Synthesis and evaluation of pantothenic acid analogues as potential inhibitors of malaria parasites and bacteria

by
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Summary

A lot of progress has been made in the development and utilization of antimalarial and antibacterial drugs. However, reports of increasing resistance to these antimicrobial agents are threatening to reduce their overall efficacy. In view of this, the need for new antimicrobials is of the paramount importance. Since pantothenate promotes the growth of microbes, analogues of this compound that may act as antimetabolites have been synthesized and tested for their inhibitory properties against microbial growth.

N-substituted pantothenamides is a class of pantothenate analogues previously synthesized in our group that have shown good antibacterial activities, as well as promising inhibition of the proliferation of malaria parasites. In this study the chemical and structural diversity of these analogues were expanded by preparing *N*-substituted pantothenamides in which a methyl functionality was introduced on either the α- or β-carbon of the β-alanine moiety of pantothenate. These compounds were synthesized and purified in parallel, resulting in the production of 40 *N*-substituted α- and β-methylpantothenamides which were investigated as potential antibacterial and antimalarial agents.

The growth inhibitory activities of these compounds were first investigated on *S. aureus*, since in the previous study it was shown that N-substituted pantothenamides demonstrated remarkable inhibitory activities against this microbe. Next, the impact of these compounds on the proliferation of malaria parasites was also investigated to determine if the introduction of the methyl group could improve on the poor inhibition exhibited by the first series of pantothenamides tested against this organism.

Finally pantoyltauramides, sulfonamide analogues of the pantothenamides, were also synthesized and tested for their inhibitory activities against proliferation of malaria parasites *P. falciparum in vitro*. The results show that additional functionalization of pantothenic acid may prove to be a viable strategy for improving the inhibitory activity of these antimetabolites.

Opsomming

Alhoewel goeie vordering gemaak is in die ontwikkeling en gebruik van middels teen malaria en bakteriële infeksies, dreig toenemende weerstand teen sulke verbindings om hul algehele doeltreffendheid te verminder. In die lig hiervan, is die behoefte vir 'n nuwe antimikrobiese middels is van die allergrootste belang. Aangesien pantoteensuur die groei van mikrobes bevorder, is strukturele analoë van hierdie verbinding wat kan optree as antimetaboliete gesintetiseer en getoets vir hul inhiberende eienskappe teen mikrobiese groei.

N-Gesubstitueerde pantoteenamiede is 'n klas van pantoteensuur-analoë wat voorheen in ons groep gesintetiseer is en goeie antibakteriese aktiwiteite, sowel as belowende inhibisie van die vermeerdering van malaria-parasiete, getoon het. In hierdie studie is die chemiese en strukturele diversiteit van hierdie analoë uitgebrei deur die bereiding van *N*-gesubstitueerde pantoteenamiede waarby 'n metielgroep funksie op die α- of β-koolstof van die β-alanien gedeelte van pantoteensuur aangebring is. Hierdie verbindings is in parallel gesintetiseer en gesuiwer om sodoende 40 gesubstitueerde α- en β-metielpantoteenamiede te produseer wat gevolglik as moontlike antibakteriese en anti-malaria agente ondersoek is.

Die groei-inhiberende aktiwiteite van hierdie verbindings is eers op *S. aureus* ondersoek, aangesien 'n vorige studie het getoon dat die *N*-gesubstitueerde pantoteenamiede merkwaardige inhiberende aktiwiteite teen hierdie bakterie toon. Daarna was die impak van hierdie verbindings ook op die vermeerdering van malaria-parasiete ondersoek om te bepaal of die installering van die metielgroep die swak inhibisie wat die eerste reeks van pantoteenamiede teen hierdie organisme getoon het, kon verbeter.

Ten slotte is pantoïeltauramiede, sulfoonamied-analoë van die pantoteenamiede, ook gesintetiseer en getoets vir hul effek op die *in vitro* vermeerdering van *P. falciparum* malaria-parasiete. Die resultate toon dat die funksionalisering van pantoteensuur 'n lewensvatbare strategie vir die verbetering van die inhiberende aktiwiteit van hierdie antimetaboliete kan wees.

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List of abbreviations

ACPs	Acyl carrier proteins
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ASKHA	Acetate and sugar kinase/hsp70/actin superfamily
ATP	Adenosine 5'-triphosphate
CoA	Coenzyme A
CoaA	Pantothenate kinase
CoaB	Phosphopantothenoylcysteine synthetase (also PPC-S)
CoaBC	Phosphopantothenoylcysteine synthetase/ Phosphopantothenoylcysteine decarboxylase (also Dfp)
CoaC	Phosphopantothenoylcysteine decarboxylase (also PPC-DC)
CoaD	Phosphopantetheine adenylytransferase (also PPAT)
CoaE	Dephospho-coenzyme A kinase (also DPCK)
DAST	Diethylaminosulfur trifluoride
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPA	Diphenylphosphorylazide
ESI-MS	Electronspray Ionization Mass Spectroscopy
FAS	Fatty acid synthase
k_{cat}	Turnover number
K_a	Affinity constant
K_M	Michaelis constant
LC-MS	Liquid Chromatography Mass Spectroscopy
MIC	Minimum inhibitory concentration
NADH	Nicotinamide adenine dinucleotide (reduced)
NPP	New permeability pathway
NMR	Nuclear Magnetic Resonance Spectroscopy
OD	Optical density
PanK	Pantothenate kinase
RNA	Ribonucleic acid
TLC	Thin layer chromatography

Chapter 1

A General Introduction

It has been estimated that about one million deaths are attributed to malaria per year, with Africa and Asia registering high rates of mortality and morbidity (*1, 2*). Malaria is caused by a protozoon of the genus *Plasmodium*, of which five species have been identified to cause infections in humans (*1, 2*). Of these, *P. falciparum* causes more deaths followed by *P. vivax* (*1, 2*). Infection with malaria occurs when parasites carried by a female anopheles mosquito are transmitted to humans by injection into the blood stream during a blood meal (*1, 3, 4*).

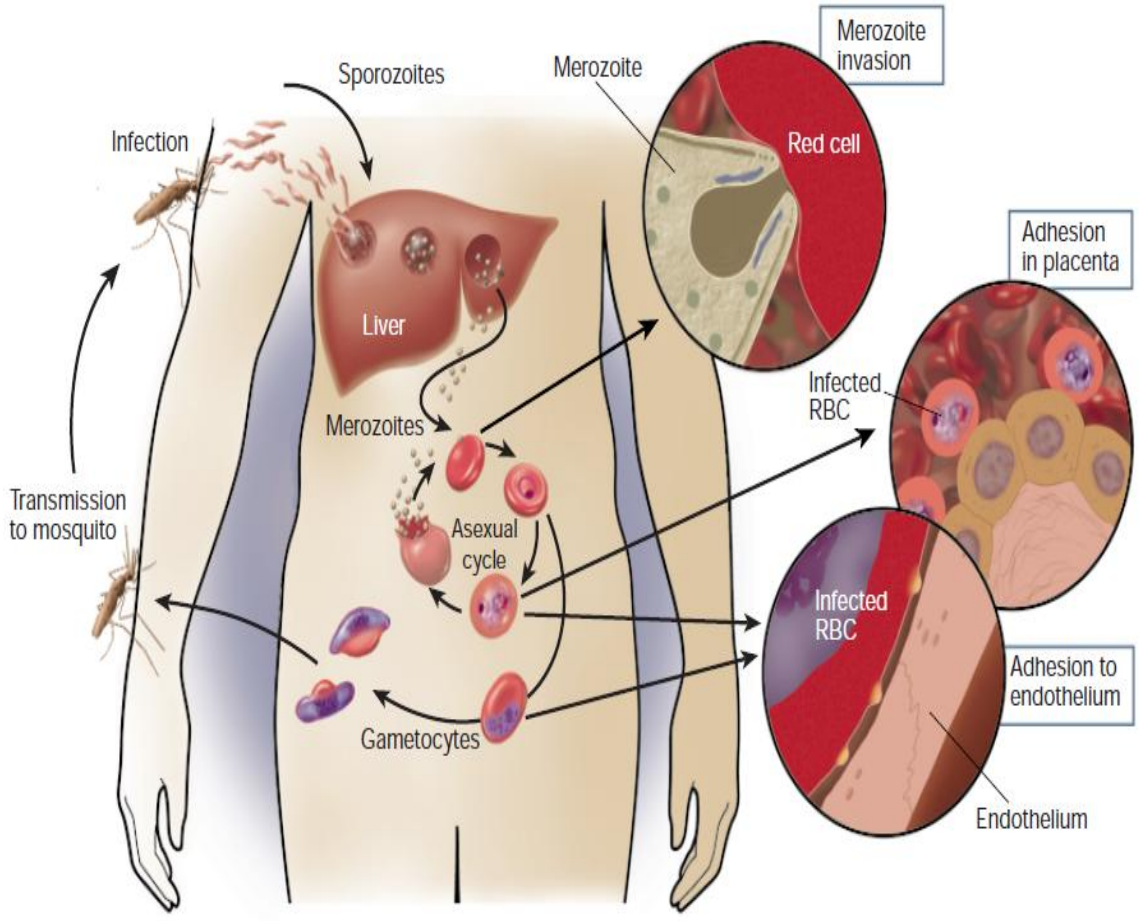

Figure 1.1. Life cycle of the malaria parasite *P. falciparum*, and its pathogenesis. Figure reproduced from Ref. 3.

While in the blood stream, the parasites in the form of sporozoites invade liver cells and take some time to develop and multiply to generate thousands of merozoites (Fig. 1.1) (*3*, *4*). This creates pressure in the liver cells which as a result swell and burst, thereby forcing the merozoites out and into circulation. While in the blood stream, the merozoites then attack the erythrocytes of the host, and in the process the asexual replication cycle (intraerythrocytic stage) begins (Fig. 1.2) (*1*, *3*-*5*). This stage of the parasite life cycle is characterized by onset of clinical symptoms including fevers and anemia, amongst others (*1*, *3*).

1.1 Asexual (intraerythrocytic) cycle of *P. falciparum*

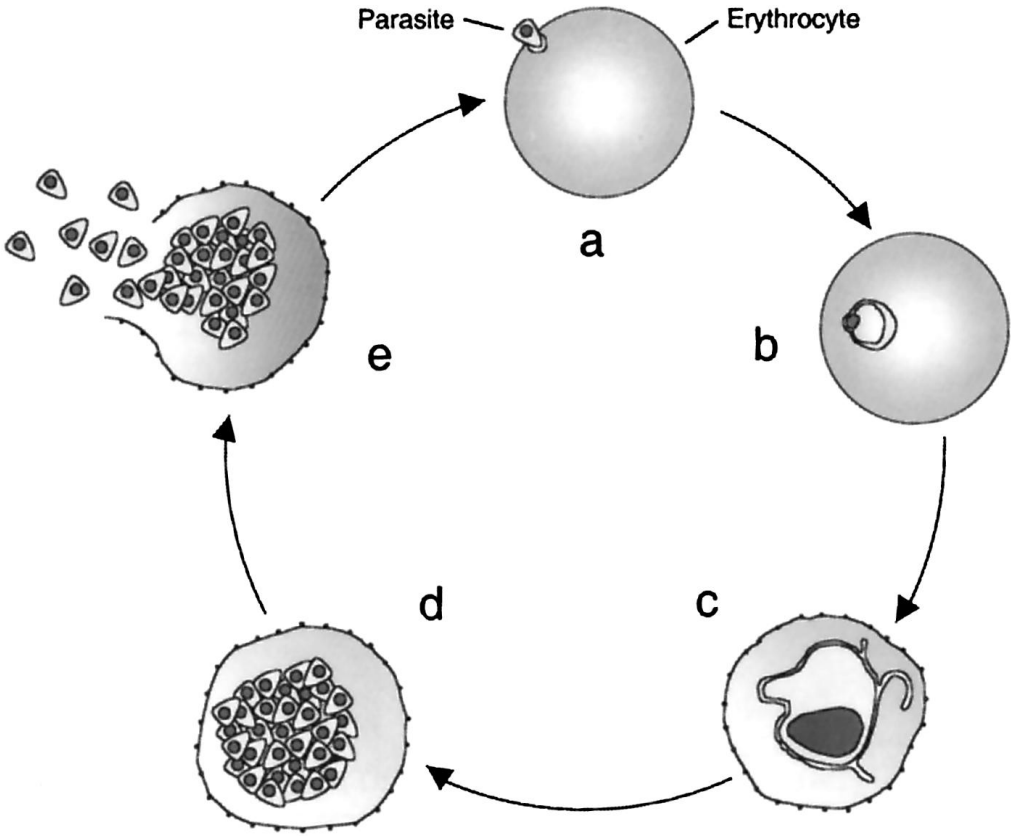

Figure 1.2. Different stages of the asexual cycle of *P. falciparum* (*5*). The cycle is initiated by merozoites after invading the erythrocyte (a). The parasite settles into the erythrocyte cytosol in such a way that it appears as a ring and this is therefore called the ring stage (b). The ring stage parasite develops into a trophozoite, which causes the erythrocytes lose its usual biconcave shape (c). The trophozoite then develops into a schizont (d), which is characterized by multiplication of the parasite into merozoites, which forces the host erythrocyte to swell and burst, releasing the merozoites back into circulation (e). Figure reproduced from Ref. 5.

Various studies aimed at developing a vaccine for malaria have been carried out, and many more are ongoing. However, malaria vaccine development still remains at an early stage due to several factors that have hindered its progress (*1, 2*). This includes the complex life cycle of the parasite as well as the difficulty in selecting a suitable target for vaccine development (*2*). Currently, the only means of controlling malaria is by means of anti-malarial drugs to check and treat the disease (*1, 2*), and through the use of insecticides and mosquito nets to prevent contact between mosquitoes and humans (*6*).

Although a lot of progress has been registered in the development and use of anti-malarial drugs, increasing resistance is threatening to reduce their overall efficacy (*1, 2, 6*). Previously, chloroquine was regarded as the best anti-malarial drug available on the market; however, the emergence of chloroquine drug resistance has rendered the drug to be non-effective (*7*). In fact, it has been reported that *P. falciparum* has developed resistance to almost all the classes of anti-malarial drugs with the exception of artemisinin (*6*), and because of this, anti-malarial chemotherapy is increasingly relying on it as a "treatment of last resort" (*7*). However, in recent studies cases of reduced sensitivity to artemisinins were also reported. This implies that the utility of the artemisinins may soon also be reduced as far as treating malaria is concerned (*8, 9*).

With this background, there is a need to develop new anti-malarial drugs that could be co-administered with artemisinin, so as to increase its life span as an effective drug (*9*). Since there is high possibility that new drugs developed against existing targets will be disposed to the already available resistance as well, emphasis is being directed at developing drugs that would act against novel targets (*1*).

Various studies have targeted the asexual erythrocytic stage of *P. falciparum* (Fig. 1.2) for anti-malarial drug development. This is because during this stage of the life cycle, parasites can be maintained in continuous culture *in vitro (5, 10).* In addition, studies have also shown that the parasite replicates during this stage of the cycle, and therefore requires a vibrant metabolism. During this phase of the life cycle, the parasite has to synthesize DNA, RNA, proteins and lipids (*1, 5*) through a variety of metabolic processes, many of which have been targeted as novel targets for anti-malarial drug development. However, for metabolism to take place in the parasites, uptake of certain essential nutrients is a pre-requisite. One such

important nutrient is pantothenate (vitamin B_5) (Fig. 1.3), (*1*, *6*) the precursor of the essential cofactor coenzyme A in all organisms, including *P. falciparum*.

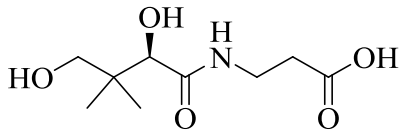

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Figure 1.3. Structure of pantothenate.

1.2 Pantothenate utilization as a target for antimalarial drug development

Studies have highlighted the importance of pantothenate through *in vivo* and *in vitro* studies with avian malaria parasites. In this study, it was found that pantothenate was required for their survival (*11*). Other *in vitro* studies showed that supplementation with calcium pantothenate promoted the growth of erythrocytic stage *P. lophurae* within chick and duck erythrocytes (*11*). Further studies implicated pantothenate for worsening *P. gallinaceum* infection in chickens fed on a diet supplemented with pantothenate, implying that pantothenate promoted survival of the parasites, and hence the severity of the infection (*12*). Furthermore, it was also shown that extracellular supply of pantothenate *in vitro* was responsible for multiplication of erythrocytic stage of *P. falciparum* within human erythrocytes. In this study, growth of parasites was monitored through the incorporation of [^{3}H] hypoxanthine (Fig. 1.4) over a 96 hour period (*13*).

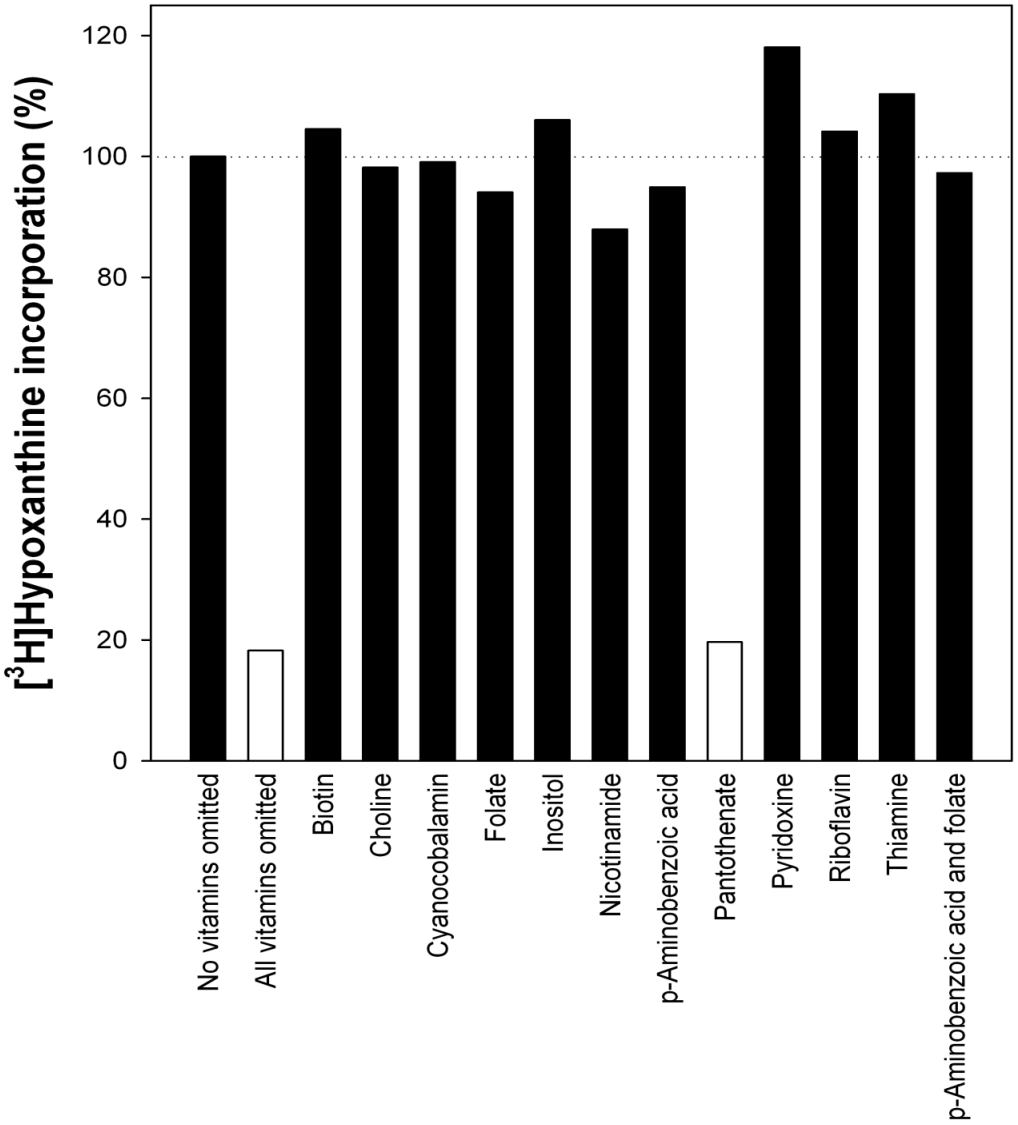

Figure 1.4. [^{3}H]Hypoxanthine incorporation by *P. falciparum* growing intra-erythrocytically over 96 hour in culture medium lacking one or more vitamins (*13*).

As illustrated in Fig. 1.4 above, growth of parasites was inhibited by 82% when all vitamins were omitted. On the other hand, growth of parasites was normal when each of the water-soluble vitamins were excluded one by one from the extracellular solution, except in the case of pantothenate. When this vitamin was excluded from the extracellular solution, growth of parasites was inhibited by 80% (*13*), signifying the importance of pantothenate for growth and survival of the parasites. Importantly, because of this discovery, recent studies have targeted pantothenate utilization for anti-malarial drug development.

Objective and outline

The main objective of this study was the synthetic preparation and biological evaluation of pantothenate analogues – specifically pantothenamides and pantoyltauramides – as potential growth inhibitors of malaria parasites and selected bacteria. This work is based on the ongoing studies in our group that focuses on the potential of CoA biosynthesis and utilization for antimicrobial drug development.

The thesis consists of five chapters:

- Chapter 1 provides a general introduction to the study, its objective and its outline.

- Chapter 2 is a review of the relevant literature, and is presented in two parts. The first part is an overview of CoA metabolism and its importance in living systems. It also describes and compares the biosynthesis of CoA from pantothenate as a precursor in various organisms. In the second part a summary of the pantothenate analogues that have been synthesized to date will be provided, with a specific emphasis on the analogues that were prepared in the search for anti-malarial drugs. The most recent results of the studies from our group on the preparation of pantothenamide-based antimalarials are also described. This chapter concludes with a description of the specific aims that we set out to accomplish in this study.

- Chapter 3 reports on the results of the synthetic work and on the findings on the biological evaluation studies. A discussion of these results is also provided.

- Chapter 4 is a description of all the materials and experimental methods used in this study.

- Finally, chapter 5 provides a conclusion. The main findings of this study will be summarized, and where appropriate, future prospects of this study will be highlighted.

Chapter 2

LITERATURE REVIEW - PART 1:

COENZYME A METABOLSIM AND ITS IMPORTANCE IN LIVING SYSTEMS

2.1 Coenzyme A as a metabolic cofactor

Enzymes are important proteins involved in the catalysis of a variety of metabolic reactions. In some reactions, however, participation of a cofactor is a prerequisite. These cofactors may be organic molecules, or metal ions in other cases. CoA (Fig. 2.1) is one such cofactor the main function of which is to act as a transporter of acyl groups (*14*). In addition, CoA is also important in the activation of acyl groups in numerous biological transformations. Finally, CoA has been shown to be the source of 4-phosphopantetheine, a prosthetic group utilized by carrier proteins of fatty acid, polyketide and non-ribosomal peptide synthases. Although CoA was discovered in 1945 by Lipmann, its structure was only reported in 1953 (figure 2.1) (*14*).

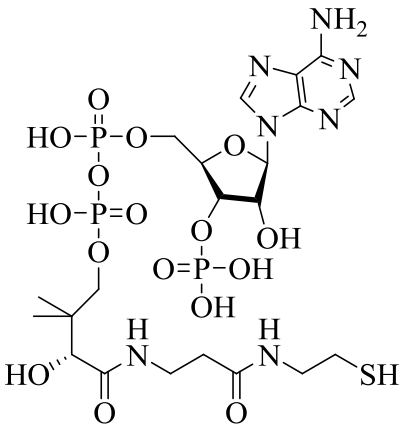

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Figure 2.1. Structure of Coenzyme A

The enzymatic reactions of CoA are dependent on the thiol group (sulfhydryl group), which is the functional group of the molecule, whereas its adenine portion functions as a recognition site for enzymes that bind to CoA (*14*). CoA esters are actively involved in important processes such as fatty acid biosynthesis and degradation, and transcription among others, where it serves as substrate for these reactions (*14*). In addition, long chain CoA esters are

involved in the regulation of various cellular functions. One of the acyl derivatives of CoA, also known as acetyl-CoA, has been implicated in the resistance to antibiotics such as chloramphenicol through acylation. CoA and its acyl derivatives play a pivotal role in metabolic reactions (*14*).

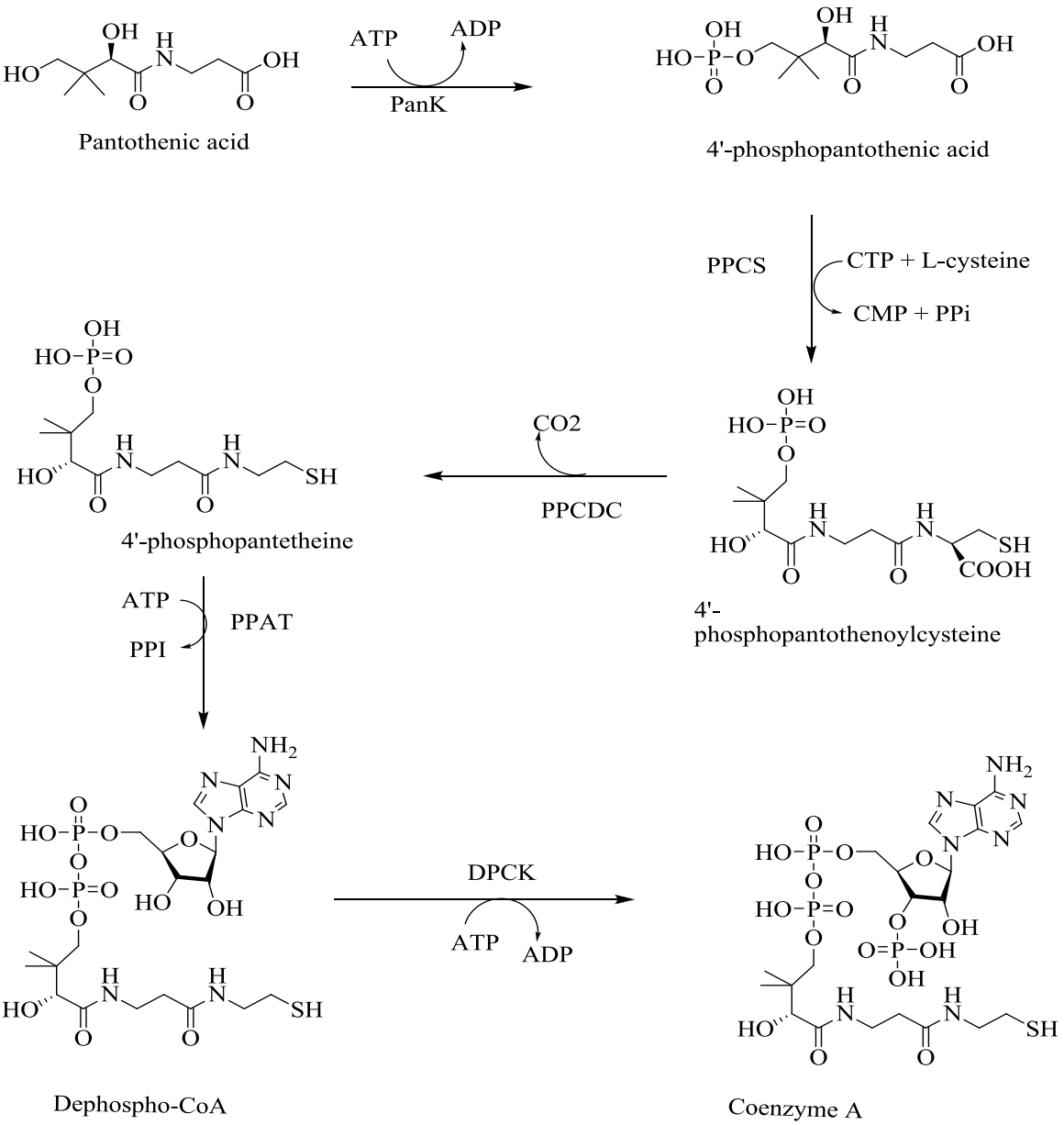
2.2 Pantothenate as CoA pre-cursor

Pantothenate (**1**) (Fig. 1.3), or vitamin B_5 as it is sometimes called, is an important small molecule involved in nutrition (*15*), and has been shown to form the core of the structure of CoA. Industrially, it is used as a food supplement, whereas the esters and derivatives of its reduced form (pantothenol) are used as additives in hair care products (*15*). Large scale production of pantothenate and its derivatives occur via chemical synthesis. In the 1990s, the global market production of pantothenate was in excess of 7×10^6 kg per annum, with most of the produced pantothenate being in the form of calcium pantothenate (about 6×10^6 kg). Pantothenol and its derivatives made up the rest (*15*).

The word "pantothenate" comes from the Greek "pantothen", meaning "from everywhere" due to the fact that most bacteria, including *Escherichia coli*, *Salmonella typhimurium*, and *Corynebacterium glutamicum*, species are capable of synthesizing pantothenate *de novo.* Similarly, plants and fungi, including *Neurospora crassa* and *Aspergillus nidulans* also synthesize pantothenate *de novo*. However, some microbes are unable to synthesize pantothenate, including *Streptococcus pneumoniae* and *Lactobacillus lactis* among others. Likewise, studies have shown that most animals are also not capable of synthesizing pantothenate and as a result rely upon an exogenous source for pantothenate requirements. However, due to its ubiquitous nature, no cases of vitamin B_5 deficiency have been reported (*16*). In other studies, it was demonstrated that survival of *E. coli* is not entirely dependent on pantothenate biosynthesis because of the fact that the microbe is capable of also using exogenous supplies of the vitamin (*17*). Almost all bacteria contain pantothenate transport systems, however, this system is vital for those bacteria that mainly lack the machinery to biosynthesize pantothenate *de novo,* as is the case with *Streptococcus pneumonia* and *Haemophilus influenza (6, 17)*. In *E. coli*, the pantothenate uptake system is mediated by a high affinity (K_M ~0.4 µM) Na^+-stimulated pantothenate transporter which is able to transport pantothenate at a maximal velocity of 1.6 pmol/min/10^8 cells (*6*).

2.3 CoA biosynthesis in bacteria

CoA is biosynthesized in five enzyme-catalyzed steps from pantothenate (Scheme 2.1) (*16*). The first and key step in the pathway involves formation of 4'-phosphopantothenate, a reaction catalyzed by pantothenate kinase (PanK). Next, L-cysteine condenses with 4'-phosphopantothenate producing 4'-phosphopantothenoylcysteine which is subsequently decarboxylated to form 4'-phosphopantetheine (*16*). Two enzymes, 4'-phosphopantothenoylcysteine synthase (PPCS) and 4'-phosphopantothenoylcysteine decarboxylase (PPCDC) catalyse the second and third reactions respectively. The next step involves addition of the AMP moiety of ATP to 4'-phosphopantheine to form dephospho-CoA, a step catalysed by phosphopantetheine adenylyl transferase (PPAT). This is followed by phosphorylation by dephospho-CoA kinase (DPCK) at the 3'-OH of the ribose moiety resulting in the production of CoA (*16*).

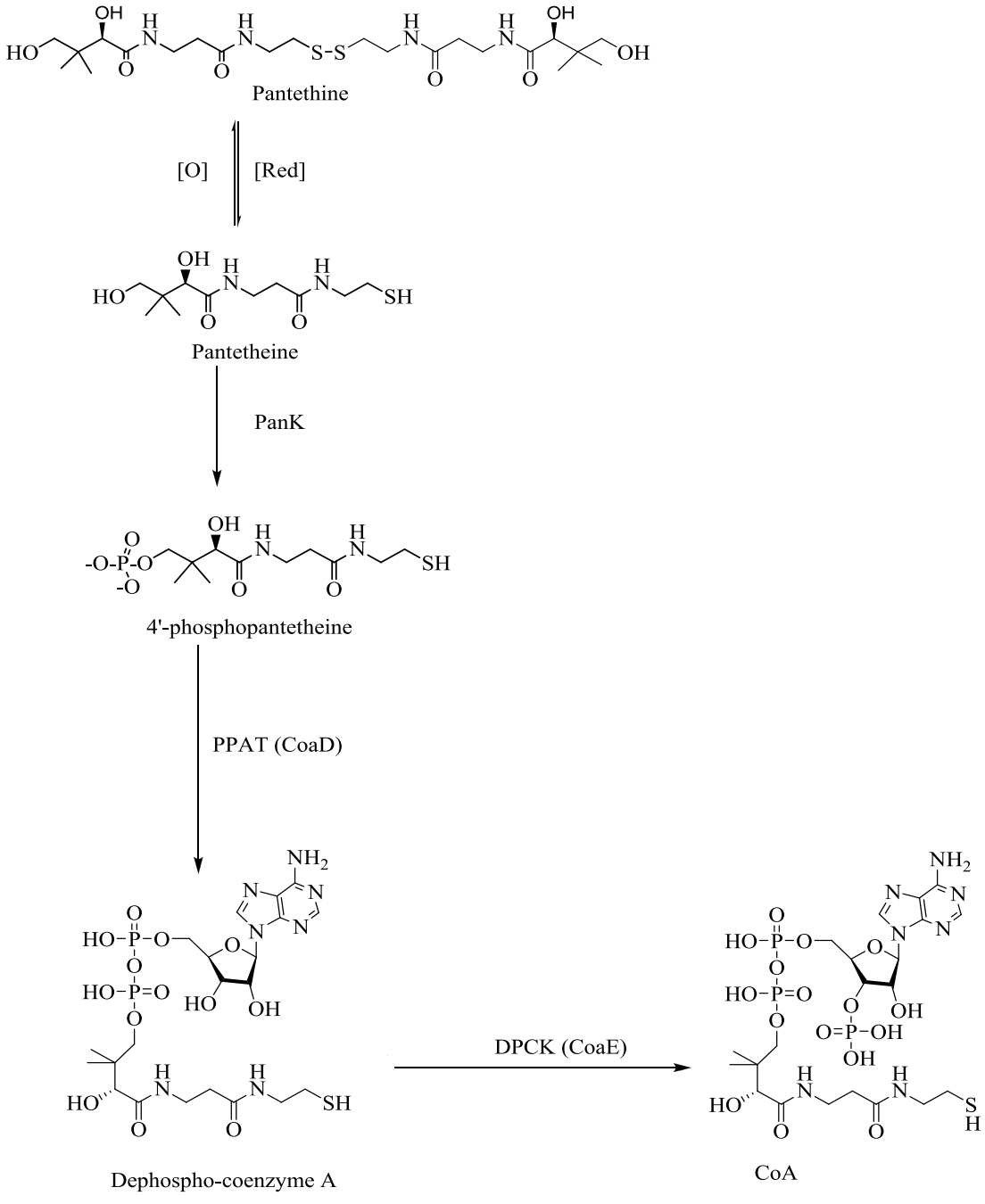


Scheme 2.1. Pathway for biosynthesis of CoA from pantothenate (vitamin B_5)

2.4 Pantetheine as CoA precursor

In bacteria, pantetheine, or its oxidized form, pantethine, is also utilized to produce CoA in the so-called CoA salvage pathway (Scheme 2.2). In this pathway, pantetheine is phosphorylated by PanK to produce 4'-phosphopantetheine, followed by adenylylation and phosphorylation by PPAT and DPCK to form CoA (*6*). The discovery of this pathway was based on studies that showed that pantetheine has growth-promoting activities in lactic acid

bacteria grown in the absence of pantothenic acid (*18, 19*). It has also been reported that *E. coli* has the ability to synthesize CoA by using dietary pantetheine (*6, 20*). Recently, *E. coli* PanK has been shown to catalyze the phosphorylation of pantetheine to 4'-phosphopantetheine, with subsequent conversion to dephospho-CoA and CoA using PPAT and DPCK respectively (*21*).

