Investigation of Coenzyme A levels in *Plasmodium falciparum* to ascertain the mode of action of new antimalarial candidates

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signed:                      Date:  March 2017
The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.
Summary

The malaria parasite, *Plasmodium falciparum*, has become increasingly resistant to all commercially available drugs used in the treatment of malaria, and as such, the development of new antimalarial drugs with novel targets is of great importance. The coenzyme A (CoA) biosynthesis pathway is one such novel target since CoA and its precursor, pantothenate, have been shown to be essential for organism survival.

*N*-phenethyl-α-methyl pantothenamide, a pantothenate analogue, has been shown in a previous study to inhibit growth in both bacteria and *Plasmodium* parasites, however the mode of action of this pantothenamide in *Plasmodium* is still unknown, and was thus investigated in this study.

First, *Plasmodium*’s requirement for pantothenate was investigated. We determined that parasites could survive without an extracellular source of pantothenate for up to eight days, however this contradicted what was found in literature, and was likely to be due to a Mycoplasma infection found late in the study. Secondly, it was investigated whether *N*-phenethyl-α-methyl pantothenamide can be metabolized to its CoA antimetabolites by the CoA biosynthetic enzymes present in *P. falciparum*. This was done by investigating the metabolism of the compound in both cell lysates and in *in vivo* *P. falciparum* cell cultures and it was found that *PfPanK* and *PfDPCK* is active in parasite lysates, while *PfPPAT* is inactive in parasite lysates. We could therefore not determine if the pantothenamide under investigation is being metabolized in the parasite by using lysates, but this is the first demonstration of the activity of *PfDPCK* in parasites lysate. Finally, we wanted to investigate the effect of tricyclic methylthiophenyl propanamide (TMP), a non-pantothenate analogue that inhibits the CoA biosynthesis pathway in other organisms, on *P. falciparum* proliferation. TMP was synthesized to use as a tool to investigate the mechanism of action of *N*-phenethyl-α-methyl pantothenamide to support that pantothenamides do not inhibit pantothenate kinase, as is known for TMP, but are rather metabolized downstream in the pathway. TMP was successfully synthesized and purified, however yields were too low to test TMP as an inhibitor of *P. falciparum* proliferation.

Not only did the work done in this study shed more light on the mode of action of pantothenamides in *P. falciparum*, but also gave valuable insight into parasite biochemistry.
Die malaria parasite, *Plasmodium falciparum*, het tot dus ver weerstand opgebou teen alle kommersiële beskikbare middele vir die behandeling van malaria. Die ontwikkeling van nuwe middele teen malaria wat ’n uitwerking het op nuwe teikens in the parasite is van die uiterste belang. Die koënsiem A (KoA) biosintese padweg is een so ’n spesifieke teiken, aangesien KoA en sy voorloper, pantoteensuur, essensieël is vir die organisme se oorlewing.

*N*-fenetiel-α-metielpantoteenamied, ’n pantoteensuur-analoog wat reeds in vorige studies inhibisie van bakteriële- en *Plasmodium* parasiet-groei tot gevolg gehad het is in hierdie studie ondersoek. Meer spesifiek, die metode van werking van hierdie pantoteenamied, wat tans nog onbekend is, was die fokus gedurende die betrokke studie.

Eerstens het ons *P. falciparum* se pantoteensuur vereistes ondersoek. Ons het bepaal dat die parasiete tot en met agt dae kan oorleef sonder ’n ekstrasellulêre bron van pantoteensuur, maar dit is teenstrydig met wat ons in die literatuur vind, en is waarskynlik as gevolg van ’n Mikoplasma infeksie wat ons eers laat in die studie ontdek het. Tweendens het ons bepaal of *N*-fenetiel-α-metielpantoteenamied omgesit word na die ooreenstemmende KoA-antimetaboliete deur die ensieme van die KoA biosintese padweg teenwoordig in *P. falciparum*. Die bepaling is gedoen deur die aktiwiteit van die ensieme te ondersoek in beide sellisaat en in *in vivo* *P. falciparum* selkulture. Daar is bevind dat *PfPanK* en *PfDPCK* in parasiet sellisaat aktief is, terwyl *PfPPAT* onaktief is. Ons kon dus nie bepaal of die pantoteenamied wat ons ondersoek deur die parasiet lisaat gemetaboliseer word nie, maar dit was die eerste bewyse van *PfDPCK* in parasiet sellisaat. Laastens, is die effek van trisikliese metieltiofeniel-propaanamied (TMP), ’n nie-pantoteensuur analoog wat die KoA biosintese padweg inhibeer in ander organismes, ondersoek as inhibitor van *P. falciparum*. TMP is gesintetiseer ter ondersteuning van die meganisme van aksie van *N*-fenetiel-α-metielpantoteenamied. Spesifiek wou ons toon dat die pantoteenamiede nie pantoteensuurkinase inhibeer soos TMP nie, maar eerder verder af in die padweg gemetaboliseer word. Die sintese en suiwering van TMP was suksesvol, maar die opbrengs was te laag om TMP te toets as ’n inhibitor van *P. Falciparum* groei.

Hierdie studie sal meer lig werp op die metode van werking wat van toepassing is op pantoteenamiede en meer inligting verskaf omtrent die biochemie van die parasite.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>APAD</td>
<td>3-acetylpyridine adenine dinucleotide</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
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<tr>
<td>B(OCH$_2$CF$_3$)$_3$</td>
<td>Tris(2,2,2-trifluoroethyl) borate</td>
</tr>
<tr>
<td>B$_2$O$_3$</td>
<td>Boron trioxide</td>
</tr>
<tr>
<td>CAF</td>
<td>Central Analytical Facility</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CPM</td>
<td>7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin</td>
</tr>
<tr>
<td>CPS</td>
<td>Counts per second</td>
</tr>
<tr>
<td>crypto-ACPs</td>
<td>crypto-acyl carrier proteins</td>
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<td><em>Escherichia coli</em> dephospho-coenzyme A kinase</td>
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<td>EcPPAT</td>
<td><em>Escherichia coli</em> phosphopantetheine adenyllyltransferase</td>
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<tr>
<td>EDC</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HBTU</td>
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<td>PES</td>
<td>Phenazine ethosulphate</td>
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<td>Abbreviation</td>
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<tr>
<td>PfPanK</td>
<td><em>Plasmodium falciparum</em> pantothenate kinase</td>
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<tr>
<td>PPAT</td>
<td>Phosphopantetheine adenylyltransfease</td>
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<tr>
<td>PPCDC</td>
<td>Phosphopantothenoylcysteine decarboxylase</td>
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<td>PPCS</td>
<td>Phosphopantothenoylcysteine synthetase</td>
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<td><em>Staphylococcus aureus</em> pantothenate kinase</td>
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Chapter 1: Coenzyme A biosynthesis as an antiplasmodial drug target

1.1 Cause and Effect of Malaria as a Disease

Malaria is a disease that is caused by a parasitic, single celled organism from the genus *Plasmodium*. Of the approximately 250 known species of *Plasmodium*, five of these species are able to infect humans, namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and the most recently discovered *P. knowlesi* [1].

Globally, in 2015, it is estimated that 2.1 billion people were at risk of contracting malaria, with approximately 214 million new cases leading to around 438 000 deaths. Although these numbers have decreased drastically over the last 15 years, malaria remains a worldwide problem [1]. *P. vivax* and *P. falciparum* are two of human malaria parasites that are of high interest. While *P. vivax* is more widely spread than *P. falciparum* and is responsible for most infections and many deaths outside of the African continent, it often has a dormant liver stage that can last for months, or occasionally years, before its symptoms present. *P. falciparum*, however, is rampant in Africa and is the greatest contributor to deaths caused by malaria, with approximately 90% of malaria-related deaths occurring in Africa [1]. In Sub-Saharan Africa, the individuals most affected by malaria and contributing most to the malaria death toll are pregnant women and children under the age of 5, however in areas with major health burdens, attempts to decrease malaria-related mortalities are also a challenge [1].

*Plasmodium* parasites usually have three main life stages of which the first one occurs in mosquitoes and the last two stages (i.e. liver and blood-stages) occur in humans (Figure 1.1). The female *Anopheles* mosquito is the vector that causes the spread of this deadly disease and forms the first part of the parasite life cycle; during a blood meal, the mosquito takes up the blood of an infected individual, containing parasites known as gametocytes, and then becomes a carrier of the parasite. Parasites can be taken up as male (microgametocytes) and female (macrogametocytes) gametocytes inside the mosquito where they undergo sexual reproduction in the sporogonic cycle, to ultimately mature into sporozoites that are then injected into another individual during the mosquitoes’ next blood meal [2]. Once a human is infected, the malaria parasite first goes through a liver stage, otherwise known as the hepatic stage, where they replicate inside the liver cells and further mature into millions of merozoites. These merozoites are then released into the blood stream where they invade the red blood cells – also known as the erythrocytic stage. It is during this stage that the symptoms, such as fever, diarrhea, headaches and nausea, are present [2, 3]. During the erythrocytic stage some parasites can differentiate into male and female gametocytes that are then again ingested by an *Anopheles* mosquito during a blood meal to complete the parasite life cycle [3].
Figure 1.1: The life cycle of a malaria parasite, such as *P. falciparum*, going through mosquito stage, hepatic stage and erythrocytic stage. See text for details. (Reproduced with permission from [3], Copyright Massachusetts Medical Society.)

1.2 Efforts to Control and Treat Malaria

The World Health Organization (WHO) has set out strategies to combat malaria to decrease the number of annual malaria cases and to lower malaria-related mortality. The newest strategy set out...
by the WHO for 2016-2030, called the Global Technical Strategy for Malaria, attempts to decrease malaria incidences and mortality by 90%, with the first milestone aimed at a 40% decrease by 2020. This is to be achieved by making sure that those at risk have access to preventative measures, diagnosis and treatment, and to increase funding available for the control and elimination of malaria [1].

The attempts to combat malaria occur predominantly in two ways: the first is focused on minimizing contact between mosquitoes, the parasite carriers, and humans, while the second is focused on using drugs to prevent or cure malaria once infection has taken place. However, adding to the difficulty of controlling the spread of malaria is the issue of resistance; not only are mosquitoes becoming increasingly resistant to insecticides, but certain strains of the malaria parasite itself are resistant to all current forms of drug-based treatment [1, 4].

1.2.1 Insecticides
The efforts made to minimize human contact with mosquitoes (vector control) include the widespread distribution of bed nets and clothing treated with insecticides, window screens and insect repellents, as well as spraying interior wall cavities of homes with insecticides. In addition to this, efforts are being made to control the breeding of the *Anopheles* mosquito in an attempt to limit the spread of the disease [1].

Although insecticides, such as organochlorine, organophosphate, carbamate and pyrethroid have thus far aided in controlling the spread of malaria by controlling the mosquito population, the insecticides are becoming less effective as *Anopheles* mosquitoes are developing resistance towards them. Approximately 77% of monitored countries have reported noted resistance to at least one type of insecticide and 63% of the monitored countries noted resistance to more than one insecticide over the last 6 years. An example of this resistance is towards the insecticide pyrethroid which was reported in 2014 by 75% of the monitored countries to no longer be effective [1].

1.2.2 Drugs and Treatment
Where prophylaxis and treatment of the disease are concerned, efforts are being made on various fronts to achieve malaria eradication world-wide. The development of a recombinant *P. falciparum* vaccine against malaria has recently been achieved; however, this vaccine is currently in stage III of clinical trials and not yet commercially available [1]. The vaccine, named RTS,S/AS0, was used in a 4-year pilot study in Africa on children between the ages of 5-18 months during which it was administered in 4 doses over a period of 20 months. It was found to be 39% effective against clinical malaria, and 31.5% effective against severe cases of malaria. Further tests are however still being conducted [1].
Although vaccines are an excellent strategy in the prevention of the disease, the limited success of RTS,S/AS0 clearly supports the ongoing desperate need for the development of new antimalarial drugs. Over the years, many effective drugs have been used to combat malaria, however malaria parasites are showing marked resistance against them, with certain strains of *P. falciparum* currently showing resistance to all known drugs [1, 4]. An example of this resistance is against the well-known antimalarial, chloroquine. Chloroquine was used mostly during the 1940s and 1950s, however resistance to chloroquine started to develop presenting in Africa around the 1970s causing an increased spread of malaria [1]. Chloroquine-resistance in *P. vivax* has been confirmed in multiple countries, including Brazil and Thailand [1].

After resistance to chloroquine became prevalent, treatment with drugs such as sulphadoxine-pyrimethamine, mefloquine and quinine became common [4]. However, with resistance building up against all these drugs, artemisinin-based combination therapies were adopted as a first line of treatment, and were shown to be effective against *P. falciparum* and *P. vivax*. When used as a monotherapy, there is a high rate of treatment failure because of the short half-life of artemisinin and its derivatives, however, when used in combination therapies with mefloquine, lumefatrine, amodiaquinem, piperaquine or pyronaridine (compounds with longer half-lives), there was a prolonged antimalarial pressure, increasing the efficacy of artemisinins and reducing the risk of developing resistance [5, 6]. Recently, *P. falciparum* has been found to start showing resistance towards artemisinins in some countries such as Thailand and Cambodia [1], however treatment failure in Africa is still less than 10% for the artemisinin, artesunate-amodiaquine, which is used as a first or second-line treatment.

With the two *Plasmodium* strains that are the greatest contributors to mortality caused by malaria showing increasing resistance to known forms of treatment, the development of novel drugs targeting new biochemical pathways or processes in the parasite is becoming a necessity.

### 1.3 Essential Nutrients for Malaria Survival

Several *in vitro* studies have been done to ascertain vitamin and amino acid requirement in different species of *Plasmodium* to determine which of these nutrients are essential for the survival and proliferation of these parasites. It was shown that the core nutrients needed for the survival of *P. falciparum in vitro* are the amino acids isoleucine, cysteine, glutamate, glutamine, proline, tyrosine and methionine, as well as nutrients pyridoxine, glutathione, potassium chloride, sodium chloride, disodium phosphate, calcium nitrate, magnesium sulfate, glucose, folic acid, riboflavin, hypoxanthine, *p*-aminobenzoic acid (PABA), biotin, human serum and pantothenate [7].
Biotin (vitamin B₇, Figure 1.2) is generally biosynthesized in bacteria and plants but cannot be biosynthesized in humans, thus the uptake of biotin in humans is solely through diet. Like humans, *Plasmodium* parasites are unable to biosynthesize this vitamin, yet possess the acetyl-coenzyme A carboxylase enzyme [8]. Acetyl-coenzyme A carboxylase is a biotin-dependent enzyme to which biotin is covalently attached by the enzyme biotin-protein ligase. Trager showed that when chickens infected with *Plasmodium* were fed a biotin-deficient diet, the parasitemia in the erythrocyte stage were almost half of that found in chickens that were fed a diet supplemented with biotin, highlighting the parasites’ requirement for biotin [9].

The role of thiamine (vitamin B₁, Figure 1.2) in parasite proliferation has been investigated recently [10]. This vitamin was originally not believed to be essential for parasite survival, however it was shown that complete depletion of thiamine from erythrocytes is detrimental to parasite growth. *Plasmodium* parasites are capable of thiamine synthesis, however it seems as if the demand in the parasite for thiamine is higher than production, therefore thiamine is also sourced from the host [10, 11].

Pantothenate (vitamin B₅ or pantothenic acid in its non-ionized form) cannot be biosynthesized by *Plasmodium* parasites (Figure 1.2) de novo. This was discovered in 1946 by Brackett et al., who studied avian malaria parasites, and found that when chickens infected with *Plasmodium gallinaceum* were fed a diet that lacked pantothenate, the blood pantothenate levels of the chickens decreased, leading to the inhibition of the blood-stage malaria parasites. This indicated that the parasites not only needed pantothenate to survive, but also that they could not produce it themselves [2]. It has since been shown that pantothenate is the only water-soluble vitamin that erythrocyte stage *P. falciparum* parasites are unable to survive without [2]. It has also been shown that pantothenate is readily taken up into *Plasmodium*-infected red blood cells, but that pantothenate is not taken up into uninfected red blood cells [12]. The uptake into the parasitized cells happens via the ‘new permeation pathway’ (NPP) that the parasite includes within the erythrocyte [12], whereby pantothenate is then taken into the cell (in humans) by a low affinity, H⁺-coupled pantothenate transporter [13].
Even though it seems as if various vitamins play an important role in the proliferation of *Plasmodium*, Divo et al. established *P. falciparum*'s absolute requirement for extracellular pantothenate by \[^3\text{H}\]hypoxanthine incorporation, in which an 80% decrease was seen in parasite proliferation when pantothenate was not present in growth media [7]. These results were confirmed by Saliba et al., who also showed that *P. falciparum* does not have an absolute requirement for any other water-soluble vitamin [13], thereby highlighting the absolute necessity of pantothenate within the parasite.

1.4 Coenzyme A and Malaria

1.4.1 Coenzyme A biosynthesis in *Plasmodium*

Pantothenate is metabolized in living organisms to produce coenzyme A (CoA), an essential cofactor in both prokaryotes and eukaryotes that is synthesized in bacteria, plants and mammals [14-16]. The biosynthesis of CoA from pantothenate takes place through a universal five-step enzymatic pathway (Figure 1.3). The five enzymes that catalyze this pathway are pantothenate kinase (PanK), phosphopantothenoylcysteine synthetase (PPCS), phosphopantothenoylcysteine decarboxylase (PPCDC), phosphopantetheine adenylyltransferase (PPAT) and dephospho-coenzyme A kinase (DPCK).

![Figure 1.3: The universal, five-step enzyme mediated CoA biosynthesis pathway, from pantothenate to CoA. See text for details.](Stellenbosch University https://scholar.sun.ac.za)

In the first step, pantothenate is phosphorylated by PanK, yielding 4'-phosphopantothenate, which subsequently undergoes a condensation reaction (amide formation) with a cysteine molecule, mediated by PPCS, to form 4'-phosphopantothenoylcysteine. In most eukaryotes, PPCS has a preference for ATP for this conversion, where in bacteria, such as *Staphylococcus aureus*, it has a preference for CTP instead, however it is unknown which *Pf*PPCS prefers. PPCDC decarboxylates the cysteine moiety of 4'-phosphopantothenoylcysteine to form 4'-phosphopantetheine, which, in the fourth step, undergoes the addition of an adenylyl group by PPAT forming dephospho-CoA. In the
final step, DPCK catalyzes the phosphorylation of dephospho-coenzyme A at the 3’ position on the ribose to form the final product, CoA [4].

