SCREENING OF PRENYLDIPHOSPHATE MIMETICS FOR THEIR POTENTIAL TO INHIBIT 4-HYDROXYBENZOATE OLIGOPRENYL TRANSFERASE

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Institute of Pharmacy

by Amina Msonga

Born on 16.01.1980 in Nzega, Tanzania

Halle (Saale), Germany

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vorgelegt

von Frau Amina Msonga

geboren am 16.01.1980 in Nzega, Tanzania

Gutachter:

> Prof. Dr. L. A. Wessjohann
> (Martin-Luther-University, Halle-Wittenberg (& Leibniz-IPB))
>
> Prof. Dr. M. Pietzsch
> (Martin-Luther-University, Halle-Wittenberg)
>
> Prof. Dr. O. Kayser
> (Technical University, Dortmund)

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ABSTRACT

4-Hydroxybenzoate oligoprenyl transferase (UbiA-prenyltransferase) (EC 2.5.1.39) is an important enzyme in ubiquinone biosynthesis in *E. coli*. It is a membrane-bound enzyme consisting of 290 amino acids (32.5 kDa). It catalyzes prenylation of 4-hydroxybenzoate (4-HB) at position 3 to form 3-oligoprenyl-4-hydroxybenzoate. In this study some prenyldiphosphate mimetics prepared by Vasilev (2015) at IPB were tested *in vitro* for their inhibitory potential against UbiA-prenyltransferase catalysed geranylation of 4-HB.

The UbiA membrane protein was prepared from *E. coli* C41 (DE3):pALMU3 which contains the wildtype UbiA construct. The effect of Mg^{2+} ion on UbiA activity was checked whereby there was no formation of GHB in absence of Mg^{2+} ion supporting the fact that Mg^{2+} ion is essential for UbiA activity. In addition, the effect of EDTA on UbiA activity was also checked. It was found that as concentration of EDTA increases, the amount of GHB formed decreases because EDTA chelates Mg^{2+} ion from the reaction mixture, thus, rendering the solution with little or no available Mg^{2+} ion.

Among the tested compounds, **11**, **26**, **4**, **28** and **36** showed inhibitory activity at 1 mM. IC_{50} values were determined for **26**, **4**, **28** and **36** and were found to be 1.51 ± 0.14 mM, 0.75 ± 0.02 mM, 0.69 ± 0.03 mM and 1.25 ± 0.03 mM, respectively. The IC_{50} value of **11** was not determined due to limited availability of the substance. Most of the potential inhibitors were FPP analogues. Thus, effect of FPP, also a native substrate on UbiA, versus geranylation was also checked and it showed significant competition at 1 mM; its IC_{50}-like value was found to be 0.88 ± 0.06 mM.

Results from this study show that inhibitory potential of the tested compounds depends on chain length, head group and chain linker.

ZUSAMMENFASSUNG

4-Hydroxybenzoat-Oligoprenyl-Transferase (UbiA-Prenyltransferase) (EC 2.5.1.39) ist ein wichtiges Enzym in der Ubichinon-Biosynthese von *E. coli*. Es ist ein membrangebundenes Enzym, das aus 290 Aminosäuren (32,5 kDa) aufgebaut ist. Es katalysiert die Prenylierung von 4-Hydroxybenzoat (4-HB) an Position 3 zu 3-Oligoprenyl-4-Hydroxybenzoat. In dieser Studie wurden Prenyldiphosphat-Mimetika, die von Vasilev (2015) am IPB hergestellt wurden, *in vitro* auf ihr inhibitorisches Potential gegen die UbiA-Prenyltransferase-katalysierte Geranylierung von 4-HB getestet.

Das UbiA Membranprotein wurde aus *E. coli* C41 (DE3):pALMU3 isoliert, welcher ein Plasmid enthält, das Wildtyp UbiA kodiert. Die Wirkung von Mg^{2+}-Ionen auf UbiA Aktivität wurde geprüft, wobei keine Bildung von GHB in Abwesenheit von Mg^{2+}-Ionen stattfand. Dies unterstützte die Tatsache, dass Mg^{2+}-Ionen für die UbiA Aktivität wesentlich sind. Darüber hinaus wurde die Wirkung von EDTA auf die UbiA Aktivität geprüft. Es konnte gezeigt werden, dass mit steigender Konzentration von EDTA, die Menge an gebildetem GHB abnimmt, da EDTA Mg^{2+}-Ionen aus dem Reaktionsgemisch chelatiert, wodurch nur wenige oder keine Mg^{2+}-Ionen in der Lösung verfügbar sind.

Unter den getesteten Verbindungen zeigten **11**, **26**, **4**, **28** und **36** inhibitorische Aktivität bei 1 mM. IC_{50} Werte wurden für **26**, **4**, **28** und **36** ermittelt und betrugen 1,51 ± 0,14 mM, 0,75 ± 0,02 mM, 0,69 ± 0,03 mM beziehungsweise 1,25 ± 0,03 mM. Der IC_{50} Wert von 11 wurde wegen der begrenzten Verfügbarkeit der Substanz nicht bestimmt. Die meisten der potentiellen Inhibitoren waren FPP Analoga. Daher wurde die Wirkung von FPP, ebenfalls natives Substrat von UbiA, gegen Geranylierung geprüft. FPP zeigte eine signifikante Kompetion bei 1 mM und der gemessene IC_{50}-ähnliche Wert betrug 0,88 ± 0,06 mM.

Die Ergebnisse dieser Studie zeigen, dass das inhibitorische Potenzial der getesteten Verbindungen von Kettenlänge, Kopfgruppe und Ketten-Linker abhängt.

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LIST OF ABBREVIATIONS

EDTA   Ethylenediaminetetraacetic acid

SDS   Sodium dodecyl sulfate

GHB   3-Geranyl-4-hydroxybenzoic acid

FPP   Farnesyldiphosphate

FHB   3-Farnesyl-4-hydroxybenzoic acid

4-HB   4-Hydroxybenzoic acid (4-hydroxybenzoate)

LC-MS   Liquid chromatography-mass spectrometry

$MgCl_2$ Magnesium chloride

GPP   Geranyldiphosphate

DMSO   Dimethyl sulfoxide

UbiA   4-Hydroxybenzoate oligoprenyl transferase (UbiA-prenyltransferase)

ESI-MS   Electrospray ionization-mass spectrometry

CID   Collision-induced dissociation

HESI   Heated electrospray ionization

UHPLC   Ultra high performance liquid chromatography

SRM   Selected reaction monitoring

IC_{50} Inhibitor concentration giving 50% inhibition

HPLC   High-performance liquid chromatography

DTT   1,4-Dithio-D-threitol

ULC/MS   Ultra liquid chromatography / mass spectrometry

IPTG   Isopropyl β-D-1-thiogalactopyranoside

OD_{600} Optical density at 600 nm

LB   Luria-Bertani (nutrient medium)

NaOH   Sodium hydroxide

NaCl	Sodium chloride
rpm	Revolutions per minute
NMR	Nuclear magnetic resonance
E. coli	*Escherichia coli*
ITC	Isothermal titration calorimetry
WT	Wildtype
DAAD	*Deutscher Akademischer Austauschdienst* (German Academic Exchange Service)
MoEVT	Ministry of Education and Vocational Training, Tanzania
IPB	Leibniz Institute of Plant Biochemistry
SD	Standard deviation
S.E	Standard error
IT	Information technology

1 INTRODUCTION AND LITERATURE REVIEW

1.1   General Introduction

A series of well-coordinated chemical reactions takes place in living organisms and life is based on these reactions (Copeland, 2000). They are catalysed by enzymes that occur naturally in living organisms to speed up the process as most of them proceed too slowly in absence of catalysts to support life (Copeland, 2000; Segel, 1993). The fact that enzymes retain their catalytic power even after being isolated from their respective organisms persuaded man to exploit various enzymes for commercial use (Copeland, 2000). The earliest known enzymes from history are those involved in cheese, bread and alcohol fermentation (Copeland, 2000; Segel, 1993). Nowadays, enzymes are fundamental commercially, particularly in food and beverage processing industries. They are also included as ingredients in commercial products such as detergents. Furthermore, enzymes today find application in pharmaceutical industries whereby potential inhibitors of enzymes that trigger/mediate disease are developed into drugs (Copeland, 2000). In the ancient societies, enzymes were used commercially as whole organisms for example in bread and wine fermentation as well as meat tenderizer and in dairy products industries (Copeland, 2000). However, these applications were based on empirical observations rather than systematic studies. Isolation and purification of enzymes dates back to 19th century when Bertrand partially purified laccase from tree sap (Copeland, 2000). In addition, Buchner revealed that alcohol fermentation could be performed even in absence of living yeast cells when he used a yeast filtrate (Copeland, 2000; Segel, 1993; Mäntsälä and Niemi, http://www.eolss.net). Advances in molecular biology have enabled scientists to clone and overexpress enzymes in foreign host organisms resulting in relatively higher yield compared to the amount produced in their natural hosts (Copeland, 2000). Molecular biology tools have facilitated isolation,

identification and characterization of numerous novel enzymes. Furthermore, molecular biology tools have made it possible to manipulate amino acid sequence through site-directed and deletion mutagenesis allowing identification of chemical groups involved in ligand binding and in specific chemical steps in the course of enzyme catalysis (Copeland, 2000). Nowadays, enzymes have a wide range of applications such as stereospecific chemical synthesis, laundry detergents, and cleaning kits for contact lenses. Not only enzymes but also inhibitors have fascinated scientists in pharmaceutical industry since they are applied as drugs in human and veterinary medicine (Copeland, 2000).

Enzymes are very specific for the reactions they catalyze, their specificity is due to the fact that they catalyze either one chemical reaction or a very few closely related reactions (Copeland, 2000; Mäntsälä and Niemi, http://www.eolss.net). This specificity is determined by the precise structure and active site of the enzyme (Mäntsälä and Niemi, http://www.eolss.net). Studies of enzymes deal with identification of the catalytic activity, purification of the enzyme, characterization of the enzymatic activity, determination of the enzyme's chemical and physical properties and integration of the protein characteristics and its enzymatic nature (Copeland, 2000; Tan, 2011). Identification of the catalytic activity involves development of an assay to quantify the activity. The assay should be simple and fast. The enzyme can be completely or partially purified. The most important thing to consider is to ensure that there is no more than one enzyme in the preparation with similar activity and endogenous inhibitors or activators are either removed or identified (Tan, 2011). Enzyme kinetics discloses important aspects of the enzymes catalytic mechanism, how its activity is controlled, and the role it plays in metabolism and the way a drug or poison can inhibit its activity (Segel 1976; Segel 1993; Bisswanger, 2008; Tan, 2011). The factors to be considered in enzyme characterization include: factors affecting its activity (e.g. pH and temperature), rate of reaction with different substrates, effect of inhibitors or activators and

kinetic parameters. The enzyme kinetic parameters that are used to characterize an enzyme are K_m (substrate concentration at half maximum velocity) and maximum velocity (Segel 1976; Bisswanger, 2008; Tan, 2011). The chemical and physical properties of the enzyme to be determined include composition, molecular weight, amino acid sequence and secondary and tertiary structure. However, the most important factor is its stability under experimental conditions (Copeland, 2000; Tan, 2011). Integration of the protein characteristics and its enzymatic nature involves identification of the amino acid residues constituting the active site and crystallography that identifies substrate orientation in the active site (Copeland, 2000; Tan, 2011). With consideration to UbiA which is the enzyme used in this study, its characterization was done in previous studies (Bräuer *et al*., 2004; Bräuer *et al*., 2008).

However, the activity of enzymes can be affected by salt concentration, temperature, pH, substrate concentration and level of macromolecular crowding (Tan, 2011; Bisswanger, 2008; Copeland, 2000; Minton, 2001). The majority of enzymes are affected with extremely high salt concentrations because the ions interfere with the relatively weak ionic bonds of proteins. However, ions seem to be necessary for activity of some enzymes, in this case, the acceptable range of ion concentration in an assay is 1-500 mM (Tan, 2011). Since enzymes are biomolecules found in living organisms, they work in a particular temperature specific to that organism (Bisswanger, 2008; Tan, 2011). Under certain circumstances such as changes in intracellular conditions, cells have an adjustment mechanism whereby allosteric regulation is used to respond to the changes. On the other hand, some enzymes' catalytic potential is regulated by cofactors such as metal ions or some small molecules (Mäntsälä and Niemi, http://www.eolss.net). Likewise, any *in vitro* assay should consider a suitable temperature under which a particular enzyme performs best. Higher temperatures may denature the enzyme leading to a decreased reaction rate. pH affects enzymes due to their protein nature, it can denature the 3D structure of the enzyme thereby breaking ionic and hydrogen bonds

(Copeland, 2000). Generally, many enzymes work at a pH range of 6.0 to 8.0. Macromolecules have been reported to alter rates and equilibrium constants by macromolecular crowding (Minton 2001; Tan, 2011). However, protein degradation is considered to be a means of regulating enzyme levels (Mäntsälä and Niemi, http://www.eolss.net).

Based on their functions, enzymes fall under six major classes; oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases/synthetases (Adugna *et al*., 2004; Detsch 2008; Kumar and Choudhary, 2012; Leisola *et al*., http://www.eolss.net).

Oxidoreductases catalyze oxidation-reduction reactions with lactate dehydrogenase being an example of this group. Transferases catalyze transfer of a group from donor molecule to acceptor molecule. For example, prenyltransferases, which are the focus of this study, catalyze the transfer of a prenyl moiety from a prenyl diphosphate compound to an acceptor molecule that can be a prenyl diphosphate also (like in synthesis of farnesyl diphosphate) or an aromatic compound (e.g. transfer of a prenyl moiety to 4-hydroxybenzoic acid by UbiA). Hydrolases catalyze hydrolysis of ester, ether, peptide, glycosyl, acid-anhydride, C-C, C-halide or P-N-bonds by using water or a similar (nucleophilic) solvolytic agent. An example of this group is β-galactosidase that acts on glycosyl bonds of galactose-conjugates. Lyases are the enzymes that catalyze elimination of groups from compounds by a mechanism different from hydrolysis resulting in double bonds. This class comprises enzymes acting on C-C, C-O, C-N, C-S and C-halide bonds with fumerase being an example. Isomerases are the enzymes that catalyze interconversion of optical, geometric, or positional isomers. Example of this group is triose-phosphate isomerase that catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Ligases or synthetases catalyze the linkage of two compounds coupled to the hydrolysis of a

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pyrophosphate ester bond of adenosine triphosphate (ATP) or a similar nucleotide like guanosine-5'-triphosphate (GTP) or uridine-5'-triphosphate (UTP). This group comprises enzymes catalyzing formation of C-O, C-S, C-N (e.g. glutamine synthetase) and C-C bonds (Adugna *et al.*, 2004; Detsch 2008; Leisola *et al.*, http://www.eolss.net).

1.2 Literature Review

1.2.1 Prenyltransferases

Prenyldiphosphate converting enzymes is a general term for enzymes that modify naturally occurring isoprenoids. This group of enzymes comprises terpene synthases, transferases and hydrolases or isomerases (Brandt *et al.*, 2009). Transferases (generally called prenyltransferases) are further divided into aromatic prenyltransferases, oligoprenyldiphosphate synthases and protein prenyltransferases. In addition to these are geranylgeranyl hydrogenase or squalene epoxidase that do not fall under either of the aforementioned groups (Brandt *et al.*, 2009).

Prenyltransferases are a class of enzymes that transfer allylic prenyl groups from allylic isoprenyl diphosphates like dimethylallyl diphosphate (DMAPP; C_5), geranyl diphosphate (GPP; C_{10}) and farnesyl diphosphate (FPP; C_{15}) to acceptor molecules (Heide, 2009). They are found in all living organisms but differ in type from one organism to another. For example, *trans* polyprenyl diphosphate synthases and aromatic prenyltransferases of lipoquinone biosynthesis (e.g. 4-hydroxybenzoate octaprenyltransferase (UbiA)) are found in all living organisms while aromatic prenyltransferases of prenylflavonoid (e.g. Naringenin 8-dimethylallyltransferase (N8DT)) and fungal indole prenyltransferases (e.g. Dimethylallyl tryphtophan synthase) are found only in plants and fungi, respectively (Heide, 2009; Li, 2009). In nature, reactions catalyzed by prenyltransferases lead to a diversity of prenylated

primary and secondary metabolites (Saleh *et al.*, 2009). Below is an example of the reactions catalysed by various prenyltransferases (Fig. 1).

trans-Prenyltransferase
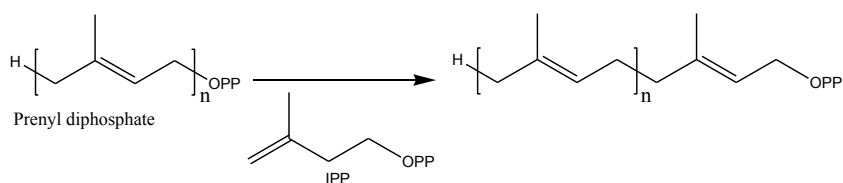
cis-Prenyltransferase

n=2: short chain *cis*-prenyltransferases

n=9 or 10: medium chain *cis*-prenyltransferases

n=13 to 23: long chain *cis*-prenyltransferases
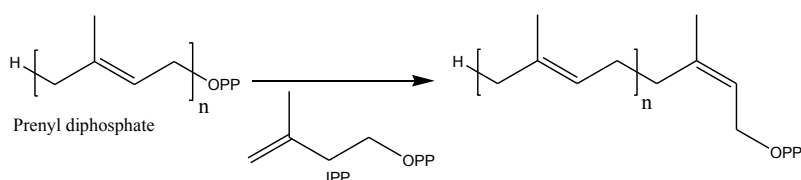
Protein prenyltransferase
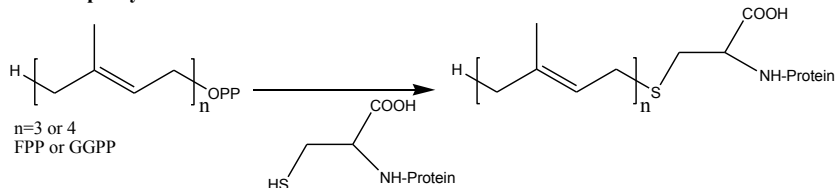
Aromatic prenyltransferase, e.g.

4-Hydroxybenzoic acid octaprenyltransferase (UbiA)
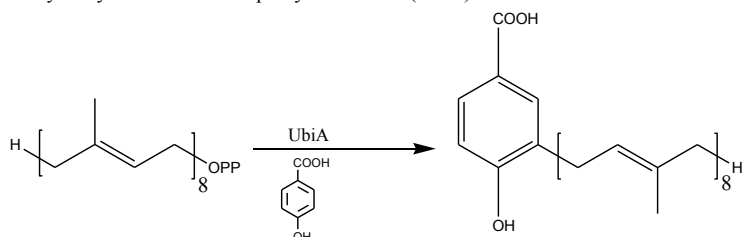
Flavonoid prenyltransferase (N8DT)
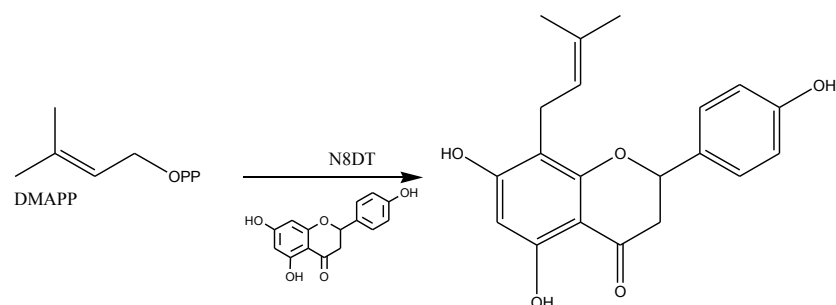
Figure 1: Some examples of diverse prenyltransferases (Li, 2009).

Some prenyltransferases are membrane-bound enzymes and they contain one or more prenyl diphosphate binding motif, (N/D)DxxD, in their sequences while some are soluble (Grundmann and Li, 2005). Membrane-bound prenyltransferases comprise those, which are involved in ubiquinone, menaquinone, plastoquinone and tocopherol biosynthesis with UbiA-prenyltransferase being a good example of this group (Yazaki *et al.*, 2002; Grundmann and Li, 2005; Haug-Schifferdecker, 2010). Soluble prenyltransferases include the PT barrel proteins that lack the (N/D)DxxD motifs for binding of the isoprenoid substrate (Grundmann and Li, 2005; Haug-Schifferdecker, 2010). A few examples of the soluble prenyltransferases are dimethylallyltryptophan synthase (DMATS) which catalyses the prenylation of tryptophan at position C-4 of the indole nucleus during ergot alkaloid biosynthesis in the fungus *Claviceps*, CloQ and LtxC from *Streptomyces roseochromogenes* and *Lyngbya majuscula* respectively (Grundmann and Li, 2005).

Based on the nature of the acceptor substrate to which the prenyl group (Fig. 2) is transferred prenyltransferases fall into two groups; protein prenyltransferases, which catalyze the transfer of an isoprenyl pyrophosphate (e.g. farnesyl pyrophosphate) to a protein or a peptide and the C-C-coupling prenyltransferases which include: (a) prenyl pyrophosphate synthases (IPPSs), which catalyze chain elongation of allylic pyrophosphate substrates (e.g. DMAPP, GPP, FPP) via consecutive head to tail condensation reactions with isopentenyl pyrophosphate (IPP) to generate linear polymers with defined chain lengths; (b) aromatic prenyltransferases that transfer intermolecularily to aromatic systems (c) terpene synthases (cyclases) that are intramolecular prenyltransferases, which catalyze the cyclization of isoprenyl pyrophosphates (Liang *et al.*, 2002; Dessoy, 2003; Degenhardt *et al.*, 2009). C-C Coupling reactions can be between allylic and homoallylic isoprenyl diphosphates where they occur in four different ways or between C-1 or C-3 of the isoprenoid substrate and one of the aromatic carbons of the acceptor substrate if the acceptor molecule is an aromatic compound (Saleh *et al.*, 2009).

CH₃

CH₃
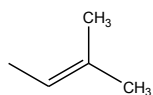
2-methylbut-2-ene

Figure 2:   Prenyl group (Botta *et al.*, 2005).

1.2.1.1   Isoprenyl Diphosphate Synthases

Isoprenyl diphosphate synthases are indispensable in formation of linear prenyl chains of all isoprenoid compounds (Wang and Ohnuma, 2000; Liang *et al.*, 2002). Up to now, there are over 20 identified isoprenyl diphosphate synthases catalyzing reactions that result in different prenylated products with various chain lengths depending on enzyme's specificity (Wang and Ohnuma, 2000). As examples on how these enzymes result in products with various chain lengths, geranyl diphosphate synthase catalyzes the formation of a C_{10} compound, while natural rubber synthase catalyzes the condensation of over 5000 isoprene units to make rubber (Kellogg and Poulter 1997; Wang and Ohnuma, 2000). These prenyltransferases can be classified into [*cis* (or Z) and *trans* (or E)] depending upon the stereochemistry and chain length of the resulting products (Kellogg and Poulter 1997; Wang and Ohnuma, 2000; Fujihashi *et al.*, 2001; Kharel and Koyama, 2003; Kharel *et al.*, 2006). Examples of *trans*-prenyltranferases are farnesyl diphosphate synthase and geranylgeranyl pyrophosphate synthase; example of *cis*-prenyltransferases is dehydrodolichyl diphosphate synthase and undecaprenyl diphosphate synthase (Wang and Ohnuma, 2000). Mutagenesis studies and their x-ray structures show that *cis*-isoprenyl diphosphate synthases do not contain aspartate-rich DDxxD motifs although they require Mg^{2+} ion for their catalysis (Fujihashi *et al.*, 2001; Brandt *et al.*, 2009) while *trans*-isoprenyl diphosphate synthases contain two aspartate-rich DDxxD motifs involved in binding of a cluster of three magnesium ions (Wang and Ohnuma, 2000; Brandt *et al.*, 2009). Avian farnesyl pyrophosphate synthase (FPPS) and undecaprenyl

diphosphate synthase (UPPS) are representatives of *trans*- and *cis*- isoprenyl diphosphate synthases respectively (Fujihashi *et al.*, 2001; Brandt *et al.*, 2009). However, the catalytic mechanism is similar for both types (Brandt *et al.*, 2009).

Ogura and Koyama (1998) classified further the isoprenyl diphosphate synthases into 4 subgroups; short chain prenyl diphosphate synthases, medium chain prenyl diphosphate synthases, long-chain (*E*)-prenyl diphosphate synthases and *Z*-polyprenyl diphosphate synthases. This classification is based on product chain length, quaternary structure and stereochemistry (Ogura and Koyama 1998; Wang and Ohnuma, 2000).

Short chain *E*-isoprenyl diphosphate synthases which require divalent ions such as Mg^{2+} and homodimer formation for their catalytic activity (e.g. FPP and GGPP synthases); medium chain *E*-isoprenyl diphosphate synthases which differ in quaternary structure from the short chain enzymes and require a second subunit in addition to the one whose active centre is conserved in a *E*-isoprenyl diphosphate synthases. The second subunit is thought to be involved in the removal of the hydrophobic products from the active site to a hydrophilic environment; long chain *E*-prenyl diphosphate synthases which do not require the second subunit but a prenyl carrier proteins or detergent to get rid of the hydrophobic products from the active site. Reactions catalyzed by this third subgroup result in compounds with chains longer than C_{40} (Wang and Ohnuma, 2000). On the other hand, reactions catalyzed by the *Z*-prenyltransferases result in compounds with chain lengths of C_{50}-C_{100} (Wang and Ohnuma, 2000). However, Guo *et al.*, (2004) stated that *cis*-prenyltransferases generally make products with chain lengths of C_{55}-C_{120}. Studies show that isoprenyl diphosphate synthases depend on Mg^{2+} ion for their catalytic activity (Wang and Ohnuma, 2000; Fujihashi *et al.*, 2001; Kharel *et al.*, 2006; Brandt *et al.*, 2009); moreover, identified crystal structures show that the two

conserved DDxxD motifs in *trans*-prenyltransferases interact with two or three Mg^{2+} ion to facilitate binding to the diphosphate group of the allylic substrate (Wang and Ohnuma, 2000; Brandt *et al*., 2009) while an aspartate plays the same role in the conserved P-loop of *cis*-prenyltransferases (Fujihashi *et al*., 2001; Brandt *et al*., 2009). In addition to this, a study by Lu and colleagues (2009) suggested that *trans*-prenyltransferase catalyzed reactions undergo a sequential mechanism, whereas *cis*-prenyltransferase catalyzed reactions undergo a concerted mechanism. A sequential mechanism is defined as a sequential ionization-condensation-elimination mechanism (S_N1-type) whereby allylic substrate releases its diphosphate to form a carbocation intermediate that is attacked by IPP and a proton is removed from IPP C2 to form the adduct. A concerted mechanism is the one where ionization of allylic substrate and condensation of IPP occurs simultaneously (S_N2-type, Lu *et al*., 2009). Considering the mechanism of isoprenyl diphosphate synthases to regulate chain length of the product, reports suggest that it's the rate of allylic carbocation formation and affinity of a synthase for its substrate that determines chain length (Wang and Ohnuma, 2000).

As stated in the classification of prenyltransferases, they play a role in chain elongation of isoprenoid compounds which are the major group of natural products with diverse structures consisting of various numbers of five carbon isopentenyl diphosphate (IPP) units (Liang *et al*., 2002). Prenyltransferases have drawn attention of scientists as they catalyze prenyl chain elongation in a very fascinating way. The reaction in most of them is terminated at a specific chain length and the product has a definite stereochemistry according to the enzyme's specificity (Ogura and Koyama, 1998; Fernandez *et al*., 2000). Over 23,000 isoprenoid compounds identified so far serve a wide range of important biological functions in living organisms especially Eukarya, Bacteria and Archaea (Liang *et al*., 2002).

