55.0 (CH, C5), 47.6 (*C*H, C9), 46.8 (CH_2, C19), 46.5 (C_{quart}, C17), 44.1 (CH_2, C1), 42.3 (*C*H, C18), 42.2 (C14, C_{quart}), 39.6 (C_{quart}, C8), 39.5 (C_{quart}, C4), 38.2 (C_{quart}, C10), 34.2 (CH_2, C21), 33.1 (CH_3, C29), 32.4 (CH_2, C7), 32.3 (CH_2, C22), 30.9 (C_{quart}, C20), 29.5 (CH_2, C31), 24.0 (CH_2, C11), 23.6 (CH_2, C16), 23.8 (CH_3, C30), 21.3 (CH_3, Ac), 21.0 (CH_3, Ac), 18.3 (CH_2, C6), 17.7 (CH_3, C26), 17.1 (CH_3, C24), 16.6 (CH_3, C25) ppm; MS (ESI, MeOH): m/z = 594.5 (91%, $[M+H]^+$), 616.5 (39%, $[M+Na]^+$), 1186.9 (24%, $[2M+H]^+$), 1209.2 (100%, $[2M+Na]^+$); analysis calculated for $C_{37}H_{55}NO_5$ (593.84): C 74.83, H 9.34, N 2.36; found: C 74.61, H 9.27; N 2.23.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.047.

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Supplementary material

Towards cytotoxic and selective derivatives of maslinic acid

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Annexin V – Assay: Detailed information

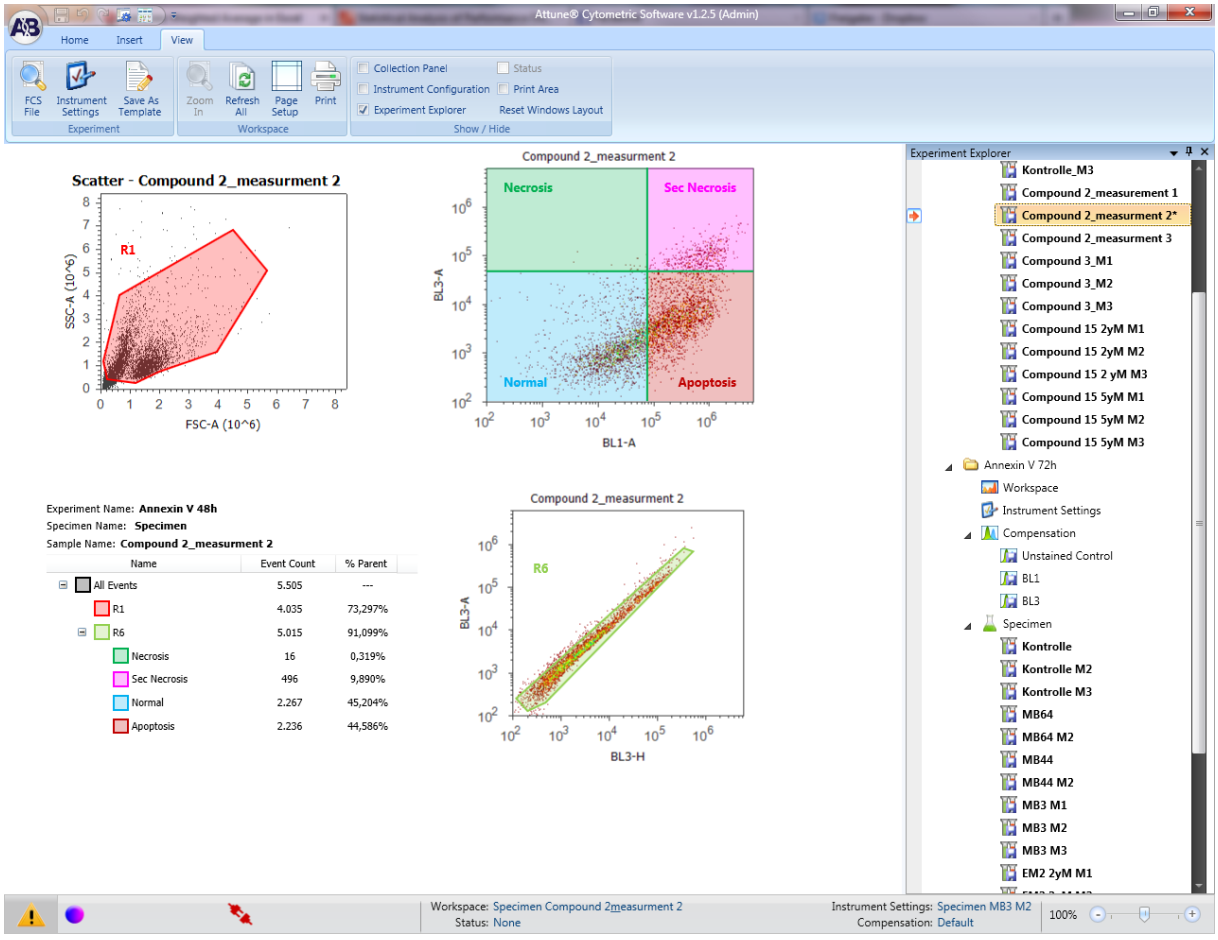


Figure 1: Screenshot of the Annexin V-Determination

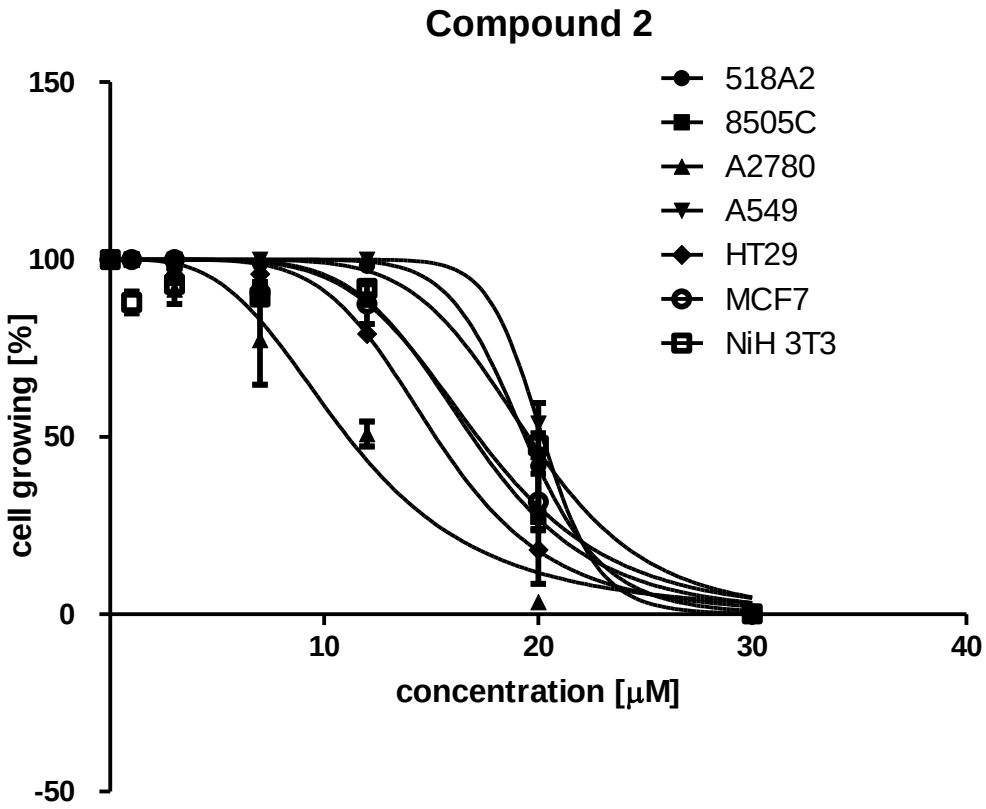
In table 1 are given the arithmetic means of the population distribution [%]. Distribution of each population in percent was calculated via Attune® Cytometric Software v1.2.5 (See figure 1). The standard deviation was calculated via Excel (STDEV function) from three experiments.

		necrosis	SD	sec necrosis	SD	normal	SD	apoptosis	SD
48h	control	6,9	0,5	12,0	1,1	64,9	1,0	16,2	0,8
	2	0,4	0,1	9,4	0,6	45,9	1,4	44,3	1,1
	3	0,9	0,1	6,3	0,0	45,8	1,1	47,1	1,0
	15	13,2	1,0	15,7	1,5	55,4	1,2	15,7	0,8
	15*	8,8	1,2	17,9	0,9	50,0	1,5	23,3	1,8
72h	control	4,7	1,4	19,4	2,1	67,1	1,9	8,8	1,1
	2	6,1	0,9	21,8	5,5	46,4	4,7	25,6	9,4
	3	6,0	1,0	16,0	2,4	44,3	1,8	33,6	1,8
	15	6,6	2,1	28,7	1,4	49,8	5,4	15,0	2,5
	15*	8,2	1,2	29,4	1,4	46,2	3,3	16,2	0,9

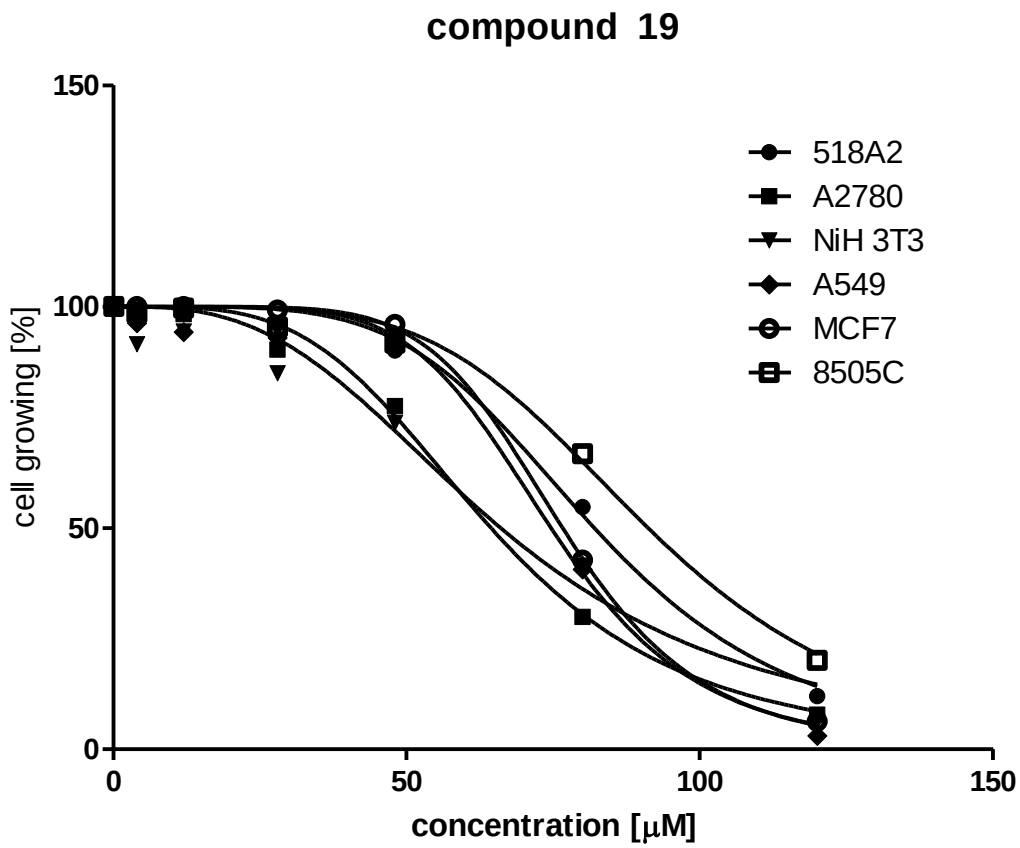
Table 1: Annexin V assay. Ovarian cancer cells (A2780) were treated for 48 and 72 h with **2** (30 μM), **3** (25 μM), **15** (2 μM) and **15*** (5 μM). The averaged values for each population are given in [%] together with the standard deviation.

