

The Development of Biocatalytic Methods for the Production of CoA analogues

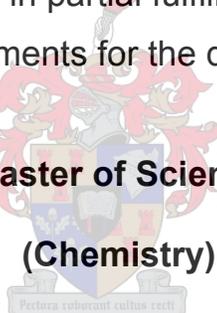
by

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Thesis

submitted in partial fulfilment of the
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Declaration

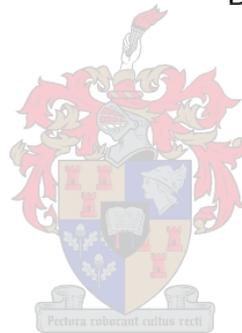
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Summary

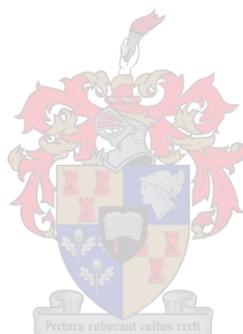
This work focuses on the biocatalytic production of coenzyme A (CoA) analogues with different tether lengths in its pantetheine moiety, and on analogues where the cysteamine moiety has been replaced with a range of other amines. An attempt was made to develop a simple biocatalytic method for the optimum production of such CoA analogues by chemo-enzymatic means.

Pantothenic acid ethyl thioesters with different tether lengths were first synthesized as substrates of the CoA biosynthetic enzymes, CoaA, CoaD and CoaE. The acceptability of these compounds as substrates for the pantothenate kinase (CoaA) from prokaryotic and eukaryotic organisms was investigated through kinetic studies. These substrates were subsequently exposed to CoaA, CoaD and CoaE to produce various general CoA synthons (ethyl pre-CoAs). Finally aminolysis of these ethyl pre-CoAs by cysteamine and homocysteamine gave the various CoA analogues of different tether lengths in their pantetheine moiety. The identical production of a second type of CoA synthon (phenyl pre-CoA) from pantothenic acid phenyl thioesters was also investigated as a means to increase reactivity of the thioester substrates. Aminolysis of the phenyl pre-CoA produced the corresponding CoA derivative, but reactivity was lower than expected.

A second strategy was also developed where the pantothenic acid phenyl thioesters were first aminolyzed, resulting in various pantothenamide intermediates. Aminolysis was attempted with thiol-bearing amines such as cysteamine and homocysteamine as well as with amines without sulfhydryl functionalities. These pantothenamide intermediates were then used in the biosynthesis of the corresponding CoA analogues by addition of CoaA, CoaD and CoaE.

The ideal method of CoA analogue production will utilize a continuous bioreactor system in which these analogues can be prepared on large scale. However, to construct a bioreactor the enzymes involved need to be immobilized on a matrix in

order to transform substrate to product. The enzymes CoaA, CoaD and CoaE can be immobilized on cellulose via a cellulose binding domain (CBD) affinity tag. Various types of CBDs were investigated and used in the construction of suitable expression vectors. Optimum expression conditions to obtain soluble CBD-fused enzymes were developed.



Opsomming

Hierdie studie fokus op die produksie van verskeie koënsiem A (KoA) analoë wat verskil op grond van hulle pantoteenamied funksionaliteite of in die lengte van die pantoteenamiedketting. 'n Poging is aangewend om optimum kondisies te ontwikkel vir die biokatalitiese produksie van hierdie KoA analoë, deur gebruik te maak van 'n chemo-ensiematiese metode.

Verskeie pantoteensuur etieltioesters met verskillende kettinglengtes is gesintetiseer om as substrate vir die KoA biosintetiese ensieme, CoaA, CoaD en CoaE, te dien. Die verskeie tioesters is geanaliseer in die aanwesigheid van prokariotiese en eukariotiese pantoteensuurkinases om te bepaal of hierdie verbindings substrate is vir hierdie ensieme. Die tioesters is vervolgens blootgestel aan CoaA, CoaD and CoaE in die biosintese van 'n algemene KoA sinton (etiesel pre-KoA). Daar is bewys dat indien hierdie etiel pre-KoAs blootgestel word aan verskeie tipes amiene, dit die verwagte KoA analoë tot gevolg het. 'n Meer reaktiewe tioester is ook bestudeer wat gelei het tot die biosintese van 'n tweede KoA sinton (feniel pre-KoA). Alhoewel die verwagte produkte gevorm het, was die reaktiwiteit van the feniel tioester laer as wat verwag is.

'n Tweede strategie in die sintese van die verlangde KoA analoë is aangepak. In hierdie metode word die fenieltioester eers aan verskeie amiene blootgestel. Hierdie reaksies het verskeie pantoteenamied-intermediêre opgelewer wat gebruik is in 'n biosintese reaksie (deur addisie van CoaA, CoaD en CoaE) om die verskeie analoë te berei.

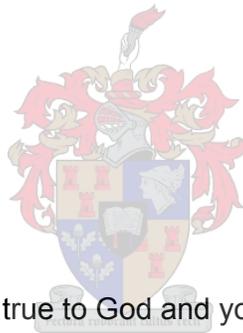
'n Bioreaktorsisteem sal die ideale wyse wees waarop KoA analoë op groot skaal geproduseer kan word. Om egter 'n bioreaktor te ontwikkel moet die ensieme van belang geïmmobiliseer word op 'n vaste matriks. Dit sal die ensieme die vermoë gee om 'n substraat om te skakel na produk in 'n kontinue kolomsisteem. Die ensieme CoaA, CoaD en CoaE kan op 'n sellulose matriks geïmmobiliseer word deur 'n sellulose-bindingsproteïen. Hierdie bindingsproteïen kan aan die

onderskeie ensieme geheg word wat dan affiniteitsbinding van die ensiemkompleks aan sellulose tot gevolg het. Verskeie tipes sellulose bindingsproteïene is ondersoek en gebruik om geskikte uitdrukkingsvektore te kloneer. Optimum uitdrukkingskondisies is ontwikkel om oplosbare ensieme te berei wat gebruik kan word in hierdie bioreaktorsisteem.



*He who trusts in his own heart is a fool,
but whoever walks wisely will be delivered.*

Proverbs 28:26



Be true to God and yourself,
for whatever is meant to happen, will happen...

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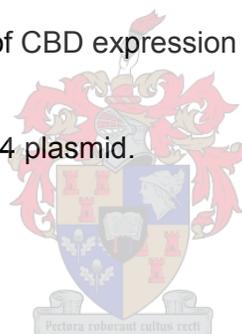


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List of Abbreviations

A	Absorbance
aa	Amino acid
ACPs	Acyl carrier proteins
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine 5'-triphosphate
<i>Bs</i>	<i>Bacillus subtilis</i>
BSA	Bovine serum albumin
CBDs	Cellulose binding domains
CoA	Coenzyme A
CoaA	Pantothenate kinase
CoaB	Phosphopantothenoylcysteine synthetase (also PPC-S)
CoaBC	Phosphopantothenoylcysteine synthetase/ Phosphopantothenoylcysteine decarboxylase (also Dfp)
CoaC	Phosphopantothenoylcysteine decarboxylase (also PPC-DC)
CoaD	Phosphopantetheine adenylytransferase (also PPAT)
CoaE	Dephospho-coenzyme A kinase (also DPCK)
Cip	Cellulose-integrating proteins
Cbp	Cellulose-binding proteins
<i>Clos</i>	<i>Clostridium</i>
<i>C. cellulovorans</i>	<i>Clostridium cellulovorans</i>
<i>C. thermocellum</i>	<i>Clostridium thermocellum</i>
<i>C. cellulolyticum</i>	<i>Clostridium cellulolyticum</i>
CTP	Cytidine 5'-triphosphate
Cys	Cysteine

DECP	Diethyl cyanophosphonate
DMF	<i>N,N</i> -Dimethylformamide
DPPA	Diphenylphosphorylazide
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i> (also <i>Ec</i>)
Ek	enterokinase
ESI-MS	Electrospray Ionization Mass Spectroscopy
FAS	Fatty acid synthase
FMN	Flavin mononucleotide
Gly	Glycine
GuHCl	Guanidine hydrochloric acid
HATs	Histone acetyltransferases
HEPES	<i>N</i> -2-Hydroxyethylpiperazine- <i>N'</i> -2-ethane sulphonic acid
His	Histidine
HPLC	High Performance Liquid Chromatography
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl-thiogalactoside
Kan ^R	kanamycin resistant
k_{cat}	Turnover number
K_a	Affinity constant
K_d	Dissociation constant
kDa	Kilodalton
K_M	Michaelis constant
LC-MS	Liquid Chromatography Mass Spectroscopy
Lys	Lysine
LB	Luria Bertani
NA	Not active
NADH	Nicotinamide adenine dinucleotide (reduced)
NH ₄ OAc	Ammonium acetate
NMR	Nuclear Magnetic Resonance Spectroscopy
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis

PanK	Pantothenate kinase
PCPs	Peptide carrier proteins
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PT-linker	Proline-threonine linker
PUFAs	Polyunsaturated fatty acids
<i>S. aureus</i>	<i>Staphylococcus aureus</i> (also <i>Sa</i>)
Ser	Serine
SDS	Sodium dodecyl sulphate
Tb	Thrombin
<i>t</i> -BOC	<i>tert</i> -butoxy carbonyl
Thr	Threonine
TRIS-HCl	Tris(hydroxymethyl)aminomethane-HCl
V_{\max}	Maximal velocity
Xa	Factor Xa.



The importance of coenzyme A analogues

1.1 The importance of coenzyme A in living systems

Enzymes can catalyze a wide variety of chemical transformations by using functional groups of their component amino acids in various capacities. However, for certain reactions enzymes require the participation of additional small molecules known as cofactors, whether it is metal ions or organic molecules (1). One such cofactor, coenzyme A (CoA 1.1, figure 1.1), is ubiquitous and essential in metabolism and along with its thioesters, CoA is in great demand as substrates for approximately 9% of all enzyme activities (2).

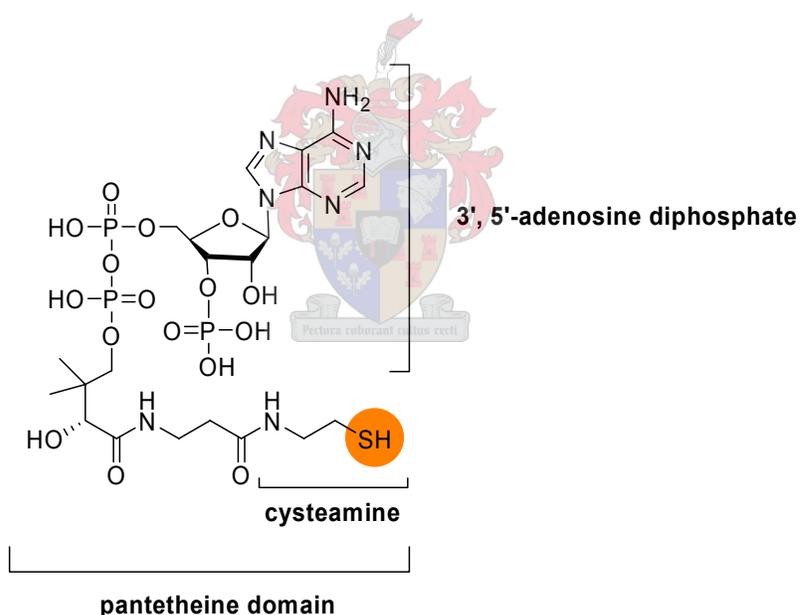


Figure 1.1 The structure of coenzyme A (1.1).

Lippmann (3, 4) discovered coenzyme A in 1945. The covalent structure of CoA was determined by hydrolysis and chemical analysis by Baddiley *et al.* (4, 5). The structure of CoA consists of a 3'-phosphoadenosine moiety and pantetheine (derived from pantothenic acid), which is linked by a pyrophosphate group.

CoA is an essential cofactor utilized by all organisms and has to be biosynthesized by the organism itself. Pantothenic acid is the most advanced CoA precursor that can be taken up by cells (6, 7) and is produced by bacteria and plants. However, in mammals this precursor has to be supplied through dietary sources (6). Coenzyme A is biosynthesized in five steps from pantothenic acid, also known as pantothenate or vitamin B₅. This biosynthetic pathway will be reviewed in Chapter 2.