1.2.1.2   Protein Prenyltransferases

A number of intracellular proteins undergo posttranslational modification such as glycosylation, prenylation, proteolysis and methyl esterification (Kang *et al*., 1995; Winter-Vann and Casey, 2005; Liu *et al*., 2010). Protein prenylation is the attachment of an isoprenoid tail particularly a farnesyl (15 carbons) or a geranylgeranyl (20 carbons) group to the end of a substrate protein, at the carboxyl terminus of the protein (Maurer-Stroh *et al*., 2003; Brandt *et al*., 2009). Most studied protein prenyltransferases are from rats and some from human, they are divided into: farnesyltransferase (FTase), geranylgeranyltransferase I (GGTase-I) and geranylgeranyltransferase II (GGTase-II) (Maurer-Stroh *et al*., 2003; Brandt *et al*., 2009). Both farnesyltransferase and geranylgeranyltransferase 1 recognize the same CaaX motif (s. below) in their substrates whereby they share the α-subunit but differs in β-subunits (which deternine substrate specificity) hence, they are known as CaaX prenyltransferases (Lee *et al*., 2010; Liu *et al*., 2010; Brandt *et al*., 2009), while geranylgeranyltransferase 2 (also known as Rab) is a non-CaaX prenyltransferase, instead, it recognizes another motif (Maurer-Stroh *et al*., 2003; Brandt *et al*., 2009). CaaX stands for: "C" denotes cysteine, the "a" residues are usually aliphatic, and "X" represents any amino acid (Winter-Vann and Casey, 2005; Brandt *et al*., 2009; Lee *et al*., 2010). The isoprenoid tail is linked to the thiol group of a C-terminal cysteine under the influence of the CaaX motif (Maurer-Stroh *et al*., 2003; Lee *et al*., 2010). Whether the protein is geranylgeranylated by GGTase-I or farnesylated by FTase is determined by the amino acid that substitutes X in the CaaX box. Geranylgeranylation occurs when leucine, phenylalanine, isoleucine, or valine substitutes X, otherwise farnesylation takes place. On the other hand GGTase-II geranylgeranylates proteins with a CC, CXC, CCX or CCXX motif at the C-terminus (Lee *et al*., 2010; Brandt *et al*., 2009). H-RAS and RHOA are examples of farnesyltransferase and geranylgeranyltransferase 1 substrates respectively. So far, there are over 100 protein

substrates for FTase and GGTase-I with RAS proteins being the most studied one because of its role in cancer development (Winter-Vann and Casey, 2005; Lee *et al*., 2010; Liu *et al*., 2010).

Prenylation of proteins helps in protein-protein interaction and facilitates their localization to the cell membrane, the process being aided by the lipophilic isoprenoid tail that plays a role in attaching the protein to the phospholipid bilayer. Membrane localization is essential for proper functioning of the respective proteins especially those involved in cellular signalling and trafficking pathways (Hougland and Fierke, 2009; Liu *et al*., 2010).

1.2.1.3   Terpene Synthases

Terpene synthases (cyclases) also referred to as intramolecular prenyltransferases, (Chappell, 1995; Christianson, 2008) are the key enzymes in the formation of low-molecular-weight terpene metabolites. They catalyze formation of hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}) or diterpenes (C_{20}) from DMAPP, GPP, FPP or GGPP, respectively (Tholl, 2006; Davis and Croteau 2000). Several terpene synthases have been reported from plant species, a few microbial terpene synthases have been functionally characterized though (Tholl, 2006; Pinedo *et al*., 2008; Agger *et al*., 2009). Their role of catalyzing cyclization and rearrangement of prenyl diphosphate chains with 10, 15 or 20 carbons results in diverse terpene structures (Agger *et al*., 2009; Gilg *et al*., 2009). The reaction catalysed by all prenyltransferases starts with the cleavage of a diphosphate group leaving a prenyl moiety to form an allylic prenyl cation. This intermediate is used by terpene synthases to add intermolecularily as electrophile to a C=C double bond (Brandt *et al*., 2009). However, terpene synthases differ in their specificity of prenyl diphosphate chain lengths of the product, the mechanism for cyclization and ability to synthesize a narrow or broad range of cyclization products chain (Agger *et al*., 2009). The x-ray structure shows that they have the DxxxD

motif with two aspartates binding Mg^{2+} ion, which is essential in fixing and activating the diphosphate moiety inside the active site (Brandt *et al.*, 2009).

1.2.1.4   Aromatic Prenyltransferases

Aromatic prenyltransferases catalyse the transfer of a prenyl moiety from a prenyl donor, e.g. DMAPP, to an aromatic substrate such as benzoic acids, naphthalenes, flavonoids, coumarins or indoles in the primary and secondary metabolism (Li, 2009; Kuzuyama *et al.*, 2005; Heide, 2009).

Based on folding or sequence homology, aromatic prenyltransferases can be grouped into: (a) all-α-helical enzymes that contain an aspartate-rich motif which catalyze mostly C-prenylations and require divalent metal ions, their x-ray structure is not known (Brandt *et al.*, 2009; Bräuer *et al.*, 2008) (b) soluble enzymes that do not contain an aspartate-rich motif although NphB requires Mg^{2+} ion for its activity and is the only member of this group with an x-ray structure. Members of this group have an ABBA-fold and some of them (CloQ, NphB and Fnq26) are biochemically characterized (Tello *et al.*, 2008; Brandt *et al.*, 2009). They transfer the prenyl chain to a carbon atom of the aromatic molecule producing a C–C coupling (Brandt *et al.*, 2009). However, Fnq26 and NphB catalyze also O-prenylations, whereby the prenyl chain is transferred to an oxygen atom of the aromatic molecule (Brandt *et al.*, 2009; Heide, 2009). The x-ray structure of NphB is a representative of the group of enzymes that lack the DxxxD motif. One arginine and two lysine residues and a magnesium ion are involved in recognition of the diphosphate. Tyr121 and to some extent Tyr175 do recognize the geranyl moiety (Brandt *et al.*, 2009); (c) soluble fungal indole prenyltransferases, which also do not have an aspartate-rich motif and their activity does not depend on Mg^{2+} ion. They catalyze C- and N-prenylations, however, their x-ray structure is not known (Grundmann and Li, 2005; Brandt *et al.*, 2009; Li, 2009). (d) In addition to these enzymes (e.g. LtxC), there are

prenyltransferases whose sequences are not related to any of the above-mentioned groups suggesting that there are more classes of aromatic prenyltransferases (Edwards and Gerwick, 2004; Brandt *et al.*, 2009).

Prenylation of aromatic compounds contributes to the diversity of plant secondary metabolites due to differences in prenylation position on the aromatic ring. Various lengths of prenyl chain and further modifications of the prenyl moiety e.g. cyclization and hydroxylation, results in a number of prenylated compounds in plants (Sasaki *et al.*, 2008; Heide, 2009; Saleh *et al.*, 2009). It is the prenyl moieties of the secondary metabolites that play a key role to the diversification of their chemical structures and biological activities (Koji *et al.*, 2005). In addition to this, it has been reported that prenylation provides a higher level of bioactivity compared to non-prenylated compounds as it increases affinity for biological membranes and interactions with cellular targets (Tello *et al.*, 2008).

Prenylated indole derivatives are a large class of alkaloids containing a tryptophan moiety substituted with isoprene moieties, and are mainly found in fungi especially *Aspergillus* and *Penicillium* (Grundmann and Li, 2005). In fungi, the prenylation of aromatic substrates results in prenylated indole alkaloids, which form an important class of their strongly bioactive secondary metabolites (Heide, 2009). Biosynthesis of prenylated indole alkaloids has been studied by feeding experiments with isotope-labelled precursors. There are a few reported enzymatic studies though, however, there is little information about the enzymes involved (Grundmann and Li, 2005). So far, ABBA family aromatic prenyltransferases were identified in *Sclerotinia sclerotiorum* 1980, *Aspergillus terreus* NIH2624 and *Penicillium marneffe* (Tello *et al.*, 2008).

Prenyl transfer and prenyl chain elongation are electrophilic reactions whereby an electron-rich acceptor molecule is alkylated by the hydrocarbon moiety of allylic isoprenoid

diphosphates like geranyl diphosphate (Phan and Poulter, 2001; Kuzuyama *et al*., 2005; Brandt *et al*., 2009). Well known acceptor molecules are carbon-carbon double bonds, for example in the synthesis of isoprenoid chains; aromatic rings for example in the synthesis of respiratory quinones, vitamins E; amino groups as occurs in the modification of tRNAs, and sulfhydryl moieties as occurs in the modification of proteins. With reference to *Escherichia coli*, *in vivo*, 4-hydroxybenzoate oligoprenyl transferase transfers diphosphorylated acyclic oligoprenyl moieties to the *meta*-position of 4-hydroxybenzoic acid (Zakharova *et al*., 2004). The enzyme was identified in 1972 in *Escherichia coli* cell extracts as a membrane-bound enzyme being encoded by the ubiA gene and it consists of 290 amino acid residues (32.5 kDa) (Young *et al*., 1972; Nishimura *et al*., 1992; Dessoy, 2003; Brandt *et al*., 2009). Characterization of UbiA-prenyltransferase showed that it requires magnesium ions for catalysis (Bräuer *et al*., 2004). Until now there is no x-ray structure of UbiA-prenyltransferase from *Escherichia coli* (EcUbiA) yet (Bräuer *et al*., 2004; Bräuer *et al*., 2008), however, crystal structures of archaeal UbiA homolog from *Aeropyrum pernix* (ApUbiA) and *Archaeoglobus fulgidus* (AfUbiA) have been recently reported (Cheng and Li, 2014; Huang *et al*., 2014). Below is an illustration of the reaction it catalyzes *in vitro* (Fig. 3).
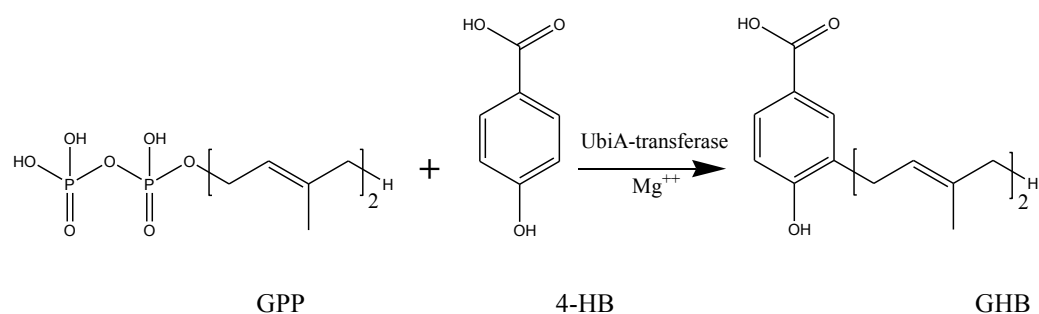
Figure 3: UbiA-prenyltransferase catalyzed formation of 3-geranyl-4-hydroxybenzoic acid
(Bräuer *et al*., 2004).

Aromatic prenylation of 4-hydroxybenzoate (4-HB) leads to biosynthesis of ubiquinones, vitamin E, shikonin and secondary metabolites like conocurvone, boviquinones and tridentoquinone (Wessjohann and Sonntag, 1996; Boehm *et al.*, 1997; Yazaki *et al.*, 2002). 4-Hydroxybenzoate oligoprenyl transferase (UbiA-prenyltransferase; EC 2.5.1.39) is involved in initial steps of ubiquinone biosynthesis in *E. coli* (Nishimura *et al.*, 1992). Ubiquinone is an essential electron carrier in both, prokaryotic (especially gram-negative bacteria) and eukaryotic organisms (Bräuer *et al.*, 2008; Hedrick and White, 1986). UbiA-prenyltransferase catalyzes prenylation of 4-hydroxybenzoate by transferring diphosphorylated oligoprenyl moieties to position 3 using oligoprenyl diphosphates (Bräuer *et al.*, 2004; Zakharova *et al.*, 2004; Saleh *et al.*, 2009). It is also involved in biosynthesis of bactoprenol, which is necessary for bacterial cell wall biosynthesis (Brandt *et al.*, 2009). Apart from 4-hydroxybenzoic acid which is the natural substrate, *in vitro* UbiA accepts also 4-amino benzoic acid, 2,4-dihydroxy benzoic acid, 3,4-dihydroxybenzoic acid, 3-amino-4-hydroxybenzoic acid, various halogenated 4-hydroxybenzoic acids and some other phenolic acids as aromatic substrates (Wessjohann and Sonntag, 1996). On the other hand, the enzyme accepted and successfully transformed prenyl diphosphate substrates with varying chain lengths (Wessjohann and Sonntag, 1996; Meganathan, 2001). However, *in vitro* a C_{10} prenyl diphosphate substrate (GPP) was more favourable than longer chain prenyl diphosphate substrates such as octaprenyl diphosphate (Wessjohann and Sonntag, 1996). Furthermore, the enzyme does not accept 2-*cis* isomers as its substrates due to significant steric interactions or even overlap of the prenyl chain with protein side chains (Bräuer *et al.*, 2004). Nevertheless, UbiA-prenyltransferase has been reported in literature to be successfully applied in organic synthesis as a biocatalyst catalyzing C-C bond formation under mild reaction conditions (Dessoy, 2003).

In addition to ubiquinone, there are other bacterial respiratory quinones like menaquinone and desmethylmenaquinone (also known as demethylmenaquinone, fig. 4) for anaerobic respiration (Hedrick and White, 1986; Søballe and Poole, 1999; White *et al.*, 2005; Lee *et al.*, 2008; Fujimoto *et al.*, 2012). However, in the present work, we are assessing the potential of some prenyldiphosphate mimetics to inhibit 4-hydroxybenzoate oligoprenyl transferase.
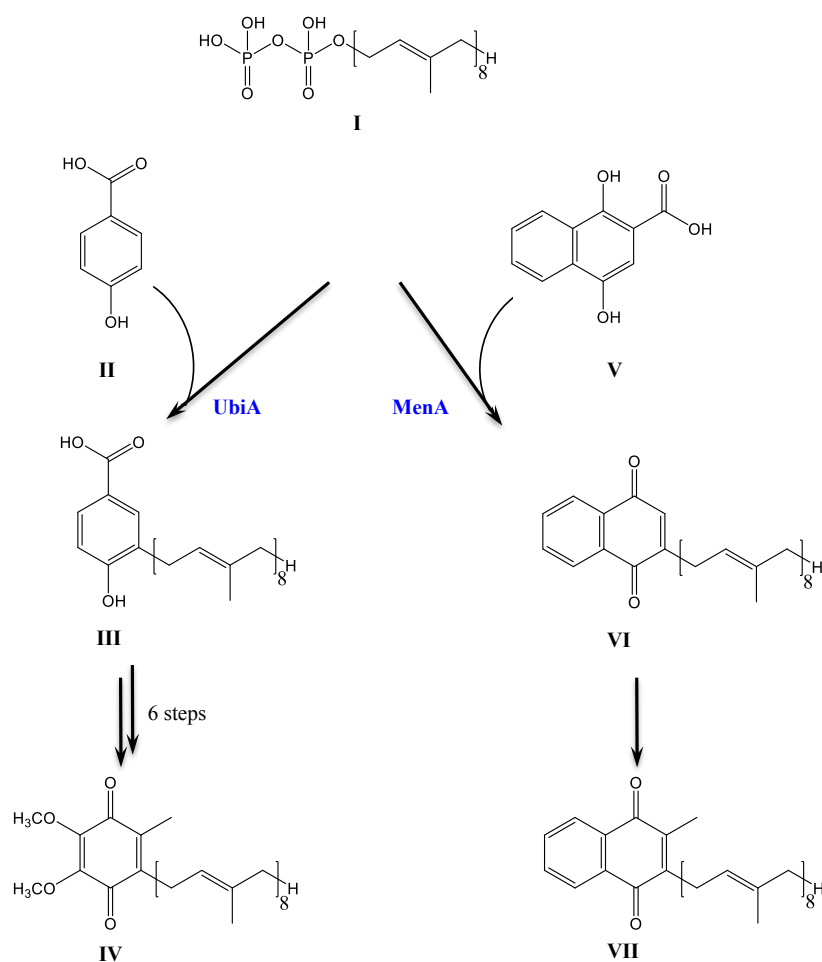
Figure 4:   Simplified native *E. coli* ubiquinone and menaquinone pathways (Lee *et al.*, 2008).

I: Octaprenyl diphosphate
II: 4-Hydroxybenzoic acid
III: 3-Octaprenyl-4-hydroxybenzoic acid
IV: Ubiquinone-8
V: 1,4-Dihydroxy-2-naphthoic acid
VI: Demethylmenaquinone-8
VII: Menaquinone-8

1.2.1.5   Medicinal and Commercial Relevance of Prenyltransferases

As stated earlier, a number of prenylated compounds, especially prenylated flavonoids are reported as active components in medicinal plants with biological activities, such as anticancer, anti-androgen, anti-leishmania, anti-bacterial, anti-fungal, anti-oxidant, anti-tumor, anti-skin aging, estrogenic activities (Sasaki *et al*., 2008; Sasaki *et al*., 2011), or e.g. anti-nitric oxide production which helps in regulating blood pressure and prevents hardening of veins (Murakami *et al*., 2000; Han *et al*., 2006). Following beneficial effects for human health, prenylated flavonoids have been attractive as lead compounds for the production of new drugs and functional foods. Since prenylation of flavonoids results in increased lipophilicity and membrane permeability; this might be the possible reason for enhanced biological activities of prenylated flavonoids compared to their unprenylated counterparts (Maitrejean *et al*., 2000; Ahmed-Belkacem *et al*., 2005; Botta *et al*., 2005; Sasaki *et al*., 2008; Sasaki *et al*., 2011).

In pharmaceutical industry, prenyltransferases are used as targets for cancer therapy. The modifications made to a cysteine residue are important for the biological activities of the prenylated proteins. On this basis, the CaaX protein prenyltransferases, especially protein farnesyltransferase, have been the target of drug-discovery programmes for more than 20 years (Lee *et al*., 2010; Fernández-Medarde and Santos, 2011; Takashima and Faller, 2013; Downward, 2003; Winter-Vann and Casey, 2005). Interest in farnesyltransferase inhibitors (FTIs) aroused since the RAS oncogenes are farnesylated CaaX proteins and inhibition of farnesylation affects negatively the activity of RAS proteins thereby reducing the risk of cancer development (Downward, 2003; Winter-Vann and Casey, 2005; Lee *et al*., 2010; Liu *et al*., 2010; Fernández-Medarde and Santos, 2011; Takashima and Faller, 2013). In early preclinical studies they were shown to have high efficacy and significantly low toxicity (Downward, 2003; Winter-Vann and Casey, 2005; Lee *et al*., 2010; Liu *et al*., 2010;

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Takashima and Faller, 2013). A genetic study on the functional relevance of the protein prenyltransferases in skin keratinocytes was conducted and revealed that protein farnesyltransferase and protein geranylgeranyltransferase type I (GGTase-I) play role in homeostasis of the skin keratinocytes in mice (Lee *et al*., 2010) as well as they are therapeutic targets for cancer and progeria (Yang *et al*., 2006; Kieran *et al*., 2007; Lee *et al*., 2010; Yang *et al*., 2010). FTIs have also shown efficacy on some leukemias in humans, tumors and vascular disease in mice (Lee *et al*., 2010; Liu *et al*., 2010: Kieran *et al*., 2007).

Commercially, prenyltransferases can be applied in rubber synthesis whereby *trans*-prenyl transferase, *cis*-prenyl transferase and rubber transferase are responsible (Cornish 1993; Wang and Ohnuma, 2000; Kharel and Koyama, 2003; Punetha *et al*., 2010; Schmidt *et al*., 2010). Thus, based on their role in nature, prenyltransferases can be used industrially as biocatalysts to facilitate similar reactions or as drug targets in the pharmaceutical industry.

1.2.2   Enzyme Kinetics

Kinetic parameters (i.e. K_m and V_{max}) are usually used to characterize enzyme-catalysed reactions (Yoshino, 1987). These kinetic parameters show how fast the enzyme is saturated with a particular substrate, and the maximum rate it can achieve. If K_m is greater than substrate concentration then, the velocity is very sensitive to changes in substrate concentrations and vice versa (Segel, 1976; Brooks *et al*., 2012). K_m and V_{max} play role on identification of inhibitors and their classification as competitive or non-competitive (Ritchie and Prvan, 1996; Tan, 2011). Furthermore, kinetic data in combination with other information such as structures and active conformations of substrates and the enzyme and mechanism of action of the latter gives insight into its biological functions. Understanding an enzyme's biological functions can provide some ideas on how to modify it for use in therapeutics (Tian *et al*., 2002; Tan, 2011). Apart from that, K_m value is also useful when working with crude

enzymes. It helps in validating enzymatic purity and identity (Scott and Williams, 2012). K_m value equal to ten or more than ten-fold difference from literature value and deviation of the plot of initial velocity vs. substrate concentration from single-site rectangular hyperbolic curve indicate that the enzyme is more likely contaminated or misidentified (Scott and Williams, 2012).

Enzyme-catalyzed reactions exhibit saturation kinetics whereby a plateau is observed at maximum reaction velocity (Copeland, 2000). Principally, the rate of reaction increases linearly with increasing enzyme and substrate concentration but at relatively high substrate concentration almost all enzyme active sites are occupied and the reaction rate reaches maximum (Bisswanger, 2008; Tan, 2011; Bisswanger, 2014).

In enzyme kinetics, the rate of reaction is usually measured by following changes in the concentration of either substrate or products with time (Bisswanger, 2008; Tan, 2011; Brooks *et al*., 2012; Bisswanger, 2014). At the beginning of the enzyme reaction the rate of product formation is linear. Later, the rate decreases as the substrate is consumed or the product accumulates making a plateau of the progress curve (Copeland, 2000; Bisswanger, 2008; Tan, 2011; Bisswanger, 2014). The slope of the linear part of the curve is the initial velocity (also known as steady state since the enzyme-substrate complex does not change) expressed as amount of product per time. Duration of the initial rate period depends on the assay conditions and can range from milliseconds to hours (Copeland, 2000; Bisswanger, 2008; Tan, 2011). Factors like enzyme or substrate concentration, temperature, pH, and other solution conditions may affect drastically the duration of the linear phase. Low substrate concentration for example, may shorten the duration of the linear phase and make its detection difficult or sometimes it disappears completely (Copeland 2000; Bisswanger, 2008; Tan, 2011). To determine the kinetic parameters, kinetic studies focus on initial velocity, which is fitted as a

function of substrate concentration (Bisswanger, 2008; Tan, 2011; Bisswanger, 2014). However, there is quite a number of different methods of evaluating kinetic parameters from the concentrations of substrates and products. These include: initial rate experiments, progress curve experiments, transient kinetics experiments and relaxation experiments (Tan, 2011).

1.2.2.1   Initial Rate Experiments

Rates are measured for a short period after attaining the quasi-steady state, usually by monitoring product formation with time. This is the most widely used method because it is simple and it overcomes problems like back-reaction and enzyme degradation (Tan, 2011).

1.2.2.2   Progress Curve Experiments

Kinetic parameters are determined from expressions for substrate or product concentrations as a function of time for a long time, enough to let the reaction approach equilibrium. This method is nowadays not usually used but was widely used earlier (Copeland, 2000; Tan, 2011).

1.2.2.3   Transient Kinetics Experiments

The reaction is followed at the initial fast transient phase as the intermediate reaches the steady-state kinetics. However, the disadvantage of this method is that it is difficult to perform (Tan, 2011).

1.2.2.4   Relaxation Experiments

This method involves disturbing of the equilibrium of enzyme, substrate and product, for example by temperature, pressure, or pH, and then the return to equilibrium is monitored.

Experiments of this kind need consideration of fully reversible reaction, however, the method is insensitive to mechanistic details and is not usually used to identify mechanism of reactions (Tan, 2011).

In this study kinetics of UbiA were determined as a preliminary to screen for inhibitors in order to provide a basis for substrate concentration to be employed during screening for inhibitors.

1.2.3   Enzyme Inhibition

Usually enzymologists study enzyme inhibition for the purpose of evaluating presence and magnitude of drug-drug interaction. Enzyme inhibition may also play role in predicting changes in drug metabolism by relating *in vitro* and *in vivo* findings within or between species (Kakkar *et al*., 1999). Enzyme inhibitors are the substances that reduce or inhibit completely the catalytic activity of the enzyme by binding to it and affect its interaction with a substrate. They are applied in pharmaceutical industry as drugs acting on enzymes involved in triggering certain diseases (Procopiou *et al*., 1994; Gordon *et al*., 2012). Some drugs that act by inhibiting enzymes include antibiotics like sulfonamides, sedatives, stimulants (Billiet, 2008), β-lactam antibiotics (Copeland, 2000) and anti-inflammatory drugs like indomethacin which acts competitively, while aspirin, betamethasone and dexamethasone act non-competitively (Penning *et al*., 1984). Increasing antibiotic resistance has prompted scientists to develop new potent antibiotic drugs. One approach in drug discovery is to target certain enzymes involved in a particular disease development or enzymes involved in respiration or enzymes that confer antibiotic resistance (Penning *et al*., 1984, Daigle *et al*., 1997; Tuquet *et al*., 2000).

Enzyme inhibitors are separated into two categories; reversible and irreversible inhibitors (Tan, 2011). Reversible inhibitors are further classified as competitive, uncompetitive and non-competitive and irreversible inhibitors are further classified as suicide substrates and transition state analogs. However, there are other categories also like allosteric, partial, tight-binding, and time-dependent inhibition (Yoshino, 1987; Kakkar *et al*., 1999; Bisswanger, 2008; Strelow *et al*., 2012).

1.2.3.1   Reversible Inhibitors

Generally reversible inhibitors bind non-covalently to the enzyme and it can reversibly dissociate from the enzyme. Usually, competitive inhibitors are structurally similar to the enzyme's substrate. They bind competitively at the same active site as the substrate and in this case, inhibition can be overcome in excess of substrate concentration (Bisswanger, 2008; Strelow *et al*., 2012; Mäntsälä and Niemi, http://www.eolss.net/).

1.2.3.1.1   Competitive Inhibitor

A competitive inhibitor binds on the same active site of the enzyme at which a substrate binds. It binds to a free enzyme only, that is, it does not associate with an enzyme-substrate complex. Often, competitive inhibitors are structurally similar to the substrate. The effect of competitive inhibitor on substrate can be overcome at higher substrate concentrations where it loses its potency as inhibitor. Competitive inhibition takes place in different ways: substrate and inhibitor may compete for the same active site or the inhibitor binds to the site other than the active site but hampers substrate binding or sites for substrate and inhibitor binding are overlapping or the binding pocket on the enzyme and are shared by the substrate and the inhibitor, or inhibitor binding can cause changes in conformation of the enzyme which

prevents the substrate from binding (Segel, 1976; Copeland, 2000; Bisswanger, 2008; Mäntsälä and Niemi, http://www.eolss.net).

1.2.3.1.2   Non-Competitive Inhibitors

In contrast to competitive inhibitors, non-competitive inhibitors bind to both, free enzyme and the enzyme-substrate complex. The inhibitor binds at a site different from the active site where the substrate binds. This type of inhibition does not affect K_m of the enzyme's substrate. However, sometimes non-competitive inhibitor is considered mixed inhibition if affinity of the inhibitor to bind to the free enzyme differs from its affinity to the enzyme-substrate complex (Segel, 1976; Bisswanger, 2008).