Detailed data from the SRB assay:

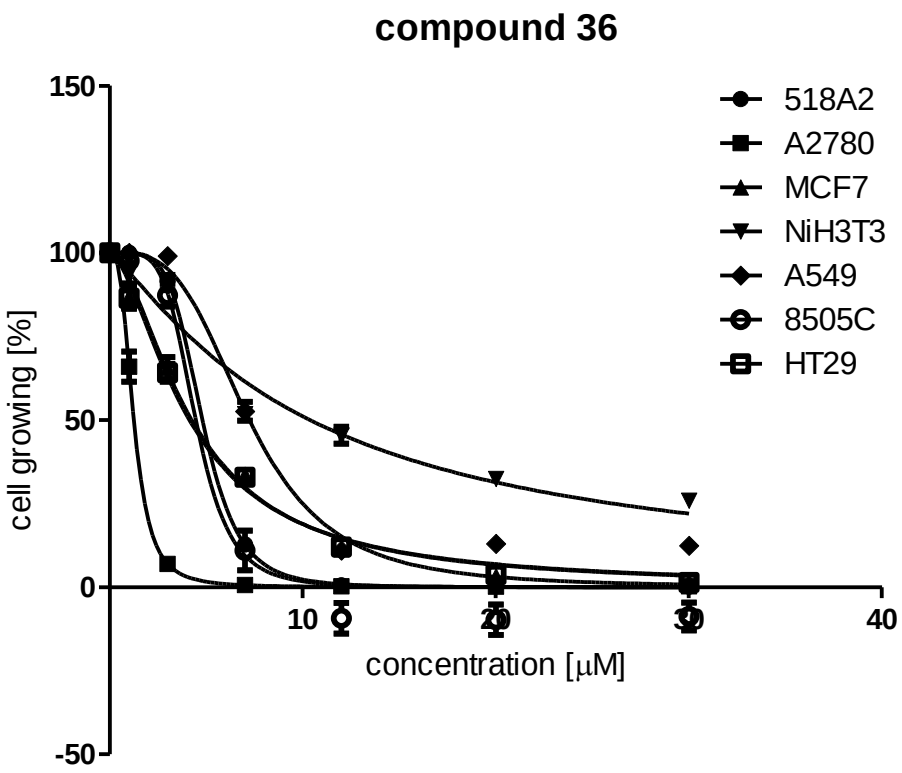
For selected compounds out of every series detailed data are given below.



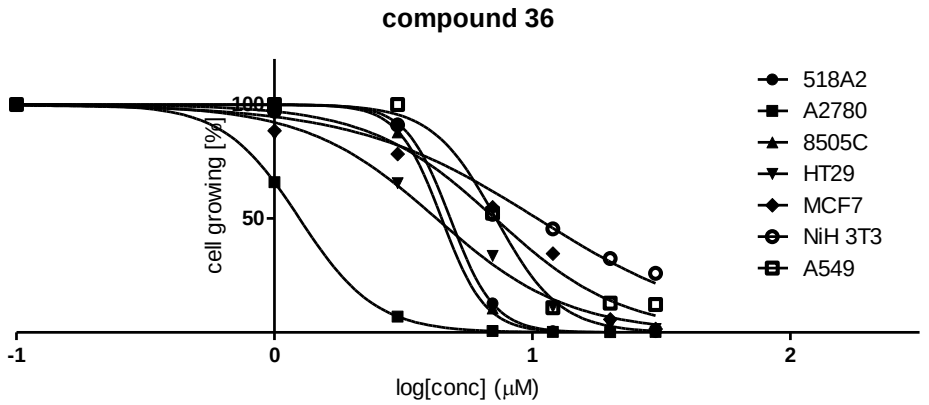
Nonlin fit Table of results	A 518A2 (MBZ43)	B 8505C' (MBZ49)	C A2780 (MBZ43)	D A549 (MBZ43)	E HT29 (MBZ43)	F MCF' (MBZ49)	G NiH3T3 (MBZ43)
	Y	Y	Y	Y	Y	Y	Y
Inhibition vs. response -- Variable slope (two parar							
Best-fit values							
Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
IC50	19.38	16.90	11.10	20.19	15.20	17.18	19.56
Hillslope	10.60	5.929	3.427	15.65	5.563	5.466	6.981
Span	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
Std. Error							
IC50	0.8068	0.8777	0.6039	0.006925	0.2238	0.5322	0.5322
Hillslope	11.68	1.335	0.5805	0.5283	0.3097	0.7268	1.907
95% Confidence Intervals							
IC50	17.69 to 21.07	15.07 to 18.74	9.835 to 12.36	20.18 to 20.21	14.73 to 15.67	16.06 to 18.29	18.44 to 20.67
Hillslope	-13.85 to 35.05	3.135 to 8.724	2.212 to 4.642	14.55 to 16.76	4.915 to 6.211	3.945 to 6.987	2.989 to 10.97
Goodness of Fit							
Degrees of Freedom	19	19	19	19	19	19	19
R square	0.9375	0.9233	0.9488	1.000	0.9941	0.9703	0.9487
Absolute Sum of Squares	1925	2474	1813	0.1111	190.9	886.1	1228
Sy.x	10.07	11.41	9.768	0.07648	3.169	6.829	8.041
Constraints							
Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
Number of points							
Analyzed	21	21	21	21	21	21	21



Nonlin fit Table of results	A 518A2 (MBZ57)	B A2780 (MBZ57)	C NiH3T3 (MBZ57)	D A549 (MBZ57)	E MCF' (MBZ57)	F 8505C' (MBZ57)
	Y	Y	Y	Y	Y	Y
Inhibition vs. response -- Variable slope (t						
Best-fit values						
Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
IC50	82.01	64.38	66.04	74.61	76.59	91.17
Hillslope	4.718	3.796	2.949	5.958	6.379	4.709
Span	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
Std. Error						
IC50	0.7718	0.8942	2.468	1.261	0.2267	0.9739
Hillslope	0.2119	0.1683	0.3020	0.6222	0.1447	0.2321
95% Confidence Intervals						
IC50	80.39 to 83.62	62.51 to 66.25	60.87 to 71.20	71.98 to 77.25	76.12 to 77.07	89.14 to 93.21
Hillslope	4.274 to 5.161	3.444 to 4.149	2.317 to 3.581	4.656 to 7.260	6.076 to 6.681	4.223 to 5.195
Goodness of Fit						
Degrees of Freedom	19	19	19	19	19	19
R square	0.9955	0.9946	0.9627	0.9871	0.9995	0.9925
Absolute Sum of Squares	91.04	137.3	790.2	326.7	11.74	118.8
Sy.x	2.189	2.688	6.449	4.146	0.7859	2.500
Constraints						
Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
Number of points						
Analyzed	21	21	21	21	21	21



	Nonlin fit Table of results	A 518A2 (MBZ8_2)	B A2780 (MBZ8_2)	C MCF7 (MBZ8_2)	D NiH3T3 (MBZ8_2)	E A549 (MBZ8_2)	F 8505C(B108)	G HT29 (MBZ9_1
		Y	Y	Y	Y	Y	Y	Y
1	Inhibition vs. response -- Variable slope (two parar							
2	Best-fit values							
3	Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
4	Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
5	IC50	4.801	1.251	4.250	10.37	7.229	4.455	4.130
6	Hillslope	5.111	2.956	1.706	1.193	3.391	4.993	1.651
7	Span	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
8	Std. Error							
9	IC50	0.05168	0.02931	0.1844	0.6766	0.3052	0.3343	0.1641
10	Hillslope	0.1332	0.2110	0.1071	0.1077	0.4891	0.8900	0.09214
11	95% Confidence Intervals							
12	IC50	4.693 to 4.910	1.190 to 1.312	3.864 to 4.636	8.959 to 11.79	6.590 to 7.868	3.755 to 5.155	3.787 to 4.474
13	Hillslope	4.832 to 5.390	2.514 to 3.398	1.482 to 1.930	0.9677 to 1.418	2.368 to 4.415	3.130 to 6.856	1.458 to 1.844
14	Goodness of Fit							
15	Degrees of Freedom	19	19	19	19	19	19	19
16	R square	0.9994	0.9959	0.9882	0.9576	0.9739	0.9718	0.9903
17	Absolute Sum of Squares	29.12	123.4	358.9	750.8	904.5	1479	288.2
18	Sy.x	1.238	2.549	4.346	6.286	6.900	8.822	3.895
19	Constraints							
20	Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
21	Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
22	Number of points							
23	Analyzed	21	21	21	21	21	21	21
24								



Nonlin fit Table of results	A 518A2	B A2780	C MCF7	D NiH 3T3	E A549	F 8505C	G HT29
	Y	Y	Y	Y	Y	Y	Y
Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
logIC50	0.6816	0.09727	0.8553	1.019	0.8586	0.6482	0.6284
HillSlope	-5.106	-2.957	-1.768	-1.226	-3.514	-5.162	-1.706
EC50	4.803	1.251	7.167	10.44	7.221	4.449	4.250
Span	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
Std. Error							
logIC50	0.001496	0.001105	0.04606	0.04973	0.03284	0.06552	0.02746
HillSlope	0.04261	0.02290	0.3042	0.1975	0.9590	1.856	0.1561
95% Confidence Intervals							
logIC50	0.6777 to 0.6854	0.09443 to 0.1001	0.7369 to 0.9737	0.8909 to 1.147	0.7742 to 0.9430	0.4798 to 0.8167	0.6578 to 0.6990
HillSlope	-5.216 to -4.997	-3.015 to -2.898	-2.550 to -0.9860	-1.734 to -0.7181	-5.980 to -1.049	-9.933 to -0.3901	-2.107 to -1.305
EC50	4.761 to 4.846	1.243 to 1.259	5.456 to 9.413	7.779 to 14.02	5.945 to 8.771	3.018 to 6.557	3.613 to 5.001
Goodness of Fit							
Degrees of Freedom	5	5	5	5	5	5	5
R square	1.000	1.000	0.9742	0.9653	0.9770	0.9727	0.9933
Absolute Sum of Squares	0.2619	0.1275	240.9	210.7	267.2	499.2	66.92
Sy.x	0.2289	0.1597	6.941	6.491	7.311	9.992	3.658
Constraints							
Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
Number of points							
Analyzed	7	7	7	7	7	7	7

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Original article

Membrane damaging activity of a maslinic acid analog

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ABSTRACT

Close inspection of human ovarian cancer cells A2780 in the course of an antitumor screening using maslinic acid analogs revealed for one of the compounds, 4-oxa-4-phenyl-butyl 2,3-dihydroxy-olean-12-en-28-oate (**1**), an unusual behavior. During the incubation of the cells with **1**, at the perimeter of the cells or close by crystals were formed consisting of cholesterol and excess **1**. Compound **1** was incorporated into the cell's membrane followed by an extrusion of cholesterol from the lipid rafts. As a consequence of the alterations of the cell membrane, a volume decrease was initiated that triggered apoptosis; this extends previous models on apoptosis initiating mechanisms.

1. Introduction

Since the industrial world reached the third stage of epidemiologic transition [1], cardiovascular and neoplastic diseases are the major causes of human mortality. Pharmaceutical, technical and medicinal improvements transferred the developed countries into Gaziano's [2] "fifth stage of epidemiologic transitions" – the age of obesity and inactivity diseases. Thus, life expectancy was increasing during the last decades while most of the causes of death remained the same. Multidrug resistances, fall into relapses and undesired side effects of treatments, however, are still unresolved problems. For example, in case of cancer, one of the milestone compounds and a gold standard of modern chemotherapy, cis-platin [3], leads to a controlled cell death of tumor cells by a tissue independent intercalation into the DNA: However, a low selectivity between tumor and non-tumor cells can be gained [4,5] due to intrinsic mechanisms and the pharmacokinetic of this compound.

On the other hand natural products have been used to treat human diseases for thousands of years. The success of these "treatments" was by and large mediocre, but nowadays natural products serve as an inspiration for the development of new and selective anti-cancer active compounds. Among them, the triterpenoic acids are regarded as most promising candidates.