1.1.1 Function of CoA

CoA is functionally a simple molecule. The sulfhydryl group of the cysteamine moiety is the functional group that is directly involved in the enzymatic reactions for which CoA serves as cofactor (8). In most instances CoA participates in a variety of acyl transfer reactions (9), where it acts as carrier of an acyl group by forming a thioester between the carboxylate of the substrate and the thiol of the CoA cofactor.

The acylation of the thiol group of CoA gives an acyl derivative (for example acetyl-CoA 1.2) that is activated in two ways. The thioester can react as an electrophile toward attack by a nucleophilic substrate or the thioester α -carbon can react as nucleophile upon deprotonation (figure 1.2) (9).

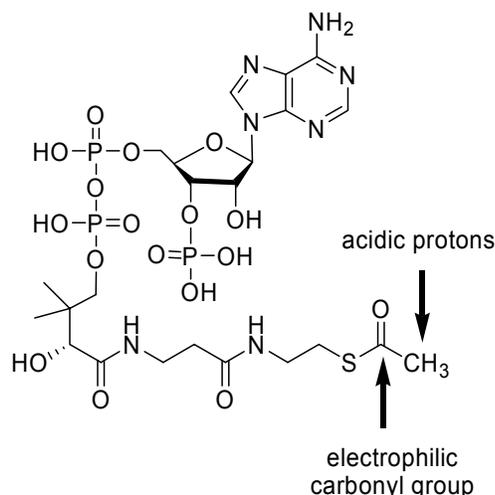


Figure 1.2 Two general modes of reactivity of acetyl-CoA (1.2) upon acetylation.

1.1.2 The importance of CoA in thioester formation

Acyl-group transfers at physiological pH are involved in many reactions in metabolism. Esterification of these acids to the thiol group of CoA is the predominant means by which these acids are activated (8). The activation of these acids is a necessary prerequisite for the Claisen condensation reactions that occur between various types of CoA derivatives in metabolism.

In biological systems, thiol esters are preferentially used in Claisen condensation reactions. This occurs mainly due to the special characteristics of thiol esters in comparison to oxygen esters. The first of these is based on size: the sulphur atom of thiol esters is larger than the oxygen atom in oxygen esters, leading to C-S bonds to be longer than corresponding C-O bonds. Sulphur utilizes 3*p* valence electrons rather than 2*p* electrons that lead to much less orbital overlap between the sulphur and the carbonyl carbon than in the oxygen system. Consequently, because the carbon and sulphur nuclei are further apart and allow less π -overlap, sulphur forms double bonds to carbon less readily than oxygen. This lowers the level of resonance stabilization observed in thiol esters compared to oxygen esters (figure 1.3), because resonance forms involving the sulphur of the thiol ester do not contribute extensively to the resonance stabilization (11). As a result, the carbonyl group of the thioester acts more like a ketone in terms of reactivity (10).

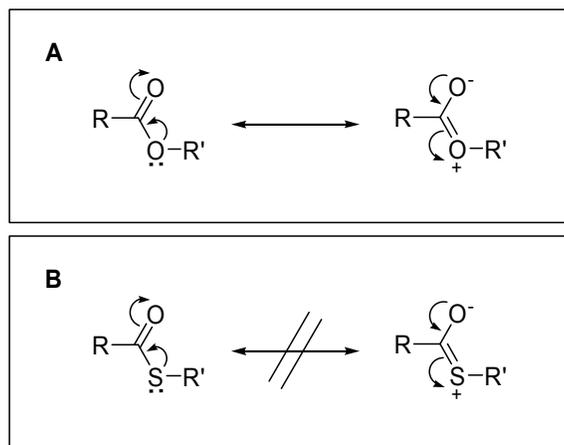


Figure 1.3 Contribution to resonance stabilization of oxygen esters (A) vs. thiol esters (B) (11).

Another factor that has to be considered is the ability of these esters to form carbanions. In thiol esters, carbanion formation at the α -carbon atom is also more favourable than for oxygen esters. The resonance stabilization is essentially the same for both the carbanions (figure 1.4). However, the carbonyl group of the thiol ester has more double bond character than the oxygen ester. The resonance stabilization (shown in figure 1.4) will thus be more favourable for thiol than for oxygen esters (11) leading to more reactivity for thiol esters in comparison to oxygen esters.

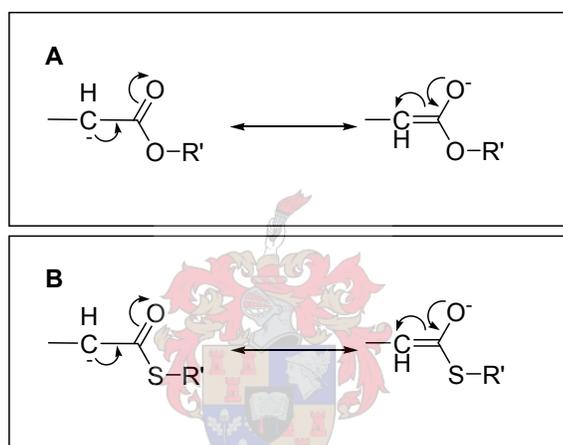


Figure 1.4 Carbanion stabilization of oxygen (A) vs. thiol esters (B) (11).

In conclusion, because sulphur does not donate electrons to an attached carbonyl group as well as oxygen does, thiol esters are better acyl transfer agents than oxygen esters. Thiol esters also contain a greater proportion of the enol tautomer at equilibrium (12). All these properties are apparent in acetyl-CoA and allow this compound and similar CoA derivatives to be involved in Claisen condensation reactions. Claisen condensation reactions occur in the condensation of acetyl-CoA with oxaloacetate in the tricarboxylic acid cycle, in fatty acid biosynthesis catalyzed by fatty acid synthases and in the formation of the broad variety of polyketide natural products produced by a diverse group of micro-organisms (13).

1.2 Applications of CoA analogues

CoA analogues can be defined as molecules that have the same basic building blocks as CoA, but certain functional groups on these basic moieties differ from CoA. CoA and its acyl derivatives already play an important role in biological systems. For example, CoA esters are involved in various essential processes like fatty acid biosynthesis and degradation, cell-cell mediated recognition and numerous other metabolic processes. Acetyl-CoA is involved in antibiotic resistance via enzyme-catalyzed acylation. Along with other examples of CoA derivatives, these compounds play a central role in many diverse areas of biology (1).

Due to the importance and ubiquity of these compounds, production of different kinds of CoA analogues could be beneficial for future research in metabolism. Two kinds of CoA analogues have already been identified. The first group consists of analogues showing antimetabolite characteristics, like ethyldethia-CoA and butyldethia-CoA (14-16), while the second group is used as mechanistic probes in enzyme-catalyzed reactions (for example in fatty acid biosynthesis (17)).

1.2.1 Utilization of CoA analogues as antimetabolites

Influence of CoA analogues on acyl carrier proteins (ACPs)

CoA is required for the synthesis of ACP, the acyl group carrier in bacterial fatty acid biosynthesis, by serving as precursor for ACP (1). *E. coli* holo-ACP synthase catalyzes the transfer of the phosphopantetheine moiety from CoA to the serine 36 hydroxyl group of the apo-form of ACP. As a result the active holo-form of ACP is formed (1). This reaction is illustrated in figure 1.5. ACP synthase is the product of the *acpS* gene, which was identified by Polacco and Cronan (7).

Although ACP is larger than CoA, it uses the phosphopantetheine group as a functional group for essentially the same purpose as CoA.

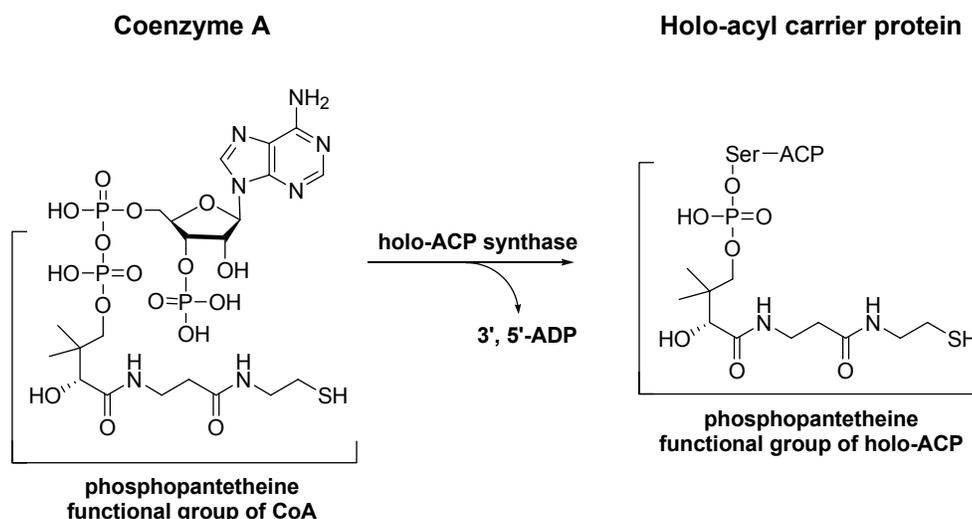


Figure 1.5 Enzymatic synthesis of holo-acyl carrier protein from CoA with the elimination of 3', 5'-ADP. The phosphopantetheine moiety is functionally the same in both molecules.

ACP is a necessary component for all the reactions of fatty acid biosynthesis. The phosphopantetheine functional group is used to esterify fatty acids to the sulfhydryl group of the cysteamine moiety. The resulting thioester can then undergo various different modifications. For example these thioesters can be used to produce desaturated fatty acids (figure 1.6).

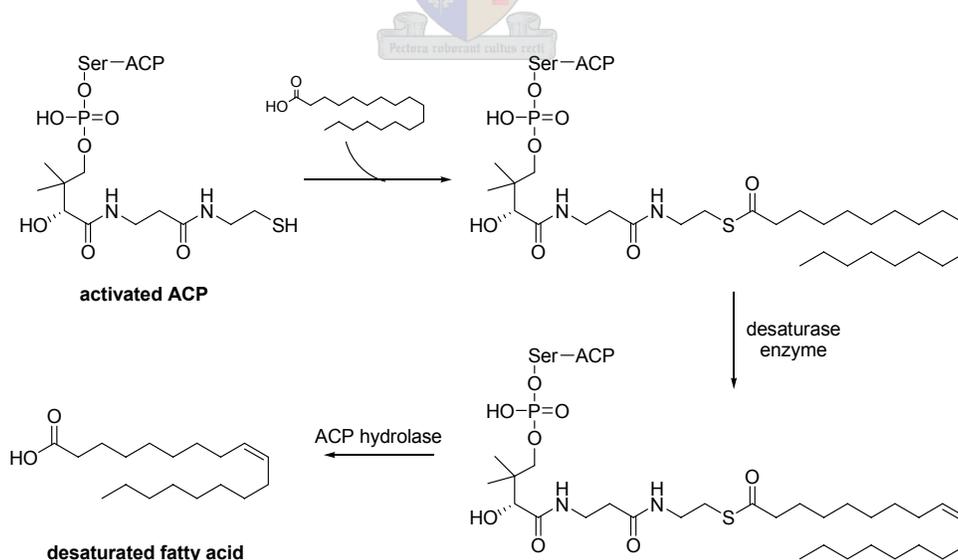


Figure 1.6 Function of holo-ACP in the desaturation of fatty acids

ACP also accepts acyl derivatives of CoA to form the corresponding ACP thioesters. These acyl-ACPs have been used for polyketide synthases in the catalysis of the biosynthesis of bacterial aromatic polyketides (1, 18). Proteins similar to ACP, so-called peptide carrier proteins (PCPs), play a role in non-ribosomal peptide synthetases where it is used in the non-ribosomal biosynthesis of peptide antibiotics by multimodular synthetases (1, 19). The proteins carry amino acids as their acyl derivatives.