Like competitive inhibition, non-competitive inhibition also happens in different ways. The inhibitor can bind at a different site from the binding site of the substrate and the catalytic centre of the active site. E.g. if a conformational change of the catalytic centre is provoked when the substrate is bound, this conformational change does not occur when the inhibitor binds, and thus this renders the enzyme inactive. Another way of inhibition is that binding and release of the substrate can be hampered sterically by the inhibitor and in this case, substrate and inhibitor which can bind on the enzyme at the same time (Segel, 1976; Copeland, 2000; Bisswanger, 2008; Mäntsälä and Niemi, http://www.eolss.net).

1.2.3.1.3   Uncompetitive Inhibitor

An uncompetitive inhibitor only binds to the enzyme-substrate complex resulting in an inactive enzyme-substrate-inhibitor complex. Usually, it does not interfere with binding of the substrate to the free enzyme. This type of inhibition decreases K_m of the substrate (Segel, 1976; Copeland, 2000; Bisswanger, 2008).

1.2.3.2   Irreversible Inhibitors

Irreversible inhibitors bind covalently to the enzyme and inactivate the enzyme permanently by either binding on its active site and block access of the substrate or by changing the enzyme's conformation (Copeland, 2000; Bisswanger, 2008; Segel, 1976; Mäntsälä and Niemi, http://www.eolss.net).

1.2.3.2.1   Suicide Substrates

Some members of this category are toxins like diisopropyl phosphorofluoridate (DFP) (Fig. 5B) that binds tightly to serine in serine proteases and acetylcholinesterase. These inhibitors are used sometimes to preserve the enzymes they inhibit; for example, phenylmethanesulfonyl fluoride most often is used to inactivate them in order to prevent them from degrading (Fig. 5A) (Bisswanger, 2008).
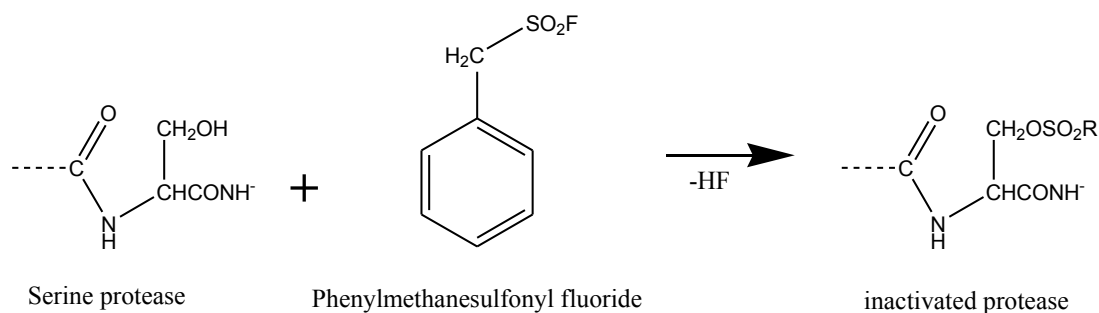
A: Reaction of a serine protease with phenylmethanesulfonyl fluoride, R=phenyl–CH2–,.
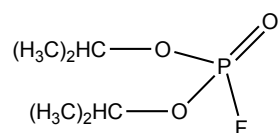
B: Diisopropyl phosphorofluoridate.

Figure 5:   Examples of suicide substrates (Bisswanger, 2008).

1.2.3.2.2 Transition State Analogues

These are compounds that mimic the substrate in its transition state and they are bound more tightly than the substrate in its free form. An example of a transition state analogue is 3,4-dihydrouridine (Fig. 6A), an analogue of cytidine deaminase in the reaction where cytidine is a substrate (Bisswanger, 2008). Transition state analogues are used to distinguish the substrate in the transition state from the substrate in the ground state (Wolfenden, 1969; Wolfenden, 1976; Bisswanger, 2008). Scholten *et al.*, (1996) successfully constructed peptide like transition state analogues by attaching the ras C-terminal tripeptide and the farnesyl group with phosphonic or phosphinic linker which are reported to be very potent against the FPTase (Fig. 6B).

A: 3,4-dihydrouridine
   (R is ribosylphosphate)

B: Peptide mimetic
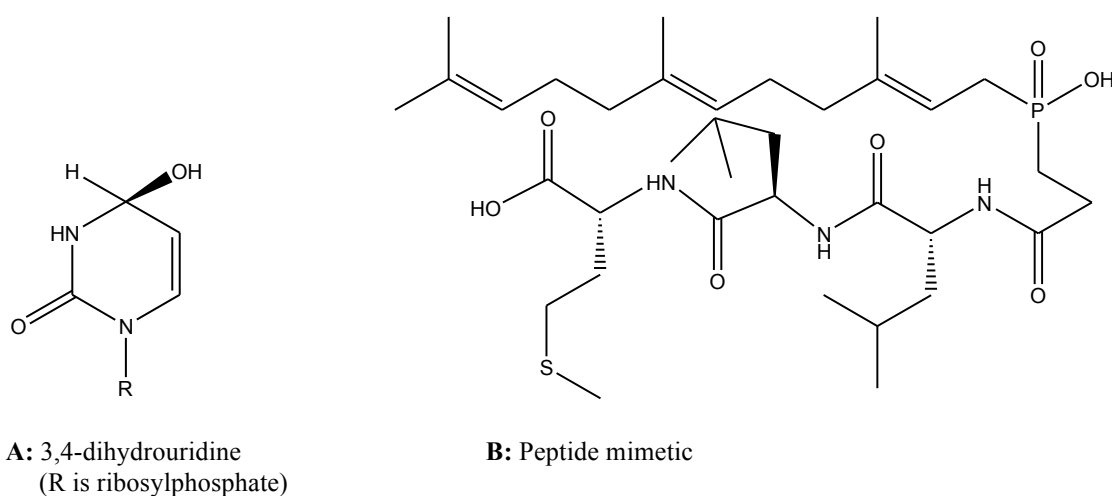
Figure 6:   Examples of transition state analogues (Scholten *et al.*, 1996; Bisswanger, 2008).

1.2.3.3   Inhibition of 4-Hydroxybenzoate Oligoprenyltransferase by Prenyldiphosphate Mimetics

A number of research groups have prepared prenyltransferase inhibitors by substituting the diphosphate group with less reactive or unreactive diphosphate mimics. However, structures of these analogues do not exactly resemble their substrates (Phan and Poulter, 2001). There

are numerous reports on inhibitory activity of prenyl diphosphate analogues, which have been investigated mainly for developing them for use as drugs, e.g. cancer drugs which inhibit protein farnesylation (Singh, 1993; Singh *et al*., 1993; Kang *et al*., 1995; Scholten *et al.,* 1996; Holstein *et al.,* 1998; Yang *et al*., 2006; Yang *et al*., 2010). Different classes of inhibitors that mimic the diphosphate moiety include phosphonophosphates, phosphonylphosphinates, bisphosphonates and phosphonophosphinates (Parker *et al*., 1978; McClard *et al*., 1987; Holstein *et al*., 1998; Phan and Poulter, 2001). However, inhibitory potential of these compounds against prenyltransferases is reported to range from moderate to poor although the differences in structures between them and the normal substrate do not contribute to their poor inhibitory activity (Phan and Poulter, 2001). Likewise, poor inhibitory activity of the diphosphate esters is not attributed by their structures rather their acidity since the bridging oxygen atoms in the diphosphate group are replaced by less electron-withdrawing carbons (Phan and Poulter, 2001). McClard *et al*., 1987, also described this phenomenon that difference in acidity between the substrate and the inhibitor affects binding of the inhibitor. Alternatively, inhibitors resembling their substrates were successfully synthesized whereby the diphosphate oxygen atom attached to the isoprenoid moiety was substituted with sulfur. A good example is (S)-geranyl thiodiphosphate that was found to be an excellent inhibitor of avian farnesyl diphosphate synthase (Phan and Poulter, 2001).

Another class of diphosphate analogues is phosphonylphosphinyl compounds that were found potent against avian liver farnesyl diphosphate synthetase. In the diphosphate moiety, the bridging oxygen between the two phosphorus atoms and the one connecting the diphosphate moiety to the alkyl moiety are both replaced by methylene groups (McClard *et al*., 1987; Stowell *et al*., 1989**)**.

However, not all diphosphate mimetics inhibit enzymes acting on diphosphate substrates. For example, phosphinyl phosphonate compounds are diphosphate mimetics that inhibit phosphate transport proteins. These inhibitors can be applied clinically to reduce serum phosphate levels to save patients susceptible to hyperphosphatemia, chronic renal failure and diseases that can be treated by inhibiting the phosphate uptake from the intestines (Jozefiak *et al.*, 2006).

The previous study by Zakharova *et al.*, 2004, also synthesized and evaluated the inhibitory potential of diphophate mimetics against UbiA. Criteria they used to synthesize the diphosphate mimetics were; affinity of the candidates to a prenyl diphosphate synthase, stability of the candidates under study conditions, particularly against hydrolysis and they should be easily synthesized. Based on these criteria they synthesized non-hydrolysable diphosphate analogues with phosphorus based acidic moieties (Fig. 7B, Parker *et al.*, 1978; Phan and Poulter, 2001; Zakharova *et al.*, 2004) and diphosphate analogues with non-phosphorus acidic moieties (Fig. 7A, Singh *et al*, 1993; Singh, 1993; Zakharova *et al.*, 2004). In addition to the two classes of inhibitors, they also attempted the intermediate compounds (compounds between class I and class II) with β-hydroxyphosphonic acid being an example (Zakharova *et al.*, 2004). Some examples of non-hydrolysable diphosphate mimetics include: phosphonophosphates (Parker *et al.*, 1978; Phan and Poulter, 2001; Zakharova *et al.*, 2004), phosphonophosphinates (Phan and Poulter, 2001; Zakharova *et al.*, 2004) and diphosphonates (Stremler and Poulter, 1987; Zakharova *et al.*, 2004), whereby the methylene group replaces the oxygen between phosphorus and carbon and the bridging oxygen between the two phosphorus atoms. Likewise, chaetomellic acid isolated from *Chaetomella acutiseta* is an example of diphosphate mimetics with non-phosphorus acidic moieties; it is a natural inhibitor of farnesyl-protein-transferase (Singh *et al*, 1993; Zakharova *et al.*, 2004). However,

most of the candidates tested in this study are diphosphate analogues with non-phosphorus acidic moieties.

Our interest in this study was to test the unexplored prenyl diphosphate mimetics that might inhibit UbiA. They were prepared by Vasilev (2015) at the IPB varying in chain length, functional groups and the way the prenyl chain is linked to the functional group.
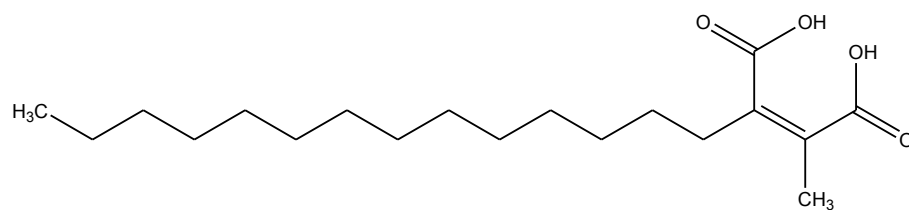
Chaetomellic acid A
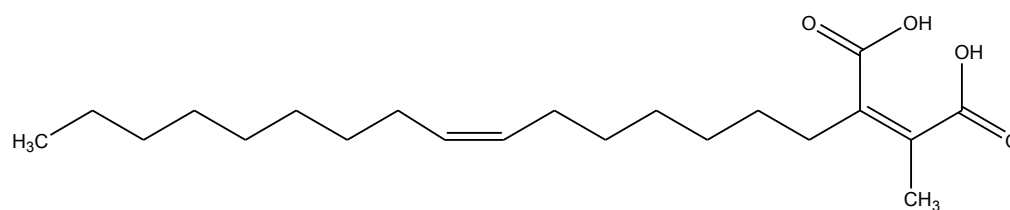
Chaetomellic acid B

A: Natural non-phosphorus FPP mimetics (Singh, 1993).
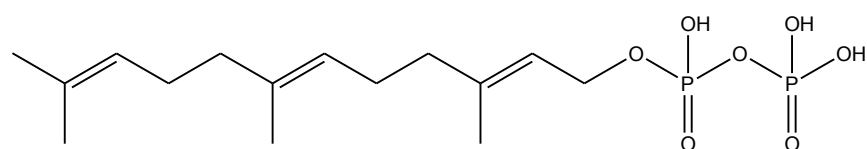
Farnesyl diphosphate (FPP), the native substrate.
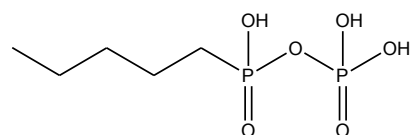
Pentyl phosphonophosphate
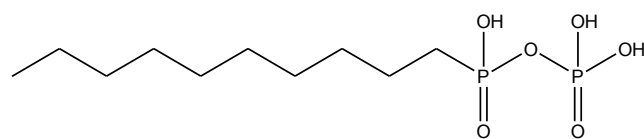
Decyl phosphonophosphate
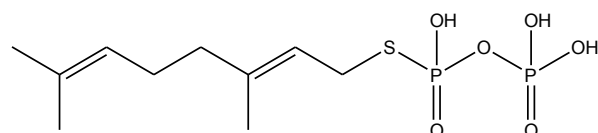
(S)-geranyl thiodiphosphate

B: Phosphorus FPP mimetics (Parker *et al*., 1978; Phan and Poulter, 2001).

Figure 7: Examples of diphosphate mimetics.

1.3   Objectives

1.3.1   General Objective

Screening of prenyldiphosphate mimetics for their potential to inhibit 4-hydroxybenzoate oligoprenyl transferase.

1.3.2   Specific Objective

To determine prenyldiphosphate mimetics that can inhibit 4-hydroxybenzoate oligoprenyl transferase and determine the inhibition curves and IC_{50} values.

1.4   Significance of the Study

In nature, enzyme inhibitors play part in the control of biological processes like homeostasis, which is regulated when there is balanced relative concentrations of proteases and their respective inhibitors (Segel, 1976; Copeland, 2000). Studies of enzyme inhibitors in basic research are crucial for determining mechanism of enzyme's catalytic activity (Segel, 1976). Enzyme inhibition studies provide insights to the structure of the active site without the use of x-ray crystallography or NMR spectroscopy. Apart from that, they are applied industrially for example as insecticides, weed killers, parasites and pests control agents in agricultural industry due to their specific action to the parasite's or pest's enzyme without exacting any effect on the host organism (Segel, 1976; Copeland, 2000). As stated in the introduction, among the approaches in drug discovery is to target enzymes involved in a particular disease development or enzymes involved in respiration or enzymes that confer antibiotic resistance (Penning *et al*., 1984; Daigle *et al*., 1997; Copeland, 2000; Tuquet *et al*., 2000). Thus, of all the industrial applications of inhibitors, relevant to this study is their use as drugs targeting enzymes involved in respiration/prenylating enzymes involved in crucial metabolic processes.

Animal cells synthesize only ubiquinone (Søballe and Poole, 1999), additionally the electron transport chain in human does not utilize menaquinone despite the fact that menaquinone-2 to menaquinone-13 have been found in animal tissues (including human) (Kurosu *et al*., 2007; Kurosu and Begari, 2010). This makes enzymes (especially MenA) involved in menaquinone biosynthesis potential targets for development of antibacterial drugs because the host cell could not be affected (Kurosu *et al*., 2007; Kurosu and Begari, 2010). However, there are pathogens, which depend entirely on ubiquinone for respiration (e.g. *Haemophilus vaginalis*, Collins and Jones, 1981). This obliges researchers to focus on enzymes involved in ubiquinone biosynthesis as potential targets for antibacterial drugs development.

There are a number of enzymes that are involved in the ubiquinone biosynthetic pathway namely; UbiA, UbiD, UbiE, UbiB, UbiH, UbiF and UbiG (fig. 8). However, mutants blocked in the oxygenases (i.e. ubiB, ubiH, and ubiF) have shown to synthesize ubiquinone under anaerobic conditions, suggesting that there are other hydroxylases, which can synthesize ubiquinone anaerobically (Meganathan, 2001). Mutants blocked in the non-hydroxylating reactions of the pathway such as ubiA, ubiD and ubiE are deficient in ubiquinone under both, aerobic and anaerobic conditions suggesting that these genes and the enzymes they encode are involved in both conditions (Meganathan, 2001). Therefore, in order to block ubiquinone biosynthesis at least one of these enzymes (fig. 8) must be blocked. UbiA is the enzyme that has been mostly studied (Heide, 2009), thus, this study focused on inhibiting it rather than UbiD or UbiE. Additionally, it has been reported that a ubiquinone intermediate accumulating in a ubiF mutant was able to support aerobic respiration with NADH, D-lactate and α-glycerophosphate. Therefore, inhibiting UbiA will ensure absence of any intermediate capable of participating in electron transfer processes (Wallace and Young 1977a; Wallace and Young, 1977b; Søballe and Poole, 1999).

Potential inhibitors of UbiA prenyltransferase can be developed as antibiotics against pathogens that depend entirely on ubiquinone for respiration. Inhibition of UbiA-prenyltransferase will interrupt respiration, hence, affecting bacterial growth making the inhibitors potential drug candidates. In addition to this, they can be developed as new drugs to overcome antibiotic resistance (e.g ethambutol resistance in *Mycobacterium tuberculosis*) that is influenced by UbiA expression (He *et al*., 2015). Furthermore, they can be applied in research as affinity-based probes to fish out similar prenyltransferases from other organisms.
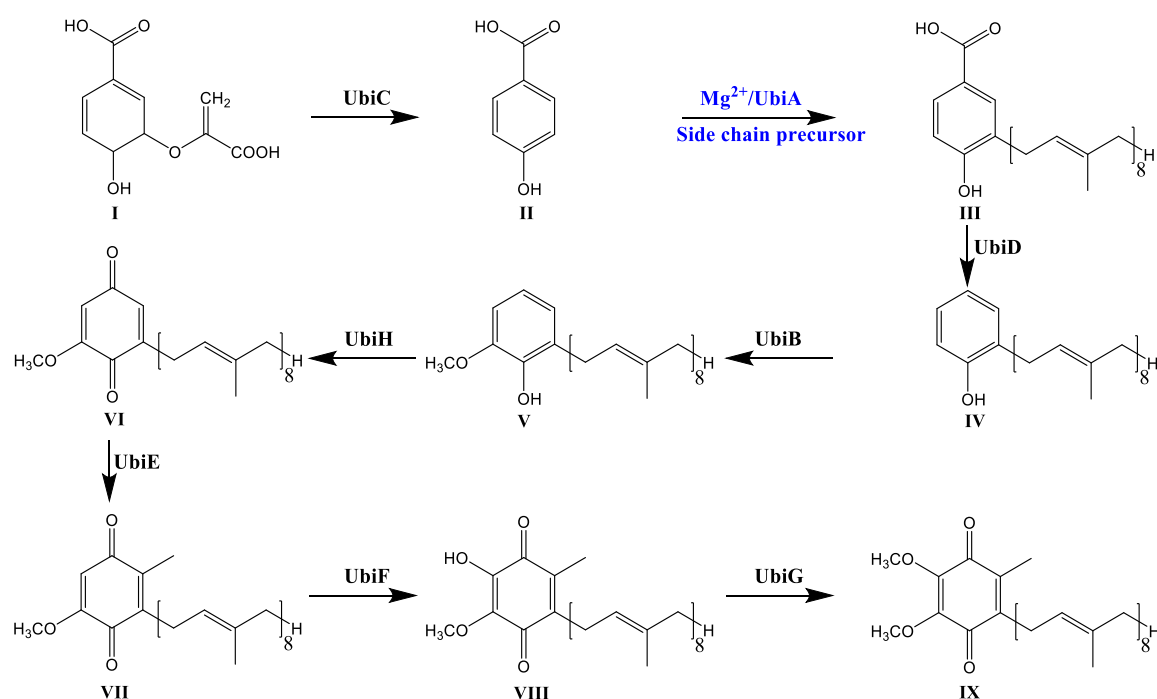
Figure 8:   The pathway for biosynthesis of ubiquinone in *E. coli* (Young *et al*., 1973).

I. chorismic acid.
II. 4-hydroxybenzoic acid.
III. 3-octaprenyl-4-hydroxybenzoic acid.
IV. 2-octaprenylphenol.
V. 2-octaprenyl-6-methoxyphenol.
VI. 2- octaprenyl-6-methoxy-1,4-benzoquinone.
VII. 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone.
VIII. 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone.
IX. ubiquinone.

2 MATERIAL AND METHODS

2.1 Strain, Plasmid and Transformation

2.1.1 Strain

Escherichia coli (*E. coli*) is the most efficient and widely used host for heterologous protein production because it is very well-established as a cell factory (Terpe, 2006; Rosano and Ceccarelli, 2014). The major factors contributing to its use as an expression host are well-known genetics, high transformation efficiency, cultivation simplicity, rapidity and inexpensiveness (Saïda *et al*., 2006; Rosano and Ceccarelli, 2014). *E. coli* OverExpress C41(DE3), is among the BL21 (DE3) derivatives that are effective in expressing toxic and membrane proteins (Miroux and Walker, 1996; Dumon-Seignovert *et al*., 2004; Terpe, 2006; Rosano and Ceccarelli, 2014). This strain is a lysogen of λDE3 and it carries a chromosomal copy of the T7 RNA polymerase gene. The T7 RNA polymerase is produced from the lysogenic λ prophage DE3 and it is expressed under the control of the IPTG-inducible lacUV5 promoter. *E. coli* OverExpress C41(DE3) is suitable for production of protein from target genes cloned into T7-driven expression vectors (Miroux and Walker, 1996; Studier, 2005; Dumon-Seignovert *et al*., 2004).

Like in the parental BL21(DE3) strain, C41(DE3) is deficient in the lon protease, which degrades abnormal/foreign proteins and ompT, the outer membrane protease whose function is to degrade extracellular proteins (Rosano and Ceccarelli, 2014; Terpe, 2006). This minimizes the risk of proteolysis in the expressed protein, hence, providing higher levels of expression (Rosano and Ceccarelli, 2014; Wang *et al*., 2011).

In this study *E. coli* strain C41(DE3) was used for expression of UbiA in order to ensure relatively high amount of the protein because it is a membrane-bound enzyme.

2.1.2 Plasmid

The wild type UbiA construct in plasmid pALMU3 (fig. 9) was kindly provided by Dr. Julia Kufka (IPB, Halle). The plasmid pALMU3 contains a 1.86kb SacI fragment from the pALMUl insert, ligated into the SacI site of pTZ19R (Siebert *et al*., 1994). It has a multiple cloning site (in frame with *lacZ* gene, fig. 9) making it a universal cloning and expression system. It also has an ampicillin resistance gene (amp, fig. 9), thus, ampicillin is used in expression of the enzyme to prevent growth of plasmid-free cells (Mead *et al*., 1986; Bräuer, 2006; Detsch 2008). Strains transformed with this plasmid for overexpression of UbiA membrane fraction, resulted in significantly high enzyme activity compared to enzyme activity in their untransformed counterparts (Melzer and Heide, 1994) suggesting that it is efficient in overexpression of the UbiA membrane protein. Thus, it was preferred for overexpression of the UbiA membrane protein in this study also.
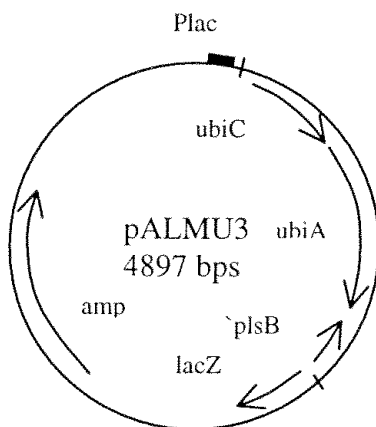
Figure 9:   A physical map of the pALMU3 cloning vectors.

2.1.3 Transformation

The plasmid pALMU3 containing the wild type UbiA prenyltransferase construct was introduced into *Escherichia coli* (*E. coli*) OverExpress C41 (DE3), (Lucigen®Corporation, Middleton, USA). Transformation of *E. coli* OverExpress C41 (DE3) strain was carried out as

follows: *E. coli* C41 OverExpress competent cells were removed from -80 °C and were thawed compeletely on wet ice for 15 minutes. A 2 µl of miniprep DNA sample was added to 50 µl cells on ice. The cells were stirred briefly with a pipet tip. The cells were incubated on ice for 30 minutes then, they were placed in a 42 °C water bath (immersion thermostat: A100, bath: 011 (Lauda-Königshofen)) for 45 s (heat shock). The cells were placed on ice again for 2 minutes. A 950 µl of room temperature Expression Recovery Medium (Lucigen) was added to the cells in the culture tube. The tubes were placed in a shaking incubator (Infors HT Ecotron Bottmingen, Schweiz) at 250 rpm for 1 h at 37 °C. A 100 µl of transformed cells were plated on LB (10 g/L NaCl (Carl Roth, Kalsruhe), 10 g/L Trypton (Carl Roth, Kalsruhe), 5 g/L Yeast extract (Carl Roth, Kalsruhe)) plate containing 100 µg/ml ampicillin. The plates were incubated overnight at 37 °C. The transformed colonies were further grown in LB medium for protein expression. A stock culture was also prepared and stored at -80 °C. pH of the medium was adjusted with NaOH (Roth) to 7.0 and it was autoclaved (Varioklav) at 121 °C for 20 min.

2.2 UbiA Protein Expression and Preparation

The UbiA wild-type construct cloned in the pALMU3 vector was expressed in *E. coli* C41 (DE3) cells. A pre-culture was prepared in four 100 ml volume conical flasks whereby each flask contained 50 ml LB medium (containing 100 µg/ml ampicillin; Carl Roth, Kalsruhe and 0.2% D (+) glucose; Carl Roth, Kalsruhe) at 37 °C overnight with shaking at 250 rpm. Expression culture was prepared in four 2 l volume conical flasks whereby each flask contained 1 l LB medium. A 50 ml pre-culture was added in 1 l fresh LB medium (containing 100 µg/ml ampicillin) and then, the cultures were incubated at 37 °C until optical density at 600 nm (OD_{600}, Eppendorf BioPhotometer Plus) reached 0.8-1. UbiA protein expression was induced by adding 1 ml 1 M IPTG (Carl Roth, Kalsruhe) in each 1 l expression culture. The

cultures were further incubated for 8 h at 37 °C with shaking at 250 rpm and the cells were harvested by centrifugation for 10 min at 4 °C and 10000 rpm (Beckman Coulter, JA-10). The supernatant was discarded and then, the pellet was resuspended in 20 ml of a 50 mM Tris/HCl pH 7.8, containing 10 mM DTT (freshly prepared; Carl Roth, Kalsruhe). The French press was rinsed with 20 ml NaOH (0.2 N at room temperature) at 1.80 kbar followed by 20 ml milli-Q water at 1.30 kbar followed by 20 ml extraction buffer (on ice) at 1.30 kbar. French pressing was performed on a Constant Systems Ltd (Low March Daventry, Northants, England) at 4 °C and a pressure of 2.0 kbar. The cells were disrupted twice; between the disruption cycles and during disruption the lysate was kept on ice (flake ice machine: Scotsman AF100). The lysate was centrifuged for 15 min at 4 °C and 15000 rpm (Beckman Coulter, JA-16.250) to remove cell debris. The membrane fraction was prepared by subjecting the supernatant to ultracentrifugation for 75 min at 4 °C and 40000 rpm (Beckman, Ti 70). The combined pellets containing the membrane fraction were resuspended in 40 ml of a 50 mM Tris/HCl pH 7.8, containing 2 mM DTT (freshly prepared) and aliquotes of 500 µl were stored at -20 °C.