The β-amyrin-type triterpenoid maslinic acid (**MA**, Fig. 1) is known as an apoptosis inducing compound. For this compound a caspase 8 supported intrinsic mechanism [6–10] has been claimed. In addition, a tumor specific mode of action was observed [11,12] but a missing knowledge of the initial death signal prevents developing a target based rational approach. Moreover, for the majority of the naturally occurring triterpenoic acids, multi-target activity is assumed; this explains the broad range of biological activities of triterpenoids, among them an anti-malaria, anti-inflammatory and anti-cancer activity have been reported [13]. During our ongoing research in the field of antitumor active triterpenoic acids an unprecedented mode of action of one of our compounds now allowed new insights into the early steps of their biological activity.

2. Results

As already known from other triterpenoic acids, the generation of an amphiphilic character [14] seems mandatory for achieving significant cytotoxicity. Thus, maslinic acid (**MA**) was converted into its 4-oxa-4-phenyl-butyl ester **1** by treating **MA** with potassium carbonate and (3-bromopropoxy) benzene in DMF. Paralleling the behavior of glycyrrhetinic acid analogs [15], this kind of derivatization gave an increase in cytotoxicity. Thus, for compound **1**, an approximately two-fold better cytotoxicity (Table 1) was established in sulforhodamine B (SRB) colorimetric assays using several human cancer cell lines and non-malignant mouse fibroblasts (NiH 3T3). This was accompanied, however, with a loss of tumor/non-tumor cell selectivity.

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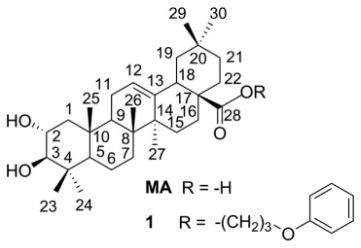

Fig. 1. Structure of maslinic acid (**MA**) and of derivative **1**.

Nevertheless, careful microscopic investigation of the cells revealed an unusual, surprising behavior. In a concentration (Fig. 2) and time dependent manner (data not shown) growth of crystals was observed – independently from the cell line used. In an extra solubility assay the solubility of **1** was determined and found to be 109.5 ± 15 μM. In addition, no crystals were found under these conditions in pure cell medium.

The crystals were located on the perimeter of the cells (or at least very close by). These crystals were collected and investigated in more detail using several analytical techniques. A solution of the crystals was subjected to an HPTLC analysis as well as to an ESI-MS investigation. From the data of these experiments the presence of **1** together with cholesterol was deduced. To support these findings, Raman spectra (using a Renishaw inVia spectrometer equipped with a Leica confocal microscope) were obtained from crystals still attached to A2780 ovarian cancer cells. These experiments, too, showed the crystals as a mixture of cholesterol and **1**. The data from these experiments are compiled in the supplementary material. The development and growth of extracellular cholesterol-containing crystals at the perimeter of cells after their incubation with triterpenoids is unprecedented.

Together with the growth of these crystals, cell shrinking, chromatin condensation and an early detachment were observed (Fig. 2A). These properties [16] are regarded as hallmarks of apoptosis. This kind of programmed cell death has been shown for these compounds to be favored. To investigate whether compound **1** acted cytostatic or cytotoxic, the cell cycle distribution of the living cells was investigated (Fig. 3). While high concentrations (24 or 60 μM) of **1** led to an early detachment of the cells (A2780, starting after approx. 6 h using a concentration of 24 μM or 60 μM; this process was finished after 24 h), the application of a lower concentration (12 μM of **1**) led to a significant G1/G0 arrest (12 h after treatment). Treatment of the cells with a concentration of 24 μM of **1** for 6 h led to a S-phase accumulation. These results confirmed the data from the SRB assay, and we assumed that **1** exhibited only a weak cytostatic behavior.

Table 1
Cytotoxicity for maslinic acid and analog **1** (IC_{50} values in μM from SRB assays); independent experiments were at least performed in triplicate employing human tumor cell lines (518A2, 8505C, A2780, A549, HT29, MCF7) and non-malignant mouse fibroblasts NiH 3T3.

IC_{50}	Maslinic acid	1
518A2	13.7	12.6
8505C	17.0	13.3
A2780	19.5	10.6
A549	23.4	13.7
HT29	28.8	12.5
MCF7	n.D.	13.6
NiH 3T3	21.1	12.7

Several apoptosis assays were performed to gain a deeper insight. In detail, the cells were treated with a double IC_{50} or a six-fold IC_{50} concentration of **1** for 6, 12 and 24 h, respectively, and then subjected to a dye-exclusion assay (Fig. 2A), extra DNA-laddering experiments (supplementary part), an annexin V assay (Fig. 4 and supplementary part) as well as to a caspase assay (Fig. 3 and supplementary part). Staurosporine (being known for its very fast and unique apoptosis inducing ability in a wide variety of mammalian cells; in addition staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms) was used as a positive control in these experiments. The results from all of these assays unambiguously gave evidence for **1** triggering apoptosis in an only weak caspase-dependent manner. In addition, a high number of secondary necrotic cells was observed in the annexin V assay (Fig. 4). This gave a reason to assume a membrane modulating process – being due to the treatment of the cells with compound **1**. The significant cell shrinking (as observed by close microscopic investigation) was additionally confirmed by FACS measurements [17–19] (forward scatter, Figs. 2B and 4).

3. Discussion

Considering the cell shrinking, the condensation of the chromatin and the DNA degradation as well as the positive annexin V assay and an increased caspase activity, an apoptotic cell death can safely be assumed. Furthermore, the delay in the activation of the caspases indicated a caspase-mediated but not a caspase-induced mode of action. The data available from literature and the unprecedented growth of cholesterol-containing crystals at the perimeter of the cells justified the discussion of an alternative mode of action of compound **1**.

Extracellular growth of crystals in cell cultures having been treated with triterpenoids is unprecedented so far: Lees and Thompson [20], however observed crystal growth in plant cells as early as 1980, and in 1994 the formation of cholesterol monohydrate crystals in macrophage-derived foam cells was shown [21]. Recently, Grebe and Latz [22] reviewed the correlation between cholesterol crystals and inflammation processes, and a clear correlation between the crystallization of cholesterol and the apoptosis of macrophages after treating human THP-1 foamy macrophages with 7-ketocholesterol was shown [23]. For this process involving caspases 3 and 8 it was assumed that an increased oxysterol incorporation into the cellular membrane should result in a subsequent release of large amounts of free cholesterol into the medium – followed by its crystallization due to supersaturation [23].

The anti-malarial activity of triterpenoic acids may be the result of their acting as cholesterol analogs [24]. Several derivatives of betulinic acids affected the invasion and growth of the parasites, and it was shown [24] that treatment of the cells with these compounds modulated the shape of the erythrocytes depending on the hydrogen bonding properties of the compounds. A proton donating group (*e.g.* a carboxylic group) at position C28 led to the formation of an echinocytic cell shape (burr cells) whereas ester moieties induced the formation of stomatocytic cell shape. Among others [25–30], Prades et al. [31] revealed the incorporation of triterpenoic acids into lipids, and a modulation of the mitochondrial membrane as a starting point for triggering apoptosis was assumed. **MA** [7,9,10,32–36] has been shown to trigger apoptosis *via* the activation of caspases.

To sum up, compound **1** seemed to incorporate into the cell membrane, it remained there, and competed with membrane located cholesterol. This resulted in a release of surplus cholesterol into the medium and explained the formation and growth of

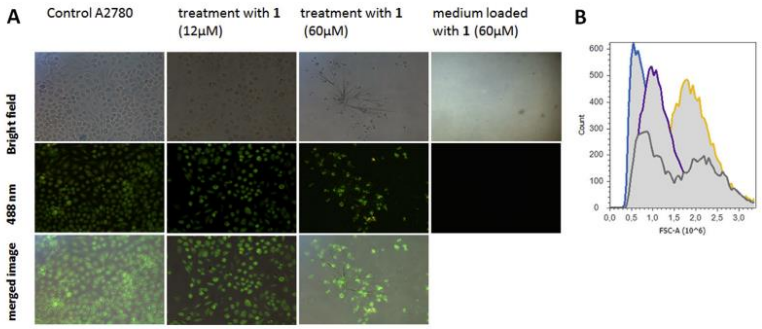

Fig. 2. A. *Dye-exclusion assay* (AO/PI), living ovarian cancer (A2780) cells treated with **1** for 24 h. B. *Representative cell size distribution*: the yellow line represents control cells after 24 h, the violet line represents cells treated with staurosporine (STP, 1 μM), gray line: cells treated with **1** (24 μM), blue line: cells treated with **1** (60 μM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

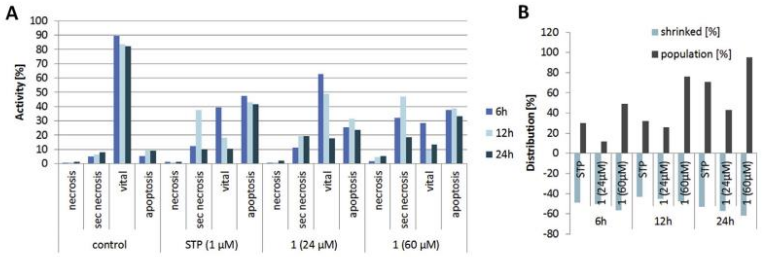

Fig. 3. A. *Caspase assay*: Ovarian cancer cells (A2780) were treated with the concentrations as indicated. After several time points all cells were harvested and treated with green-DEVD-FMK, green-IETD-FMK and red-LEHD-FMK; staurosporine (STP) was used as positive reference. B. *Cell cycle analysis*: Ovarian cancer cells (A2780) were treated with the concentrations as indicated. After several time points the adherent cells were harvested and treated with PI/RNAse for 30 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

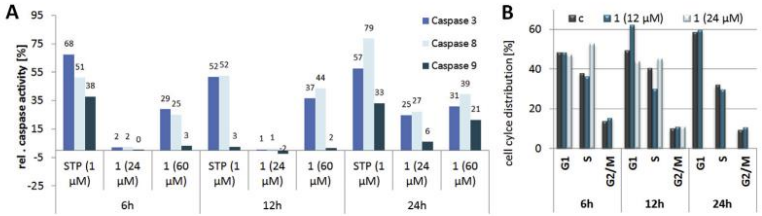

Fig. 4. A. *Annexin V/PI Assay*: Ovarian cancer cells were treated with the concentrations as indicated. After several time points all cells were harvested, treated with annexin V–FITC and propidium iodide for 15 min and subjected to an FACS measurement. B. *Cell size distribution*: Ovarian cancer cells were treated with the concentrations as indicated. After several time points all cells were harvested and subjected to FACS measurements. Comparisons of the FCS led to a relative size distribution.

cholesterol crystals at the perimeter of the cells. Replacement of cholesterol in the membrane by **1** altered form and function of (parts of) the membrane thus explaining the early detachment of the cells during this experiment. However, this process of competition between cholesterol and **1** is not to be expected to be selective; this explains the loss of tissue selective toxicity of **1**. The cell death would now occur *via* an activation of the caspase machinery as a consequence of the structural changes in the membrane. Caspases 3 and 8 were shown to be activated following an incubation with **1**. A DNA laddering by a caspase 3 activated DNAse, however, was found after an incubation period of >24 h, whereas for 80% of the cells after having been treated with **1** for 6 h (60 μM) an annexin V assay gave evidence for a translocation of phosphatidylserine onto the outer side of the membrane. Usually this translocation [37] occurs in a late phase of the apoptotic process. As a consequence, we assumed that the high percentage of phosphatidylserine expression is caused by an alteration of the structure of the membrane. Triterpenes have been shown [38–45] to alter the activity of several ion channels. As a consequence, a volume decrease took place.

Shrinking of cells is usually regarded [37] as a hallmark of apoptosis. This apoptotic volume decrease occurs very early in the

process of apoptosis, and it remains unclear [46–48] whether this process triggers apoptosis or has to be regarded as a result of an already initiated apoptotic process. It was shown for HeLa cells that shrinking of the cells (with participation of ion channels) occurs prior to an activation of caspases – leading to the assumption [49] of a "cell-shrinked induced" cell death. As far as ion channels are concerned, voltage gated, ATP-sensitive and Ca^{2+} activated channels have been discussed [50] in this context. Also, chloride channels [51,52], the voltage-dependent anion channel, the cystic fibrosis trans-membrane regulator, the outwardly rectifying chloride channel and calcium dependent chloride channels have been in the focus [49,51–55] of several investigations. These channels (as well as the Na^+/K^+-ATPases) are located in the lipid rafts of the cell membranes; cholesterol stabilizes these channels, and its depletion causes a change [50,56–63] in the opening/closing behavior of some of these channels.