Putative genes encoding all five enzymes required for CoA formation from pantothenate have been identified in the *P. falciparum* genome [2]. The first four enzymes are predicted to localize to the *P. falciparum* cytosol. The last enzyme in the pathway, DPCK, is predicted to localize to the apicoplast, the non-photosynthetic, plastid relic present in most apicomplexan parasites [2]. None of the enzymes encoded by these genes have thus far been characterized in any detail, since they are notoriously difficult to overexpress and purify from bacterial systems. Various attempts by different research groups have been made to produce pure *PfPanK* using cell-free protein expression systems, heterologous expression from codon-harmonized and -optimized genes and by removal of expression-hampering sequences [17]. Unfortunately, none of these have been effective to date. Nevertheless, some information has been gathered for the activity of PanK in *P. falciparum* lysates (to be discussed in detail in Chapter 3).

Currently, all the information known regarding pantothenate utilization—and consequently CoA biosynthesis in *Plasmodium*—is based on knowledge obtained from the blood-stage of the parasites. No information regarding the liver and mosquito stages is currently available.

### 1.4.2 Functions of CoA in *Plasmodium*

Little is known about the specific functions of CoA in the erythrocytic stage of the parasites' life cycle. In general, it is known that CoA acts as an acyl group carrier in all living organisms, since it contains a thiol group that can undergo esterification with carboxylic acids, thereby activating them for nucleophilic attack or for enolate formation. This is important in many cellular metabolic processes such as the all-important first step of the Krebs cycle, fatty acid biosynthesis and regulation, biosynthesis of sterols and amino acids, and the post-translational modifications of proteins, among others [17, 18]. It is estimated that up to 9% of enzymes in all living organisms make use of CoA or one of its thioesters [2].

Although CoA is important in the Krebs cycle, it is known that blood-stage *Plasmodia* do not make use of the Krebs cycle as its primary source of energy. Instead, these parasites rely on glycolysis for energy production [19]. It was shown by Sharma et al. that glucose consumption in parasite-infected red blood cells are up to 100-fold higher than for the uninfected red blood cells, supporting the parasites' dependence on glycolysis [19].

*P. falciparum* possesses its own fatty acid biosynthesis pathway, where it uses CoA and pyruvate as substrates. It was therefore proposed that in the erythrocytic stage, where fatty acids are important for protein post-translational modifications and membrane biosynthesis, the parasite’s absolute requirement for CoA in this stage is due to these functions [17]. It was however recently discovered
that fatty acid biosynthesis can also be blocked in malaria, by targeted deletion of a crucial enzyme involved in fatty acid biosynthesis (FabB/F), without any effect on the blood-stage replication. This renders fatty acid biosynthesis non-essential for the survival of the parasite, thus making it a poor drug target in malaria [20].

In addition to the biosynthesis of fatty acids, CoA is also involved in fatty acid modification. This occurs when the cofactor is attached to fatty acids by acyl-CoA synthetase causing the activation of the fatty acids. The *P. falciparum* genes encoding for the acyl-CoA synthetases are expressed during the erythrocytic stage of the parasites’ lifecycle. Although red blood cells also contain active acyl-CoA synthetase, the activity of this enzyme is up to 20-times higher in a parasite-infected erythrocytes, indicating its possible importance in fatty acid activation, rather than in its ability to biosynthesize fatty acids [17].

Recently, however, an analysis of *P. yoelii*’s (a rodent malaria parasite) proteome and transcriptome in liver stage found that type II fatty acid synthesis enzymes were present in the liver stage proteome. Type II fatty acid synthesis gene transcription has also been shown to be upregulated in liver stage compared to levels in the erythrocytic stage. In addition, it was found that an inhibitor of FabG, an important enzyme in fatty acid chain elongation, was able to inhibit *in vitro* parasite proliferation, thus suggesting that fatty acid biosynthesis might still be a possible target in liver stage of *Plasmodium* parasites [20].

Taken together, the universal occurrence of the CoA biosynthesis pathway and the importance of CoA in all living organisms, in addition to the needs of the *Plasmodium* for this cofactor in different life stages, make this biosynthetic pathway an attractive target for antimalarial drug development.

### 1.5 Targeting CoA Biosynthesis in *Plasmodium*

The aim to develop new antimalarials has led to a search for new possible drug targets. Different approaches have been used to illustrate that CoA biosynthesis is a novel drug target in *P. falciparum*. One such approach is based on the concept of chemical rescue where assays have been developed to screen for inhibitors (varying in their chemical structure) of vital metabolic pathways and then rescuing the antimalarial effect by the addition of CoA or metabolites of the pathway [21]. An alternative approach to validate the CoA biosynthesis pathway as a potential antiplasmodial drug target, has been to synthesize pantothenate analogues using the core structure of pantothenate, the native substrate of the CoA biosynthesis pathway and then testing these compounds for an antimalarial effect. The synthesis of pantothenate analogues should allow these compounds to interfere with *Plasmodium*’s ability to utilize the vitamin. The inhibition of parasite growth by pantothenate analogues is based on the essentiality of pantothenate for blood-stage parasite survival *in vitro*. The various compounds that have been tested in the past by various studies, some
of which have been developed to be vanin resistant in order to retain their potency in blood serum, are discussed in the following section.

1.5.1 Pantothenate Analogues

1.5.1.1 Pantothenol

Pantothenol (Figure 1.4), or provitamin B₅, is a pantothenate analogue in which the carboxylic acid group is reduced to a primary hydroxyl group. It is a compound commonly used in consumer products from multivitamins to cosmetic products. It has been shown that no significant adverse effects are noted, even after long term use, even when used or ingested in high concentrations by humans [21]. However, in vitro studies have shown that it prevents proliferation in a variety of different bacteria [22]. Consequently, pantothenol was tested by Saliba et al. on blood-stage P. falciparum parasites and shown to have antimalarial activity in vitro [23].

![Figure 1.4: The structure of pantothenol. This pantothenate analogue merely has a hydroxyl group in the position where pantothenate has a carboxyl group (indicated in orange).](image)

Since pantothenol can act as a provitamin in some organisms by being oxidized to pantothenate, Saliba et al. tested to see whether this was the case in Plasmodium as well; however, blood-stage parasites could not survive in pantothenate-free media that was supplemented with pantothenol. They found that instead of supporting the proliferation of the parasite, pantothenol actually inhibited parasite proliferation by competing with pantothenate in the conversion to its phosphorylated form, thus implicating the possible inhibition of PanK [13].

1.5.1.2 CJ-15,801

The pantothenate analogue, CJ-15,801 (Figure 1.5) is a compound isolated from the fungus Seimatosporium sp., CL28611. The only difference in structure between CJ-15,801 and pantothenate is the inclusion of a double bond in the β-alanine moiety of the vitamin.

![Figure 1.5: The structure of CJ-15,801. This pantothenate analogue has a double bond between carbons 2 and 3 as indicated in orange, where pantothenate has a single bond.](image)

CJ-15,801 was previously shown to have antibacterial effects, even on multi-drug resistant S. aureus [24]. It was therefore investigated by Saliba et al. for its antiplasmodial properties against P. falciparum [23]. Blood-stage parasites were incubated with CJ-15,801 in the presence of physiological concentrations of pantothenate (1 µM), and it was found that CJ-15,801 inhibits
parasite proliferation at concentrations above 250 µM, without an effect on the host cell line [23]. However, the antiplasmodial effect of CJ-15,801 was reversed when pantothenate concentrations were increased, but it was only completely reversed at very high pantothenate concentrations (around 100-200 µM) [23]. This confirmed that this compound is targeting the biosynthesis pathway that utilizes pantothenate in order to form CoA.

1.5.1.3 Pantoyltauramides

One of the first pantothenate analogues to be tested against malaria was pantoyltauramide (Figure 1.6 A), an analogue in which the carboxylic acid has been substituted for a sulphonamide group. When tested in vivo against different types of avian malaria parasites such as *P. relictum*, *P. gallinaceum*, and *P. lophurae*, this compound was found to be almost inactive when incorporated into the birds' diet [25]. However, when administered intravenously at a dose of 400 mg/kg body weight, proliferation of *P. gallinaceum* was inhibited in chickens [26].

![Figure 1.6: (A) The structure of pantoyltauramide, one of the first pantothenate analogues to be tested for its antiplasmodial activity, (B) the most potent N-substituted pantoyltauramide in the study done by Trager in 1971 [27] and (C) the structure of N-substituted pantoyltauramides (where R- groups are specified in the grey block).](image)

In an attempt to make the pantoyltauramides more active when orally administered, the pantoyltauramide structure was modified further by inclusion of additional groups to the sulfonamide functionality, thus producing a library of new compounds called the *N*-substituted pantoyltauramides (Figure 1.6 C) [27]. The most potent of the *N*-substituted pantoyltauramides in this library (Figure 1.6 B) was found to be approximately four times more potent than quinine when tested against *P. gallinaceum* in chickens [28].
1.5.2 \textit{N}-substituted Pantothenamides

Another class of pantothenic acid analogues is the \textit{N}-substituted pantothenamides (PanAms) (Figure 1.7A).

\begin{center}
\includegraphics[width=0.8\textwidth]{figure17}
\end{center}

\textit{Figure 1.7: The structure of (A) a synthetic general \textit{N}-substituted pantothenamide and (B) naturally occurring pantetheine. Pantetheine is essentially an \textit{N}-substituted pantothenamide with an ethylthiol moiety as the R-group.}

Although these compounds are technically pantothenate analogues (they have an amide functionality instead of a carboxylic acid), they are structurally more closely related to the natural occurring pantothenamide, pantetheine (containing a cysteamine moiety, Figure 1.7 B). Pantetheine is present in certain bacteria and is believed to be a degradation product of CoA, however, it also has the ability to be phosphorylated by PanK to feed back into the biosynthesis of CoA in a salvage pathway to reproduce CoA (Figure 1.8) [29]. Currently it is still unknown if pantetheine is present in \textit{Plasmodium} parasites.

\begin{center}
\includegraphics[width=0.8\textwidth]{figure18}
\end{center}

\textit{Figure 1.8: The CoA salvage pathway, starting with natural compound, pantetheine, which also undergoes phosphorylation by PanK after which PPAT and DPCK convert it to CoA.}

In the past a large number of \textit{N}-substituted pantothenamides were tested on blood-stage parasites by Jackowski \textit{et. al} [30], Park \textit{et. al} [31], Strauss \textit{et. al} [32], Saliba \textit{et. al} [33-35] and others [36]. Fortunately, many of these analogues had an effect on \textit{Plasmodium}'s proliferation and, because of
their similar structure to pantothenate, some of them have been shown to interfere with the phosphorylation of pantothenate by PanK [33]. This in turn supports the theory that pantothenate analogues target the CoA biosynthesis pathway. In fact, a study by Spry et al. [33] found that PanAms (Figure 1.7 A) are the most potent pantothenate analogues against *P. falciparum*, with N-phenethyl-PanAm (N-PE-PanAm) being the most potent *in vitro*, with an IC$_{50}$ of 20 ± 3 nM. This IC$_{50}$ is comparable to chloroquine, an antimalarial used from 1947 until the late 1950s [37], with an IC$_{50}$ of 15 nM [33, 34, 38].

### 1.5.3 Pantetheinase (Vanin)

Unfortunately, the use of PanAms for clinical treatment of malaria is not possible since these compounds are prone to degradation by vanins (hydrolase enzymes that target non-peptide carbon-nitrogen bonds), specifically pantetheinases, in the blood serum [39]. When looking at *in vivo* conditions, pantetheine gets degraded to form pantothenate and cysteamine by the hydrolysis of the scissile amide bond (Figure 1.9). Since the PanAms have the same core structure as pantetheine, containing an amide bond in the same position, these compounds are also susceptible to degradation, thus rendering them ineffective as antiplasmodials in the presence of blood serum [33].

Various strategies have been employed to overcome this shortcoming. One specific strategy is combination therapies of PanAms with pantetheinase inhibitor compounds (Figure 1.10). These compounds, which are also PanAm analogues, but which have the scissile amide bond replaced by a ketone, act as selective, competitive vanin inhibitors that inhibit vanin activity in rat plasma as low as nanomolar concentrations [40]. The potential of these compounds to also inhibit *P. falciparum* parasite proliferation was also investigated and it was found that they do have antiplasmodial activity, however with less potency (low micromolar range) than existing PanAms [41].
Figure 1.10: Structure of a pantothenate analogue, RR6, which acts as the most potent vanin inhibitor.

1.5.4 PanAm Analogues Resistant to Pantetheinase-mediated Degradation

A second strategy in circumventing the pantetheinase degradation effects on PanAms, is the design of compounds that are resistant to degradation by this blood serum enzyme. This has the added advantage that only one specific therapy needs to be used, i.e. without the added complication of having two compounds that could potentially be toxic towards humans. Various degradation-resistant modifications to the PanAm structure have been attempted by investigators, with the following modifications showing the most promise.

1.5.4.1 Sterically hindered N-substituted PanAms

It was found that modification of PanAms by substituting the β-alanine moiety for a glycine to produce α-PanAms (Figure 1.11 B), or substituting the β-alanine moiety for γ-aminobutyric acid to produce HoPanAms (Figure 1.11 C), makes these compounds less susceptible to pantetheinase-mediated degradation by shifting the position of the amide bond. This resistance to degradation however comes at the cost of a loss in potency [34]. This becomes evident when studying the antiplasmodial activity of N-PE-PanAm: when testing the antiplasmodial activity in vitro with no pantetheinase present, this compound has an IC\(_{50}\) of 20 ± 3 nM, however, when pantetheinase is present in the media, a shift in IC\(_{50}\) to 53 ± 11 μM is observed [33]. The α- and HoPanAms have been shown to have increased IC\(_{50}\)s of 3.4 ± 0.8 μM and 2.1 ± 0.1 μM, respectively, in the absence of pantetheinases [34], which suggests the core pantothenate structure is necessary in order for the PanAm to be potent. This realization led to further modification of the N-PE-PanAm structure in order to produce PanAms that are stable in serum while retaining their antiplasmodial activity.

Figure 1.11: The structures of synthesized pantothenamides (A) N-PE-PanAm, and its (B) α-PanAm and (C) HoPanAm counterparts. The β-alanine moiety is highlighted in orange, the glycine moiety highlighted in blue and the γ-aminobutyric acid highlighted in green.
To overcome the loss of potency, N-PE-PanAm was modified to include a methyl substituent adjacent next to the amide that is normally cleaved by the vanins, forming N-phenethyl-α-methyl-PanAm (N-PE-α-Me-PanAm) (Figure 1.12). This methylated compound was then tested in the presence of pantetheinases and shown to be more resistant to degradation while still retaining its potency (0.06 ± 0.02 μM) [42]. Importantly, methylated-PE-PanAm was significantly more resistant to degradation by pantetheinase compared to N-PE-PanAm, and, crucially, still acted on-target, as demonstrated by the fact that addition of excess extracellular pantothenate antagonized its antiplasmodial activity [2]. Furthermore, it has limited cytotoxicity to mammalian cells [36]. However it must be noted that the compound tested was the mixture of the two epimers, (R)- and (S)-N-PE-α-Me-PanAm. The specific activities of the two compounds have not been investigated to date.

![Figure 1.12: The structure of the synthetic PanAm, N-PE-α-Me-PanAm, that was shown to be more resistant to degradation by pantetheinases. The yellow highlighted group indicates the methyl group added to the compound to prevent cleavage of the amide bond adjacent to it.](image)

1.5.4.2 N-Substituted triazole bio-isosteres

Recently, another strategy to increase the resistance of PanAms towards pantetheinase-mediated degradation has been illustrated by exchanging the scissile amide bond with a triazole bio-isostere [35]. In total 19 triazoles were synthesized and tested for antiplasmodial activity against *P. falciparum* with all compounds tested having sub-micromolar IC₅₀ values, with 2 of them having IC₅₀ values of ~50 nM against blood-stage parasites (Figure 1.13), only slightly higher than that of chloroquine [35]. These compounds have been shown to be on target after the addition of extracellular pantothenate shifted their antiplasmodial activity and it has been speculated that they have an effect on PanK phosphorylation [35].

![Figure 1.13: The structure of the N-substituted triazole bio-isosteres with IC₅₀ values of ~50 nM against blood-stage *P. falciparum* parasites. N5-triazole has an IC₅₀ of 56 ± 5 nM and N6-triazole has an IC₅₀ of 55 ± 5 nM.](image)
1.5.4.3 \(N\)-Substituted PanAm bio-isosteres with an inverted amide group

A recent patent application on PanAms as antimicrobials has employed an additional strategy to produce stable PanAms in the presence of pantetheinase. In this simple, yet elegant modification, the scissile amide bond has been replaced by an amide bond in the inverse orientation when compared to that of normal PanAms. The resulting PanAm bio-isosteres are resistant to degradation by pantetheinases, and when compared to the parent compound, have no loss of potency. A small library of compounds has been tested, including the inverted amide variant of \(N\)-PE-PanAm (Figure 1.14 B) which had an \(IC_{50}\) of 0.12 µM against \(P. falciparum\). The ortho-fluoro derivatives of \(N\)-PE-\(\alpha\)-Me-PanAm were also tested as the individual \(R\)- and \(S\)-stereoisomers (Figure 1.14 C and D), with \(IC_{50}\) values of 0.004 µM and 0.21 µM against \(P. falciparum\) being reported. This makes the \(R\)-stereoisomer derivative of \(N\)-PE-\(\alpha\)-Me-PanAm the most potent of these compounds, when tested against \(P. falciparum\), with compound CXP18.6-052 (Figure 1.14A), the second most potent with an \(IC_{50}\) of 0.008 µM [32].