Several compounds are biosynthesized from the *N*-alkylpantothenamide class of antibacterials, which have shown to be active against bacteria. These CoA analogues like ethyldethia-CoA and butyldethia-CoA have shown *in vitro* inhibitor characteristics of CoA utilizing enzymes like acyl carrier proteins (1, 14-16). These compounds have an alkylpantothenamide moiety instead of a terminal sulfhydryl like in CoA. The biological effects of these compounds are exerted through the transfer of the inactive 4'-phosphopantothenamide moiety from the CoA analogues to the ACP. This causes accumulation of inactive ACPs and also results in cessation of fatty acid synthesis (16). These analogues are thus inhibitors of CoA and acetyl-CoA utilizing enzymes due to the fact that there is no terminal thiol group to allow esterification, therefore leaving the ACP inactive after transfer of the phosphopantothenamide moiety (figure 1.7) (15). This characteristic thus allows these compounds to act as antimetabolites.

Chapter 1 - The importance of coenzyme A analogues.

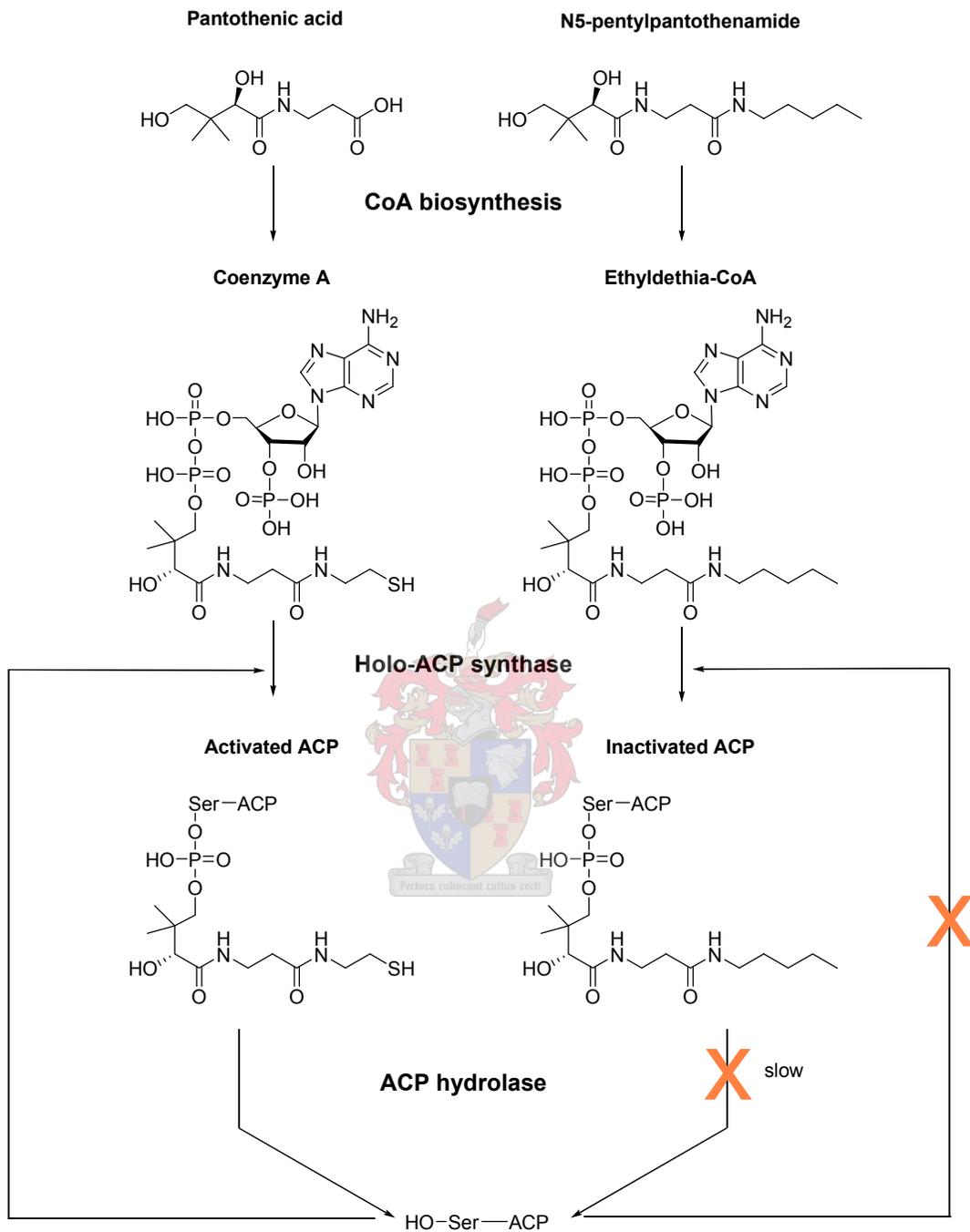


Figure 1.7 The metabolism of pantothenic acid and pantothenamide in *E. coli*. Pantothenic acid and *N*-pentyl pantothenamide are converted to CoA and ethyldethia-CoA, respectively. The pantotheine moieties from each molecule are then transferred to create the holo-ACP molecule. In the case of the pantothenamide analogue, the holo-ACP is inactive due to the lack of the sulfhydryl group for acyl chain attachment. The prosthetic group of ACP is hydrolyzed by ACP hydrolase for the pantotheine-ACP, whereas the inactivated ACP is not a good substrate for this enzyme (15).

Inhibitors of the acetyl-CoA-dependent histone acetyltransferases

It has been shown that reversible protein acetylation is a major mechanism for the regulation of gene expression and chromatin remodelling (20, 21). The histone acetyltransferases (HATs) catalyze the transfer of the acetyl group from acetyl-CoA to the ϵ -amino lysine group in histones and other proteins. These enzymes are critical to transcriptional control in a range of pathways. It has also shown to be important in normal development and disease. Inhibitors for this enzyme would be very useful as a biological tool and may have therapeutic value (20).

Despite significant efforts, potent and specific inhibitors of HATs have not been reported until recently (20). In 2003, several bisubstrate inhibitors of the acetyl-CoA-dependent histone acetyltransferases were developed (21). One of these substrates, Lys-CoA (Figure 1.8; 1.3), has been shown to block the activity of HATs and this compound has been used by a number of groups to evaluate the function of HATs. A series of Lys-CoA derivatives has also been under investigation as inhibitors of this enzyme (20).

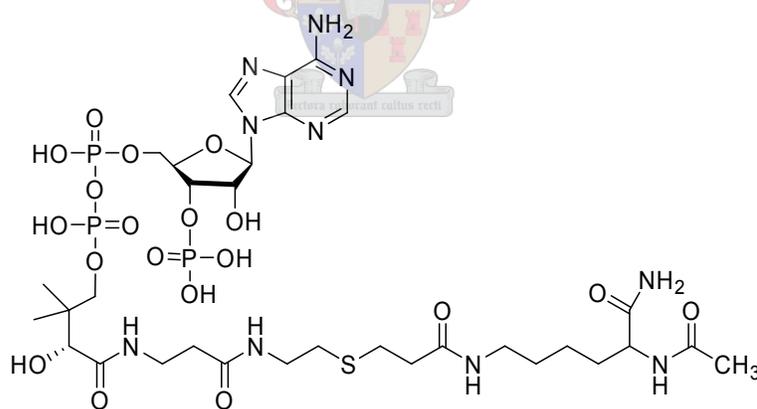


Figure 1.8 The structure of Lys-CoA (1.3) (20)

1.2.2 Biocatalysis of fatty acids via CoA analogues

CoA has many functions in living systems and one of its tasks is to assist in fatty acid biosynthesis. Fatty acids are usually esterified to the phosphopantetheine moieties of the acyl carrier protein (ACP) or coenzyme A (CoA) (figure 1.6). It also occurs as the major constituent of various types of lipids and thus rarely occurs in cells in the free acid form (7). It is in one of these forms that fatty acids are biosynthesized, metabolized and modified. An important modification which saturated fatty acids can undergo is regio- and stereospecific desaturation. This results in the conversion of carboxylic acids into a variety of mono- and poly unsaturated fatty acids. Unsaturated fatty acids are important for normal cell function, whether it as constituents of the cell membrane or as precursors of other essential biomolecules such as prostaglandins. In mammals, these compounds cannot be biosynthesized by the organism itself and thus have to be provided in dietary sources.

Certain long chain polyunsaturated fatty acids (PUFAs) recently raised interest due to their elucidation of their biological role in clinical conditions and emerging therapeutical roles. It is anticipated that the current sources of these acids (seed oils, marine fish and certain mammals) will be inadequate for the PUFA market. Therefore, as a result of the biomedical and nutraceutical interests alternative sources need to be explored to help provide an adequate supply of these compounds for the future market. The additional discovery of methods in which to produce novel mono-unsaturated fatty acids would also be of great interest.

The biosynthetic pathway of CoA could play an important role as target for the production of these compounds by utilizing it in the production of different analogues of CoA that will differ in the length of the pantetheine moiety. The production of unsaturated fatty acids can be achieved by using these specific analogues in combination with a biocatalyst. Such a biocatalyst will be based on soluble desaturase enzymes, such as the acyl-ACP Δ^9 desaturase from the castor oil plant (22). Regardless of the source of the desaturase enzymes, these enzymes are all quite specific as to the regio- and stereospecificity of the fatty

acids on which they act. However, a change in regioselectivity may be achieved by using specially modified ACPs to which the fatty acids are subsequently esterified. Such modified ACPs can be prepared from modified CoAs, which will act as the source of the phosphopantetheine moiety in the reaction catalyzed by ACP synthetase (phosphopantetheine transferase) enzyme. These enzymes have previously been shown to accept a variety of CoA analogues as donor molecules and as a result transfers modified phosphopantetheine group to the ACP (figure 1.9) (17).

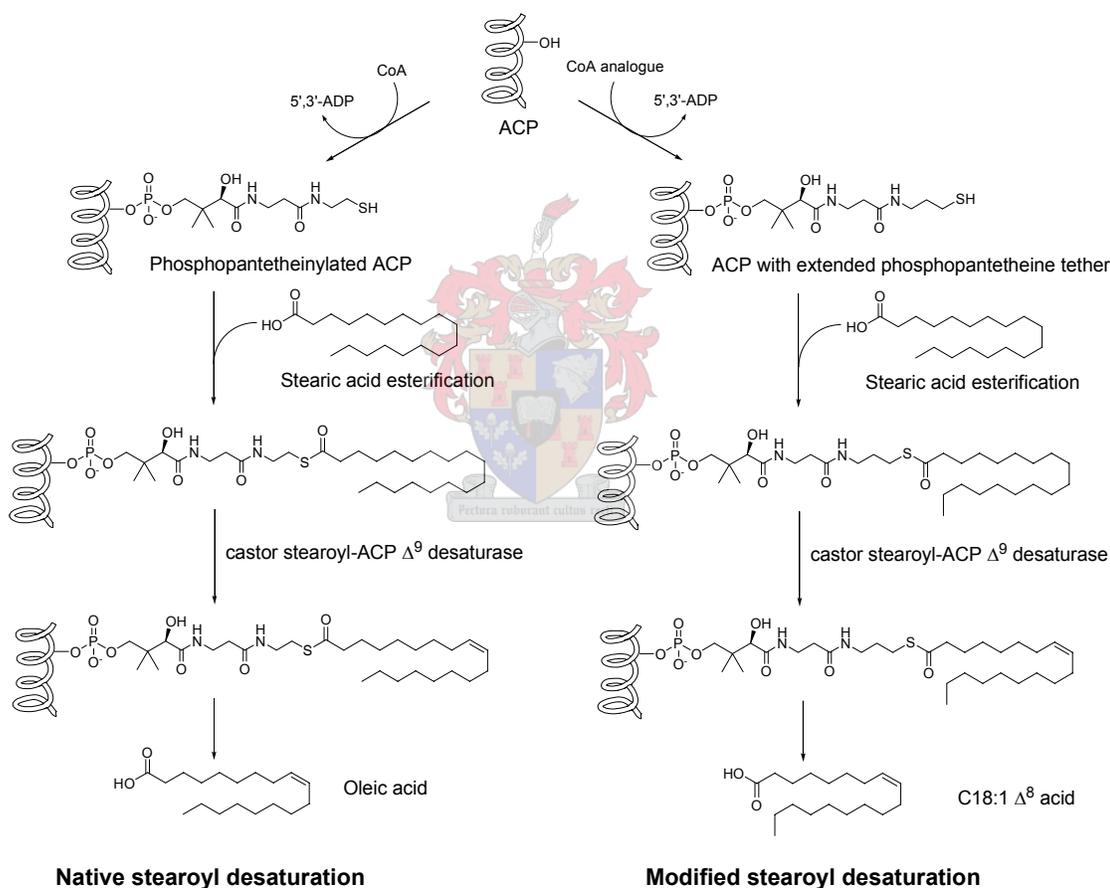


Figure 1.9 Fatty acid desaturation as catalyzed by a typical desaturase enzyme and modification of the process using CoA analogues.