4. Conclusions

As an alternative to established triggers of apoptosis, we suggest an extension of apoptosis initiating mechanisms. The incorporation of the triterpenoid **1** into the cell membrane caused a depletion of cholesterol in the lipid rafts, and cholesterol from the membrane was released into the medium. As a result, a crystallization process of cholesterol (together with excess **1**) took place at the perimeter of the cells. As a consequence of this depletion a decrease in the volume of the cells occurred, and cell shrinking was observed. This process was non-selective with respect to the cell line and hence explains a lowered tumor/non-tumor selectivity of **1**. Furthermore, the incorporation of an ester group resulted in an increase in cytotoxicity, but this loss in selectivity is unwanted. Thus, small alterations of the molecules structure led to an altered mode of action. Triterpenoids have been shown to interfere with different processes in the cancer cells, among them the initiation of reactive oxygen species (ROS) [33], by direct interaction with the cytochrome *c* release machinery [35], by blocking the sonic hedgehog pathway [64] as well as by acting as proteasome-inhibitors [65–67]. Our findings suggest an incorporation of the substances into the membrane, and – as a consequence – the induction of apoptosis. Moreover, the ability of triterpenoids to alter membranes should be considered for the development of new strategies for the synthesis of antitumor/antimicrobial/antiviral compounds.

5. Experimental part

5.1. General – chemistry

Reagents were bought from commercial suppliers without any further purification. Melting points were measured with a LEICA hot stage microscope and were not corrected. NMR spectra were recorded on VARIAN Gemini 2000 spectrometer at 27 °C (chemical shifts δ are given in ppm and *J* in Hz). Mass spectra were taken on a FINNIGAN MAT TSQ 7000 (electrospray, voltage 4.5 kV, sheath gas nitrogen) instrument. Elemental analyses were measured on a Foss–Heraeus Vario EL unit. IR spectra were recorded on a Perkin Elmer FT-IR spectrometer Spectrum 1000, optical rotations on a Perkin Elmer 341 polarimeter (1 cm micro cell, 25 °C) and UV–vis spectra on a Perkin Elmer unit, Lambda 14. TLC was performed on silica gel (Merck 5554, detection with cerium molybdate spray reagent). Solvents were dried according to usual procedures. The purity of the compounds was checked by HPLC/DAD (Prontosil C18, MeOH/H_2O 95/5, 1% H_3PO_4) and found to be >98% for each compound. The Raman spectra were recorded using a Renishaw inVia Raman microscope equipped with a CCD camera and a Leica microscope.

5.2. Biological material and procedures

5.2.1. Cell lines and culture conditions

The cell lines 518A2, 8505C, A2780, A549, HT29, MCF7, NiH 3T3 and WW030272 were included in this study. Cultures were maintained as monolayer in RPMI 1640 (PAA Laboratories, Pasching, Germany) supplemented with 10% heat inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

5.2.2. Cytotoxicity assay, dye-exclusion assay, DNA-laddering and apoptosis assay

Instrumentation, cell lines and culture conditions, cytotoxicity assay, dye-exclusion tests and DNA fragmentation were performed as previously described [68].

5.2.3. Cell cycle investigation

Approximately 1×10^6 cells (HT29, A2780 or NIH 3T3) were seeded in cell culture flasks (25 cm^2), and the cells were allowed to grow for 24 h. After removing of the used medium, the substance loaded medium was reloaded (or a blank fresh medium as a control). After 24 or 48 h, the living cells were harvested, washed with PBS (with Mg^{2+} and Ca^{2+}) twice and fixed with ethanol (70%, 4 °C, 1 h). After removing of the fixation and permeabilization agent, the cells were washed with PBS buffer (with Mg^{2+} and Ca^{2+}, containing 1% BSA and 0.1% NaN_3, 3×1 mL, 1000 rpm) and adjusted to 1×10^5 million cells. The pellet was gently suspended in staining buffer (PBS buffer containing BSA, RNAse, NaN_3 and PI following the procedure of Darzynkiewicz et al. [69]) and incubated for 30 min at 37 °C. Analyses were performed using the Attune® FACS machine; collecting data from the BL-2A channel. Doublet cells were excluded from the measurements by plotting BL-2A against BL-2H. For each cell cycle distribution 20,000 events were collected. Distribution was calculated [70] by the method of Dean et al.

5.2.4. Annexin V/PI assay

Approx. 500,000 cells (A2780) were seeded in cell culture flasks and were allowed to grow for 1 day. After removing of the medium, the substance loaded medium was added, and the flasks were incubated for about 24 h. All cells were harvested, centrifuged (1200 rpm, 5 min) and washed twice (PBS (*w*/*w*)). Approx. 100,000 cells were washed with annexin V bounding buffer (BD), and treated with a propidium iodide solution (3 μL, 50 μg/mL) and annexin V (5 μL, life technologies™) for 15 min at 25 °C in the dark. After adding annexin V bounding buffer (400 μL), the suspension was submitted to a FACS measurement. Calculation was performed as suggested by the supplier (BD Biosciences®).

5.2.5. Caspase assay

Approx. 1×10^6 cells were seeded in cell culture flasks (25 cm^2), and the cells were allowed to grow for 24 h. After removing of the used medium, the substance loaded fresh medium was reloaded (or a blank new medium as a control). After indicated time points, all cells were harvested and centrifuged (300*g*, 4 °C), the pellet was gently suspended in phosphate-buffered saline (PBS, with Ca^{2+}, Mg^{2+}, 1 mL) and centrifuged again. The PBS was removed, and the pellet gently suspended in PBS (100 mL). Approx. 300,000 cells were treated with the corresponding caspase substrates and incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 50 min. After washing with buffer following the protocol suggested by the supplier (Promokine), the cells were subjected to a FACS supported measurement.

5.3. Solubility assay

To a stock solution (10 μM) of **1**, buffer (190 μL) was added, and the mixture was shaken at 200–300 rpm at 24 °C for 90 min. After centrifugation (10,000 rpm), the supernatant (160 μL) was diluted with MeCN and subjected to a HPLC measurement (as described above). The solubility was determined following the MultiScreen® Solubility Protocol (Millipore Corporation, Billerica, MA, USA).

5.4. Maslinic acid (*MA*)

MA was isolated from olive pomace as described before, and it was obtained as a colorless solid; m.p. 263–267 °C.

5.5. (4-Oxa-4-phenyl-butyl) 2,3-dihydroxy-olean-12-en-28-oate (*1*)

Maslinic acid (**MA**) (150 mg, 0.3 mmol) was dissolved in dry DMF (5 mL), and finely grounded potassium carbonate (200 mg, 1.45 mmol) was added. After 60 min of stirring at 25 °C, (3-bromopropoxy)-benzene (150 mg, 0.7 mmol) was added, and stirring was continued for another 18 h. The mixture was poured into ice cold hydrochloride acid (5%, 50 mL), and the white precipitate was filtered off. Chromatographic purification (silica gel, hexane/ethyl acetate, 7:3) followed by recrystallization (ethanol) gave the product; yield: 150 mg, 83%; m.p. 166–170 °C; $R_F = 0.4$ (*n*-hexane/ethyl acetate, 1:1); $[\alpha]_D = +45.8°$ (*c* 0.33, $CHCl_3$); IR (KBr): $\nu = 3424vs$, 2946*vs*, 2930*vs*, 2878*m*, 2864*m*, 1726*vs*, 1602*m*, 1498*m*, 1470*s*, 1458*m*, 1384*s*, 1364*m*, 1242*s*, 1202*m*, 1180*m*, 1172*m*, 1162*s*, 1124*m*, 1080*m*, 1052*s*, 1034*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.30–7.24$ (*m*, 2H, CH_{aromat}), 6.94 (*dd*, $J = 7.3, 7.3$ Hz, 1H, CH_{aromat}), 6.88 (*d*, $J = 8.0$ Hz, 2H, CH_{aromat}), 5.25 (*dd*, $J = 3.4, 3.4$ Hz, 1H, *CH* (12)), 4.27–4.14 (*m*, 2H, CH_2 (31)), 4.03 (*ddd*, $J = 6.1, 6.1, 1.5$ Hz, 1H, CH_2 (33)), 3.68 (*ddd*, $J = 11.5, 9.7, 4.4$ Hz, 1H, *CH* (2)), 2.99 (*d*, $J = 9.5$ Hz, 1H, *CH* (3)), 2.87 (*dd*, $J = 13.8, 3.8$ Hz, 1H, *CH* (18)), 2.18 (*brs*, 2H, *OH*), 2.15–2.06 (*m*, 2H, CH_2 (32)), 2.00–1.86 (*m*, 2H, CH_a (16) + CH_a (1)), 1.83 (*dd*, $J = 8.8, 3.4$ Hz, 2H, CH_2 (11)), 1.75–1.66 (*ddd*, $J = 13.8, 13.8, 4.4$ Hz, 1H, CH_a (7)), 1.67–1.58 (*m*, 3H, CH_a (19) + CH_a (15) + CH_b (16)), 1.57 (*m*, 1H, *CH* (9)), 1.54–1.45 (*m*, 2H, CH_a (22) + CH_a (6)), 1.43–1.35 (*m*, 1H, CH_b (7)), 1.33–1.23 (*m*, 2H, CH_a (21) + CH_b (6)), 1.22–1.14 (*m*, 3H, CH_b (19) + CH_b (21) + CH_b (22)), 1.11 (*s*, 3H, CH_3 (27)), 1.02 (*s*, 3H, CH_3 (23)), 1.05–0.95 (*m*, 1H, CH_b (15)), 0.92 (*s*, 3H, CH_3 (25)), 0.90 (*s*, 3H, CH_3 (30)), 0.89 (*s*, 3H, CH_3 (29)), 0.88–0.80 (*m*, 1H, CH_b (1)), 0.81 (*s*, 3H, CH_3 (24)), 0.80 (m, 1H, *CH* (5)), 0.66 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (100 Hz, $CDCl_3$): $\delta = 177.8$ (*C*=O, C28), 158.9 (C_{aromat}, C34), 144.0 (*C*=CH, C13), 129.6 (CH_{aromat}, C35), 122.4 (*CH*=C, C12), 120.9 (CH_{aromat}, C36), 114.5 (CH_{aromat}, C37), 84.1 (*CHOH*, C3), 69.1 (*CHOH*, C2), 64.3 (CH_2, C31), 61.1 (CH_2, C33), 55.4 (CH, C5), 47.7 (*CH*, C9), 46.9 (C_{quart}, C17), 46.5 (CH_2, C1), 46.0 (CH_2, C19), 41.9 (C_{quart}, C14), 41.4 (CH, C18), 39.5 (C_{quart}, C8), 39.3 (C_{quart}, C4), 38.4 (C_{quart}, C10), 34.0 (CH_2, C21), 33.2 (CH_3, C30), 32.7 (CH_2, C7), 32.6 (CH_2, C22), 30.9 (C_{quart}, C20), 28.8 (CH_2, C32), 28.8 (CH_3, C23), 27.7 (CH_2, C15), 26.1 (CH_3, C27), 23.8 (CH_3, C29), 23.6 (CH_2, C11), 23.1 (CH_2, C16), 18.5 (CH_2, C6), 17.1 (CH_3, C26), 16.9 (CH_3, C24), 16.7 (CH_3, C25) ppm; MS (ESI, MeOH, source CID): $m/z = 607.3$ (20%, $[M + H]^+$), 629.3 (100%, $[M + Na]^+$), 929.3 (60%, $[3M + K + H]^{2+}$); analysis for $C_{39}H_{58}O_5$ (606.87): C 77.18, H 9.63; found C 77.03, H 9.71.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.12.031.

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Supplementary material

Membrane damaging activity of a maslinic acid analogue.