Protein content determination was carried out following standard Bradford (1976) assay with Roti®-Quant reagent (CarlRoth, Karlsruhe) according to the manufacturer's instructions. The proteins in the enzyme solution were detected at 595 nm in a photometer based on color change of the Coomassie Brilliant Blue G-250 dye. The standard curve for estimation of protein content was prepared using bovine serum albumin (BSA) at a concentration range of 1 - 20 µg/ml and protein content in the sample was calculated using equation 1.

$$c \ (\mu g/ml) = (OD_{595}/m) \ x \ DF \qquad\qquad\qquad\qquad\qquad\qquad \textbf{(1)}$$

c - protein concentration
m - slope of the standard curve
DF - dilution factor

2.2.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To check the presence of the UbiA protein, an SDS-PAGE was performed according to Laemmli (1970). The separation of the proteins was carried out in 10% acrylamide at a constant voltage of 250 V for 2:30 h. After electrophoresis the gel was stained with Coomassie blue staining reagent (0.25% Coomassie Brilliant Blue G-250 (w / v), 40% H_2O, 50% MeOH, 10% acetic acid (v / v)) by immersing it in the reagent and heating in a microwave (30 s, 360 watt). It was then placed on a rotating platform until the bands became visible. The staining reagent was damped off and the gel was rinsed with water. Decolorization was carried out with decolorizing solution (10% acetic acid, 30% MeOH, 60% H_2O (v / v)) by heating in the microwave (30 s, 360 watt) followed by rinsing with water.

2.3 Bioassays

In all experiments in this study samples were assayed in replicates. This is due to the fact that it is inevitable to control random errors in scientific measurements that lead to some uncertainties (Malo *et al*., 2006). These uncertainties may affect measurements precision resulting in data values that are higher or lower relative to their true values (Malo *et al*., 2006; Serrano *et al*., 2015). Potential sources of random errors include biological, instrument and human-related influences and compound-related problems involving chemical properties and activity, for example, stability, solubility and degradation (Malo *et al*., 2006). These random errors may accumulate as a collection of several minimal differences across assays, for example, solution dispensing differences and reagent or sample preparation and handling (Malo *et al*., 2006). Therefore, assaying samples in replicates helps to increase precision and minimize extraneous variation due to sample handling and processing (Malo *et al*., 2006). It also allows a minimum of statistical analysis and gives confidence in validity of data (Malo *et al*., 2006; Serrano *et al*., 2015).

2.3.1 UbiA Assay

The prenyl diphosphate substrates (GPP and FPP) were synthesized at IPB. Pippeting was done using pippetes from Eppendorf Research (0.1-2.5 µl, 0.5-10 µl, 10-100 µl and 100-1000 µl). Reactions were performed in microtubes 1.5 ml SafeSeal (Sarstedt, Nümbrecht).

The reaction mixtures contained 10% (v/v) DMSO (Acros Organics New Jersey, U.S.A.), varying concentrations (mM) of GPP (0.2, 0.5, 0.8, 1, 1.5, 2, 2.5) and 4-HB (0.4, 0.6, 0.8, 1, 2, 3, 5; (Serva, Heidelberg)) at 5 mM 4-HB and 1 mM GPP, respectively, 12.5 µl UbiA membrane fraction (protein concentration of 1.5 mg/ml) in 50 mM Tris/HCl (pH 7.8) containing 2 mM DTT, 5 mM $MgCl_2$ (Fluka, Buchs, Switzerland) and was filled up with 50 mM Tris/HCl (pH 7.8) to a final volume of 100 µl. Unless stated otherwise GPP, 4-HB and $MgCl_2$ were dissolved in 50 mM Tris/HCl (pH 7.8). For these kinetic experiments, geranyl diphosphate was the last component to be added in the reaction mixtures. A reaction mixture without enzyme was also included as a negative control. Samples were assayed in duplicate. The reaction mixtures were vortexed (VWR MiniStar silverline) for 10 s prior to incubation. Incubation was carried out for a maximum of 7.50 min at 37 °C and 250 rpm in a thermomixer (BioShake iQ, Analytik Jena/Biometra) with sampling at regular time intervals. The reactions were stopped by addition of 2 µl formic acid (EMSURE® ACS, Reag. Ph Eur, Merck KgaA, Darmstadt) and vortexed for 10 s. Products were extracted with 500 µl ethyl acetate (distilled for purity), vortexed (VWR Mixer Mini Vortex 230 V EU, USA) for 1 min followed by centrifugation for 3 min at 14000 rpm on a table centrifuge (Hettich MIKRO 120, Tuttlingen). Ethyl acetate was then removed under nitrogen stream and the remainders were dissolved in 100 µl methanol (ULC/MS, Biosolve, Dieuze, France) containing 100 µM p-hydroxybiphenyl (Fluka, Buchs, Switzerland) as a HPLC internal standard. P-hydroxybiphenyl was used as a HPLC internal standard because its retention time is different from retention time of the substrate and products (i.e. there is clear separation of the signals).

The internal standard helps to cancel out any shift in signal in both substrate and products due to measurement anomalies; consequently, the percentage inhibition is not affected. The products were then analyzed by HPLC for the formation of GHB.

2.3.1.1   HPLC Analysis

HPLC analysis (1.0 mm x 5 cm, Ascentis Express 2.7 µm, C18, Supelco, USA) was performed on an Agilent 1100 instrument (Agilent, USA) with an integrated Agilent 1100 multi wavelength detector. Samples were eluted with methanol:water each containing 0.1% aqueous formic acid (gradient: 50% MeOH > 3 min > 80% > 2 min > 100% (1 min)). The HPLC was operated at a flow rate of 50 µl min^{-1}. 0.2 µl of reaction products were injected and detected at a wavelength of 260 nm.

2.3.1.2   Determination of K_m Values

Initial velocities (calculated as slopes, equation 2) for each of the reaction progress curve were determined at the linear portion (steady state phase) of the reaction progress curve and plotted against substrate concentration (Brooks *et al*., 2012; Sancenon *et al*., 2015).

$$v = \frac{\Delta P}{\Delta t}$$

(2)

v - initial velocity
ΔP – change in product formed
Δt - change in time

K_m values were calculated by fitting the data to a nonlinear regression analysis using a single-site rectangular hyperbola model, equation 3 (Kakkar *et al*., 1999; Scott and Williams, 2012;

Brooks *et al.*, 2012; Sancenon *et al.*, 2015) in SigmaPlot graphing software (version 12.0). Linear transformations of the data were avoided as in many literatures they are reported to bias the error (Strelow *et al.*, 2012; Ritchie and Prvan, 1996; Dowd and Riggs, 1965).

$$v = \frac{V_{max}[S]}{[S] + K_m} \qquad (3)$$

v - initial velocity

V_{max} - maximum velocity

[S] - substrate concentration

K_m - Michaelis-Menten constant

2.3.2   Optimization

2.3.2.1   4-HB Conversion

To establish suitable conditions for UbiA inhibition assay substrate (4-HB) conversion was optimized. Two experiments were performed to improve the assay conditions that will result in high substrate (4-HB) conversion. In one experiment the reaction mixtures were incubated at 37 °C for 2 h and in the other, at 28 °C for 6 h. Likewise, two different combinations of substrate concentrations (1 mM GPP and 1 mM 4-HB; 2.5 mM GPP and 2 mM 4-HB) were checked with the same purpose. In these experiments, 50 µl UbiA membrane fraction (protein concentration of 1.4 mg/ml) were used. Other procedures and analysis of reaction products remained the same as described in section 2.3.1. Samples were assayed in triplicate.

Data were presented as percent substrate conversion (% C). The percent conversion (% C) of substrate to product was calculated as the relative amount of the product (P) divided by the

sum of the relative amounts of the substrate (S) and product multiplied by 100%, equation 4 (Greis *et al.*, 2006; Thibodeaux *et al.*, 2013).

%C = [P/(S+P)] x 100% **(4)**

%C - percent conversion
S - substrate
P - product

A statistical test was conducted to determine if there is any difference in percent substrate conversion between the two substrate combinations and incubation temperatures. An unpaired t-test was used to determine the statistical significance because the data are independent (Normando *et al.*, 2010; Khusainova *et al.*, 2016).

Statistical analysis of the data was done on SigmaPlot software (version 12) using the *t* test (Fields, 2011) with a level of significance (α) of 0.05. The level of significance is the value set within the specified significant limits and together with the P-value (p) it helps to make a statistical decision. The P-value reflects the measure for deciding for or against a null hypothesis (du Prel *et al.*, 2009; du Prel *et al.*, 2010). It is a probability that is calculated by that particular statistical test. It suggests whether the null hypothesis should be retained or rejected. Small P-values give a great chance to retain the null hypothesis. However, to decide acceptance or rejection of the null hypothesis a level of significance (α), which in most cases is set to 0.05 should be considered. If the P-value is less than the level of significance (i.e. $P < 0.05$), then, the null hypothesis is rejected and the findings are considered statistically significant (du Prel *et al.*, 2009; du Prel *et al.*, 2010). The hypotheses below were formulated and statistical decision (conclusion about the results) was made based on them.

1. Difference in substrate conversion between the two-substrate combinations

- **Null:** There is no significant difference in substrate conversion between the two-substrate combinations (i.e. 1 mM GPP + 1 mM 4-HB and 2.5 mM GPP + 2 mM 4-HB).

- **Alternative:** There is a significant difference in substrate conversion between the two-substrate combinations (i.e. 1 mM GPP + 1 mM 4-HB and 2.5 mM GPP + 2 mM 4-HB).

2. Difference in substrate conversion between the two-incubation temperatures

- **Null:** There is no significant difference in substrate conversion between the two temperatures (i.e. 37°C and 28°C).

- **Alternative:** There is a significant difference in substrate conversion between the two temperatures (i.e. 37°C and 28°C).

2.3.2.2 Progress Curve Analysis

A further experiment for the purpose of checking relative and linear substrate conversion was carried out with two different concentrations of enzyme. In this experiment, 50 µl or 25 µl UbiA (membrane fraction; protein concentration of 1.4 mg/ml) were added in the reaction mixture (10% DMSO, 5 mM Tris/HCl buffer (pH 7.8), 2 mM HBA, 2.5 mM GPP and 5 mM $MgCl_2$). Other reaction conditions and analysis of reaction products remained the same as described in section 2.3.1; incubation was carried out at 37 °C for 2 h with sampling at regular time intervals and in another assay incubation was carried out at 37 °C for 10 min with sampling after every minute. Samples were assayed in duplicate.

2.3.3   UbiA Inhibition Assay

The masses of the test compounds (listed in table 5) were verified by ESI-MS prior to inhibition assay. The ESI mass spectra of the compounds were measured under an ion spray voltage of 5.5 kV and -4.5 kV at 400 °C on an LCMS API-150EX single quadrupole system (Applied Biosystems). Solvent system was methanol:water (6:4), flow rate of 250 µl min^{-1}.

In this experiment samples were assayed in triplicate. The reaction mixtures contained DMSO or organic soluble inhibitors in DMSO (final concentrations of inhibitors: blank, 0.01 mM, 0.1 mM or 1 mM), or water soluble inhibitor in Tris/HCl buffer (pH 7.8, final concentrations: 0.01 mM, 0.1 mM or 1 mM), 2 mM 4-HB and 25 µl of UbiA (membrane fraction; protein concentration of 1.4 mg/ml or 1.5 mg/ml) in 50 mM Tris/HCl pH 7.8 containing 2 mM DTT, 2.5 mM GPP and 5 mM MgCl$_2$ and was filled up with 50 mM Tris/HCl (pH 7.8) to a final volume of 100 µl. The inhibitor solution was vortexed for 10 s before adding in the reaction mixture. Unless otherwise stated, final DMSO concentration in the assay was 10%. The reactions were initiated by addition of geranyl diphosphate followed by MgCl$_2$. The reaction mixtures were vortexed for 10 s before incubation and then, incubated at 37 °C and 250 rpm for 10 min. The reactions were stopped by addition of 2 µl formic acid and vortexed for 10 s. Products were extracted with 500 µl ethyl acetate, vortexed for 1 min followed by centrifugation for 3 min at 14000 rpm on a table centrifuge. Ethyl acetate was then removed under nitrogen stream and the remainders were dissolved in 100 µl methanol containing 100 µM p-hydroxybiphenyl as a HPLC internal standard. The products were then analyzed by HPLC for the formation of GHB.

Positive and negative controls were also included in the assay and they were processed and analyzed in the same way as samples. A reaction mixture without inhibitor was considered a positive control. In this assay, there were two negative controls; (i) a reaction mixture with

inhibitor but without enzyme (ii) a reaction mixture without both, inhibitor and enzyme. The positive control determines if the assay is working and is used to normalize the substrate conversion across experiments while the negative control is used for comparison (Al-Ali *et al*., 2014; Haas *et al*., 2012). In this study, no product formation is expected in the negative controls; hence, they serve as reactions where there is 100% inhibition. In addition to this, they help to verify that there is no non-enzymatic product formation. Apart from this, the negative control without both, inhibitor and enzyme helps to verify that there is no residual enzyme in the reaction mixtures or any other contamination (e.g. presence of an enzyme acting on the same substrates leading to formation of an unexpected product).

The inhibition data were normalized to the positive control and presented as percent relative substrate conversion (% RC); that is the ratio of GHB formed in presence of the test compound (inhibitor) to the GHB formed in absence of the test compound multiplied by 100%, equation 5 (Greis *et al*., 2006).

%RC = (GHB formation with inhibitor/GHB formation with no inhibitor) x 100%   **(5)**

%RC - percent relative conversion

2.3.3.1   Selection of Active Compounds

Generally, in enzyme inhibitor screening assays a number of compounds are screened and those which show biologically relevant effect (e.g. >50% inhibition) are selected for further studies like determination of IC_{50} in order to assess their potency (Shun *et al*., 2011; Gufford *et al*., 2014). Selection of the active compounds is based on a cutoff point that is set based on the inhibitory effect of the test compounds (Brideau *et al*., 2003; Inglese *et al*., 2007; Blucher

and Mcweeney, 2014). For example, Inglese and colleagues (2007) considered inhibitors with greater than 30% inhibition as effective while in their study, Rudolf *et al*., (2014) considered inhibitors with the highest inhibition rate as effective and their IC_{50} values were determined.

In screening enzyme inhibitors there is no a pre-defined cutoff for selecting active compounds contrary to the basic statistical methods where the significant level (α) for rejection or acceptance of the null hypothesis is commonly 0.05 (Quinn and Keough, 2002). This is because a statistically significant effect may not be biologically significant as well (Quinn and Keough, 2002; Haas *et al*., 2012). A biologically significant effect varies with the questions under study. For example, small changes in the concentration of a toxin in body tissues may be enough to cause mortality while the same changes may not have significant impact on ecological processes under field conditions. However, there are modern statistical methods that are applied mostly in bioassays especially in high-throughput screening for data normalization and hit (active compounds) selection (Brideau *et al*., 2003; Siqueira-Neto *et al*., 2010). These methods are categorized into controls-based and non-controls-based methods (Brideau *et al*., 2003; Malo *et al*., 2006; Shun *et al*., 2011; Blucher and Mcweeney, 2014; Penã *et al*., 2015). Controls-based-normalization is based on percent inhibition/activation and it includes positive and negative controls in the bioassay (Brideau *et al*., 2003; Pereira *et al*., 2009). It is widely used because it is more natural for bioassays than some of the other statistical methods and it works well, for example, it adjusts both, additive and multiplicative differences between assays (Brideau *et al*., 2003; Blucher and Mcweeney, 2014). Furthermore, Blucher and Mcweeney, (2014) conducted a study on exploratory data analysis and results showed that percent inhibition was the most appropriate normalization method in comparison to z-score. If a positive control is analyzed with all compounds within the run, each compound will be equally affected (Haas *et al*., 2012). What is important is to monitor the controls for shifts in order to minimize variation between different runs (Brideau *et al*.,

2003; Haas *et al*., 2012). On the other hand, non-controls-based statistical methods include; median-based activities, Z scores, B scores and BZ score and they are commonly used in high throughput screening especially the Z-score (Brideau *et al*., 2003; Malo *et al*., 2006; Shun *et al*., 2011; Blucher and Mcweeney, 2014). These methods differ from the control-based-methods in some aspects; for example, the median calculation adjusts multiplicative variations only while the statistical scoring methods are based on the observation that potent compounds are outliers compared to the majority of non-potent compounds.

Conventionally, an arbitrary percent activity/inhibition is chosen as a selection criterion for active compounds based on the observed effect of a compound (potency) (Brideau *et al*., 2003; Malo *et al*., 2006; Siqueira-Neto *et al*., 2010; Penã *et al*., 2015). Apart from this, a combination of *in vitro* and *ex vivo* (cell-based assays) or *in vivo* (whole organism) study helps in selecting a cutoff point because in *ex vivo*/*in vivo* study the test compounds act directly on the target cell/tissue/organism (Inglese *et al*., 2007; Leuchowius *et al*., 2010; Hughes *et al*., 2011). For example, if the inhibitors are intended to affect the growth of a particular organism (e.g. a pathogen) by blocking its respiratory pathway, then, the lowest concentration that effectively reduces cell viability can be considered as a cutoff (selection criteria). Alternatively, a reference compound (inhibitor) whose desired inhibitory effect at a specified concentration is known can be used to select active compounds. This is done by comparing the effect of the test compound to the effect of the reference compound; those which show effect similar to or greater than the reference are considered active compounds (Siqueira-Neto *et al*., 2010; Romanha *et al*., 2010; Sancenon *et al*., 2015; Katsuno *et al*., 2015).

2.3.3.2 Determination of IC$_{50}$ Values

For the compounds that showed inhibition (relevant inhibitory effect), the range of concentration was broadened to determine IC$_{50}$ values. IC$_{50}$ value is the concentration at which the compound inhibits 50% of the enzyme activity (Krohn and Link, 2003; Haas *et al.*, 2013). It is the statistical parameter that estimates potency of a test compound and it is generally derived from concentration-response assays (curves) designed to measure activation, inhibition or modulation of targets and pathways of pharmacological significance (Haas *et al.*, 2013). In this study the IC$_{50}$ values were derived from concentration-response curves for estimating potency of the test compounds as inhibitors. The maximum tested inhibitor concentrations were 10 mM. Samples were assayed in triplicate. IC$_{50}$ values were calculated by using the SigmaPlot graphing software (version 12.0), standard curve analysis was done using the four-parameter logistic equation (equation 6) and a curve fit tolerance of 1 x 10^{-10} (Bapat *et al.*, 2010; Fields, 2011; Beck *et al.*, 2012):

$$y = Min + \frac{Max - Min}{1 + (x/EC_{50})^{Hill\ slope}}$$

(6)

Definition of the four parameters in the formula (http://www.sigmaplot.com; Devanarayan *et al.*, 2012).

x - concentration of inhibitor
y - relative conversion in percent
Min - bottom of the curve
Max - top of the curve
EC$_{50}$ - the x value for the curve point that is midway between the max and min parameters.
- In this case EC$_{50}$ in the formula represents IC$_{50}$.
Hill slope - characterizes the slope of the curve at its midpoint.

The four-parameter logistic nonlinear regression model (4PL) is the best and most common equation fit to *in vitro* concentration-response data (Brooks *et al*., 2012; Auld *et al*., 2012; Haas *et al*., 2012). It is used when the raw data is not a linear function of the biological response (Campbell *et al*., 2012). Important things to consider for a concentration response curve are; suitability of the concentration-response model for the biological effects under study and appropriate concentration range for the model. In order to fit well the four-parameter logistic model, a wide concentration-range enough for well-defined tops and bottoms is required (Bapat *et al*., 2010; Auld *et al*., 2012; Campbell *et al*., 2012; Haas *et al*., 2012).

2.3.3.3   Hypotheses Formulation and Testing

If a reference compound were available, hypotheses could be formulated and based on an appropriate statistical test decision about the potency (effect level) of the test compounds in comparison to the reference compound could be made. A null hypothesis would state, "There is no significant difference in IC_{50} values between a test compound and control" and an alternative hypothesis would state, "There is a significant difference in IC_{50} values between a test compound and the control. However, there was no reference compound in this study that would lead to hypothesis testing. An unpared t-test would be appropriate for hypothesis testing in this case.

2.3.4   Effect of Concentration of Magnesium Chloride (Mgcl₂) on UbiA Activity

The reaction mixtures (100 µl) contained: 45 mM Tris/HCl (pH 7.8), 10% DMSO, 2 mM 4-HB, 25 µl UbiA (membrane fraction; protein concentration of 1.5 mg/ml), 2.5 mM GPP, and various concentrations (mM) of $MgCl_2$ (0.00, 0.05, 0.50, 1.00, 2.50, 5, 10, 20, 50, 80). The

reaction mixtures were incubated at 37 °C and 250 rpm for 10 min. Another experiment was performed whereby the reaction mixtures (100 µl) contained: 45 mM Tris/HCl (pH 7.8), 10% DMSO, 2 mM 4-HB, 25 µl UbiA (membrane fraction; protein concentration of 1.5 mg/ml), 2.5 mM GPP, 5 mM $MgCl_2$ and 1 mM or 5 mM or 10 mM EDTA (Carl Roth, Kalsruhe). The reaction mixtures were incubated at 37 °C and 250 rpm for 10 min. Other procedures and analysis of reaction products remained the same as described in section 2.3.1.

2.3.5   Effect of inhibitors on UbiA Activity Without GPP

In this experiment the reaction mixtures (100 µl) contained: 45 mM Tris/HCl (pH 7.8), 10% DMSO, 2 mM 4-HB, 25 µl UbiA (membrane fraction; protein concentration of 1.5 mg/ml), 1 mM inhibitor and 5 mM $MgCl_2$. In addition to this, in some samples GPP and inhibitor were excluded from the reaction mixture to check if there might be residual GPP in the enzyme preparation that can lead to product formation. The reaction mixtures were incubated at 37 °C and 250 rpm for 10 min. Other procedures and analysis of reaction products remained the same as described in section 2.3.1.

2.3.6   Effect of Farnesyl Diphosphate (FPP) on Formation of GHB

In this competition experiment the reaction mixtures (100 µl) contained: 45 mM Tris/HCl (pH 7.8), 10% DMSO, 2 mM 4-HB, 25 µl UbiA (membrane fraction), 0.01, 0.1, 1 or 10 mM FPP, 2.5 mM GPP and 5 mM $MgCl_2$. In addition to this, FPP alone was applied as an isoprenyl substrate. Reaction mixtures for these samples contained 10 mM FPP plus the aforementioned reaction components. The reaction mixtures were incubated at 37 °C and 250 rpm for 10 min. Other procedures remained the same as described in section 2.3.1. Products were analysed by HPLC for the formation of GHB and FHB. Formation of the farnesylated product was confirmed by liquid chromatography-mass spectrometry (LC-MS).

2.3.6.1 LC-MS Analysis of FHB

The negative ion ESI mass spectra and the collision-induced dissociation (CID) mass spectra were obtained from a TSQ Quantum Ultra AM system equipped with a hot ESI source (HESI, electrospray voltage 3.0 kV, sheath and auxillary gas: nitrogen; vaporizer temperature: 50 ^{o}C; capillary temperature: 270 ^{o}C; The MS system is coupled with a UHPLC system Accela 1250 (Thermofisher Scientific), equipped with a Syncronis™ C18 column (50 x 2.1 mm, 1.7 μm, Thermo Scientific). For the UHPLC a gradient system was used starting from water:acetonitrile 60:40 (each of them containing 0.2% formic acid) to 0:100 within 30 min and then hold on 5% for further 30 min; flow rate 150 μl min^{-1}. The CID mass spectra and the selected reaction monitoring (SRM) measurements of the samples were recorded during the UHPLC run with collision energy of 25 eV (collision gas: argon; collision pressure: 1.5 mTorr).

3 RESULTS AND DISCUSSION

3.1 UbiA Protein Expression

The heterologous gene expression of the UbiA prenyltransferase gene in *E. coli* expression strain BL41 (DE3) using the pALMU3 cloning vector was successful as the band (in a blue box) with expected molecular weight ($\approx$ 32.5 kDa) was observed on SDS-PAGE (Fig. 10).
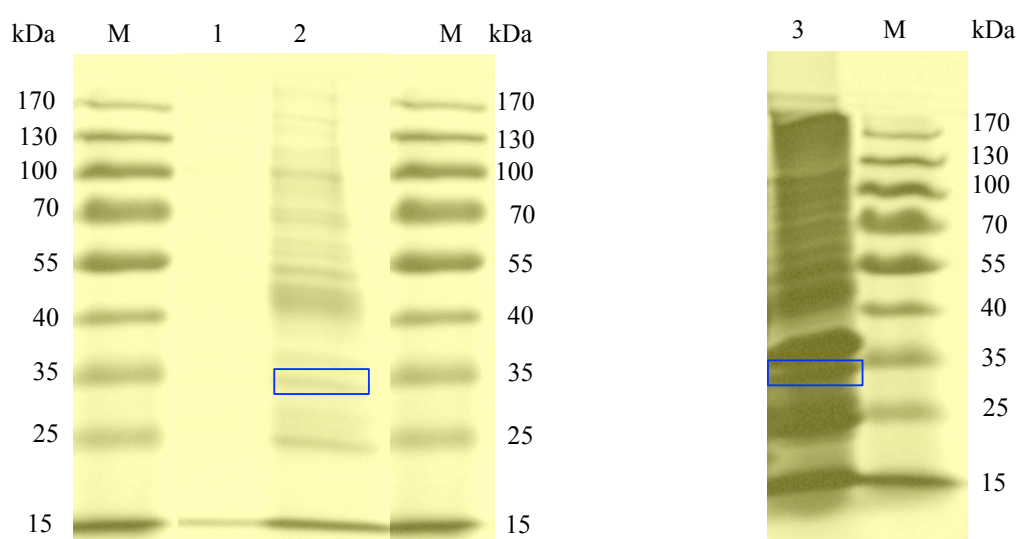

Figure 10: **SDS-PAGE gel analysis of UbiA enzyme preparation**.

M: Molecular marker **1:** Before IPTG induction **2:** After IPTG induction **3:** Membrane fraction

3.2 Enzyme Kinetics Data

Kinetic studies showed that K_m values for GPP and 4-HB were 548 $\pm$ 111 μM and 362 $\pm$ 97 μM respectively (Table 1). In literature, it is stated that K_m values for many enzymes generally range from 10^{-6} to 10^{-2} M, (Tan, 2011; Segel, 1993) thus, K_m values obtained in this study, are within this range. Enzymes with larger K_m values are thought to dissociate aversively from the substrate (Mäntsälä and Niemi, http://www.eolss.net).

Table 1:   Kinetic data.