Some successes have already been reported in this area: for example, the castor stearoyl-ACP Δ^9 desaturases which reacts on 18:0 fatty acids was modified to act on 16:0 acids. This was done without loss of catalytic activity (23). In this case

substrate specificity of the enzyme was altered by modification of the fatty acid binding pocket. A patent related to this work has already been granted, in which it was suggested that the enzyme's regiospecificity could also be modulated in a similar manner (24).

The development of an appropriate system based on the strategy above can have large impact in the field of essential fatty acid production and its application.

1.3 Objectives of this study

CoA analogues can be generally obtained in three ways. A first approach relies on the preparation of analogues by using conventional methods to synthesize the product from different available substrates. This non-enzymatic method often provides more practical and versatile routes for the preparation of CoA analogues, but is tedious and time-consuming (1). In the second approach, the native CoA molecule can be modified chemically to form new analogues. For example, derivatives of acyl-CoA can be prepared by acylation of the free thiol of CoA activated esters (9). A variety of CoA thioesters, CoA thioethers, desulpho-CoA and CoA sulfoxides and sulphones can also similarly be prepared by both enzymatic and non-enzymatic reactions. This specific method however is not the best approach as side reactions can occur at functional groups that are not targeted by the transformation. Activated esters also show non-specific reactivity, which can be a problem (1). A third approach depends on a combined chemical and enzyme-based synthetic strategy to produce CoA analogues. In this strategy, purified recombinant *E. coli* enzymes can be used for the modification of synthesized pantothenate derivatives. Synthesis of these pantothenate derivatives, followed by a one-pot assembly into CoA scaffold has already been attempted, but has the disadvantage of feedback inhibition by *E. coli* pantothenate kinase (the enzyme that catalyzes the first step in the biosynthesis of CoA), which lowers the yield of the product (9).

In this study we will aim to use the chemo-enzymatic synthetic approach in the production of different kinds of CoA analogues. A strategy will be developed to produce CoA analogues in a simple, clean method that does not utilize long reaction times, harsh conditions or complicated synthesis.

1.3.1 Objective 1: Production of CoA analogues via a bioreactor system.

The first objective of this study was to investigate the feasibility of constructing a bioreactor for the production of CoA analogues. This strategy entails the immobilization of the enzymes of the biosynthetic pathway of CoA on solid support, for the production of these various analogues in column format. The enzymes of biosynthetic pathway of CoA can be immobilized on cellulose via a cellulose-binding domain (CBD). This small peptide is a fusion protein that will serve as an affinity tag for enzyme immobilization. The immobilized proteins can then be used affixed to a cellulose column, where the protein becomes an important tool to be used in a bioreactor system in the production of the CoA analogues of choice. This strategy will be discussed in Chapter 3.

1.3.2 Objective 2: CoA analogues containing different tether lengths

Various studies have been done on the production of CoA analogues by using a general synthetic approach to prepare these compounds by using a combination of enzymatic and non-enzymatic reactions (25-27). In general these studies used complicated and time-consuming synthetic strategies.

In this study an attempt was made to produce CoA analogues that differ in the length of the pantetheine moiety of the CoA molecule by chemo-enzymatic synthesis, with the ultimate goal of using these analogues in the modified desaturase system described above. These different molecules have an added or removed methylene unit to the distal portion of the molecule as seen in the examples in figure 1.10. An approach similar to the strategy of Martin *et al.* (26) was used in the production of these analogues. Pantothenic acid thioesters were prepared with different pantetheine tether lengths. These substrates were then used in combination with enzymes from the biosynthesis of CoA to produce a

thioester CoA synthon. This thioester CoA synthon differs from CoA by replacing the amide functional group of CoA by a thioester. Aminolysis of this thioester bond with either cysteamine or homocysteamine formed the analogue of preference with varying tether lengths as seen in figure 1.11, Strategy 1.

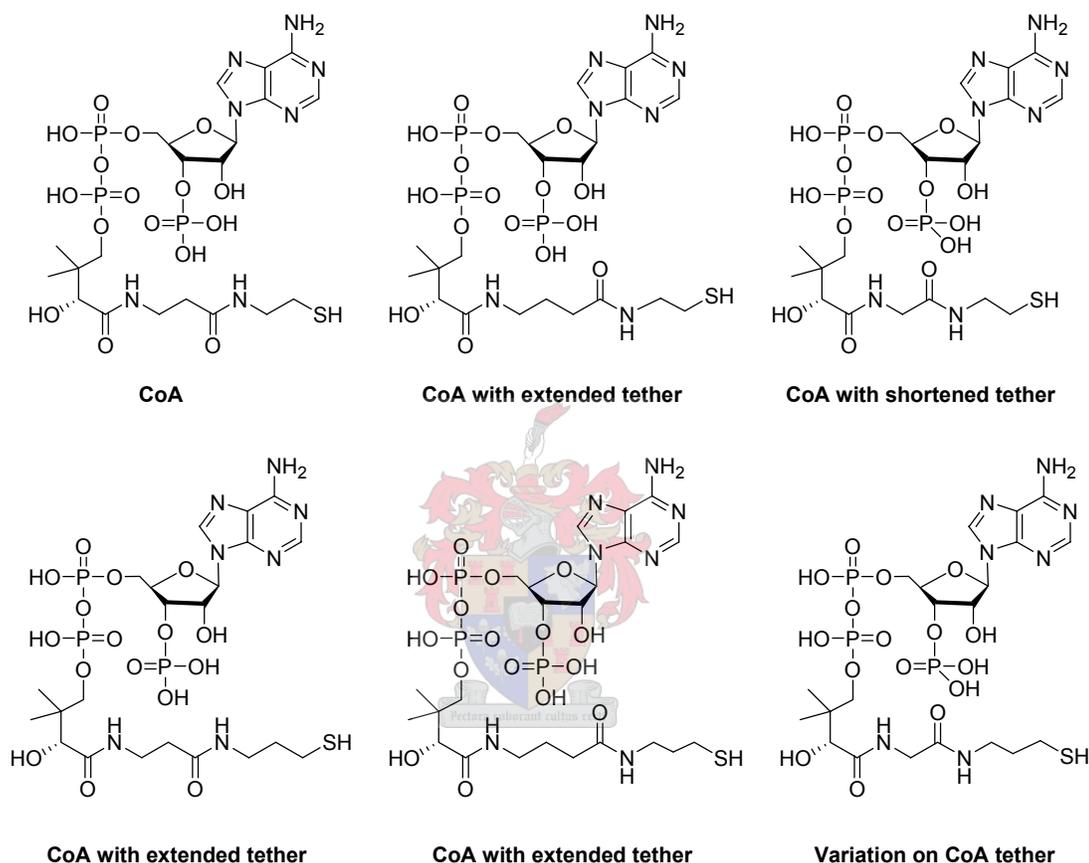


Figure 1.10 CoA compared to its proposed analogues with different tether lengths.

This strategy was developed as described in chapter 4. The aminolysis reaction of the thioester CoA synthon with cysteamine and homocysteamine was optimized by investigating different thioesters as activated substrates. In combination with these results a second strategy was investigated (strategy 2; figure 1.11). In this strategy, aminolysis of the thioester-activated substrate was attempted first followed by the biosynthesis of the CoA analogue from the corresponding pantothenamide analogue. These findings are discussed in chapter 5.

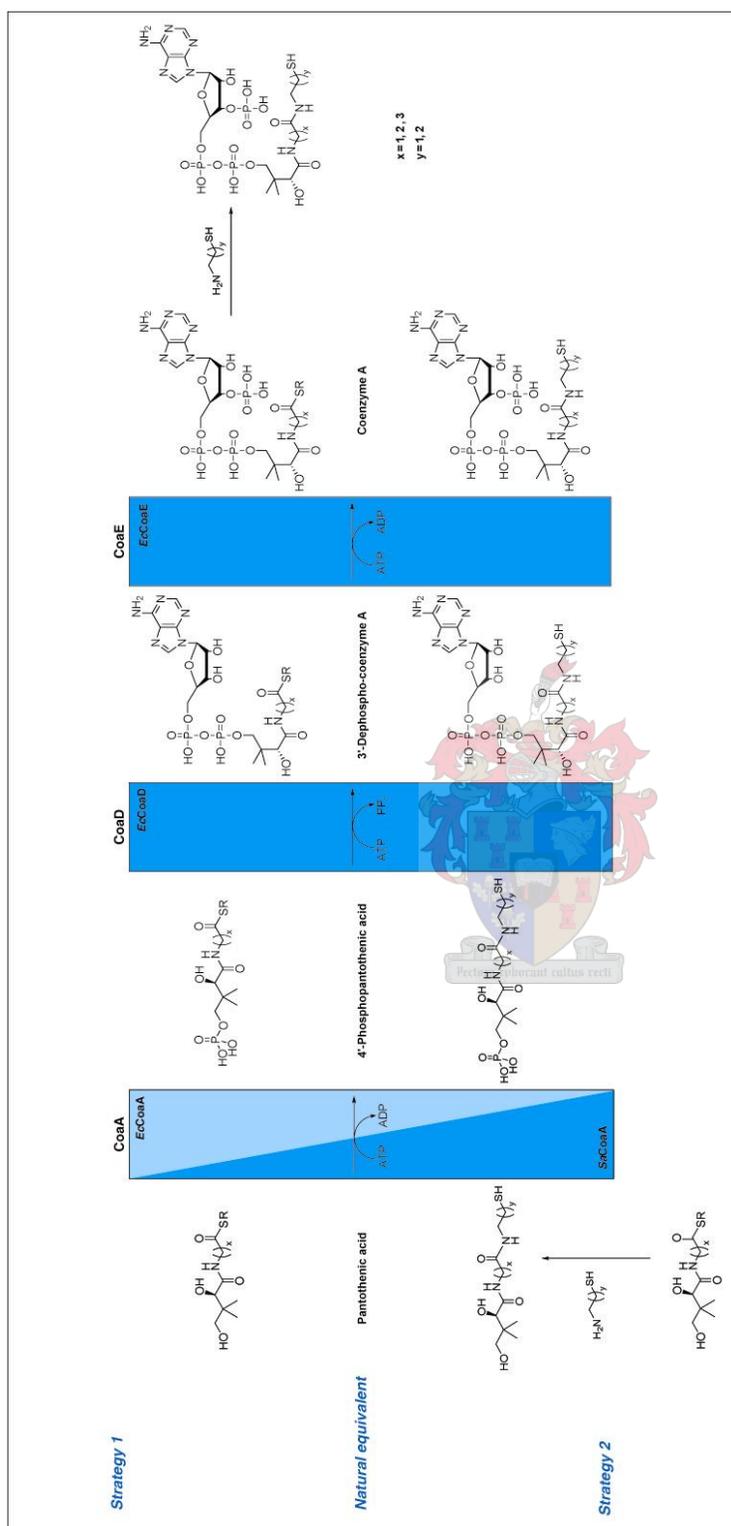


Figure 1.11 Different strategies used in the production of CoA derivatives.

1.3.3 Objective 3: Optimization of CoA analogue production

As an expansion of objective 2, the last objective of this particular study was to develop a method for the chemo-enzymatic synthesis of various CoA analogues with a variety of functional groups. This would allow the production of various types of CoA analogues and not just thiol bearing analogues. Therefore, strategy 2 (figure 1.11) was expanded to include amines with a variety of functional groups, including alcohols, aromatic rings and alkyl groups, to prepare the corresponding pantothenamide analogues. These different pantothenamide analogues were then used in the biosynthesis of the corresponding CoA analogues.