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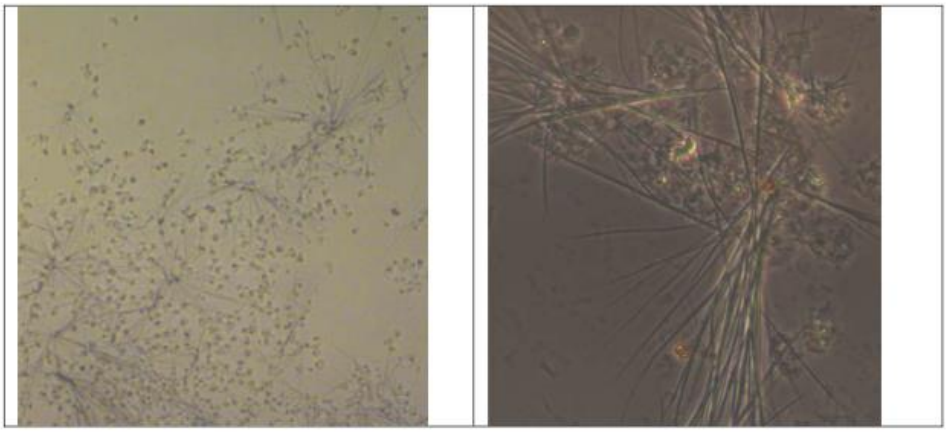
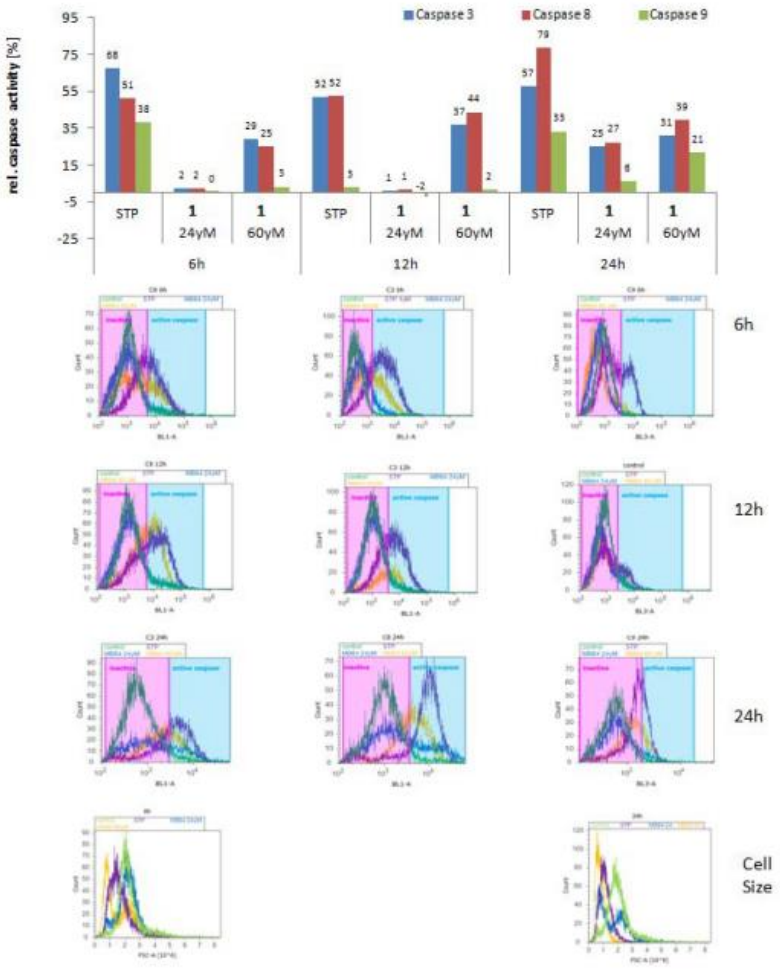
1. Microscopy:

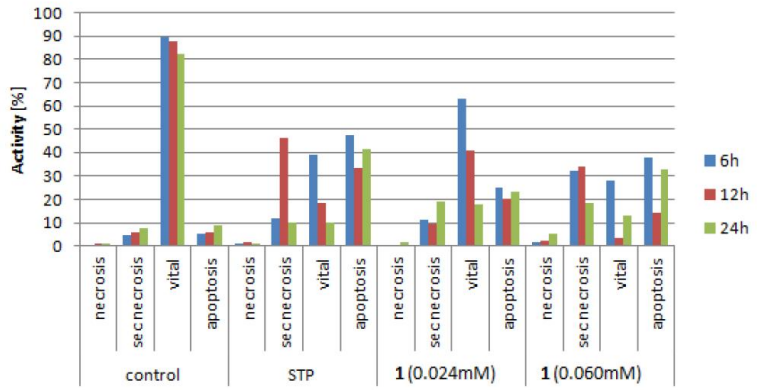


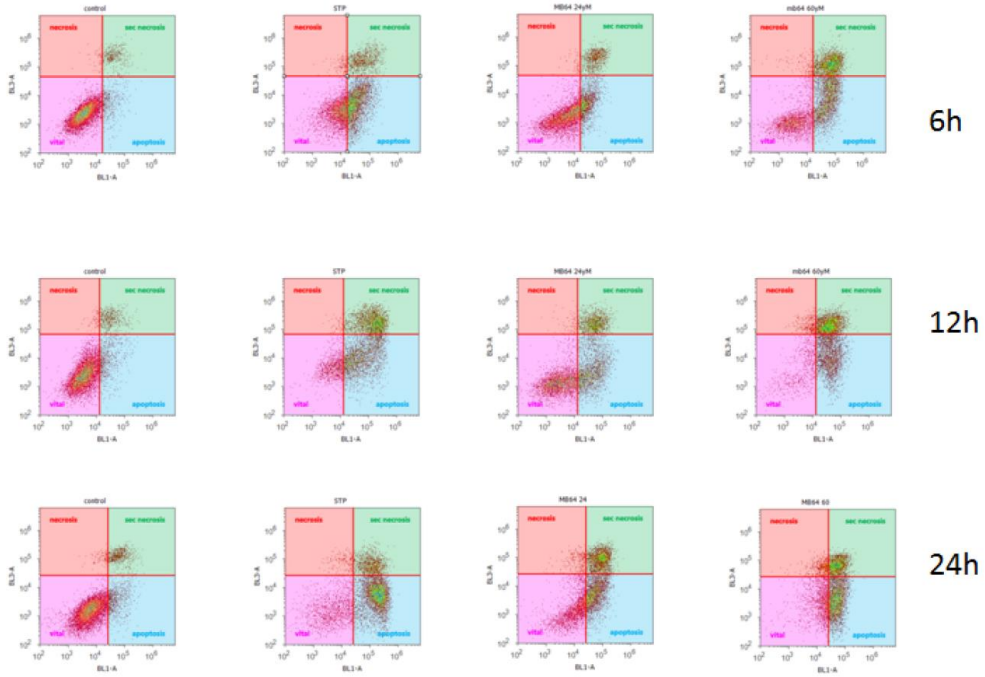
Left part: treatment of ovarian cancer cells A2780 with **1** (24 h, 60 µM), 10-fold magnification; **right part:** treatment of ovarian cancer cells A2780 with **1** (24 h, 60 µM), 40-fold magnification.

Representative results of the caspase assay:

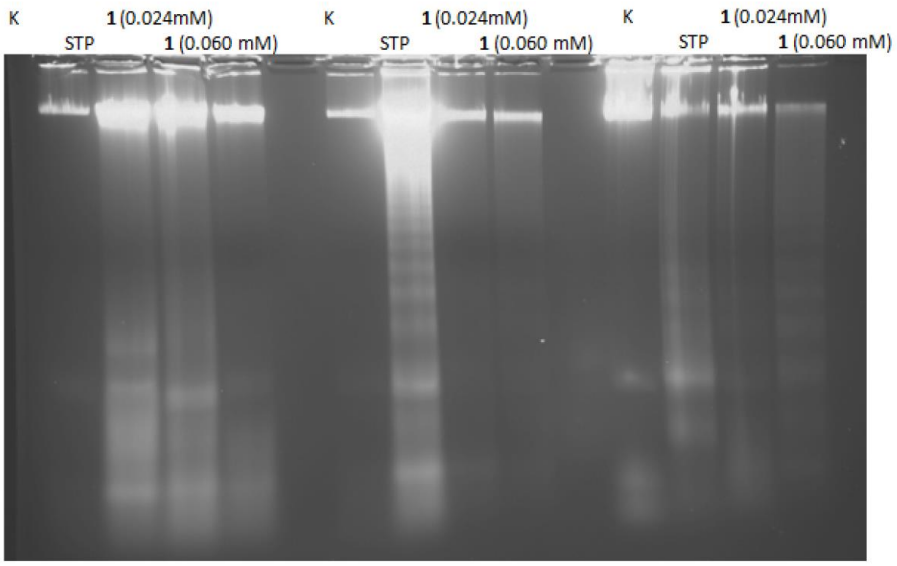


Representative results of the Annexin V Assay





DNA-Laddering Assay



Detailed results from the SRB assay

Cytotoxicity for maslinic acid and analog **1** (IC_{50} values in μM from SRB assays; independent experiments were at least performed in triplicate; confidence interval (95 %) is given employing human tumor cell lines (518A2, 8505C, A2780, A549, HT29, MCF7) and non-malignant mouse fibroblasts NiH 3T3.

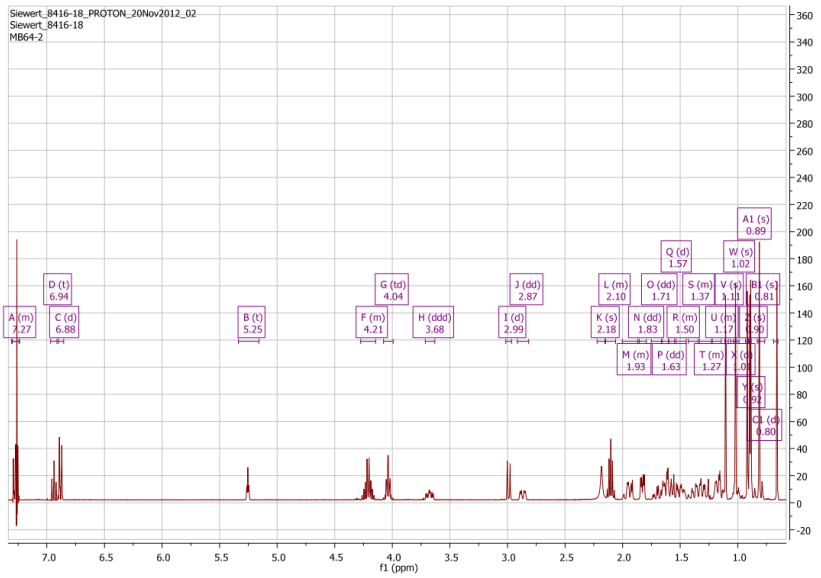
IC_{50}	maslinic acid		1	
518A2	13.7	1.9 1.7	12.6	10.0 5.6
8505C	17.0	2.1 1.9	13.3	1.0 0.9
A2780	19.5	1.8 1.7	10.6	1.2 1.1
A549	23.4	0.6 0.6	13.7	4.7 3.5
HT29	28.8	1.0 0.9	12.5	1.4 1.3
MCF7	n.D.		13.6	4.7 3.5
NiH 3T3	21.1	3.0 2.6	12.7	0.7 1.1

Raman shifts (cm^{-1}) of the extracellular crystals, cholesterol and of analog 1.