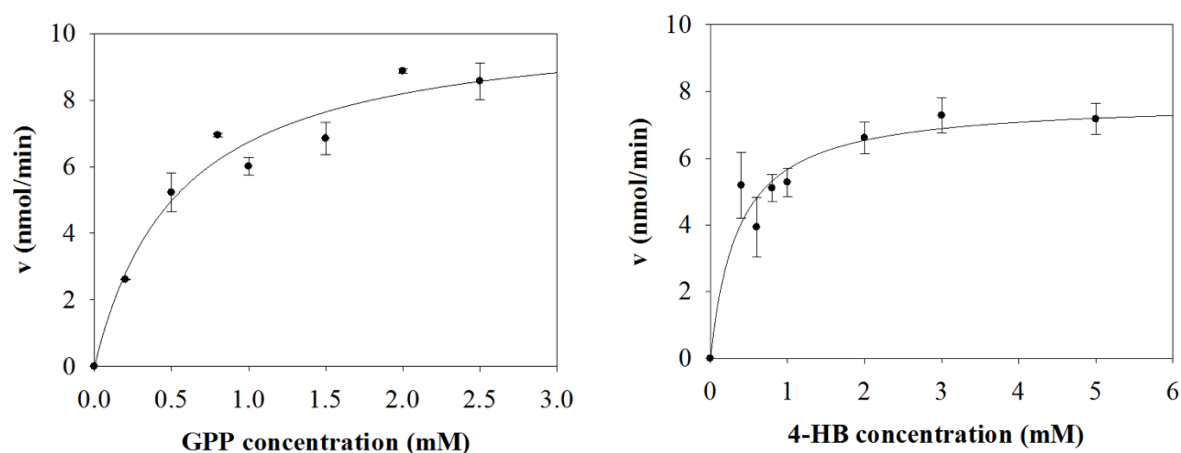| Parameter	K_m (µM)
GPP	548 ± 111
4-HB	362 ± 97

K_m value ± S.E.

Figure 11:   Plot of the initial velocities (v), measured as the slopes of the linear portions of reaction progress curves, as a function of substrate concentrations. Average of duplicate data ± SD.

Results from this study show a similar trend like those observed by Melzer and Heide (1994). Melzer and Heide (1994) showed that K_m for GPP and 4-HB were 255 ± 52 µM and 188 ± 40 µM respectively suggesting that the enzyme has high affinity to 4-HB (like observed in this study). However, the values obtained in this study cannot be directly compared to those obtained by Melzer and Heide (1994) because the enzyme was prepared differently and the experimental conditions are not the same despite the fact that they also kept GPP and 4-HB constant at 1 mM and 5 mM respectively, e.g. the addition of DMSO by us. The values are acceptable though because the deviation is less than ten times of the values reported in literature (i.e. reported by Melzer and Heide, 1994) (Brooks *et al*., 2012). In addition to this, the plots follow a single-site rectangular hyperbolic curve (Fig. 11), this shows that, the data fit the Michaelis-Menten equation.

Since the K_m values obtained in this study differed from those reported in literature experiments were conducted to find out which combination of substrate concentration will result in higher substrate conversion. The chosen combinations of concentrations were 1 mM GPP and 1 mM 4-HB that was used by Dessoy (2003) and Zakharova *et al*., 2004 and 2.5 mM GPP and 2 mM 4-HB, the concentrations that are about ten times K_m values reported by Melzer and Heide (1994).

3.3   Optimized Reaction Conditions

3.3.1   4-HB Conversion

Between the two tested combinations of substrate concentration, 2.5 mM GPP + 2 mM 4-HB resulted in higher 4-HB conversion than 1 mM GPP and 1 mM 4-HB at both incubation temperatures (Table 2). Based on statistical test results show that there was a statistically significant difference in 4-HB conversion between the two combinations of substrate concentration ($p < 0.05$; Table 3). The higher 4-HB conversion in the reactions with 2.5 mM GPP and 2 mM 4-HB compared to 1 mM GPP and 1 mM 4-HB (Fig. 12) suggests that 2.5 mM GPP and 2 mM 4-HB is the suitable substrate combination for subsequent assays. However, there was with 2.5 mM GPP + 2 mM 4-HB no statistically significant difference in 4-HB conversion between 37 °C and 28 °C ($p > 0.05$; Table 4) and therefore, 37 °C was preferred as the incubation temperature because it was already in the standard protocol. Also, 37 °C was advantageous because incubation time is 2 h compared to 6 h at 28 °C.

Table 2:   4-HB conversion.

Substrate combination	Conversion in %	
	37 °C	28 °C
1 mM GPP + 1 mM 4-HB	44.21±1.34	36.40±1.79
2.5 mM GPP + 2 mM 4-HB	67.48±3.08	63.58±4.13

Average of triplicate data ± SD.

Table 3: Comparative summary of combination of substrate concentrations.

Temperature (°C)	1 mM GPP + 1 mM 4-HB/ 2.5 mM GPP + 2 mM 4-HB (P-value)	Significance
37	0.001	Yes
28	0.001	Yes

Table 4: Comparative summary of temperature.

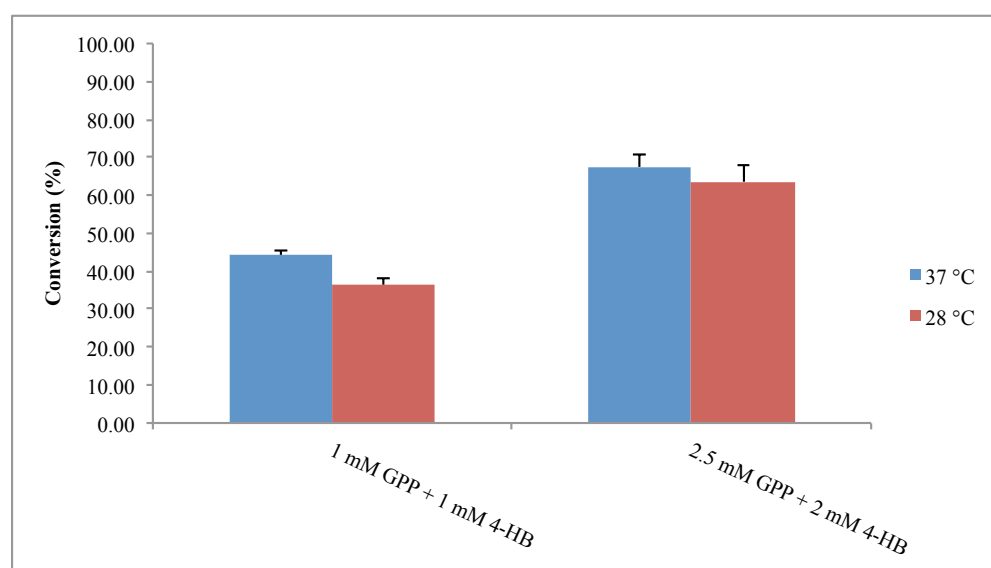| Substrate combination	37°C /28°C (P-value)	Significance
1 **mM** GPP + 1 **mM** 4-HB	0.004	Yes
2.5 **mM** GPP + 2 **mM** 4-HB	0.260	No

Figure 12: 4-HB conversion by UbiA at different incubation temperatures.

3.3.2 Progress Curves

Following progress curve analysis, it was observed that the reaction mixtures incubated for 2 h resulted in a completely saturated progress curve for both, 50 and 25 µl UbiA. The reaction mixtures incubated for 10 min resulted in a progress curve that is not completely saturated. Furthermore, the progress curve for 25 µl UbiA was more linear than the one for 50 µl UbiA

(insert in Fig. 13). Evaluation of inhibitors (especially substrate analogues as most likely they are competitive inhibitors) is recommended to be performed at the linear portion of the progress curve (Brooks *et al*, 2012; Strelow *et al*., 2012) because this is where the impact of the inhibitor is well determined, therefore, it was decided to use 25 µl UbiA membrane fraction and incubate the reaction mixtures for 10 min.
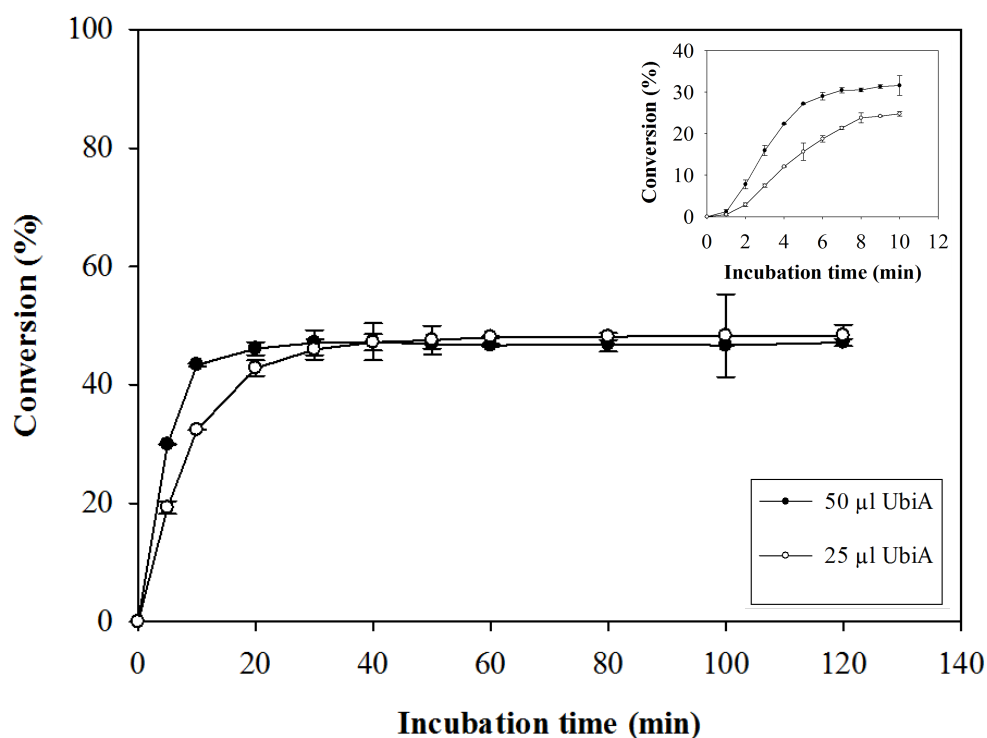

Figure 13:   Saturation curves at different enzyme concentrations. Average of duplicate data ± SD.

In summary, the reaction conditions for subsequent assays were chosen to be: 10 % DMSO, 2.5 mM GPP, 2 mM 4-HB, 5 mM $MgCl_2$ and 25 µl UbiA membrane fraction (for activity normalization see details below); filling up with 50 mM Tris/HCl (pH 7.8) to a final volume of 100 µl, incubation at 37 °C for 10 min with shaking at 250 rpm. Since UbiA is a membrane bound enzyme, control from one preparation to another was done based on substrate conversion (%). Therefore, whenever a new preparation of UbiA was used, controls were

done by testing GPP conversion and a compound that has already being tested to see if degree or trend of inhibition will be the same (i.e. reproducibility).

3.4   UbiA Inhibition

Below, the structures of the compounds that were tested in this study are given (Table 5). As stated in section 2.3.3, data were normalized to the positive control and presented as percent relative substrate conversion (% RC). Some compounds showed various degree of inhibition, some did not inhibit while others showed slightly enhanced activity. Of all compounds tested, only eight, showed inhibition at 1 mM (summary see Fig. 23). Among the compounds that showed slightly enhanced activity, range of concentration for compound **2** and **31** as representatives, was broadened to see if they could inhibit at concentrations higher than 1 mM. However, there was no inhibition even when tested at 10 mM. As stated in the methodology (section 2.3.3.1), in this study no *in vivo* assay was conducted following *in vitro* screening neither a reference compound was available. Thus, an arbitrary percent activity of 75% substrate conversion (i.e. 25% inhibition) at 1 mM was chosen as a cutoff (Fig. 22) for selecting active compounds. Table 6 shows the data for all tested compounds; only figures for the compounds that showed inhibition (Fig. 14-21) are presented. Activities of all compounds at 1 mM are presented in figure 22 whereby the yellowish green bars represent active compounds (i.e. inhibitors).

As an example, the product peak height in fig. 36 is relatively lower than in fig. 35. The reduced product peak height in fig. 36 is due to the inhibitory effect of compound **28**. Based on the negative controls, results show that there is neither residual enzyme in the reaction mixtures nor non-enzymatic product formation. This is because no product was formed in both negative controls. Fig. 39 is the chromatogram of the negative control without both, enzyme and inhibitor; it can be seen that there is no peak for product.

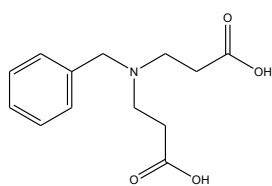**Table 5:   Structures of all potential GPP mimetics tested.**

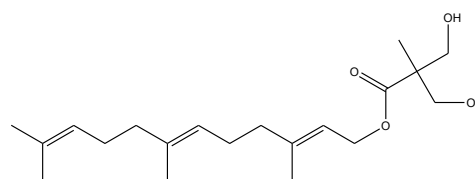Compounds were synthesized by Vasilev (2015).
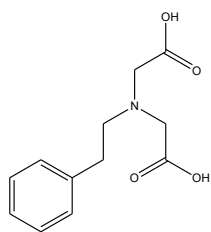
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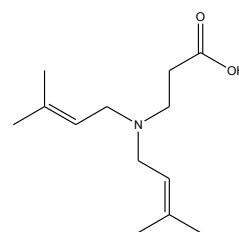
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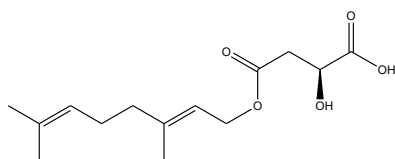
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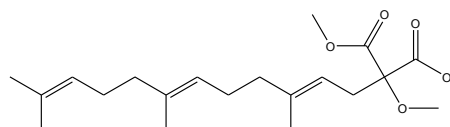
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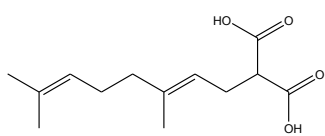
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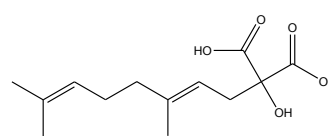
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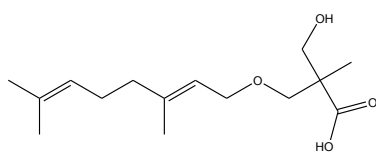
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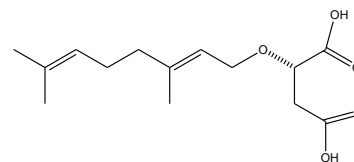
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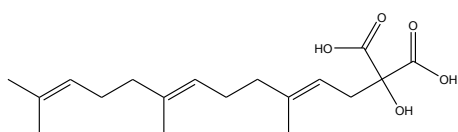
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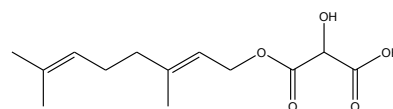
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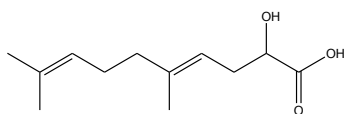
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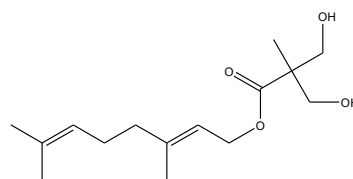
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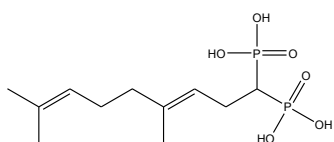
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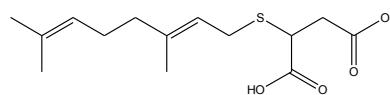
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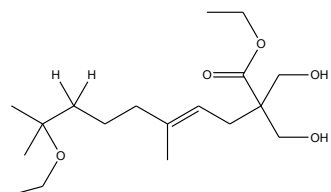
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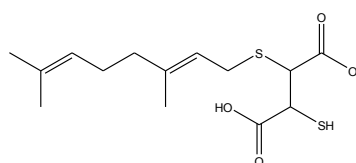
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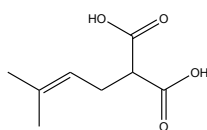
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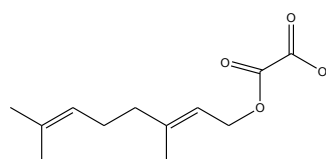
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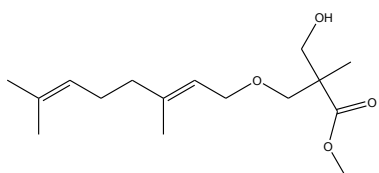
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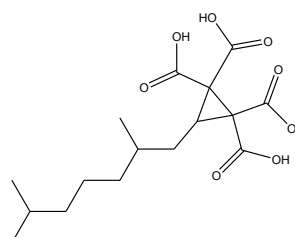
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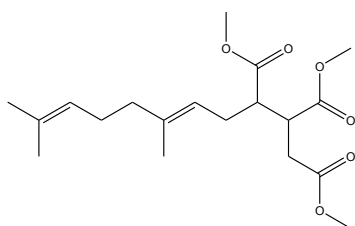
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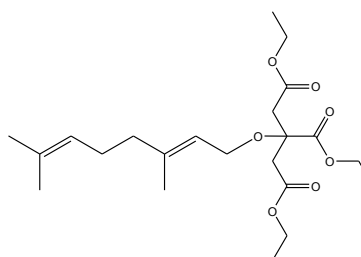
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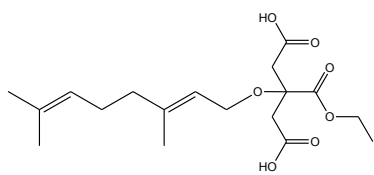
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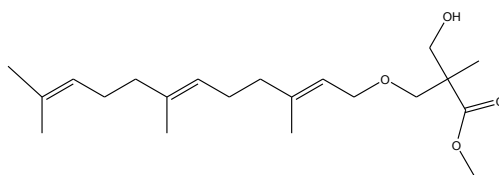
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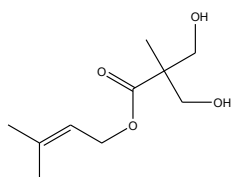
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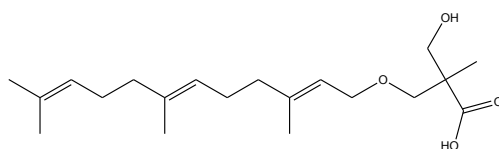
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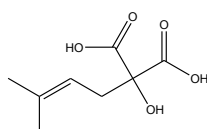
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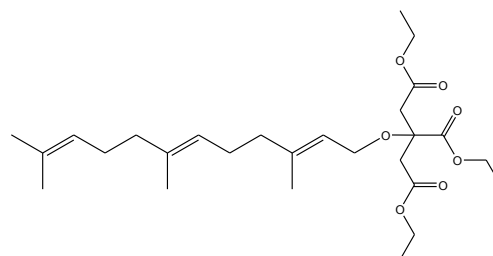
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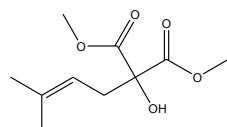
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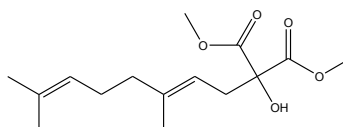
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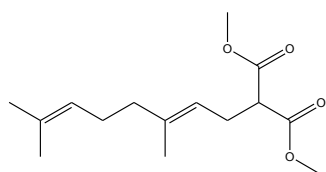
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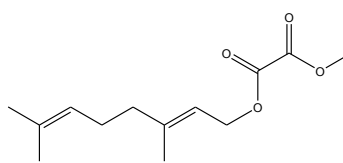
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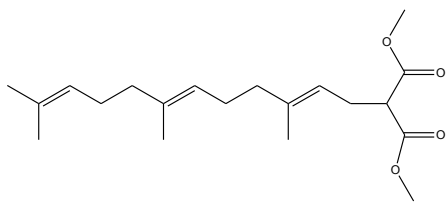
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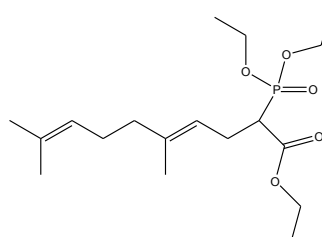
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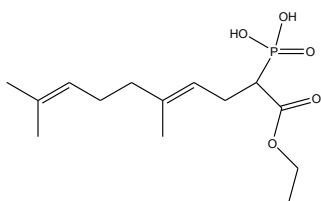
37

Table 6:   Substrate conversion by UbiA in presence of different compounds.

Inhibitors of relevance (< 75% conversion at 1 mM, except FPP an alternative substrate) are highlighted with a grey background. Grey values are outliers, probably measurement errors.

Compounds	Relative conversion (%)			
	0.00 mM	**0.01 mM**	**0.10 mM**	**1.00 mM**
1	100.00 ± 3.87	99.02 ± 0.80	102.27 ± 2.46	101.63 ± 3.77
2	100.00 ± 3.87	98.97 ± 5.06	93.05± 3.48	113.11 ± 3.09
3	100.00 ± 2.01	93.41 ± 1.13	93.54 ± 4.58	94.66 ± 1.22
4	100.00 ± 2.01	97.52 ±1.26	94.45 ±5.31	31.73 ± 4.78
5	100.00 ± 2.01	94.82 ± 2.19	89.35 ± 5.02	84.77 ± 3.50
6	100.00 ± 3.48	94.91 ± 1.71	88.53 ± 1.48	58.57 ± 1.55
7	100.00 ± 3.48	103.15 ± 3.12	106.50 ± 3.53	103.21 ± 5.52
8	100.00 ± 3.48	97.23 ± 0.54	94.85 ± 2.40	88.76 ± 2.09
9	100.00 ± 4.42	89.79 ± 3.21	98.74 ± 1.96	100.68 ± 1.15
10	100.00 ± 4.42	99.15 ± 3.75	96.72 ± 3.85	89.53 ± 3.30
11	100.00 ± 4.42	93.09 ± 1.20	92.43 ± 5.98	42.00± 1.68
12	100.00 ± 4.42	97.22 ± 5.23	96.90 ± 1.83	74.39 ± 1.55
13	100.00 ± 4.42	93.50 ± 3.58	94.26 ± 3.05	90.84 ± 6.71
14	100.00 ± 0.84	99.80 ± 3.13	100.71 ± 3.48	94.25 ± 1.03
15	100.00 ± 0.84	98.60 ± 0.99	99.83 ± 1.72	93.02 ± 3.29
16	100.00 ± 0.84	93.72 ± 2.00	93.45 ± 3.02	93.73 ± 2.43
17	100.00 ± 5.42	105.36 ± 2.42	102.27 ± 3.11	100.75 ± 3.13
18	100.00 ± 0.84	99.96 ± 2.73	95.44 ± 0.91	85.95 ± 2.43
19	100.00 ± 3.77	96.30 ± 2.88	105.24 ± 2.44	105.04 ± 2.96
20	100.00 ± 5.42	102.54 ± 0.96	101.98 ± 1.27	91.70 ± 5.64
21	100.00 ± 3.83	97.19 ± 4.60	98.31 ± 3.81	87.78 ± 1.11
22	100.00 ± 5.42	100.75 ± 3.19	101.64 ± 2.36	84.91 ± 3.91
23	100.00 ± 5.42	104.01 ± 0.41	100.35 ± 4.70	93.89 ± 1.49
24	100.00 ± 3.83	99.35 ± 3.79	91.31 ± 3.34	80.63 ± 5.06
25	100.00 ± 2.25	93.66 ± 2.77	98.16 ± 2.83	101.67 ± 3.87
26	100.00 ± 9.43	108.21 ± 7.02	105.15 ± 6.68	75.61 ± 3.65
27	100.00 ± 3.77	104.16 ± 1.75	107.49 ± 1.67	110.73 ± 2.42
28	100.00 ± 9.43	101.03 ± 6.97	96.07 ± 1.43	27.10 ± 8.15
29	100.00 ± 3.77	107.15 ± 5.41	103.88 ± 4.51	108.74 ± 2.49
30	100.00 ± 9.43	103.11 ± 2.79	104.08 ± 9.14	106.08 ± 7.00
31	100.00 ± 2.25	99.28 ± 9.65	102.54 ± 4.37	110.32± 0.85
32	100.00 ± 2.66	102.82 ± 4.70	107.33 ± 2.24	105.27 ± 3.81
33	100.00 ± 2.66	108.41 ± 1.68	109.03 ± 2.24	99.52 ± 2.45
34	100.00 ± 2.66	99.70 ± 4.60	118.28 ± 3.75	134.09 ± 1.86
35	100.00 ± 2.25	92.79 ± 4.18	82.85 ± 3.34	68.50 ± 2.90
36	100.00 ± 2.04	100.44 ± 1.38	101.85 ± 2.20	44.71 ± 6.18
37	100.00 ± 2.04	103.93 ± 1.46	108.63 ± 2.20	98.82 ± 2.88
FPP	100.00 ± 2.68	95.24 ± 9.55	94.37 ± 1.80	48.79 ± 2.62