![Figure 1.14](https://scholar.sun.ac.za)

**Figure 1.14:** (A) The structure of compound CXP18.6-052, the most potent of the reversed amides tested against \(P. falciparum\), (B) the structure of the inverted amide for of PE-PanAm, and (C &D) \(R\)- and \(S\)-stereoisomers of the ortho-fluoro derivative of \(N\)-PE-\(\alpha\)-Me-PanAm.

1.6 Mode of Action of the PanAms

The mode of action of pantothenate analogues in \(P. falciparum\) is still currently unknown. It is also possible that different pantothenate analogues have different modes of action involving different targets in the biosynthesis and/or utilization of CoA. For this particular study our main focus is on the mode of action of PanAms, and therefore possible targets of these compounds will be discussed in this regard.

Previous studies have shown that the addition of pantothenate to the parasites extracellular environment reverses the PanAms’ antiplasmodial activity [34, 36]. This suggests that the PanAms target the enzymes involved in CoA biosynthesis and/or the downstream processes that use CoA or
its thioesters [43]. Since no other information regarding the mode of action of PanAms is known for *P. falciparum* parasites, we considered what is known for these compounds when studied in bacteria.

The antibacterial mode of action of the PanAms has mainly been studied in *Escherichia coli* and *S. aureus*. Also, although various PanAms inhibited bacterial growth, most of these studies focused on *N*-pentyl pantothenamide (*N*5-Pan) and *N*-heptyl pantothenamide (*N*7-Pan). Three possible modes of action have been proposed for the PanAms in bacteria, illustrated in Figure 1.15 for *N*5-Pan: (1) PanAms inhibit the PanK, the first enzyme in the CoA biosynthesis pathway, inhibiting the phosphorylation of pantothenate and therefore shutting down the CoA biosynthesis pathway; (2) when PanAms act as competing substrates, they can be phosphorylated by PanK and metabolized by PPAT and DPCK downstream in the pathway. This allows for the formation of CoA antimetabolites. These antimetabolites will either compete with the production of CoA and lower CoA intracellular concentrations to levels that are insufficient to sustain bacterial growth, or cause the inhibition of CoA-dependent processes and enzymes; (3) a specific CoA-dependent process, fatty acid biosynthesis is inhibited. This occurs by the formation of *crypto*-acyl carrier proteins (*crypto*ACPs) where the 4'-phosphopantothenamide moiety is transferred to an acyl carrier protein (ACP) instead of the 4'-phosphopantetheine moiety it usually needs from CoA. These *crypto*-ACPs are inactive due to the lack of a terminal thiol (which they usually obtain from CoA) necessary for fatty acid biosynthesis [33, 44, 45]. This last option was supported by a previous study that indicated that the PanAms do not inhibit bacterial PanK, since the PanAms are still phosphorylated once taken up into the bacterial cell, specifically in *E. coli*. In this organism it was therefore found that the main growth inhibition takes place via inhibition of fatty acid biosynthesis [30, 33]. However, recent findings have also indicated that PanK in *S. aureus* is partially inhibited by PanAms via trapping of the phosphorylated product [31, 34] and once the phosphorylated product is released (which takes place very slowly) they can be metabolized further to inhibit fatty acid biosynthesis [30]. The specific mode of action for this inhibition of fatty acid biosynthesis is not clearly defined yet.
Fig 1.15: The three main biological targets for PanAms in bacteria, illustrated with N5-Pan: (1) PanK, the first enzyme in the CoA biosynthesis pathway; (2) the CoA-dependent enzymes and processes, once the antimetabolite is formed; and (3) fatty acid biosynthesis, when the antimetabolite formed acts as a substrate for the enzyme AcpS to form the inactive crypto-ACP instead of the catalytically active holo-ACP [46] (Reproduced with permission from [33], Copyright John Wiley & Sons, Inc.).

When extrapolating this knowledge to *P. falciparum*, there are four proposed modes of action for PanAm inhibition (Figure 1.16). In Target 1 PanAms could interfere with the transport of pantothenate by inhibiting the essential primary pantothenate transporter in *P. falciparum*, leading to the deprivation of the essential vitamin. This is not considered a target in bacteria, since bacteria have the means to synthesize pantothenate *de novo* which is not the case for *P. falciparum* parasites. It has been recently discovered by Kehrer et al. that in *P. berghei* the pantothenate transporter, PAT, is essential for the secretion of vesicles needed for the transmission of the parasite between the human host and the mosquito [48]. This supports pantothenate transport as possible target. Target 2 involves the inhibition of PanK, the first CoA biosynthetic enzyme, which would shut down CoA production. Alternatively, PanAms can be phosphorylated by PanK as substrates. This allows for these compounds to become metabolically activated as substrates for PPAT and DPCK, converting them into CoA antimetabolites. Such a transformation could result in growth inhibition in two ways: (Target 3) the decrease of CoA levels to concentrations below what is required to sustain parasite survival or (Target 4) the direct inhibition of one or more CoA-requiring processes by the CoA antimetabolites. It has been shown that fatty acid biosynthesis is not essential in blood-stage *P. falciparum* parasites [47, 48] and blood-stage parasites rely exclusively on glycolysis for their energy needs. Consequently, these parasites do not use the Krebs cycle (with its many acyl-CoA
intermediates) in this life-stage [19]. It is therefore unlikely that fatty acid biosynthesis and the Krebs cycle are targets for CoA antimetabolites. However, since there are various processes relying on CoA, other targets might come into play. In addition, fatty acid biosynthesis has been implied to be important in the liver stage of *P. falciparum* [20] and therefore these compounds might act on different targets in the different life stages of the parasite.

Currently it is unknown which of the four targets is the main point of inhibition for PanAms. Various studies have shown the inhibition of pantothenate uptake and phosphorylation by pantothenate analogues [13, 23]. A recent study has shown that PanAms inhibit pantothenate uptake and phosphorylation by these compounds competing with pantothenate uptake and phosphorylation [49]. It has also been shown that pantothenamides are phosphorylated by *PfPanK* present in parasite lysate; therefore, it seems as if these compounds act as substrates for PanK [42, 49]. These findings eliminates target 1 and 2 as possible targets for PanAm inhibition and elude to target 3 and 4 as possibilities. Therefore further research is needed to investigate whether the antimetabolites are actually formed, and if so, what the effects of these compounds are on the biochemistry of the malaria parasite.

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**Figure 1.16:** The proposed mechanisms of action for the PanAms in *P. falciparum*: (1) Interference with the pantothenate transporters; (2) inhibition of PanK; (3) CoA antimetabolites could cause a decrease in CoA levels to concentrations lower than the parasite needs to survive or (4) the CoA antimetabolites inhibit one or more downstream CoA-requiring processes directly.
1.7 Aims of this Study
The purpose of this project is to shed light on the mode of action of PanAms’ inhibition of \(P. falciparum\) parasite proliferation. Three main aims were set out for this study in order to lay groundwork for future research in finding the exact point of action of PanAm inhibition. Not only will this allow CoA biosynthesis to be considered as a new drug target in \(P. falciparum\), but importantly, it will also increase knowledge on unknown parasite biochemistry.

**Aim 1: Establish the minimum extracellular pantothenate concentration needed for parasite survival**

Ultimately we want to determine what the critical amount of CoA is for parasite survival. In doing so we can determine the threshold needed for PanAms to interfere with CoA biosynthesis. However, this is not an easy determination to make since parasites obtain CoA from pantothenate received from the host red blood cell that in turn obtains pantothenate from extracellular sources. We therefore set out to determine the minimum required pantothenate needed for the survival of blood-stage \(P. falciparum\) parasites.

**Objective 1: Determine parasite survival without pantothenate present**

In order to achieve this, custom RPMI media without pantothenate present must be obtained since all commercially available RPMI-1640 media contains ~1 µM pantothenate. \(P. falciparum\) 3D7 is then cultured in this media under otherwise normal culturing conditions to determine how long they can survive without any extracellular pantothenate available to them.

**Objective 2: Determine the minimum amount of extracellular pantothenate necessary for \(P. falciparum\) survival**

Once we know how long blood-stage parasites can survive without pantothenate, we can determine the minimum amount of pantothenate needed for parasite survival by again culturing \(P. falciparum\) 3D7 strain in pantothenate-free media, supplemented with varying concentrations of pantothenate.

Ultimately these two experiments will lay a foundation for future studies to determine the minimum amount of CoA required for parasite survival.

**Aim 2: Determine if \(N\)-PE-\(\alpha\)-Me-PanAm is metabolized by CoA biosynthetic enzymes as substrate to form CoA antimetabolites**

Evidence that the PanAms can be converted to the corresponding CoA antimetabolites by the CoA biosynthetic enzymes present in \(P. falciparum\) parasites will support the mode of action of PanAms that is based on either the lowering CoA levels, or the inhibition of processes in the parasite that depend on CoA.
Objective 1: Validate an analytical technique for CoA antimetabolite detection

Several analytical chromatography techniques are available in our laboratory for the detection of CoA and intermediates of the pathway. Our first objective was therefore to determine which technique would suite our application (using parasite lysate) the best.

Objective 2: Analysis of PanAm conversion in P. falciparum lysate

In this case we analysed the conversion of N-PE-α-Me-PanAm to the corresponding CoA antimetabolites in the presence of lysate from isolated P. falciparum parasites.

Objective 3: Analysis of N-PE-α-Me-PanAm conversion in P. falciparum cultures in vitro.

We also investigated the conversion of PanAms in P. falciparum cultures treated with a non-lethal dose of PanAm to determine if any conversion of the compound is taking place under culture conditions.

Aim 3: Synthesis and evaluation of the effect of a PanK inhibitor that is not a pantothenate analogue.

To support that PanAms do not inhibit PanK, but are rather metabolized downstream in the pathway we set out to prepare a PanK inhibitor that is not a pantothenate analogue. Recently studies have shown that human PanK can be inhibited by tricyclic methylthiophenyl propanamides (TMPs) that in structure are not related to pantothenate at all [50]. TMPs bind to the ATP-PanK complex to induce their inhibitory effect and were shown to impair CoA levels at increasing concentrations of TMPs [50]. We therefore want to prepare a TMP analogue and test it on P. falciparum to see if it has any inhibitory effect on PfPanK and parasite proliferation. If so, it should inhibit by lowering CoA levels since it shuts down CoA biosynthesis at the first enzyme. This can then be used for future studies as a tool to compare to PanAm as inhibitors in order to determine if PanAms lower CoA levels in a different way—the difference being that the TMP analogue cannot act as alternative substrates and the pantothenamides can, since they are pantothenate analogues.

Objective 1: Synthesis and purification of TMP

In order to test TMPs as a P. falciparum inhibitor, one of these compounds had to be synthesized. This was attempted by using published protocols in literature [50].

Objective 2: Test TMP as P. falciparum PanK inhibitor

TMP was established in literature as human PanK inhibitors by using a discontinuous radioactive assay. These results should be confirmed by testing TMPs on human PanK 3, which is available in our laboratory. Once this has been established we can test the effect of this compound on P. falciparum in parasite lysate using the same discontinuous radioactive assay.
Objective 3: Test TMP as inhibitor of *P. falciparum* proliferation

TMP will be tested as inhibitor of *P. falciparum* parasite growth in culture and if it is an inhibitor the IC$_{50}$ will be determined.

The aims and objectives set out in this study will form the pilot study in the quest to determine the specific mode of action of PanAms in order to develop these compounds further as inhibitors of *P. falciparum* in various stages of the parasites life cycle in future studies to come.
1.8 References:


Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General reagents
All general reagents and chemicals, solvents, silica and thin-layer chromatography (TLC) plates used for synthesis were purchased from Sigma-Aldrich. SYBR Safe fluorescent DNA stain (10 000 × concentrate in DMSO) for *P. falciparum* growth assays were acquired from Invitrogen. Novagen’s KOD Hot start DNA Polymerase Kit was bought from Merck. Mycoplasma removal agent (MRA) was from Biorad. Mycoplasma DNA was a kind gift from Dr. Annelise Botes (Department of Biochemistry, Stellenbosch University). Nancy-520 gel dye was obtained from Sigma-Aldrich.

2.1.2 Plastics and glassware
For culturing, Kymex and Falcon 15 ml and 50 ml centrifuge tubes as well as Greiner Cellstar ® 75 cm² (250 ml) tissue culture flasks and NEST 25 cm² (60 ml) tissue culture flasks were used for all in vitro *P. falciparum* culturing. For all the assays, cultures were incubated in sterile, clear, NEST 96-well flat bottom cell culture plates and fluorescent assays analyzed in Grenier Bio One black 96-well flat bottom plates. For the malstat assays, clear Grenier Bio One 96-well flat bottom plates were used. 1 ml, 5 ml, 10 ml and 60 ml Surgi Plus syringes were purchased from Stellenbosch Medical Suppliers and 1.5 ml and 2.0 ml centrifuge tubes where obtained from Scientific Specialties, Inc.

2.1.3 Culture medium and media components
Standard RPMI-1640 culture media as well as HEPES, sodium bicarbonate, glucose and hypoxanthine used to supplement the media were purchased in powder form from Sigma-Aldrich and were all suitable for cell culture. The gentamycin was purchased from Sigma-Aldrich in liquid form, and Albumax II serum acquired from Professor Peter Smith, Division of Pharmacology at the University of Cape Town, who acquires it from Thermo Fisher Scientific Inc. The custom made, pantothenate free media was first made up using components bought from Sigma-Aldrich. Later it was custom made by an American based company, AthenaES and was acquired in liquid form (containing Glutamax instead of L-glutamine). Other components used in assays were purchased in powder form from Sigma-Aldrich and the low oxygen gas mixture used to gas cultures was obtained from Afrox.

2.1.4 Human erythrocytes
Human A⁺ blood was used for all malarial cell cultures and was obtained biweekly from the Western Cape Blood Bank at Vergelegen Medi-Clinic, Somerset West, South Africa. The work performed in this study was performed in the laboratory of the co-supervisor, Prof Erick Strauss. Prof. Strauss has ethical clearance from the The Health Research Ethics Committee of Stellenbosch for use of human blood in cell culture medium.
2.1.5 *Plasmodium falciparum* parasites

The *P. falciparum* strain 3D7 parasites used in this study were available in the laboratory of Prof. Erick Strauss (co-promotor to this study) in the Department of Biochemistry at Stellenbosch University.

2.1.6 *N*-substituted Pantothenamides and CoA biosynthesis metabolites

*N*-PE-α-Me-PanAm used in this study was synthesized by Dr. Leanne Barnard (Department of Biochemistry, Stellenbosch University) as part of another study [1]. Its purity was confirmed by $^1$H, $^{13}$C NMR and High Resolution Mass Spectrometry (HRMS) analysis prior to being stocked. These compounds are usually stored at -20 °C at a concentration of 200 mM in either DMSO or 50% acetonitrile/water.

Pantothenate, pantetheine, dephospho-CoA, and CoA, were obtained from Sigma-Aldrich. 4'-phosphopantetheine was synthesized from pantetheine using a *S. aureus* PanK enzyme.

2.1.7 Bacterial Enzymes

*S. aureus* PanK and *E. coli* PPAT and DPCK were already available in the laboratory of Prof. Erick Strauss and were expressed and purified from *E. coli* according to published procedures [2, 3].

2.1.8 Spectrophotometry and fluorimetry

All spectrophotometric and fluorometric readings were done in 96-well plates with a Varioskan multimode reader (Thermo Fisher Scientific Inc.)

2.1.9 High Performance Liquid Chromatography (HPLC)

HPLC was performed using the Opti-guard® 1 mm C18 guard column from Sigma-Aldrich, in combination with the Phenomenex Luna 5u C18(2) 100A 250 x 4.60 mm column from Separation Sciences. These were used on an Agilent/Hewlett Packard Series 1100 HPLC system from Cohesive Technologies and Agilent Technologies with in line FLD-fluorescence and UV detectors. HPLC-grade CHROMASOLV solvents (acetonitrile and methanol) used for HPLC were acquired from Sigma-Aldrich.

2.1.10 Liquid chromatography Mass Spectrometry (LCMS)

All LCMS and Electron Spray Ionisation Mass Spectrometry (ESI-MS) analyses were done at the Central Analytical Facility (CAF) at Stellenbosch University using a Waters 2690 Separations Module with a Waters 996 Photodiode Array Detector for the liquid chromatography separations, linked to a Waters Micromass Quattro mass spectrometer for mass analysis. The data were then analyzed using MassLynx software.

2.1.11 Nuclear Magnetic Resonance (NMR) Spectroscopy

All NMR analyses were done at CAF (Stellenbosch University) on a Varian VXR 300 MHz “R2-D2” NMR spectrometer and a Varian Inova 400 MHz NMR spectrometer. The data were then analyzed using MestReNova software. The analyses were run in deuterated solvents (chloroform-d, dimethyl sulfoxide-d6 and deuterium oxide) obtained from Sigma-Aldrich.
2.2 Methods

2.2.1 Preparation of erythrocytes for *P. falciparum* cultures and experiments

A fresh bag of A+ red blood cell concentrate (3-5 days old) was collected biweekly and stored at 4 °C. Before use of the blood in experiments or cultures, it was washed to ensure that any leukocytes or proteins still present were removed. This was performed by mixing equal parts blood and RPMI-1640 media, supplemented with 25 mM HEPES, 23.8 mM sodium bicarbonate, 11.1 mM glucose, 200 µM hypoxanthine (dissolved in 0.5 M NaOH), 24 µg/ml gentamycin and 0.6% m/v Albumax II serum, at pH 7.4 (hereafter called RPMI complete media), in a sterile 50 ml Falcon tube and centrifuging it at 1200 x g for 3 min. Supernatant was then discarded and the washing step repeated one more time. Thereafter 5 ml media was added to the erythrocytes and they were stored at 4 °C and used for no longer than 14 days.