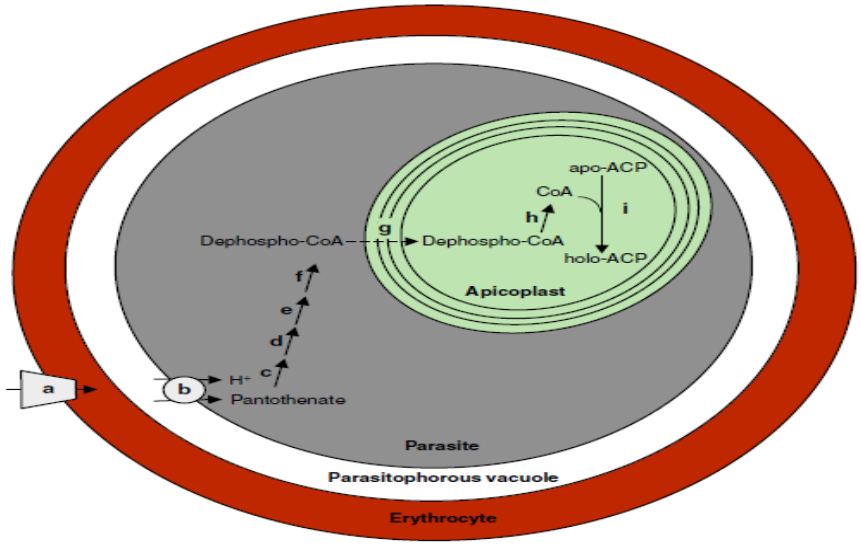


Scheme 2.2. CoA salvage pathway using pantetheine as a precursor

2.5 CoA biosynthesis in malaria parasites

As discussed in chapter 1, pantothenate is an essential nutrient of the intra-erythrocytic stage of the human malaria parasite *Plasmodium falciparum*. However, it has been shown that pantothenate is not capable of traversing through the membranes of normal uninfected human erythrocytes, but is permeable to *P. falciparum*-infected erythrocytes. This is due to the presence of the "new permeability pathways" (NPP), which are induced by the parasite upon infection of the host cell (*22, 23*). Other studies have also shown that the NPP possesses broad specificity in that it is permeable to neutral and anionic substances, and consequently makes the cell membrane more permeable to a variety of nutrients and metabolic waste products. However, NPP do not occur in normal and uninfected erythrocytes (*24, 25*).

When pantothenate reaches the inside of the host erythrocyte, it is thought to traverse into the parasitophorous vacuole through the membrane that surrounds it. At this stage, it is taken up by the parasite with the aid of a transporter. This is supported by studies done by Saliba and Kirk who demonstrated that uptake of pantothenate by the intracellular parasite occurs via an H^+-coupled transporter. They showed that this transport system possesses a low affinity for pantothenate (K_m ~23 mM), and that the transport of pantothenate occurs in a 1:1 stoichiometry with H^+ (*23*). Once in the parasite cytosol, pantothenate is phosphorylated by PanK (*Pf*PanK) (*26*) with subsequent conversion to dephospho-CoA (scheme 2.3). It is presumed that dephospho-CoA then finally enters the apicoplast where it gets converted to CoA. (*6*).



Scheme 2.3. Pantothenate uptake and utilization by *P. falciparum*-infected human erythrocyte. Figure reproduced from Ref. 5

2.6 CoA biosynthesis as a target for anti-microbial drug development

Due to its essential role in metabolism, and the fact that all organisms have to produce their own CoA *de novo*, CoA biosynthesis has been considered an important potential target for drug development. Three processes have been identified as specific targets:

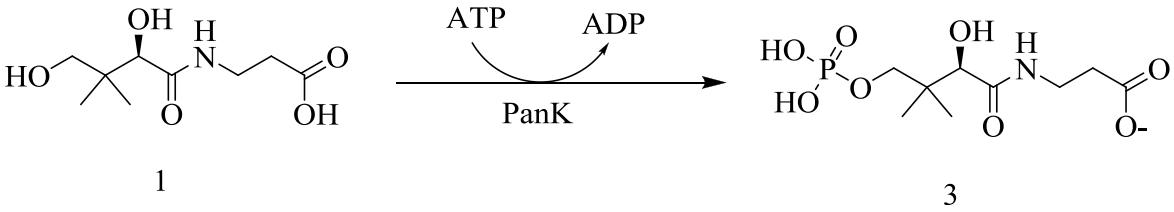
1. Pantothenate uptake
2. Pantothenate phosphorylation
3. CoA utilization

2.6.1 Pantothenate uptake as target

As discussed before (*22*), normal human erythrocytes are impermeable to pantothenate. However, recent studies have shown that pantothenate can traverse through the membranes of normal human erythrocytes, although the uptake process is still very slow as compared to the speedy uptake of pantothenate by *P. falciparum*-infected erythrocytes via the "NPP" (*27*). In addition, comparison of the mammalian pantothenate transport system to that of *P. falciparum* showed that mammalian cells take up pantothenate via a Na^+: pantothenate symport system, compared to the H^+:pantothenate system in *P. falciparum*. Furthermore, it was also shown that the mammalian pantothenate transport systems have high affinity for substrates with a K_M of 2-5 µM (*28-30*) in contrast with pantothenate transport system in *P. falciparum* which exhibits low affinity for its substrate (K_M ~ 23 mM) (*23*). Since the pantothenate requirement of the infected erythrocyte is different from health erythrocytes, these remarkable differences can be exploited as a target for drug design.

2.6.2 Pantothenate phosphorylation via pantothenate kinase (PanK) as target

As previously stated, pantothenate kinase (PanK) is an enzyme that catalyses the phosphorylation of pantothenate to form 4-phosphopantothenate in the CoA biosynthetic pathway. The process is ATP-dependent, and is the first committed step in the biosynthesis of CoA (scheme 2.4) (*16*).



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Scheme 2.4: Conversion of pantothenate to 4-phosphopantothenate by PanK.

Three types of PanK can be distinguished based on the differences in their primary sequences, structural motifs and folds, and kinetic properties (*31*). Importantly, despite the fact that the eukaryotic and prokaryotic PanKs are structurally different, they are both feedback-inhibited by CoA or its thioesters, thereby acting as regulators of homeostasis and CoA biosynthetic processes in the cell (*32*, *33*). Interestingly, some prokaryotes contain genes that encode for two different PanK's, as in the case of *Bacillus subtilis*, and *Mycobacterium tuberculosis (31)*, which contain both type I and III PanKs.

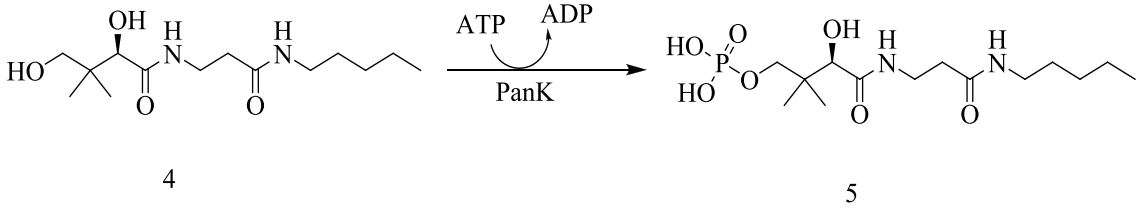
The fact that CoA biosynthesis depends on PanK as a key rate-determining enzyme suggests that targeting this enzyme for drug development may be possible. Moreover, since the human and *P. falciparum* PanK (*Pf*PanK) have low sequence similarity, targeting *Pf*PanK for drug development seems like an attractive target. However, structurally both the human PanK and *Pf*PanK are type II classes of PanKs, indicating that the design of selective inhibitors targeting *Pf*PanK may be a challenge.

The biology of PanK types, which has been comprehensively reviewed by Strauss *et al* (*34*), is discussed in the following subsections.

2.6.2.1 Type I Pantothenate Kinase (PanK-I)

Type I pantothenate kinases (PanK-I) are commonly found in eubacteria species. The best characterized member of this group is the *Escherichia coli* enzyme (*31*). PanK-I is encoded by *coaA* gene (*33*). Structurally, bacterial PanK-I has been shown to be a member of the P-loop kinase superfamily (*32*). *E. coli* PanK-I is a 36 kDa dimer containing identical subunits (*33*), with an A-type ATP binding consensus sequence GXXXXGKS (*35*). Studies have shown that PanK-I is inhibited by CoA, and to a lesser degree by its thioesters *in vitro* (*36*). In addition, non-esterified CoA is thought to be most effective inhibitor of *E. coli* PanK, whereas acetyl-CoA (*37*), and malonyl-CoA are the most potent regulators in eukaryotic

systems. Moreover, it has been reported that PanK-I enzymes are capable of phosphorylating pantothenate analogues known as N-alkylpantothenamides (*32*). For example; the phosphorylation of *N*-pentyl pantothenamide (N5-Pan) (scheme 2.5) demonstrates the reactivity of PanK-I towards pantothenamides (*34*).



4 5

Scheme 2.5. Phosphorylation of *N*-pentyl pantothenamide by PanK-I

Kinetic analysis studies have revealed that CoA exerts feedback inhibition through competitive inhibition of the ATP binding site (*38*). The ATP binding site has been shown to contain lysine 101 as a key residue implicated in the nucleotide substrate and inhibition binding (*39*). Studies on carbon source shift experiments in *E. coli* demonstrated that CoA is the most effective inhibitor of PanK activity *in vivo (40).*

2.6.2.2 Type II Pantothenate Kinase (PanK-II)

These enzymes are mainly found in eukaryotes including plants (*Arabidopsis thaliana*) and fungi (*Aspergillus nidulans*), among others (*31*). In addition, it has been reported that the mammalian PanK belongs to this group, and the best characterized member of the group is the murine PanK. Recently, a prokaryotic type II enzyme was identified in *Staphylococcus aureus* (*S. aureus*) (*41*). Contrary to PanK-I enzymes which belong to P-loop family of kinases, bioinformatics studies predicted that PanK-II is a member of ribonuclease H-like family of kinases (*42*). The R-nase H-like group is slightly related to the ASKHA (acetate and sugar kinase/hsp70/actin superfamily) (*31*). Studies of the crystal structures of the *S. aureus* PanK-AMPPNP complex confirmed these predictions (*42*). PanK-I and PanK-II share little sequence similarity, although both are regulated through feedback inhibition by CoA and its thioesters (*41*), with acetyl-CoA demonstrating high potency. Like PanK-I enzymes, PanK-II also recognize pantothenamides as alternative substrates, phosphorylating them to allow the formation of inactive CoA analogues (*33*).

2.6.2.3 Type III Pantothenate Kinase (PanK-III)

The most recent discovery has been the identification of PanK-III in bacteria. This enzyme is encoded by the *coaX* gene, a terminology used to distinguish this enzyme from the PanK-I gene (*coaA*) (*32*). This enzyme is most commonly found in pathogens such as *Helicobacter pylori* and *Pseudomonas aeruginosa (33),* and also in *Bacillus subtilis (42).* PanK-III differs from PanK-I and PanK-II in that it is not inhibited by CoA or its thioesters, a property that is unique among organisms which contain this type of enzyme (*31, 32*). Although the K_M-values of PanK-I and PanK-II for their substrates are similar (in the micromolar range), PanK-III enzymes show a remarkable high K_M for ATP (up to ~10 mM) (*33*), which in some cases constitutes an increase of 30- to 100-fold over PanK-I and PanK-II (*31*). PanK-III enzymes are not inhibited by N5-Pan, nor do they act on it as an alternative substrate (*42*).

Apart from having an extraordinarily high K_M for ATP, CH_2Cl_2Pank-III enzymes also have a requirement of monovalent cations (such as NH_4^+ or K^+) for activity (*32*). Structural studies have shown that PanK-III belongs to the ASKHA superfamily, as confirmed by crystal structures of *Thermotoga maritima* PanK at 2.0-Å resolution (*31*). Additionally, crystal structures of the PanK-III from *P. aeruginosa* (*Pa*PanK) in complex with pantothenate (PaPanK-Pan) also confirmed this (*42*). The main characteristics of PanK enzymes are summarized in table 2.1 (*34*).

Table 2.1. Comparison of the main characteristics of the three PanK enzymes.

Attribute	**Type I PanK**	**Type II PanK**		**Type III PanK**
	(*E. coli* PanK)	(*A. nidulans* PanK)	(*S. aureus* PanK - atypical)	(*H. pylori* PanK)
K_M (pantothenate	36 ± 4 µM	60 µM	23 µM	5.5 µM
K_M (ATP)	136 ± 15 µM	145 µM	34 µM	7.9 mM
k_{cat}	0.30 ± 0.13 s^{-1}	1.95 s^{-1}	1.65 ± 0.09 s^{-1}	2.09 ± 0.26 s^{-1}
Cofactor requirements	Mg^{2+}	Mg^{2+}	Mg^{2+}	Mg^{2+}, K^+, or NH_4^+
Feedback inhibitor	CoA (less by CoA thioesters)	Acetyl-CoA	None	None
Pantothenamides	Are substrates	Are substrates	Are substrates	No effect
Structural fold	P-loop kinase	ASKHA superfamily	ASKHA superfamily	ASKHA superfamily

2.6.3 CoA utilization as target

Fatty acids play a crucial role in cells as precursors for metabolic processes. They are synthesized by elongation of acyl chains using malonyl-CoA as a precursor, and fatty acid synthase (FAS) catalyzes the elongation process. Previously, it was thought that *P. falciparum* was unable to synthesize fatty acids *de novo*, instead it survived by scavenging fatty acids from the host erythrocyte. However, the discovery of the apicoplast in *P. falciparum* overturned this assumption (*43*). Molecular and cellular biological analyses aimed at understanding the cells of the parasite, as well as the completion of the genome sequences of *P. falciparum* produced results that showed that *Plasmodium* parasites have the ability to synthesize fatty acids *de novo*. In addition, it was also shown that the parasites contain the genes encoding type II FAS, which catalyzes the synthesis of these lipids (*44*). Furthermore, bioinformatics studies showed that the *Plasmodium* genome contain genes encoding proteins with primary sequences showing homology to the orthologs in bacteria and plants that are involved in the *de novo* synthesis of fatty acids. In plants and bacteria, these enzymes form a complex that functions as an FAS, known as the type II FAS (*44*). Studies by Waller and co-workers (*43*) showed that *P. falciparum* relies on type II FAS for *de novo* fatty acid synthesis. On the other hand, FAS in animals including humans appears as a single large polypeptide multifunctional protein belonging to type I FAS (*43*, *44*). Interestingly, the fact that type II FAS is not available in humans, suggests that the corresponding pathway can be investigated as a possible target for antimalarial drug design.

2.7 Conclusion

All the three targets in CoA metabolism that have been identified for potential drug development, namely pantothenate uptake, pantothenate phosphorylation by PanK and CoA utilization may potentially be exploited by the use of compounds that mimic the structure of pantothenate. Such pantothenate analogues can act as antagonists (antimetabolites) of CoA by virtue of their chemical relationship with the metabolite, or mimic its activities, or inhibit enzyme reactions involved in its synthesis or utilization. In the next section the pantothenate analogues that have been synthesized and evaluated as inhibitors to date will be reviewed.

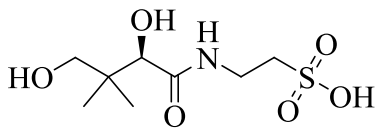
LITERATURE REVIEW - PART II:

PANTOTHENIC ACID ANALOGUES AS ANTIMICROBIAL AGENTS

Studies focusing on pantothenic acid analogues as inhibitors of microbial growth were initiated in early 1940, and since then many analogues of pantothenic acid have been synthesized and tested for their inhibitory properties (*6*). While many were found to lack activity, some promoted the growth of microbes (specifically those that rely on dietary supply of pantothenic acid) while other analogues were effective inhibitors (*6*). A summary of these analogues, thoroughly reviewed by Spry et al (*6*) will be discussed below. An important aspect of the study of the inhibitory effects of pantothenate analogues related to the extent to which any observed inhibition can be reversed by the addition of pantothenate; in cases where an analogue binds to its target less effectively than pantothenate, such a reversal will be easy to accomplish with even small amounts of additional pantothenate. In the following summary the extent to which inhibition was reversed in this manner will also be highlighted in each case.

2.8 Pantoyltaurine and related compounds

Pantoyltaurine, **6,** is an analogue of pantothenic acid, **1**, containing a sulphonic acid group (Fig. 2.2) instead of the carboxylate of pantothenic acid. The most basic of these analogues was named pantoyltaurine by Barnette and Robinson in 1942 (*45*).



6

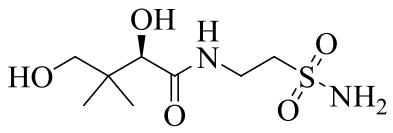
Figure 2.2: Structure of pantoyltaurine

This analogue was found to be effective at inhibiting growth of bacteria which rely on dietary supply of pantothenic acid, including *Lactobacilus arabinosus* and *Streptococcus lactis*,

among others. However, **6** did not negatively affect growth of microbes which synthesize their own pantothenic acid, such as *E. coli* and *S. Aureus (46, 47)*. There was an observed variation in potency of **6** against different microbes. This is demonstrated by the fact that *Lactobacillus arabinosis* is fully inhibited at pantoyltaurine concentration of 1000-fold the concentration of pantothenate in the medium, while *Leuconostoc mesenteroides* only showed growth inhibition at pantoyltaurine concentration of 162000-fold that of pantothenate (*6*).

Other studies demonstrated that **6** negatively affected growth of bacteria *in vivo*, through administration of large subcutaneous dose of this compound to rats. On further examination, the analogue was found to protect rats from infection caused by a virulent strain of *Streptococcus haemolyticus (48)*. In addition, simultaneous administration of pantothenate to rats resulted in reversing the therapeutic effects of **6**, showing that antibacterial properties were due to pantothenate dependent processes. Interestingly, despite a fairly high dose of **6** administered to rats, the analogue was shown to be non-toxic, and the rats responded well by increasing their weight normally throughout the treatment (*48*).

Since compound **6** demonstrated inhibitory characteristics both *in vivo* and *in vitro*, and considering the fact that the analogue was easily synthesized, this was enough motivation to prepare more analogues similar to **6** structurally. As a result, pantoyltauramide **7**, (Fig. 2.3), a sulphonamide analogue of pantothenic acid was prepared (*45, 49*). Other compounds which resulted from substitutions of sulphonic acid group of pantoyltaurine with a thiol, a disulphide, and a sulphone, were also synthesized. The majority of these compounds were effective at inhibiting bacterial growth *in vivo*, of microbes which need supply of exogenous pantothenic acid such as *Streptococcus haemoliticus*, and *Lactobacillus arabinosus* among others; and *in vitro*, in strains such as *Corynebacterium diptheriae*. However, the effectiveness of these analogues as inhibitors was reversed by pantothenic acid in all cases (*50*).



7

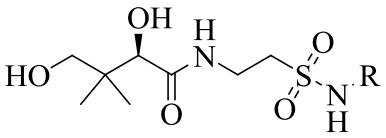
Figure 2.3: Structure of pantoyltauramide

Since the discovery that pantothenic acid is required for the survival of the intra-erythrocytic stage of malaria parasites, studies have been intensively conducted in search of compounds with antimalarial activity that would affect the utilization of pantothenic acid by the parasites. The quest for these compounds led to the synthesis of pantothenic acid analogues such as pantoyltaurine and pantoyltauramide which were then tested against malaria parasites.

When pantoyltaurine was tested against *P. relictum*-infected canaries, *P. lophurae*-infected ducks and *P. gallinaceum*-infected chickens for its antiplasmodial activities, the analogue demonstrated inactivity. Another analogue, pantoyltauramide, **7**, (Fig. 2.3) was shown to inhibit the growth of *P. gallinaceum* after intravenous administration of this analogue. However, the antiplasmodial activity of the analogue was reversed in the presence of increased concentration of pantothenate (*51*).

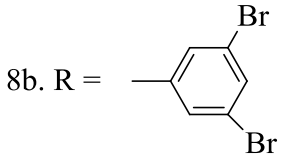
In other studies, *N*-substituted pantoyltauramides, **8**, were prepared (Fig. 2.4), and when tested for antiplasmodial activity in *P. gallinaceum* infected chicks through intravenous administration, it was found that compound **8a** inhibited growth of the parasites. However, the activity of these analogues was less as compared to the activity of pantoyltauramide (*51*).

In another attempt, Winterbottom *et al* synthesized other *N*-substituted pantoyltauramides (Fig. 2.4) which were found to be more active against *P. gallinaceum*-infected chickens than pantoyltaurine and pantoyltauramide (*51*).

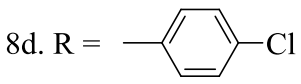


8

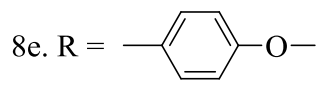
8a. R = Me



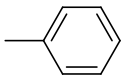
8b. R =



8d. R =



8e. R =



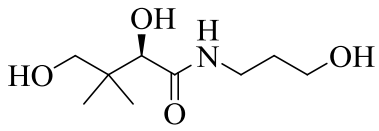
8c. R =

Figure 2.4: *N*-substituted pantoyltauramides

According to Brackett et al (*12*), a few of the *N*-substituted pantoyltauramides (Fig. 2.4) demonstrated higher activity compared to the activity of the antimalarial quinine, and it was also found that the most active compound, **8d,** was four times more active than quinine, and sixteen times more active against *P. gallinaceum* blood-induced infection. In addition, **8d** suppressed the growth of *P. lophurae in vitro*, when incorporated into the culture at a concentration of 360 µM. Nevertheless, supplementation of 40 µM pantothenate to the culture medium reversed the antiplasmodial activity (*12*). Furthermore, **8d** also inhibited the proliferation of the monkey malaria parasite *P. coatneyi* and the human malaria parasite *P. falciparum* within monkey and human erythrocytes respectively. When the cultures were incubated with **8d** in concentrations of between 220 and 820 µM for two days, there was a remarkable reduction in parasitemia levels of these parasites. However, the antiplasmodial activity of *P. coatneyi* was to some extent reversed in the presence of 40-65 µM pantothenate in the cultures (*12*).

2.9 *N*-Pantoyl-substituted amines

N-Pantoyl substituted amines constitute all analogues in which the β-alanine moiety of pantothenate has been replaced by another amine. The most important of these is pantothenol, **9**, (the alcohol analogue of pantothenic acid) first prepared by Pfaltz in1943.



9

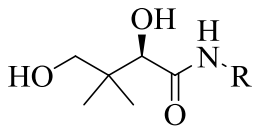
Figure 2.5: Structure of pantothenol.

This analogue was successful in preventing achromatrichia (loss of pigment in hair) of black rats, the activity attributed to the oxidation of **9** to pantothenic acid in the animal. In addition, compound **9** demonstrated inhibitory activities towards growth of lactic acid bacteria including *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, and *Lactobacillus arabinosus in vitro.* However, in a manner similar to pantoyltaurine, growth inhibition was competitive with respect to pantothenic acid (*52, 53*). Further studies with compound **9** demonstrated that the analogue was inefficient at inhibiting the growth of the yeast *Saccharomyces cerevisiae*. On the other hand, at 300 times the concentration of pantothenate,

the analogue demonstrated more potency as compared to pantoyltaurine when tested against *Leuconostoc mesenteroides* and completely inhibited the growth of this organism (*52, 53*).

Since compound **9** can be converted back to pantothenic acid in some organisms, and since the analogue has been shown to be effective at inhibiting growth of different bacteria *in vitro*, Saliba *et al* (2005) evaluated the effect of this analogue as an inhibitor of the growth of the malaria parasite *P. falciparum in vitro* (*54*). In this study, compound **9** was administered orally to mice suffering from murine parasite *Plasmodium vinckei vinckei* infection. Results showed that the analogue was effective at inhibiting growth of the parasites *in vitro*. Additionally, the analogue was also able to suppress multiplication of parasites *in vivo* through reduction of parasitemia levels in mice subjected to daily oral administration of pantothenol for 4 days after infection (*54*).

In other studies, other *N*-pantoyl-substituted amines, **10**, (Fig. 2.6) were also prepared as pantothenic acid analogues.



10

Figure 2.6: Structure of *N*-pantoyl substituted amine

These analogues were prepared by reacting pantolactone with a variety of amines including hydroxyamines, alkylamines, as well as amino acids (*6*). Many of these compounds demonstrated inhibitory activities against lactic acid bacteria, but growth inhibition occurred in a competitive manner with respect to pantothenic acid (*6*).

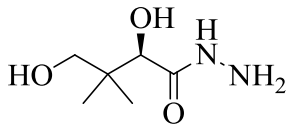
In studies conducted by Fissekis *et al* (*6, 55*), analogues of pantothenic acid were prepared by reacting pantolactone with a variety of arylalkylamines. These *N*-pantoylaryl alkylamines had inhibitory activities against growth of lactic acid bacteria, and again it was found that the inhibition was overcome by increasing pantothenate concentration (*6, 55*).

Further studies in search of pantothenic acid analogues were conducted by Parker et al (*56*). In this study, a variety of functional groups including chloro- and nitro- groups were attached

to the phenyl group of *N*-pantoylarylalkylamines, forming analogues called *N*-pantoyl (substituted-phenyl) alkylamines (*56*). By using two microorganisms, *Lactobacillus arabinosus* and *Leuconostoc mesenteroides*, the potency of these analogues was evaluated. Further analysis showed that all analogues were effective at inhibiting growth of lactic acid bacteria. However, growth inhibition was competitive with respect to pantothenic acid. Additionally, it was further demonstrated that analogues with chloro- and nitro-substituents were more potent against these two microorganisms (*56*).

2.10 Pantoylhydrazide and related compounds

In an attempt to produce more potent analogues of pantothenic acid, other groups (*49*) generated various analogues, including pantoylhydrazide, **11** (Fig. 2.7).



11

Figure 2.7: Structure of pantoylhydrazide

When compound **11** was tested on microbes, it demonstrated slightly more potent inhibitory activities against the growth of *Lactobacillus casei in vitro* than pantoyltaurine in the presence of 10 µM pantothenate. However, when the extracellular supply of pantothenate was reduced to 0.1 µM, the analogue demonstrated 50 times more potency, clearly showing that the activity was antagonized by pantothenate (*49*). Furthermore, **11** negatively affected growth of *Streptococcus pyogenes in vitro*, but failed to reverse bacterial activity in rats infected with *Streptococcus pyogenes* infection after subcutaneous administration in four doses of 1 g/kg body mass over a 12 hour period after infection (*49*).

2.11 Pantothenones and related compounds

In other studies, other groups substituted the carboxyl group of pantothenic acid with a variety of alkylketones. In this manner, two pantothenic acid ketone analogues were prepared; first a methyl ketone analogue called methylpantothenone, **12a**, was synthesized (Fig. 2.8) which demonstrated toxic effects towards the growth of the bacterium

Lactobacillus casei and the yeast S*accharomyces cerevisiae*, with microbial growth lowered to 50% at concentrations of **12a** between 460 and 2300 µM. Importantly, the activity of the analogue was not affected by pantothenic acid (*57*).

12a 12b

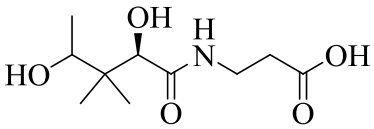
Figure 2.8: Structures of methylpantothenone and phenylpantothenone

Secondly, a phenylketone analogue termed phenylpantothenone, **12b**, (Fig. 2.8) was synthesized and showed growth inhibition of several bacteria that depends upon availability of exogenous pantothenic acid for survival (such as *L. casei*), as well as a variety of microorganisms which are capable of synthesizing their own pantothenic acid, such as *E. coli*. It was shown that at 0.2 µM pantothenic acid concentrations, growth of *L. casei*, *E. coli*, and *S. aureus* was lowered to half maximum at phenylpantothenone concenterations of 190, 7200, and 500 µM respectively. When compared to **12a**, the inhibitory properties of **12b** were reversed by addition of pantothenic acid (*57*).

2.12 Analogues of pantothenic acid with a modified pantoyl moieties

Most pantothenic acid analogues have been produced as a result of structural modifications to the β-alanine moiety of pantothenic acid. However, some analogues have been produced as a result of structural modification to the pantoyl group of pantothenic acid (*6*).

One such pantoyl-modified analogue, ω-methylpantothenic acid, **13**, proved to be an effective antagonist of pantothenic acid, and inhibited growth of lactic acid bacteria *in vitro (58).*



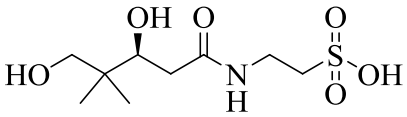
13

Figure 2.9: Structure of ω-methylpantothenic acid.

Furthermore, compound **13** also showed activities that prevented the proliferation of β-haemolytic *Streptococcus* in mice when administered in the diet (*59*). Since **13** was seen to be effective at inhibiting bacterial growth *in vitro*, and growth of β-haemolytic Streptococci *in vivo*, a variety of ω-substituted analogues were prepared by replacing the methyl group of **13** with alkyl or aryl groups. Although the resulting analogues demonstrated growth inhibition against lactic acid bacteria, they were less active as compared to ω-methylpantothenic acid (*60*).

In another effort, Fissekis et al (*61*) prepared an analogue in which the methyl group of ω-methylpantothenic acid was replaced with a cyclopentane ring. The resulting compound demonstrated inhibitory activities against the growth of bacteria including *Streptococcus lactis* and *Lactobacillus arabinosus*, however, supplementation of pantothenate reversed the inhibitory activities of the compound (*61*).

When modifications made to the pantoyl-moiety of pantothenic acid were incorporated into inhibitory pantothenic acid analogues whose β-alanine moiety was already modified, mixed results were obtained. For example; homopantoyltaurine, **14**, (Fig. 2.10), containing an extra carbon unit introduced into pantoyl group of pantoyltaurine, was shown to inhibit pantothenic acid utilization (*45*, *62*).

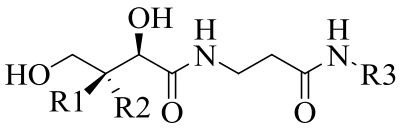


14

Figure 2.10: Structure of homopantoyltaurine

In addition, the analogue also inhibited growth of *Streptococcus haemolyticus*, although its activity was lower than that of pantoyltaurine. Furthermore, homopantoyltaurine inhibited growth of *Diplococcus pneumonia* and certain strains of *Corynebacterium diptheriae*, with less activity than pantoyltaurine (*62*).

Recently, Akinusi and co-workers (2011) synthesized a small library of *N*-substituted pantothenamides, **15**, by replacing the geminal dimethyl groups on the pantoyl moiety with various alkyl substituents (Fig. 2.11) (*63*).



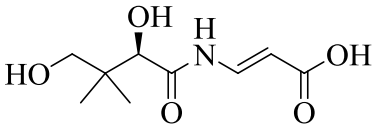
15

Figure 2.11; Modifications to the germinal dimethyl groups on the pantoyl moiety of N-substituted pantothenamides. R1,R2, R3 = alkyl substituents

When these analogues were tested for their biological activity, it was found that N5-Pan (R1 and R2 = Me, R3 = pentylamine) inhibited the growth of *S. aureus* with a MIC activity of 7 µM. However, when the pro-*R* methyl group (R2 substituent) was substituted with an allyl group, the resulting analogue was twice as potent as the parent N5-Pan, and inhibited the growth of *S. aureus* with an MIC of 3.2 µM. Substitution of both the geminal dimethyl groups with allyl substituent produced a diallyl-substituted derivative with weak activity, having an MIC-value of 376 µM. Further attempts to vary R1 with various substituents proved futile, as the resulting analogues were all found to be devoid of activity. In this study, most of the analogues were produced as a result of single substitution at R2, with most derivatives showing increased activity (*63*).

2.13 Analogues of pantothenic acid with a modified β-alanine moiety

An interesting pantothenic acid analogue extracted from the fungus *Seimatosporium* sp strain CL28611 was found to have antibacterial activities against *S. aureus*. This analogue, called CJ-15,801, **16**, (Fig. 2.12), differs from the parent pantothenic acid structure simply by possessing a double bond between the α- and β-carbons on the β-alanine moiety of pantothenic acid (*64*).