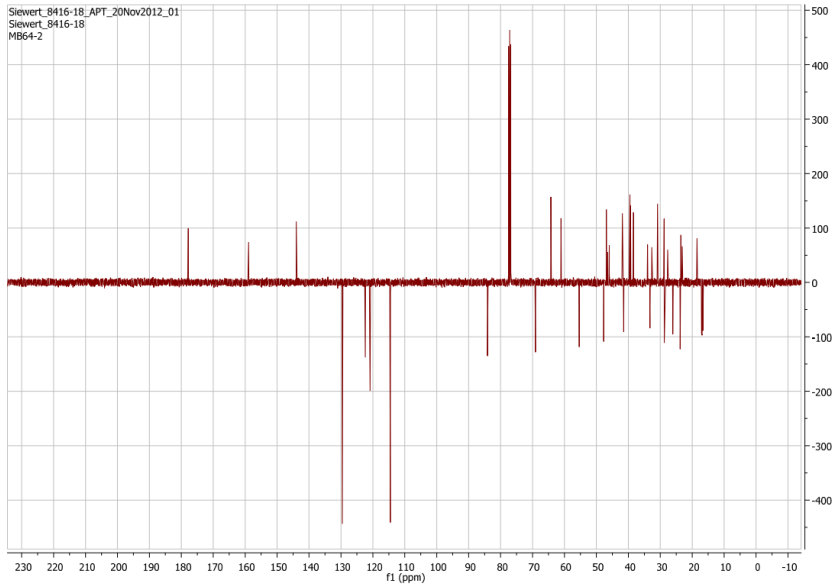
cholesterol	compound **1**	crystals
	140.9	
147.6		147.8
	607.0	606.4
	613.2	
		618.9
	671.7	671.5
	679.9	680.8
701.3		702.3
	734.7	733.0
	999.8	999.9
	1028.6	1028.0
	1173.0	
	1293.5	
1437.0	1437.0	1440.0
1438.8		1445.6
1462.6		1468.0
	1470.5	1472.2
	1586.9	1599.8
	1601.5	1665.6
	1666.1	1668.3
1672.8		1673.8

NMR spectra of compound 1, *(4-oxa-4-phenyl-butyl) 2,3-dihydroxy-olean-12-en-28-oate*

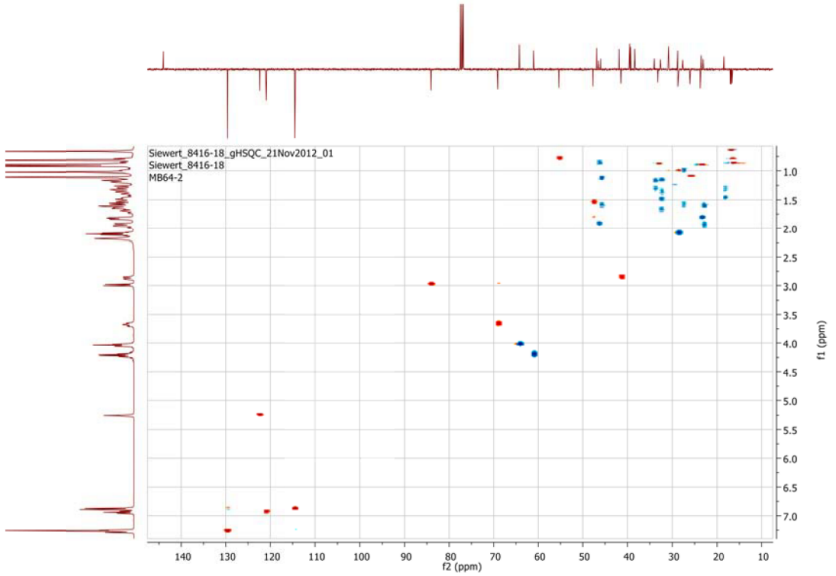
^{1}H NMR ($CDCl_3$)



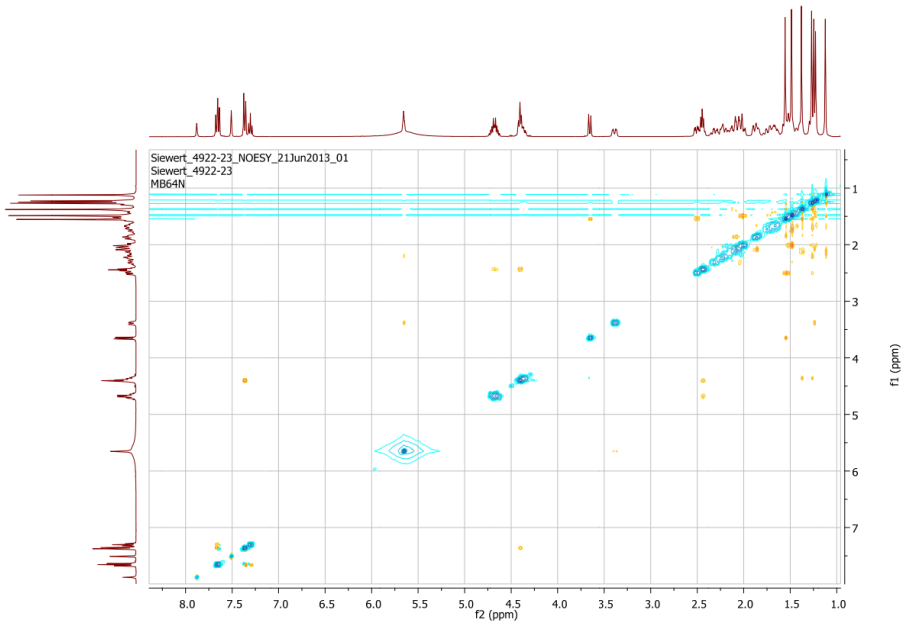
^{13}C-APT NMR ($CDCl_3$)



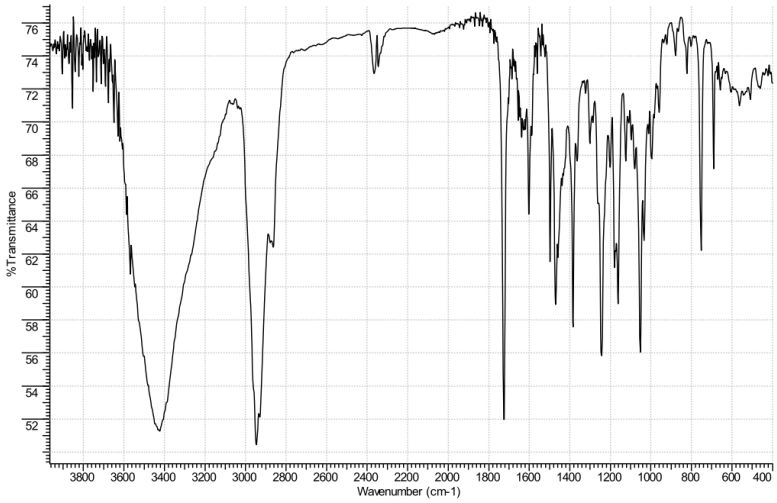
gHSQC ($CDCl_3$)



H,H Noesy (d_5-pyridine)



IR-Spectrum of compound 1



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PUBLIKATIONEN

In der nachfolgenden Liste sind chronologisch alle Publikationen aufgeführt, welche im Zeitraum von 2008 bis 2013 entstanden sind. Von den zwanzig nachfolgend aufgelisteten Publikationen sind bisher sechszehn in *peer review* Zeitschriften angenommen, die restlichen wurden in ebensolchen eingereicht. Die farblich markierten sind dabei jene Publikationen, welche Grundlage der vorliegenden Arbeit bilden.

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TAGUNGSBEITRÄGE

Während den Jahren 2009 bis 2013 war es mir durch die finanzielle Förderungen (Stipendien) dankbarerweise möglich, die Ergebnisse meiner Arbeit auf verschiedensten Konferenzen vorzustellen. Im Anschluss sind die Konferenzbeiträge chronologisch aufgeführt, welche Teile der Arbeit und/oder im Rahmen des Promotionsstudiums entstandene, eigene Daten enthalten. Der beitragsvorstellende Autor wurde mit einem Stern (*) markiert. Auf den Seiten A-208 bis A-210 sind ausgewählte Tagungsbeiträge zu sehen.

07/2013	"Intracellular drug tracking by a "click" chemistry based approach", Siewert, B.*; Csuk, R.; Poster, **Challenges in Chemical Biology (ISACS11)**, Boston, *2013*, 23-26. Juli
06/2013	"To avoid forgetfulness: don´t forget our purines", Schwarz S.*, Teixeira, V.H.*, Machuqueiro, M., Siewert, B., Csuk, R., Rauter, A.P., **Frontiers in Medicinal Chemistry,** San Francisco, *2013*, 23.-26. Juni
06/2013	"It´s just one nitrogen!" Siewert, B*; Pianowski, E; Csuk, R.; Poster P1.149, **14th Tetrahedron Symposium: Challenges in Organic and Bioorganic Chemistry**, Wien, *2013*, 25-28. Juni
09/2012	"Semi-synthetic maslinic acid derivates with promising antineoplastic activity" Siewert, B.*; Bittmann, E.; Csuk, R.; Poster P474, **XXIInd International Symposium on Medicinal Chemistry (EFMC-ISMC 2012),** Berlin, *2012*, 02.-06. Sep
07/2012	"A convenient separation of Ursolic and Oleanolic acid and their potential against cancer diseases" Siewert, B.*, Csuk, R.; Poster 200, **19th international Conference on Organic Synthesis**, Melbourne, *2012*, 01.-06 Juli.
07/2012	"Purine Nucleosides with anti-Alzheimer and anti-Tumour Potential"; Schwarz, S.*; Teixeira, V., H.; Machuqueiro, M.; Mathon-Claudon, L.; Siewert, B.; Csuk, R.; Rauter, A., P. Poster, **6th Spanish Portuguese Japanese Organic Chemistry Symposium**, Lissabon, *2012*, 18.-20.Juli
08/2011	"QSAR - studies on antineoplastic maslinic acid derivates" Siewert, B.*, Schäfer, R., Csuk, R.; Oral presentation, IUPAC179, **43 rd IUPAC World Chemistry Congress**, San Juan, *2011*, 31. Juli -5. August.
08/2011	"Synthesis and cytotoxic activity of ring A modified glycyrrhetinic acid derivatives" Schwarz, S.*; Siewert, B., Csuk, R.; Oral presentation, IUPAC448, **43 rd IUPAC World Chemistry Congress**, San Juan, *2011*, 31. Juli-5. August.
07/2011	"Antitumor activity of some glycyrrhetinic acid glycosides" Schwarz, S.*; Siewert, B.; Csuk, R.; Xavier, N., M.; Jesus, A., R.; Rauter, A., P., **16 th European Carbohydrate Symposium**, Sorrento-Neapel, *2011*, 3.-7. Juli.

08/2010	“Derivatives of Glycyrrhetinic acid induce apoptosis in tumor cells”; Siewert, B.*, Schwarz, S., Csuk, R.; Poster 319, **18th International Conference on Organic Synthesis**, Bergen, *2010*, 01. - 06.August.
03/2010	“New anticancer active derivatives from glycyrrhetinic acid”; Siewert, B.*, Schwarz, S., Csuk, R.; Poster CAN 12, **Frontiers in Medicinal Chemistry**, Münster, *2010*, 14.-17. März.

A CONVENIENT SEPARATION OF URSOLIC AND OLEANOLIC ACID AND THEIR POTENTIAL AGAINST CANCER DISEASES

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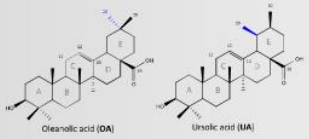
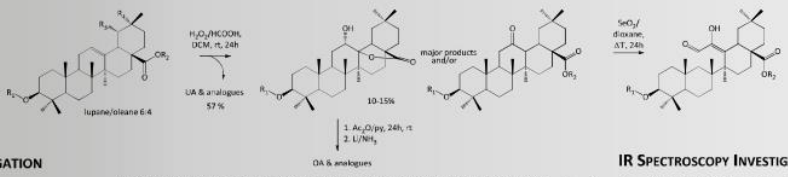
INTRODUCTION

The chiral pool of natural compounds seems to be a "*gold-mine*" for medicinal chemists as long as isolating and purifying is convenient.
Ursolic acid (**UA**) - a representative pentacyclic triterpene - was obtained from the leaves of bearberry (*Arctostaphylos uva-ursi*) by Trommsdorff[1] as early as 1854 and it shows - like other members of this class - some interesting biological activities, including anti-malaria, anti-diabetis and anti-neoplastic properties[2].
Its separation from accompanying oleanolic acid (**OA**) however, is challenging because of structure similarity. Although many analytical methods have been elaborated, separation of these two compounds on a preparative scale remains by and large an unresolved problem.