2.2.2 Continuous *P. falciparum* cultures

*P. falciparum* 3D7 strain was used for all in vitro experiments containing malaria parasites. These parasites were maintained in a continuous culture, using similar methods to those described by Trager in 1976 [4]. Briefly, the *P. falciparum* parasites were cultured in A+ human erythrocytes (4% hematocrit) in RPMI complete media. The cultures were incubated in ventilated culture flasks (small cultures had a volume of 5 ml culture in a 60 ml culture flask and standard cultures had a volume of 50 ml culture in a 250 ml culture flask), in a low oxygen gas mixture environment (1% oxygen, 3% carbon dioxide, 96% nitrogen). Cultures were laid horizontally for maximum surface area exposure to the gas mixture, and incubated at 37 °C on a rotary shaker (50 rpm) to encourage synchronous cell culture growth. The media on the cultures was changed daily and cultures split on days when cultures were in trophozoite stage and parasitemia was high, to prevent cell death and stress caused by overpopulation. The cultures were maintained with parasitemia between 5-20%. To split, cultures were centrifuged at 750 x g for 3 min, the media removed, and of the pellet, 0.25 – 1 ml was transferred to a new falcon tube (dependent on parasitemia), followed by the addition of 1 – 1.75 ml washed blood, to maintain the 4% hematocrit in the culture. The rest of the volume was then made up to 50 ml by pre-incubated (37°C) RPMI complete media.

2.2.3 Synchronization of *P. falciparum* cultures

*P. falciparum in vitro* cultures do not stay in a synchronous phase like they do in vivo, and thus cultures need to be synchronized ~4 days before using cultures for experiments. Methods adapted from a published protocol by Lambros and Vanderberg [5] were used for this purpose. Briefly, the culture media was removed and the erythrocyte and parasite pellet treated with 10 volumes of 5% (m/v) filtered, prewarmed D-sorbitol at 37 °C for 10 min, homogenizing the suspension every 2-3 min for best synchronization. This process would lyse all parasites in trophozoite stage, thus only leaving parasites in ring stage.
2.2.4 Determination of parasitemia

Before cultures were split or experiments were started using *P. falciparum*, the percentage of parasite-infected erythrocytes (parasitemia) was determined. This was done manually by taking culture samples and making blood slides. Cells were fixed with methanol and the cells then stained with Giemsa working stain (10 ml 10x Giemsa stock in 90 ml 1x PBS buffer). The slides were then viewed under a light microscope with a 1000x magnification and the number of parasites (stained blue) divided by the number of red blood cells (not stained because they do not have DNA) calculated as percentage parasitemia.

2.2.5 Testing and treatment of cultures for Mycoplasma infection

Mycoplasma infections are common in cell cultures and thus *P. falciparum* cultures need to be tested regularly for Mycoplasma infections.

2.2.5.1 Testing for Mycoplasma infection

A few set of primers are needed to test for the presence of Mycoplasmas in *P. falciparum* cultures. These primers are 5' primers Myc51 (seq: CGCCTGAGTAGTACGTCGC), Myc52 (seq: CGCCTGAGTAGTACGTCGC), Myc53 (seq: TGCGTGGGTAGTACATTCGC), Myc54 (seq: TGCGTGGGTAGTACATTCGC), Myc55 (seq: CGCCTGGGTAGTACATTCGC, Myc56 seq: CGCCTGAGTAGTATGCTCGC, and 3' primers Myc31 (seq: GCGGTGTGTAAGACCCGA), Myc32 (seq: GCGGTGTGTAAGACCCGA) and Myc33 (seq: GCGGTGTGTAAGACCCGA). A primer mix containing 2 µM of each of the 9 primers was prepared.

When changing media in cultures (section 2.2.2) an aliquot of the old media was dispensed into a centrifuge tube after centrifugation of the blood culture. This supernatant was then heated to 95 °C for 15 min and then placed on ice for 5 min to cool. Once cooled, it was centrifuged in a microcentrifuge at top speed (16 606 x g) for 3 min and at least 50 µL of this supernatant collected. PCR reaction mixtures containing 2.5 µl 10x PCR reaction buffer, 1.75 µl 25 mM MgSO₄, 5 µl 2 mM dNTPs, 12.25 µl sterile MilliQ water from Novagen’s KOD Hot start DNA Polymerase Kit and 2.5 µl 2 µM primer mix per sample was made up. 24 µl of this PCR mix was then aliquoted into PCR tubes and 1 µl sample supernatant added. In addition to the sample from the blood culture, 2 negative controls (one containing no KOD polymerase and one containing no DNA template) were set up, as well as one positive control containing 17.1 pg Mycoplasma DNA. Once heated, 1 µl KOD polymerase also from Novagen’s KOD Hot start DNA Polymerase Kit was added to each sample. The PCR program ran a 2 min pre-cycle at 95 °C, followed by 40 cycles of 95 °C for 20 seconds, 52 °C for 10 seconds, 74 °C for 32 seconds and then finally ended on 4 °C. Once the PCR was complete, the DNA was run on a 1% agarose gel with TAE buffer, containing 5 µl Nancy-520 gel dye. The entire PCR samples with 5 µl loading buffer each were loaded onto the gel, along with 10 µl of the Universal Ladder (Kapa Biosystems) and the gel run for 65 min at 80 volts in TAE buffer and then imaged on a blue light trans-illuminator once completed.
2.2.5.2 Treatment of Mycoplasma infection in *P. falciparum* culture

If the *P. falciparum* cultures tested positive for Mycoplasma infection, parasitemia of the culture should be determined (section 2.2.4) and a 5 ml culture split into a 60 ml cell culture flask, containing 4% hematocrit and 2% parasitemia. This culture is then treated with 50 µl Mycoplasma removal agent (MRA). The media was changed daily (section 2.2.2) and 50 µl MRA added to the culture every time media is changed. The culture was treated for a week before it was tested again for the presence of Mycoplasma (section 2.2.5.1), and only if it tests negative is the culture increased to a 50 ml culture again. Two weeks after the Mycoplasma removal was successful, the cultures were tested a second time for the presence of Mycoplasma (section 2.2.5.1) to confirm that all Mycoplasmas were removed from the culture during treatment.

2.2.6 *P. falciparum* parasite isolation and lysate preparation

For experiments that made use of *P. falciparum* cell lysates, cultures were grown to a high parasitemia (~10-20%) and parasites isolated on a day when they were in trophozoite stage. For isolation, the *Plasmodium* infected erythrocytes, still suspended in media, were subjected to 0.05% (m/v) saponin. Saponin is a plant-derived compound, attacking the cholesterol of cell membranes. Since the erythrocyte cells have cholesterol in their cellular membranes where the parasite cell membranes lack cholesterol, parasite cells remain intact, while pores form in the erythrocyte cell membranes, thus lysing them. The treated cultures are then immediately centrifuged at 2000 x g for 8 min at 4 °C after which the supernatant was removed and discarded. The remaining cell pellet, consisting of predominantly parasite cells, was washed with malaria saline (125 mM sodium chloride, 25 mM HEPES, 1 mM magnesium chloride, 5 mM potassium chloride, and 20 mM glucose) to remove any cell debris still in the pellet. The mixture was centrifuged for 30 seconds at 16 606 x g at 4 °C and the supernatant removed. This step was repeated a further 3-4 times until the supernatant was clear after the centrifugation step.

Once isolated, the parasites in the pellet were resuspended in 500 µl malaria saline. Of this, 20 µl was transferred to another centrifuge tube and diluted 50x with malaria saline; this was used for the purpose of cell counts (Section 2.2.6). The remaining 480 µl of parasite suspension was centrifuged one last time for 30 seconds at 16 606 x g at 4 °C, the supernatant removed, and the pellet resuspended in cold lysis solution (10 mM Tris, pH 7.4). The parasite cells were then lysed by trituration (~20x) through a 25-gauge needle. The lysed suspension was then centrifuged 3 times for 30 min at 16 606 x g at 4 °C, each time transferring the supernatant to a new centrifuge tube and discarding the pellet. The lysate was stored at -20 °C until used for experiments.

2.2.7 Cell Counts for lysate preparation

The 50x dilution of the parasite cells before lysis was used to determine the number of trophozoites by manually counting them on a Neubauer hemocytometer at a 100x magnification under a light microscope to determine the cell counts in cells/ml.
2.2.8 Custom preparation of Pantothenate-free media

Since standard commercially available media contains 1 µM pantothenate, and pantothenate free complete media was needed for experiments where pantothenate had to be excluded, two sources of pantothenate free media were used for experiments. First, pantothenate free complete media was prepared in house by using component information and concentrations of RPMI-1640 media available from the Sigma-Aldrich website [6]. A 50x RPMI-1640 amino acid mix was commercially available which was used, but further, stocks were made up of the other components: (1) a 20x inorganic salt stock containing calcium citrate (8.47 mM), magnesium sulfate (8.12 mM), potassium chloride (107.31 mM), sodium chloride (2.053 M), and sodium phosphate dibasic (112.71 mM), (2) a 2500x vitamin mix containing D-biotin (2.05 mM), choline chloride (53.72 mM), folic acid (5.66 mM), myo-inositol (485.68 mM), niacinamide (20.47 mM), p-aminobenzoic acid (18.23 mM), pyridoxine (12.16 mM), riboflavin (1.33 mM), thiamine (7.41 mM), and vitamin B\textsubscript{12} (first made up as a 250 000x stock and then added to the mixture) (9.00 nM), and separate stocks of (3) D-glucose (22.2 mM), (4) glutathione (500x, 1.63 mM), (5) phenol red dissolved in ethanol (250x, 3.52 mM), and (6) L-glutamine (250x, 513.21 mM). While most of the stocks could be stored at 4 °C and reused whenever fresh media was made, glutathione and L-glutamine stocks had to be made up on the day of the experiment. Once all the stocks were added together it was supplemented with 25 mM HEPES, 23.81 mM sodium bicarbonate, 11.1 mM glucose, 200 µM hypoxanthine (dissolved in 0.5M NaOH), 24 µg/ml gentamycin and 0.6% (m/v) Albumax II serum, and the pH adjusted to 7.4, after which the pan-free media was filtered with a 0.2µ syringe filter under sterile conditions and stored at 4 °C till needed.

Later we sourced commercially available custom-made pantothenate free media (Custom RPMI medium 1640, without pantothenate, with Glutamax) from AthenaES (USA). This media did not require the additional addition of L-glutamine, since it contains Glutamax.

2.2.9 *P. falciparum* survival without pantothenate present

Parasites were cultured as described in section 2.2.2. Once parasites were in the synchronized ring stage, 5 ml cultures in 60 ml culture flasks with 2% hematocrit and 1% parasitemia were maintained in custom made pantothenate free RPMI-1640 media supplemented with 25 mM HEPES, 23.81 mM sodium bicarbonate, 11.1 mM glucose, 200 µM hypoxanthine (dissolved in 0.5M NaOH), 24 µg/ml gentamycin and 0.6% (m/v) Albumax II serum, with pH 7.4 (here on called Pan-free complete media). Infected erythrocytes (2% hematocrit, 1% parasitemia) in Pan-free complete media supplemented with 1 µM pantothenate were used as a control to estimate the growth pattern when the parasites were growing under normal conditions. These cultures were also gassed with a low oxygen gas mixture (1% oxygen, 3% carbon dioxide, 96% nitrogen) and incubated at 37 °C on a rotary shaker (50 rpm) to encourage synchronous cell culture growth. The media in these cultures were changed every day (Section 2.2.2) for 8 days, and each day a blood slide made and parasitemia determined (section 2.2.4). In addition, an aliquot was taken and frozen at -20 °C for a SYBR Safe and Malstat
assay analysis (sections 2.2.8 and 2.2.9). The experiment was performed twice as separate experiments each performed in triplicate.

2.2.10 P. falciparum growth assays for minimum pantothenate requirement

Parasites were cultured as described in section 2.2.2. Once parasites were in the ring stage, the assay was set up in a sterile 96-well cell culture plate with a 2% hematocrit and 1% parasitemia in Pan-free complete media. A two-times serial dilution of the pantothenate-containing wells was done in triplicate, with the final pantothenate concentration ranging between 1 µM and 1.9 pM, with a final volume of 200 µl per well. Infected erythrocytes (2% hematocrit, 1% parasitemia) in Pan-free complete media supplemented with 1 µM pantothenate were used as a control to estimate 100% parasite growth. Uninfected erythrocytes with a hematocrit of 2% in RPMI complete media, as well as Plasmodium infected erythrocytes with 1 µM chloroquine in RPMI complete media were both used as a negative control (to subtract background), with the uninfected erythrocytes control serving as a check that the parasites were not becoming chloroquine resistant. In addition to this, parasite infected erythrocytes with a hematocrit of 2% and 1% parasitemia in Pan-free complete media served as an extra control to confirm whether the parasites actually died within the incubation period with no pantothenate present. Wells not being used were filled with 200 µl media. These plates were then incubated at 37 °C for 2 parasite life-cycles (96 hours) inside an air-tight desiccator that had been flushed with a low oxygen gas mixture (1% oxygen, 3% carbon dioxide, 96% nitrogen). These assays were then analyzed by means of the SYBR Safe assay (see section 2.2.12) as well as the Malstat assay (See section 2.2.13).

2.2.11 SYBR Safe assay

For all SYBR Safe assay analyses used to determine parasite viability, 100 µl SYBR Safe solution, which consists of 2 µl SYBR Safe in 10 ml lysis buffer (20 mM Tris, 5 mM EDTA, 0.008% (m/v) saponin, 0.08% (v/v) TritonX-100, pH 7.5), was aliquoted into each well of a black 96-well plate. From the original assay 96-well plate, 100 µl of the homogenous sample of each well was transferred to the SYBR Safe solution and mixed gently. Fluorescence was measured on a Varioskan multimode reader (Thermo Fisher Scientific Inc.) with excitation and emission wavelengths at 490 nm and 520 nm, respectively.

2.2.12 Malstat assay analysis

Before the Malstat analysis was done, culture samples were subjected to freeze-thaw cycles (3x) to lyse all the red blood cells. Thereafter, to a clear 96-well plate, 100 µl Malstat reagent (0.2% (v/v) Triton X-100, 220 mM lactic acid, 41.9 mM Tris, 0.17 mM 3-acetylpyridine adenine dinucleotide (APAD), pH 9.0), 25µl NBT/PES solution (1.96 mM nitro blue tetrazolium, 0.24 mM phenazine ethosulphate) and 15 µl homogenous blood culture sample were added. Once mixed, the plates were incubated in the dark (the reagents are light sensitive) for 30 min before they were spectrophotometrically analyzed at 620 nm with a Varioskan multimode reader (Thermo Fisher Scientific Inc.).
2.2.13 Data analysis for Malstat and SYBR Safe assays

For SYBR Safe and Malstat assays data were collected in triplicate. The average of the blank was thus subtracted from all the data points and the average of each point plotted as a percentage of total growth (compared to the 100% control). Two repeats were done of each experiment, and error calculated (standard error of the mean) by the standard deviation of the average of the repeats, divided by the number of repeats (in this case 2).

2.2.14 Absorption profile of $N$-PE-$\alpha$-Me-PanAm

The absorption profile of 500 µM $N$-PE-$\alpha$-Me-PanAm (dissolved in water) was recorded between 200 and 400 nm on a Varioskan spectrophotometer.

2.2.15 Biosynthesis of CoA analogues with $P$. falciparum cell lysate

Reaction mixtures (250 µl final volume) containing $P$. falciparum cell lysates (section 2.2.6) were used to carry out the conversion of the $N$-PE-$\alpha$-Me-PanAm, the substrate, to its corresponding CoA antimetabolites. Cell lysate stored in 10 mM HEPES buffer (pH 7.6) had a concentration of ~1.975 x 10^9 cells/ml and lysates stored in 10 mM Tris (pH 7.6) had a concentration of ~3.128 x 10^9 cells/ml. The reactions consisted of 16.5 mM ATP (pH 7), 10 mM MgCl₂, 125 µl cell lysate in HEPES or Tris buffer, 50 mM HEPES or Tris (pH 7.6) and 5 mM $\alpha$-Me-N-PE-PanAm. The reactions were incubated overnight at 37 °C before analyzing via HPLC (See section 2.2.21.2).

2.2.16 $N$-PE-$\alpha$-Me-PanAm metabolism in $P$. falciparum cultures

$P$. falciparum cultures (8 x 50 ml) were cultured to a parasitemia of 10-15% (section 2.2.2) and treated in the ring stage with 52 nM of the racemic mixture of $N$-PE-$\alpha$-Me-PanAm. Another set of $P$. falciparum cultures (8 x 50 ml) were cultured with the same parasitemia, however these cultures were not treated with $N$-PE-$\alpha$-Me-PanAm, but served as a control. All cultures were incubated for 24 hours, after which the parasites were isolated and lysed (section 2.2.6). The lysate was then heated to 95 °C for 15 min and kept on ice for 5 min before centrifugation at 16 606 x g to pellet the proteins and enzymes. The supernatant was then analyzed by LC-MS (see section 2.2.20 for conditions).

2.2.17 Conversion of $N$-PE-$\alpha$-Me-PanAm to antimetabolites by bacterial enzymes for LC-MS method validation

An enzymatic reaction, using $N$-PE-$\alpha$-Me-PanAm as a substrate, was set up using active isolated bacterial enzymes from $S$. aureus and $E$. coli. The reactions, with a volume of 250 µl each, were set up by adding 60 mM HEPES (pH 8.0), 16.5 mM ATP, 10 mM MgCl₂, and 10 mM $N$-PE-$\alpha$-Me-PanAm followed by the addition of 51 µg of each enzyme, SaPanK, EcPPAT and EcDPCK. The reaction mixtures were incubated for 24 hours at 37 °C after which they were diluted with 250 µl 10 mM ammonium acetate, pH 6.0. Proteins were then removed by heat precipitation at 95 °C for 5 min, followed by 2 x 30 min centrifugation at 16 606 x g to pellet the proteins and the supernatant transferred to a new centrifuge tube each time. The supernatant was then analyzed by LC-MS (see section 2.2.20 for conditions).
2.2.18 Biosynthesis of CoA and intermediates with *P. falciparum* cell lysate for LC-MS analysis

To check whether the *P. falciparum* lysate enzymes were active, biosynthetic reactions were set up in lysates prepared in Tris and HEPES buffer respectively using CoA biosynthesis natural substrates, pantetheine and dephosphoCoA. Pantetheine was prepared by reducing, 25 mM pantetheine (commercially available disulfide of pantetheine) in 37.5 mM TCEP for 10 min at room temperature. 5 mM substrate (either pantetheine or dephosphoCoA) was then incubated in the presence of 16.5 mM ATP, 10 mM MgCl$_2$, 125 µl cell lysate in HEPES (10 mM, pH 7.6) and 2.5 mM HEPES buffer (pH 7.6) in a final volume of 250 µl. Another set of reactions were set up, replacing the cell lysates stored in HEPES with the lysate stored in 10 mM Tris (pH 7.6) and the HEPES buffer with 2.5 mM Tris (pH 7.6). All the reactions were incubated for 24 hours at 37 °C after which proteins were then removed by heat precipitation at 95 °C for 5 min, followed by 2 x 30 min centrifugation at 16 606 x g to pellet the proteins and the supernatant carried over to a new centrifuge tube each time. The supernatant was then analyzed by LC-MS (see section 2.2.19 for conditions).