16

Figure 2.12: Structure of CJ-15,801

In a study conducted by Saliba and Kirk (2005), compound **16** was shown to inhibit proliferation of *P. falciparum* at the intraerythrocytic stage of the life cycle. In this study,

growth inhibition was monitored through incorporation of [^{3}H]hypoxanthine (*64*). It was demonstrated that the analogue inhibited growth of the parasites with an IC_{50} of 39 ± 3 µM, however, this occurred in the presence of 1 µM of pantothenic acid (*64*). Furthermore, it was also shown that the proliferation of the parasites was completely inhibited when the concentration of the analogue was increased to more than ~250 µM. When the same concentration of the analogue was used on a mammalian (rat hepatoma; HTC) cell line, no inhibitory activities were observed (*64*). It was also demonstrated that the antiplasmodial effect of CJ-15,801 was reversed in the presence of increased concentrations of pantothenic acid in the medium, for example, 100 to 500 µM. This suggests that the antiplasmodial effects of CJ-15,801 are exerted by blocking utilization of pantothenic acid by the parasites (*64*).

2.14 Pantothenamides as antimicrobial agents

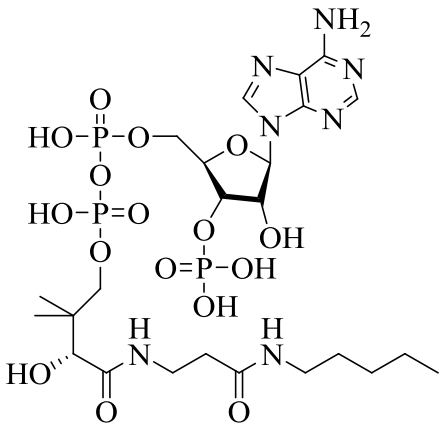
Pantothenamides are important pantothenic acid analogues in which the carboxylate has been replaced by *N*-substituted amides, as in the case of *N*-pentylpantothenamide (N5-Pan) and *N*-heptylpantothenamide (N7-Pan) for example (*65*). These analogues generally referred to as *N*-substituted pantothenamides have been utilized in many studies as inhibitors of bacterial growth. It has been shown that these analogues have an effect down-stream of CoA biosynthesis, as they are converted by the CoA biosynthetic enzymes into corresponding CoA analogues which act as inhibitors of CoA-dependent enzymes. (*66*). specifically, these analogues all lack a sulfhydryl (-SH) group that can act as a functional group. This renders them inactive, since they cannot transfer acyl groups from one reaction to the next. Consequently, fatty acid metabolism is inhibited, since inactive prosthetic groups are transferred to the acyl carrier proteins (ACPs) which play a pivotal role in these processes (*66*).

In studies conducted by Clifton *et al* in 1970 (*67*), a variety of *N*-substituted pantothenamides were prepared. When these analogues were tested for activity, it was shown that they negatively affected the growth of lactic acid bacteria including *Lactobacillus arabinosus* and *Lactobacillus casei*. However, its activity was reversed by pantothenic acid in a competitive manner (*67*). Of the synthesized compounds, *N*-nonylpantothenamide and *N*-phenylpantothenamide were shown to be highly active and caused complete inhibition of growth of *Lactobacillus casei* at a concentration of ~20 µM (*67*). In addition, other

pantothenamides were shown to inhibit the growth of *E. coli*, including *N*-pentylpantothenamide and *N*-heptylpantothenamide. It was also shown that these analogues exhibited exceptional properties in that at lower concentration of the analogues, their inhibition activities were reversed by pantothenic acid, whereas the analogues were irreversibly toxic at higher concentrations. *N*-pentylpantothenamide exhibited the highest potency against *E. coli*, inhibiting growth completely at 2 µM (*67*).

A variety of pantothenic acid analogues structurally similar to pantothenamides were prepared by Sergent *et al* (1975) by substituting the terminal amide functionality with an *N*-substituted carbamate or ureido group. The resulting analogues demonstrated inhibitory activities against lactic acid bacteria including *Lactobacillus plantarum* and *Pediococcus cerevisiae*, but did not inhibit *E. coli (6).* However, the inhibitory activity of the analogues was antagonized by pantothenic acid in a competitive manner. On assessing the efficiency of these analogues as inhibitors, it was shown that the carbamate analogues were more successful as inhibitors than the ureido analogues, completely inhibiting growth of *Lactobacillus plantarum* at a concentration of 2 µM (*6*).

Studies conducted by Strauss and Begley (2002) showed that the analogue *N*-pentylpantothenamide (N5-Pan) did not act as an inhibitor of the CoA biosynthetic enzymes PanK, PPAT, and DPCK in the CoA biosynthetic pathway, but rather acted as a substrate of these enzymes which resulted in its conversion to the CoA analogue ethyldethia-CoA, **17**, (Fig. 2.13) (*68*).



17

Figure 2.13. Structure of the inactive CoA analogue, ethyldethia-CoA (*6*).

In the same study, they demonstrated that the conversion of N5-Pan to **17** occurred 10.5-fold faster than biosynthesis of CoA from pantothenic acid, implying that biosynthesis of ethyldethia-CoA could be competitive with respect to conversion of pantothenate to CoA in the cell (*68*). With this result, Strauss and Begley therefore suggested that the toxic effects of N5-Pan were as a result of the analogue's conversion to compound **17**, which exerts its influence by inhibiting CoA and acetyl-CoA utilizing enzymes (*68*). N5-Pan and N7-Pan efficiently inhibited growth of bacteria, and are believed to act through conversion of these analogues by PanK to intermediates which later are implicated in the production of CoA analogues which are not active (*35*).

In other studies, Ivey *et al* (2004) demonstrated that N5-Pan and N7-Pan were successful competitive inhibitors of *E. coli* PanK with an IC_{50} of 60 µM, and the inhibitors were shown to be competitive with respect to pantothenate (*35*). Determination of an experimental K_M for N5-Pan and N7-Pan as substrates for PanK revealed that these analogues were alternative substrates, showing K_M values of 140 and 124 µM respectively, as compared to the K_M for pantothenate which was 41 µM (*35*).

In other studies, N5-Pan and N7-Pan demonstrated inhibitory activities against *S. aureus* PanK with an IC_{50} in the low micromolar range (*69*). Similarly, Leonardi *et al* (2005) demonstrated the inhibitory activities of N5-Pan and N7-Pan on *S. aureus* PanK with IC_{50} values of 3.5 and 4.8 µM respectively. In addition, these analogues were found to be both inhibitors and substrates for *S. aureus* PanK (*70*). By using broth microdilution assays, MIC values of 0.16 and 25 µM of N5-Pan and N7-Pan were determined against *S. aureus* strain RN4220. Supplementation of the growth medium with 50 µM pantothenate resulted in a reduced efficacy of these analogues, showing that the primary target of these analogues is pantothenate metabolism (*70*). Subsequently, it has been demonstrated that the toxic effects of N7-Pan in *S. aureus* is a result of fatty acid synthesis inhibition (*70*).

Studies performed by Brand and Strauss (*71*) and Hong *et al* (*33*), which involved characterization of the type-III PanK enzymes expressed by *Bacillus subtilis*, *Helicobacter pylori*, and *Pseudomonas aeruginosa*, found that these enzymes' activity was not inhibited by N5-Pan. In addition, these enzymes do not allow N5-Pan as alternative substrate (*33*, *71*). The inhibitory activity of N5-Pan was also shown to be ineffective against growth of *P.*

aeruginosa. In addition, when the *P. aeruginosa coaX* gene was expressed in *E.coli* strain DV70 containing a a temperature sensitive PanK which is inactivated at 42^0C, the microbe demonstrated resistance to growth inhibition by N5-Pan at the said temperature (*33*).

Based on the promising results of pantothenamide inhibitor studies, van Wyk and Strauss (2008) developed a method for parallel production of a variety of pantothenamides for inhibitor identification purposes. Based on the known reactivity of activated thioesters towards amines, they prepared *S*-phenyl thiopantothenate, *S*-phenyl thio-α-pantothenate (with one methylene group less in the β-alanine moiety)), and *S*-phenyl thiohomopantothenate (with one three methylene more in the β-alanine moiety) as activated precursors of pantothenic acid. Reaction of these compounds with a variety of amines produced a library of pantothenamides, **18**, as products (Fig. 2.14) (*66*).

When these analogues were tested against malaria parasites *P. falciparum*, the results showed that **18b** demonstrated poor inhibitory activity against the parasites with a best inhibitor concentration being 53 µM, whereas **18a** and **18c** registered highest inhibitory capabilities, with the best α- and homopantothenamides inhibiting at a concentration of 4.6 and 2 µM respectively. However, despite demonstrating inhibitory activities against *P. falciparum*, concentration-response curves of **18a** and **18c** showed a significant lowering of their antiplasmodial activity in the presence of an increased pantothenate concentration in the extracellular medium. This demonstrated that the activities of these analogues were competitive with respect to pantothenate utilization, and that their effect was antagonized in the presence of increased pantothenate. Importantly, the same shift was not seen in the case of the normal pantothenamide series **18b**.

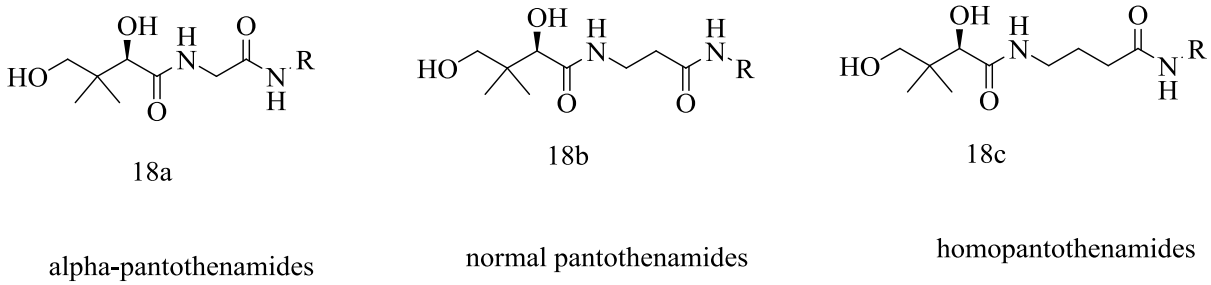


Figure 2.14: Structures of *N*-substituted pantothenamides with varied β-alanine moiety.

In the same study, it was found that **18b** negatively affected growth of *S aureus*, with the best hit inhibiting at a concentration of 1.5 µM, whereas **18a** and **18c** were not active at concentrations as high as 200 µM.

2.15 Conclusion

As this literature review has shown, pantothenic acid analogues have a rich history in antibacterial and antimalarial drug discovery studies, which recently have received renewed interest with the rediscovery of the pantothenamides as potential antimicrobial agents. With the ability to inhibit all three CoA-related targets (pantothenate uptake, pantothenate phosphorylation and CoA utilization), such analogues definitely deserve further study.

Achieving the objective of this study: Specific Aims

The main objective of this study is the synthesis and characterization of new pantothenic acid analogues as potential antibacterial and antimalarial agents. Specifically, we wanted to:

1. Expand the current structural diversity of normal-type pantothenamides (i.e. those prepared from pantothenate, see Fig. 2.14) by the addition of methyl groups in the β-alanine moiety of pantothenate to determine if such a change will improve the antiplasmodial activity of these compounds.

2. Prepare and test pantoyltauramides as antimicrobial agents, including the analogues of the best pantothenamide inhibitors identified in previous studies in our group, and those that showed promising inhibitory effects against avian malaria parasites in the studies highlighted above.

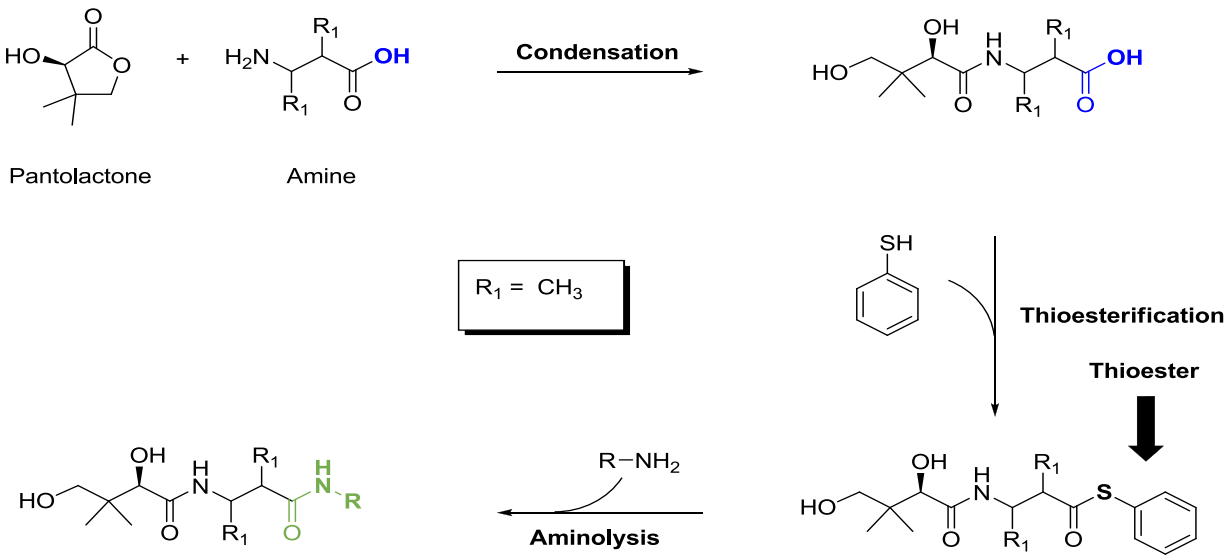
Chapter 3

Results and Discussion

AIM 1: Preparing and characterizing *N*-substituted α- and β-methylpantothenamides as antimicrobial agents

For the first aim of this study we set out to expand the structural diversity of the normal-pantothenamides previously prepared in our group (see section 2.14) by introducing methyl substituents on the α- and β-carbons of the β-alanine moiety of the pantothenate molecule. The strategy behind this modification was to determine whether the increase of steric bulk would increase the biological stability of these compounds, as one possible reason for their poor inhibitory activity could be due to the hydrolysis of the pantothenamide amide bond. Such a hydrolysis reaction, which could be enzyme-catalyzed, would release pantothenate and the amine substituent, and would render the pantothenamide harmless. If such an analysis is correct, the introduction of steric bulk close to the amide carbonyl may reduce the rate of pantothenamide hydrolysis, and increase the potency of the compounds. However, such modifications could also negatively impact on the potency if they reduce the compounds' binding to either the CoA biosynthetic enzymes that transform them into CoA metabolites, or to the actual target of inhibition. We therefore decided to prepare the α- and β-methylated analogues of the normal-pantothenamides that were previously found to be the best inhibitors of plasmodial and bacterial growth, and to test these to see if their inhibition profile is improved.

To prepare these methylated pantothenamides, a parallel synthesis method that was previously developed on our group was used (*66*). In this method, the methylated pantothenic acids are prepared, and then converted to their corresponding activated thioesters. The thioesters subsequently undergo aminolysis by reaction with the appropriate amines to give the required pantothenamides (Scheme 4.1).



Scheme 3.1: General approach for the parallel synthesis of methylated pantothenamides

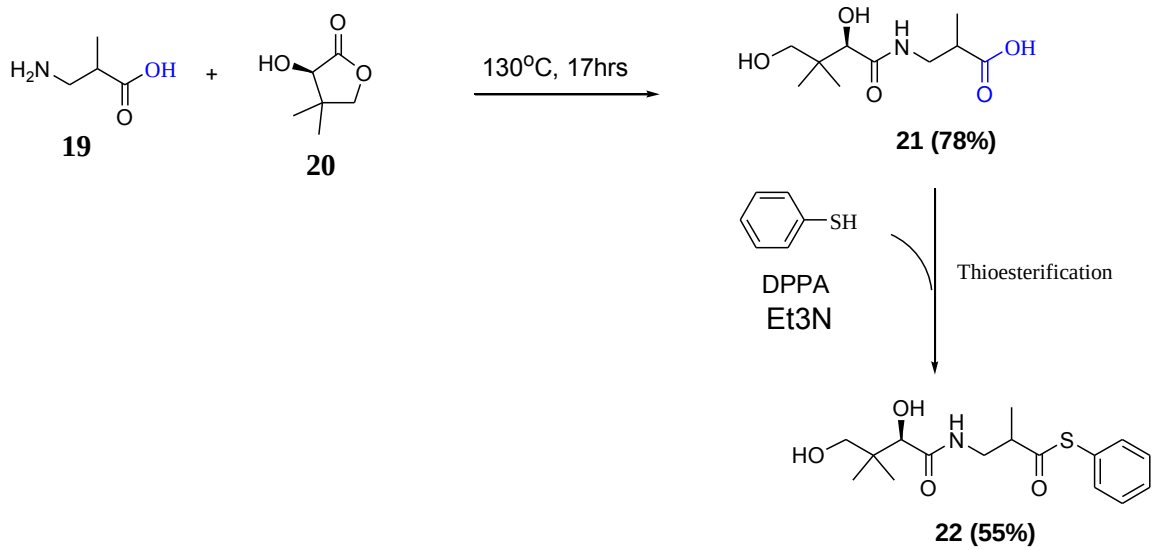
3.1 Parallel Synthesis of α- and β-methyl pantothenamides

3.1.1 Synthesis of α- and β-methyl *S*-phenylthiopantothenate

The synthesis of the α- and β-methyl pantothenate thioesters was performed as shown in scheme 3.2. Since the corresponding pantothenic acids were not commercially available, these were prepared first. Isoaminobutyric, **19**, was condensed with pantolactone, **20**, to produce α-methyl pantothenic acid, **21**, in 78% yield. Similarly, β-methyl pantothenic acid, **24**, was also prepared by condensing 3-aminobutyric acid, **23**, with **20**, and was obtained in 54% yield. ^{1}H NMR spectroscopic analysis confirmed formation of these acids.

In the next step, the α- and β-methyl pantothenic acids **21** and **24** were then coupled to thiophenol in the presence of diphenylphosphoryl azide (DPPA) and triethylamine (Et_3N) to obtain the corresponding *S*-phenyl α-methylthiopantothenate, **22**, and *S*-phenyl β-methylthiopantothenate, **25**, in 55% and 47% yield respectively. ^{1}H NMR and MS analysis confirmed formation of these thioesters.

alpha-methyl N-substituted pantothenamides



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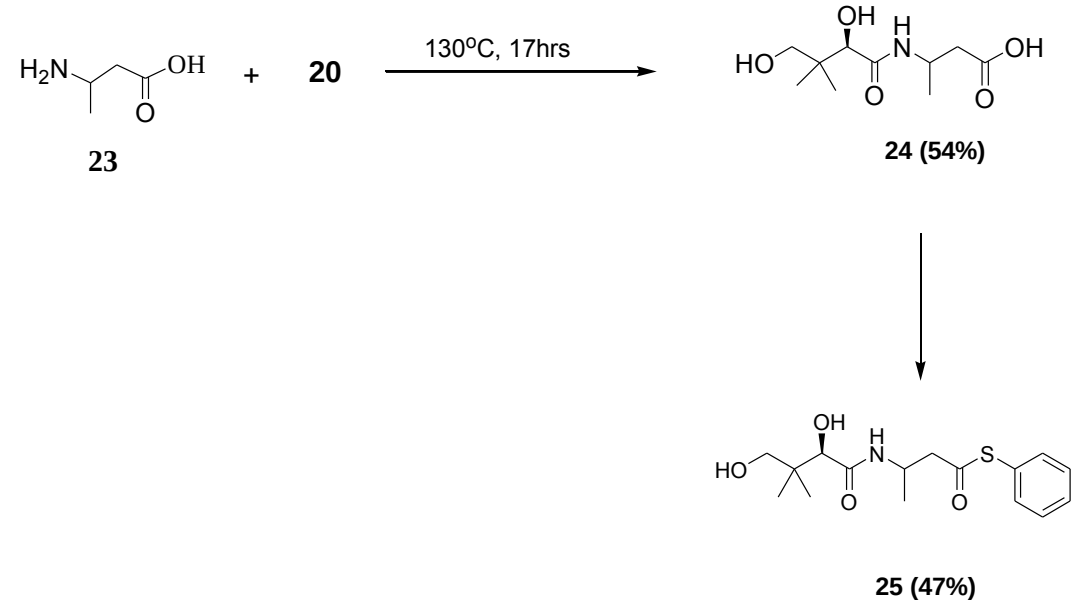
beta-methyl N-substituted pantothenamides

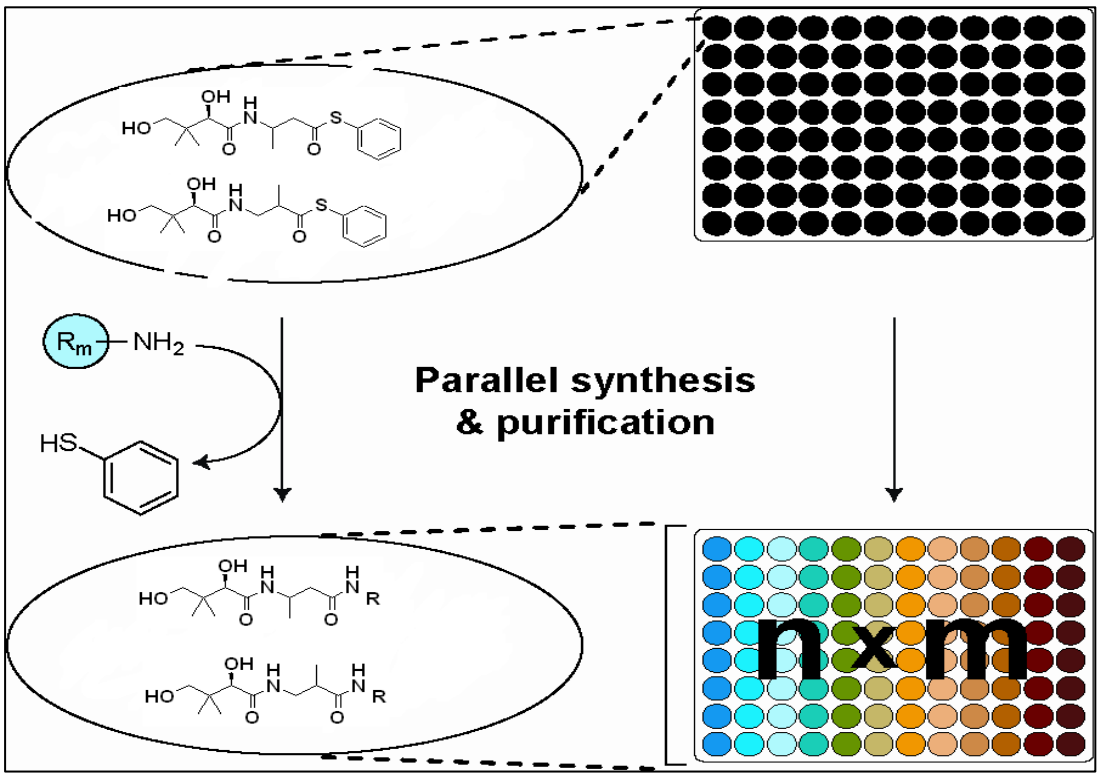


Scheme 3.2. Synthetic route for preparation of *S*-phenyl α- and β-methyl thiopantothenate esters.

It is important to note that while the established reaction conditions were initially used in the preparation of these thioesters, the introduction of the methyl groups clearly influenced the reactivity of the starting materials, and therefore the yields of the reaction. However, optimization of the reaction conditions allowed for the successful preparation of these thioesters as described.

3.1.2 Synthesis and purification of alkyl N-substituted pantothenamides

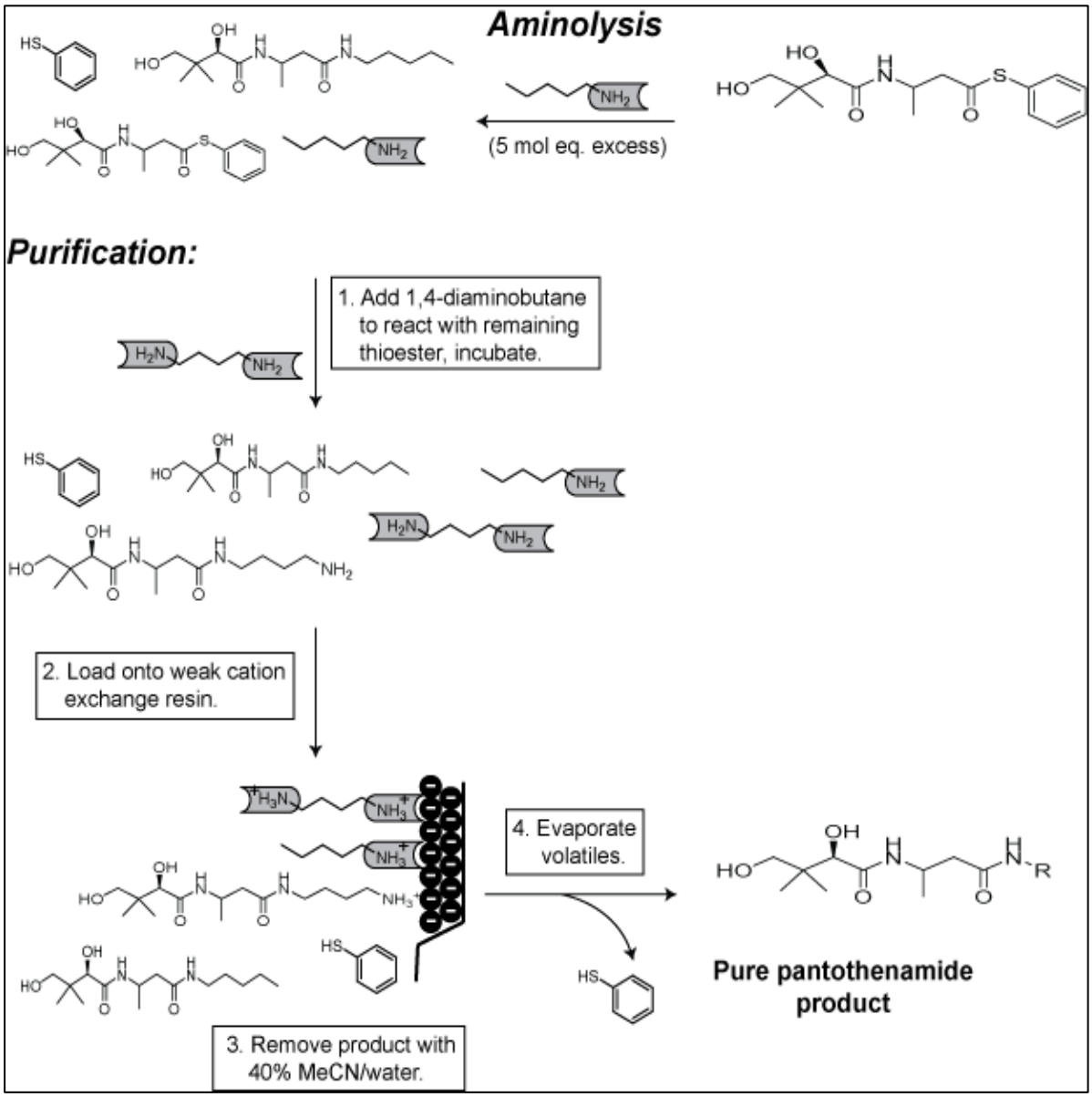
In order to synthesize the *N*-substituted α- and β-methyl pantothenamides, the activated thioesters **22** and **25** were subsequently reacted with a diverse group of amines in parallel. For the synthesis and purification of these pantothenamides the method previously developed in our group was used (Scheme 3.3) (*66*).



Scheme 3.3. Parallel synthesis and purification of *N*-substituted α- and β-methyl pantothenamides using *S*-phenyl thioesters **22** and **25** (n=2) as precursors in aminolysis reactions with m number of amines, producing a library with n x m members.

First, the *S*-phenyl α- and β-methylthiopantothenates were separately subjected to aminolysis reactions with a variety of amines to obtain the desired corresponding pantothenamides (Scheme 3.4). The aminolysis reactions were performed in 96-well 2 mL deep-well plates, and involved incubating the thioesters in the presence of five equivalents of amine for a period of 3 hours at moderate temperature (30°C), followed by addition of 1,4-diaminobutane to each well. The 1,4-diaminobutane reacts with any unreacted thioester to yield *N*-(4-amino-butyl) α- and β-methylpantothenamides. The pantothenamides produced were subsequently purified according to the established method (*66*). The reaction mixtures were transferred to a new 96-well filter plate preloaded with weakly acidic cation exchange resin, and allowed to

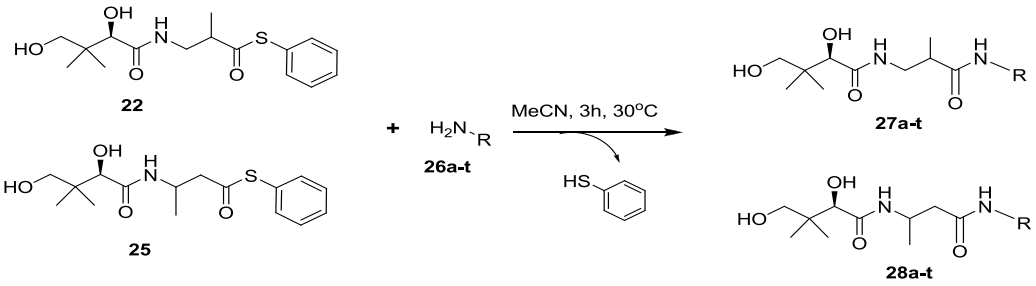
elute under gravity. This allowed the resin to remove excess amine as well as any *N*-(4-amino-butyl) pantothenamides produced by reaction with unreacted thioester. The resin was thoroughly rinsed with aqueous acetonitrile, after which the solvent and thiophenol was simultaneously removed from the eluents by evaporation.

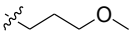
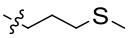
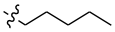
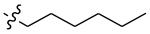
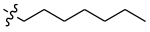
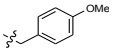
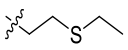
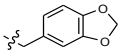
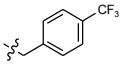
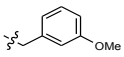
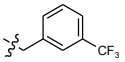


Scheme 3.4. Purification of *N*-substituted α- and β-methylpantothenamides produced by parallel aminolysis of activated thioesters.

Table 3.1 shows the structures of the 20 amines (**26a-t**) that were used in the aminolysis of *S*-phenyl α- and β-methyl thiopantothenate **22** and **25**, as well as the corresponding yields of the *N*-substituted α-methylpantothenamides **27a-t**, and *N*-substituted β-methylpantothenamides **28a-t** obtained after purification.

Table 3.1. Aminolysis of *S*-phenyl α- and β-methylthioesters **22** and **25** with a variety of amines (**26a-t**) to form the corresponding *N*-substituted α-methyl pantothenamides (**27a**-t) and *N*-substituted β-methyl pantothenamides (**28a-t**). Yields were determined by weight determination of each purified compound, followed by their characterization by ^{1}H NMR.



Amine	R-group	% Yields α-Me-Pan 27a-j	% Yields β -Me-Pan 28a-j	Amine	R-group	% Yields α-Me-Pan 27k-t	% Yields β -Me-Pan 28k-t
26a		89	85	**26k**		77	85
26b		80	85	**26l**		91	89
26c		83	86	**26m**		48	82
26d		74	85	**26n**		100	100
26e		88	86	**26o**		65	91
26f		75	87	**26p**		89	97
26g		82	87	**26q**		83	94
26h		87	96	**26r**		82	100
26i		65	87	**26s**		38	51
26j		89	89	**26t**		59	79

The results show that we have prepared and successfully purified in parallel the *N*-substituted α- and β-methylpantothenamides **27a-t** and **28a-t** respectively by following a procedure outlined in scheme 3.4. Overall, the parallel syntheses of these pantothenamides were judged to have been successful since the yields of most of the purified products are above 70%, with only five pantothenamides (**27i**, **27m**, **27o**, **27s** and **28s**) that gave yields lower than this benchmark. Of these, four are α-methyl pantothenamides, indicating that the position of the methyl group on the β-alanine moiety of the pantothenate thioester definitely influences the reactivity of the carbonyl group, as expected. Moreover, the lowest yields of 38% (**27s**) and

51% (**28s**) being obtained for the products of the aminolysis with the same amine (**26s**), suggesting that it may not be as reactive or pure as anticipated.

3.2 Inhibitory effects of *N*-substituted α- and β-methylpantothenamides against bacterial growth[1]

To determine the effect of the modification of the pantothenamide backbone on the antibacterial potency of these compounds, we set out to test all 40 *N*-substituted α- and β-methylpantothenamides against the *S. aureus* RN 4220 strain. Inhibition assays were performed at a single concentration of 200 µM methylpantothenamide as an initial screen for inhibitory activity. The potency of each pantothenamide was evaluated by comparing the growth *S. aureus* grown in 1% tryptone (the positive control, which showed 100% growth) to its growth in the same medium with the pantothenamides added. The results for the α- and β-methyl pantothenamides are summarized in the figures **3.1** and **3.2** respectively. These show that in most cases both sets of pantothenamides exhibit poor inhibition of *S. aureus* with growth inhibition ranging between 10-30% at the test concentration of 200 µM.

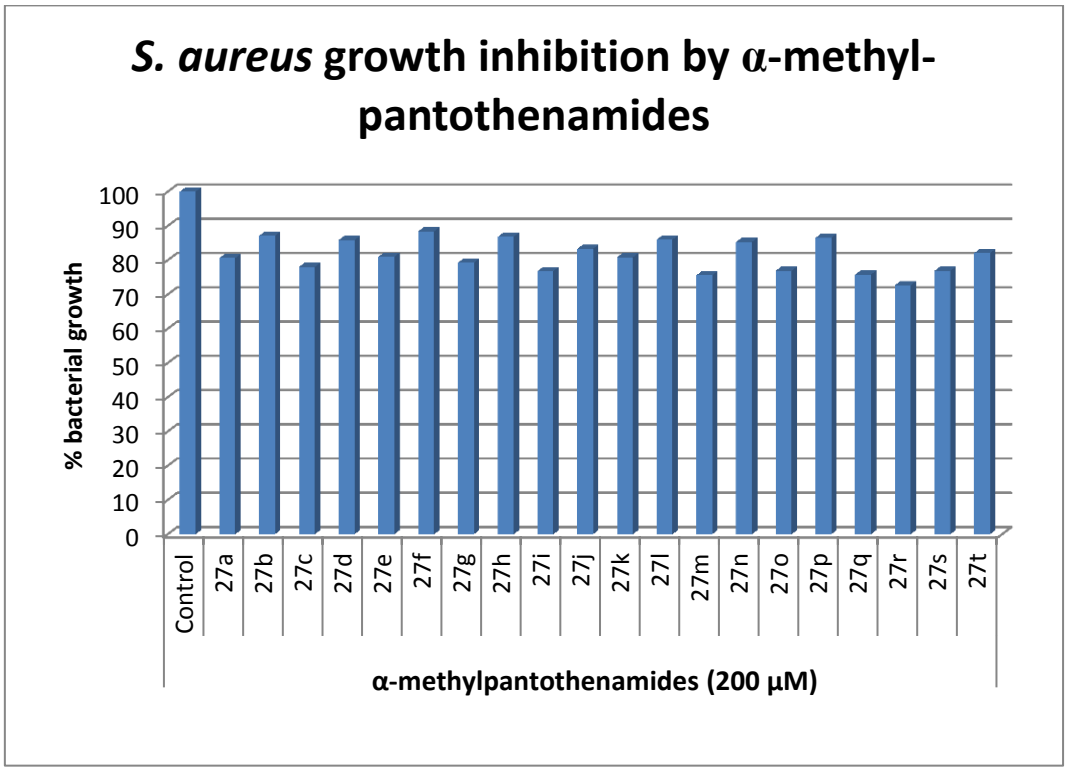


Figure 3.1. Growth of *S. aureus* in the presence of 200 µM of the α-methylpantothenamides **27a-t**.