1.4 Conclusion

Coenzyme A and its derivatives play very important roles in biological systems. It is expected that both new and existing CoA analogues will continue to be very valuable in future research. These compounds have been studied intensively, but convenient methods for the synthesis of such analogues are only beginning to be developed. The general aim of this study was to develop a method to produce CoA analogues via chemo-enzymatic synthesis. This method utilized short reaction times with mild reaction conditions. A bioreactor system will also be constructed in which this chemo-enzymatic method will be applied to produce these compounds.

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Chapter 2

A review of Coenzyme A and its biochemical pathway

2.1 Introduction

Coenzyme A (CoA; **1.1**) is an indispensable cofactor in all living systems, where it has various functions such as being an acyl group carrier and carbonyl-activating group in a number of central biochemical transformations, including the tricarboxylic acid and fatty acid metabolism (1). Along with its thioesters, CoA is in great demand as substrates for approximately ~9% of all enzyme activities (2), where it participates in a variety of acyl transfer reactions. CoA is involved in over 100 different reactions in intermediary metabolism (3).

Interest in CoA has been renewed the past few years due to the fact that the biosynthetic pathway is a good target for antibacterial drug discovery and from the unexpected association of a human neurodegenerative disorder with mutations in pantothenate kinase, the enzyme that catalyzes the first step in the biosynthesis of CoA (1). Coenzyme A has also shown significant importance in the pharmaceutical, nutraceutical and cosmetic industries (4). The biosynthesis of CoA has various aspects of interest that will be explored and reviewed in this chapter. The characteristics of CoA, its precursors and the different enzymes involved in the five step biosynthesis which produces CoA in living systems will be summarized. Only proteins of interest to this particular study will be reviewed.

2.2 Structure and biosynthesis of coenzyme A

Closer investigation makes it clear that CoA (figure 2.1) is a structurally complex molecule (5). A phosphorylated pantotheine domain, derived from pantothenic acid (a member of the vitamin B family) makes up one part of the molecule, while the other part consists of 3',5'-adenosine diphosphate joined to 4'-phosphopantotheine in a phosphoric anhydride linkage. The pantotheine moiety can also be divided into two functionalities. The distal portion consists of a cysteamine moiety, also known as β -mercaptoethylamine, and pantothenic acid. The sulfhydryl group of the cysteamine is directly involved in the acyl transfer reactions, which is one of CoA's main functions. The adenine moiety of CoA acts as a recognition site, increasing the affinity of CoA binding to its enzyme (6).

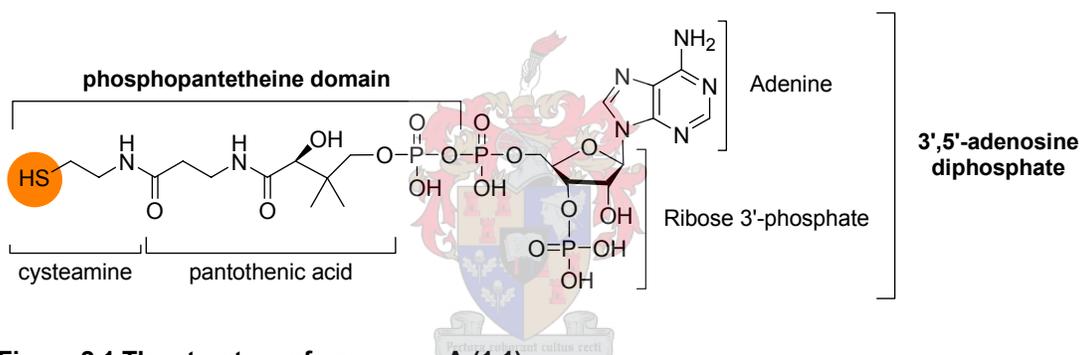


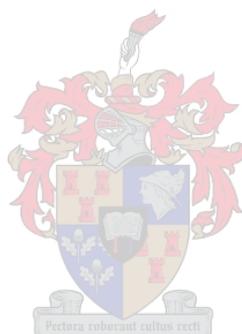
Figure 2.1 The structure of coenzyme A (1.1)

The biosynthesis of CoA (figure 2.2) is a universal pathway in prokaryotes and eukaryotes and is essential in all organisms (1). All the CoA biosynthetic genes in bacteria, plants and mammals are known. The biosynthesis of CoA proceeds in five enzymatic steps from its vitamin precursor, pantothenic acid (also known as Vitamin B₅). Pantothenic acid (2.2) is first phosphorylated by pantothenate kinase (PanK; CoaA) with the consumption of ATP to yield 4'-phosphopantothenic acid (2.3). L-cysteine is then coupled to this compound by 4'-phosphopantothenoylcysteine synthetase (PPC-S; CoaB). The intermediate 4'-phosphopantothenoylcysteine (2.4) is decarboxylated by 4'-phosphopantothenoylcysteine decarboxylase (PPC-DC; CoaC) to yield 4'-phosphopantetheine (2.5). Dephospho-CoA (2.6) is formed by

Chapter 2 - A review of Coenzyme A and its biochemical pathway.

phosphopantetheine adenyltransferase (*PPAT*; *CoaD*), which couples an AMP moiety from ATP to the phosphate of 4'-phosphopantothenic acid, with the concomitant formation of inorganic pyrophosphate. The 3'-hydroxy group is phosphorylated by dephospho-CoA kinase (*DPCK*; *CoaE*) to yield CoA (1.1). The overall process requires four equivalents of ATP, one of which provides the adenine moiety of CoA (1, 7, 8).

All of the genes encoding for the enzymes in the CoA biosynthetic pathway have been identified, characterized and overexpressed during the past few years and the structures of several of these enzymes were determined.



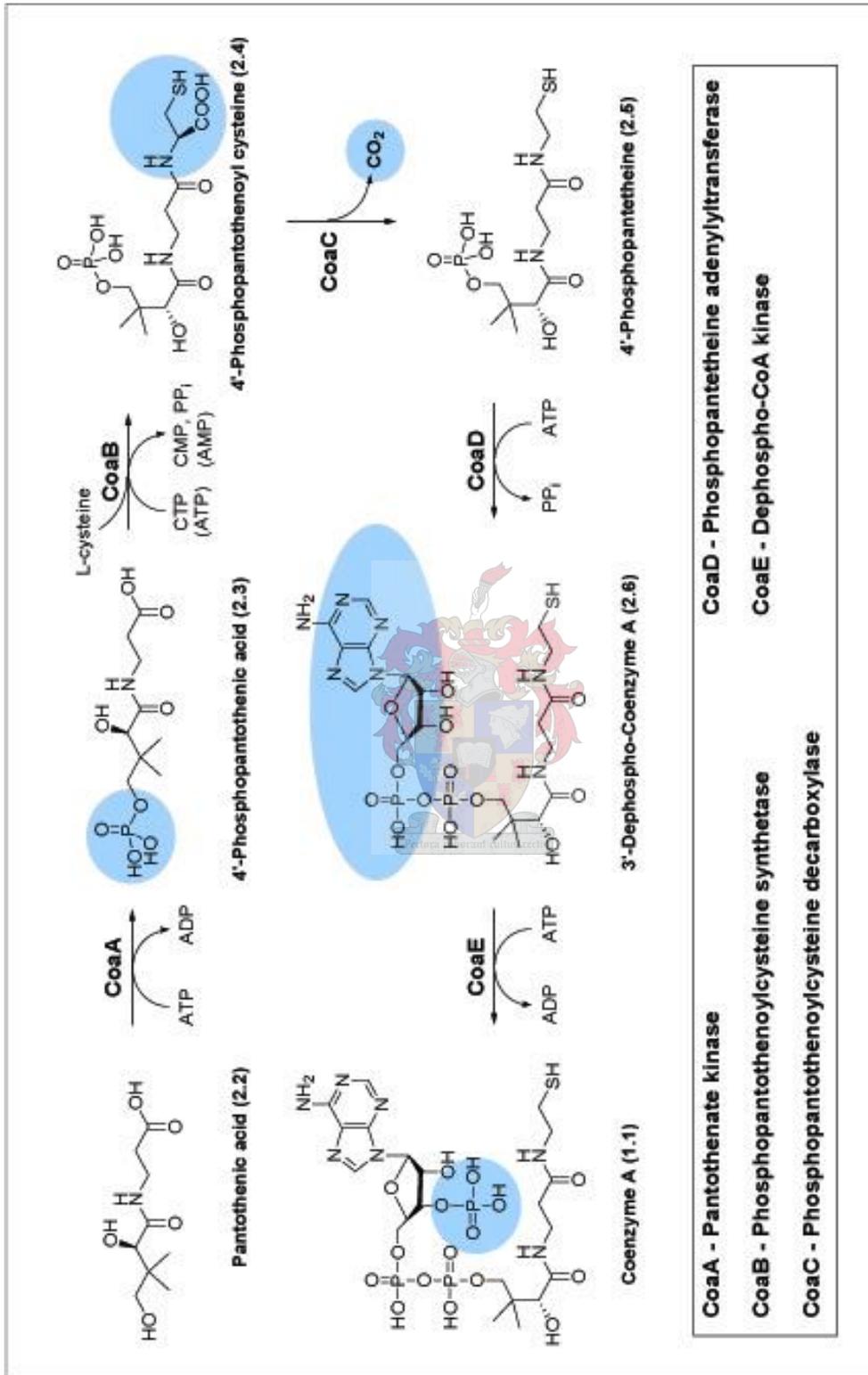


Figure 2.2 the biosynthesis of coenzyme A

2.3 Pantothenate as precursor of coenzyme A

Pantothenate (**2.2**), vitamin B₅, is one of several small molecules that are essential for animal nutrition. The biochemical role of this precursor in all organisms is to form the core of the structure of coenzyme A. Bacteria divert amino acids and their biosynthetic intermediates to produce pantothenate, which also plays a role in the synthesis of many secondary metabolites including lignin (7). Certain organisms, like animals and some microbes lack the capacity to synthesize pantothenate and are totally dependant on the uptake of exogenous pantothenate (1). However, pantothenate is readily available to these organisms through dietary sources. Pantothenate is found virtually everywhere due to the fact that most bacteria (e.g. *Escherichia coli*) and fungi (e.g. *Neurospora crassa*) have the ability to synthesize this precursor from β -alanine and D-pantoate.

E. coli is capable of *de novo* pantothenate biosynthesis or can import pantothenate from the medium via a sodium-dependant active transport process (9). In general, this organism produces and secretes 15 times more pantothenate than required for intracellular CoA biosynthesis. Plants also synthesize pantothenate *de novo* and along with bacteria it is the major source of vitamin B₅ in the diet of mammals. A controlled study showed that sufficient quantities of pantothenate are obtained from ruminant micro-organisms to maintain animals without pantothenate supplementation. Due to the ubiquitous nature of this compound no vitamin B₅ deficiency in humans has been reported (1).

Phosphopantetheine (**2.5**) is incorporated into the prosthetic group of acyl carrier proteins in fatty acid synthetases, polyketide synthetases and non-ribosomal peptide synthetases and is directly derived from pantothenate that accentuates the importance of this substrate in living systems.

2.4 Enzymes of the CoA biosynthetic pathway

2.4.1 Pantothenate kinase (PanK; CoaA)

Pantothenate kinase catalyses the ATP-dependant conversion of pantothenic acid to 4'-phosphopantothenic acid that forms the first committed and most regulated step in the five-step biosynthesis of CoA. The *coaA* gene encodes pantothenate kinase, which is also known as CoaA or PanK. This gene was first identified in *Salmonella typhimurium* and *E. coli* and subsequently in numerous other bacteria by comparative genomics. CoaA proteins from *E. coli* and *Staphylococcus aureus* have been expressed and purified in various previous studies and was used in this particular investigation. The *E. coli* enzyme has been extensively characterized by previous studies and is considered the prototypical bacterial CoaA. It is also structurally distinct from the eukaryotic counterparts. However, the CoaA from *S. aureus* and the putative CoaA from *Bacillus anthracis* are moderately related to the eukaryotic PanK proteins and unrelated to the *E. coli* CoaA (1).