2.2.19 LC-MS conditions for analysis of biosynthesized CoA analogues

LC-MS analysis was performed at CAF (Stellenbosch University) as described previously [7]. LC-MS analysis was performed with 10 mM ammonium acetate, pH 6.5 (solution A) and 1% formic acid in acetonitrile (solution B) on a Waters Synapt G2 system on a Waters HSS C18 column (2.1 x 150 mm) with a flow rate of 0.3 ml/min. The column was first equilibrated in 98% solution A and 2% solution B. This was followed by elution with 98% solution A (0-1 min, isocratic), a linear gradient increasing solution B to 20% (1-10 min), a linear gradient increasing solution B to 100% (10-15 min), an isocratic elution at 100% solution B (15-16 min) and a linear gradient returning to 98% solution A (16-20 min, isocratic). Electrospray Ionization (ESI) was applied in the positive mode and negative modes at a capillary voltage of 2.5 kV, cone voltage of 15 V, desolvation temperature of 275 °C and desolvation gas setting of 650 L/h, with the remaining settings optimized for optimal sensitivity. The instrument was calibrated with sodium formate, and leucine enkaphelin was used as lock mass for accurate mass determinations. The MS acquisition method consisted of a low energy function at a trap voltage of 6 V and a high energy function where the trap collision energy was ramped from 15 to 60 V to generate fragmentation data (MSE).

2.2.20 Derivatization of thiol containing substrates and products with CPM

Cell lysate reactions prepared as in section 2.2.19 were derivatized with 7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) before HPLC analysis. The reactions were stopped after 24 hour incubation step by heat precipitation of the *P. falciparum* cell lysate proteins at 95 °C for 5 min, followed by centrifugation twice for 30 min at 16 606 x g. The supernatant was then filtered with 0.2 gauge syringe filter and the supernatant CPM derivatized. This was done by setting up 140 µl derivatization reactions, containing 44.62 µl lysate reaction supernatant, 67.6 nM TCEP, 3.62 mM Tris (pH 7.6), 1.45 mM, MgCl$_2$ and 1.45 mM KCl. Subsequently, reactions were allowed to incubate
for 10 min at room temperature before the addition of 4.5 nM CPM. This mixture was incubated overnight at room temperature before analysis via HPLC.

2.2.21 HPLC analyses

2.2.21.1 HPLC analysis of CPM derived CoA metabolites and intermediates
Analysis of CPM derivatized CoA analogues and intermediates was done using a previously published method [2]. Briefly, once the metabolites were labelled with CPM (section 2.2.20), they were injected (5 µl injection) onto an Agilent Series 1200 HPLC system on a 5µM SUPELCOSIL™ LC-DP column (4.6 x 250 mm) with a flow rate of 1.0 ml/min. HPLC analyses were performed with 50 mM potassium phosphate (pH 6.8) (solution A), 60% acetonitrile (solution B) and 100% acetonitrile (solution C). The column was first equilibrated in 50% solution A and 50% solution B. This was followed by elution with 50% solution A and 50% solution B (0-5 min, isocratic), a linear gradient increasing solution B to 60% (5-25 min), a linear gradient increasing solution C to 60%, keeping solution A at 40% (25-26 min), an isocratic elution at 40% solution A/60% solution C (26-33 min) and a linear elution increasing solution C to 90%, with solution A decreasing to 10% (33-36 min). This was followed by an isocratic elution at 10% solution A/90% solution C (36-42 min), a linear gradient returning to 50% solution A/50% solution C (42-52 min), ending with an isocratic elution at 50% solution A/50% solution C (52-57 min).

2.2.21.2 HPLC analysis of the N-PE-α-Me-PanAm and its CoA antimetabolites
Analysis of N-PE-α-Me-PanAm and its corresponding CoA antimetabolites was done using the following method: samples were injected (35 µl injection) onto an Agilent Series 1100 HPLC system on a Phenomenix Luna C18 100A 250 x 4.60 mm column with a flow rate of 1.0 ml/min. HPLC analysis was performed with 10 mM ammonium acetate buffer, pH 5.5 (solution A) and acetonitrile (solution B). The column was first equilibrated in 95% solution A and 5% solution B. This was followed by elution with 95% solution A (0-5 min, isocratic), a linear gradient increasing solution B to 20% (5-6 min), a linear gradient increasing solution B to 40% (6-10 min), an isocratic elution at 40% solution B and 60% solution A (10-15 min) a linear gradient increasing solution B to 60% (15-16 min), and an isocratic elution at solution B to 60% (16-20 min). UV detection of products was done at 220 nm and 254 nm.

2.2.22 Synthesis of tricyclic methylthiophenyl propanamide

2.2.22.1 Compound 1
2-Chloro-3-pyrimadinecarbonitrile (0.660 g, 4.76 mmol) was dissolved in 10 ml ethanol, followed by the addition of 5 equivalents of hydrazine hydrate (1.49 ml, 23.8 mmol). This was refluxed overnight under nitrogen gas, resulting in a bright yellow reaction mixture which was concentrated in vacuo. Thereafter, 5 ml water was added and the resulting mixture was stirred at room temperature for 1 hour. The suspension was then filtered and the filtrate dried in vacuo to yield a yellow residue (0.890 g, 66% yield). The residue was used in the next step without further purification. ¹H NMR (300 MHz, DMSO-d6) δ 11.91
(s, 1H), 8.33 (dd, J = 4.6, 1.6 Hz, 1H), 8.11 (dd, J = 7.9, 4.6 Hz, 1H), 6.94 (dd, J = 7.9, 4.6 Hz, 1H),
5.54 (s, 2H), 3.41-3.17 (m, 2H). \(^1\)H NMR is consistent with literature data [8].

2.2.21.2 Compound 2

Compound 1 (0.948 g, 5.56 mmol) was dissolved in 8 ml ethanol and methyl 4-acetyl-5-oxohexanoate (1.85 ml, 10.6 mmol) was added. The mixture was refluxed overnight under nitrogen, resulting in a clear orange mixture. After cooling, the mixture was concentrated \(\textit{in vacuo}\), dissolved in ethyl acetate and dried \(\textit{in vacuo}\) again. This was repeated three times. The resulting residue was resuspended in 10 ml diethyl ether and the final product filtered, and the precipitate washed (3 x 10 ml) with diethyl ether. The final product was obtained in 49% yield (1.54 g). \(^1\)H NMR (300 MHz, chloroform-\(d\)) \(\delta\) 8.91 (dd, J = 4.4, 1.8 Hz 1H), 8.61 (dd, J = 8.1, 1.8 Hz, 1H), 7.20 (dd, J = 8.1, 4.4 Hz, 1H), 3.74 (s, 3H), 3.24 (dd, J = 9.2, 7.1 Hz, 2H), 3.05 (s, 3H), 2.81 (s, 3H), 2.66 – 2.61 (m, 2H). \(^1\)H NMR is consistent with literature data [8].

2.2.22.3 Compound 3

Compound 2 (0.050 g, 0.18 mmol) was mixed with 3 equivalents 1M aq. NaOH (1.48 ml) and 5 ml tetrahydrofuran (THF). This was left to stir overnight at room temperature. After the reaction was deemed complete by TLC (5:1 ethyl acetate:hexane), the THF was removed \(\textit{in vacuo}\) and the remaining residue dissolved in 5 ml water. It was then washed three times in a separating funnel, with 5 ml ethyl acetate and the product freeze dried to yield a white powder (0.305 g; >99% yield). \(^1\)H NMR (300 MHz, D\(_2\)O) \(\delta\) 8.57 (dd, J = 4.5, 0.9 Hz, 1H), 8.13 (d, J = 8.1 Hz, 1H), 7.11 (dd, J = 8.1, 4.5 Hz, 1H), 2.92 (dd, J = 9.8, 6.9 Hz, 2H), 2.67 (s, 3H), 2.55 (s, 3H), 2.37 (dd, J = 9.7, 6.9 Hz, 2H). \(^1\)H NMR is consistent with literature data [8].

2.2.22.4 Compound 5

Method 1:

Compound 3 (0.100 g, 0.370 mmol) was added to 2.1 equivalents of thionyl chloride (0.059 ml, 0.41 mmol) in 3 ml dimethylformamide (DMF). This mixture was stirred overnight while heating from room temperature to 70 °C under nitrogen. Product formation was determined by TLC (16:2:1:1 ethyl acetate:butanol:acetic acid:water, Rf value 0.45), however no visible change was seen on TLC. It was thus followed by the addition of 2 equivalents of triethylamine (0.061 ml, 0.40 mmol) and stirred it at room temperature for 4 hours under nitrogen, after which the DMF and excess amines were removed \(\textit{in vacuo}\), resulting in a residue (0.152 g, <99% yield).

Method 2:

Compound 3 (0.150 g, 0.560 mmol) mixed with 12.5 equivalents of thionyl chloride (0.350 ml). This was cooled to 0 °C, after which 2.5 ml dry DMF was added drop-wise and the mixture stirred for 5 min after which the reaction was refluxed overnight. Product formation was determined by TLC (5% methanol/DCM, Rf value 0.66). The reaction was deemed complete when no starting reagent was visible on TLC. The DMF and excess thionyl chloride were removed \(\textit{in vacuo}\), followed by co-
evaporation with 10 ml toluene and 10 ml diethyl ether, respectively. This resulted in a brown residue (0.248 g, <99% yield). No further purification was done and the resulting residue was used as is in the next step.

### 2.2.2 Compound 4

#### Method 1: HBTU coupling

Compound 4 (0.153 g, 0.106 mmol) was added to 1.3 ml dry DMF to form a suspension. Subsequently, 2 equivalents $N,N,N',N'$-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (0.074 g, 0.21 mmol) and 1.5 equivalents (31 µl, 0.16 mmol) triethylamine were added and allowed to stir for 5 min. Thereafter, 3.6 equivalents 3-(methylthio)aniline (65 µl, 0.38 mmol) were added to the reaction mixture and stirred for 4 hours at room temperature under nitrogen gas. The reaction was monitored by TLC (5% methanol/dichloromethane) and subsequently the reaction mixture was concentrated in vacuo (0.980 g crude). The resulting residue was purified on a silica column (5 % methanol/dichloromethane, 20 % methanol/dichloromethane) and the fractions analyzed on TLCs (5 % methanol/dichloromethane). The desired fractions (Rf value 0.41) were pooled and dried (<5 mg, therefore enough to do NMR analysis but not to pursue further). $^1$H NMR (300 MHz, DMSO-d6) $\delta$ 9.99 (s, 1H), 8.81 (dd, J = 3.2 Hz 1H), 8.57 (ddd, J = 8.1, 1.8, 0.6 Hz, 1H), 7.53 (t, J = 1.8 Hz, 1H), 7.31-7.28 (m, 1H), 7.24 – 7.19 (m, 2H), 6.91 (ddd, J = 7.7, 1.8, 1.1 Hz, 1H), 3.32 – 3.02 (m, 4H), 2.92 (s, 3H), 2.73 (s, 3H), 2.68 – 2.57 (m, 2H), 2.42 (s, 1H). $^1$H NMR is consistent with literature data [8].

In an alternative work-up, after the reaction was complete, the resulting residue was dissolved in 10 ml ethyl acetate and washed with 5 % (m/v) citric acid (2 x 5 ml), 1 M NaHCO$_3$ (2 x 5 ml), and brine (1 x 5 ml). The organic layer was dried over NaSO$_4$, filtered and concentrated in vacuo. The crude mixture was purified by flash column chromatography (10% ethyl acetate/dichloromethane followed by 5 % methanol/dichloromethane). The partially purified product was dried in vacuo to yield a white solid (<5 mg). However, NMR spectroscopy indicated this was not the desired product. Unfortunately the NMR spectra was inconclusive to identify peaks present.

#### Method 2: EDC Coupling

3-(Methylthio)aniline (21 µl, 0.17 mmol) was added to 2.5 ml dry DMF and this mixture cooled to 0 °C. $N,N$-diisopropylethylamine (DIPEA) (30 µl, 0.17 mmol) was added dropwise to this cooled mixture as not to raise the temperature above 5 °C. Hydroxybenzotriazole (HOBt) (0.005 g, 3 nmol), compound 3 (0.050 g, 0.19 mmol) and $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide hydrochloride (EDC) (0.026 g, 0.68 mmol) were added. This mixture was then stirred overnight at room temperature. Product formation was determined by TLC (5% methanol/dichloromethane, Rf...
value 0.59) but this showed the coupling to be unsuccessful and thus the method was not pursued further.

**Method 3: B(OCH$_2$CF$_3$)$_3$ amidation**

B$_2$O$_3$ (12.0 g, 172 mmol) and 2,2,2-trifluoroethanol (25 ml, 347 mmol) were refluxed overnight, after which the mixture was filtered to remove excess boric anhydride and the filtrate purified by distillation (80 °C under a vacuum). The resulting B(OCH$_2$CF$_3$)$_3$ could then be stored at room temperature for up to 4 months.

Next, the amidation reaction was performed by refluxing the compound 3 (0.104 g, 0.385 mmol) with 2 equivalents of B(OCH$_2$CF$_3$)$_3$ (0.048 g, 0.77 mmol), 1 equivalent 3-(methylthio)alanine (5 µl, 0.4 mmol) and 2 ml acetonitrile overnight. After completion of the reaction, the product was diluted with ethyl acetate (3 ml) and water (0.5 ml). Amberlyst A-26(OH), Amberlyst 15 and Amberlite IRA743 resins were added (50 ml dry volume of each). This resulting slurry was stirred for 30 min and magnesium sulphate added to remove excess water. The resulting solid was washed 3 times with 5 ml ethyl acetate and filtered, then the filtrate concentrated in vacuo. TLCs were run in 5% methanol/dichloromethane and 1:9 hexane/ethylacetate but many impurities were present and suggested co-elution if purified on a silica column, thus the method was abandoned.

**Method 4: Amidation using acid chloride derivative**

Compound 4 (0.062 mg, 0.22 mmol) was dissolved in 2.5 ml dichloromethane (DCM) and divided into 4 round bottom flasks. To each flask, 4 equivalents of the amine (0.56 mmol) in 500 µl 4-picoline (5.14 mmol) were added separately to each flask. The amines coupled were 3-(methylthio)aniline, 3-fluoroaniline, 3-(trifluoromethyl)aniline and 4’-aminoacetanilide. After stirring for 4 hours under nitrogen gas, the mixtures were concentrate in vacuo. The reactions were followed by TLC (5% methanol/dichloromethane) to determine which reaction was the most successful. The reaction of 3-fluoroaniline was chosen for purification by silica plug using 5%-10% methanol/DCM to yield a partially purified product (0.630 g – not dry). $^1$H NMR (400 MHz, DMSO-d6) showed all peaks present that were present in the NMR spectrum of the HBTU coupling product, however many impurities resulted in additional peaks.
2.3 References


Chapter 3: Results and Discussion

3.1 Introduction

Most of the efforts to curb the malaria-caused death toll are aimed at prevention. However treatment of this disease is still critical, especially now that *Plasmodium* has exhibited resistance to all current drugs used for the treatment of malaria [1]. New antimalarial drugs with novel modes of action are therefore still desperately needed. In order to develop new drugs, alternative drug targets need to be identified. This is a challenge because limited knowledge is available about the metabolic processes of *Plasmodium*, and thus even an increased knowledge of such processes and parasite biology could aid in the discovery of novel drug targets.

The CoA biosynthetic pathway is one of the pathways which is crucial to the survival of the parasite [2] and many attempts are currently being made to target this pathway [3-7]. Presently the most potent inhibitors of the CoA biosynthesis pathway are PanAms [4], however the specific mode of action of these compounds are still unknown. The purpose of this project was therefore to better understand parasite biology in terms of CoA biosynthesis and to pinpoint how biological processes are affected within the parasite. This will aid future studies to determine the exact mode of action for the PanAms.

3.2 Results

3.2.1 Establishing the minimum extracellular pantothenate needed for parasite survival

PanAms target CoA biosynthesis and/or utilization in *P. falciparum*, however the critical amount of CoA needed for parasite survival is still unknown. By determining the critical amount of CoA the parasite needs for survival, we can determine the threshold needed for PanAms to interfere with CoA biosynthesis. However this is not an easy determination to make since parasites obtain CoA from pantothenate received from the host red blood cell that in turn obtains pantothenate from extracellular sources. Based on current data, the amount of pantothenate available to *P. falciparum* in the host blood stream is ~2.5 µM [8]. *P. falciparum* is cultured in the presence of 1 µM pantothenate, which correlates well with levels available in the human host. These culture conditions are similar to *in vivo* conditions, thus allowing us to perform experiments to determine the minimum required pantothenate needed for the survival of blood stage *P. falciparum* parasites.

3.2.1.1 Parasite survival without pantothenate present

In order to determine the minimum amount of pantothenate needed for survival, the first objective was to determine how long *P. falciparum 3D7* parasites cultured in the laboratory could survive without pantothenate present in the culturing media, in order to determine the timeframe for the pantothenate requirement assay. For this purpose the parasites were cultured in pantothenate-free
media. Since all commercially available RPMI-1640 media contains pantothenate, we prepared RPMI-media without any pantothenate present in-house. To achieve this, the component information of RPMI-1640 media available from Sigma-Aldrich was used as reference [9]. See section 2.2.8 for details.