[1] The growth inhibition tests were performed with the assistance of Mr. Cristiano Macuamule, a PhD candidate in the Strauss group

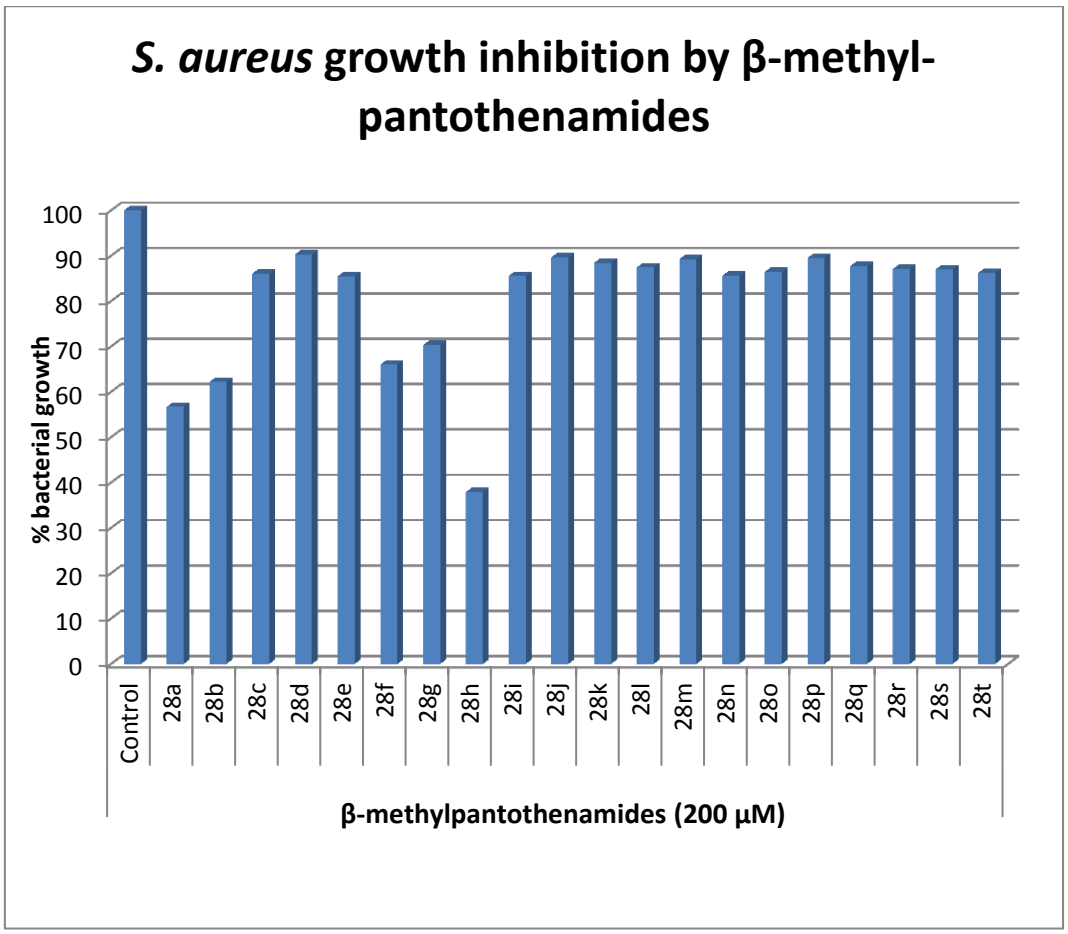
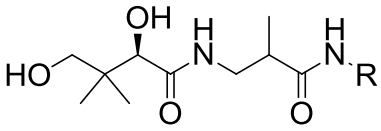
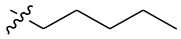
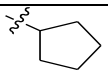
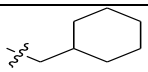
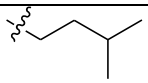
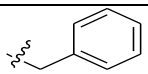
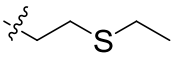
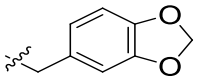
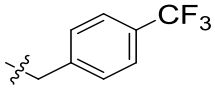


Figure 3.2: Growth of *S. aureus* in the presence of 200 µM of the β-methylpantothenamides **28a-t**.

The inhibition results of the methylpantothenamides showing the best inhibition were subsequently compared to the inhibition data obtained for the normal pantothenamides previously prepared and tested in our group.

Table 3.2. Antistaphylococcal activities of selected α-methylpantothenamides

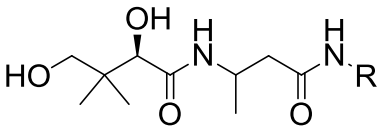
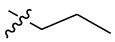
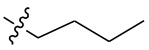
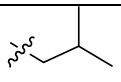
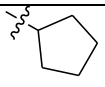
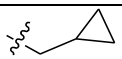
		α-methylpantothenamides 
A	**R-group**	**% inhibition at 200 µM**
27c		22
27g		21
27i		23
27m		24
27o		23
27q		24
27r		27
27s		23

From the the inhibition of the α-methylpantothenamides (Table 3.2) it can be seen that *N*-pentyl α-methylpantothenamide (**27c**) and *N*-cyclopentyl α-methylpantothenamide (**27g**) inhibited the growth of *S. aureus* by only 22 and 21% respectively. Previous studies showed that the normal pantothenamides with the corresponding R-groups had relatively low antistaphylococcal activity. *N*-piperonyl α-methylpantothenamide (**27r**) is the analogue that shows the best growth inhibition among the α-methylpantothenamides, with a modest value of 27%. Likewise, the corresponding normal *N*-piperonylpantothenamide demonstrated a relatively poor antibacterial activity in the previous study. The other α-methylpantothenamides (**27i, 27m, 27o, 27q,** and **27s**) inhibited growth of *S. aureus* in the range between 23 and 24%, whereas the corresponding normal pantothenamides had the same poor activities. On the other hand, α-methylpantothenamides with aliphatic R-groups, such as pentyl (**27c**), hexyl (**27d**) and heptyl (**27e**) inhibited growth of *S. aureus* by only 22, 14, and 18% respectively (Fig. 3.1), although the data from the previous study showed that normal *N*-

pentyl, *N*-hexyl, and *N*-heptyl pantothenamides potently inhibited the growth of *S. aureus*. Based on these results, we can conclude that the introduction of the methyl group on the α-carbon of the β-alanine moiety of normal pantothenamide has not enhanced the anti-staphylococcal activity of the resulting analogues, as evidenced by the poor showing of these analogues as potential inhibitors.

Among the β-methylpantothenamides tested, five (**28a, 28b, 28f, 28g,** and **28h**) showed better inhibitory activities between 30-60%, with the best analogue (**28h**) inhibiting *S. aureus* growth by 62% (Table 3.3). This result is in agreement with the previous inhibition results which showed that the *N*-substituted normal pantothenamide with a methylcylopropyl group showed improved inhibition. Similarly, the results show that *N*-cyclopentyl β-methylpantothenamide (**28g**), and *N*-isobutyl β-methyl pantothenamide (**28f**) inhibited proliferation of *S. aureus* by 30 and 34% respectively. In the previous study it was shown that normal pantothenamides containing cyclopentyl and isobutyl R-groups demonstrated better activities against *S. aureus*. From the results, it can also be seen that *N*-propyl β-methylpantothenamide (**28a**) inhibited by 43%, whereas normal propyl pantothenamide demonstrated activity against *S. aureus* with MIC value of 50 µM in the previous study.

Table 3.3: Antistaphylococcal activities of a selected β-methylpantothenamides

		β-methylpantothenamides 
Entry	**R-group**	**% inhibition at 200 µM**
28a		43
28b		37
28f		34
28g		30
28h		62

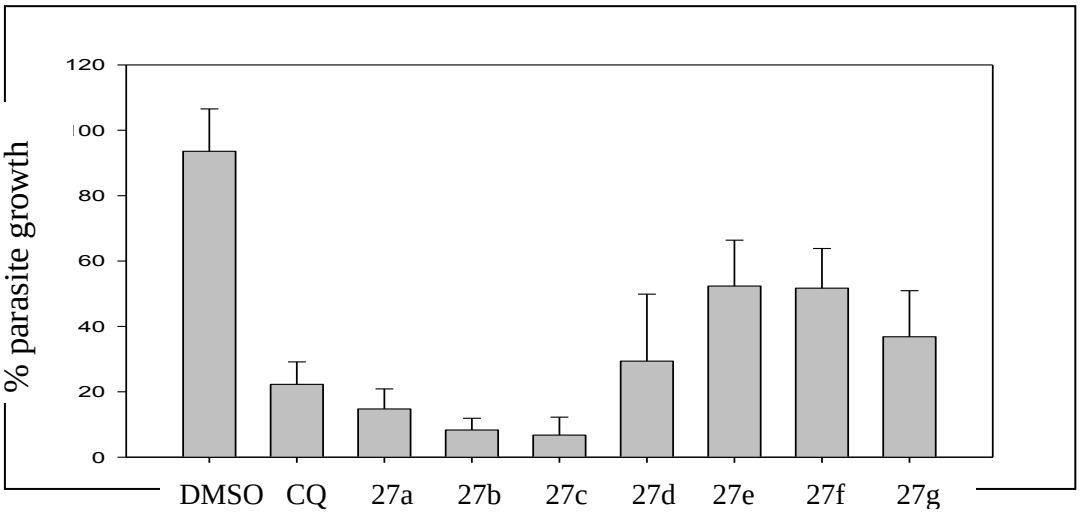
The data presented in table 3.3 suggests that the β-methylpantothenamides, especially **28h**, may in fact be promising anti-staphylococcal agent. However, since the data from the previous study in our lab showed that normal pantothenamides with R-groups similar to the ones shown in table 3.3 also demonstrated remarkable activities against *S. aureus*, we can conclude that the anti-staphylococcal activities demonstrated by the β-methylpantothenamides is not due to the methyl group which was introduced on the β-alanine moiety of normal pantothenamides, but is attributed to the nature of R-groups on these analogues. We can therefore conclude that the β-methyl functionality has no additional effects as far as inhibitory activities of the pantothenamides are concerned.

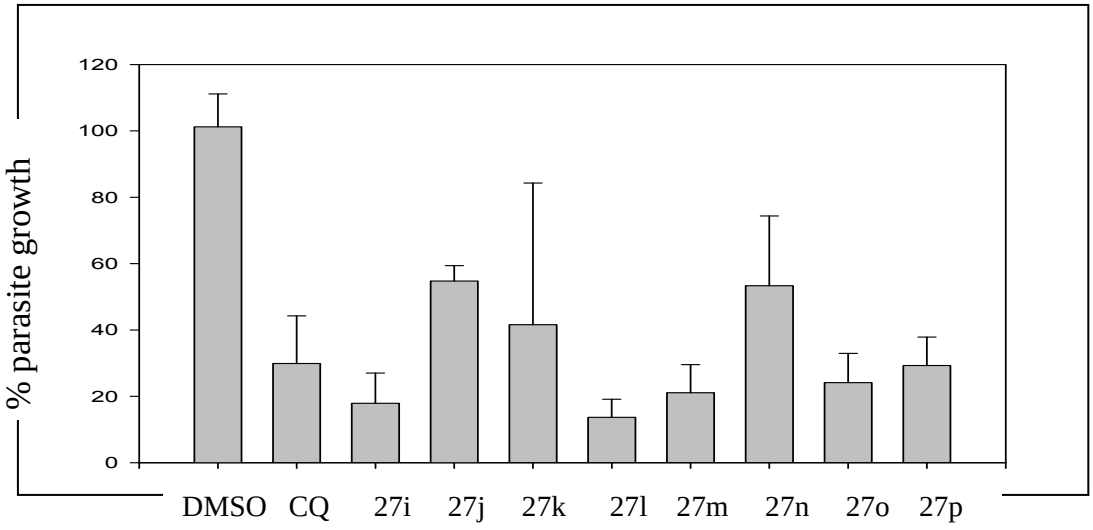
3.2.1 Inhibitory effects of *N*-substituted α- and β-methylpantotenamides against *P. falciparum* growth[2]

In previous studies done in our lab, it was found that normal pantothenamides were poor inhibitors of *P. falciparum*. In fact, there was little or no inhibitory activity when most of the analogues were tested at a concentration of 200 μM (M. de Villiers, PhD dissertation, 2009).

In the current study, α- and β-methyl pantothenamides were tested for growth inhibition of *P. falciparum* (3D7 strain). Growth assays were done at single concentrations of 200 μM to determine if any of the compounds gave good inhibition at this concentration. Uninfected erythrocytes were included to act as control (0% parasite growth). Infected red blood cells without any added analogues also acted as a negative control (100% parasite growth). Since the pantothenamides were dissolved in DMSO, inhibition by DMSO was tested as well. A known antimalarial, chloroquine, was included as a positive control. The results for the α- and β-methylpantothenamide inhibition are summarized in figure 3.3a and b respectively.

[2] The growth inhibition tests were performed with the assistance of Mr. Cristiano Macuamule, a PhD candidate in the Strauss group





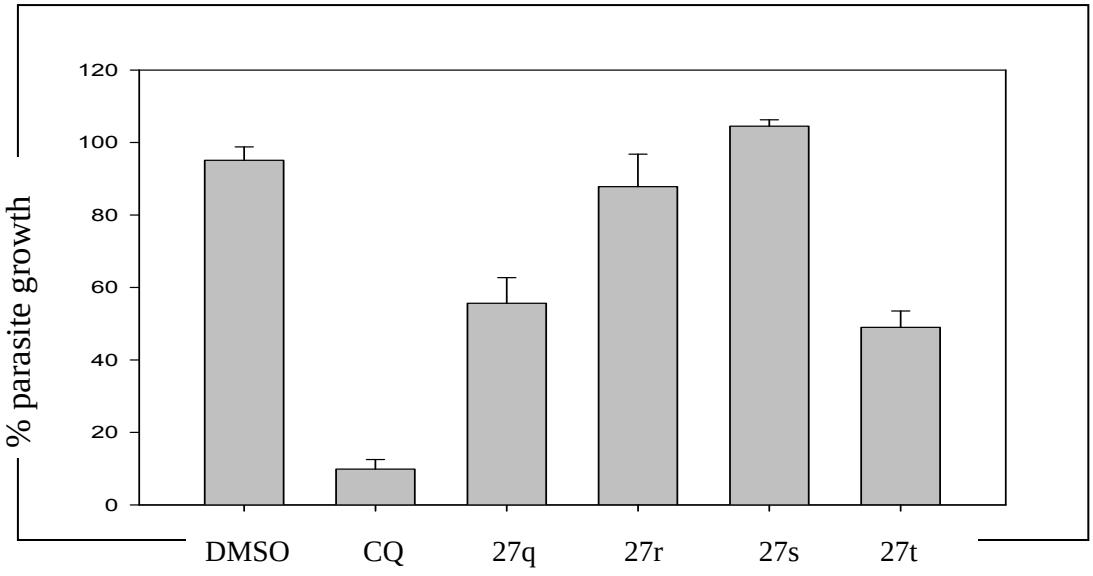


Figure 3.3a. Growth inhibition of *P. falciparum* in the presence of 200 µM of α-methylpantothenamides.

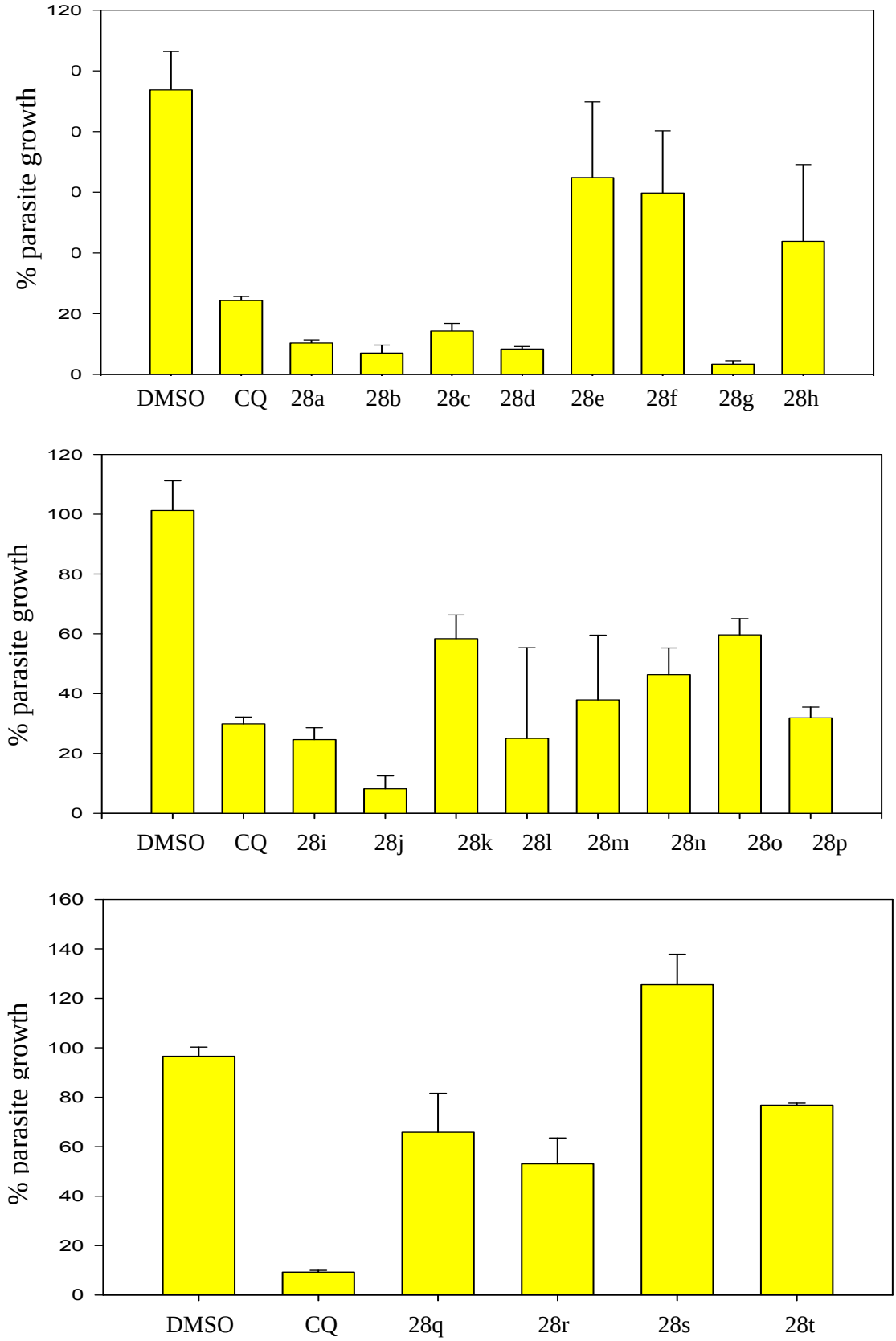


Figure 3.3b. Growth inhibition of *P. falciparum* in the presence of 200 μM of β-methylpantothenamides.

The results show that the parasites registered 100% growth in DMSO, implying that DMSO does not have any effect on the inhibitory activities of the analogues. Parasites cultured in media containing chloroquine only registered 20% growth, as expected. In general, the α-methyl pantothenamides (figure 3.3a) showed better inhibition, with the most active inhibitors, *N*-propyl α-methylpantothenamide (**27a**), *N*-butyl α-methylpantothenamide (**27b**) and *N*-pentyl α-methylpantothenamide (**27c**), *N*-cyclohexylmethyl α-methylpantothenamide (**27i**), *N*-3-methylbutyl α-methylpantothenamide (**27m**), inhibiting proliferation of the parasites by more than 80%. Other active analogues which also demonstrated promising activity include: *N*-hexyl α-methylpantothenamide (**27d**), *N*-benzyl α-methylpantothenamide (**27o**) and *N*-4-methoxyaminobenzyl α-methylpantothenamide (**27p**). These analogues inhibited proliferation of malaria parasites by 60%. Overall, of the twenty α-methylpantothenamides tested, six analogues, (**27e**, **27f**, **27j**, **27n**, **27r** and **27s)** demonstrated poor activity as they only registered growth inhibition of less than 50% at 200 μM.

The best β-methylpantothenamide inhibitors (figure 3.3b) were *N*-propyl β-methylpantothenamide (**28a**), *N*-butyl β-methylpantothenamide (**28b**) and *N*-pentyl β-methylpantothenamide (**28c**), *N*-hexyl β-methypantothenamide (**28d**), *N*-cyclopentyl β-methylpantothenamide (**28g**), and *N*-3-methoxybenzyl β-methylpantothenamide (**28j**) inhibiting proliferation of the parasites by more than 80%. Additionally, the analogues **28h**, **28i**, **28l**, **28m**, **28n** and **28p** demonstrated some activity by inhibiting growth of malaria parasites by more than 50%. However, of the tested β-methylpantothenamides, compounds **28e**, **28f**, **28k**, **28o**, **28q**, **28s**, and **28t** showed poor inhibition (less than 50%).

Comparing the antiplasmodial activities of the α- and β-methylpantothenamides, the data shows that methylated pantothenamides containing aliphatic R-groups such as propyl (**27** and **28a**), butyl (**27** and **28b**), pentyl (**27** and **28c**), and hexyl (**27** and **28d**) were among the best active inhibitors. On the other hand, the normal pantothenamides with corresponding aliphatic R-groups displayed poor activities against proliferation of *P. falciparum* in the previous study. There is also an interesting trend on the poor activities of some of the methylated pantothenamides. For example, compounds such as **27** and **28e**, **27** and **28f**, **27** and **28q**, and **27** and **28s,** which have similar R-groups, all show poor activity. The poor inhibitory activity of these analogues can therefore be attributed to the nature of these R-groups.

In the same study, the methylated pantothenamides which inhibited proliferation of malaria parasites by more than 50% were selected and subjected to a secondary screen at 50 μM. The results for the selected methylated pantothenamides are summarized in the figure **3.4** and table **3.4** respectively.

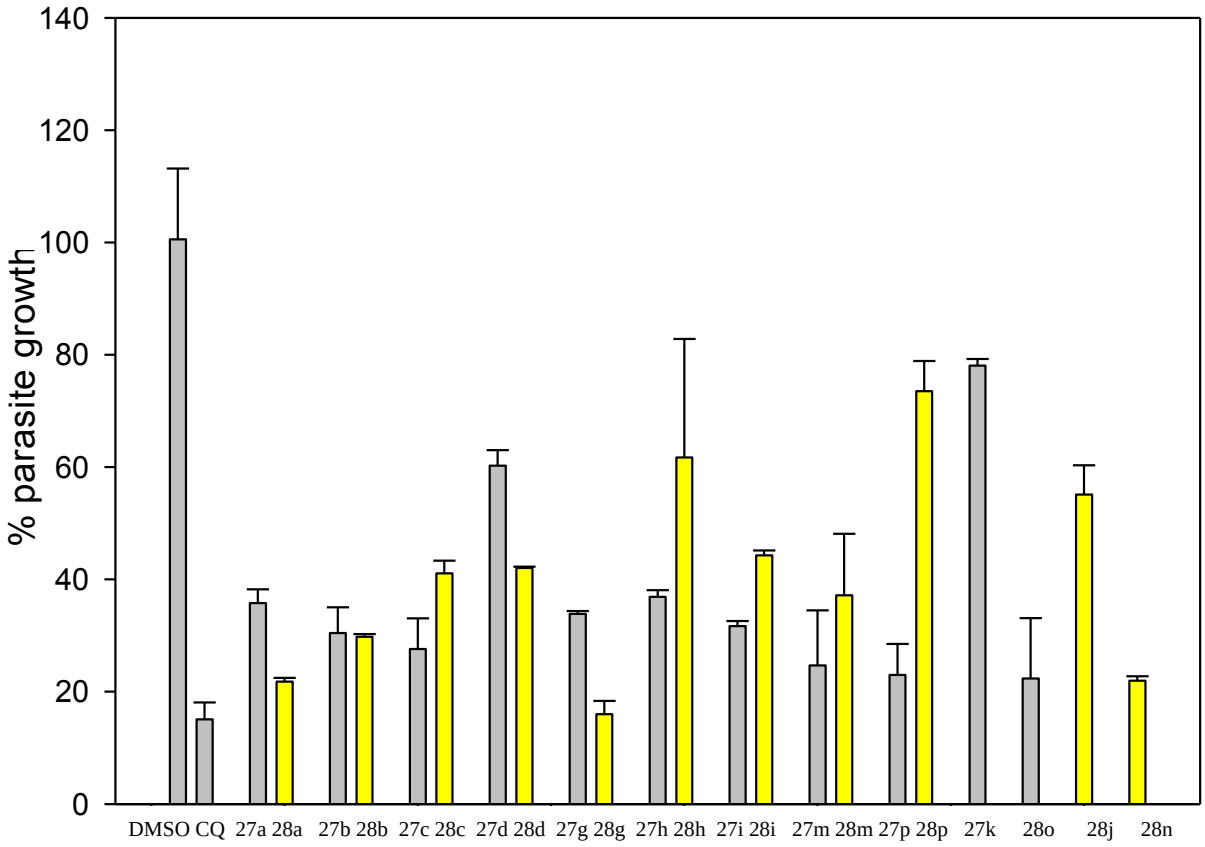
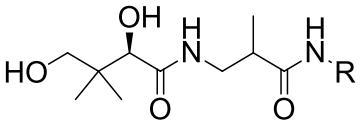
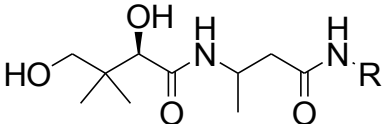
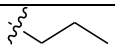
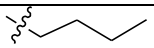
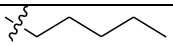
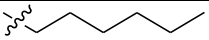
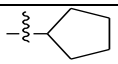
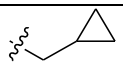
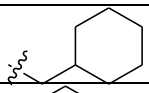
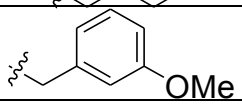
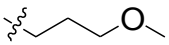
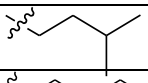
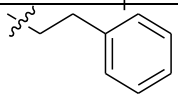
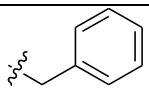
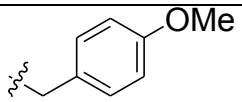


Figure 3.4. Growth inhibition of *P. falciparum* in the presence of 50 μM selected α- and β-methylpantothenamides

As can be seen from the results in figure 3.4, the majority of the selected methylated pantothenamides inhibited growth of malaria parasites with growth inhibition ranging between 50-80%. This is a clear demonstration that these analogues may be potential antimalarials. As summarized in table 3.4 below, certain pantothenamides such as **27d**, **27k**, **28h**, **28j**, and **28p** displayed poor antiplasmodial activity at 50 μM.

Table 3.4: Antiplasmodial activity of a selected α- &β-pantothenamides at 50 µM.

		α-methylpantothenamides 		**β-methylpantothenamides** 
Entry	**R-group**	**% inhibition at 50 µM**		**% inhibition at 50 µM**
a		65		78
b		70		70
c		72		58
d		40		56
g		65		82
h		62		38
i		68		52
j		NT[a]		46
k		20		NT
m		75		60
n		NT		78
o		78		NT
p		77		34

[a]NT, not tested.

Based on these results it is clear that, unlike in the case of bacteria, the antiplasmodial potency of the methylpantothenamides are not based on their *N*-substituents, but on the introduced methyl groups in the β-alanine moiety. These results therefore indicate that the introduction of the methyl groups may indeed have had the desired effect in improving the biological stability of the pantothenamides, and suggest that especially the *N*-substituted methylated pantothenamides deserve further investigation as these showed a definite improvement in their inhibition capabilities.

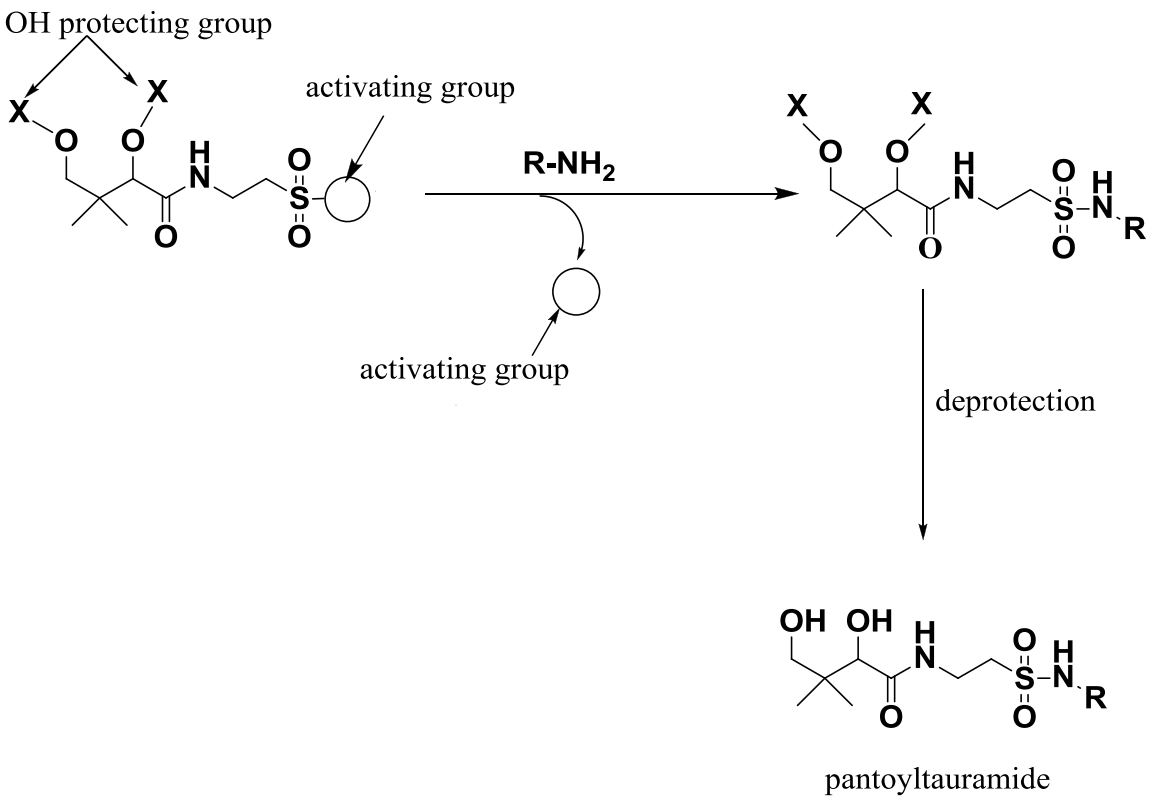
Aim 2: Preparing and characterizing *N*-substituted pantoyltauramides as antimicrobial agents

Sulfonamides are a group of pharmaceutical compounds that is receiving much attention because of its biological activities. A large number of sulfonamides are used in clinical medicine among others as antibacterial, diuretics and HIV protease inhibitors (*72-74*). Additionally, some are used in the agricultural industry as herbicides and plaguicides which are important for protecting vines from attacks caused by insects and weeds (*73, 74*).

According to the literature, sulfonamides have been synthesized using a variety of different methods, with the most general involving nucleophilic attack by ammonia or amines on sulfonyl chlorides in the presence of a base. However, despite the effectiveness of this method, it requires the availability of sulfonyl chlorides, which in the case of alkyl sulfonic acids may be difficult to prepare and handle. Recently, sulfonamides have been prepared from thiols as substrates using a H_2O_2-$ZrCl_4$ reagent system which converts them into sulfonyl chlorides; these then act as precursor for the synthesis of these sulfonamides (*73*). However, regardless of the specific method (which often is laborious and time-consuming), they only allow the synthesis of sulfonamides one at a time.

In an effort to develop new routes for the synthesis of pantoyltauramides as potential antimicrobial compounds, our approach was to develop a method that entails parallel synthesis and purification in a manner similar to that used to prepare the pantothenamides. The library of sulfonamides produced by such a method would subsequently be used for inhibitor screens. To the best of our knowledge, such a parallel synthetic method for the preparation of sulfonamides has not been reported in the literature.