The CoaA protein from *E. coli* (EcCoaA) was first identified as a mixture of two peptides, which are both active and the protein primarily exist as a homodimer of the larger 36kDa subunits, but can also function as a heterodimer. The phosphorylation reaction proceeds by an ordered sequential mechanism (10), with ATP binding to the active site of the enzyme before pantothenic acid. Pantothenic acid forms a phosphoric anhydride linkage with the enzyme-ATP complex and after elimination delivers 4'-phosphopantothenic acid as product (figure 2.3). The binding of ATP is highly cooperative (1). The CoaA activity is inhibited *in vivo* and *in vitro* by unacylated CoA and less efficiently by CoA thioesters resulting in feedback inhibition by these compounds, which will be discussed later.

The *S. aureus* CoaA protein (SaCoaA) has a distinct primary sequence that has limited homology with the mammalian PanK proteins but does not resemble the prototypical bacterial CoaA of *E. coli*. In contrast to all known PanK's, the activity of SaCoaA is not regulated by feedback inhibition through CoA and CoA thioesters. The absence of feedback inhibition results in accumulation of high concentrations of intracellular CoA due to the absence of regulation at the PanK

step of CoA biosynthesis (1). This characteristic makes this enzyme particularly suited to biocatalytic applications.

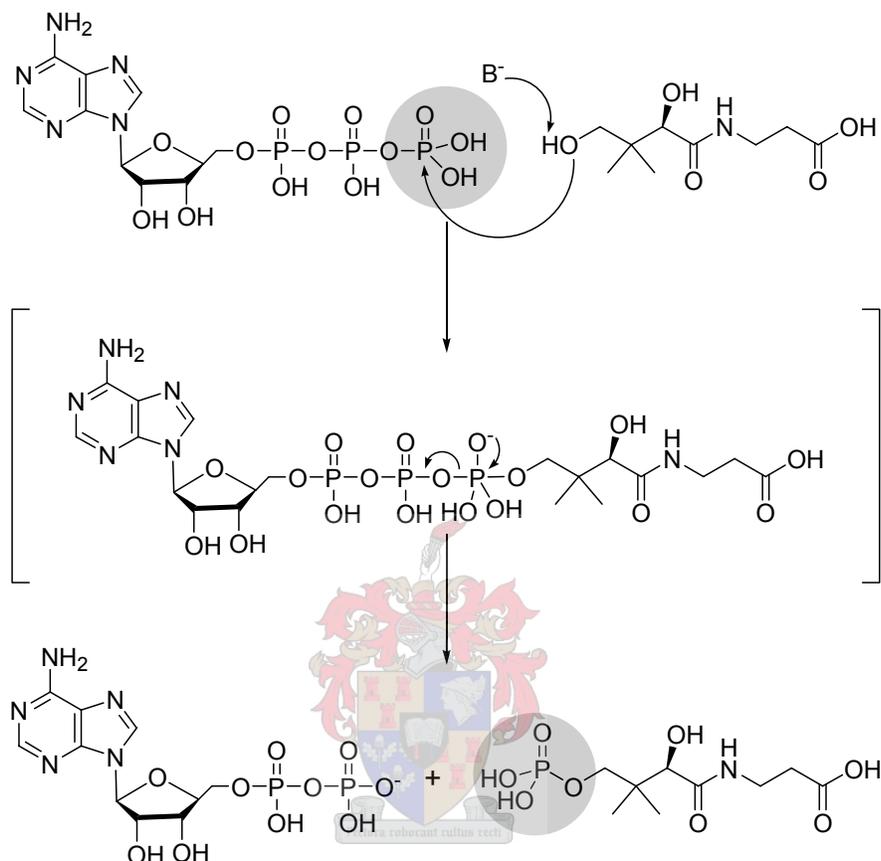


Figure 2.3 The sequential mechanism of the first step in CoA biosynthesis.

Pantothenate kinase is also known in fungi (*Aspergillus nidulans*), plants (*Arabidopsis thaliana*) and in mammals. These PanK's however were not used in this study and will not be discussed.

2.4.2 4'-Phosphopantoenoylcysteine synthetase (PPC-S; CoaB) and 4'-Phosphopantoenoylcysteine decarboxylase (PPC-DC; CoaC)

The 4'-phosphopantoenoylcysteine decarboxylase activity in *E. coli* was initially associated with a 35 kDa protein reported to contain a covalently bound pyruvoyl group. Later, it was discovered that a flavin mononucleotide (FMN)-containing bifunctional enzyme is responsible for both the 4'-phosphopantoenoylcysteine

synthetase and the 4'-phosphopantothenoylecysteine decarboxylase activities. The *dfp* gene was responsible for the encoding of this new enzyme, renamed CoaBC. These activities are fused together in almost all prokaryotes except in *Streptococci* and *Enterococci*, which possess separate genes predicted to encode CoaB and CoaC proteins (1).

E. coli CoaBC (*EcCoaBC*) is a homododecamer protein of 43.8 kDa subunits and the two individual activity domains have been expressed and purified. The carboxy terminal domain (CoaB) encompasses residues 191-406. The first part of this bifunctional enzyme catalyzes the formation of 4'-phosphopantothenoylecysteine from 4'-phosphopantothenate and cysteine. This particular reaction in *E. coli* requires CTP and occurs via an activated acyl-cytidylate intermediate (figure 2.4). This intermediate is then attacked by an amino group of the cysteine molecule. Site-directed mutagenesis identifies Asn²¹⁰, Arg²⁰⁶ and Ala²⁷⁶ as residues involved in the second half-reaction and these residues are proposed to bind cysteine (1).

The use of CTP is a characteristic feature of bacterial CoaB proteins because human and partially purified rat liver 4'-phosphopantothenoylecysteine synthetases use ATP (1, 3, 8). There is little sequence similarity between prokaryotic CoaB domains and their monofunctional eukaryotic counterparts. The Lys²⁸⁹ and Asn²¹⁰ residues are strictly conserved and responsible for the 4'-phosphopantothenoylecysteine synthetase activity (1).

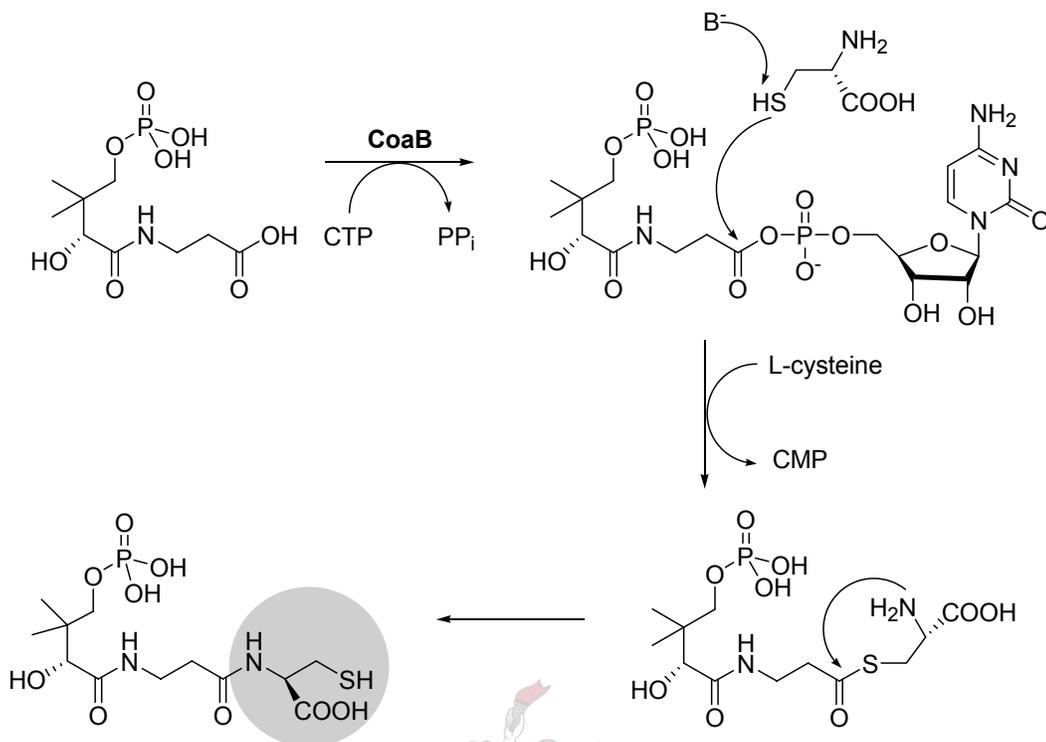


Figure 2.4 The mechanism of the activity of CoaB on 4'-phosphopantothenate.

The amino terminal domain (CoaC) includes residues 1-190 and contains a bound FMN. This part of the enzyme possesses 4'-phosphopantothenoic-L-cysteine decarboxylase activity. Site-directed mutagenesis within these protein regions identifies critical residues for enzymatic activity such as Gly¹⁴, Asn¹²⁵ and Cys¹⁵⁸. The decarboxylation reaction catalyzed by the CoaC domain proceeds via a thioaldehyde intermediate formed by the FMN-dependant oxidation of the cysteine moiety of 4'-phosphopantothenoic-L-cysteine as illustrated in figure 2.5. The catalytic cycle is complete when this intermediate is decarboxylated to 4'-phosphopantothenoic-L-aminoethanethiol followed by the reduction through flavin to produce 4'-phosphopantetheine (1, 11).

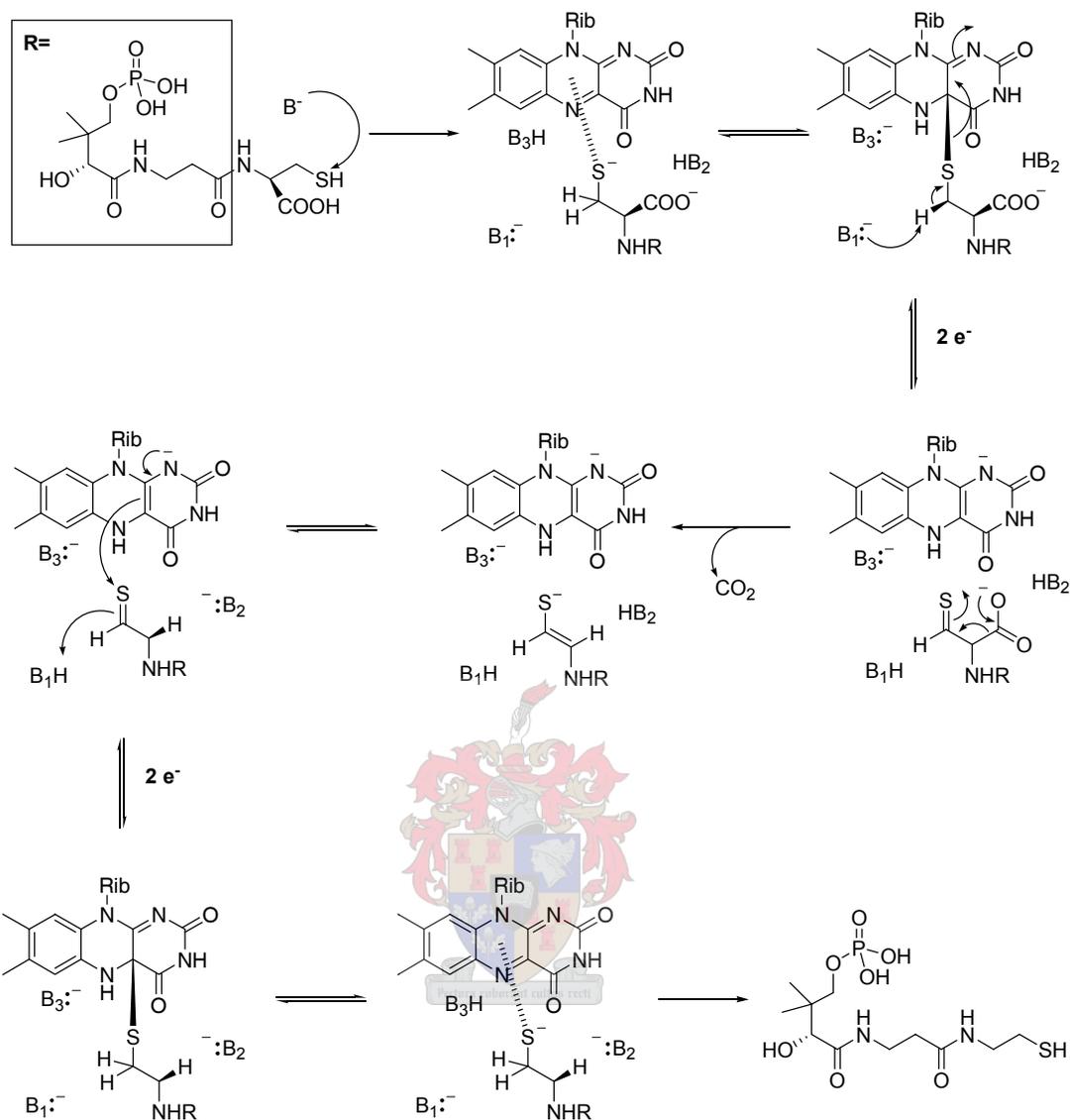


Figure 2.5 The decarboxylation of phosphopantothienoylcysteine by CoaC. This figure is adapted from Strauss et. al. (11).