After the successful preparation of custom-made pantothenate-free media (Pan-free complete media), *P. falciparum* parasites were cultured in this media for six days to observe their ability to grow without pantothenate present. Parasite survival was determined by using a Malstat assay which measures a colourimetric change caused by the production of pyruvate from lactate by lactate dehydrogenase enzymes, which leads to the reduction of NTB, which causes a colour change from yellow to blue. Control experiments were also performed in parallel. The first control was *P. falciparum* parasites cultivated in Pan-free complete media supplemented with 1 μM pantothenate (comparable to commercial complete RPMI-media) that was used to determine normal parasite growth. In addition a culture containing 1 μM chloroquine was used as positive control for no parasite growth.

Surprisingly, all cultures (including the negative control that contained sufficient pantothenate) showed a decline in parasite growth over six days (Figure 3.1A). We therefore concluded that the quality of some of the components sourced for media preparation was not suitable for *P. falciparum* *in vitro* culture. As an alternative, commercial Pan-free media was therefore sourced from an American-based company, AthenaES, which had the same components as the RPMI-1640 media, except for pantothenate. This media was used to repeat the above experiment of the controls to determine whether the media prepared in-house was the problem. Here, the controls were incubated for seven days after which the Malstat assay was used to determine parasite survival of each control (Figure 3.1B)
Figure 3.1: (A) Parasite survival assay controls over 6 days in a 96-well microtiter plate, determined by use of a Malstat assay. The symbols in red indicate parasite-infected erythrocytes in complete media, the symbols in blue indicate the negative control where parasite-infected erythrocytes are cultured in media containing chloroquine, and the yellow symbols indicate parasite-infected erythrocytes cultures in Pan-free media. Data are the average of one experiment performed in duplicate with errors bars indicating SD. (B) Malstat Assay determination of parasite survival (assay controls) of parasites cultured in a 96-well microtiter plate, in commercially bought custom made Pan-free media (yellow bar) and complete media (red bar) after a period of seven days. Data are the average of one experiment performed in triplicate with errors bars indicating SD.

In this experiment the iRBCs in complete media showed the highest growth (assumed to be 100% growth) while the iRBCs with chloroquine in complete media had little to no growth and could be subtracted as a blank. This is an indication that under normal culture condition in the commercially sourced media, parasites proliferate normally. However, the iRBCs were still showing >90% growth even after 7 days in Pan-free complete media, which indicated that there were alternative factors that interfered with the assay. Confident that the commercially bought media was of good quality, we set out to determine how long our parasites could survive in Pan-free complete media. Consequently, 5 ml *P. falciparum* cultures were cultivated over time, in both Pan-free and complete media to determine parasite survival in a pantothenate-free environment in comparison to when there was 1 μM pantothenate present. Three methods were used to determine parasite survival in order to compare the results from different assays: 1) Giemsa-stained blood slides which allows manual counting of viable parasites, 2) a SYBR Safe assay which relies on SYBR safe dye to fluorescently
stain parasite DNA for fluorometric measurement, and 3) the Malstat assay that measures lactate dehydrogenase activity present in parasites.

**Figure 3.2**: Parasite survival determined over time in Pan-free media using three different methods, starting with a 2% hematocrit and 1% parasitemia. Culture samples analyzed by (A) manual counting of cells on a blood slide, (B) SYBR Safe assay measuring fluorescence of SYBR safe dyed parasite DNA, and (C) Malstat assay measuring the colourimetric change of NTB from yellow to blue. Red circles indicate parasite-infected erythrocytes cultures in RPMI complete media and yellow symbols indicate parasite-infected erythrocytes cultures in Pan-free media. Data are the average of two independent experiments performed in triplicate with errors bars indicating range/2.
Although the different methods of data collection resulted in different curve shapes (Figure 3.2), there is still a general correlation for parasite survival between the different methods used to determine survival. All cultures were started with a 1% parasitemia for both cultures in Pan-free complete media and RPMI-complete media, however the parasites in the complete media proliferated at a much faster rate than those in the Pan-free media as expected. All three assays indicated maximum parasitemia in the RPMI-complete media between days 5 and 6, before rapidly decreasing again. For the parasites grown in Pan-free complete media, the parasitemia first increased slightly after ~3-4 days, before decreasing again, reaching zero after eight days.

Other groups have performed similar experiments in Pan-free complete media. Saliba et al. used a hypoxanthine assay in order to determine parasite survival in the absence of pantothenate [1]. This assay measures the level of radioactivity of the $[^3H]$-hypoxanthine incorporated into nucleic acids of the parasites when they proliferate, therefore the higher the level of radioactivity, the more parasites are present. They performed the experiments over 96 hours in 96-well microtiter plates at 1% parasitemia and 1% hematocrit, in RPMI-1640 media that was devoid of vitamins, supplemented with 25 mM sodium bicarbonate, 25 mM HEPES, 2.4 µM hypoxanthine, 24 µg/mL gentamicin, 11 mM glucose and 6 g/L Albumax serum. After the 96 hour incubation without pantothenate, the parasite proliferation was determined by measuring the $[^3H]$hypoxanthine incorporation. In these experiments, the parasites only survived approximately 48 hours in the absence of pantothenate. Another study investigated the *P. falciparum* pantothenate transporter and its importance to parasite survival [10]. *P. falciparum* parasites were cultured in the absence of pantothenate, starting with a 2% hematocrit and a 1% parasitemia and parasite survival was determined daily by manual counting of parasitemia. In this study, the parasites only survived for three days in the absence of pantothenate. It is evident in our results, even when using a variety of assay methods, that the apparent time period that parasites survive without pantothenate is much longer than found in these studies.

### 3.2.1.2 The minimum amount of extracellular pantothenate necessary for *P. falciparum* survival

In order to determine the minimum extracellular pantothenate needed by parasites to survive, assays were set up in a 96-well microtiter plate, where parasites were incubated in varying concentrations of pantothenate, between 1 µM (the concentration in standard RPMI-1640 culture media) and 1.907 pM by means of a two-fold serial dilution. These plates were then incubated for 96 hours before analyzing the parasite survival at each concentration by means of the SYBR Safe assay. The growth seemed to stay constant at 70% in media containing between 1 µM and 7.9 nM pantothenate compared to standard RPMI-complete media used for culturing, before the parasite survival started to be affected (Figure 3.3). From 7.9 nM, the parasite survival drops, however ~25%
of the parasites still survived at very low concentrations of pantothenate present. These results align with survival experiments in section 3.2.1 where parasites are able to survive on no or very low levels of pantothenate present after 96 hours.

![Graph showing parasite survival vs. pantothenate concentration](image)

Figure 3.3: Parasite survival in varying concentrations of pantothenate after 96 hours. A serial dilution was done of the pantothenate between a concentration of 1 µM and 1.907 pM. Data were normalized to RPMI-complete media as which was considered 100% growth. Data are the average of one experiment performed in triplicate with errors bars indicating SD.

### 3.2.1.3 The influence of Mycoplasma infections on parasite survival

Since *P. falciparum* parasites cultured were found to survive for 8 days compared to the 3-4 days reported in previous studies [1, 10], we investigated possible causes for the prolonged survival without pantothenate. One option was to test cultures for Mycoplasma infection. Mycoplasma infections have become a common problem in *P. falciparum* culture procedures. It is also known that Mycoplasmas can lead to various unwanted effects in eukaryotic cell cultures like impaired growth and abnormalities in various biochemical processes [11]. Therefore we tested our cultures for the presence of Mycoplasmas by using a routine PCR method for this purpose. Briefly, a PCR is performed using media that was incubated with the parasites for 24 hours, along with 9 primers, 6 forward and 3 reverse primers, specific for a broad range of Mycoplasma species.
Figure 3.4: Detection of Mycoplasma in *P. falciparum* cultures before and after treatment with Mycoplasma Removal Agent. (A) PCR products run on a 1% agarose gel, showing a positive test for the presence of Mycoplasma from cultures. The lanes are as follows: (1) Universal Ladder (KAPA), (2) negative control (no enzyme), (3) negative control (no template), (4) positive control (50x dilution Mycoplasma MS02 DNA), (5) culture supernatant, (6) freezer stocks culture supernatant. (B) PCR run on a 1% agarose gel, testing negative for the presence of Mycoplasma from cultures after treatment with the Mycoplasma Removal Agent. The lanes are as follows (1) Universal Ladder, (2) positive control (50x dilution Mycoplasma MS02 DNA, (3) negative control (no enzyme), (4) negative control (no template), (5) treated culture supernatant, (6) treated freezer stocks culture supernatant, (7) RPMI-1460 media used, (8) fresh bottle of RPMI-1640 media without Albumax II serum, (9) Fresh RPMI-1640 media containing Albumax II serum, (10) supernatant from washed blood stock.

The PCR (Figure 3.4A) test was positive for the presence of Mycoplasmas in both the cultures (one of which was recently started from freezer stocks and the other which had already been cultured for a few weeks) with at band 700 bp comparing well with the positive control, *MS02*. This indicated that the Mycoplasma infection was present not only in the actively growing cultures but in the freezer stocks too. Consequently, a culture was treated with Mycoplasma Removal Agent (MRA) for a period of five days before the PCR was repeated and amplified DNA run on a 1% agar gel containing Nancy-520 fluorescent DNA stain. From the results (Figure 3.4B), it can be concluded that the cultures were Mycoplasma free. In addition, all the components used to culture, such as the RPMI-1640 media, the Albumax-II serum, and the washed human erythrocytes, were also tested and confirmed to be Mycoplasma free.

After the removal of Mycoplasma, the survival experiment for *P. falciparum* parasite in Pan-free complete media as well as the experiment to ascertain the minimum amount of pantothenate required for survival were repeated, however the results were inconclusive. Unfortunately, within the timeframe allowed for this project, we were unable to perform either of the experiments again to allow conclusions to be drawn and these experiments need to be repeated in future work.
3.2.2 Metabolism of PanAms by the CoA biosynthetic enzymes of *P. falciparum*

Although we were unable to establish the minimum requirement for pantothenate in parasites, previous experiments have clearly shown that the PanAms have an influence on pantothenate metabolism. We wanted to determine whether PanAms could be converted to the corresponding CoA antimetabolites by the CoA biosynthetic enzymes present in *P. falciparum* parasites. Such evidence would lend support to the PanAms having a mode of action that is downstream in the pathway, and affecting the parasite by either lowering CoA levels (by competitive formation of CoA antimetabolites) or by forming CoA antimetabolites that can possibly inhibit downstream processes in the parasite. In this study we specifically chose *N*-PE-α-Me-PanAm which was the most potent vanin resistant inhibitor in our laboratory [12]. To determine the fate of this PanAm in the CoA biosynthesis pathway (Figure 3.5) we followed two approaches: 1) expose the PanAm to enzymes present in parasite lysate prepared from isolated *P. falciparum* parasites or 2) incubate *P. falciparum* parasites with a non-lethal dose of PanAm after which they can be isolated and lysed for analysis. In both these approaches HPLC analysis was used to detect antimetabolite formation.

![Figure 3.5: The biosynthesis of CoA antimetabolites from a PanAm. The conversion of *N*-PE-α-Me-PanAm by the CoA pathway enzymes PanK, PPAT and DPCK is shown, forming the antimetabolite intermediates 4'-phospho-*N*-PE-α-Me-PanAm, dephospho-*N*-PE-α-Me-CoA and *N*-PE-α-Me-CoA [13].](image-url)
3.2.2.1 Method development for CoA antimetabolite detection

3.2.2.1.1 Detection of N-PE-α-Me-PanAm via UV absorbance

We have various methods available in our laboratory for the detection of PanAms and their corresponding metabolites [13-16]. However, most of these methods were used to analyze samples that made use of purified CoA biosynthesis enzymes from bacteria and therefore allowed the preparation of antimetabolites at high concentrations. Since we do not have the ability to express and purify these proteins from \textit{P. falciparum}, we had to identify which of the HPLC methods available would allow for detection of the presumed CoA antimetabolites in parasite in much lower concentrations than what is possible when working with purified protein.

First, we wanted to determine at what concentration we would see adequate signal via UV detection of N-PE-α-Me-PanAm. The amide bonds present in PanAms usually allow detection at 214 nm [13], however the baseline of chromatograms obtained at this wavelength can contain a lot of background noise. Since N-PE-α-Me-PanAm has an aromatic group in the phenethylamide moiety of the molecule, we also investigated whether UV detection was possible at ~254 nm, the wavelength usually used for detection of aromatic compounds. The UV absorption profile of the molecule was determined spectrophotometrically to obtain the absorption maxima of the compound. N-PE-α-Me-PanAm has an absorption maxima of 220 nm and 254 nm.

Next, we analyzed N-PE-α-Me-PanAm by HPLC (see section 2.2.21.1 except these were not derivatized) using a Luna 5u C18(2) 100A 250 x 4.60 nm column with 50 mM phosphate buffer (pH 6.8) and acetonitrile as eluents to determine whether this method would result in sufficient detection of N-PE-α-Me-PanAm at the absorption maxima determined. We injected 5 µM, 50 µM and 500 µM of N-PE-α-Me-PanAm to determine the differences in absorption at the various concentrations. At both wavelengths the peak for N-PE-α-Me-PanAm was visible at 50 min. As expected the baseline for the absorption at 254 nm was more stable than for the chromatogram at 220 nm (Figure 3.6 A and B), however the intensity of the absorbance at 254 nm was 2.5 times lower than at 220 nm. Since we were uncertain how much conversion to product we would be able to detect when using parasite lysate, we used 220 nm as wavelength for detection in further experiments. In addition, the differences in absorbance between 5 µM, 50 µM and 500 µM N-PE-α-Me-PanAm were also not very pronounced, therefore we also performed all future reactions using 5 µM of N-PE-α-Me-PanAm.
Figure 3.6: HPLC analysis of different concentrations of \( N\)-PE-\( \alpha \)-Me-PanAm at a wavelength of (A) 220 nm and (B) 254 nm: 5 \( \mu \)M PanAm (in blue), 50 \( \mu \)M PanAm (in green), and 500 \( \mu \)M PanAm (in red). The \( N\)-PE-\( \alpha \)-Me-PanAm peak eluted at \( \sim \)50 min.
3.2.2.1.2 Analysis of \textit{N}-\textit{PE-\(\alpha\)}-\textit{Me-PanAm} metabolism via UV absorbance

\(\textit{N}-\textit{PE-\(\alpha\)}-\textit{Me-PanAm}\) was incubated with \textit{P. falciparum} cell lysates (which should contain all the CoA biosynthetic enzymes) and three equivalents of ATP for 24 hours after which it was analyzed using HPLC while monitoring the separation at 220 nm (Figure 3.7). The same column was used as described above, however the eluents were changed to 10 mM ammonium acetate (pH 5.5) and methanol to improve separation and baseline stability, and the solvent gradient was adapted to allow for shorter analysis time (see section 2.2.21.2). Standards of \(\textit{N}-\textit{PE-\(\alpha\)}-\textit{Me-PanAm}\) and ATP were injected to allow for substrate identification. Although peaks were visible for both ATP and \(\textit{N}-\textit{PE-\(\alpha\)}-\textit{Me-PanAm}\), (Figure 3.7) neither compound eluted as one single peak. ATP eluted in three overlapping peaks between 2.1 and 2.65 min, while the \(\textit{N}-\textit{PE-\(\alpha\)}-\textit{Me-PanAm}\) standard contained four peaks between 10.5 and 16 min, with the major peak eluting at 13.6 min. Only two very small peaks not present in the standards were visible at 3.53 min and 9.86 min, suggesting that antimetabolites were not formed under these conditions.

Since we do not know the specific concentration or activity of the CoA biosynthetic enzymes present in parasite lysate, the lack of product peaks could be due to very low turnover of substrate to product which might then fall under the detection limit of UV absorbance at 220 nm. We therefore explored the use of an alternative detection method.

\[\text{Graph: UV chromatogram at 220 nm of } \textit{N}-\textit{PE-\(\alpha\)}-\textit{Me-PanAm} \text{ incubated in the presence of parasite lysate and ATP for 24 hours. Peaks indicated in green were identified as ATP (between 2.15 min and 3.40 min) and in red represents PanAm (between 10.58 min and 15.50 min) as compared to provided standards.}\]
3.2.2.2 Analysis of N-PE-α-Me-PanAm metabolism via LCMS

Since we needed to use a more sensitive method for the analysis of our reactions we reverted to detection with mass spectrometry (MS). To validate a liquid chromatography mass spectrometry (LCMS) method used in another study where other PanAm analogues were investigated in S. aureus [17], we determined if all the expected antimetabolites that can form are detected by this method. Standard reactions were set up with E. coli and S. aureus CoA biosynthetic enzymes that were obtained by recombinant expression in E. coli. Consequently, SaPanK, EcPPAT and EcDPCK were incubated with 5 mM N-PE-α-Me-PanAm and 10 mM ATP for 24 hours before the reaction was stopped by heat precipitation of the proteins and the samples submitted for LCMS analysis (ESI positive and negative mode) at CAF (Figure 3.8).

Figure 3.8: LCMS chromatogram (TOF-MS, ESI positive, BPI) N-PE-α-Me-PanAm incubated with SaPanK, EcPPAT and EcDPCK and ATP. The peaks were identified as (1) ATP and ADP at 1.68 minutes (mass found: 508.00 and 428.04, mass expected: 507.99 and 428.03), (2) 4’-phospho-N-PE-α-Me-PanAm at 12.13 minutes (mass found: 417.18, mass expected: 417.17), (3) N-PE-α-Me-PanAm at 12.99 minutes (mass found: 337.21, mass expected: 337.21), (4) N-PE-α-Me-dephosphoCoA at 10.84 minutes (mass found: 746.23, mass expected: 746.22), and (5) N-PE-α-Me-CoA at 8.96 minutes (mass found: 826.20, mass expected: 826.19). Peaks after 12.99 minutes were not identified and are unknown.