3.3 Parallel synthesis of pantoyltauramides

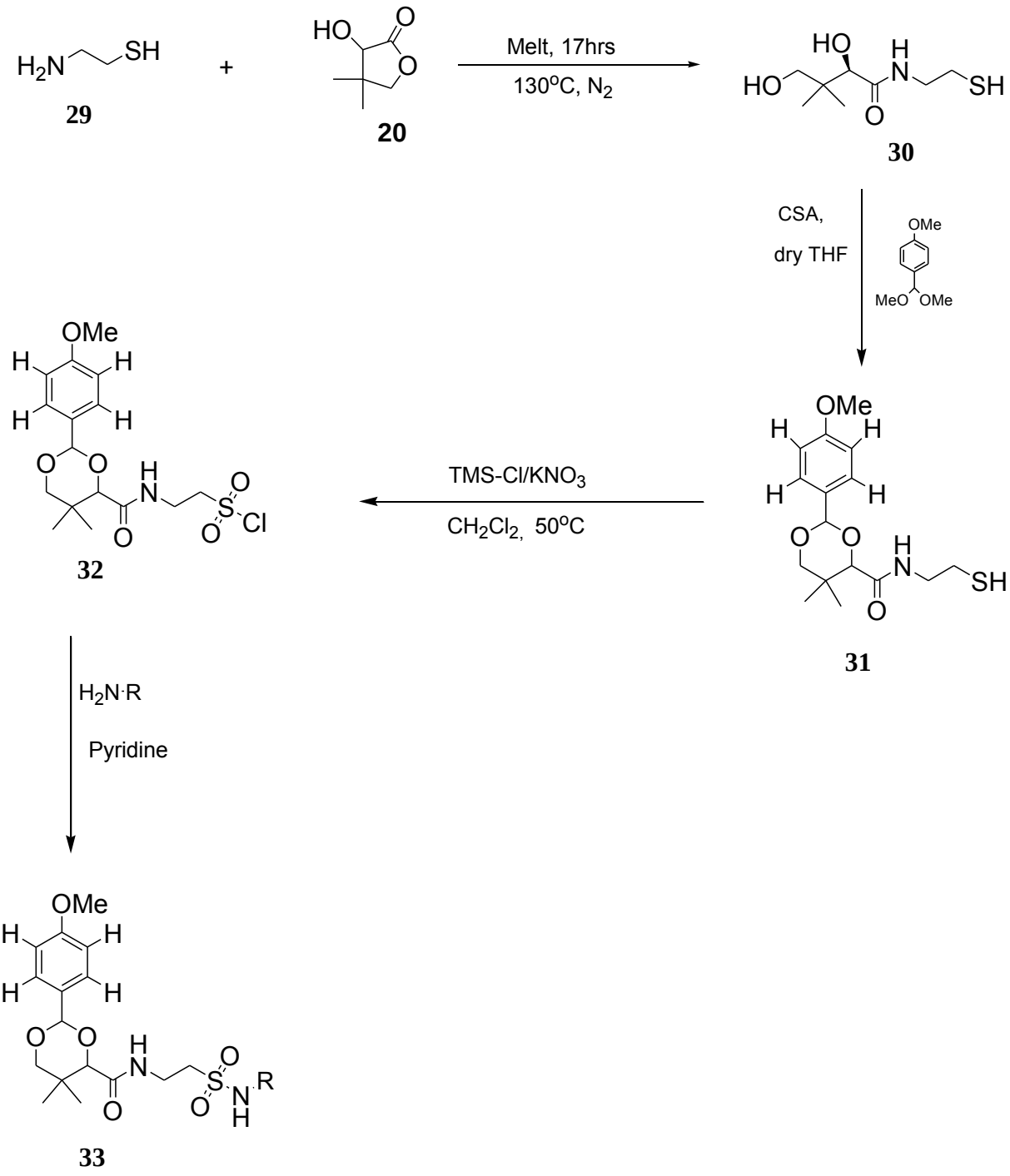


Scheme 3.5. Proposed strategy for the parallel synthesis of pantoyltauramides

In the process of developing a method for the parallel synthesis of pantoyltauramides, we realized there were several challenges that could hamper our progress in the synthesis of these compounds. The first challenge was the choice of protecting groups for the 2´- and 4´-OHs of the pantoate moiety, since these functional groups would likely interfere in the activation of the sulfonic acid or preparation of the sulfonamide. The second challenge was related to the choice of activating group for the sulfonic acid (scheme 3.5). In an attempt to address these challenges, we set out to investigate a number of reactions that would involve different protecting and activating groups in our quest to develop this method. Our strategy was to use pantoylcysteamine or pantoyltaurine as substrates, and to determine if these could be used for the successful development of the proposed method.

3.3.1 Attempt 1: Synthesis of pantoyltauramides from pantoylcysteamine via the corresponding sulfonyl chloride

Our first attempt at the synthesis of the pantoyltauramides involved using pantoylcysteamine **30** as substrate (Scheme 3.6**).**



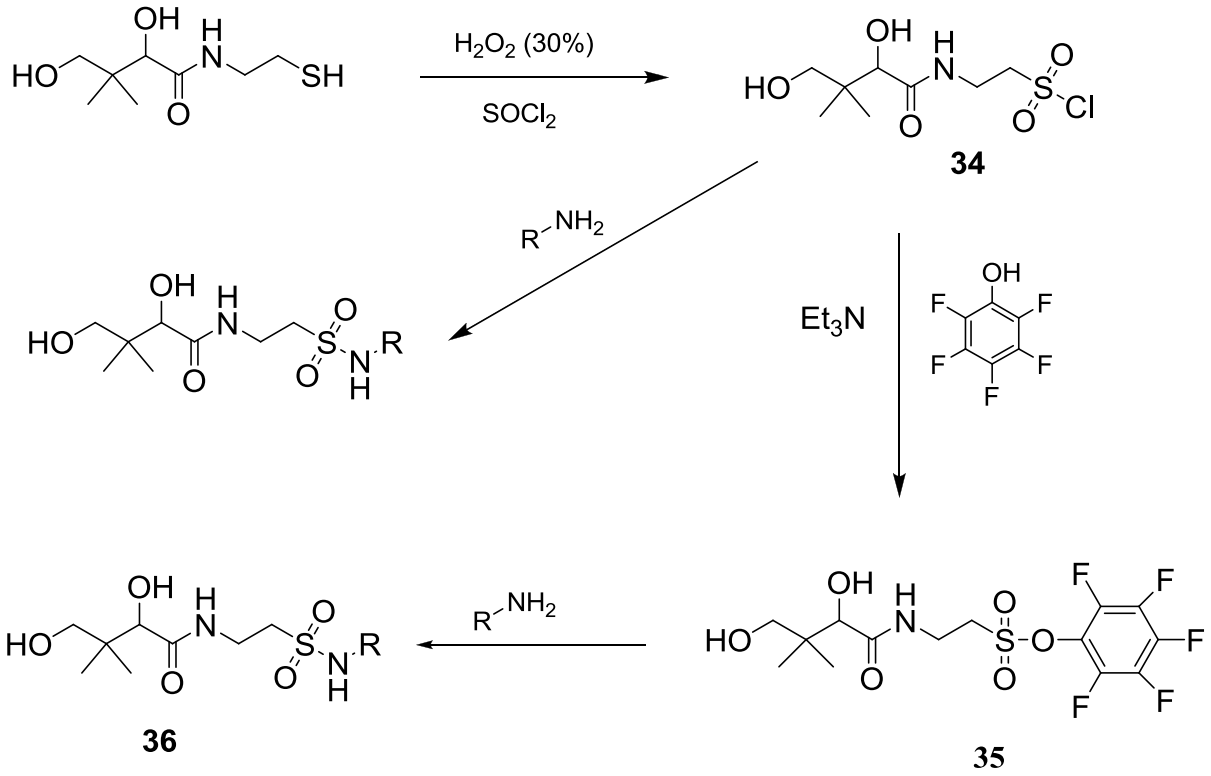
Scheme 3.6: Proposed route for the synthesis of PMB-protected pantoyltaurines from pantoylcysteamine

Pantoylcysteamine **30** was prepared by condensing pantolactone, **20** with cysteamine, **29**, which yielded **30** in 75% yields. The structure of the product was confirmed by ^{1}H NMR analysis. For protection of the 1,3-diol, we first attempted to use a *p*-methoxybenzyl (PMB) acetal as a protecting group. The protection reaction was performed in the presence of 10-camphor sulfonic acid (CSA) as a catalyst to obtain the protected thiol, **31**, in 55% yield. The ^{1}H NMR spectroscopic analysis of the protected molecule showed that the product was successfully prepared.

After successful introduction of the protecting group, we set out to investigate the conversion of the thiol to a sulfonyl chloride as an activating group. Such a conversion was first used by Prakash *et al* (*75*) in a study in which he utilized the oxidizing properties of TMS-Cl/KNO_3 reagent system to convert thiols to sulfonyl chlorides. The reaction was performed as described (*75*) by dissolving the protected thiol in CH_2Cl_2, and subjecting it to oxidation by TMS-Cl/KNO_3. Immediately after adding the reagent to the reaction mixture, we observed the formation of a viscous suspension. In addition, even after 24 hour of stirring, the reagent did not completely dissolve. ^{1}H NMR spectroscopic analysis showed that the desired sulfonyl chloride, **32**, was not obtained. Our results are in contradiction to the literature results (*75*), because by using the similar method, Prakash *et al* obtained the desired products within 2-3 hours of reaction. Our results showed that this reagent was not suitable for the oxidation and activation of pantoyltaurine protected with a PMB group.

3.3.2 Attempt 2: Synthesis of pantoyltauramides from pantoylcysteamine via the corresponding sulfonyl chlorides and pentafluorophenol ester

In a second effort to develop a method for parallel synthesis of sulfonamides, we decided to explore the viability of converting pantoyltaurine directly to the sulfonyl chloride and subsequently to the pentafluorophenol (PFP) ester, without protection. Such (PFP) could then be used in aminolysis reactions to produce sulfonamides. Our aim was to investigate the applicability of utilizing pentafluorophenol as an activating group. First, we used a procedure developed by Bahrami et al (*73*) which utilizes H_2O_2 in combination with $SOCl_2$ for direct conversion of thiols to the corresponding sulfonyl chlorides through oxidative chlorination (Scheme 3.7).

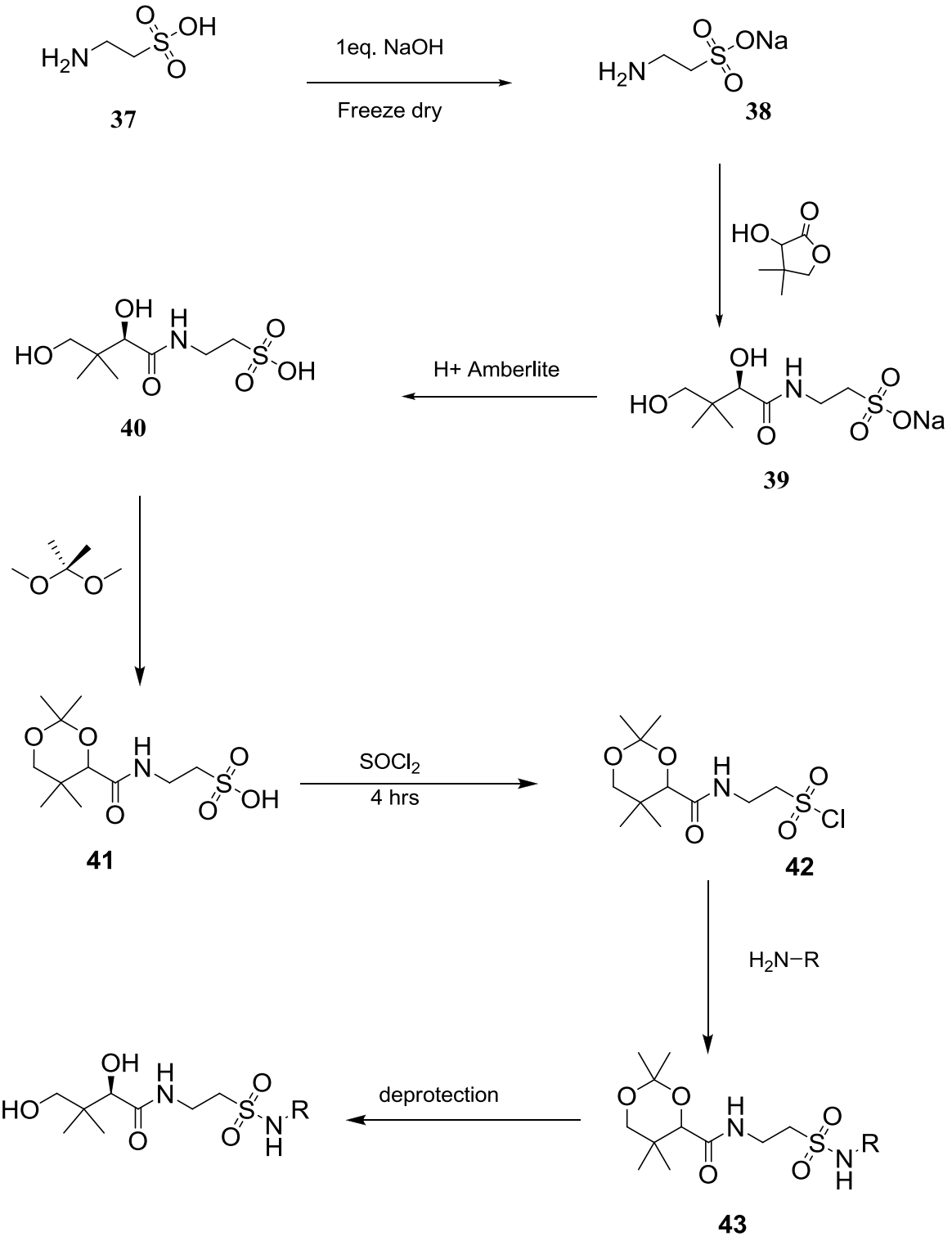


Scheme 3.7. Proposed synthetic route leading to formation of pantoyltaurines from the corresponding pentafluorophenol esters.

A reaction mixture consisting of pantoylcysteamine **30**, H_2O_2 (30%), and $SOCl_2$ was stirred in MeCN at 25°C for 5 minutes, according to the literature (*73*). After completion of the reaction time, however, we noticed that the thiol did not properly dissolve and that a colloidal suspension formed. According to TLC analysis, there was nothing shown on the TLC plate as an indication of the formation of product. We attempted to add a solution of pentafluorophenol in Et_3N to the colloidal suspension. However, only a viscous suspension resulted. Spectroscopic analysis by ^1H NMR did not show the formation of any of the desired products. From these results, it is clear that failure to protect the 1,3-diol of pantoylcysteamine may have detrimental consequences in the reactivity of the compound. According to the literature procedure, sulfonyl chlorides were obtained within 2 minutes of the reaction. However, in their case only aromatic thiols that had no multiple hydroxyl functionalities were used. We utilized an aliphatic thiol with free hydroxyl groups as a starting material, so we suspect that the most likely problem is the possibility of $SOCl_2$ reacting with the unprotected alcohols. Consequently, similar chemoselectivity was clearly not achieved.

3.3.3 Attempt 3: Synthesis of pantoyltauramides by activation of pantoyltaurine

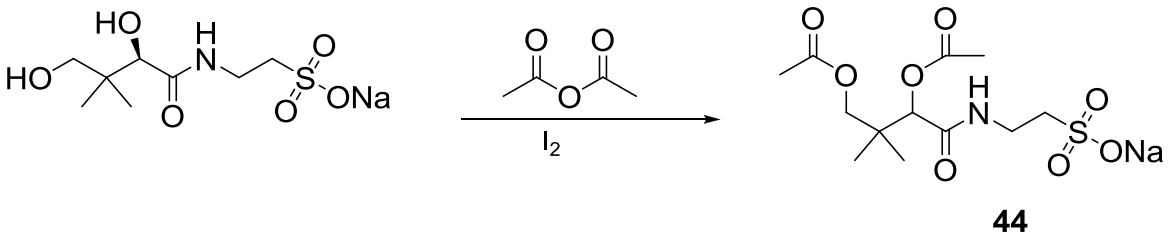
A third strategy involved attempting to synthesize sulfonamides from pantoyltaurine by following the synthetic route depicted in the scheme 3.8. First, pantoyltaurine **40** was synthesized using taurine as a starting material. Taurine, **37**, was dissolved in one equivalent NaOH (1M) to produce the tauryl sodium salt **38**, which was then condensed with pantolactone **20** upon heating to produce pantoyltaurine sodium salt **39**. The salt produced was then passed through the acidic resin which exchanges Na^+ for H^+ to produce (*R*)-pantoyltaurine **40** in 75% yield. ^{1}H NMR analysis confirmed the formation of this product.



Scheme 3.8. Proposed route for the synthesis of pantoyltauramides from pantoyltaurine.

To protect the 1,3-diol moiety of pantoyltaurine, we decided to use 2,2-dimethoxy propane to convert this group to the dimethyl acetal **41**. However, when the reaction was complete, ^{1}H NMR spectroscopic analysis of the reaction mixture showed that instead of the expected product, spectral data correlated well with that of the starting material, indicating that the protection reaction was not successful.

We also attempted to protect the 1,3-diol groups of commercially obtained (*R*,*S*)-pantoyltaurine sodium salt by using acetic anhydride to convert these to the corresponding acetates. The protection reaction (scheme 3.9) was performed by suspending the pantoyltaurine sodium salt in acetic anhydride with a catalytic amount of iodine (I_2) and then stirring the mixture at 0^oC for 2 hours followed by stirring at room temperature for 18 hours with subsequent removal of solvent *in vacuo*.

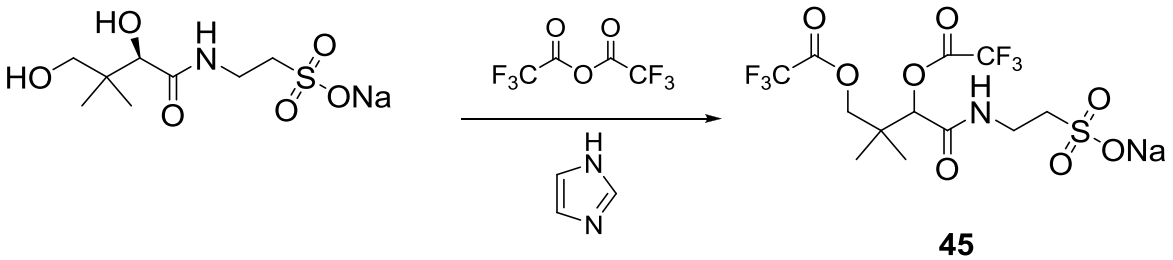


44

Scheme 3.9. Proposed protection of the 1,3-diol of pantoyltaurine sodium salt using acetic anhydride.

However, during the course of the reaction, we noticed that pantoyltaurine sodium salt did not properly dissolve in acetic anhydride; much of the salt remained undissolved after 18 hours of stirring. Spectroscopic analysis of the product by ^1H NMR showed that acetylation of pantoyltaurine sodium salt did take place but only in low conversion.

The ability of trifluoroacetic anhydride to introduce protecting groups was also investigated. In this strategy, we again attempted to protect commercially obtained pantoyltaurine sodium salt. The protection was conducted by adding trifluoroacetic anhydride drop wise over 5 minutes to a stirred suspension of pantoyltaurine sodium salt in dry THF (Scheme 3.10).



45

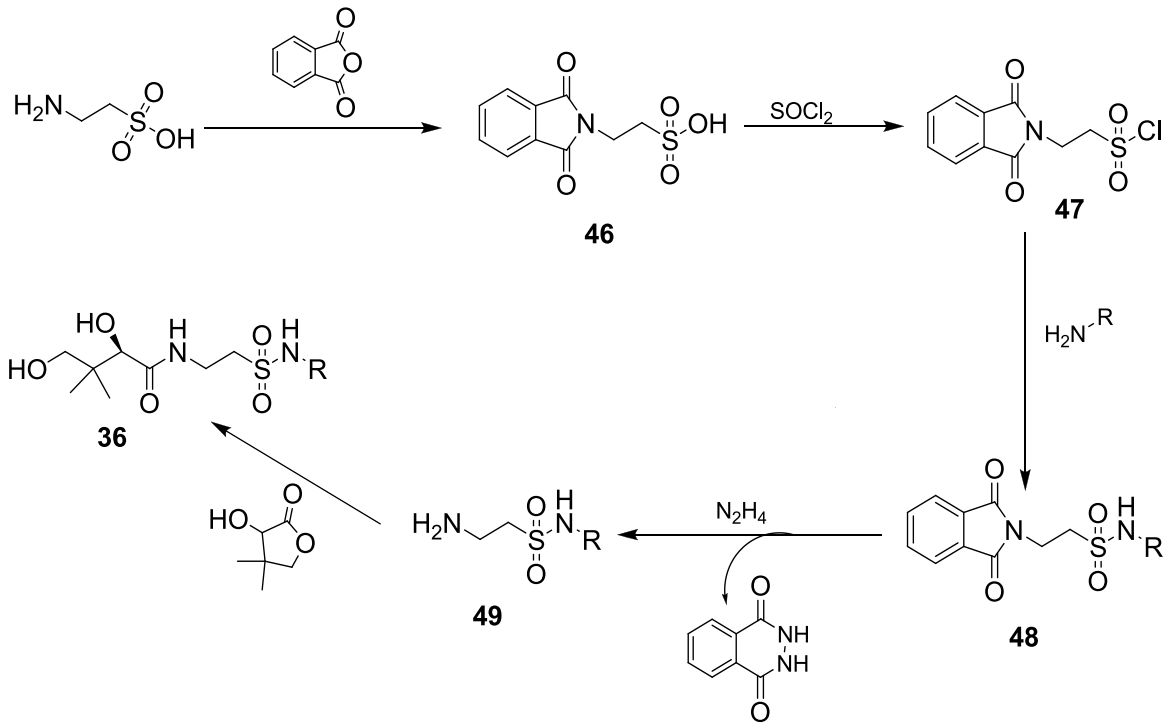
Scheme 3.10. Proposed protection of 1, 3-dihydroxyl groups of pantoyltaurine sodium salt using trifluoroacetic anhydride.

Upon addition of trifluoroacetic anhydride the reaction was stirred for over 24 hours; however, the pantoyltaurine sodium salt again did not dissolve, and the procedure was abandoned.

The attempted synthesis of sulfonamides using methods of our own design proved to be unsuccessful in our hands. We therefore decided to revisit the methods that were originally reported for the preparation of pantoyltaurines in the first studies aimed at investigating these important compounds as antiplasmodial agents.

3.3.4 Synthesis of pantoyltauramides by modification of taurine: original synthetic methods

In the original studies of pantoyltauramide by Winterbottom and co-workers (*51*), a reversed strategy was used for the preparation of these compounds. Instead of activating and reacting the sulfonic acid group of pantoyltaurine with amines to prepare the pantoyltauramides, they first prepared the tauramides and subsequently reacted these with pantolactone to give the desired products (Scheme 3.11). They achieved this by using phthalic anhydride to protect the free amine of taurine, followed by its reaction with thionyl chloride. The resulting sulfonyl chloride was subsequently reacted with a variety of amines to obtain phthalimide-protected tauramides. Deprotection with hydrazine hydrate (N_2H_4) released the amino group of the tauramide **49** (*76*), which was finally condensed with pantolactone to produce the desired sulfonamides.



Scheme 3.11. Synthetic route used for the first and original synthesis of **36.**

Based on the apparent success of this method, we decided to investigate it for the synthesis of pantoyltauramides in our case. However, we decided to employ an amine protecting group other than phthalimide since its deprotection requires hydrazine, which is highly toxic and dangerous, and since such a procedure would not be suitable for parallel synthesis. Also, we wanted to investigate other methods for the activation of the sulfonic acid.

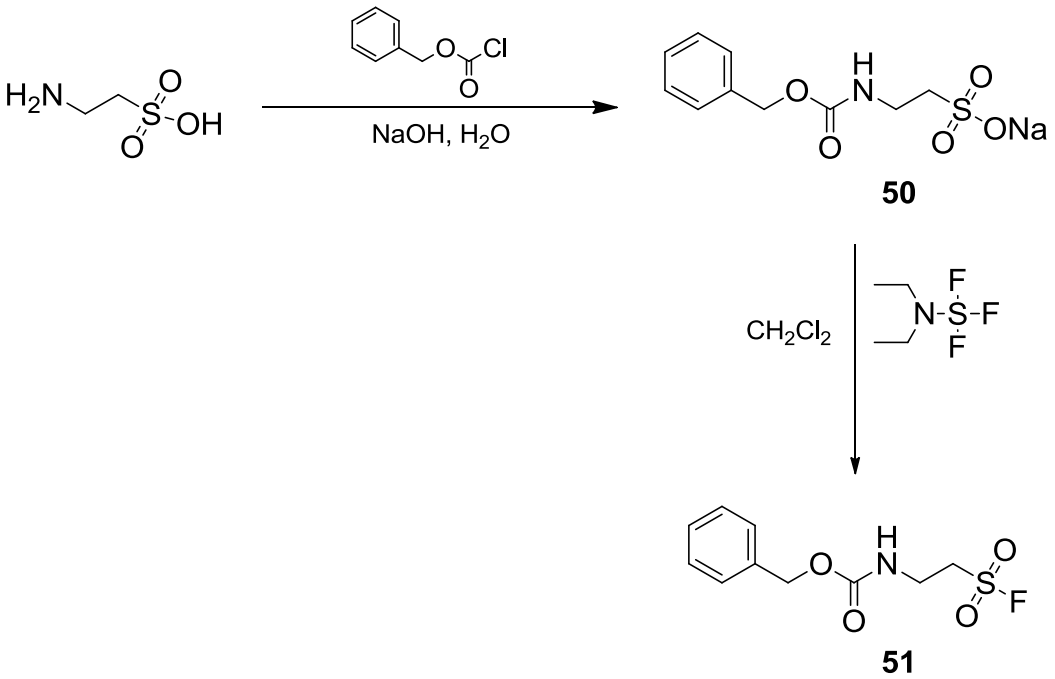
In view of this background, we decided to explore other published methods used in the synthesis of tauramides, and to apply these in a modification of the original pantoyltauramide synthesis. We therefore attempted to use a method which involves using benzyl carbamates (Cbz) and sulfonyl fluorides as protecting and activating groups respectively.

3.3.5 Attempt 4: Synthesis of pantoyltauramides by modification of taurine: using modified protection and activation strategies

3.3.5.1 Protection and activation of taurine

The modified strategy for the synthesis of protected and activated tauramides (Scheme 3.12) relied on published methods. A method for the protection of the amino group of taurine **37** using Cbz-Cl was developed by Brouwer et al in 2000 (*77*). We attempted to use this method

in our effort to synthesize tauramides **49** from taurine. With slight modification to the method, the protection of taurine with Cbz-Cl was conducted as follows: taurine was dissolved in aqueous NaOH (1 M) followed by addition of a solution of Cbz-Cl in dioxane with stirring for 1 hour (Scheme 3.12). The reaction mixture was then extracted with EtOAc to get rid of unreacted Cbz-Cl, followed by evaporation of solvent *in vacuo*. While the literature procedure made use of P_2O_5 to dry the crude sodium sulfonate salt **50**, we instead freeze dried the product, which was obtained in 90% yield.



50

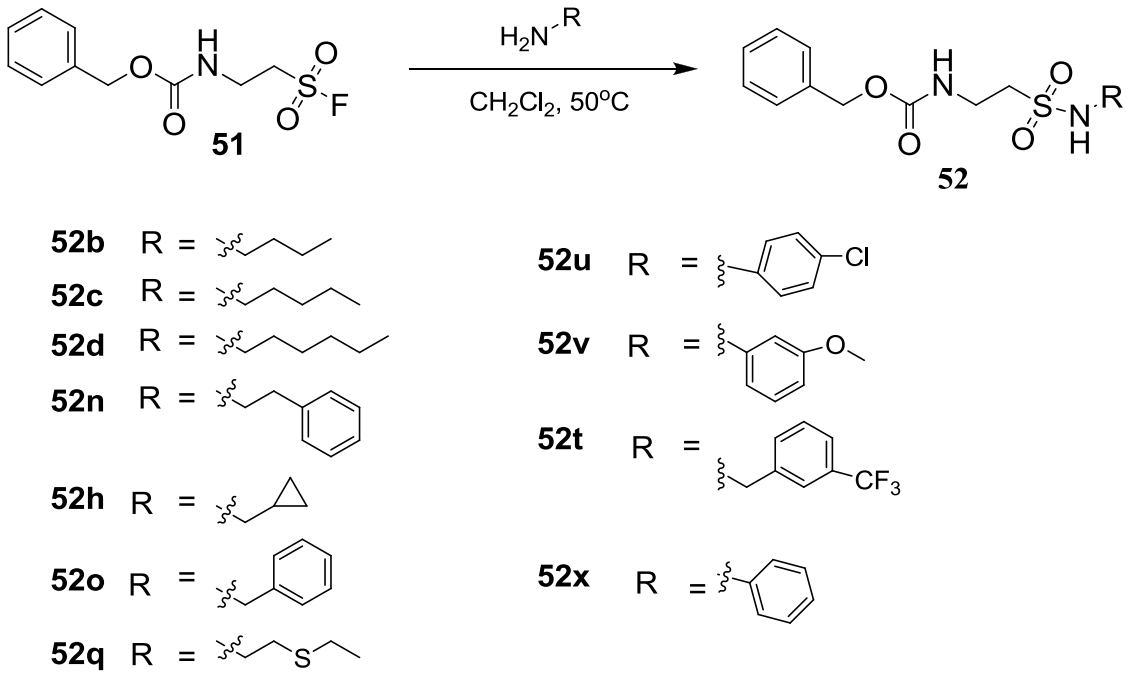
51

Scheme 3.12. Protection of taurine with Cbz-Cl, followed by activation of the sulfonic acid by reaction with DAST to form the sulfonyl fluoride.

In our effort to activate the sulfonate salt prepared above for aminolysis, we explored the practicability of using diethylaminosulfur trifluoride (DAST) as an activation agent , as described in the literature (*78*). With a minor modification to the method, a solution of DAST was added to stirred slurry of the sodium sulfonate **50** in CH_2Cl_2 at 0^oC under a N_2 environment. Product formation was followed by TLC analysis. The product was obtained after stirring for 2 hours at 0^oC, followed by solvent evaporation and purification by flash chromatography. The resulting fluoride **51** (Scheme 3.12) was characterized by ^1H NMR and ^{19}F NMR to verify its structure. ^{19}F NMR spectral data showed the presence of a fluorine peak at 56.90 ppm. This shows that the fluorination of **50** was successfully achieved with the product **51** being obtained in good isolated yield of 44%.

3.3.5.2 Synthesis of Cbz-tauramides by aminolysis of Cbz-tauryl fluoride

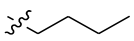
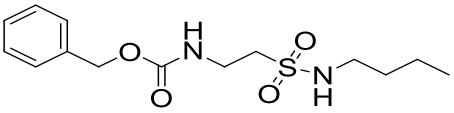
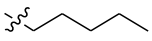
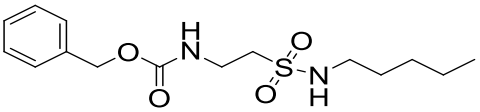
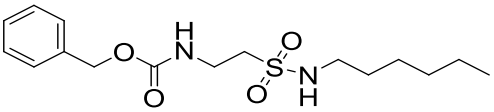
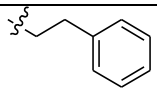
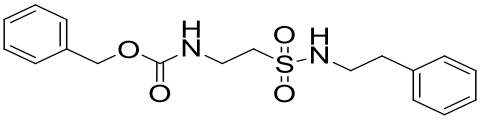
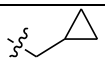
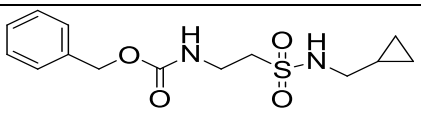
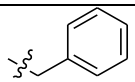
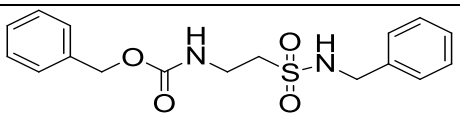
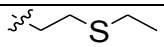
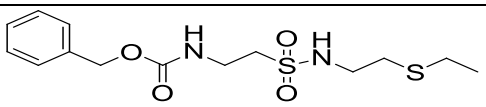
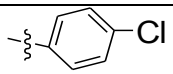
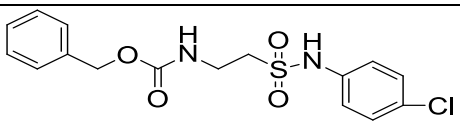
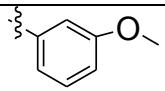
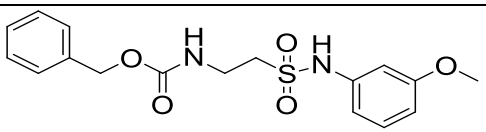
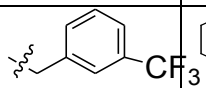
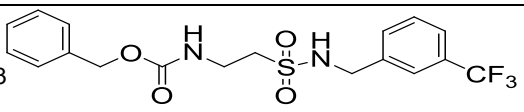
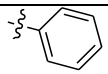
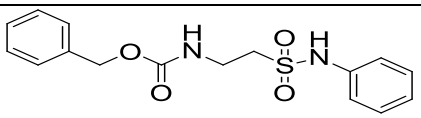
In our attempt to subject the Cbz-tauryl fluoride **51** to aminolysis, we again used a method available in the literature (*79*). Conversion of Cbz-tauryl fluoride to Cbz-tauramides **52** was done by reaction of a variety of amines with the fluoride dissolved in CH_2Cl_2, at 50ºC (Scheme 3.13).



Scheme 3.13. Reaction of sulfonyl fluoride with various amines

The formation of product was monitored by TLC, ^{1}H NMR, as well as ^{19}F NMR. In the case of ^{19}F NMR spectroscopic analysis, the formed products failed to show a fluorine peak, as expected. In reactions where no product was obtained, ^{1}H NMR spectroscopic analysis of the reaction mixtures did not show the formation of any structures that correspond with the expected products. In addition, ^{19}F NMR spectroscopic analysis showed the presence of fluorine peaks, showing the aminolysis reaction did not take place in these reactions.