Mammalian CoaB and CoaC were identified, where 4'-phosphopantothienoylcysteine synthetase (PPC-S; CoaB) is located on chromosome 1, whereas 4'-phosphopantothienoylcysteine decarboxylase (PPC-DC; CoaC) is located on chromosome 15. Human PPC-S (also known as *HsCoaB*) catalyzes the synthetase reaction separately from the decarboxylation reaction by utilizing ATP for the activation of substrate in the ligation reaction four times more efficiently than CTP. This is in contrast to the *E. coli* CoaBC

bifunctional enzyme, which shows a distinct preference for CTP (3). The *HsCoaB* protein is a dimer of identical monomers. The structure of human phosphopantothencysteine synthetase was determined at 2.3 Å resolution. This structure predicts a ping-pong mechanism with initial formation of an acyl-adenylate intermediate, instead of an acyl-cytidylate intermediate as is the case for *EcCoaB* (8). The release of pyrophosphate and the binding of cysteine are followed by the formation of the 4'-phosphopantothencysteine and AMP as products (1).

The human CoaC is a 22kDa protein of 204 amino acids. Investigation of the human PPC-DC protein structure is still in progress. The protein structure reveals that it is a trimer with each monomer binding the flavin mononucleotide cofactor (1). The decarboxylation reaction catalyzed by *HsCoaC* occurs in similar fashion as in *E. coli*.

The differences between the genes, encoding the CoaB and CoaC enzymes, from bacteria versus higher organisms raise the possibility of exploiting the selective inhibition of the bacterial enzyme in the development of new antibiotics (3).

2.4.3 4'-Phosphopantetheine adenylyltransferase (PPAT; CoaD)

This enzyme is involved in the fourth step of the biosynthesis of CoA and is also termed CoaD or PPAT. 4'-Phosphopantetheine adenylyltransferase catalyzes the Mg^{2+} -dependant reversible transfer of the adenylyl group of ATP to 4'-phosphopantetheine. This enzyme was first isolated as a trimer from *Corynebacterium ammoniagenes* and has K_M constants of 0.19 and 0.53 mM for 4'-phosphopantetheine and ATP respectively. The gene responsible for the encoding of *E. coli* 4'-phosphopantetheine adenylyltransferase is the *kdtB* gene and the enzymes was renamed as CoaD (1).

CoaD purified from *E. coli* contains 0.5 mole of CoA per mole of enzyme and exists in solution as a homohexamer of 17.8 kDa subunits, arranged as a dimer of trimers. Various kinetic experiments of the reverse reactions reveals that a ternary

complex between CoaD and both ATP and phosphopantetheine is formed before catalysis, but it is not known if the order of substrate binding is important in this reaction (1).

CoaD displays a dinucleotide-binding fold. The binding of ATP to CoaD supports a model in which the α -phosphate of ATP undergoes nucleophilic attack by the phosphate of 4'-phosphopantetheine in an in-line displacement mechanism. His¹⁸, Ser¹²⁸, Arg⁹¹ and Ser¹²⁹ are conserved in all known PPAT sequences. The His¹⁸ residue lends stabilization to the corresponding pentacoordinate transition state in the reaction, while hydrogen bonding to Lys⁴² and Thr¹⁰ appears to be crucial for orienting the nucleophile. Arg⁹¹, Ser¹²⁸, Ser¹²⁹ and Ser¹³⁰ direct the β - and γ -phosphates of ATP. This arrangement of the active site residues allows effective interaction between the substrate without the need for a direct involvement in acid-base or covalent catalysis (12, 13).

Phosphopantetheine adenylyltransferase is fused to dephospho-CoA kinase and exists as a bifunctional enzyme in humans. This bifunctional enzyme does not share significant sequence with its prokaryotic counterpart. The availability of a crystal structure makes the bacterial CoaD an attractive target for antibacterial drug design (1).

2.4.4 Dephospho-CoA kinase (DPCK; CoaE)

The gene encoding *E. coli* dephospho-CoA kinase, also known as DPCK or CoaE, was identified as *yacE*. The *E. coli* CoaE protein utilizes ATP to phosphorylate dephospho-CoA produced by CoaD. The 3'-hydroxyl group of the ribose moiety takes up a phosphate group from ATP to result in coenzyme A and ADP as products (1).

The K_M value for the phosphorylation of dephospho-CoA by CoaE is 0.74 and 0.14 mM for dephospho-CoA and ATP. The enzyme was originally isolated as a 22 kDa monomer. The sulphate ions however promote the formation of trimers. Two proline residues at position 90 and 134 are highly conserved. Other conserved

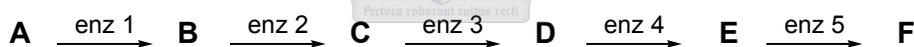
residues are also involved in the reaction. The side chain of Arg¹⁴⁰ interacts with the adenine base of ATP. Thr⁸ is probably involved in dephospho-CoA binding and Asp³³ is proposed to activate the 3'-OH group of the ribose for the attack on the γ -phosphate of ATP (1).

Information on plant and human dephospho-CoA kinase is also available but not of interest in this study.

2.5 Regulation of coenzyme A levels in living systems

Due to the fact that CoA is involved in numerous metabolic pathways intracellular CoA levels need to be controlled by the modulation of several key enzymes activities in the pathway. The levels of CoA in living systems are mainly regulated by feedback inhibition, but can also be regulated by CoA utilization.

Feedback inhibition is a form of allosteric regulation which acts to modulate enzymes situated at key steps in metabolic pathways. The biosynthesis of CoA for example can be simplified in the following pathway where A is the precursor, pantothenic acid and CoA the end product F.



The end product F inhibits enzyme 1 which catalyzes the first step in the pathway. Therefore, when sufficient F is synthesized, it blocks further synthesis of itself and this phenomenon is then called feedback inhibition (6).

Feedback inhibition occurs mainly in the first step of the biosynthetic pathway where pantothenate kinase phosphorylates pantothenic acid. Some regulation also plays a role in step 4, the transfer of the adenylyl group of ATP to 4'-phosphopantetheine by CoaD (1). The CoA levels can also be controlled by acyl carrier proteins (ACP) and fatty acid synthases (FAS).

2.5.1 Feedback regulation of pantothenate kinase

The first step of the biosynthesis of CoA is the primary rate-determining step of the pathway and is controlled by CoA and CoA thioesters. The utilization of pantothenic acid in this step is shown to control the rate of CoA biosynthesis rather than to be regulated by the amount of pantothenic acid supplied as substrate (14). Feedback regulation of CoaA by different type of CoA molecules controls overall CoA availability in response to a cell's metabolic status. Different CoA analogues can result in feedback inhibition. In *E. coli* the CoA pool is made up of mainly acetyl-CoA followed by non-esterified CoA, succinyl-CoA and malonyl-CoA. The relative distribution of CoA varieties and the total amount of CoA are functions of the carbon source in which the *E. coli* bacterium is cultured. *E. coli* produces 15 times more pantothenate than the amount actually utilized to synthesize CoA, releasing the excess pantothenate into the medium. This illustrates that the CoaA enzyme limits the rate of pantothenate conversion into CoA (1).

CoA inhibition is competitive with ATP binding and both ligands bind to kinetically distinguishable sites on the enzyme. This allows that the CoaA biosynthetic activity can be coordinated with the energy state of the cell through the utilization of ATP. The reduction in the ATP level would allow for more binding of the feedback inhibitor (CoA), which would lead to more regulation of CoA biosynthesis (1).

The gene sequence of pantothenate kinase from *S. aureus* has little if any homology to *E. coli* CoaA and only 18% homology with the eukaryotic PanK's. SaCoaA is not regulated by feedback inhibition by CoA or CoA thioesters. The intracellular CoA level is not limited except by the amount of input pantothenate, which enables the bacterium to achieve very high levels of CoA that function in maintaining the intracellular redox state. However, one disadvantage to unregulated CoA levels is a depletion of the pantothenate supply. Reduction of the CoA levels and pantothenate depletion does not result in cell death, but rather the cell goes into a growth status that is similar to metabolic dormancy associated with reduction in the free CoA level in *Bacillus megaterium* (1).

2.5.2 Regulation by 4'-phosphopantetheine adenylyltransferase

Pantothenate and 4'-phosphopantetheine are the two intermediates in the CoA biosynthesis that are detected in the highest concentrations. Primarily regulation of CoA levels occurs at the first step of CoA biosynthesis, but secondary regulation is evident at the CoaD reaction (15). This is clear from the release of 4'-phosphopantetheine from the bacteria to the outside medium. CoaD becomes more important when regulation at the CoaA site is disrupted or when the CoaA protein is overexpressed. Under both circumstances the levels of intracellular and extracellular 4'-phosphopantetheine increases. This reflects the restriction of the rate of flux through the CoA biosynthetic pathway at the CoaD reaction. 4'-Phosphopantetheine cannot be transported back into the cells and the CoaD proteins are probably feedback regulated by free CoA in a similar way as in the case of CoaA. CoA remains bound to the CoaD protein in a ratio of 1 mole per 2 moles of protein when purified from *E. coli*. The crystal structure of bound CoA to CoaD shows the inhibitor bound in the 4'-phosphopantetheine site (1).

In *S. aureus* however, the regulation at the CoaD step is not evident when the cells are radiolabelled with a pantothenate precursor. This implies that the homologous protein in this bacterium is significantly different from the *E. coli* enzyme (1).

2.5.3 Regulation of CoA levels by CoA utilization.

CoA can be degraded in two ways: CoA can be dephosphorylated to yield dephospho-CoA or it can be hydrolyzed by cleavage of the phosphodiester bond to yield 4'-phosphopantetheine and 3', 5'-adenosine mononucleotide (1).

However, two additional regulatory mechanisms in the utilization of CoA have been characterized. In the first mechanism the 4'-phosphopantetheine moiety of CoA can be transferred to carrier proteins such as acyl carrier proteins (ACP) of bacteria or fatty acid synthase (FAS) in eukaryotes. The 4'-phosphopantetheine is the prosthetic group that activates these proteins and enables them to form thioester linkages with carboxylic acids. ACP synthase catalyzes the formation of

the active holo-ACP. The prosthetic group is removed by ACP phosphodiesterase. This enzyme is still uncharacterized. The active mammalian FAS, is formed by a cytoplasmic 4'-phosphopantetheinyl transferase with broad substrate specificity. This mechanism provides a method to coordinate the level of active ACP or FAS with the level of CoA. These proteins are available in considerably lower amounts than CoA. ACP is the essential acyl-group carrier that interacts with enzymes of fatty acid biosynthesis in bacteria. The FAS on the other hand is the multifunctional polypeptide that produces long-chain fatty acids from acetate and malonate in eukaryotes. The rate of new ACP protein synthesis is four times slower than the turnover of the ACP prosthetic group during recovery from CoA deprivation. The rate for the turnover of the ACP prosthetic group drops about an order of magnitude during logarithmic growth when the CoA level is high. The cycle of holo-ACP synthesis allows the phosphopantetheinyl prosthetic group to either re-enter the CoA biosynthetic pathway or exit from the cell and regulates the CoA concentration. The physiological role of this cycle is still unknown. After the transfer reaction the ACP protein with a phosphopantetheine prosthetic group is then involved in fatty acid synthesis (1).