All the antimetabolites were detected via LCMS (positive mode) with ATP and ADP co-eluting at 1.68 min, N-PE-α-Me-CoA at 8.95 min, N-PE-α-Me-dephosphoCoA at 10.84 min, 4’-phospho-N-PE-α-Me-PanAm eluting at 12.13 min and finally the substrate N-PE-α-Me-PanAm at 12.99 min. As a result, we were able to successfully validate the LCMS method for separation and detection of the PanAm antimetabolites in samples.
3.2.2.2.1 N-PE-α-Me-PanAm conversion in *P. falciparum* lysate

Since we had a confirmed method that could detect the CoA antimetabolite intermediates formed by the CoA biosynthetic enzymes, and which separates the antimetabolite intermediates well, this method was used to determine whether the CoA biosynthetic enzymes in lysate isolated from *P. falciparum* are able to convert the PanAms to their relevant CoA antimetabolites like the bacterial (*S. aureus* and *E. coli*) enzymes were found to do. For this, parasite lysates were incubated with N-PE-α-Me-PanAm for 24 hours and the resulting products submitted for analysis by LCMS (ESI positive and negative mode).

Although in the previous section the antimetabolites were detected in ESI positive mode, no peaks were identified. However, after evaluation of chromatogram in ESI negative mode, co-elution of ATP and ADP was detected at 2.03 min with a peak corresponding to the substrate N-PE-α-Me-PanAm observed at 12.68 min (Figure 3.9). The only biotransformed metabolite observed was 4’-phospho-N-PE-α-Me-PanAm, which eluted at 12.68 min although in a much smaller amount in comparison to N-PE-α-Me-PanAm. No PE-α-Me-dephosphoCoA (expected at ~9 min) or PE-α-Me-dephosphoCoA (expected at ~11 min) were detected. This could mean that too little of these antimetabolites are formed to allow for detection by LCMS, or that these antimetabolites are not formed by the *P. falciparum* enzymes implying that PPAT is inactive. Alternatively PPAT does not accept 4’-phospho-N-PE-α-Me-PanAm as substrate.

![Figure 3.9: LCMS chromatogram (TOF-MS, ESI negative, TIC) of N-PE-α-Me-PanAm incubated with P. falciparum cell lysates for 24 hours. The peaks were identified as (1) ATP and ADP at 2.03 minutes (mass found: 506 and 426, mass expected: 505.99 and 426.03), (2) 4’-phospho-N-PE-α-Me-PanAm at 12.68 minutes (mass found: 415, mass expected: 415.17), and (3) N-PE-α-Me-PanAm at 13.47 minutes (mass found: 335, mass expected: 335.21).](https://scholar.sun.ac.za)
The finding that 4’-phospho-N-PE-α-Me-PanAm is biosynthesized by PfPanK is not surprising since several other studies have shown that PfPanK in parasite lysate is active [1, 7, 12]. However, no information is known about the activity of PPAT and DPCK, since no studies have ever investigated the activity of these two enzymes in lysates prepared from P. falciparum. In order to confirm that we have active PPAT and DPCK present in the prepared lysate, an alternative possible substrate of PanK (pantetheine) and the substrate of DPCK (dephospho-CoA) was incubated in the presence of ATP and P. falciparum lysate, and subjected to LCMS analysis (ESI positive and negative mode). These reactions would result in the formation of dephospho-CoA if PPAT is active, or CoA if DPCK is active. However, none of the CoA metabolites could be detected by this method, indicating that either the antimetabolites are present in such miniscule concentrations that the method of detection is not sensitive enough, or that the P. falciparum PPAT and/or DPCK are inactive in the lysates.

An alternative analysis method in our laboratory allows for the utilization of the terminal thiol present in the natural metabolites occurring in the CoA salvage pathway (Figure 1.8) to derivatize these compounds with CPM, a thiol-reactive compound that has weak fluorescent properties until it reacts with thiols (Figure 3.10). This allows detection by fluorescence when HPLC analysis is performed [13]. We therefore reverted to this method to determine if the PPAT and DPCK present in parasite lysates are active, since fluorescent detection will increase sensitivity of detection and possible problems with ionization via MS could be excluded. P. falciparum parasite lysate was incubated with the natural substrates pantetheine (5 mM) and dephospho-CoA (5 mM) for 24 hours, after which the samples were derivatized with CPM and analyzed via HPLC with fluorescent detection at an excitation and emission of 387 nm and 465 nm, respectively.

![Chemical structure of CPM labeling of thiolated compounds](https://scholar.sun.ac.za)

**Figure 3.10:** The chemistry of CPM labeling of thiolated compounds. CPM is a thiol-reactive compound that has weak fluorescent properties until it reacts with free thiols.
From the HPLC analysis (Figure 3.11A), we see that 4’-phosphopantetheine (retention time of 4.27 min) is produced by \( PfPanK \) from pantetheine, however no dephospho-CoA (retention time 4.92 min) or CoA (retention time of 4.15 min) is produced (expected product elution times were confirmed by spiking samples with known samples of 4’-phosphopantetheine, dephospho-CoA and CoA). This indicates that PPAT in \( P. falciparum \) lysate is inactive. Interestingly, analysis of the reaction mixture with dephospho-CoA as substrate indeed showed the formation of CoA, illustrating that \( PfDPCK \) is active. We next investigated if the Tris buffer used in the reaction was the cause for the poor PPAT activity, and therefore repeated both reactions with pantetheine and dephospho-CoA in HEPES buffer at pH 8. The same results were obtained as for the reactions performed in Tris buffer with 4’-phosphopantetheine formation visible from pantetheine, and CoA from dephospho-CoA. However, no PPAT activity was observed (data not shown).

![Figure 3.11 Fluorescence chromatogram (with excitation and emission wavelengths at 387 nm and 465 nm respectively) of HPLC injected cell lysates incubated with (A) pantetheine and lysates stored in Tris buffer, and (B) Dephospho-CoA with lysates stored in Tris buffer, for 18-24 hours. Peaks are identified as (1) TCEP, (2) 4’-phosphopantetheine, (3) pantetheine, (4) Dephospho-CoA, (5) CoA and (6) cysteine. Although no peak is visible on the chromatogram, CoA was present but omitted from the chromatogram due to a system overload at 4.15 min (indicated by blue line) because of a high metabolite concentration.](https://scholar.sun.ac.za)
3.2.2.2 Analysis of N-PE-α-Me-PanAm conversion in P. falciparum cultures in vitro.

To further investigate whether *P. falciparum* metabolizes N-PE-α-Me-PanAm downstream from *Pf* PanK we investigated the fate of this compound in parasite cultures *in vitro*. Cultures were treated with a non-lethal dose of N-PE-α-Me-PanAm (i.e. a concentration equivalent to the IC\textsubscript{50} of 52 nM ± 6 nM determined previously [6]) for 24 hours, after which the parasites were isolated, lysed, proteins precipitated and removed, and the cellular contents submitted for LCMS analysis. Although pantetheine, 4′-phosphopantetheine and a very small amount of CoA were detected as evidenced by the peaks at 15.85 minutes, 12.99 and 15.18 minutes respectively (Figure 3.12), dephospho-CoA was not detected. In addition, no N-PE-α-Me-PanAm or any of its corresponding antimetabolite intermediates were detected by LCMS in either ESI positive or negative modes.

![LCMS chromatogram](Stellenbosch University https://scholar.sun.ac.za)

**Figure 3.12:** LCMS chromatogram (TOF-MS, ESI positive, BPI) of N-PE-α-Me-PanAm incubated with *P. falciparum* whole cells in culture for 24 hours. The peaks were identified as (1) 4′-phospho-pantetheine at 12.99 minutes (mass found: 359, mass expected: 359.10), (2) CoA at 15.18 minutes (mass found: 767, mass expected: 767.12), and (3) Pantetheine at 15.85 minutes (mass found: 278, mass expected: 279.13). Non-labelled peaks were not identified and are unknown.

3.2.3 Synthesis and evaluation of the effect of a PanK inhibitor that is not a pantothenate analogue on *P. falciparum* CoA biosynthesis.

To confirm that PanAms do not inhibit *Pf*PanK, but are rather metabolized by this enzyme to affect targets downstream in the pathway, we wanted to prepare a PanK inhibitor that is not an analogue of the natural substrate of PanKs, pantothenate. Recently, studies by Sharma *et. al* have shown that
human PanK can be inhibited by tricyclic methylthiophenyl propanamides (TMPs), which are not structurally related to pantothenate [18]. TMPs have been found to bind to the ATP-PanK complex to assert their inhibitory effect, and it was shown that increasing concentrations of these TMPs cause a decrease in CoA levels [18].

Our aim was therefore to synthesize a TMP analogue and test it on *P. falciparum* to see if it has any inhibitory effect on *Pf*PanK and parasite proliferation. If so, it should inhibit by lowering CoA levels since it shuts down CoA biosynthesis at the first enzyme. This could then be used as a possible tool to compare to PanAms as inhibitors in order to determine if PanAms lower CoA levels in a different way—the difference being that the TMP analogue cannot act as alternative substrates and the PanAms can, since they are pantothenate analogues.

### 3.2.3.1 Synthesis and purification of TMP

Using the methods published by Sharma *et. al* [18], we set out to synthesize TMP in four steps starting from 2-chloro-3-pyrimadinecarbonitrile (Figure 3.13A). The syntheses of compounds 1 and 2 were performed according to the published method, except that instead of using a microwave-assisted procedure, the reaction mixtures were refluxed overnight. The reaction for compound 1 was successful and we obtained the product as a yellow precipitate in a yield of 40%. The structure of the product was confirmed by $^1$H NMR spectroscopy, showing a purity of >95%. Compound 2 was prepared from compound 1 by refluxing overnight under nitrogen in ethanol in the presence of methyl 4-acetyl-5-oxohexanoate. This resulted in a clear orange mixture that was concentrated *in vacuo* to obtain a yellow product without further purification in a yield of 97%. The structure of the product was confirmed by $^1$H NMR spectroscopy, which showed a purity of >95%. To obtain compound 3, hydrolysis of the methyl ester was performed with 1 M NaOH in THF overnight. After work-up the product was obtained as a white powder in a yield of >99%. The structure of the product was confirmed with $^1$H NMR and its purity estimated at >95%.

Although the first three synthetic transformations were performed successfully, significant problems were encountered in executing the last step. Since our attempts to reproduce published methods were not always successful, we also used alternative methods (Figure 3.13B) in the attempt to successfully synthesize the PanK inhibitor, TMP. Various attempts were made to couple the carboxylic acid (compound 3) with 3-(methylthio)alanine with limited success; the attempts are summarized in Table 3.1. The first attempt involved using HBTU as activating agent as per published method [19]. However, the procedure calls for the purification of the reaction mixture via preparative HPLC on a reversed phase column, and since we did not have access to such a system, we therefore had to investigate alternative means for purification. Flash column chromatography using silica and methanol/dichloromethane (5-10%) as eluent was used to attempt purification. Although purified product was recovered from the column and identified via $^1$H NMR (Figure 3.15A), too little (<2 mg)
was obtained for any further use. As an alternative the reaction was attempted a second time with an added work-up step before purification; however this attempt was unsuccessful.

Alternative activation strategies were attempted to allow more product formation in order to simplify purification. One of these attempts involved the use of EDC in the presence of HOBt as activation reagent of the carboxylic acid, however without success. Another route attempted was amidation by means of $\text{B(OCH}_2\text{CF}_3)_3$, which was prepared from $\text{B}_2\text{O}_3$ and 2,2,2-trifluoroethanol. After completion of the reaction, the resulting reaction mixture was diluted with ethyl acetate and water and stirred with Amberlyst A-26(OH) (anion exchanger), Amberlyst 15 (cation exchanger) and Amberlite IRA743 resins (chelator). These resins should remove all impurities except the expected product, which is a neutral compound, in order to simplify purification. Unfortunately this procedure also failed to produce the final product.

Figure 3.13: (A) Original synthesis of TMP as set out by Sharma et. al [18], and (B) a scheme of the alternative last step, whereby compound 3 is first converted to the acid chloride (compound 5) before the attachment of the amide to form TMP or compounds 6-8.
Finally, an alternative strategy was attempted by preparing the acid chloride in catalytic amounts of DMF, followed by the addition of 3-(methylthio)aniline dissolved in picoline (Figure 3.13B). We also investigate three other amines (3-fluoroaniline, 3-(trifluoromethyl)aniline and 4'-aminoacetanilide) to determine if the problem encountered in the coupling could be due to the structure of the amine; these alternative amines were used in the published method for the production of other TMP analogues that also gave good inhibition profiles. The reactions were followed by TLC in 10% methanol/dichloromethane. The reaction with 3-fluoroaniline seemed the most promising with a UV active spot at R$_f$ ~0.21 as possible product. This reaction was purified by means of a silica plug with 5%-10% methanol/dichloromethane, however the product could unfortunately not be isolated (Figure 3.15B).

Table 3.1: Summary of the methods investigated for TMP synthesis.

<table>
<thead>
<tr>
<th>Activation group</th>
<th>Amine</th>
<th>Product formation</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBTU</td>
<td>3-(methylthio)aniline</td>
<td>Yes</td>
<td>Partially successful</td>
</tr>
<tr>
<td>EDC</td>
<td>3-(methylthio)aniline</td>
<td>Yes</td>
<td>Partially successful</td>
</tr>
<tr>
<td>B(OCH$_2$CF$_3$)$_3$</td>
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<td>No</td>
<td>Not purified</td>
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<td>Not purified</td>
</tr>
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<td>-Cl</td>
<td>3-(trifluoromethyl)aniline</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>-Cl</td>
<td>3-fluoroaniline</td>
<td>Yes</td>
<td>Not purified</td>
</tr>
<tr>
<td>-Cl</td>
<td>4'-aminoacetanilide</td>
<td>No</td>
<td>Not purified</td>
</tr>
</tbody>
</table>

Figure 3.14: TLCs at (A) 254 nm (UV) and (B) 336 nm (fluorescence) of all 4 compounds synthesized in amidation reaction with the acid chloride. TLCs were run in 10% methanol/DCM. The spots were as follows (S) compound 4, compound 4 and all added components before the reaction was stirred, with 3-(methylthio)aniline (S1), 3-fluoroaniline (S2), 3-(trifluoromethyl)aniline (S3), and 4'-aminoacetanilide (S4) as the added amines, and the products after the reactions with 3-(methylthio)aniline (P1), 3-fluoroaniline (P2), 3-(trifluoromethyl)aniline (P3), and 4'-aminoacetanilide (P4) as the added amines. The product we are aiming to synthesize is both UV active and fluorescent. The black box indicates the reaction that seemed to have best product formation and is the reaction that was chosen for further analysis.
Figure 3.15: $^1$H NMR analysis of (A) TMP synthesized by HBTU coupling of the amine to the carboxylic acid purified on a silica column (5% methanol/DCM, 20% methanol/DCM), dissolved in DMSO ($^1$H NMR is consistent with literature data [8]), and (B) the partially purified product formed from the amidation reaction using acid chloride derivative and purification by silica plug using 5%-10% methanol/DCM, dissolved in deuterium oxide. Unassigned peaks were either impurities or unknowns.
3.2.3.2 TMP as inhibitor of PfPanK in parasite lysate and parasite growth

The last two objectives of this study (to test TMP as inhibitor of PanK-mediated phosphorylation of pantothenate and as antiplasmodial against *P. falciparum* parasites in culture) were dependent on the successful synthesis of TMP. Since we were unable to purify enough material to perform these experiments these objectives will be addressed in future work.

3.3 Discussion

3.3.1 Minimum extracellular pantothenate needed for parasite survival

A study of the nutrient requirements of malaria parasites can be complex and usually requires the preparation of custom-made culture media (for the purpose of this study, RPMI-1640 media without pantothenate present), and considering the complexity of the medium, there is high chance for error. Pan-free culture medium was prepared in-house, however parasites were not supported by this medium. This might be due to the medium either lacking an essential component for parasite growth or some of the components sourced where not of sufficient quality for cell culture.

To eliminate error with media preparation, we sourced custom made Pan-free RPMI-1640 media commercially and repeated control experiments to determine if parasite growth can be sustained in this media with the addition of pantothenate compared to when pantothenate is not present. Surprisingly, the *P. falciparum* parasites were surviving in the Pan-free media up to ~92% after 4 days in a microtiter plate (96-well). In a follow-up experiment, small-scale cultures were cultured in a Pan-free environment with culture media containing no pantothenate being replaced daily. In these experiments parasites were surviving for 7 days with pantothenate being absent in their otherwise nutrient-rich environment. This finding is in contrast to what was found by other groups that have shown that *P. falciparum* 3D7 parasites can only survive in a Pan-free environment for 3-4 days [1, 10]. In these studies either manual counting of parasitemia by light microscopy was used [10] or a radiolabeled assay that measures the incorporation of \[^{3}H\]hypoxanthine into viable parasites [1]. In this thesis we described three methods used to determine parasite survival of which one was also manual counting of parasitemia by microscopy in addition to a Malstat and SYBR Safe assay that has not been used previously for this purpose.

The first method (manual counting) show an increase in parasitemia from 1% (similarly to what was used by Mamoun et al. and Saliba et. al to initiate the experiment [10]) to ~5% at days 2 and 3, after which parasite growth decreases to 1%. This is in line with what was found by Mamoun et al. who showed that parasitemia decreased after the first 3 days. However, no parasite growth was visible after 3 days in their study whereas we still observed residual parasite growth of ~0.5% up until day 7. Manual counting of parasites is prone to human error due to parasites in ring phase, as well as
those deprived of pantothenate, being smaller and therefore more difficult to see under light microscopy. This method is also time consuming and impractical for high numbers of cultures. We therefore reverted to other assays, such as the SYBR Safe and Malstat assays, that are more sensitive.