Note: Compound **37** is the intermediate derived from compound **36** (during its hydrolysis).
Average of triplicate data ± SD. FPP is a competitive substrate and not an inhibitor.
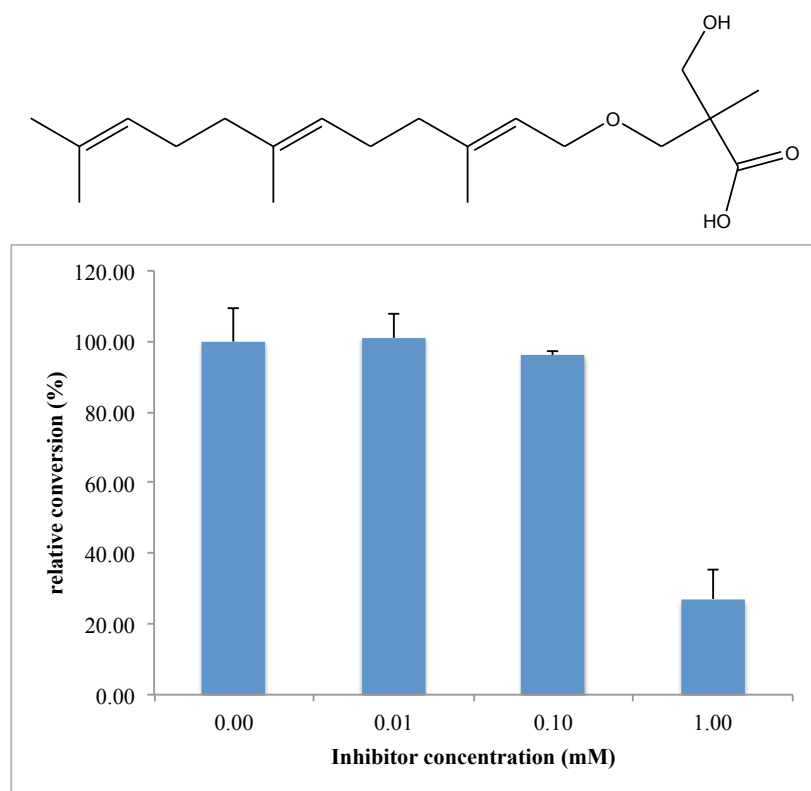
Figure 14: Effect of compound **28** on UbiA.
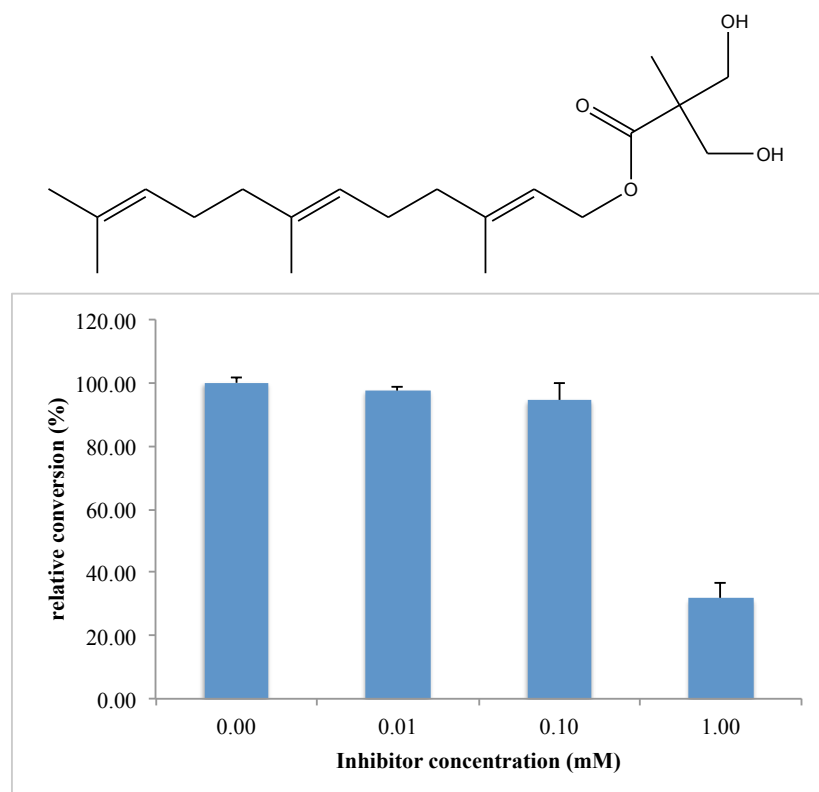
Figure 15: Effect of compound **4** on UbiA.
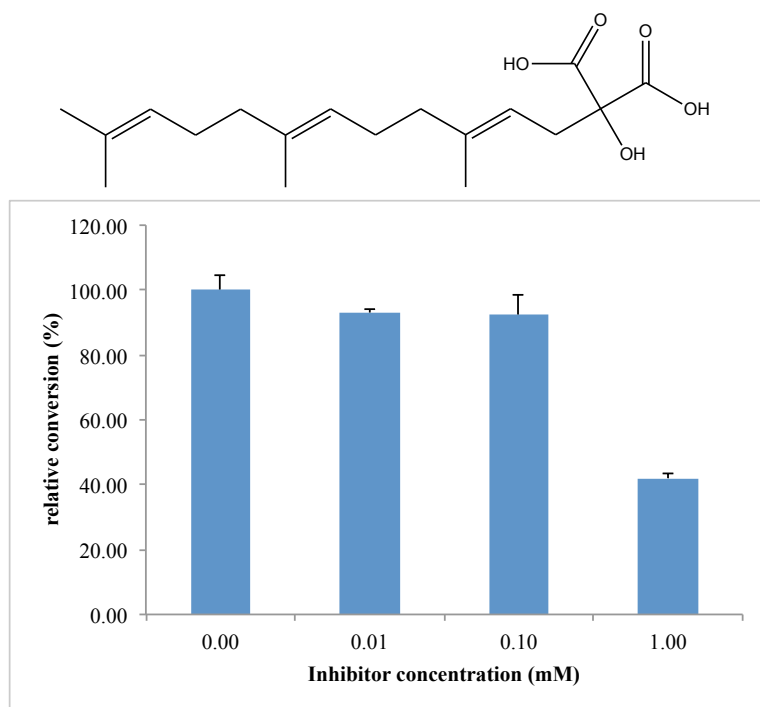
Figure 16: Effect of compound **11** on UbiA.
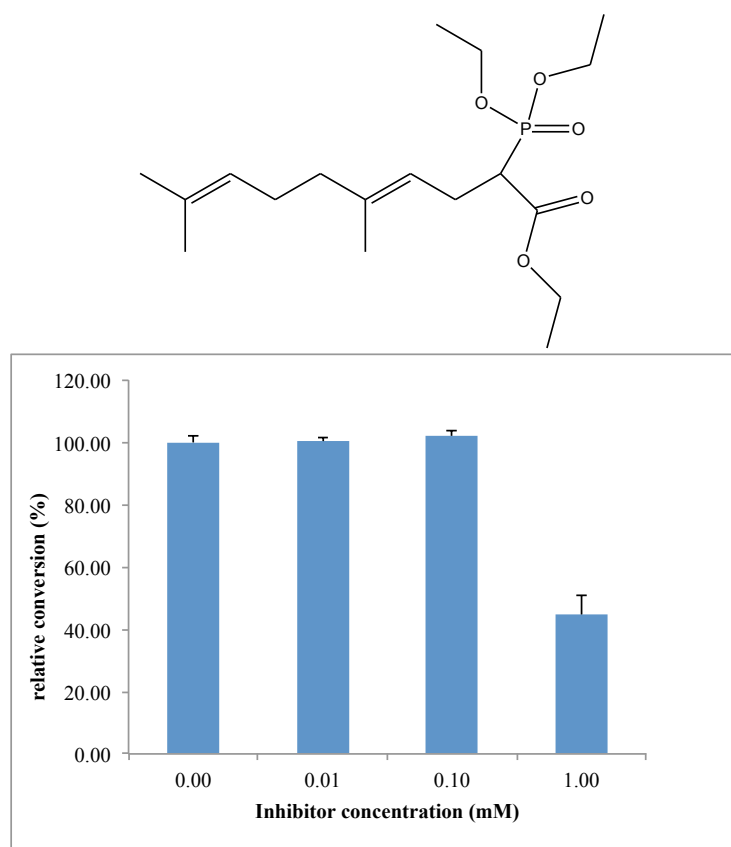
Figure 17: Effect of compound **36** on UbiA.
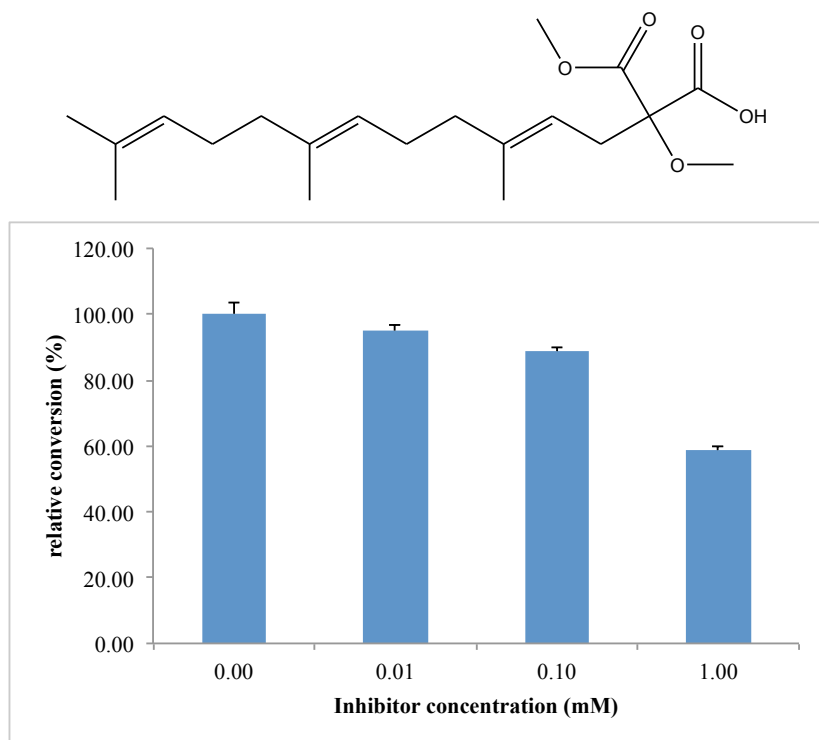
Figure 18: Effect of compound **6** on UbiA.
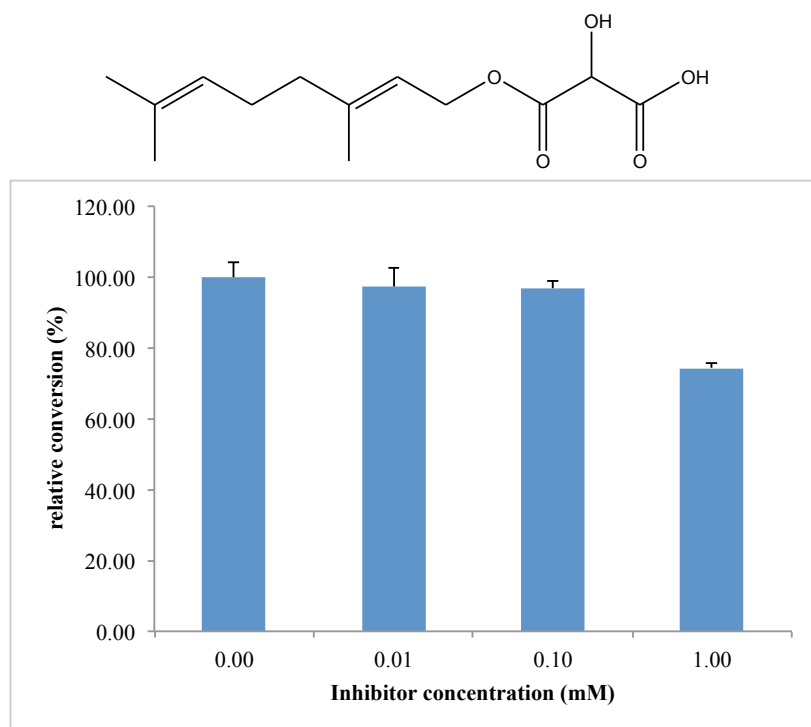
Figure 19: Effect of compound **12** on UbiA.
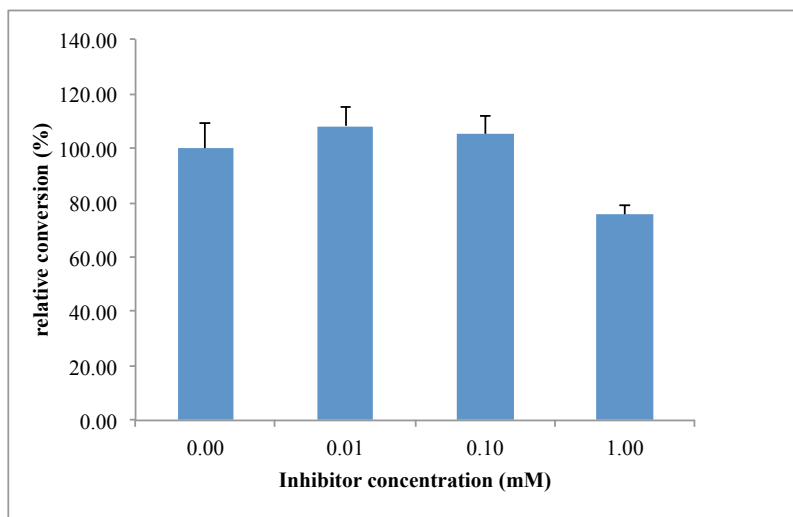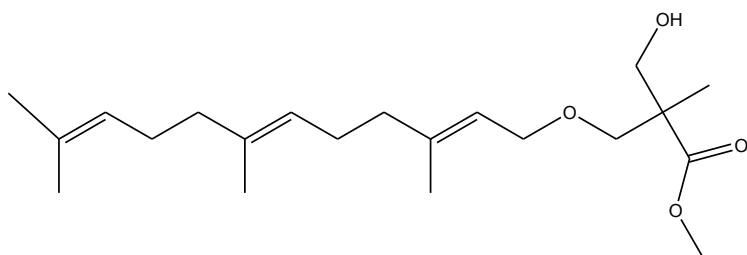
Figure 20: Effect of compound **26** on UbiA.
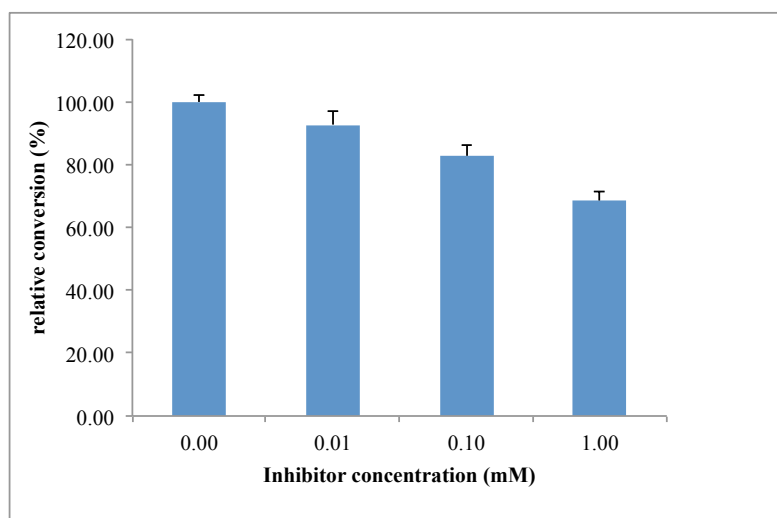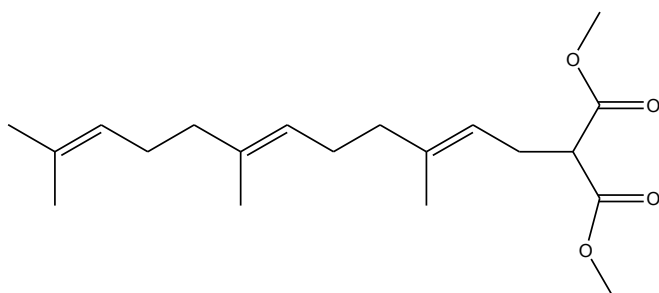
Figure 21: Effect of compound **35** on UbiA.
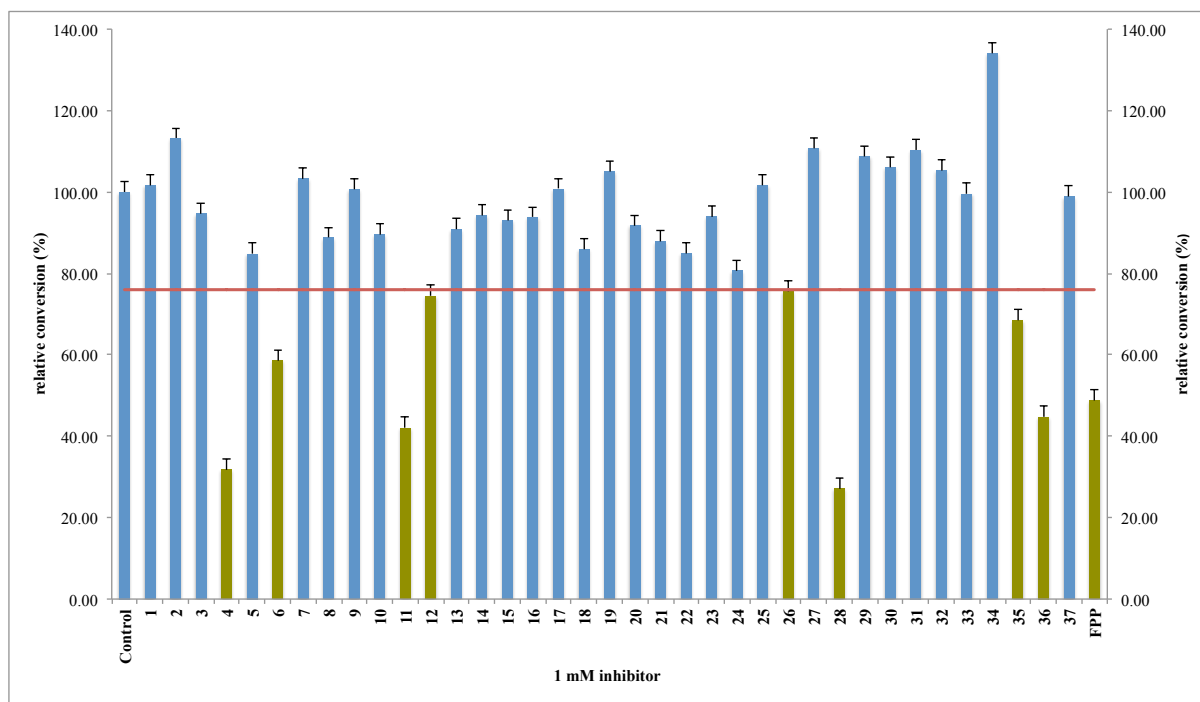
Figure 22: Summary of all tested compounds effects at 1 mM.

As a positive control, the reaction mixture did not contain any inhibitor. The red line is the cutoff.
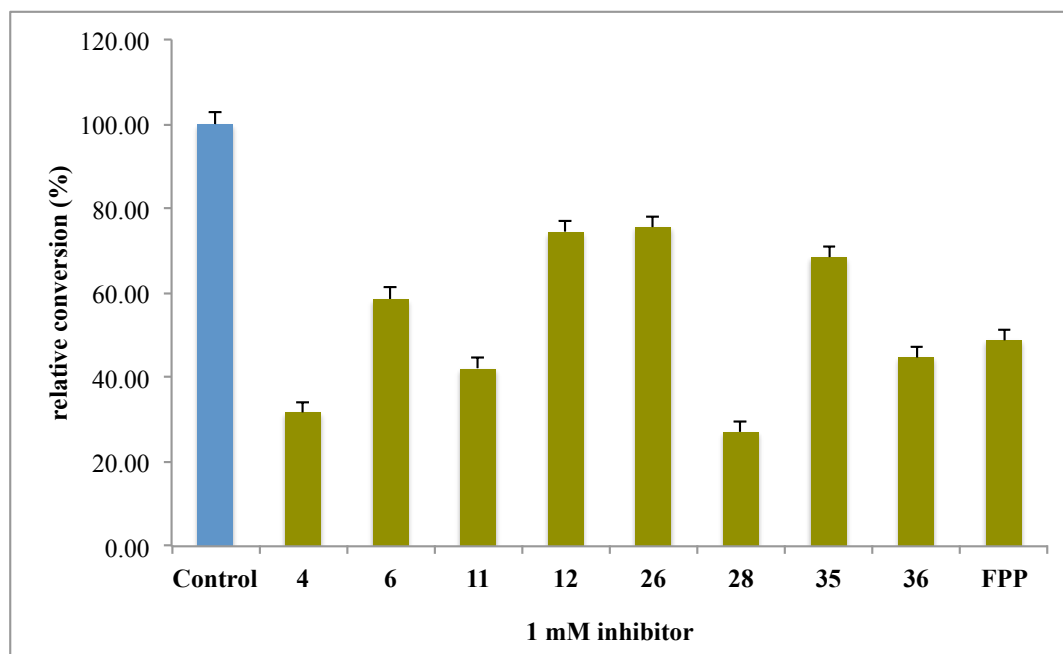
Figure 23: Summary of inhibitor effects at 1 mM.

3.4.1 Effect of Magnesium Chloride (MgCl$_2$) on UbiA Activity

It is known from literature that Mg^{2+} ion is essential for UbiA activity and standard assays use 5 mM final MgCl$_2$ concentration. However, there has been contrasting observations on effect of MgCl$_2$ on UbiA activity. For example, Dessoy (2003) observed that high MgCl$_2$ concentration (50 mM) resulted in high initial rate but caused denaturation of the enzyme much faster than at lower concentrations (1, 2.5 and 5 mM). Melzer and Heide (1994) observed maximum initial reaction velocity at between 20 and 100 mM MgCl$_2$. However, assay conditions differed between them, and also both can be true as one looked at enzyme stability over a longer period while the others at maximum turnover in the initial phase. Due to these results, the effect of MgCl$_2$ on UbiA activity under the conditions relevant for this study was determined. It was found that UbiA activity increased with increasing MgCl$_2$ up to 10 mM. Further increase in MgCl$_2$ concentration resulted in reduced activity (Fig. 24). Effects of EDTA on UbiA was investigated and results showed that UbiA activity decreased with increasing EDTA concentration (Fig. 25) supporting the fact that MgCl$_2$ is essential for UbiA activity. However, experiments were carried out with the standard 5 mM MgCl$_2$ since there was no big difference in substrate conversion (%) between 5 and 10 mM, and lower concentrations are better to avoid salt problems with UbiA (Dessoy, 2003).
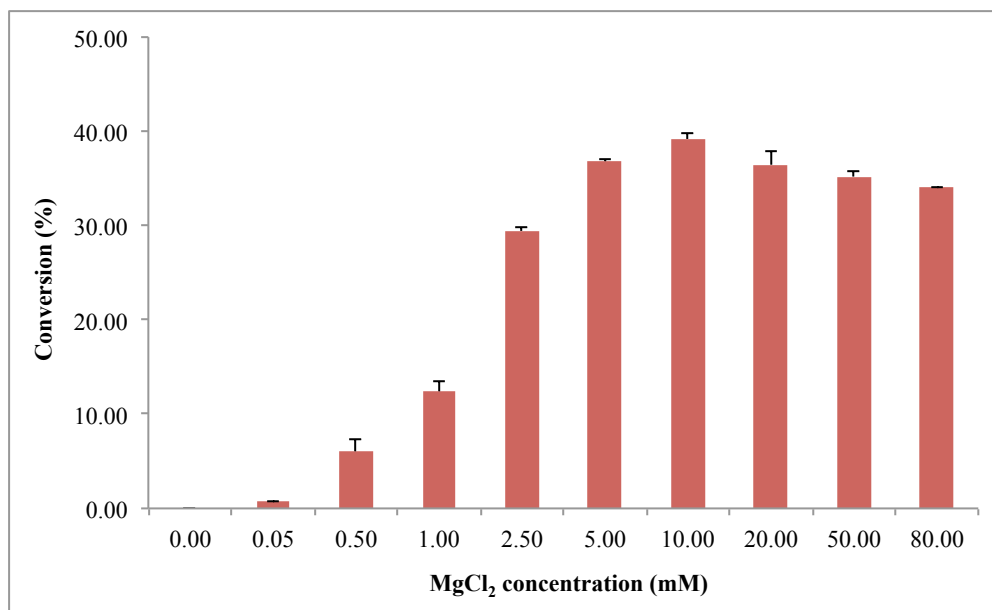
Figure 24: Effect of MgCl$_2$ on UbiA activity (after 10 min at 37 °C, with 10% DMSO).
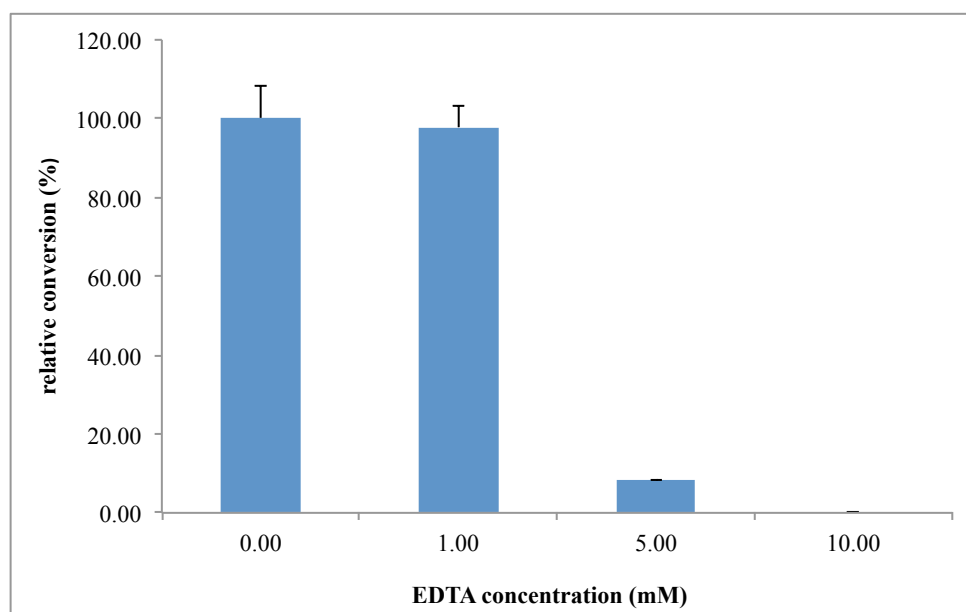
Figure 25: Effect of EDTA on UbiA activity at 5 mM Mg^{2+} concentration.

3.4.2 Effect of Inhibitors as Substrates

For the sake of completeness, the compounds were tested as explained in the methodology part to find out whether they can act as substrates. Of course, even in theory only substrates with a leaving group like geraniol esters or other O- or S-linked substrates have a remote

though unlikely chance to show any such reactivity. No product was formed implying that the tested prenyl diphosphate mimetics do not act as substrates as was anticipated. Likewise, there was no product formation when GPP was excluded from the reaction mixture implying that there is no residual GPP in the enzyme preparation.

3.4.3   Effect of Farnesyl Diphosphate (FPP) on Formation of GHB

It is known that FPP is a substrate of ubiA, fig. 38 (Melzer and Heide, 1994). Accordingly, as FPP concentration increases, the amount of GHB formed decreases (Fig. 27). GHB-formation is dropping faster than a 1:1 competition would imply. E.g. at 2.5 mM FPP and GPP each, with equal velocities a 50% rel. conversion to GHB would be expected, instead only 14% GHB is found (Table 8). This supports our observation that most potential inhibitors have long chain lengths (i.e. FPP analogues). Although FPP itself is a substrate for UbiA the amount of FHB formed is relatively very low (Fig. 26, see also fig. 37), so that FPP is a competitive substrate and, vs. GPP also an inhibitor without showing full analogous conversion. If the underlying reason is e.g. Mg-ion binding or direct binding in the active site without conversion is to be checked in future work. FHB was confirmed by LC–MS analysis. As stated in the introduction, long chain prenyl diphosphate substrates were not as well accepted as geranyl diphosphate (Wessjohann and Sonntag, 1996). Apart from that, when two substrates are simultaneously present in the reaction mixture, only one at a time can be accepted and converted to product. Usually the physiological substrate is more efficient than the other (Bisswanger 2008). From literature GPP is known to work best *in vitro* (Wessjohann and Sontag, 1996) despite the higher affinity of UbiA to FPP (Melzer and Heide, 1994). This could be an explanation why FHB was formed in relatively low amount.

However, it would have been better to not only measure endpoints but also relative kinetics of GHB and FHB formation in absence of FPP and GPP respectively and in presence of both

substrates as well (Pocklington and Jeffery, 1969; Bisswanger, 2008). This would give insight on how the initial rate of reaction for individual substrate is affected in mixture of both substrates relative to the initial rate for either substrate alone (Pocklington and Jeffery, 1969).

Table 7: **Formation of GHB and FHB at 2.5 mM GPP and different concentrations of FPP.**

[FPP] mM	Conversion (%)	
	GHB	FHB
0.00	44.73 ± 7.81	0.00 ± 0.00
0.01	38.77 ± 1.41	0.00 ± 0.00
0.10	33.07 ± 0.63	2.30 ± 0.45
1.00	19.33 ± 0.58	7.91 ± 0.12
1.50	13.89 ± 3.86	8.10 ± 0.20
2.50	7.06 ± 3.32	6.89 ± 0.44
5.00	4.60 ± 0.57	7.12 ± 0.12
7.50	1.15 ± 0.17	3.96 ± 0.34
10.00	0.00 ± 0.00	1.62 ± 1.18

Average of triplicate data ± SD.
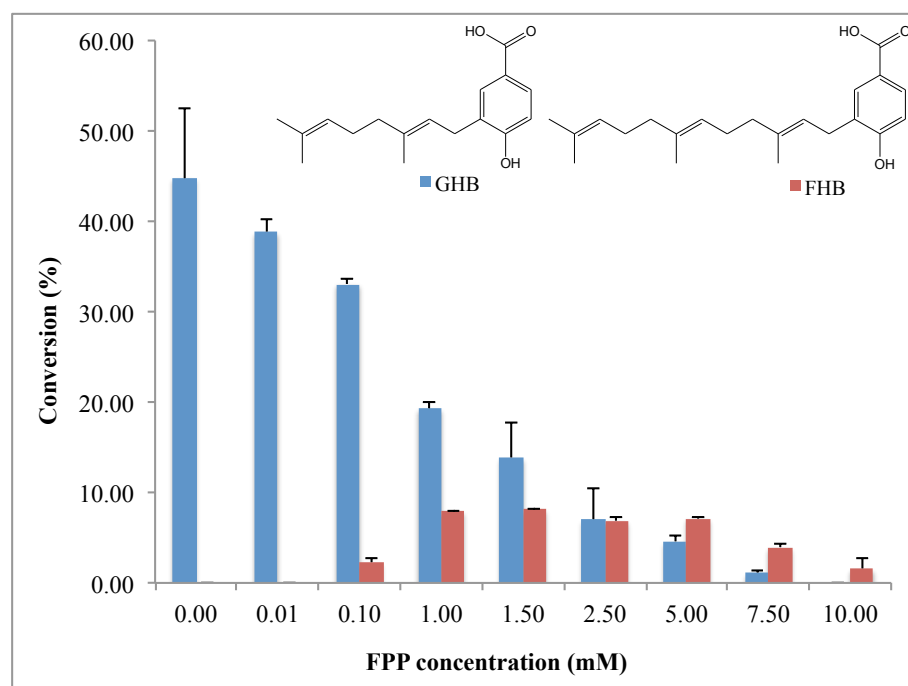
Figure 26: 4-HB conversion at 2.5 mM GPP and different concentrations of FPP. The inserts are the structures of the products.