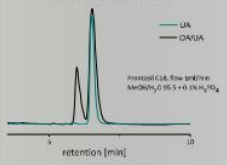


CHEMISTRY PART A - SEPARATION

Lewis' approach[3] from 1983 uses the different reactivity of **UA** and **OA** towards elemental bromine. This synthesis, however, is lengthy and yields drop significantly on scaling-up. Keeping in mind the alkenic bond between carbons C-12 and C-13 in α-amyrin-type triterpenes is less reactive than that in β-amyrins, β-amyrin-type triterpenoids are known to react quantitative with *m*CPBA and other peracids[4]. Thus, treatment with formic peracid for one day at room temperature gave pure UA and several OA oxidation products[5].



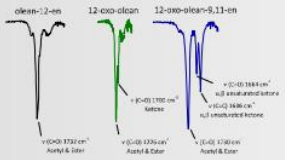
HPLC INVESTIGATION



The 12,13-olefin regeneration by metal-liquid ammonia reduction, developed by Barton[6] et al. as early as 1965, performs well for compounds holding electron-withdrawing groups at position C27, but only moderate for **OA** (20 %). Generation of 9,11-en-12-oxo system with HBr and acetic acid, developed by McKenzie[7] et al. seems to be more Rewarding: Yields are very good and the product holds one hallmark of CDDO-analogues. Additionally the treatment with SeO_2 led to a hitherto unknown c-Ring modification.

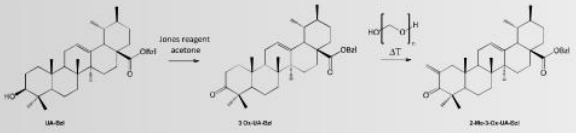
IR SPECTROSCOPY INVESTIGATION

Transformation of Methyl 3β–3–O-Acetyl-olean-11-en-28-oate

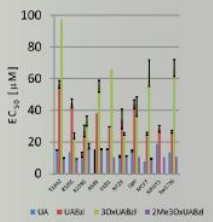


CHEMISTRY PART B – SAMPLE MODIFICATION

The presence of a 2-methylene-3-oxo moiety increases the cytotoxity of betulinic aicd analogues.[8] Hence, we became interested in investigating the influence of the carbon skeleton on cytotoxicity, and we synthesized several analogues from parent **UA** and **OA.** For one of these compounds a detailed biological evaluation is shown.

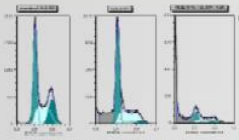


BIOLOGICAL EVALUATION



Antitumor activities were tested by the well known SRB-assay against several malignant human tumor cells and one non malignant mouse fibroblast cell line.

The kind of cytotoxicity was investigated by an AO/PI dye exclusion assay, where green cells document controlled and the red ones are significant for necrotic cell death. Typical apoptotic DNA fragmentation, as well as changes of the DNA content in the cell cycle were analyzed by FACS analysis using propidium iodide.

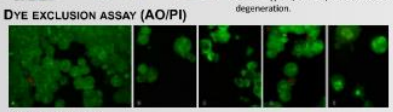


CELL CYCLE INVESTIGATION

Lung cancer cells (A549) were treated with **UA** and **2Me3OxUABzl** for 24h. The DNA amount of living cells was investigated by FACS analysis.

The results document, that **UA** as well as **2Me3OxUABzl** induced, programmed cell death typical, a G1/G0 arrest and DNA degeneration.

DYE EXCLUSION ASSAY (AO/PI)



(A) control, living A549 cells
(B) living cells after treatment with UA (30 µM, 24h)
(C) death cells after treatment with UA (30 µM, 24h)
(D) living cells after treatment with **2Me3OxUABzl** (15 µM, 24h)
(E) death cells after treatment with **2Me3OxUABzl** (15 µM, 24h)

Starting from natural occurring UA and OA mixtures leads to pure UA and pre-CDDO after max. 3 steps, resulting in promising hits for anticancer research.

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Acknowledgements: - Dr T. Müller (Dept. of Haematology/Oncology, Univ. Halle) for providing the cell lines
- Gründerwerkstatt Biowissenschaften (Univations) and cand.B.Sc E. Sorge for biological support.
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SEMI-SYNTHETIC MASLINIC ACID DERIVATES WITH PROMISING ANTINEOPLASTIC ACTIVITY

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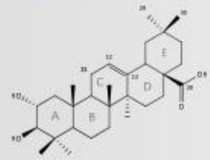
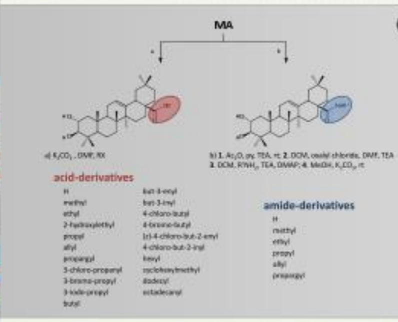
INTRODUCTION

Olives pomace, an apparently useless residue of olive oil, contains pharmacological interesting terpenes, among them the pentacyclic triterpenic acids oleanolic (**OA**) and maslinic acid (**MA**) up to 0.15 %. It seems that these acids are one cause of the low cancer mortality rate in Mediterranean countries [1].

Reyes-Zurita *et al.* [2] showed that **MA** carries the ability to induce apoptosis in several cancer cell lines. Although a certain tumor selectivity of **MA** has been established [3], little is known on structure-activity relationship of **MA** derivates. Thus, we became interested in that promising area.



Maslinic acid (MA)



CHEMISTRY PART

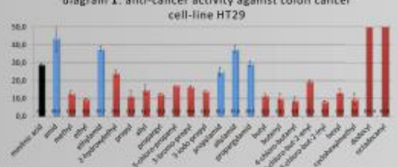
The parent compound, which was obtained from olives pomace (0.25 %) possess three different functional groups: two hydroxyl groups in ring A, a double bond in ring C and a carboxylic group at position 28. The latter one affects biological activities significantly, e.g. a 40 fold increase in anticancer activity was observed for a 4-bromobut-2-enyl ester of glycyrrhetinic acid [4], a 87.5 % apoptosis activity for the 28-benzoyl **MA** derivative [5] or a significant inhibition of glycogen phosphorylase by derivatisation of **MA** into its 4-bromo-butanyl ester [6].

Thus, to build up a systematically SAR-study **MA** was transformed into corresponding esters by treatment with several alkyl halides in the presence of sodium carbonate. Further, several amides where accessed from a four step SCHOTTEN-BAUMANN analogue reaction with an average yield of 30 %.

Structures as well as the purity of all synthesized maslinic acid derivatives were confirmed by extensive analytical investigation.

BIOLOGICAL PART A – ACTIVITY EVALUATION

The antitumor activity of the compounds was tested by an SRB-assay using up to fifteen cancer cell lines from different human tissues. The IC_{50} values are shown in diagram **1** and **2**. They were obtained from three independent experiments by non-linear regression of the semi-logarithm dose-response curve using the two parametric Hill-slope equation, and given with confidence interval (95 %). To proof the selective anti-tumor behavior, the non-maligant NiH 3T3 cell-line as well as human fibroblasts WW030272, a primary cell culture, were used (figure **1**).



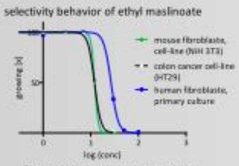
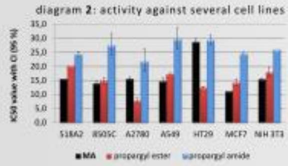


figure 1: selectivity doses-response curves



BIOLOGICAL PART B – APOPTOSIS INVESTIGATIONS

Apoptosis, the programmed cell death, is characterized by several biochemical phenomena. Firstly, membrane integrity remains stable, further e.g. occurrence of membrane blebbing due to actin degeneration, which is well observable by fluorescence microscopy. The controlled condensation of chromatin and additional DNA degeneration becomes evident in the cell cycle, as well as by DNA ladder technique. Another hall mark of apoptosis, phosphatidyl-serine expanding, could be measured with fluorescence marked Annexin V. Mostly, all biochemical changes depend on several caspases, the activity of which is depicted in figure **2D**.

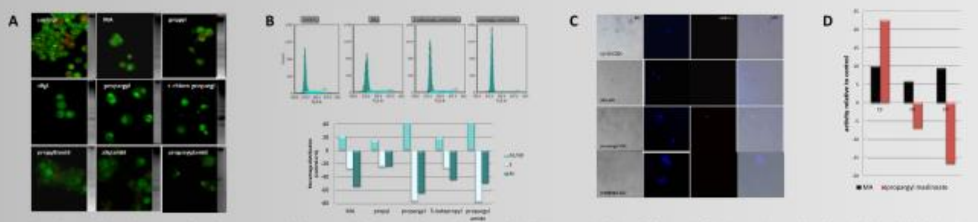


Figure 2 A AO/PI dye exclusion assay (green fluorescence marked apoptotic cells, red necrotic); B cell cycle measured with PI incorporation (living cells); C Annexin V/PI microscopic assay, blue cells carried phosphatidyl serine; D colorimetric caspase activity assay, zero percent represents the caspase activity in control cells.

CONCLUSION

Starting from industrial waste a series of several homologues esters and amides was synthesized, with >20 derivatives unknown so far. Biochemical investigations show, that a moderate lipophilic residue improved the activity, whereas amides or long hydrophobic chains lead to a decrease.

Combined with the results from the dye exclusion assay, DNA ladder technique, caspase assay as well as annexin V binding studies the anticancer activity seems to be independent from the substitution pattern at position 28, whereas modifications of this position, however, directs their way of action.

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Acknowledgements:
- Dr. T. Müller (Dept. of Haematology/Oncology, Univ. Halle) for providing the cell lines
- Gründerwerkstatt Biowissenschaften & BioSolutions GmbH Halle (BSH) providing the cell culture laboratory
- GDCh for personal financial support
- Becker und Karsten for providing the olive pomace

It´s just a Nitrogen atom!

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INTRODUCTION

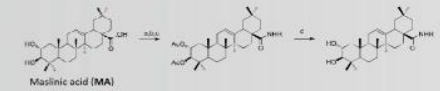
Cancer is one of the leading causes of death; around 500,000 new incidences of cancer appeared in Germany in 2008 [1]. Several new drug-like structures are designed, tested and modified every year fighting this disease. A lot of these molecules usually fail due to unwanted side-effects or non-selective behavior. Fortunately, the "natural pool" still provides substances with a promising selectivity regarding tumor cells.

A huge group of secondary plant metabolites, the pentacyclic triterpenoids, are, moreover, able to trigger the desired form of cell death - apoptosis.[2] Recent studies showed that the activity of these substances can be improved by chemical modifications.[3] Thus, we became interested in studying maslinic acid (**MA**), one of the triterpenoic acids being easily extracted from dish olives or olives pomace [4]. Improving the antitumor activity of this acid was the aim of our study.

CHEMICAL MODIFICATIONS AND STRUCTURE-ACTIVITY-RELATION STUDIES

Starting from 6 kg of olive dishes, 5.64 g of **MA** was isolated using several methanolic extraction steps and purification by column chromatography. For comparison, 3.07 g were obtained starting from 4 kg of olive pomace.[4] First, several esters of maslinic acid were prepared. In addition, amides were formed (scheme 1). A selection of the compounds as well as the results from the SAR are compiled in figure 1.

The additional synthesis of benzyl 2,3 diacetylic maslinoate allowed the comparison with the protected amides. An remarkable result is shown below.