Table 3.6. Cbz-tauramides and their corresponding yields

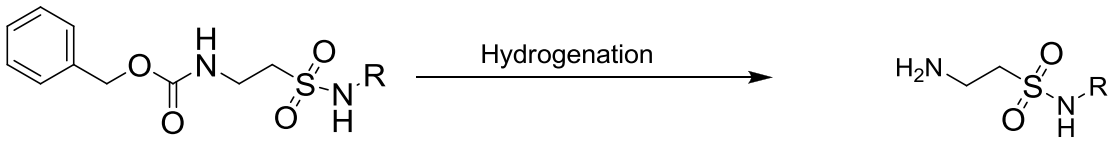
Entry	R-group	Cbz-tauramide	% yield	^{19}F NMR (presence of F-peak)
52b			68	No
52c			75	No
52d			80	No
52n			80	No
52h			69	No
52o			74	No
52q			65	No
52u			No product	Yes
52v			No product	Yes
52t			No product	Yes
52x			No product	Yes

The results of the aminolysis reaction are shown in the table 3.6, and show that the Cbz-tauramides were obtained in excellent yields and with high purity in some reactions, (**52b, 52c, 52d, 52n, 52h, 52o** and **52q)**, while no product was formed in other reactions **52u, 52v,**

52t and **52x)**. Aromatic amines (anilines) with either electron-withdrawing or electron-donating substituents (Table 3.6, entries **52u-52x**) did not react very well and did not give corresponding Cbz-tauramides. However, all aliphatic amines, even those with aromatic side chains, produced the corresponding Cbz-tauramides in good yields (Table 3.6, entries **52b, 52c, 52d, 52n, 52h, 52o** and **52q)**. This results show that Cbz-protected tauryl fluorides are ideal precursors for the parallel synthesis of tauramides.

3.3.5.3 Synthesis of tauramides by deprotection of the Cbz-tauramides

Upon realizing successful aminolysis, we then attempted to cleave off Cbz-group from Cbz-tauramides through catalytic transfer hydrogenation (Scheme **3.14**).

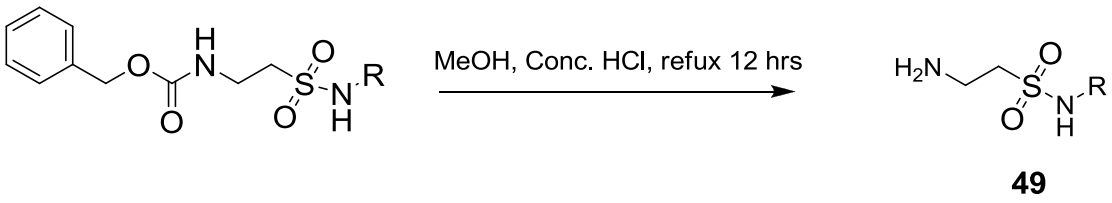


52 **49**

Scheme 3.14. Deprotection of **52b, 52c, 52d, 52n, 52h, 52o** and **52q** (table 3.6) by catalytic hydrogenation

First, we attempted to cleave off Cbz-Cl group from Cbz-tauramides **52b, 52c, 52d, 52n, 52h, 52o** and **52q** (Table 3.6) by hydrogenation under N_2 environment using 10% Pd-black with ammonium formate in methanol acting as a donor of hydrogen. However, spectroscopic analysis of the hydrogenation products by ^{1}H NMR analysis showed that spectral data obtained did not correspond with the structures of the expected tauramides. Instead, it corresponded well with the structure of the starting material, the Cbz-tauramides. This shows that the catalytic transfer hydrogenation was not successful. This can be attributed to either the ammonium formate not acting as hydrogen donor as expected, or that the palladium catalyst was not effective under these conditions.

Next we attempted to remove the Cbz-group from Cbz-tauramides by refluxing the latter using concentrated HCl in the presence of methanol (Scheme 3.15). However, ^{1}H NMR spectroscopic analysis showed that this deprotection did not work for compounds **52b, 52d, 52h** and **52o** (Table 3.6), but was successful in the case of three compounds **52c, 52n**, and **52q** (Table 3.6) with high isolated yields of about 72, 89 and 70% respectively.

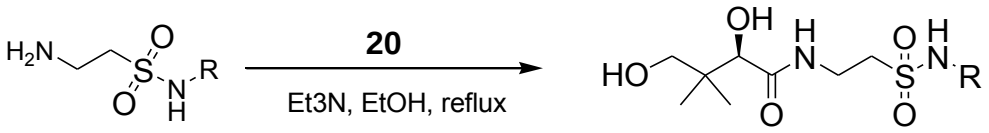


49

Scheme 3.15. Deprotection of **52b, 52c, 52d, 52n, 52h, 52o** and **52q** (table 3.6), using conc. HCl in MeOH.

3.3.5.4 Synthesis of pantoyltauramides from tauramides

With the free tauramides **49** in hand, the desired pantoyltauramides **36** could be prepared by coupling them to pantolactone (Scheme 3.16).

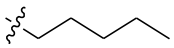
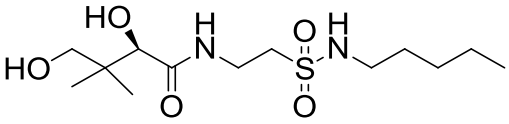
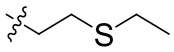
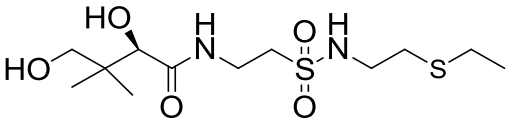
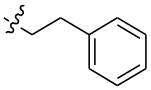
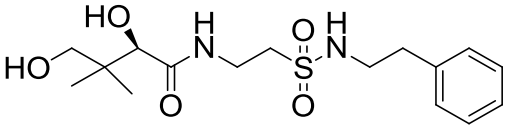


49 **36**

Scheme 3.16: Coupling of tauramides to pantolactone to form pantoyltauramides

The pantoyltauramides **36** were prepared by adding pantolactone **20** (3 equiv) and Et_3N (3 equiv) to a solution of tauramides **49** in ethanol. The reaction was allowed to stir under reflux for 7 hours, after which the solvent was evaporated, and the resulting pantoyltauramide was purified by flash chromatography (1:4 EtoAC: Hexane to 2:1 EtoAC: Hexane to obtain **36**. Spectroscopic analysis of **36** by ^{1}H NMR showed that *N*-pentyl pantoyltauramide (**36c**, table 3.7) was pure as demonstrated by its clean ^{1}H NMR spectrum. However, spectral data of *N*-2-(ethylthio)ethyl pantoyltauramide **36q**, (table 3.7), and phenethyl pantoyltauramide **36n** (table 3.7) showed that these two compounds contained some residual triethyl amine as signified by the presence of peaks at 1.35 and 3.2 ppm respectively. This was surprising, since these pantoyltauramides were subjected to several purification processes by varying the ratio of EtoAC to hexane, including dissolving of the pantoyltauramides in water with subsequent lyophilisation in order to get rid of the triethylamine.

Table 3.7: Pantoyltauramides and corresponding yields

Entry	R-group	Pantoyltauramide	Yield (%)
36c			40
36q			45
36n			52

3.3.6 Conclusion

Our attempts to develop a method for parallel synthesis and purification of pantoyltauramides proved to be unsuccessful. However, we have managed to synthesize pantoyltauramides by using methods described in the literature. Overall, we failed to synthesize as many pantoyltauramides as possible due to a number of difficulties we encountered in the synthetic process. For example, in the process of cleaving off Cbz-group from Cbz-tauramides, the procedure entailed refluxing the Cbz-tauramides in concentrated HCl, which proved to be incompatible with some amine functionalities. While the Cbz-groups could most probably have successfully been removed by hydrogenation in some of these cases, such a method would not be amenable to parallel synthesis. It will therefore be important to investigate other amine protecting groups in future work.

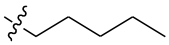
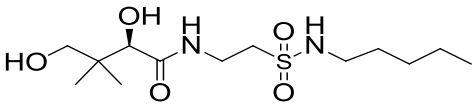
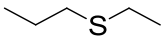
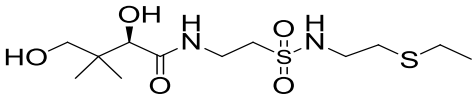
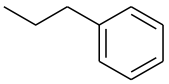
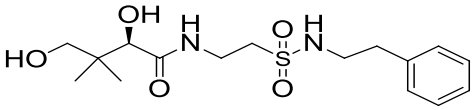
3.4 Inhibitory effects of *N*-substituted pantoyltauramides against *P. falciparum*

In studies performed in 1940's, all the pantoyltauramides prepared at that time were tested for antimalarial activity in birds. Findings showed that *N*-(4-chlorophenyl) pantoyltauramide demonstrated four times higher activity than quinine.

In this study, the three pantoyltauramides **36c**, **36q** and **36n** were tested for antiplasmodial activities against *P. falciparum*. The results show that at a concentration of 200 µM, the

pantoyltauramides demonstrated very good activities, inhibiting the proliferation of *P. falciparum* by between 67 and 78%.

Table 3.8. Aantiplasmodial activity of pantoyltauramides at 200 µM.

Entry	R-group	Pantoyltauramides	% inhibition
36c			78
36q			78
36n			67

Comparing the activities of pantoyltauramides with that of *N*-substituted pantothenamides, it can be seen that the former demonstrated better inhibition of proliferation of the malaria parasites than the latter. The pantoyltauramides seem to be promising antimalarial agents considering the fact that of all the analogues tested against *P. falciparum* in this study, the pantoyltauramides showed the best inhibitory activity. Analyzing the results, it also seems as if certain R-groups give the best the inhibitory characteristics. For example, the N-pentyl substituent imparted good inhibitory characteristics to nearly all the pantothenate analogues in which it occurs. Specifically, of all the alkyl *N*-substituted pantothenamides tested against *P. falciparum*, the *N*-pentyl α-methyl pentylpantothenamide **27c** showed the best activity, inhibiting the proliferation of parasites by 65%. Likewise, N-pentyl pantoyltauramide **36c** inhibited the growth of *P. falciparum* by 78% in vitro. This is the best activity of all the analogues tested in this study.

3.5 Conclusion

The results of this study on modified pantothenamides indicate that these compounds have a very different activity profile when acting against bacteria compared to malaria parasites. The results show that the methylated pantothenamides do not exhibit increased activity on *S. aureus* compared to previous studies. This observation indicates that the added methyl functionalities on the β-alanine moiety did not enhance the activities of these analogues in these bacteria. In contrast, the series of α-methylpantothenamides did show improved antiplasmodial activity compared to the previous study's results. This indicates that especially α-methylation of selected pantothenamides may increase the potency of these compounds against the proliferation of *P. falciparum*. The selected pantoyltauramides that were prepared and synthesized also showed good inhibition. This is encouraging, as it shows that both the methyl functionality introduced on the β-alanine moiety, as well as the sulfonamide, has impacted positively on the inhibitory activities of these analogues, possibly by increasing their biological stability. It will be important to follow up on these initial results in future work, particularly by determining MIC values for the most promising inhibitors. It will also be important to determine if the inhibitory effect of these compounds are reduced in the presence of pantothenate, to establish if they will be effective *in vivo*.

Chapter 4

Experimental

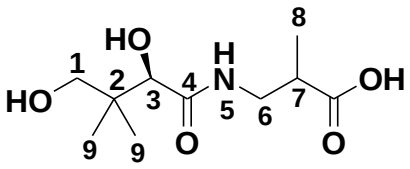
Materials and methods

All chemicals used in the synthesis of pantothenamides and sulfonamides were purchased from Sigma-Aldrich, Merck, or Acros Organics. The resin and silica used in purification processes were purchased from Sigma-Aldrich. All solvents used in the preparation and purification of these compounds were CHROMASOLV HPLC grade solvents purchased from Sigma-Aldrich. Polypropylene 2 mL 96-well filter plates purchased from NUNC were used during aminolysis reactions. AcroPrep 96-well deep-well filter pates obtained from Pall Life Sciences were used during filtration of pantothenamides for purification purposes. Polypropylene cluster tubes (Corning) were used in yield determination. Solvent from deep-well plates was evaporated on a Labconco Centrivap concentrator. SYBR green for fluorescence studies as well as Gentamycin and Albumax II for cell culturing were purchased from Invitrogen. Flat-bottomed 96-well cell culture plates from NUNC were used for growth assays as well as fluorescent measurement. All NMR analyses were performed on Varian INOVA instruments (300 MHz, 400 MHz, and 600 MHz) at the Central Analytical Facility (CAF) at Stellenbosch University. All LC-ESI-MS analyses were also done at CAF using a Waters 2690 Separations Module with a Waters 996 Photodiode Array Detector for LC separations, followed by mass analysis on a Waters Micromass Quattro mass spectrometer.

4.1 Synthetic preparation of α- and β-methyl pantothenate thioesters

4.1.1 *S*-Phenyl α-methylthiopantothenate (22)

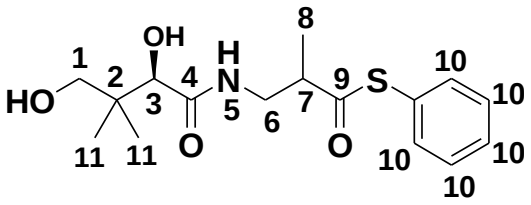
4.1.1.1 α-Methylpantothenic acid (21)



D,L-3-amino isobutyric acid (0.50 g; 4.8 mmol) was dissolved in 1M NaOH (4.8 mL) and the solution was freeze dried. Pantolactone (0.70 g; 5.28 mmol) was added to the resulting white salt and the mixture was heated under nitrogen for 17 hours at 130^{o}C. The resulting sticky oil

was dissolved in water and loaded onto a column of Amberlite IR-120 (H^+-form) ion exchange resin. The free α-methyl pantothenic acid was eluted with deionised water, and then lyophilized. The resulting residue was purified by flash chromatography (silica gel; ethyl acetate/methanol/water/acetonitrile 5:2:1:1) to remove unreacted pantolactone and amine to give the pure acid **21** as a sticky oil. (0.8 g; 70%). ^{1}H NMR (400 MHz; D_2O; 25^{o}C): δ 0.892 (3H, s, -CH_3[9]), 0.925 (3H, s, -CH_3[9]), 1.21 (3H, s, -CH3[8]), 2.76 (1H, m, -CH-[7]), 3.39 (1H, s, -CH[1]), 3.42 (1H, s, -CH[1]), 3.52 (1H, s, -CH-[6]), 3.55 (1H, s, -CH-[6]), and 4.01 (1H, br s, -CH-[3]).

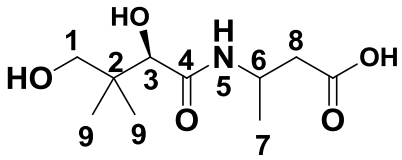
4.1.1.2 *S*-Phenyl α-methylthiopantothenate (22)



The acid **18** (0.80 g; 3.4 mmol) was dissolved in 3.4 mL DMF, followed by addition of diphenylphosphoryl azide (1.3 mL; 6.12 mmol) and thiophenol (0.42 mL; 4.08 mmol). The reaction mixture was cooled to 0^{o}C, followed by addition of triethylamine (0.854 ml; 6.12 mmol). The reaction mixture was stirred at 0^{o}C for 10 minutes, followed by stirring at room temperature for 3 hours. Ethyl acetate (50 ml) was added to the reaction mixture, followed by washing with 5% citric acid (3×10 ml), 1M $NaHCO_3$ (3×10 ml), and saturated NaCl solution (2×10 ml). The organic layer was dried over Na_2SO_4 followed by removal of the solvent *in vacuo* using a rotary evaporator. The resulting residue was purified by flash chromatography (silica gel; ethyl acetate/hexane 2:1 to 4:1) to give **22** as a sticky oil (0.60 g; 55%). ^{1}H NMR (600 MHz; $CDCl_3$; 25^{o}C): δ 0.92 (3H, s, -CH_3[11]), 1.0 (3H, s, -CH_3[11]), 1.29 (3H, d, *J* 6.98, -CH3[8]), 3.43-3.56 (4H, m, -CH2-[1+6]), 3.1 (1H, m, -CH-[7]), 4.05 (1H, s, -CH-[3]), 7.1 (1H, br s, -NH-[5]), and 7.4 (5H, m, arom.[10]); ^{13}C NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 15.0, 19.8, 22.0, 40.0, 42.5, 48.2, 78.0, 121.5, 128.0, 130.0, 135.2, 174.8, and 202.5; ESI-MS: *m/z* $[M-H]^-$ calcd for $C_{16}H_{22}NO_4S$: 324.13; found 324.1

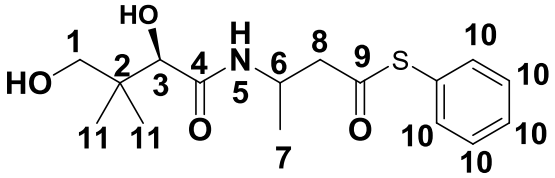
4.1.2 *S*-Phenyl β-methylthiopantothenate (25)

4.1.2.1 β-Methylpantothenic acid (24)



The synthesis, workup and purification were performed as for **21**, using (*R*/*S*)-3-Aminobutyric acid (0.50 g; 4.8 mmol) as starting material. The pure acid **24** was obtained as a sticky oil (0.6 g; 54%). ^{1}H NMR (400 MHz; D_2O; 25^{o}C): δ 0.91 (3H, s, -CH_3[9]), 0.96 (3H, s, -CH_3[9]), 1.23 (3H, s, -CH_3[7]), 2.5 (2H, t, *J* 6.5, -CH_2-[8]), 3.4 (2H, d, -CH_2-[1]), 3.53 (1H, d, *J* 10.46, -CH-[6]), 3.97 (1H, s, -CH-[3]) and 4.3 (1H, br s, -NH-[5]).

4.1.2.2 *S*-Phenyl β-methylthiopantothenate (25)

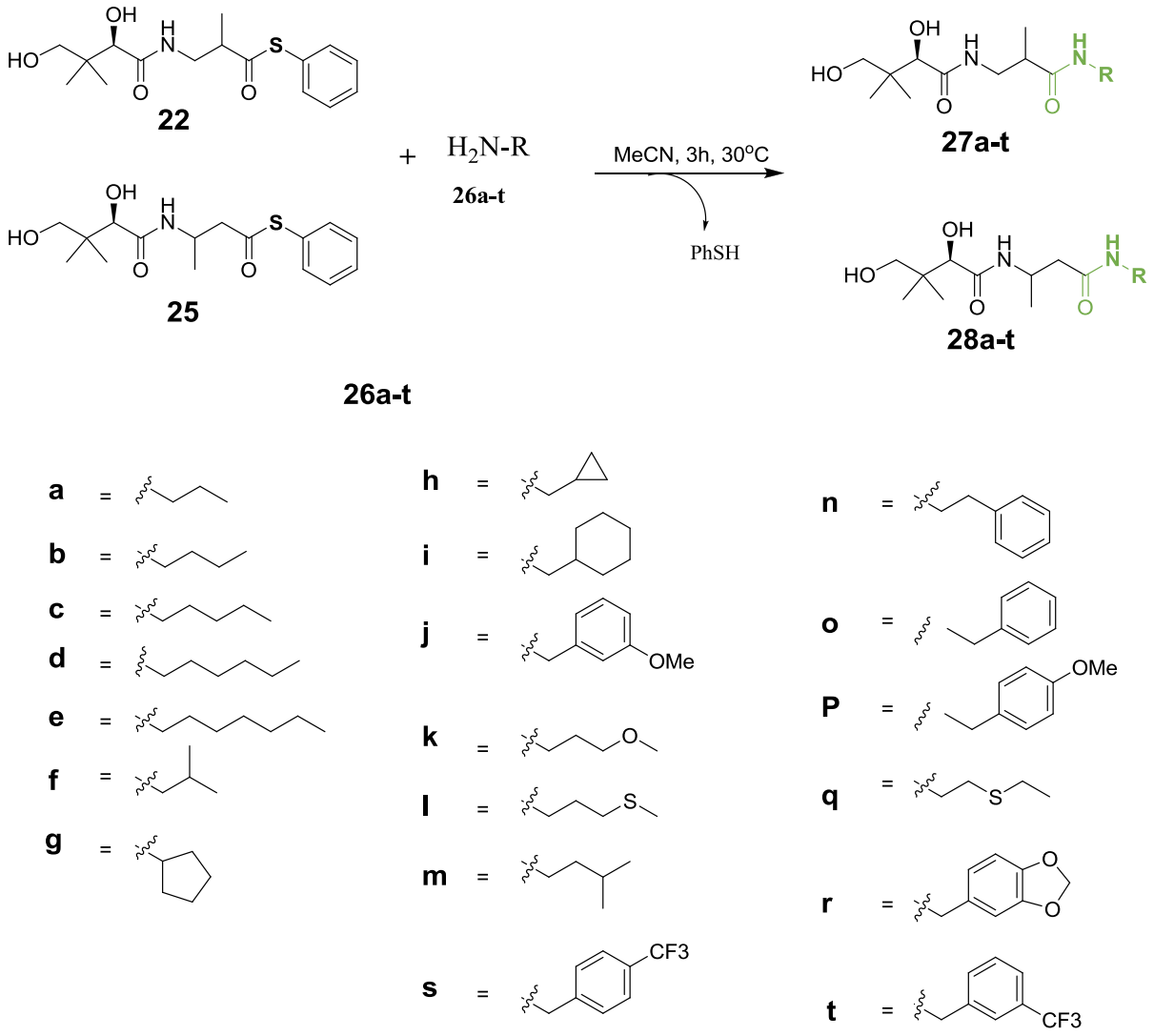


The synthesis, workup and purification were performed as for **22**, using **24** (0.60 g; 2.6 mmol) as starting material. The product **25** was obtained as a sticky oil (0.40 g, 47%). ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.9 (3H, s, -CH_3[11]), 1.0 (3H, s, -CH_3[11]), 1.3 (3H, m, -CH_3[7]), 2.86-2.91 (2H, m, -CH_2-[8]), 3.5 (2H, m, -CH_2-[1]), 3.99 (1H, d, *J* 4.7, -CH-[3]), 4.4 (1H, s, -CH-[6]), 7.1 (1H, br s, -NH-[5]) and 7.4 (5H, m, arom.[10]); ^{13}C NMR (400 MHz; $CDCl_3$; 25^{o}C); δ 20.2, 22.8, 40.1, 43.5, 49.0, 71.0, 78.0, 78.2, 121.5, 128.1, 132.2, 135.1, 174.3, and 197.0; ESI-MS: *m/z* $[M+H]^+$ calcd for $C_{16}H_{24}NO_4S$: 326.13; found 326.0.

4.2 Parallel synthesis and purification of α- and β-methylpantothenamides

4.2.1 General procedure

The thioesters **22** and **25** prepared above were subjected to parallel aminolysis using 20 different amines (Scheme 4.1), followed by parallel purification. The procedure was executed according to an established method (*66*).



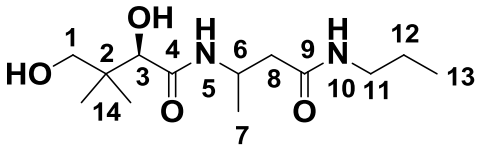
Scheme 4.1: Aminolysis of *S*-phenyl methylpantothenate esters using various amines.

The aminolysis reactions were performed by using stock solutions of the thioesters (prepared in 40% aqueous acetonitrile) and amines (prepared in 100% acetonitrile). Reactions were performed in 2 mL 96-well deep-well plates by addition of the amine (100 mM final concentration, 5 eq.) followed by addition of the thioester (20 mM final concentration, 1 eq.) The negative control reaction contained no amine. Each well had a final reaction volume of 1 mL. The deep-well plate was then capped and placed in an incubator with vigorous shaking for 3 hours at 30°C.

4.2.2 Purification method

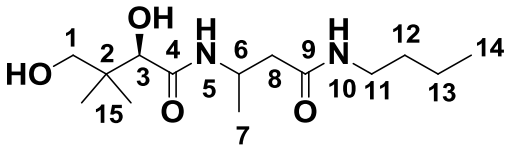
After 3 hours, 1,4-diaminobutane (100 µL; 550 mM) was added to the reaction mixtures in each well. The mixtures were incubated with shaking for 1 hour at 30°C, after which they were loaded onto pre-washed Amberlite IRC-86 weak cation exchange resin (300 mg dry weight per well) loaded in a 1 mL AcroPrep 96-well filter plate. The resin was allowed to elute under gravity, followed by washing it twice with 300 µL 40% aqueous acetonitrile. The combined eluates were dried overnight on a Centrivap concentrator under reduced pressure. After drying, the resin was washed again with another 1200 µL 40% aqueous acetonitrile and the collected eluates were added to the dried residues. The resulting solutions were transferred to individual pre-weighed 96-well cluster tubes and dried for another 3 days by centrifugal concentration to remove all solvent and thiophenol present in the product mixtures. After the final drying process, the cluster tubes containing the dried products were weighed again individually to establish the final yield of each product.

*N-Propyl β-methylpantothenamide (**28a**)*



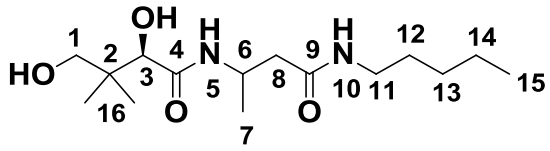
^{1}H NMR (300 MHz; $CDCl_3$; 25°C): δ 0.9 (3H, t, *J* 2.16, -CH_3[13]), 0.95 (3H, s, -CH_3[14]), 1.0 (3H, s, -CH_3[14]), 1.3 (3H, m, -CH_3[7]), 1.52 (2H, m, -CH_2-[12]), 2.5 (2H, m, -CH2-[8]), 3.2 (2H, m, -CH_2-[11]), 3.5 (2H, m, -CH2-[1]), 4.0 (1H, s, -CH-[3]), 4.4 (1H, br s, -CH-[6]), 6.1 (1H, br s, -NH-[10]) and 7.5 (1H, br s, -NH-[5]).

*N-Butyl β-methylpantothenamide (**28b**)*



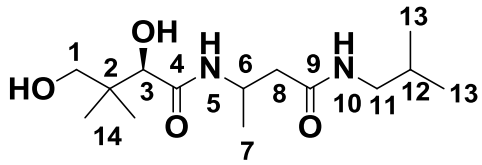
^{1}H NMR (300 MHz, $CDCl_3$; 25°C): δ 0.9 (3H, t, *J* 3.42, -CH_3[14]), 0.99 (3H, s, -CH_3[15]), 1.02 (3H, s, -CH_3[15]), 1.25 (3H, m, -CH_3[7]), 1.35 (2H, m, -CH_2-[13]), 1.5 (2H, m, -CH_2-[12]), 2.5 (2H, m, -CH_2-[8], 3.25 (2H, m, -CH_2-[11]), 3.5 (2H, s, -CH_2-[1]), 4.0 (1H, s, -CH-[6]), 4.30 (1H, br s. –CH-[3]), 6.0 (1H, br s, -NH-[10]) and 7.5 (1H, br s, -NH-[5]).

*N-Pentyl β-methylpantothenamide (**28c**)*



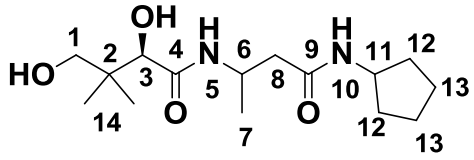
^{1}H NMR (300 MHz; $CDCl_3$; 25$_o$C): δ 0.85 (3H, m, -CH_3[15]), 0.99 (3H, s, -CH_3[16]), 1.01 (3H, s, -CH_3[16]), 1.23-1.33 (3H, m, -CH_3-[7]), 1.23-1.33 (4H, m, -CH_2-[13+14]), 1.48 (2H, m, -CH_2-[12]), 2.3-2.45 (2H, m, -CH_2-[8]), 3.4 (2H, m, -CH_2-[11]), 3.5 (2H, m, -CH_2-[1]), 4.0 (1H, s, -CH-[6]), 4.3 (1H, br s, -CH-[3]), 6.0 (1H, br s, -NH-[10]) and 7.5 (1H, br s, -NH-[5]).

*N-Isobutyl β-methylpantothenamide (**28f**)*



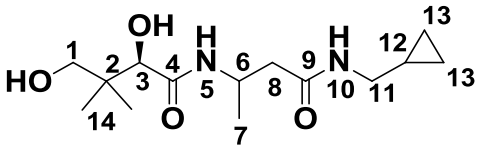
^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.89 (3H, t, -CH_3[13]), 0.9 (3H, s, -CH_3[14]), 0.92 (3H, t, -CH_3[13]), 1.0 (3H, s, -CH_3[14]), 1.23-1.27 (3H, m, -CH_3[7]), 2.5 (2H, m, -CH_2-[8]), 2.62 (1H, br s, -CH-[12]), 2.98-3.13 (2H, m, -CH_2-[11]), 3.5 (2H, m, -CH_2-[1]), 4.0 (1H, s, -CH-[6]), 4.4 (1H, br s, -CH-[3]), 6.0 (1H, br s, -NH-[10]) and 7.5 (1H, br s, -NH-[5]).

*N-Cyclopentyl β-methylpantothenamide (**28g**)*



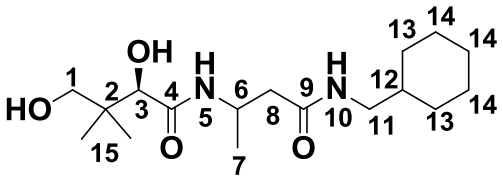
^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.96 (3H, s, -CH_3[14]), 1.02 (3H, s, -CH_3[14]), 1.25 (3H, m, -CH_3[7]), 1.32-1.40 (2H, m, -CH-[13]), 1.93-1.96 (2H, m, -CH-[12]), 2.4 (2H, m, -CH_2-[8]), 3.5 (2H, m, -CH_2-[1]), 4.0 (1H, s, -CH-[11]), 4.2 (1H, br s, -CH-[6]), 4.35 (1H, br s, -CH-[3]), 5.9 (1H, br s, -NH-[10]) and 7.5 (1H, br s, -NH-[5]).

*N-Cyclopropanemethyl β-methylpantothenamide (**28h**)*



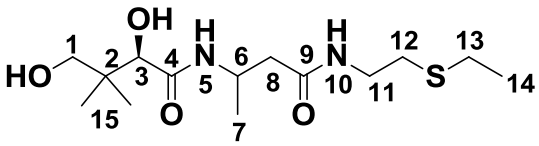
^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.2 (2H, m, -CH[13]), 0.5 (1H, m, -CH-[12]), 0.96 (3H, s, -CH_3[14]), 1.05 (3H, s, -CH_3[14]), 1.3 (3H, m, -CH_3[7]), 2.5 (2H, m, -CH_2-[8]), 3.1 (2H, m, -CH_2-[11]), 3.5 (2H, m, -CH_2-[1]), 4.0 (1H, s, -CH-[6]), 4.35 (1H, br s, -CH-[3]), 6.0 (1H, br s, -NH-[10]), and 7.5 (1H, br s, -NH-[5]).

*N-Cyclohexanemethyl β-methylpantothenamide (**28i**)*



^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.92 (3H, s, -CH_3[15]), 0.92 (3H, s, -CH_3[15]), 0.99 (3H, t, *J* 6.04, -CH_3[7]), 1.15-1.25 (6H, m, -CH_2-[14]), 1.5 (4H, br s, -CH_2-[13]), 2.4 (1H, s, -CH-[12]), 2.5 (2H, m, -CH_2-[8]), 3.1 (2H, m, -CH_2-[11]), 3.5 (2H, m, -CH_2-[1]), 4.0 (1H, s, -CH-[6]), 4.3 (1H, br s, -CH-[3]), 6.0 (1H, br s, -NH-[10]) and 7.5 (1H, br s, -NH-[5]).

*N-2-(Ethylthio)ethyl β-methylpantothenamide (**28q**)*

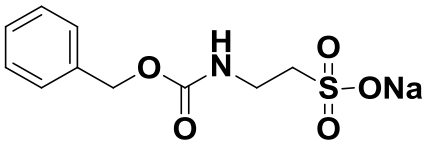


^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.98 (3H, s, -CH_3[15]), 1.01 (3H, s, -CH_3[14]), 1.22-1.27 (6H, m, -CH_3[7+14]), 2.5 (4H, m, -CH_2-[8+13]), 2.7 (2H, m, -CH_2-[12]), 3.4 (2H, m, -CH_2-[1]), 3.5 (2H, m, -CH_2-[11]), 4.35 (1H, br s, -CH-[3]), 6.4 (1H, br s, -NH-[10]) and 7.4 (1H, br s, -NH-[5]).

4.3 Synthetic preparation of pantoyltauramides

4.3.1 Synthesis and activation of *N*-carbobenzoxy taurine

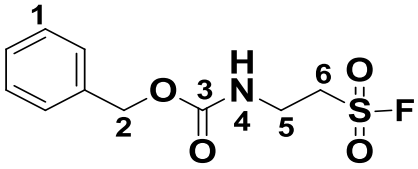
*N-Carbobenzoxy taurine sodium salt (**50**)*



50

The synthesis was performed according to a method by Brouwer *et al* (*77, 80*). Taurine (4.0 g, 32 mmol) was dissolved in 1M NaOH (32 mL). A solution of benzyl chloroformate (CbzCl) (7 mL, 48 mmol) in dioxane (42 mL) was added, followed by addition of a further portion of 1M NaOH (1M, 48 mL). The reaction mixture was stirred for 1 hour, and extracted with acetyl acetate (2×125 mL) to remove unreacted CbzCl. The aqueous layer was then concentrated, followed by sequential co-evaporation with toluene (100 mL), ethanol (100 mL), and dichloromethane (3×100 mL). The crude product was lyophilized and subsequently used in the next step without further purification.

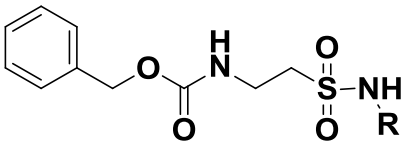
*N-Carbobenzoxy tauryl fluoride (Cbz-tauryl fluoride, **51**)*



51

The synthesis was performed by modification of an established method (*78*). To a stirred slurry of **50** (0.5 g, 1.78 mmol) in CH_2Cl_2 (20 mL) was added a solution of (diethylamino)sulphur trifluoride (DAST) (0.585 ml, 4.45 mmol). The reaction mixture was stirred at 0^{o}C for 2 hours, with product formation being monitored by TLC (10% MeOH/ CH_2Cl_2), and ^{19}F NMR in $CDCl_3$. Upon completion of the reaction the solvent was removed *in vacuo* by rotary evaporation, followed by purification using flash chromatography (silica gel; CH_2Cl_2/Hexane 1:1 to 3:1) to give **51** as a yellow powder (0.22 g, 44%). ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 3.64 (2H, m, -CH_2-[5]), 3.74 (2H, m, -CH_2-[6], 5.13 (2H, s, -CH_2-[2], 5.32 (1H, br s, -NH-[4] and δ 7.35 (5H, s, arom.[1]. ^{19}F NMR (300 MHz; $CDCl_3$): δ 56.9.