The second method used was the SYBR Safe assay, where SYBR Safe (a nucleic acid stain) binds to viable parasite DNA (erythrocytes have no DNA) and the resulting DNA-dye-complex allows for fluorescent determination of parasite survival [20]. Alternatively we also used the Malstat assay, also known as an LDH assay. Here Malstat reagent, NBT/PES solution and cultures initiate a lactate dehydrogenase reaction (measured colourmetrically) as NBT is reduced [21]. Viable parasites will still produce lactate that will allow this colour change to measure parasite growth. Both erythrocytes and *P. falciparum* produce lactate, therefore there will be a slight colourometric change even when studying uninfected erythrocytes, however the amount of lactate contributed by erythrocytes is minimal when compared to the amounts produced by the parasites. In both these assays parasite growth increased for 4 days after which a dramatic decrease in parasite growth was observed. However, residual amount of parasites still survived up until day 7 similarly to what was observed using manual counting.

The majority of *P. falciparum* parasites do not survive more than 96 hours without pantothenate present, which correlates with findings by Saliba *et al.* who used a hypoxanthine assay to determine parasite survival in the absence of pantothenate [1] by measuring the level of radioactivity of the [3H]-hypoxanthine incorporated into nucleic acids of the parasites when they proliferate. However in this study, similar to that of Mamoun et al., no residual growth was visible [1, 10]. The observed differences between parasite survival in this study compared to published data can be rationalized in various ways. First, it is possible that the *Plasmodium* strain has mutated, which has been seen to happen after many rounds of sorbitol synchronizations [22], however this is unlikely to change the capability of the parasites to survive without pantothenate since so many biological processes rely on CoA. Alternatively, there could be another source of pantothenate providing the parasites with the vitamin for survival. This seems like a more likely explanation since *P. falciparum* has no metabolic pathway to synthesize pantothenate *de novo* and thus has to gain the pantothenate from its environment.

Possible alternative sources of pantothenate could be from culture contamination by other organisms. Since no contamination of cultures was visible under light microscopy, we tested cultures for possible Mycoplasma contamination. PCR detection of Mycoplasma confirmed that the cultures were indeed infected. Mycoplasma infection of cultures can have varying effects on cell cultures, and the effects varying in severity—all dependent on the Mycoplasma species, the type of culture that is infected, and the culturing conditions [23]. Mycoplasma infections can hinder most if not all
facets of cell cultures, including cellular metabolism [11], thus although Mycoplasmas do not kill or noticeably suppress the growth of the *Plasmodium* parasites, they can interfere with the parasites’ metabolism or in this case be a source of small amounts of pantothenate that can sustain parasites survival albeit in residual amounts. This therefore highlights the need to remove these organisms from cell cultures to accurately study the parasites’ metabolism of pantothenate and its survival in a Pan-free environment.

Even though cultures were treated for the removal of Mycoplasma in order to confirm that this organism are indeed the source of residual pantothenate, these experiments were unfortunately not successful and due to time constraints were not repeated. As a results we could also not determine the minimum amount of pantothenate needed for parasite survival and therefore these experiments will be addressed in future work.

### 3.3.2 Metabolism of PanAms by the CoA biosynthetic enzymes of *P. falciparum*

It is known that in bacteria PanAms are converted to antimetabolites to exert their inhibitory effect [24]. To determine whether *N-PE-α-Me-PanAm* has a similar mode of action in *P. falciparum* we investigated whether this compound can be converted to the corresponding CoA antimetabolites by the CoA biosynthetic enzymes present in *P. falciparum* parasites. We first confirmed which analytical technique would allow sufficient sensitivity to allow the identification of substrate and antimetabolites. Two approaches were considered where either parasite lysates or cell cultures were used to determine the conversion of *N-PE-α-Me-PanAm* to its corresponding antimetabolites. However, since we are not able to perform this experiment using purified proteins or high cell numbers when using cell cultures (as is the case when working with bacteria), we needed to ascertain that our method of detection is reliable for metabolite identification. After various experiments looking at the UV absorbance of *N-PE-α-Me-PanAm* we determined that the sensitivity of this method will not be sufficient to detect product formation. As an alternative we validated a different method utilizing LCMS that was previously used for PanAm metabolism studies in *S. aureus* [17]. The LCMS method proved to have adequate sensitivity to locate the antimetabolites when incubating *N-PE-α-Me-PanAm* with recombinant CoA biosynthesis proteins from *E. coli* and *S. aureus*. In fact, we successfully showed for the first time that *N-PE-α-Me-PanAm* can be converted by enzymes from the CoA biosynthesis pathway to the corresponding CoA antimetabolites, albeit from bacteria. This gave as the tools to explore if the same process can take place in *P. falciparum*.

Consequently, the same LCMS method was used to analyse *N-PE-α-Me-PanAm* incubated with *P. falciparum* cell lysate containing CoA biosynthesis proteins. The formation of 4′-phospho-*N-PE-α-Me-PanAm* indicated that *PfPanK* is active in parasite lysate, as is known from previous studies [1, 25, 26, 27]. In addition we also demonstrated for the first time that this specific PanAm can act as
substrate for PfPanK which aligns with recent studies that found that PfPanK is the gateway for PanAm mediated inhibition of *P. falciparum* [26]. The PanAm therefore has to be metabolically activated by this enzyme to exert their inhibitory effect downstream in the pathway. However, the fate of the molecule downstream in the pathway is still unknown since no corresponding dephospho-CoA and CoA antimetabolites were observed. This could be due to several reasons. First, it might be that the metabolites fall under the limit of detection of the method, especially since very low amounts of 4′-phospho-N-PE-α-Me-PanAm are observed. Secondly, PPAT and DPCK might not be active in parasite lysate.

To further investigate the activity of PPAT and DPCK, we exposed these proteins to the natural substrate pantetheine and dephospho-CoA. Surprisingly, no activity was observed for any proteins from the CoA pathway (not even PfPanK that showed activity previously). CoA metabolites have very poor ionization capability and can have varying results when analyzing by LC-MS. We therefore reverted to a third method of detection since we were investigating the natural metabolites. This allowed the use of CPM derivatization of the terminal thiol to detect these compounds via fluorescence. No activity was found for PPAT (even performing the experiment in two different buffers); however, we found that DPCK is active in parasite lysate prepared in either Tris or HEPES buffer. This is the first experiment, to our knowledge, that successfully demonstrates DPCK activity in parasite lysate prepared from isolated parasites. This is especially important since DPCK is localized to the apicoplast of the parasite and no information regarding the status of the organelle is known in lysates. Whether the apicoplast is lysed or still intact after manual lysis is still unknown, however, we have demonstrated that DPCK present in this organelle is functional once the parasite is isolated and lysed.

The fact that PPAT was inactive supports no antimetabolite formation in the presence of 4′-phospho-N-PE-α-Me-PanAm downstream from PfPanK. However, PPAT might require different reaction conditions than those used in this study. The formation of the antimetabolites can therefore not be excluded. Alternatively, N-PE-α-Me-PanAm was incubated in cell culture and analyzed after 24 hours, however no antimetabolites (not even 4′-phospho-N-PE-α-Me-PanAm) could be detected. In this case the amount of antimetabolite formation is most likely under the limit of detection and probably requires a large amount of cell cultures to isolate enough parasites for analysis. This is a costly process and also consumes a lot of substrate that would need to be synthesized in-house.

3.3.3 Synthesis and evaluation of the effect of a PanK inhibitor on *P. falciparum* CoA biosynthesis that is not a pantothenate analogue.

To support the fact that N-PE-α-Me-PanAm exerts its effect downstream in the CoA biosynthesis pathway, we considered comparing the inhibitory effect of this pantothenate analogue to a compound that is a PanK inhibitors but is structurally not related to pantothenate. Recently it has been show
that TMP is an inhibitor of human PanK3 and exerts its inhibitory effect by lowering CoA levels. In order to use this compound for comparison to PanAms we first had to synthesize the TMP and determine if it has antiplasmodial properties.

The synthesis of TMP was performed according to published work [18]. While compounds 1-3 were synthesized and purified in good to excellent yields (40%, 97% and >99% respectively), the final product proved to be difficult to obtain. This is mainly due to the chemical properties of the final product that makes purification of the final product cumbersome, regardless of which method was used to prepare it. This led to major loss of product where purification methods were not sufficient to purify compound 5, thus yielding only a partially purified product. The original synthesis of this compound described the use of preparative HPLC for the purification of TMP. Unfortunately we did not have preparative HPLC to our disposal and had to revert to alternative purification methods like silica purification or purification by cation and anion exchange. Silica purification was not successful and proved to decrease the stability of the product in non-polar solvents. Even though cation and anion exchange purification strategies were also employed we had no success. The reason for this is unclear.

However we were successful in purifying trace amounts of TMP to confirm product formation. We were able to synthesize a small amounts of the partially purified compound (Figure 3.16B), however due to impurities we did not feel confident in testing this product further on *P. falciparum*. As a result we did not have sufficient amounts of product to test it as a PanK inhibitor on *P. falciparum*. 
3.4 References


4. 1 Conclusion

4.1.1 Establishing the minimum extracellular pantothenate needed for parasite survival

CoA plays an important role in central metabolism, and it is known that the CoA pathway is crucial for the survival of Plasmodium [1]. Since CoA cannot be taken up by the parasite and pantothenate is the substrate for the CoA biosynthesis pathway, previous research has focused much attention on pantothenate analogues as novel antimalarials. Analogues such as PanAms are known to inhibit P. falciparum parasite proliferation, however the PanAms’ mode of action is unknown [2]. However, the addition of extracellular pantothenate antagonizes the effect of PanAms on P. falciparum proliferation and thus PanAms are thought to interfere with pantothenate metabolism [1]. A key question to answer when setting out to establish what the effect of PanAms on pantothenate utilization is, is what the critical amount of CoA is needed for P. falciparum survival. This is because one of the possible modes of action would be the lowering of CoA levels; it is therefore important to know whether PanAm treatment causes CoA levels to fall to critical levels. Another way to address this is to establish how long the parasites can survive when transferred to medium without pantothenate, as this would give an indication of their ability to survive only on the pantothenate-derived metabolites that are present in the cells at the time of transfer. We first determined that the parasites can survive without pantothenate for 4 days, after which residual amounts of parasites survive for up to 8 days. The longer-than-expected survival times were not in line with the results of previous studies. Subsequent investigations found that the parasite cultures used in these tests were infected with Mycoplasma spp, and we therefore concluded that the presence of Mycoplasma in the cultures extended the survival of the parasites in Pan-free media. There are two possible ways in which we hypothesize this can occur: either by alteration of the parasites’ metabolism, leading to a lower demand for pantothenate, or by acting as a source of pantothenate for P. falciparum parasites. However these hypotheses will have to be tested in Mycoplasma-free cultures. The experiments that were executed to determine the minimum amount of pantothenate required for P. falciparum survival was consequently not conclusive due to the uncertainty about the effect of the Mycoplasma contamination on the cultures.

4.1.2 Metabolism of PanAms by the CoA biosynthetic enzymes of P. falciparum

While we know that PanAms show antiplasmodial activity and that their mode of action is connected to the CoA biosynthetic pathway, we wanted to determine whether PanAms target one of the enzymes in the CoA biosynthesis pathway directly, or whether they are converted by the CoA biosynthetic enzymes to CoA antimetabolites that affect processes dependent on CoA. After
validating HPLC and LCMS methods for the detection of the CoA metabolites and the PanAm-related CoA antimetabolites respectively, we determined that \( N\text{-PE-}\alpha\text{-Me-PanAm} \) is converted to 4'-phospho-\( N\text{-PE-}\alpha\text{-Me-PanAm} \) by \( Pf\text{PanK} \) in vitro. However, the results indicated that \( Pf\text{PPAT} \) is inactive in cell lysates, as the subsequent biosynthetic intermediates were not detected. This inactivity could be due to the enzymes being unstable in the lysis buffer, or because we do not fully understand its requirements for activity.

However, we have shown (for the first time) that DPCK is active in parasite lysate prepared from isolated \( P. falciparum \) parasites, which opens up new avenues for studies on this enzyme as a possible antiplasmodial target.

4.1.3 Synthesis and evaluation of the effect of a PanK inhibitor that is not a pantothenate analogue on \( P. falciparum \) CoA biosynthesis.

From a study done by Sharma et. al [3], TMP was shown to inhibit human PanK3 by binding to the ATP-PanK complex, which leads to a decrease in CoA levels. It was thus hypothesized that if TMP inhibits \( Pf\text{PanK} \), it can be used as a tool to show that PanAms (which are pantothenate analogues) have a different mode of action than TMP (which is not a pantothenate analogue) and do not exert their inhibitory effect on \( Pf\text{PanK} \). While we were successful in synthesizing the immediate precursor to TMP, we encountered several stumbling blocks in the last step of the synthesis. A significant problem was purification of the compound, which, due to its polarity characteristics, was not amenable to purification by normal phase chromatography. As a result we were unable to test or compare the inhibitory effect of TMP to \( N\text{-PE-}\alpha\text{-Me-PanAm} \).

4.2 Future Work

4.2.1 Establishing the minimum extracellular pantothenate needed for parasite survival

Due to the discovery of the Mycoplasma infection of the \( Plasmodium \) cultures at a late stage in this study, we were not able to repeat the appropriate experiments with cultures that were known to be Mycoplasma-free. I therefore propose that the following experiments are repeated for better and more accurate results: (1) the parasite survival in Pan-free media using Mycoplasma free cultures; this is to establish whether Mycoplasma indeed extends the parasites life-span without extracellular pantothenate added to culture media. (2) determining the minimum amount of extracellular pantothenate necessary for \( P. falciparum \) survival, since in this study we were unable to find what the lowest concentration of pantothenate is necessary to support parasite proliferation.
4.2.2 The critical amount of CoA for *P. falciparum* survival

Once the minimum concentration of extracellular pantothenate that sustains *P. falciparum* survival is determined, it will be important to establish how this correlates with the intracellular CoA concentration. Does the intracellular CoA concentration track the extracellular pantothenate concentration, or is the internal CoA concentration maintained at a certain level regardless of what it can obtain from outside sources? To determine this, cultures will be treated at the different levels of pantothenate and then analyzed by the already validated HPLC method (using CPM derivatization for fluorescence detection of CoA metabolites) to determine the CoA levels at different pantothenate concentrations. This will give an indication of the critical amount of CoA needed for parasite survival. We will also investigate whether PanAm-treated cultures display the same levels of CoA, therefore whether PanAm-treatment decreases the amount of CoA to levels that are insufficient for parasite survival.

4.2.3 Investigate the inactivity of *Pf*PPAT *in vitro*

Due to the fact that *Pf*PPAT was inactive in our *in vitro* experiments, the requirements of the enzyme in lysate should be revisited. Little information is known regarding this enzyme in *P. falciparum* even though a putative PPAT-encoding gene has been identified in the *P. falciparum* genome. However, this gene encodes a protein that does not show a high level of sequence homology to other eukaryotic PPAT enzymes, which could suggest that the parasite enzyme’s mechanism is different from that characterized for those enzymes. The enzyme’s need for specific cofactors and metal ions has to be investigated (Figure 4.1). Once it has been established what the requirements of the enzyme for activity is in lysate, parasites lysates in the presence of ATP and the required cofactors can be incubated with *N*-PE-α-Me-PanAm to establish if this PanAm is being metabolized downstream from *Pf*PanK.

![Figure 4.1: Scheme representing *Pf*PPAT’s possible need for alternative cofactors and metal ions. Not much is known about PPAT and thus it needs to be investigated.](https://scholar.sun.ac.za)
4.2.4 TMP as potential antiplasmodial

Since TMP could not be purified in this project, the last two objectives of the project could not be completed. If TMP could be successfully synthesized and purified (e.g. by preparative HPLC, which was not available in this study), TMP can be confirmed as a \( P. \ falciparum \) PanK inhibitor. As a positive control, TMP will be tested on human PanK3, after which it will be tested on PfPanK present in parasite lysates, using the same discontinuous radioactive assay that has been developed for tracking PanK activity in impure samples. This assay is a high-throughput method of monitoring the phosphorylation of small molecules, such as \( \[^{14}C\] \) pantothenate to \( \[^{14}C\]4'-phosphopantothenate \) by PanK [4]. TMP can then also be tested as inhibitor of \( P. \ falciparum \) parasite growth in culture to determine its IC\(_{50}\) value. TMP activity in \( P. \ falciparum \) can then be compared to that of \( N\)-PE-\( \alpha \)-Me-PanAm as an additional tool in our attempt to elucidate the PanAms mode of action in parasites.

4.2.5 Attempt to elucidate the differences in \( N\)-PE-\( \alpha \)-Me-PanAms enantiomer interactions

Currently, the most potent PanAm in our library is \( N\)-PE-\( \alpha \)-Me-PanAm, with an IC\(_{50}\) of 20 ± 3 nM. However this compound is currently synthesized as the diastereomeric mixture (Figure 4.2). Other members in our laboratory have recently been successful in the synthesis of the (R)- and (S)-epimers of this compound, which would allow us to establish whether the epimers are equally resistant to vanin pantetheinase degradation, and/or show equal antiplasmodial activity. Both compounds should therefore be tested for their susceptibility to vanin degradation to determine if there are any differences in their sensitivity towards pantetheinase-mediated breakdown. In addition, they should be tested for their ability to inhibit \( P. \ falciparum \) proliferation to determine if there is a difference in their antiplasmodial activity. Not only may this point to an improved potency compared to the parent compound (since it is possible that in the diastereomeric mixture, one of the epimers is less active), but may also shed light on the characteristics of the molecular target of this particular PanAm.

![Chemical structures of the \( N\)-PE-\( \alpha \)-Me-PanAm diastereomeric mixture as well as the (R)- and (S)-epimers](https://scholar.sun.ac.za)

Figure 4.2: Chemical structures of the \( N\)-PE-\( \alpha \)-Me-PanAm diastereomeric mixture as well as the (R)- and (S)-epimers. The portion in the grey blocks highlights the methyl group and its position and stereochemistry in the molecule.
4.3 Final Remark

Taken together, the scope of the work still to be done on this project illustrates the relevance of the groundwork described in this thesis. Ultimately the elucidation of the mode of action of the PanAms will not only bring us one step closer to developing these compounds as clinically relevant antimalarials but may also provide us new information on the biology of the parasite.
4.4 References