Scheme 1: Amid synthesis. a) Ac_2O, py, TEA, rt; b) DCM, oxalyl chloride, DMF, TEA; c) DCM, $R'NH_2$, TEA, DMAP; d) MeOH, K_2CO_3, rt

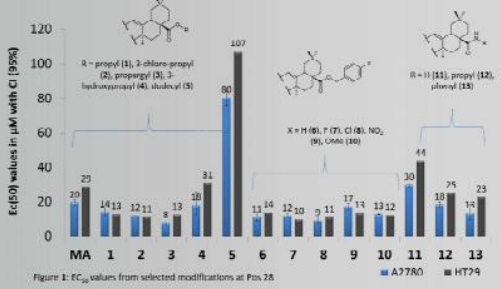


Figure 1: EC_{50} values from selected modifications at Pos 28

Replacing an oxygen by a nitrogen led to a phenomenal increase of activity:

→ Up to 60 fold more active
→ Up to 70 fold more selective for malignant tissues

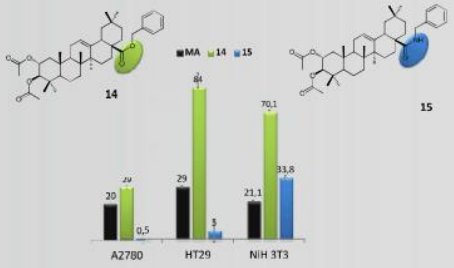


Figure 2: EC_{50} for **14** and **15** values in µM and CI (95%). NIH3T3 = non malignant cell line

Modifications at position 28 are promising if:

→ Lipophilic aromatic or alkyl chain will be introduced
→ The alkyl chain is not longer than 6 carbon atoms
→ No additional proton donator will be introduced

Following modifications had no significant influence:

→ Saturation & halides

INVESTIGATIONS OF THE BIOLOGICAL MODE OF ACTION

Motivated by these remarkable results, we took a closer look to the mode of action. Desired drug candidates should show a selectivity for malignant, but not for normal tissues.
We could demonstrate a high cytotoxicity for nine malignant cell lines. Furthermore, treatment of primary human cells showed that **15** is almost not toxic – the EC_{50} value is about 300 fold higher than for ovarian cancer cells.

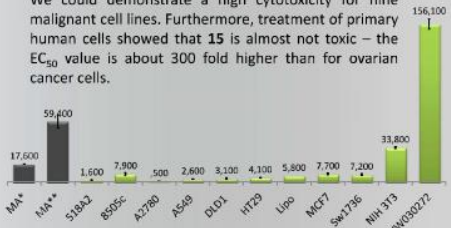


Figure 3: EC_{50} values of **15** against: 9 cancer cell lines and two non malignant cell types. NIH 3T3 the non-maligned mouse fibroblasts and WI030272 a non maligned human primary fibroblasts.

Another aim of medicinal chemistry is to create drugs being able to induce a programmed cell death (PCD) as shown for all of our compounds by a whole green stained cell population in AO/PI assays. DNA fragmentation assays gave the significant DNA laddering pattern , thus indicating an apoptotic MOA. To validate this assumption, we investigated the caspase activity (figure 6). An additional annexin V-assay (figure 5) and a cell cycle investigation (data not shown) led to the following assumption: compared to the benzyl maslinoate (**6**) the amide (**15**) acts rather cytostatic than cytotoxic.

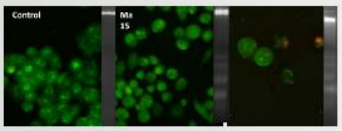


Figure 4: Dye exclusion assay. Ovarian cancer cells (A2780) were treated for 24h with the indicated compounds

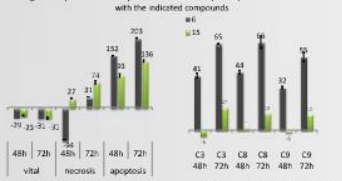


Figure 5: Vital and death cell distribution [%] (A2780 cells)

Figure 6: relative caspase activity [%] in percent with SE (A2780 cells)

CONCLUSION

We were able to turn a natural substance into a promising compound for anticancer research exhibiting a 40 fold higher activity (compared to the parent compound) as well as an outstanding selectivity. Furthermore, a shift from a cytotoxic to a rather cytostatic agent was gained. In conclusion, compound **15** was shown to be a perfect candidate for further studies regarding the antitumorigenesis potential.

Literature:
[1] Krebs in Deutschland 2007/2008, Robert Koch Institut, Berlin, 2012; [2] Salvador, J.A.R., Pentacyclic Triterpenes as Promising Agents in Cancer. Cancer Etiology, Diagnosis and Treatments. 2010: Nova Science Pub Inc.; [3] Csuk, R.; Schwarz, S.; Siewert, B.; Kluge, R.; Strohbl, D. *Eur. J. Med. Chem.* **2011**, 46, 5356. [4] Manuskript submitted June 2013

Acknowledgements:
- Dr. T. Müller (Dept. of Haematology/Oncology, Univ. Halle) for providing the cell lines
- Gründerwerkstatt Biowissenschaften (Univations)
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Intracellular drug tracking by a "click" chemistry based approach!

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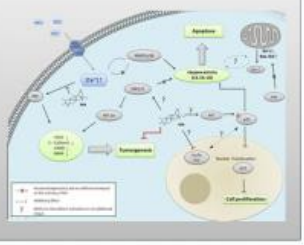
INTRODUCTION

The widely disturbed class of natural pentacyclic triterpenoids carry high promising pharmacological properties. The natural compounds and many synthetically derivates thereof are called as high promising candidates for drug-like structures again *e.g.* viral, diabetic or cancer diseases. Some of them are submitted into clinical trails.

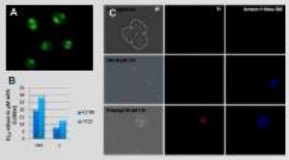
However, regarding the cellular target many approaches let to certain suggestions but less evidence. At least for the first question, act the molecule inside of the cell – no answer is existing.
Classical approaches, like isotopic marking, are problematic due to the complexity of the molecule. Thus, we became interested in a new non-radioactive approach.



THE IDEA AND CONCEPT

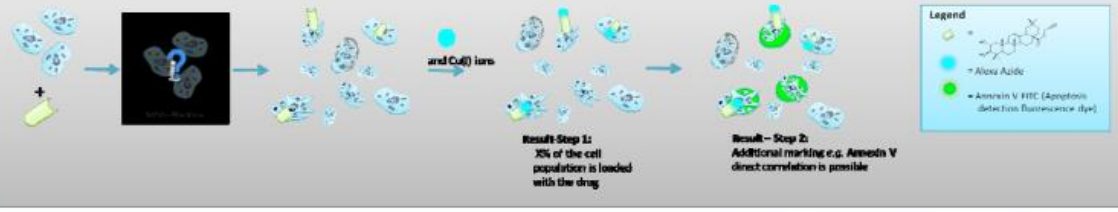
In a convenient way it is not possible to marker an active triterpenoid molecule in front of the treatment without drastical molecular changes. Thus it should be possible to marker and detect the drug after the treatment. Therefore a molecular anchor is necessary, to catch the molecule. The EdU-Assay, Click-FISH and our promising result for propargyl derivates of maslinic acid disclose a possibility – fluorescence labeling inside the cell suspension after an incubation time. Additionally, sorting and counting the cells with the FACS machine would led to statistical results.

BIOLOGICAL EVALUATION

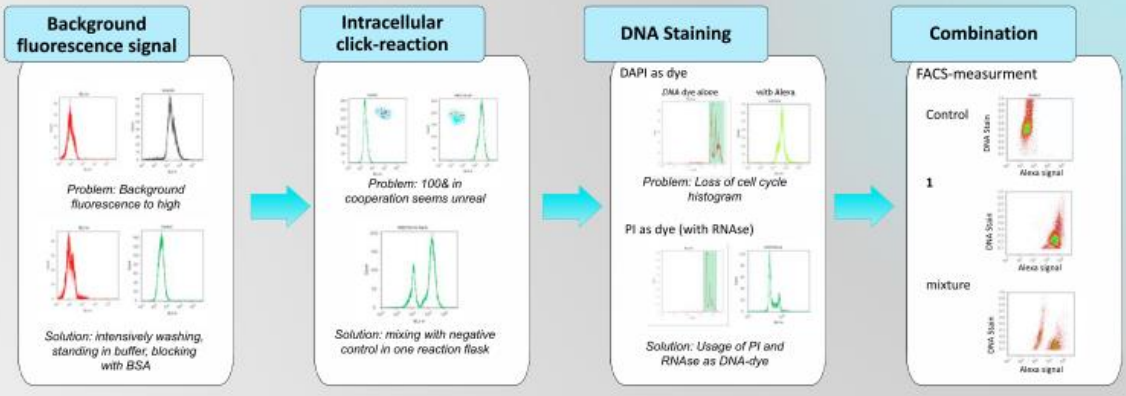


A: Dye-Exclusion assay. Propargyl maslinoate (**1**) induce a programmed cell death in colon cancer cells after 24h treatment. **B** Selected result of the cytotoxicity assay (SRB-Assay) **C** Annexin V Assay. The result showed, **MA** and **1** trigger apoptosis.

SCHEMA OF THE CONCEPT



IMPLEMENTATION OF THE CONCEPT



CONCLUSION & OUTLOOK

Our results show, that in principal the staining over click reaction inside of the cell is possible as well as the counterstaining with DNA-sensitive dyes (PI). Additionally, the most used fixation additives (paraformaldehyde and ethanol) are compatible.
Furthermore the combination with a third fluorescence dye is imaginable to measure e.g. whether **1** induced directly apoptosis, mitochondrial membrane modulation or the production of ROS. Additional fluorescence microscopic investigations might be possible, to locate **1** inside of the cell.

Literature:

Acknowledgements:
- Dr. T. Müller (Dept. of Haematology/Oncology, Univ. Halle) for providing the cell lines
- Gründerwerkstatt Biowissenschaften (Unrivations)
- Fermín Rodríguez Jiménez & Andreas Becker (AcetoVocantor.de) for providing the olive pomace
- for financial support: GDCH and Martin-Luther University of Halle -Wittenberg

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06/2013	DAAD Reisestipendium
04/2013	Frauenförderung Uni Halle
03/2013	GDCH Reisestipendium
08/2012	GDCH Reisestipendium
04/2012	DAAD Reisestipendium
03/2012	Frauenförderung Uni Halle
03/2011	Frauenförderung Uni Halle
05/2010	DOW Reisestipendium
02/2010	Frauenförderung Uni Halle
01/2010	GDCH Reisestipendium

BETREUTE ABSCHLUSSABREITEN

In der nachfolgenden Liste sind die fachlich sowie praktisch betreuten Abschlussarbeiten aufgeführt, welche in dem Zeitraum von 2011 bis 2013 durch die angegebenen Studenten angefertigt wurden.

2013	BA Felix Tzschöckell
2013	MA Sandra Tschullik
2013	MA Franziska Flemming
04/2013	„Synthese und biologische Evaluierung von Vanillyl- und Stilbenylderivaten der Glycyrrhetinsäure“, DA, Christin Klauk
09/2012	„Oxidative C-Ring-Modifikationen der Glycyrrhetinsäure“; BA, Jana Wiemann
09/2012	„Synthese von antineoplastischen Biuretderivaten der Glycyrrhetinsäure“; BA, Anja Obernauer
06/2012	„Synthese und biologische Evaluierung von Triterpencarbonsäure-Derivaten”; DA, Elke Bittmann
09/2011	„Synthese von antitumoraktiven Aminosäurederivaten der Oleanolsäure“; BA, Sophie Reimann
09/2011	„Synthese von verschiedenen Exo-Methylenderivaten“; BA, Vincent Perl
09/2011	„Synthese von antineoplastischen Zimtsäurederivaten der Oleanolsäure“; BA, Steve Neumann

Sie ist wie der Wind,
- wie der Wind von hinten -
bescheiden,
kraftvoll,
unsichtbar,
facettenreich,
und oftmals leider namenslos
- und ihr, ihr gilt mein tiefster, ehrlichster Dank!

War sie es doch, die machte, dass die Zeit verflog,
dass Regenbögen leuchteten, dass Berge und Täler passierten,
dass der Einsamkeit, der Hoffnungslosigkeit und der Dunkelheit, kein Raum gewährt wurde.

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SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Die Arbeit wurde bisher an keiner anderen Universität oder Hochschule vorgelegt.

Halle (Saale), 18.07.2013 Bianka Siewert

„[…] je näher man dem Ende kommt,

desto mehr hat man zu sagen.

Das Ende besteht nur in der Einbildung,

es ist ein Ziel,

das man erfindet,

um durchzuhalten,

doch irgendwann gelangt man zu der Erkenntnis,

dass man nie dort ankommen wird.

Vielleicht muss man aufhören,

aber nur weil die Zeit abgelaufen ist.

Man hört auf,

aber das bedeutet nicht,

dass man das Ziel erreicht hat. […]"

PAUL AUSTER

– Im Land der letzten Dinge –