4.3.2 Synthesis of *N*-carbobenzoxy *N´*-substituted tauramides

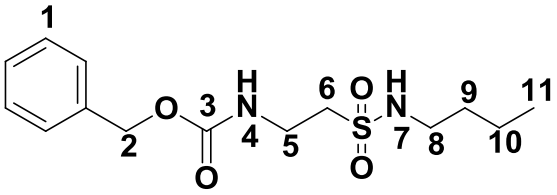


52

General method

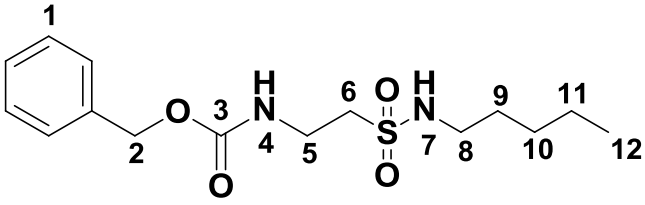
The *N*-carbobenzoxy *N´*-substituted tauramides **52** were synthesized according to a method described by Toulgoat *et al* (*79*). To a solution of **51** (0.38 mmol for **52b** and **52n,** 1.22 mmol for **52c**, 1.15 mmol for **52d**, 0.84 mmol for **52h**, 1 mmol for **52o**, and 0.69 mmol for **52q)** in DCM (3 mL) was added the appropriate amine (5 eq.) at room temperature under nitrogen. The reaction mixture was stirred at 50^{o}C for 20 hours with product formation being monitored by TLC and ^{19}F NMR (disappearance of fluorine peak). The reaction mixture was cooled to room temperature, after which 10% aqueous HCl was added. The aqueous phase was extracted with CH_2Cl_2, followed by drying of the organic layer over $MgSO_4$, filtration and rotary evaporation of the solvent *in vacuo* to obtain the respective product. The analytical data of each compound is given below.

*N-Carbobenzoxy N´-butyltauramide (**52b**)*



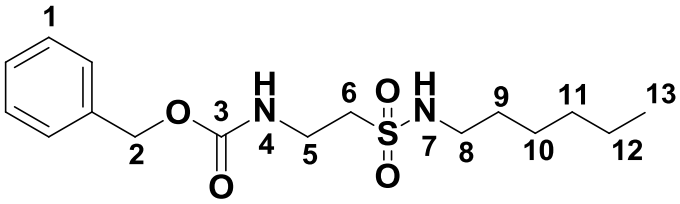
Yield: 90 mg; 68%. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.92 (3H, t, *J* 7.33, -CH_3[11]), 1.32-1.39 (2H, m, -CH_2-[10]), 1.47-1.55 (2H, m, -CH_2-[9]), 3.05-3.12 (2H, m, -CH_2-[8]), 3.19 (2H, t, *J* 6.05, -CH_2-[6]), 3.63-3.69 (2H, m, -CH_2-[5]), 4.48 (1H, br s, -NH-[7]), 5.1 (2H, s, -CH_2-[2]), 5.45 (1H, br s, -NH-[4]) and 7.34 (5H, s, arom.[1]).

*N-Carbobenzoxy N´-pentyltauramide (**52c**)*



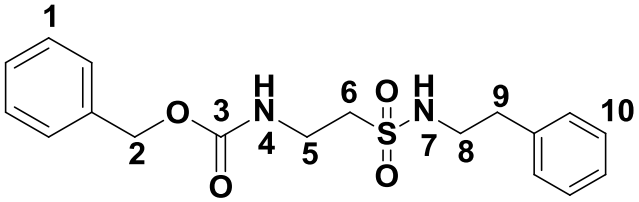
Yield: 0.30 g, 75%. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.89 (3H, t, *J* 6.84, -CH_3[12]), 1.25-1.32 (4H, m, -CH_2-[10+11]), 1.55 (2H, s, -CH_2-[9]), 3.0-3.1 (2H, m, -CH_2-[8]), 3.1 (2H, t, *J* 5.98, -CH_2-[6]), 3.63-3.69 (2H, m, -CH_2-[5]), 4.5 (1H, br s, -NH-[7]), 5.1 (2H, s, -CH_2-[2]), 5.4 (1H, s, -NH-[4]) and 7.3 (5H, s, arom.[1]).

*N-Carbobenzoxy N´-hexyltauramide (**52d**)*



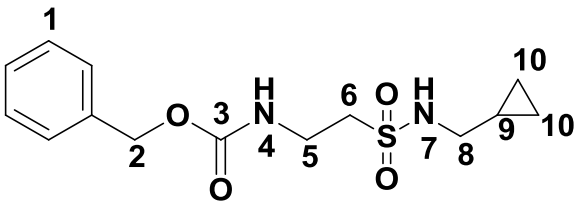
Yield: 0.32 g; 80%. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.88 (3H, t, *J* 7.06, -CH_3[13]), 1.29-1.33 (6H, m, -CH_2-[10+11+12]), 1.51-1.55 (2H, m, -CH_2-[9]), 3.08-3.11 (2H, m, -CH_2-[8]), 3.2 (2H, t, *J* 6.13, -CH_2-[5]), 3.63-3.69 (2H, m, -CH_2-[6]), 4.52 (1H, br s, -NH-[7]), 5.1 (1H, s, -CH_2-[2]), 5.46 (1H, br s, -NH-[4]) and 7.33 - 7.35 (5H, m, arom.[1]).

*N-Carbobenzoxy N´-phenethyltauramide (**52n**)*



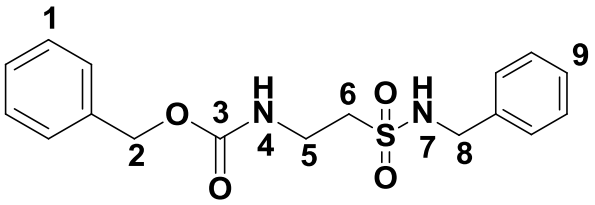
Yield: 0.11 g; 80 %. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 2.85 (2H, d, *J* 6.8, -CH_2-[9]), 3.06 (2H, t, *J* 6.2, -CH_2-[6]), 3.34-3.41 (2H, m, -CH_2-[8]), 3.52-3.58 (2H, m, -CH_2-[5]), 4.47 (1H, t, *J* 6.04, -NH-[7]), 5.09 (2H, s, -CH_2-[2]), 5.4 (1H, br s, -NH-[4]) and 7.29 - 7.35 (10H, m, arom.[1 and 10]).

*N-Carbobenzoxy N´-methylcyclopropanetauramide (**52h**).*



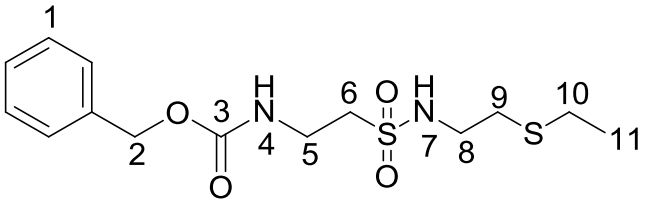
Yield: 0.18 g; 69 %. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.22 (2H, d, -H[10]), 0.54 (1H, s, -CH-[9]), 2.92-3.00 (2H, m, -CH_2-[8]), 3.22 (2H, t, *J* 6.07, -CH_2-[6]), 3.64-3.70 (2H, m, -CH_2-[5]), 4.62 (1H, br s, -NH-[7]), 5.1 (2H, s, -CH_2-[2]), 5.46 (1H, br s, -NH-[4]) and 7.32-7.35 (5H, m, arom.[1]).

*N-Carbobenzoxy N´-benzyltauramide (**52o**)*



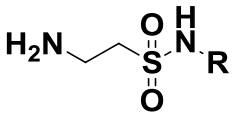
Yield: 0.26 g; 74%. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 3.1 (2H, t, *J* 5.88, -CH_2-[5]), 3.56 – 3.64 (2H, m, -CH_2-[6]), 3.72-3.78 (2H, m, -CH_2-[8]), 4.9 (1H, s, -NH-[7]), 5.1 (2H, d, *J* 9.98, -CH_2-[2]), 5.38 (1H, br s, -NH-[4]) and 7.30- 7.36 (10H, m, arom.[1 and 9]).

*N-Carbobenzoxy N´-ethylthioethyltauramide (**52q**)*



Yield: 0.15 g; 65%) ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 1.25 (3H, t, *J* 7.37, -CH_3[11]), 2.52-2.57 (2H, m, -CH_2-[10]), 2.7 (2H, d, *J* 6.52, -CH_2-[9]), 3.19-3.28 (4H, m, -CH_2-[8+6]), 3.64-3.70 (2H, m, -CH_2-[5]), 4.98 (1H, br s, -NH-[7]), 5.1 (2H, s, -CH_2-[2]), 5.45 (1H, br s, -NH-[4]) and 7.33-7.36 (5H, m, arom. [1]).

4.3.3 Synthesis of *N*-substituted tauramides

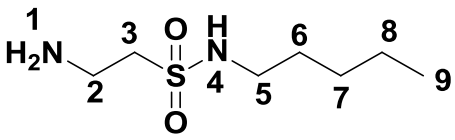


49

General method

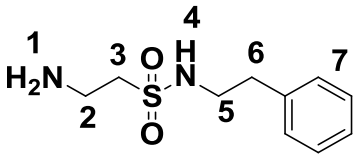
The *N*-carbobenzoxy *N´*-substituted tauramide **52** (0.20 g) was dissolved in methanol (4 mL) and the equivalent volume of concentrated HCl was added. The reaction mixture was stirred overnight under reflux, followed by addition of water (10 mL). The reaction mixture was washed with CH_2Cl_2, after which the aqueous phase was extracted and lyophilized to give the HCl salt as the product.

*N-Pentyltauramide (**49c**)*



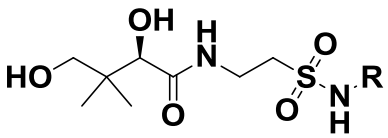
Yield: 0.10 g, 72%. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.9 (3H, t, *J* 6.5, -CH_3[9]), 1.33 (4H, m, -CH_2-[7+8]), 1.51 (2H, m, -CH_2-[6]), 2.4 (2H, m, -CH_2-[5]), 3.0 (2H, s, -CH_2-[2]), 3.6 (2H, s, -CH_2-[3]), 6.8 (1H, s, -NH-[4]), and 7.9 (1H, s, -NH[1]).

*N-Phenethyltauramide (**49n**)*



Yield: 40 mg; 89%. ^{1}H NMR (300 MHz; $CDCl_3$; 25oC): δ 2.5 (2H, br s, -CH_2-[6]), 2.9 (2H, br s, -CH_2-[5]), 3.2-3.8 (4H, m, -CH_2-[2 + 3]), 7.0 (1H, br s, -NH-[4]) and 7.9 (2H, br s, -NH[1]).

4.3.4 Synthesis of *N*-substituted pantoyltauramides

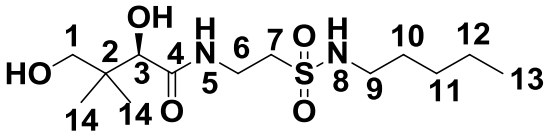


36

General method

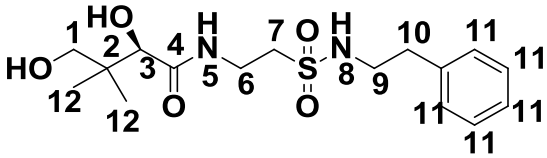
To a flask containing the *N*-substituted tauramide HCl salt **49** (0.10 g) was added pantolactone (2.5 eq.) and NEt_3 (2.5 eq.). The reaction mixture was dissolved in ethanol (3 mL) and refluxed with stirring for 7 hours. After cooling to room temperature, the solvent was evaporated and purified by flash chromatography (silica gel; 100% CH_2Cl_2) to 10% MeOH/ CH_2Cl_2) to afford the pure *N*-substituted pantoyltauramide **36**.

*N-Pentyl pantoyltauramide (**36c**)*



Yield: 90 mg, 40%. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C) δ 0.90 (3H, t, *J* 7.03, -CH_3[13]), 0.96 (3H, s, -CH_3[14]), 1.04 (3H, s, -CH_3[14]), 1.30-1.35 (4H, m, -CH_2-[11+12]), 1.55-1.62 (2H, m, -CH_2-[10]), 3.07-3.13 (2H, m, -CH_2-[9]), 3.20-3.24 (2H, m, -CH_2[1]), 3.53 (2H, s, -CH_2-[7]), 3.72-3.77 (2H, m, -CH_2-[6]), 4.0 (1H, s, -CH-[3]), 4.9 (1H, t, -NH-[8]) and 7.43 (1H, br s, -NH-[5]).

*N-Phenethyl pantoyltauramide (**36n**)*



Yield: 0.10 g; 52%. ^{1}H NMR (300 MHz; $CDCl_3$; 25^{o}C): δ 0.93 (3H, s, -CH_3[12]), 1.0 (3H, s, -CH_3[12]), 2.88 (2H, d, *J* 6.62, -CH_2-[10]), 3.0-3.1 (2H, m, -CH_2-[9]), 3.38 (2H, t, *J* 6.84, -CH_2-[1]), 3.5 (2H, s, -CH_2[6]), 3.6 (2H, t, *J* 5.47, -CH_2[7]), 4.0 (1H, s, -CH-[3]), 4.96 (1H, br s, -NH-[8]) and 7.3 (1H, s, -NH-[5]).

4.4 *P. falciparum* cell culture

All *in vitro* experiments on malaria parasite was performed using *P. falciparum* strain 3D7 by Mr. Cristiano Macuamule. The parasites were synchronized in continuous culture and maintained in that state using a method described by Trager (*81*) and Cranmer et al (*82*) with further modification. In brief, the parasites were first cultured in group O, Rh^+ erythrocytes suspended in RPMI-1640 culture medium to which was added 25 mM HEPES (pH 7.4), 11 mM glucose, 200 µM hypoxanthine, 24 µg gentamycin/mL and 0.6% w/v Albumax II (complete media) giving a final hematocrit of ~5%. Cultures were grown horizontally in sealed tissue culture flasks gassed with a low O_2 gas mix (1% O_2, 3% CO_2 in N_2) with rotary shaking. The cultures were treated with 5% (w/v) Dsorbitol solution for a minimum of 10 minutes at 37^{o}C prior to using them for experiments so as to ensure that they are completely synchronized, as described (*83*). Fully matured trophozoite stage parasitized erythrocytes were subjected to lysis process leaving all parasites remaining in the ring stage. To reduce stress as a result of overgrowth, the medium was changed every day and cultures were split when parasites were mainly in trophozoite stage. Cultures were then split by using 1.25 mL of parasite infected erythrocytes isolated by centrifugation with the addition of 2 ml of fresh blood (100% hematocrit) in a final complete media volume of 50 ml giving a final hematocrit of ~5%. The level of parasitemia was determined by light microscopy counting a minimum of 500 erythrocytes on a Giemsa-stained thin blood smear and was kept between 5-15%.

4.5 *P. falciparum in vitro* growth inhibition assays

Determination of parasite proliferation was performed with the help of Mr. Cristiano Macuamule using a SYBR green assay as described by Smilkstein et al (*84*) and Bennett et al (*85*) with further modification. Procedures were performed in 96-well plates using ring-stage parasites with a hematocrit of 1% and 1% parasitemia were used. The malaria parasites were cultured in RPMI-1640 culture medium. The medium (contained 1 µM pantothenate), with a supplementation of 25 mM HEPES (pH 7.4), 11 mM glucose, 200 µM hypoxanthine, 24 µg gentamycin/mL and 0.6% w/v albumax II (complete media). Two-fold serial dilutions of pantothenamides and sulfonamides (ranging between 0.391-200 µM) were prepared in triplicate in a final volume of 200 µl. Normal red blood cells with a hematocrit of 1% was used as a blank; however, chloroquine (500 nM) was included as positive control. Infected

parasites cultured in the absence of pantothenamides and sulfonamides were includes to estimate 100% parasite growth. The prepared plates were incubated in a desiccator cabinet flushed with N_2 gas mix (1% O_2, 3% CO_2, in N_2) for 96 h at 37^{o}C to allow the parasite to complete two life cycles.

Parasite proliferation was determined by preparing fresh 96-well plates that contained 100 µL SYBR green solution in each well, comprising of 2 µL of SYBR safe DNA gel stain (10,000X concentrate in DMSO); in 10 mL lysis solution (20 mM Tris-HCl, 5 mM EDTA, 0.008% w/v saponin, 0.08% v/v Triton X-100, pH 7.5). After 96 hour the plates containing the cultures were resuspended thoroughly and 100 µL of erythrocyte suspension was transferred to plate containing SYBR green solution, mixed properly until no visible erythrocyte sediment remained. The contents were then kept in the dark until plates were read by measuring the fluorescence, with excitation and emission wavelength bands at 490 nm and 520 nm using a Varioskan multi-detection micro plate reader from ThermoLabsystems. The background fluorescence obtained were subtracted from each well by using the wells designated as blanks (containing uninfected erythrocytes) yielding fluorescence counts for analysis.

4.6 Bacterial growth inhibition assays

Inhibition studies were performed by preparing starter cultures of *S. aureus* RN4220 in 1% tryptone by inoculation with four separate colonies grown on LB agar plates. The starter culture was grown until OD_{600} of 0.62 and then diluted 10,000-fold in the same medium. A 10% aliquot of the diluted cell suspension was used to inoculate each well of a 96-well flat-bottomed plate containing 100 µL 1% tryptone broth supplemented with a specific *N*-substituted pantothenamide in a final concentration of 200 µM. Final concentrations of compounds were between 0.039-200 µM. The plates were incubated at 37^{o}C for 20 hours before the cell densities were measured by reading the absorbance in each well at 600 nm. The extent of growth in each well was determined by normalizing the OD600 values relative to those of negative control (containing 3% acetonitrile instead of pantothenamide), which were taken as 100% growth. Each compound was tested in triplicate and all experiments were repeated at least once after the initial experiment.

Chapter 5

Conclusion

5.1 Summary of results

In this study 40 *N*-substituted α- and β-methylpantothenamides with different amide functionalities were successfully prepared by adapting an established method whereby such compounds can be prepared and synthesized in parallel. These compounds tested as potential inhibitors of bacterial growth and the proliferation of malaria parasites. The synthesis of *N*-substituted pantoyltauramides was also investigated, and three such analogues were successfully prepared and tested as inhibitors of *P. falciparum* parasites.

The results of the biological studies show that the introduction of methyl functionalities does not improve the inhibitory properties of *N*-substituted pantothenamides in respect to bacteria. However, methylated pantothenamides show promise as improved inhibitors of *P. falciparum* growth. The same observation was also made for the *N*-substituted pantoyltauramides, which exhibited the best inhibition of all the inhibitors tested in this study. Taken together, these results indicate that the inhibitory properties of pantothenate analogues that act as antimetabolites can be improved by the introduction of new functional groups, or the modification of existing ones.

5.2 Future research possibilities

In future studies, the current set of pantothenamide analogues can be expanded by increasing the structural diversity on the β-alanine moiety to include other functionalities such as trifluoromethyl groups (CF_3) or halogen functionalities (F and Cl). This could be achieved following the same strategy used in this study, where the functionality is introduced by using an appropriate β-alanine analogue to prepare the corresponding pantothenic acid analogue, the thioester, and eventually pantothenamide by parallel aminolysis and purification.

Since the three pantoyltauramides have shown the potential of possessing inhibitory activities against malaria parasite proliferation, the structural diversity of the pantoyltauramides should also be expanded.

Future work should also include the biological testing of the newly prepared methylpantothenamides on bacteria other than *S. aureus*, such as *E. coli* and other bacteria that are known to accept pantothenamides as PanK substrates. Studies will also need to be conducted to determine if these compounds have the same mode of action as the known pantothenamide inhibitors. It will also be important to evaluate the antibacterial properties of the three pantoyltauramides, since inhibitory studies were only done to investigate the antimalarial activities of these analogues on *P. falciparum*. Finally, the effect of pantothenate on the observed inhibition will have to be determined, to ensure that the improved inhibitory properties are not offset by antagonism in the presence of the vitamin.

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Appendix

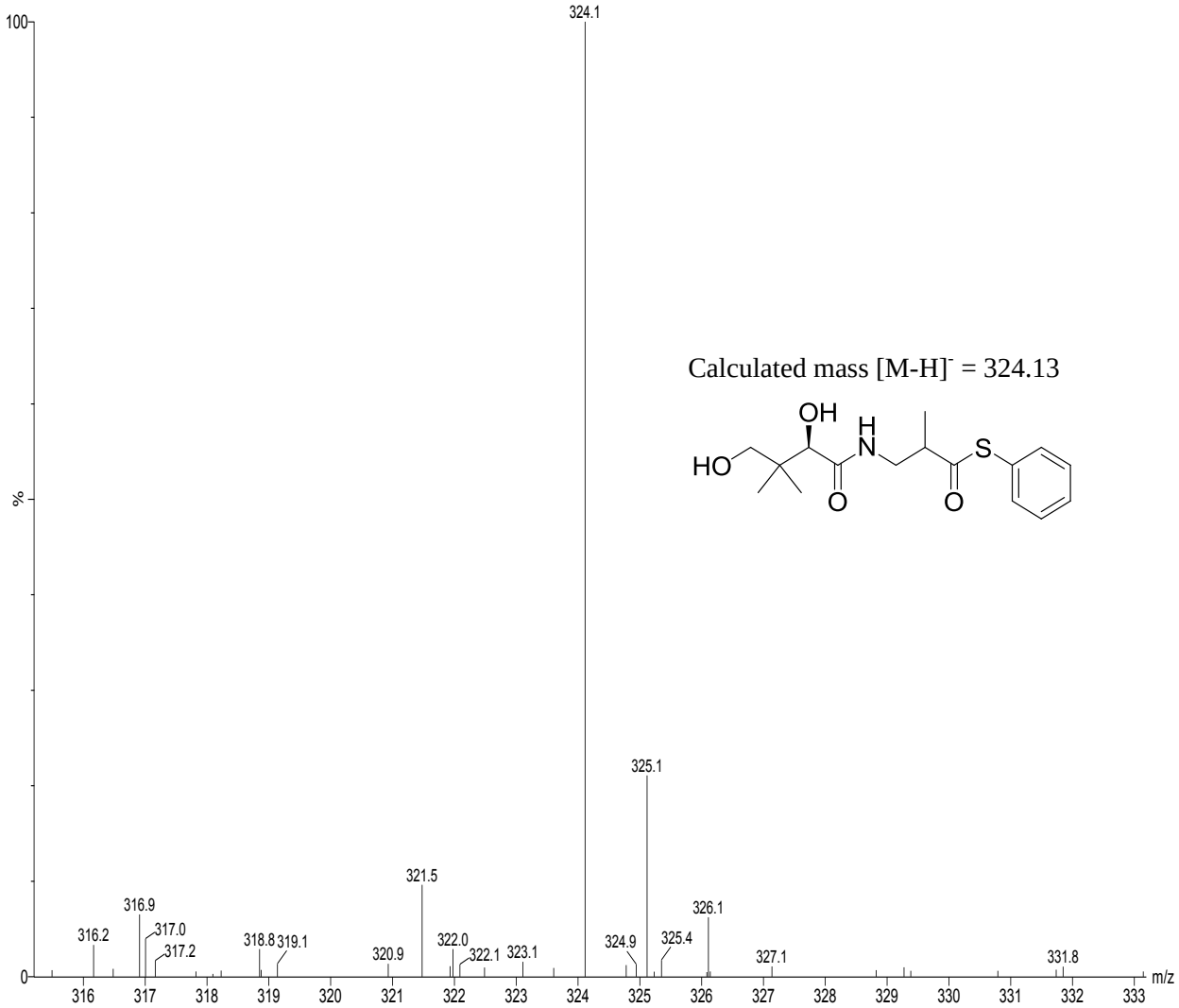
Characterization of *S*-phenyl α- and β-methylthiopantothenate

S-Phenyl α- and β-methylthiopantothenate were thoroughly characterized by ^{1}H NMR, ^{13}C NMR, and by LC-MS analysis

Characterization of *S*-phenyl α-methylthiopantothenate

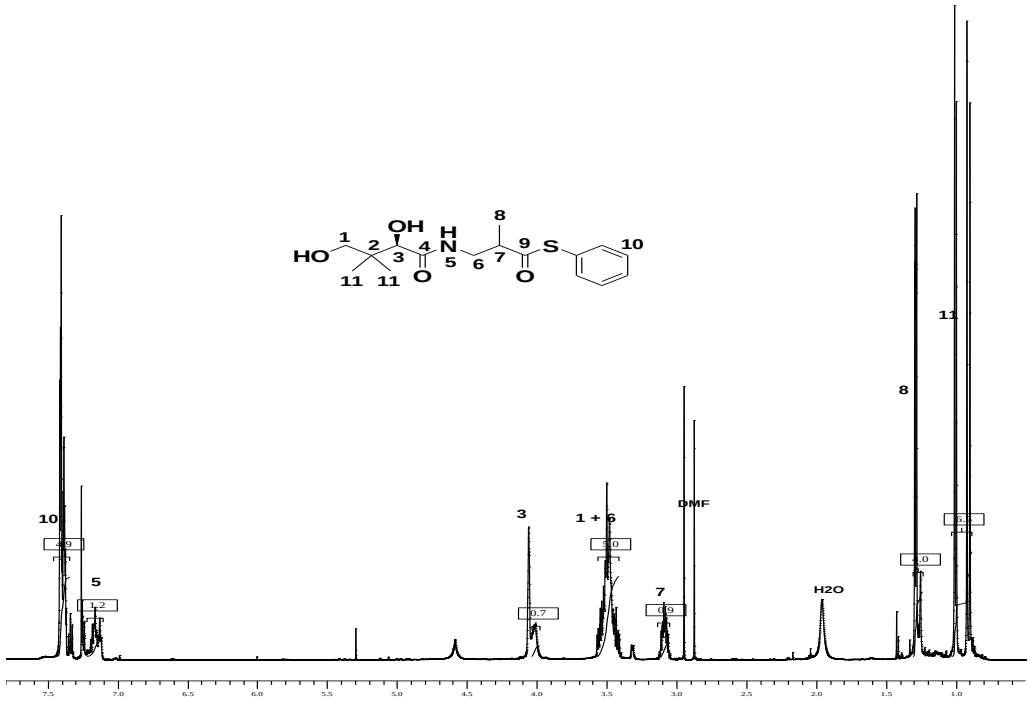
LC-MS analysis

α-methyl thioester



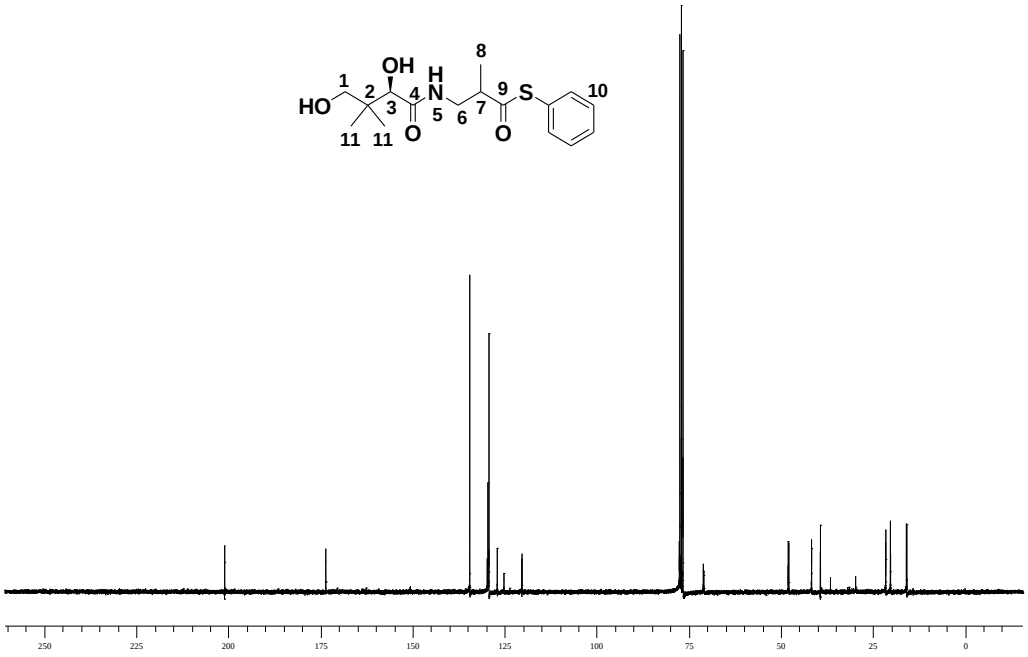
^{1}H NMR (600 MHz)

α-methyl thioester



^{13}C NMR (300 MHz)

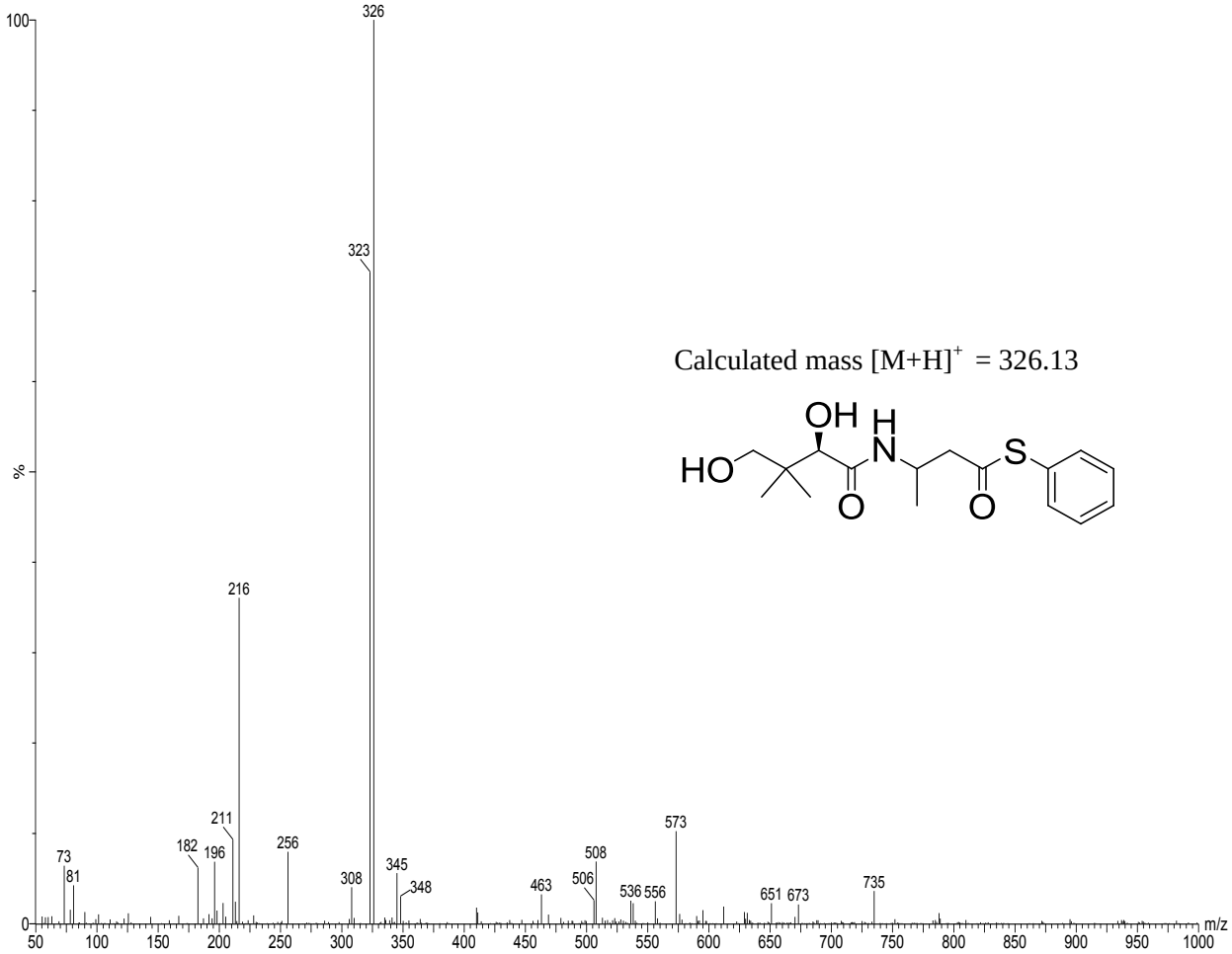
α-methyl thioester



Characterization of *S*-phenyl β-methylthiopantothenate

LC-MS

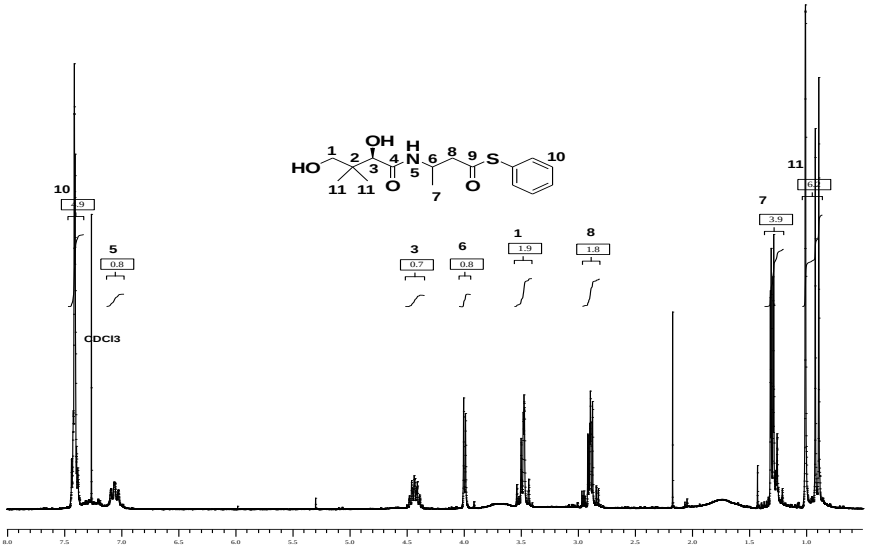
β-methyl thioester



Calculated mass $[M+H]^+$ = 326.13

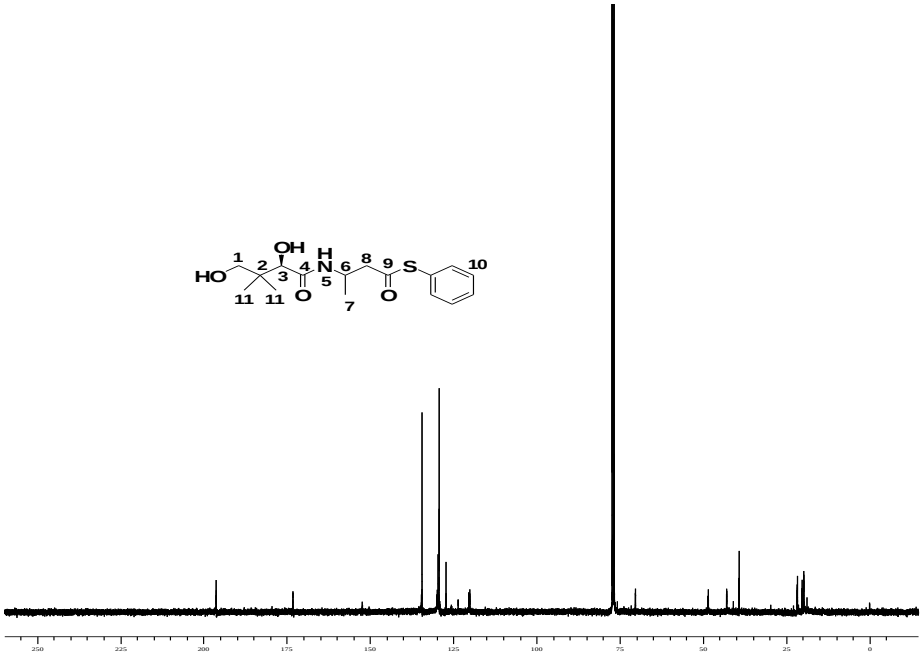
^{1}H NMR (300 MHz)