Table 8:   Effect of FPP on formation of GHB at 2.5 mM GPP.

[FPP] mM	Relative conversion in %
0.00	100.00 ± 7.30
0.01	95.24 ± 9.55
0.10	94.37 ± 1.80
1.00	44.86 ± 5.50
1.50	28.05 ± 7.80
2.50	14.41 ± 6.78
5.00	9.67 ± 1.20
7.50	2.66 ± 0.38
10.00	0.00 ± 0.00

Average of triplicate data $\pm$ SD.
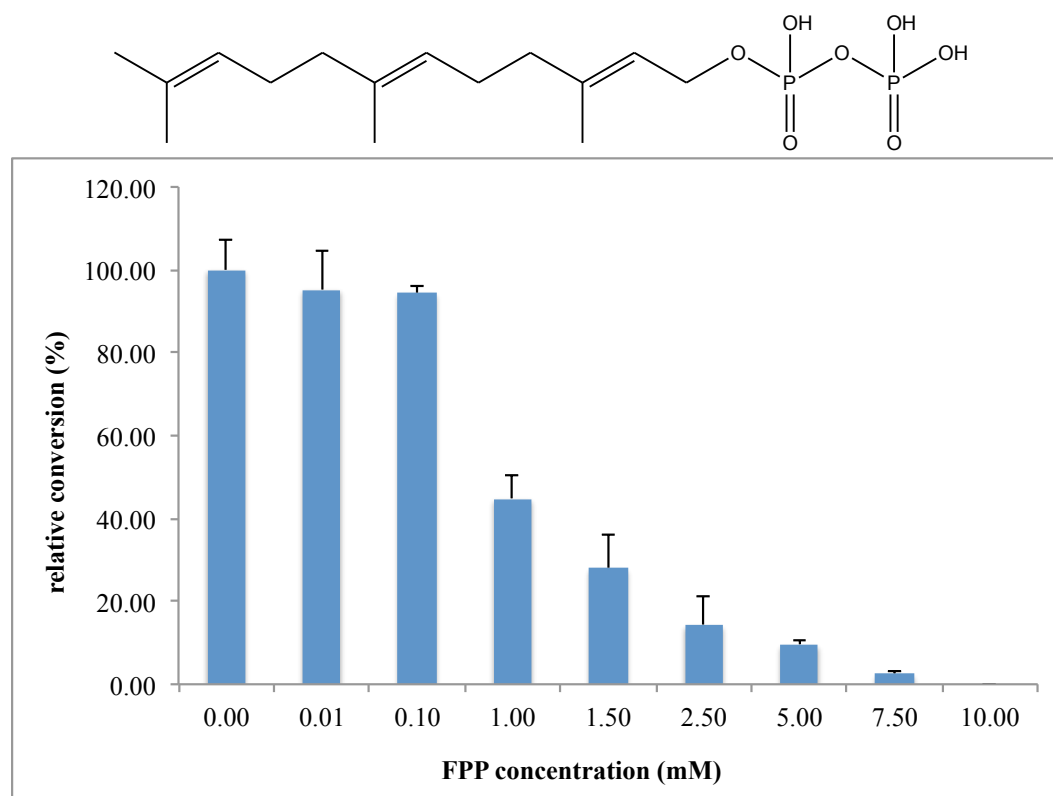
Figure 27:   Effect of FPP on formation of GHB at 2.5 mM GPP.
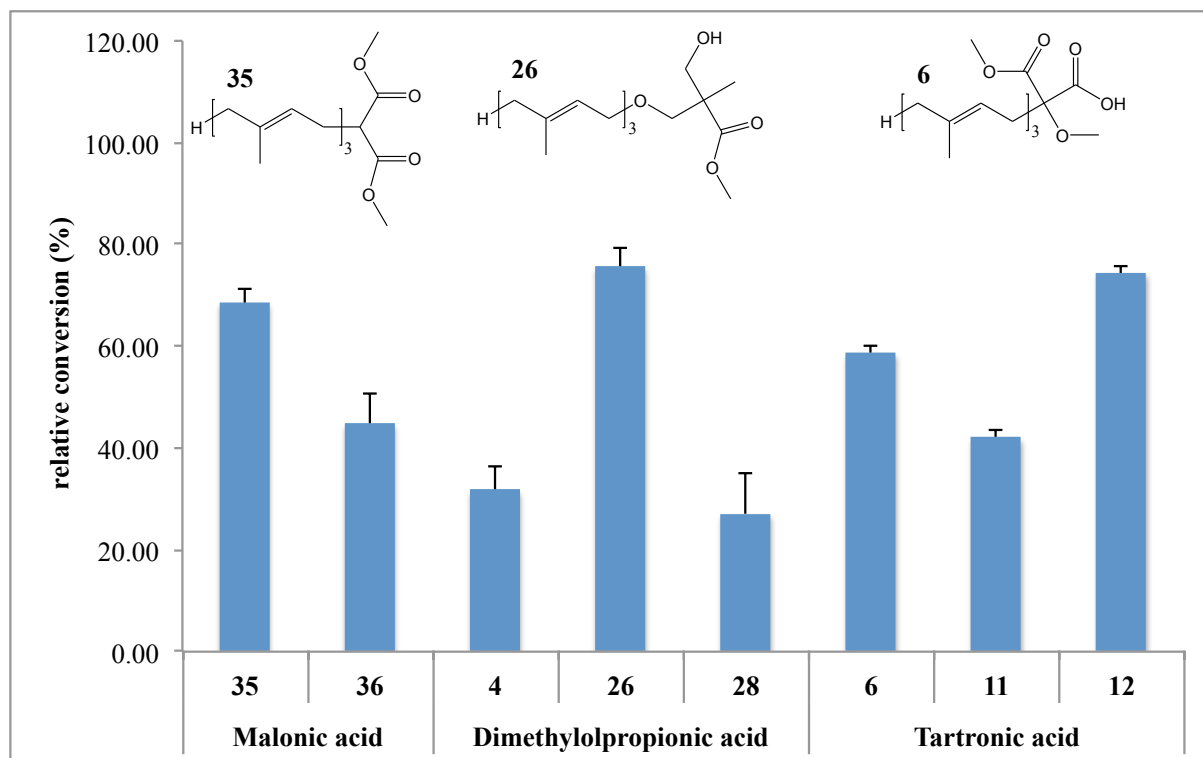
Figure 28: Influence of head group on inhibitionn of UbiA activity at 1 mM inhibitor. The inserts are the representative structures for each class.

3.4.4 Concentration Response Curves for Estimation of IC_{50} Values

Generally, the concentration-response curve should be sigmoidal with zero inhibition in absence of any test compound and near 100% inhibition at high compound concentrations and a Hill slope at or near 1 (Auld *et al*., 2012; Haas *et al*., 2012; Jang *et al*., 2014; Peñã *et al*., 2015; Katsuno *et al*., 2015). This applies if the compound inhibits only one enzyme in the preparation (Scott and Williams, 2012). However, practically, it is difficult for experimental data to yield the expected top and bottom (Haas *et al*., 2012), instead, the data can yield the top and bottom that are close to the theoretical values (Haas *et al*., 2012). In this study, the model fit yielded values close to 100% and 0% for top and bottom (fig 30-34) respectively and Hill slopes ranging from 1.58 to 3.15 (Table 9). This shows that the data from this study fulfilled the assumptions of the concentration-response curve. Validity of the data would be suspiciuos if the fit resulted in low slope or low maximum asymptote (Scott and Williams,

2012). For example, Penã *et al*., (2015) neglected the compounds whose concentration-response curves had low slope or low maximum asymptote. Low asymptote implies low potency of a compound or poor solubility at higher concentrations. On the other hand, a Hill slope that is significantly less than one (e.g. <0.8) suggests that the observed activity is most probably contributed by more than one enzyme (Scott and Williams, 2012) (Scott and Williams, 2012).

Among the four compounds whose IC_{50} values were determined; **28** was the most potent one with an IC_{50} value of 0.69 ± 0.03 mM. Potency of **4** was almost similar to **28**, its IC_{50} value was 0.75 ± 0.02 mM. Likewise, potency of **26** was almost similar to **36**, their IC_{50} value were 1.51 ± 0.14 mM and 1.25 ± 0.03 mM for **26** and **36**, respectively. Since most of the active compounds were FPP analogues and FPP also showed to inhibit formation of GHB, then a fictive (calculatory) IC_{50} value for FPP was also determined (Table 9 and Fig. 33). Though 1 mM activity appears to be very low, similar best values were observed in the group of Prof. Spíchal in Olomouc, Czech Republic, even though he used a much larger set of potential inhibitors of our compounds but also of other substance classes, showing that this is generally an unsolved problem.

However, due to lack of reference compound, it was difficult to draw conclusion (make a statistical decision) whether the potency of the test compounds based on their IC_{50} values could be considered biologically relevant to the purpose of this study. In addition to that, no *in vivo* study was conducted; this would give insight about the minimum inhibitor concentration that could give a pharmacologically relevant effect.

In a previous study by Zakharova *et al*., (2004) several prenyl diphosphate mimetics were prepared and their inhibitory potential against UbiA prenyltransferase was evaluated. Among the tested compounds were β-hydroxy carboxylic acid and difluoro phosphinophosphonate

(Fig. 34) whereby the former showed almost complete inhibition at 1 mM, the latter was not inhibitory. These compounds were not tested in this study because the current study focused on prenyl diphosphate mimetics synthesized by Vasilev (2015). However, in this study some α-hydroxy (di)carboxylic acids and diesters with various chain lengths were tested and one α-hydroxy dicarboxylic acid (FPP analogue) showed 58% inhibition (Fig. 16), unfortunately, its IC_{50} was not determined due to the amount limited available of the substance.

Table 9: **Summary of calculated IC_{50} values for different compounds.**

Compound	IC_{50} (mM)	Hill slope
26	1.51 ± 0.14	1.88 ± 0.31
28	0.69 ± 0.03	3.12 ± 0.36
4	0.75 ± 0.02	3.15 ± 0.35
36	1.25 ± 0.03	2.38 ± 0.17
FPP	0.88 ± 0.06	1.58 ± 0.26

Values ± S.E.
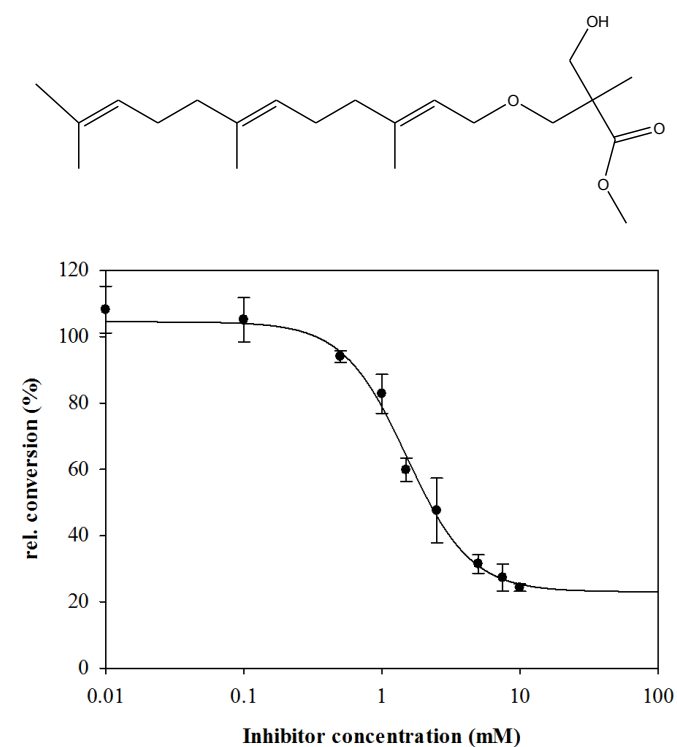
Figure 29: Concentration-response curve for compound **26**.
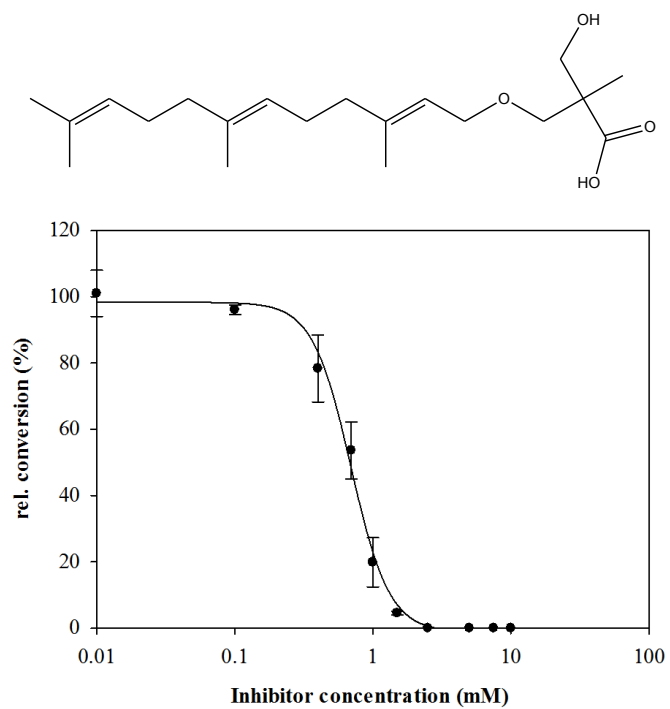
Figure 30: Concentration-response curve for compound **28**.
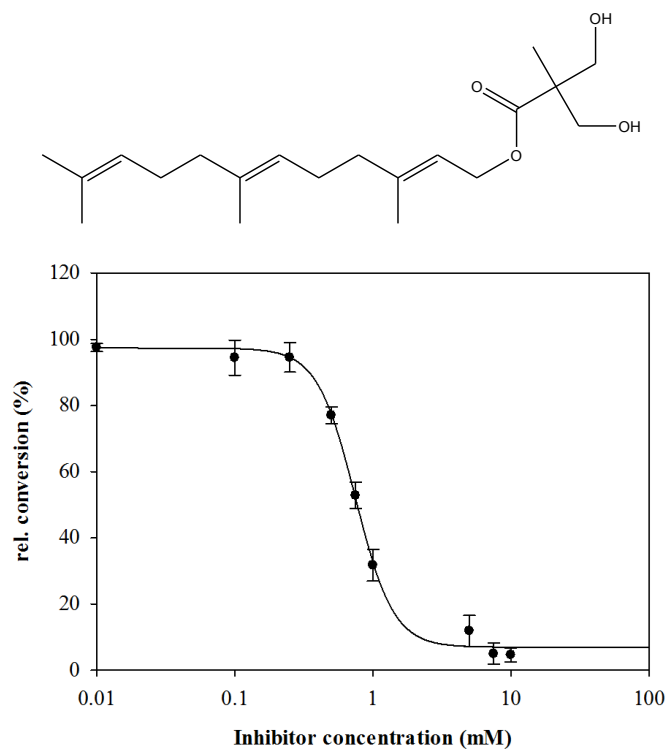
Figure 31: Concentration-response curve for compound **4**.
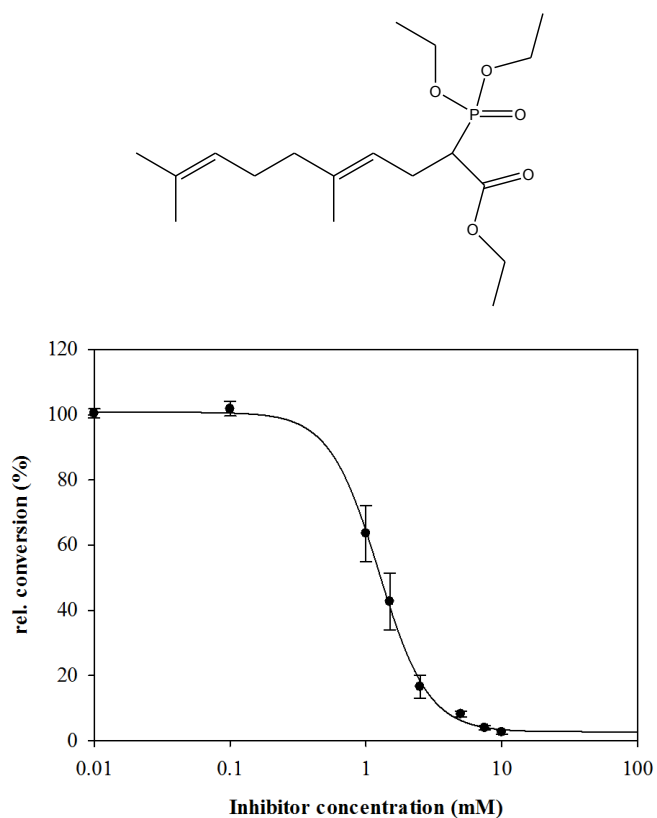
Figure 32: Concentration-response curve for compound 36.
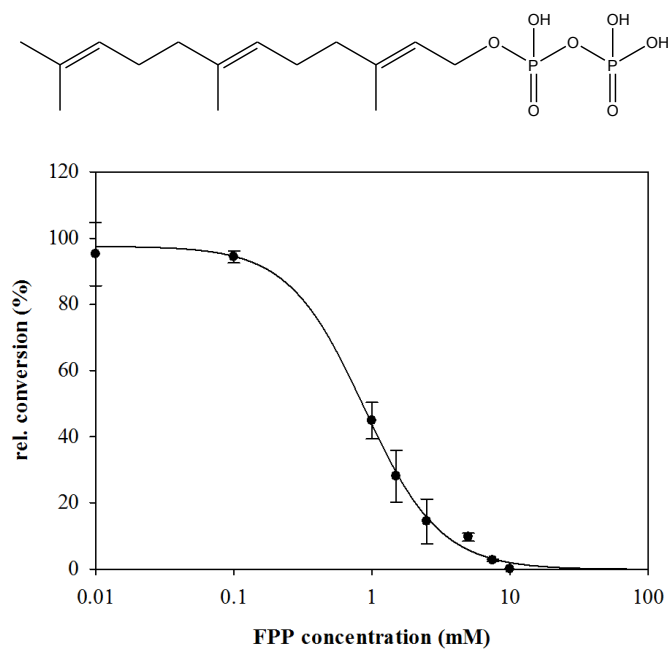
Figure 33: Concentration-response curve for FPP (competitive substrate with inhibitory properties).
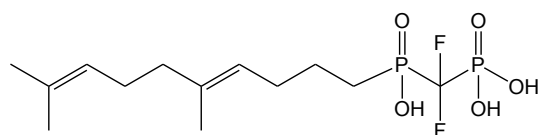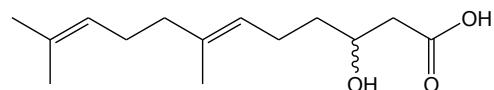
Difluoro phosphinophosphonate   β-hydroxy carboxylate

Figure 34: Diphosphate mimetics that were tested in the previous study (Zakharova *et al*., 2004).

3.5 Discussion

3.5.1 Potential Inhibitors of UbiA

Screening for inhibitors of UbiA was successful and among the tested compounds, only **11**, **4**, **28** and **36** showed over 50% inhibitory activity. At 1 mM inhibitor concentration relative conversions (%) were 42.00 ± 1.68, 31.73 ± 4.78, 27.10 ± 8.15 and 44.71 ± 6.18 for **11**, **4**, **28** and **36**, respectively (Table 6). Other compounds that showed inhibition at this concentration were **6**, **12**, **26** and **35** with relative conversions (%) of 58.57 ± 1.55, 74.39 ± 1.55, 75.61 ± 3.65 and 68.50 ± 2.90 respectively (Table 6). Relative conversion is expressed as the ratio of GHB formation in the presence of the compound to the GHB formation in the absence of the compound. Results show that inhibition depends on chain length, head group and linker of the prenyl moiety.

3.5.2 Dependency on Chain Length

The dependence of inhibition on chain length could be explained based on activity of compounds with similar head groups but various chain lengths whereby compounds with shorter chain length (i.e. containing one or two isoprene units) did not show inhibition. The compounds that were more active than others had one more isoprene unit (FPP analogues) compared to the natural substrate.

The fact that FPP analogues showed more inhibitory activity than GPP analogues can be explained as follows: It is known from literature that the hydrophobic pocket plays role in substrate binding (Tarshis *et al*., 1996). Farnesyl diphosphate synthase contains an aspartate rich motif which is crucial for catalysis and its binding pocket can accommodate both, FPP and GPP although in order for FPP to bind some side chain amino acids must undergo some orientations. Likewise for UbiA, aspartate is involved in binding of the prenyl diphosphate substrate, moreover, the farnesyl moiety is more hydrophobic than the geranyl moiety, and thus, most probably its chain length increases its affinity to the enzyme in a competitive way since the binding pocket is also hydrophobic (Tarshis *et al*., 1996; Bräuer *et al*., 2008). Furthermore, it is reported that prenyl diphosphate with long chain lengths may form micelles or vesicles thereby reducing the reaction velocity (Bräuer *et al*., 2008). Simplified one might propose now, to check even longer chains. However, such compounds would be water insoluble and thus impractical for applications.

Mechanistic studies show that magnesium ions play a role in binding of the diphosphate substrate whereby it forms a tetrahedral metal complex with the two oxygen atoms of Asp75 of the enzyme and two oxygens of the diphosphate moiety of the substrate. Furthermore, the binding is stabilized by hydrophobic interactions of the organic oligoprenyl chain, mainly with Leu141 and Leu256 of the enzyme (Bräuer *et al*., 2004). Thus, the more hydrophobic the prenyl side chain is, the more stable the binding. As described before, farnesyl diphosphate has longer prenyl chain than geranyl diphosphate and the longer the prenyl chain the more hydrophobic it is. Reed and Rilling (1976) also reported that dimethylallyl diphosphate, geranyl diphosphate and farnesyl diphosphate can be accommodated competitively on the same binding site of the avian farnesyl diphosphate synthase. Since the FPP side chain is more hydrophobic than the GPP side chain (Reed and Rilling, 1976), then, it could be that FPP analogues had higher binding affinity than GPP analogues. Therefore, the farnesyl

moiety might have stabilized binding more than the geranyl moiety. In addition to that, kinetics study for GPP and FPP showed that FPP has lower K_m value than GPP implying that UbiA has higher affinity to FPP than GPP (Melzer and Heide, 1994), so, probably the FPP analogues also. Dependency of inhibition on chain length was also observed by Parker *et al*., (1978) whereby phosphonate or phosphonophosphate with longer chain were more active than the ones with shorter chain. In addition to this, FPP has confirmed to inhibit, or better compete, with formation of 3-geranyl-4-hydroxybenzoic acid (Fig. 27). ITC could be a way to determine such better binding experimentally in the future.

3.5.3   Dependency on Head Group

The compounds which were active can be classified based on head groups into; (i) dimethylolpropionic acids which include **28**, **4** and **26**; (ii) tartronic acids which include **12**, **11** and **6**; and (iii) malonic acids which include **35** and **36** (Fig. 28).

The dependence of inhibition on the head groups can be clarified based on compounds with the same chain length but different head groups. Dimethylolpropionic acid was found to be the leading group of compounds showing inhibitory activity against UbiA with exception of **26**. The next best group was tartronic acid and malonic acid was the last (Fig. 28). Parker *et al*., (1978), also observed similar results in a phosphonate series, whereby phosphonophosphates were more potent than phosphonates. Despite the fact that most of the compounds that showed inhibitory potential were FPP analogues, compound **36** and **12** belonging to malonic and tartronic acids respectively are GPP analogues. Comparison of their inhibitory potential based on their head groups shows that malonic acid represented by a diphosphate ester **36** is more potent than tartronic acid **12**. These compounds may be considered exceptional due to the fact that, there are three **7, 33&37** and two **8&32** more GPP analogues belonging to malonic and tartronic acids respectively but they were not potential

inhibitors.

3.5.4   Dependency on the Chain Linker

The most active compound, **28** is a dimethylolpropionic acid linked to the prenyl chain through an ether bond. The second most active compound, **4** is a dimethylolpropionic acid linked to the prenyl chain through an ester bond.

The third most active compound, **11,** is a tartronic acid linked to the prenyl chain via a C-C bond. The fourth most active compound, **36,** is a diphosphate ester (malonic acid) linked to the prenyl chain through a C-C bond. The fifth most active compound, **6**, is a tartronic acid linked to the prenyl chain through a C-C bond. The sixth most active compound, **35**, is a malonic acid linked to the prenyl chain through a C-C bond. The seventh most active compound, **12** is a tartronic acid linked to the prenyl chain through an ester bond and the eighth active compound, **26** is a dimethylolpropionic acid linked to the prenyl chain through an ether bond. General observation about dependence of inhibitory activity on chain linker suggests that influence of ester and ether bond is unpredictable. The most active compound is linked to the prenyl chain through ether bond and the last active compound is also linked to the prenyl chain through ether bond. Likewise, the second most active compound is linked to the prenyl chain through ester bond and the second last active compound is also linked to the prenyl chain through ester bond. However, the type of bond on C-1 may also contribute to the inhibitory activity. For example, the (bis)phosphanates tested by Holstein *et al*., (1998) showed that inhibitory activity depends on presence or absence of a double bond at C-1 whereby the most active inhibitor lacked a double bond at C-1.

Since these are the compounds that were synthesized in IPB and it was the first time to screen them for their inhibitory potential, there are no reported IC_{50} values for comparison or

reference. Literature shows that most substrate analogues are competitive inhibitors, most probably the diphosphate mimetics tested in this study are also competitive inhibitors. Attempts to hydrolyze the esters by a colleague so that their corresponding acids can be tested were not successful. However, one ester and its corresponding hydrolysis intermediate were tested and as it is shown in the results section, the ester, **36**, showed inhibitory activity while the hydrolysis intermediate, **37**, did not. The same was later also found in the Prof. Spíchal group for other prenyltransferases and is a disturbing result, because neutral esters were considered worse mimics of diphosphate and worse binders of Mg-ion than carboxylates.

As stated in the introduction that UbiA is involved in ubiquinone biosynthesis, thus, potential inhibitors may be developed as antibiotics interrupting the respiratory chain. In literature there are a number of studies on assessment of inhibitory potential of farnesyl diphosphate analogues against farnesyl transferase. For example, β-ketophosphonic acid and chaetomellic acids A and B were found to be the best among farnesyl-derived inhibitors of *ras* farnesyl transferase (Kang *et al*., 1995; Singh 1993; Singh *et al*., 1993). These inhibitors could be developed as anti-tumor drugs for tumors mediated by *ras* proteins.