β-methyl thioester



^{13}C NMR (400 MHz)

β-methyl thioester

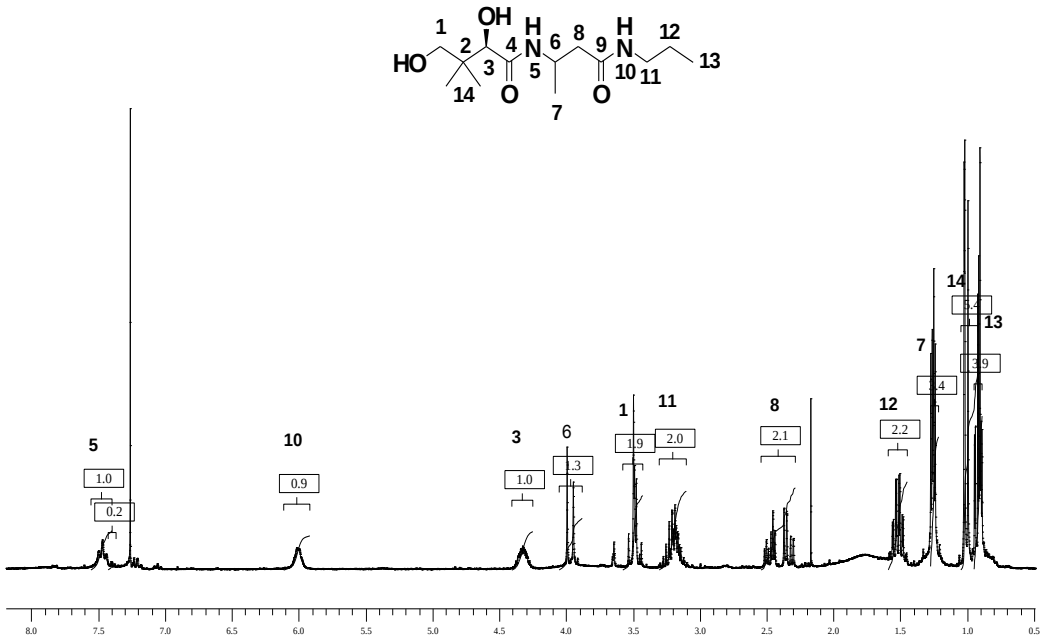


Characterization of pantothenamides

All pantothenamides were characterized by ^{1}H NMR

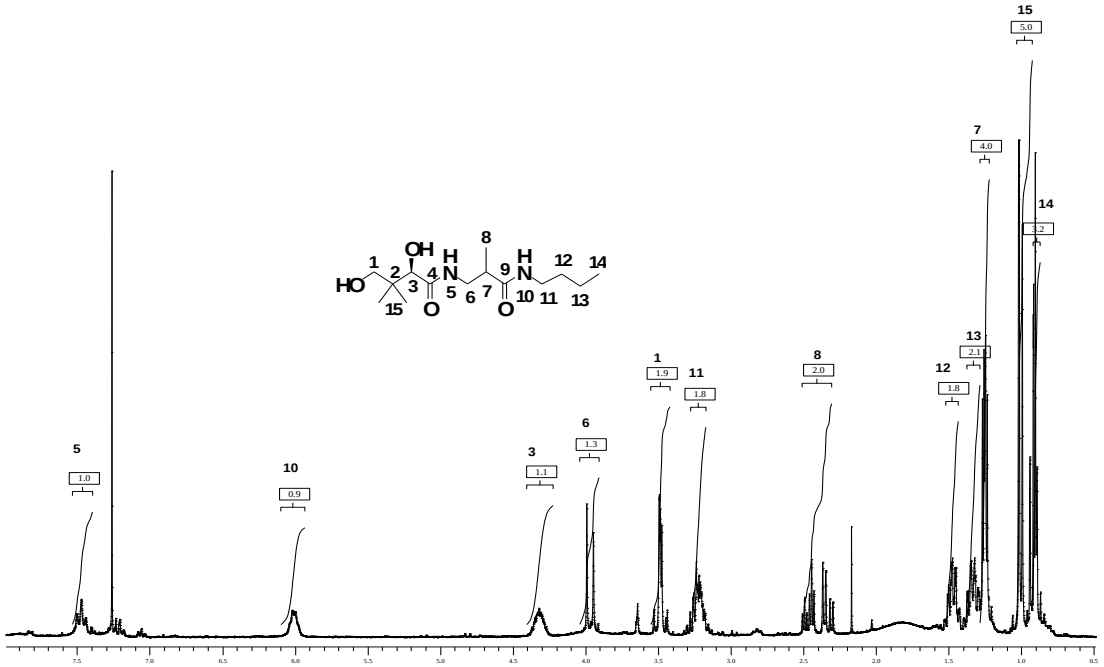
N-Propyl β-methylpantothenamide

^{1}H NMR (300 MHz)



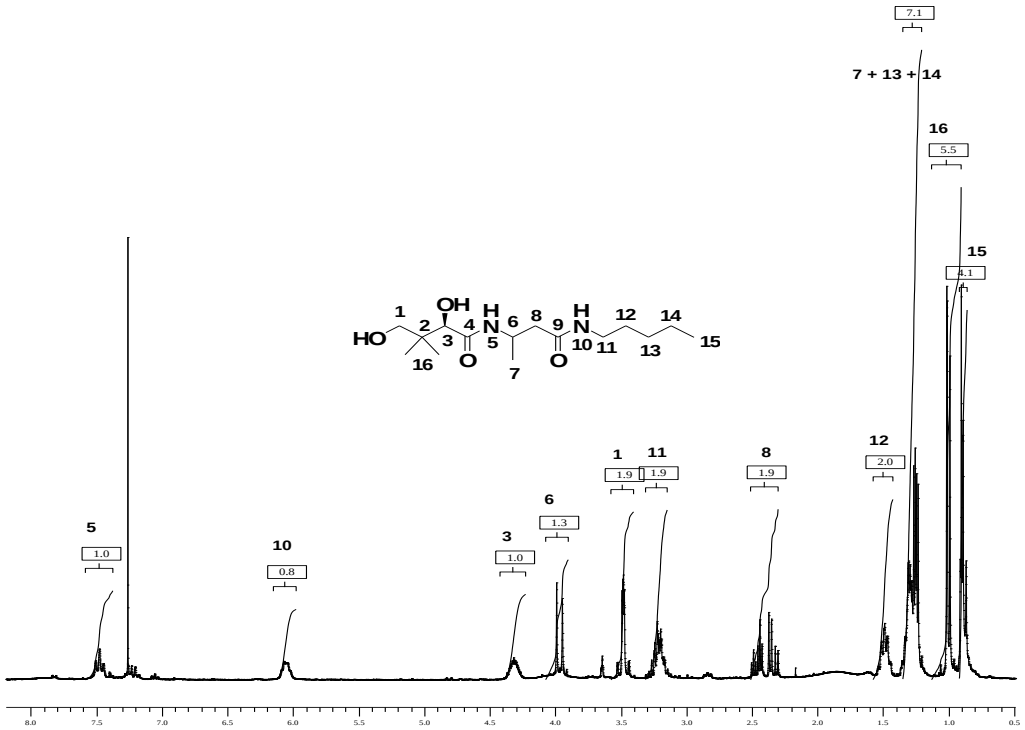
^{1}H NMR (300 MHz)

N-Butyl β-methyl pantothenamide



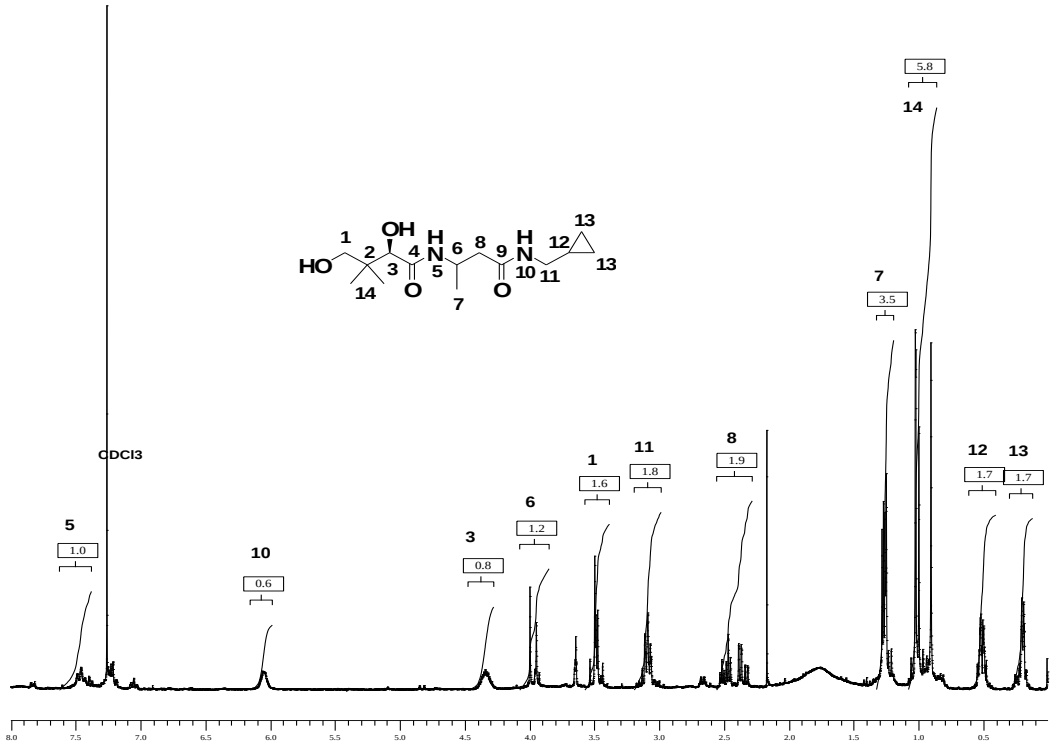
^{1}H NMR (300 MHz)

N-Pentyl β-methylpantothenamide



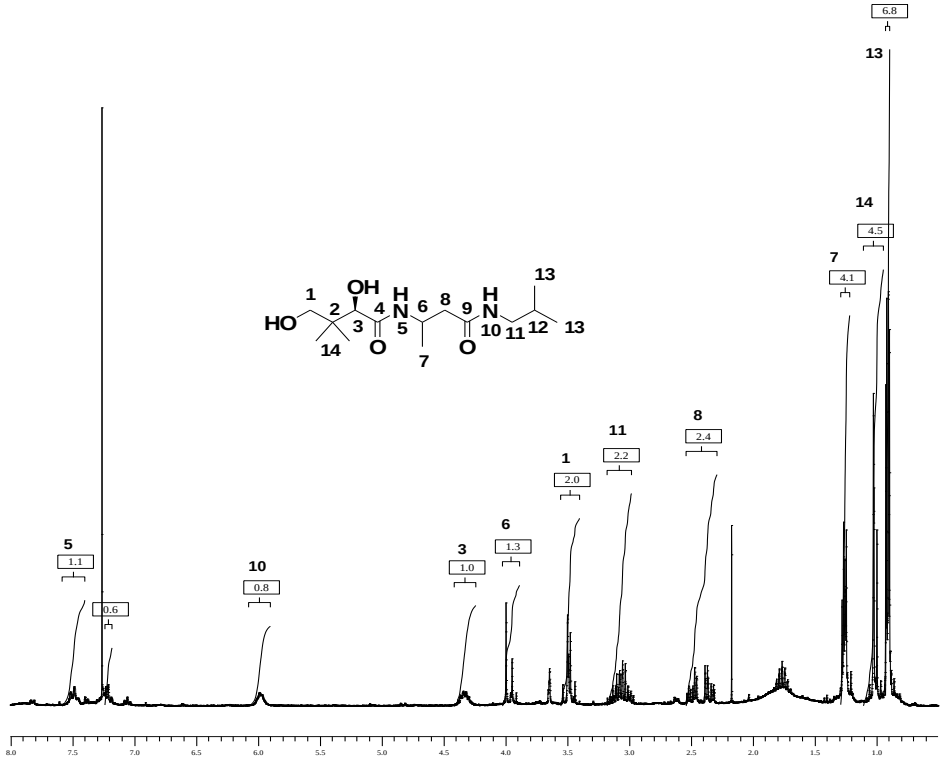
^{1}H NMR (300 MHz)

N-Cyclopropanemethyl β-methylpantothenamide



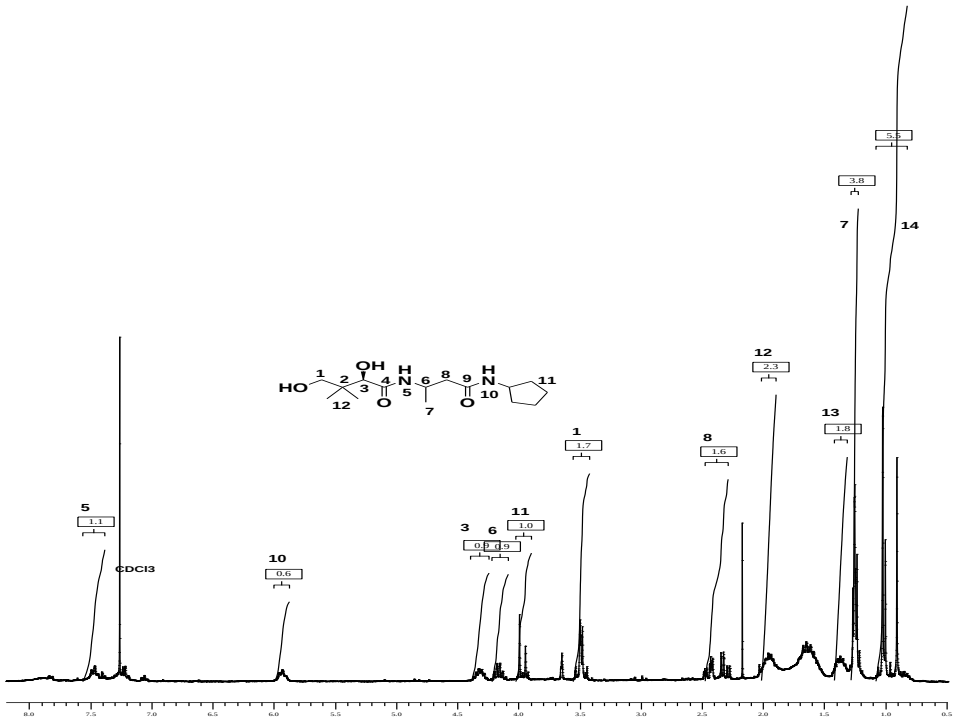
^{1}H NMR (300 MHz)

N-Isobutyl β-methylpantothenamide



^{1}H NMR (300 MHz)

N-Cyclopentyl β-methylpantothenamide

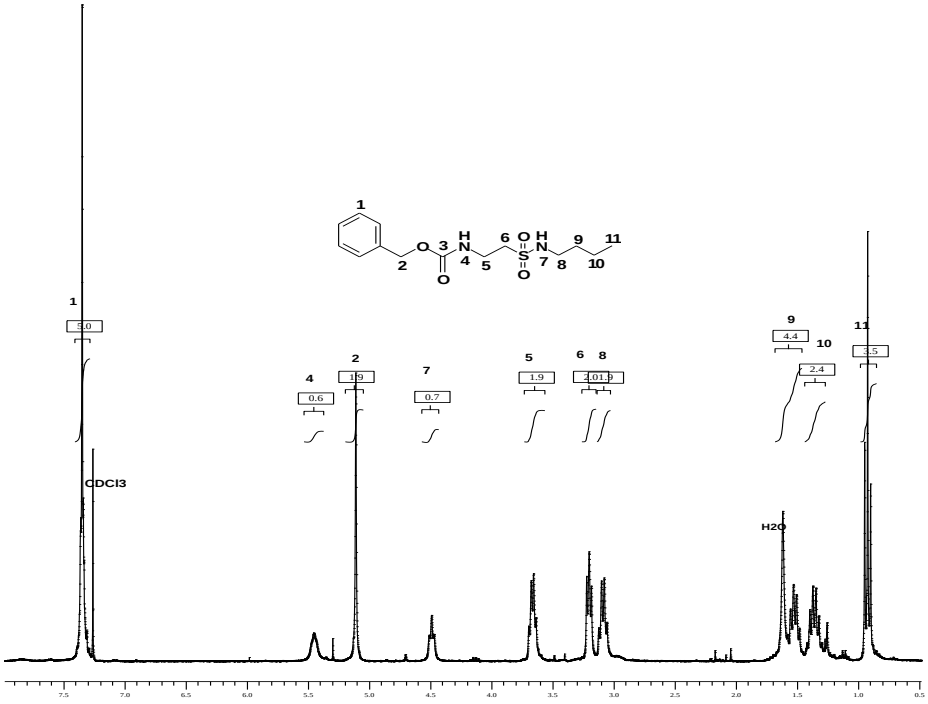


Characterization of Cbz-tauramides

All Cbz-tauramides were characterized by ^{1}H NMR

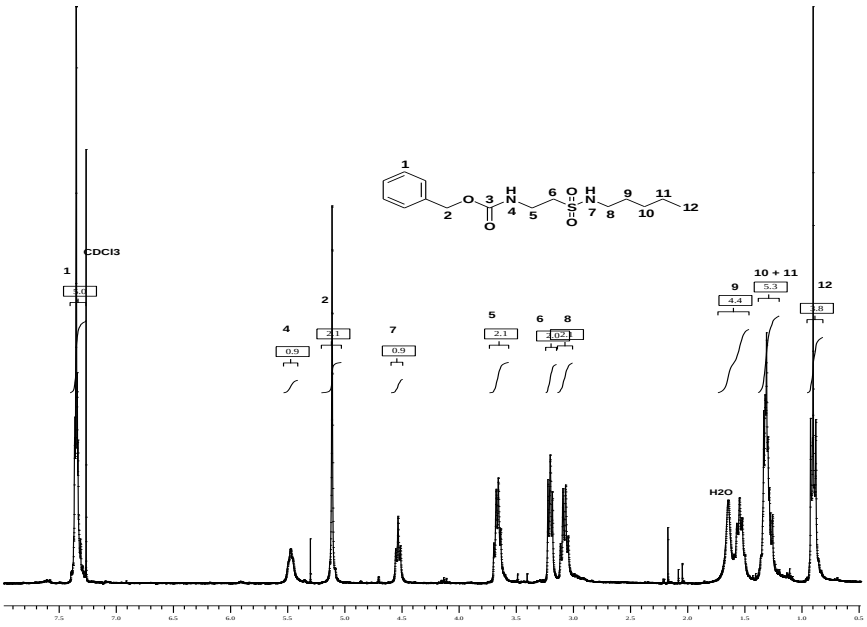
^{1}H NMR (300 MHz)

N-Butyl cbz-tauramide



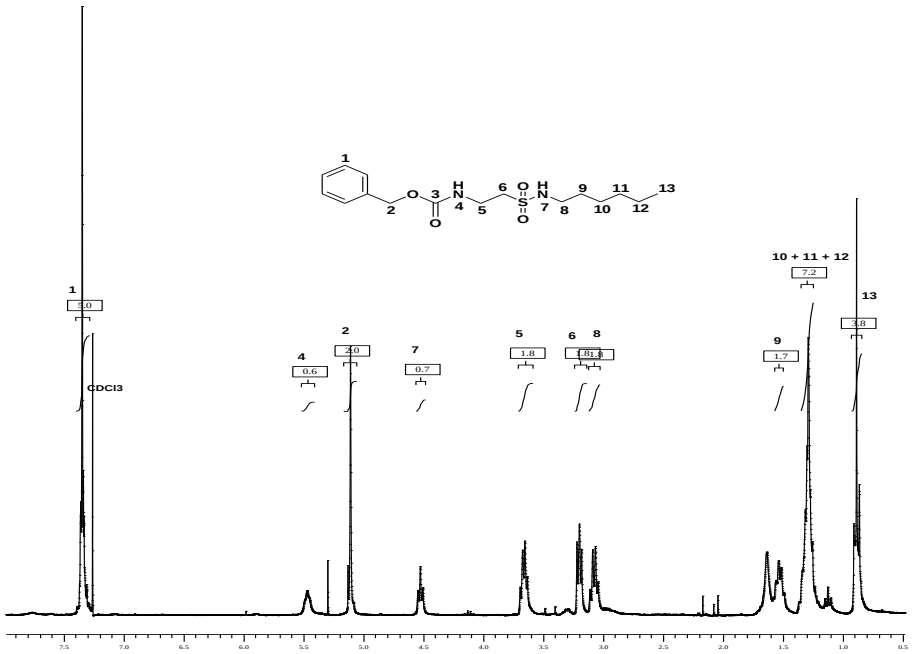
^{1}H NMR (300 MHz)

N-Pentyl cbz-tauramide



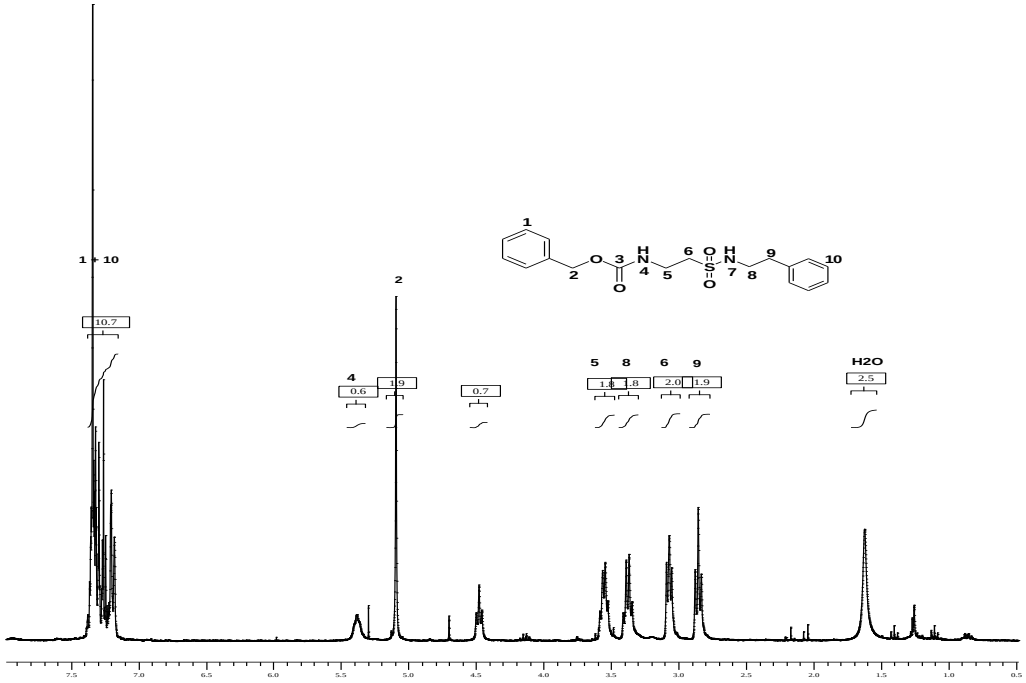
^{1}H NMR (300MHz)

N-Hexyl Cbz-tauramide



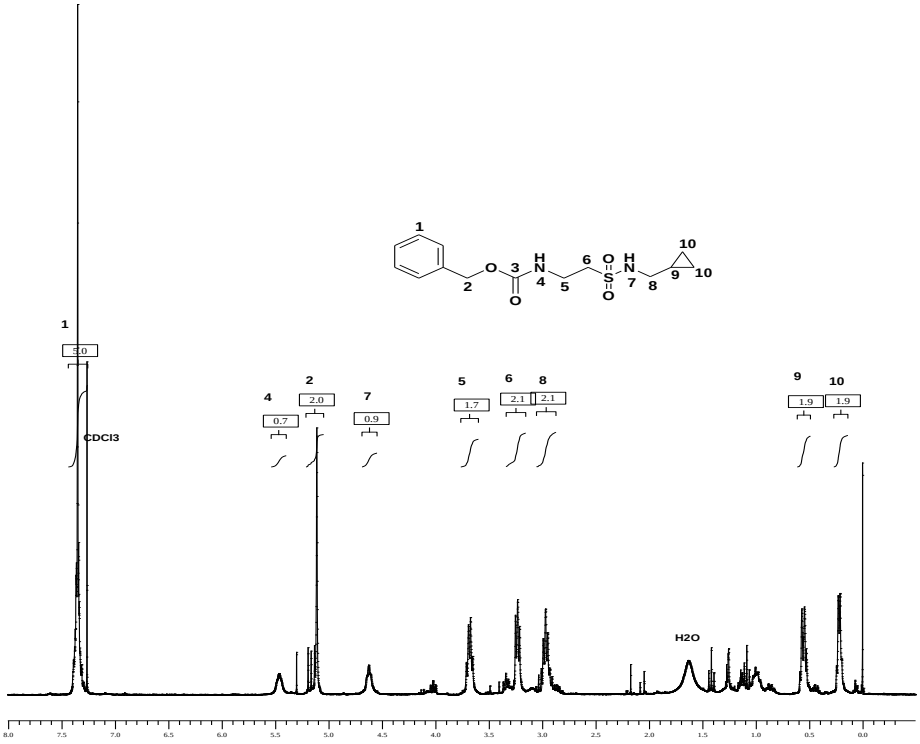
^{1}H NMR (300 MHz)

N-Phenethyl Cbz-tauramide



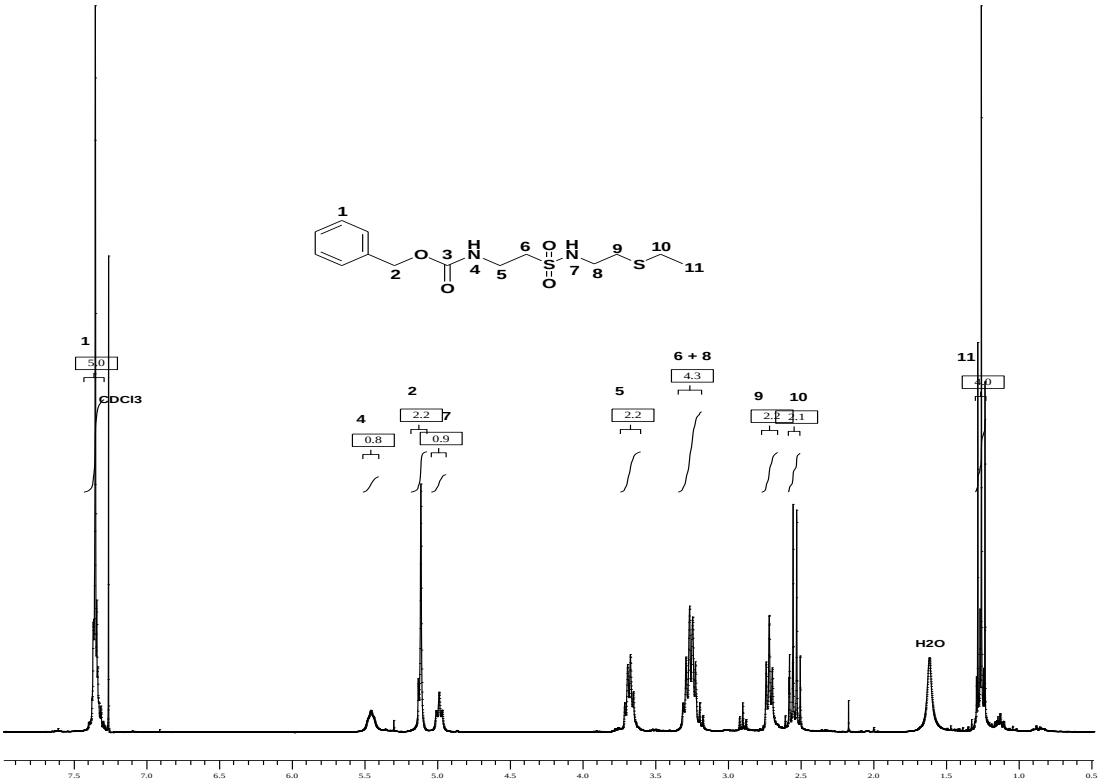
^{1}H NMR (300 MHz)

N-Cyclopropanemethyl cbz-tauramide



^{1}H NMR (300 MHz)

N-2-(Ethylthio)ethyl Cbz-tauramide

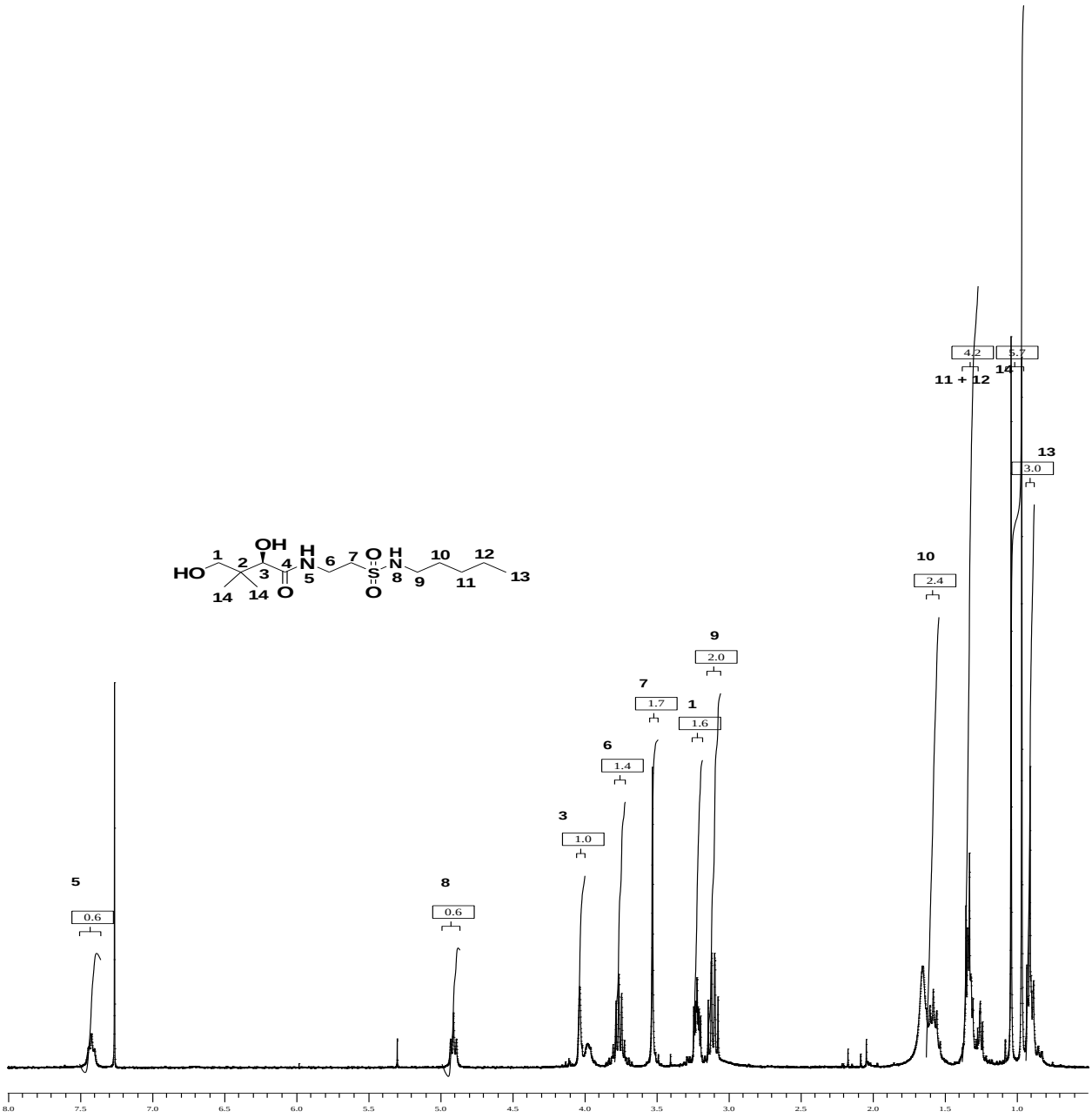


Characterization of pantoyltauramides

All pantoyltauramides were characterized by ^{1}H-NMR

^{1}H NMR (300 MHz)

N-Pentyl pantoyltauramide



^{1}H NMR (300 MHz)

N-Phenethyl pantoyltauramide