3.5.5 Dependency of UbiA Activity on Concentration of Magnesium Chloride

The dependence of UbiA activity on Mg^{2+} ion was proved by determining effect of EDTA on formation of GHB. As EDTA (chelating agent for Mg^{2+} ion) concentration increases the amount of 3-geranyl-4-hydroxybenzoic acid (GHB) formed decreases since more Mg^{2+} ion is withdrawn from the reaction mixture (Fig. 25). This observation supports the fact that Mg^{2+} ion is essential for UbiA activity. Formation of the product at 5 mM EDTA would suggest that there is some Mg^{2+} ion in the enzyme preparation itself apart from the one added in the reaction mixture because at 5 mM EDTA it is expected that all the 5 mM Mg^{2+} ion is chelated and there would be no product formation as it can be seen at 10 mM EDTA. However, these

results were controversial because when $MgCl_2$ was omitted from the reaction mixture there was no product formation, therefore, a simple explanation to this phenomenon could be that 5 mM EDTA solution that was used was not able to chelate all Mg^{2+} ion in the reaction mixture.

4 GENERAL DISCUSSION, CHALLENGES AND RECOMMENDATIONS

4.1 General Discussion

This study aimed at screening of prenyldiphosphate analogues for their potential to inhibit UbiA-prenyltransferase. However, prior to inhibition assay K_m values were determined to establish substrate concentrations that would be suitable for inhibition assay. Furthermore, conditions for UbiA inhibition assays were optimized. This is because high substrate conversion is required for better analysis of inhibition data and it is also recommended to perform enzyme inhibition assays at the linear phase of the progress curve.

4.1.1 Evaluation of Enzyme Kinetic Analyses

Enzyme kinetics analysis is underlied by the following assumptions (Copeland, 2000; Biswanger, 2008; Tan, 2011; Brooks *et al*., 2012).

i. High enough concentration of substrate should be used to ensure saturation of the reaction velocity vs substrate concentration curve. That means substrate concentration should not be a limiting factor as the curve is expected to have a hyperbolic shape (Biswanger, 2008; Brooks *et al*., 2012). It is recommended that, the highest substrate concentration should be at least 10 times the K_m (Biswanger, 2008; Tan, 2011) and there should be enough substrate concentration points below and above the K_m value (Brooks *et al*., 2012).

ii. Product formation should be linear with time. This is because initial velocity is determined at the linear phase of the progress curve.

iii. The product is formed from the activity of a single enzyme. This means that there is no more than one enzyme that can act on the same substrate (Copeland, 2000).

iv. There is no product formation in absence of the enzyme.

Although the K_m values differed from literature, the assumptions of Michaelis-Menten kinetics were fulfilled as follows:

i.   The highest substrate concentrations in this this study were 2.5 and 5 mM for GPP and 4-HB respectively. That means the highest GPP and 4-HB concentrations were almost 10 and 25 times respectively the K_m reported in literature (See section 3.2). Therefore, the assumption about the highest substrate concentration was fulfilled.

ii.   Reaction progress curves in Dessoy's thesis (2003) showed that the product was formed at a linear initial rate at the start of the reaction to approximately 20 min incubation. Based on this information 7.5 min was chosen to be the maximum incubation time for enzyme kinetics in this study because it is within the linear phase of the reaction progress curve. In addition to this, the progress curve in this study was linear to 10 min incubation (insert in fig. 13). This fulfills assumption (ii).

iii. The fact that the curves follow a single-site rectangular hyperbolic curve (Fig. 11) implies that the observed activity results from a single enzyme (Copeland 2000; Scott and Williams, 2012); hence, assumption (iii) is fulfilled.

iv.   When the enzyme was excluded from the reaction mixture, there was no product formation implying that there was no spontaneous non-enzymatic product formation; thus, assumption (iv) is fulfilled.

4.1.2   Assay Conditions

As stated in the results (section 3.3), 2.5 mM GPP + 2 mM 4-HB at 37 °C resulted in a statistically significant higher amount of product (Fig. 12) that could be easily detected and analysed by HPLC, hence, they qualified as suitable conditions for UbiA inhibition assay. Determining the full progress curve is advantageous to ensure that the reaction goes to completion and the measurements are made in the linear phase of the reaction (Copeland

2000). A typical progress curve is initially characterized by an approximately linear increase of product formation (or decrease of substrate depletion) with time. As the reaction proceeds, a curvature is observed and finally the curve plateaus due to substrate depletion (Copeland 2000; Brooks *et al*., 2012). Fig. 13 meets these criteria suggesting that the reaction went to completion. The fact that the two curves with different enzyme concentrations converge implies that the enzyme is stable under the assay conditions. If the enzyme were not stable, the two reaction progress curves would not achieve a similar maximum plateau value of product formation (Brooks *et al*., 2012). Likewise, the reaction mixtures incubated for 10 min with 25 µl UbiA membrane fraction resulted in a more linear curve (insert in Fig. 13). It is within this linear phase where testing inhibitiors is recommended (Brooks *et al*., 2012; Strelow *et al*., 2012). At this linear phase it is assumed that: (i) there is no buildup of any intermediate other than the enzyme-substrate complex (ii) concentration of the substrate does not change significantly because the product formed is very little and the reverse reaction is negligible (iii) the enzyme is catalytically active and its concentration is far less than the concentration of substrate (Copeland, 2000; Brooks *et al*., 2012; Strelow *et al*., 2012). Failuire to meet these assumptions may lead to substrate limitation, product inhibition or enzyme inactivation (Strelow *et al*., 2012), which might mislead interpretation of the level of inhibitory effect exerted by the test compounds. Therefore, with reference to the insert in fig. 13 the assumptions were fulfilled and thus, 10 min reaction time and 25 µl UbiA membrane fraction were chosen as suitable conditions for UbiA inhibition assay.

4.1.3   Evaluation of Inhbitors

Results show that most of the tested compounds were not inhibitory and those, which were inhibitory, showed inhibition only at 1 mM. In literature, reported inhibitory concentration or IC_{50} values of potent inhibitors for the structurally different protein-oligoprenyl-transferases

range from low micromolar to nanomolar, while for aromatic PTAses from Arabidopsis, values also were only down to 1 mM (Lukas Spíchal - personal communication). For protein PTases, potential inhibitors reported by Holstein *et al*., (1998) and Kang *et al*., (1995) were in micromolar units while those presented by Scholten *et al*., (1996) were in nanomolar units.

Furthermore, results from my study show that inhibition does not rely on whether the compound is an ester or an acid rather on chain length, head group and chain linker. Both esters and acids showed inhibitory activity at various degrees and almost all compounds, which showed inhibition, were FPP analogues with exception of **12** and **36**, which are GPP analogues. However, there might be in addition to the observed ones other factors also contributing to inhibitory effect. For example, these are diphosphate mimetics, thus, it might happen that they also form more stable Mg^{2+} ion complexes than GPP, thereby reducing ion concentration in the reaction mixture, and hence, induce low product formation. In connection to this, Zakharova *et al*., (2004) observed a slightly increased activity of UbiA that was dependent on enzyme and inhibitor concentration and the effect was related to low Mg^{2+} ion concentration because earlier experiments already showed that reduction in Mg^{2+} ion concentration results in increased activity. Therefore, in this case, it was hypothesized that the effect is due to reduction in Mg^{2+} ion concentration probably because of complexion with inhibitors since they are diphosphate mimetics. When Mg^{2+} ion concentration was slightly reduced by chelating it with EDTA (maximum concentration: 1.00 mM) enzyme activity increased with increasing EDTA concentration especially at low protein concentration proving the hypothesis (Zakharova *et al*., 2004). And in this study, chelating Mg^{2+} ion with EDTA reduced product formation with increased EDTA concentration.

Since most of the compounds that showed inhibition were FPP analogues, effect of FPP on GHB formation was also determined. It inhibited formation of GHB by competitive use of the

4-HB substrate but also by additional effects, whereby the degree of inhibition increased with increasing FPP concentration. Since FPP is also a substrate for UbiA-prenyltransferase although a bad one with respect to formation of FHB, the two effects cannot be clearly separated with the experiments performed. Substrate inhibition by FPP might be assumed as formation of FHB was decreasing with increasing FPP concentration at 2.5 mM GPP as a co-substrate (Fig. 26). Results in table 7 shows that there was increase of FHB formation from 0.1 mM to 1 mM FPP. From 1 mM to 1.50 mM FPP, FHB formation was almost constant and slightly dropped at 2.50 mM FPP. From 2.50 mM to 5 mM FPP, FHB formation was almost constant and drastically dropped at 7.50 mM FPP. At 10 mM FPP the amount of FHB formed was very low. Substrate inhibition occurs when a second molecule of the substrate binds on the enzyme-substrate complex forming an inactive ternary complex, enzyme-substrate-substrate (Copeland, 2000; Bisswanger 2008). This phenomenon is evidence that FPP binds to the enzyme and acts as an inhibitor with respect to formation of GHB.

4.2   Challenges

As stated in the introduction, the linear phase at low substrate (e.g. < 0.8 mM GPP) concentration was very short under the experimental conditions used in this study for enzyme kinetics.

Of all the compounds tested, ester **4** was challenging in generating reproducible data. A possible explanation for this might be instability of the compound, that is, probably the compound is not stable in the reaction mixture. Apart from that, Hill slopes characterize the steepness of the curve and the Hill slope for compound **4** is 3.15 (Table 9); according to Scott and Williams (2012), a calculated Hill slope that is significantly greater than 1 (e.g. >1.5) suggests that, either there is aggregate formation when the inhibitor is in aqueous solution leading to non-specific inhibition or the inhibitor may be chemically reacting with the enzyme

or chelating a required cofactor. Shoichet (2006a; 2006b) also stated that steep concentration-response curve is among the features of the aggregating inhibitors. Thus, it probably forms aggregates and the extent of aggregation differs between assays. It might also, be driven by Thorpe-Inogold effects, with Mg-ion Lewis acid autocleave to form the corresponding butyrolactone derivative and geraniol. Since these diphosphate mimetics were tested for the first time in this study, it was not possible to compare these findings with similar or analogous findings, as there is a dearth of information in literature for comparison of their inhibitory activity.

4.3   Recommendations

In case of further study, a reference compound should be included in the assay for comparing the effect level between the reference and test compounds. Compounds with similar or greater effects as compared to the reference compound will be further investigated. Both Ki and IC_{50} are used to measure relative potency of the inhibitor but Ki is used to determine type of inhibition also (Copeland, 2000; Cer *et al*., 2009; Strelow *et al*., 2012). Therefore, I would recommend in case of further studies to determine Ki values for the compounds whose IC_{50} values have been determined to reveal their types. This may give insight on how to modify the structures in order to increase their potency. This was not done because the focus of this study was to determine potency of the inhibitors and not their types. I therefore recommend for further studies (in case), to determine their Ki values and characterise them as competitive, non-competitive, uncompetitive or otherwise. In case of any further studies on these compounds, effect of inhibitors whose IC_{50} values were determined in this study as a function of enzyme concentration may be checked. This is because in their study, Zakharova *et al*., (2004) for example, β-hydroxy acid showed more inhibitory potential with 0.67 mg protein/ml than with 2.68 mg protein/ml. It was not done in this study because it was not the

focus of this study; I recommend it only because I found this phenomenon interesting. Potential inhibitors of UbiA that will be developed for therapeutic application should be investigated for toxicity against human cells and the inhibitor should be modified to target only bacterial UbiA. Furthermore, effects of cellular metabolism on an antibiotic (i.e. the inhibitor) should be investigated so that either its potency is not diminished or lost at all.

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6 APPENDIX

6.1   Names of the Tested Compounds

Table 10:   Names of the tested compounds.

Compound no.	Three letter code	Name using Chemdraw software, which is not always stringent on IUPAC rules)
1	VAD 252	2,2'-(phenethylazanediyl)diacetic acid
2	VAD 269	3-(bis(3-methylbut-2-enyl)amino)propanoic acid
3	VAD 270	3,3'-(benzylazanediyl)dipropionic acid
4	VAD 622	(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienyl 2,2-bis(hydroxymethyl)propanoate
5	VAD 626	(S)-3-(((E)-3,7-dimethylocta-2,6-dienyloxy)carbonyl)-2-hydroxypropanoic acid
6	VAD 439	(4E,8E)-2-(methoxycarbonyl)-2-methoxy-5,9,13-trimethyltetradeca-4,8,12-trienoic acid
7	VAD 549	2-((E)-3,7-dimethylocta-2,6-dienyl)malonic acid
8	VAD 551	2-hydroxy-2-((E)-3,7-dimethylocta-2,6-dienyl)malonic acid
9	VAD 552	3-((E)-3,7-dimethylocta-2,6-dienyloxy)-2-(hydroxymethyl)-2-methylpropanoic acid
10	VAD 592	(S)-2-((E)-3,7-dimethylocta-2,6-dienyloxy)succinic acid
11	VAD 648	2-hydroxy-2-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienyl)malonic acid
12	VAD 645	2-(((E)-3,7-dimethylocta-2,6-dienyloxy)carbonyl)-2-hydroxyacetic acid
13	VAD 644	(E)-2-hydroxy-5,9-dimethyldeca-4,8-dienoic acid
14	VAD 526	(E)-3,7-dimethylocta-2,6-dienyl 2,2-bis(hydroxymethyl)propanoate
15	VAD 558	(E)-(4,8-dimethylnona-3,7-diene-1,1-diyl)bis(phosphonic acid)
16	VAD 572	2-((E)-3,7-dimethylocta-2,6-dienylthio)succinic acid
17	VAD 679	(E)-ethyl 9-ethoxy-2,2-bis(hydroxymethyl)-5,9-dimethyldec-4-enoate
18	VAD 650	2-((E)-3,7-dimethylocta-2,6-dienylthio)-3-mercaptosuccinic acid
19	VAD 550	2-(3-methylbut-2-enyl)malonic acid
20	VAD 700	(((E)-3,7-dimethylocta-2,6-dienyloxy)carbonyl)formic acid
21	VAD 464	methyl 3-((E)-3,7-dimethylocta-2,6-dienyloxy)-2-(hydroxymethyl)-2-methylpropanoate
22	VAD 729	3-(2,6-dimethylheptyl)cyclopropane-1,1,2,2-tetracarboxylic acid

Compound no.	Three letter code	Name using Chemdraw software, which is not always stringent on IUPAC rules)
23	VAD 451	(E)-trimethyl 6,10-dimethylundeca-5,9-diene-1,2,3-tricarboxylate
24	VAD 442	triethyl 2-((E)-3,7-dimethylocta-2,6-dienyloxy)propane-1,2,3- tricarboxylate
25	VAD 666	3-((E)-3,7-dimethylocta-2,6-dienyloxy)-3-(ethoxycarbonyl)pentanedioic acid
26	VAD 467	methyl 3-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienyloxy)-2-(hydroxymethyl)-2-methylpropanoate
27	VAD 524	3-methylbut-2-enyl 2,2-bis(hydroxymethyl)propanoate
28	VAD 681	3-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienyloxy)-2-(hydroxymethyl)-2-methylpropanoic acid
29	VAD 647	2-hydroxy-2-(3-methylbut-2-enyl)malonic acid
30	VAD 445	triethyl 2-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienyloxy)propane-1,2,3-tricarboxylate
31	VAD 436	dimethyl 2-hydroxy-2-(3-methylbut-2-enyl)malonate
32	VAD 438	dimethyl 2-hydroxy-2-((E)-3,7-dimethylocta-2,6-dienyl)malonate
33	VAD 411A	dimethyl (E)-2-(3,7-dimethylocta-2,6-dien-1-yl)propanedioate
34	VAD 698	methyl (E)-3,7-dimethylocta-2,6-dienyl oxalate
35	VAD 412A	dimethyl 2-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienyl)malonate
36	VAD 414	ethyl (E)-2-(diethoxyphosphoryl)-5,9-dimethyldeca-4,8-dienoate
37	VAD 414 ITM	(E)-1-(ethoxycarbonyl)-4,8-dimethylnona-3,7-dienylphosphonic acid

Note: Compounds **1**, **3**, **15**, **24**, **33**, **36** were named using chemdraw professional software.
VAD 414 ITM is the intermediate (ITM stands for the word, "intermediate") obtained from hydrolysis of VAD 414.
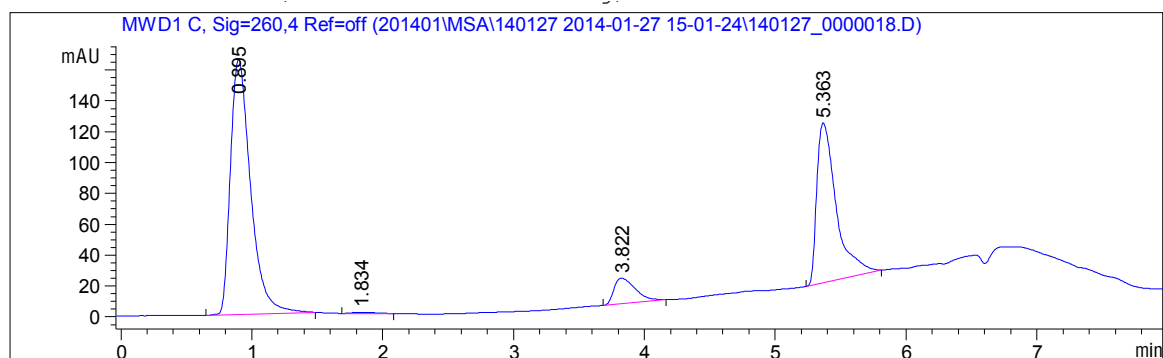
6.2 HPLC Chromatograms

Figure 35: HPLC chromatogram of UbiA standard assay (positive control).

The peak at: **0.895** - 4-hydroxybenzoic acid
3.822 - p-hydroxybiphenyl
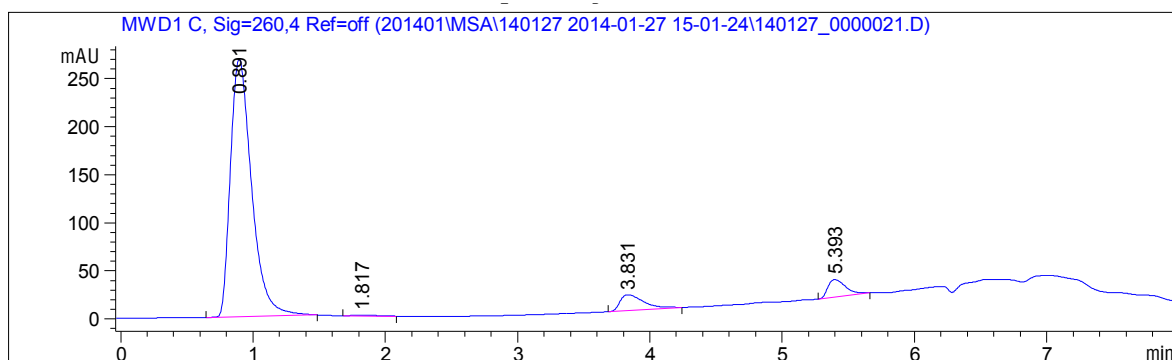**5.363** - 3-geranyl-4-hydroxy benzoic acid

Figure 36: HPLC chromatogram of UbiA standard assay plus compound **28** at 1 mM.

The peak at: **0.891** - 4-hydroxybenzoic acid
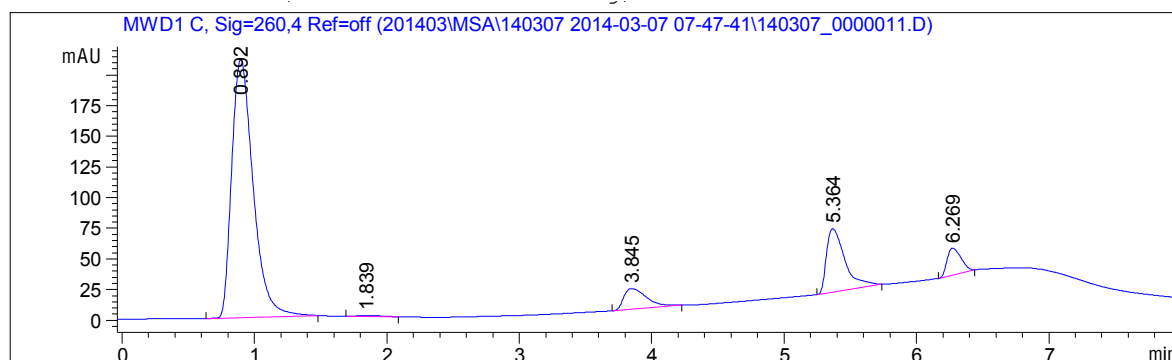**3.831** - p-hydroxybiphenyl
5.393 - 3-geranyl-4-hydroxy benzoic acid

Figure 37: HPLC chromatogram of UbiA standard assay plus FPP at 1 mM.

The peak at: **0.892** - 4-hydroxybenzoic acid
3.845 - p-hydroxybiphenyl
5.364 - 3-geranyl-4-hydroxy benzoic acid
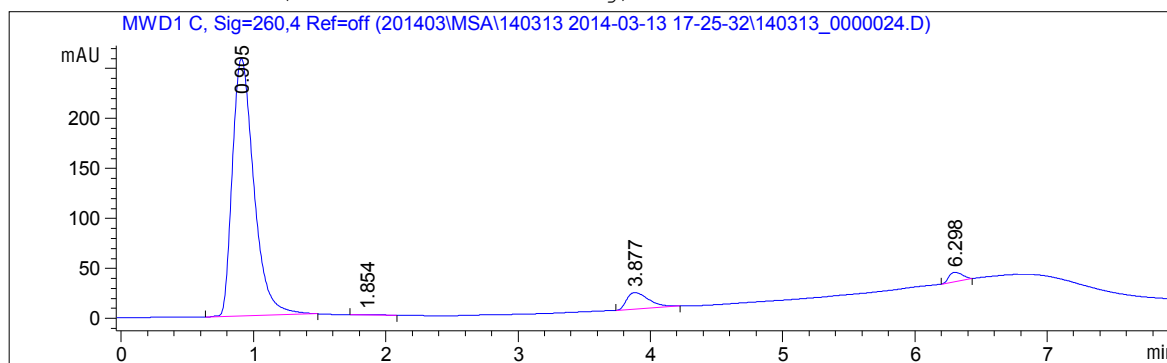**6.269** - 3-farnesyl-4-hydroxy benzoic acid

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Figure 38: HPLC chromatogram of UbiA assay at 10 mM FPP minus GPP.

The peak at: **0.905** - 4-hydroxybenzoic acid
3.877 - p-hydroxybiphenyl
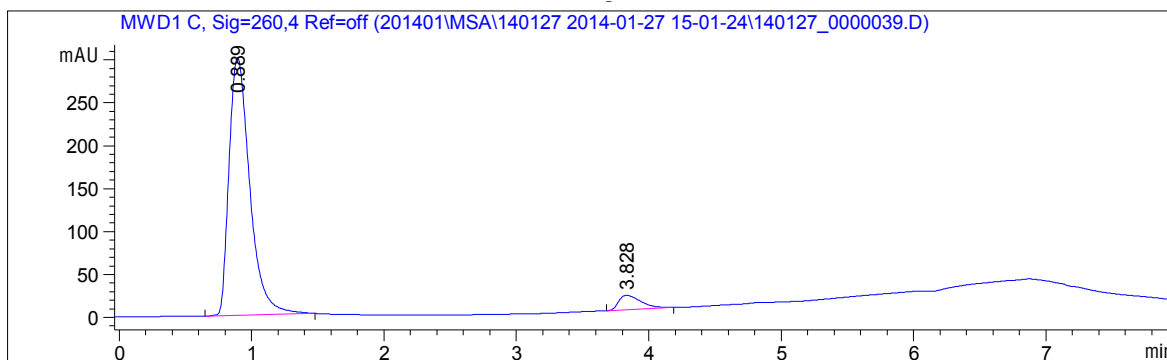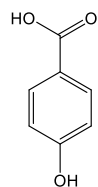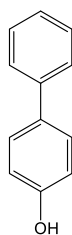**6.298** - 3-farnesyl-4-hydroxy benzoic acid

Figure 39: HPLC chromatogram for the negative control**.**

As a negative control UbiA was omitted from the reaction mixture.
The peak at: **0.889** - 4-hydroxybenzoic acid
3.828 - p-hydroxybiphenyl
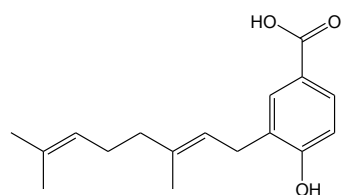no 3-geranyl-4-hydroxy benzoic acid

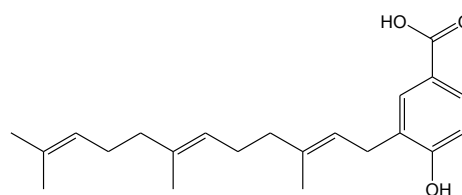Below are the structures of the compounds corresponding to the peaks on the chromatograms.

4-hydroxybenzoic acid

p-hydroxybiphenyl

3-geranyl-4-hydroxy benzoic acid

3-farnesyl-4-hydroxy benzoic acid

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Note: Standard UbiA assay (100 µl) contained:

10 µl DMSO + 10 µl (20 mM 4-HB) + 10 µl (25 mM GPP) + 10 µl (50 mM $MgCl_2$) + 25 µl of UbiA (membrane fraction) + 35 µl (50 mM Tris/HCl (pH 7.8)).

6.3   Curriculum Vitae

Personal information

Name	Amina Msonga
Nationality	Tanzanian
Date of birth	16/01/1980
Gender	Female

Work experience

Dates	02/2010 to date
Position	Assistant Lecturer, University of Dar es Salaam, Tanzania.
Main activities and responsibilities	Teaching, Research and Consultancy.
Dates	11/2007 to 01/2010.
Position	Tutorial Assistant, University of Dar es Salaam, Tanzania.

Education

Dates	10/2010 - 12/2014.
Title of qualification awarded	PhD (Biochemistry)
Thesis	*Screening of Prenyldiphosphate Mimetics for their Potential to Inhibit 4-Hydroxybenzoate Oligoprenyl Transferase.* Supervisor: Prof. Dr. L. A. Wessjohann. Martin Luther University, Halle-Wittenberg, Halle (Saale), Germany.
Dates	November, 2009.
Title of qualification awarded	Master of Science.
Thesis	Bioactivity of Secondary Metabolites from Microbes Isolated from *Idundi*, Traditionally Fermented Bananas from Tanzania. University of Dar es Salaam, Dar es Salaam, Tanzania.
Dates	November, 2006.
Title of qualification awarded	Bachelor of Science Molecular Biology and Biotechnology University of Dar es Salaam, Dar es Salaam, Tanzania.
Scholarships awarded	University of Dar es Salaam/Sida-SAREC project (MSc.: 2006-2008) and DAAD/MoEVT (PhD: 2010-2013).

7 DECLARATION / ERKLÄRUNG

I, **Amina Msonga,** hereby declare that this dissertation is my own original work done under supervision of **Prof. Dr. L. A. Wessjohann** at the Leibniz Institute of Plant Biochemistry, Halle (Saale) and that the references are appropriately cited. This work has not been presented and will not be presented to any other University for a similar or any other degree award.

*Ich, **Amina Msonga**, erkläre hiermit, dass diese Dissertation von mir unter der Leitung von **Prof. Dr. L. A. Wessjohann** am Leibniz-Institut für Pflanzenbiochemie, Halle (Saale), angefertigt wurde und die Quellen entsprechend zitiert wurden. Diese Arbeit wurde bisher nicht veröffentlicht und wird auch nicht an anderen Universitäten für diesen oder einen anderen Abschluss veröffentlicht.*

Halle (Saale), 31.03.2017 _____

Place / Ort, Date / Datum   Signature / Unterschrift

Supervisors:

Prof. Dr. L. A. Wessjohann

Prof. Dr. M. Pietzsch