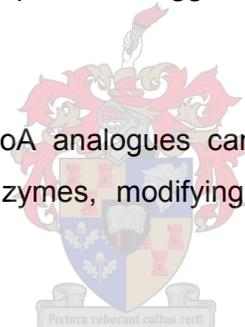
The second regulatory step is characterized to some extent in bacteria. This mechanism is mediated by a distinct phosphodiesterase. The CoA pool responds to a rapid decrease in the carbon supply by dramatic reduction of acetyl-CoA. This leads to a fleeting increase in the free CoA concentration, which is followed by CoA degradation to 4'-phosphopantetheine, which is effluxed to the culture medium. No protein prosthetic group turnover appears to be involved in this rapid adjustment of the CoA pool (1). According to Leonardi *et al.* recent data suggests that the nudix hydrolases may catalyze the reaction, which hydrolyzes CoA and acyl-CoA thioesters. This results in the production of 4'-phosphopantetheine or the acyl-phosphopantetheine product (1).

2.6 Conclusion

The requirement of CoA in numerous metabolic processes makes it essential for bacterial proliferation in the host and plays a central role in the metabolism of carboxylic acids, including short- and long-chain fatty acids. The biosynthesis of CoA occurs in five steps from pantothenic acid and the pathway intermediates are common to both prokaryotes and eukaryotes.

Coenzyme A and its biosynthesis have been studied for a number of years. In the last few years all of the genes encoding the pathway have been determined. Research interest in the area of CoA metabolism has recently accelerated. Since essential genes encode all of the steps in CoA biosynthesis, it makes them attractive targets for antibacterial drug discovery (10). The dissimilarity between bacterial and mammalian sequences suggests that unique inhibitors can be identified (1).

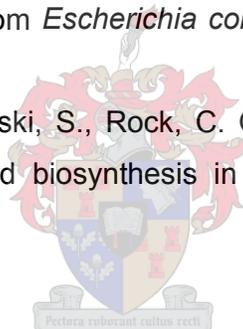
The production of various CoA analogues can also play an important role in inhibition of CoA-utilizing enzymes, modifying fatty acid synthesis and other processes related to CoA.



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Enzyme Immobilization using Cellulose Binding Domains

3.1 Introduction

Enzyme expression, purification and immobilization play an important role in biotechnology. The production of soluble proteins, which is easily purified, is very important if these proteins are to be used in industrial settings on a larger scale in order to be sold as products such as drugs, vaccines, diagnostic tools or food additives. The use of immobilized proteins has found applications in a wide range of biological processes from the production of ethanol to the degradation of phenol (1). Desired characteristics for this technique include ease of use, reproducibility, high capacity and high selectivity for target molecules (2). Gene fusion technology is providing an array of options to confer specific binding properties on proteins (3).

Various methods of immobilization have been developed in recent years, such as covalent immobilization, ionic immobilization and hydrophobic adsorption, but most of these processes require chemical modifications of the matrix on which immobilization occurs (4). These modifications often result in loss of ligand activity for the matrix and the inclusion of toxic organic compounds, which have to be removed before the method can be applied to medical or food processes. Various techniques use expensive materials such as sepharose or glass beads that require costly chemical modifications and use of highly toxic compounds to render these methods successful in protein immobilization (5).

Cellulose however, is an attractive matrix for enzyme immobilization mainly because of low cost. It can be used with cellulose binding domains (CBDs) as affinity tag to constitute an excellent tool for linking proteins to cellulose (6, 7). This small protein has the added advantage of being approved for utilization in many pharmaceutical and human applications (3). This chapter will focus on different CBDs as affinity tags and its properties. The application of CBDs will be

investigated as an affinity tag in the immobilization of proteins involved in the biosynthetic pathway of coenzyme A to construct a bioreactor for the production of coenzyme A analogues.

3.2 Cellulose Binding Domains (CBDs) and its properties

3.2.1 CBD and its function in nature

Cellulose binding domains are discrete polypeptide domains found in proteins that mediate binding to cellulose. Therefore, these proteins are classified as carbohydrate binding modules (2). Cellulolytic bacteria and fungi produce cellulases, which are responsible for the degradation of cellulose. Cellulolytic systems can be associated into multi-enzyme complexes called cellulosomes or they can be unassociated as individual enzymes (8).

In nature, cellulose binding domains are found as discrete domains in cellulases and xylanases, which are proteins that are involved in the degradation of plant biomass. In polysaccharidases, CBDs are involved in targeting of enzymes to their substrates or particular substrate regions. This increases the effective enzyme concentrations at the surface of the insoluble cellulose. CBDs are also found in proteins without hydrolytic activity such as the clostridial cellulose-integrating proteins (Cip) or cellulose binding proteins (Cbp). CBDs involved with Cip and Cbp is part of a scaffolding subunit that organizes the catalytic subunits in a cohesive multi-enzyme complex (cellulosome) (3). The cellulosome is composed of several hydrolytic enzymes attached to a large, enzymatically inactive protein termed CipA which also carries a CBD (9).

The role of CBD in the degradation of cellulose is still not clear, but one obvious function of these binding domains is to keep cellulases in close contact with their substrate. Removing CBD severely impairs the activity of cellulases on insoluble substrates. This suggests that the presence of CBD is important for efficient degradation of cellulose (10).

3.2.2 Structure and properties of CBDs

The CBD from *Clostridium thermocellum* provide a good example of the structure (figure 3.1) and function of CBD. *C. thermocellum* is an aerobic thermophilic, cellulolytic bacterium, which grows on cellulose and its degradation products (11). This particular CBD is composed of nine-stranded β -sandwiches of jellyroll topology that form two anti-parallel β -sheets. The planar face of the CBD molecule interacts with three successive chains on the cellulose surface by harbouring a planar strip of aromatic residues of amino acids aligned precisely along one of the cellulose chains. The stacking interactions performed between the planar strip residues and glucose rings along the cellulose chain are considered the major cause of the specificity and strong binding of the CBD to crystalline cellulose substrate (12-14).



Figure 3.1 Cellulase CelA from *Clostridium thermocellum* as published in Bayer *et. al.* (14). (Reprinted with permission from Elsevier from Bayer, E. A., Chanzy, H., Lamed, R., and Shoham, Y. (1998) Cellulose, cellulases and cellulosomes. *Current Opinion in Structural Biology* 8, 548-557. © 1998).

According to Boraston *et al.* (2) over 150 different CBD sequences have been identified and these sequences have been classified into a number of families based on sequence similarities. Some families contain only one or a few members, whereas others are large families of forty or more sequences. Members

of the same family are expected to have similar properties, although there may be subtle differences. Different CBDs exhibit different binding specificities, binding affinities and elution characteristics. Three families of CBDs are involved in the context of affinity purification and immobilization. Two of these families (Family II and III) are available in commercial vectors (pET vectors) designed for the production of fusion proteins. The third family, family IX, is relatively newly characterized and has novel elution properties that make this tag ideal in affinity purifications (2). Table 3.1 summarizes the relevant properties of representative CBDs from each of these families.

The two general forms of CBDs are important in affinity chromatography and protein immobilization: one illustrated by the CBDs of fungal cellulases (e.g. family I) and the other by the CBDs of bacterial cellulases (e.g. family III). The fungal CBD domains are about 33-36 amino acids long and are generally smaller than the bacterial CBD domains, which usually contain about 90-180 amino acids (15). This wide range in domain size plays a role when selecting tags to minimize interference with the biological activity of the target molecule or when selecting a fusion partner that can stabilize these target molecules, which can turn out to be a great advantage. Small tags are usually more suited for this purpose but larger CBDs can be used with equal success when a small linker sequence are introduced between the compactly folded cellulose binding domains (3).

Table 3.1 Properties of cellulose binding domains from each family (2).

Family	Family IIa	Family III	Family IX
Domain Size (avg. no. aa)	105	150	190
K_a (M^{-1}) @ 25 °C			
Crystalline cellulose	10^6	10^6	10^5
Amorphous cellulose	10^6	10^6	10^5
Glucose	Negligible	Negligible	10^4
Binding: Reversibility	Irreversible	Irreversible	Reversible
Range	2 < pH < 9	2 < pH < 10	
	4° < T < 50 °C	4° < T < 50 °C	

Elution	Ethylene glycol and GuHCl; distilled water (in certain cases)	Ethylene glycol and GuHCl	1 M glucose
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(Avg. no. aa = average number of amino acids)

Cellulose binding domains have many highly desirable attributes and thus provide a new class of affinity tags based on these qualities. Firstly, it provides a readily available, low cost affinity matrix in the form of cellulose with inherent low non-specific protein binding characteristics. Secondly, it has high affinity binding that facilitates rapid purification from dilute and/or complex samples. It also has efficient release under non-denaturing conditions and fourthly CBD can be effectively immobilized to cellulose-based matrices without the need for covalent cross-linking (16).

3.3 Choice of cellulose matrix

Cellulose is the most abundant renewable source of carbon and energy on earth (6) and has the primary function in nature to impart strength and rigidity to plants (17). Algae, various plants, certain bacteria and amoebae naturally produce this compound. Not only is this very stable polymer a naturally abundant compound, it is also a low cost affinity matrix with inherently low, non-specific binding characteristics (1). The chemical structure of cellulose (figure 3.2) is very simple, but the diverse origins of cellulose results in variety of complex physical forms (16). The structure of cellulose can be describe as several thousand D-glucose units linked by 1, 4'- β -glycoside bonds, with degrees of polymerization ranging from about 100 to greater than 10 000. Different cellulose molecules interact to form a large aggregate structure held together by hydrogen bonds. Crystalline cellulose is resistant to the action of many β -1, 4'-glucanases and the hydrogen bonding in the closely packed cellulose chains stabilize the polymer chains to form a tight, regular array which shields many of the glycoside bonds from enzymatic attack (18). These specific properties account for its contribution to modern society in the form of paper and as cellulosic waste (6).

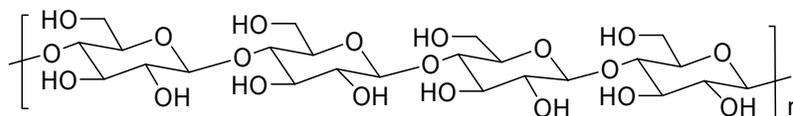


Figure 3.2 The structure of cellulose (17).

In general CBD has a high affinity for crystalline forms of cellulose. The dissociation constants (K_d) of CBD for cellulose are in the range of 1.0×10^{-5} M to 2.9×10^{-7} M and depend on the actual cellulosic substrate that is used (15). Cellulose is available in a variety of forms, which are appropriate for purification of CBD hybrid proteins. The description of cellulosic substrates includes such qualities as size, shape, porosity, and surface area, association with non-cellulosic components, molecular conformation and crystallinity (19). The most commonly used celluloses are CF1, AvicelTM and beaded cellulose and these forms are considered the most effective, but even among these types of cellulose each has its most appropriate application. CF1 and AvicelTM are considered to be very similar in their general properties, but in its application with CBD, CF1 is better for larger columns due to its packing characteristics. AvicelTM is more effective in smaller columns. Beaded cellulose is better characterized than particular cellulose and has the advantage that it generally has a higher capacity. It also can be utilized with higher flow rates and little preparation of cellulose is required prior to pouring a column. Beaded cellulose also shows better packing by gravity and is less prone to damage by chaotropic agents (15).

The binding capacities of CF1 and AvicelTM are approximately 40 mg of target protein per gram cellulose. To ensure complete binding 2-4 times the amount of cellulose required to bind the estimated amount of protein is needed. The binding capacity of beaded cellulose can vary greatly from 40 mg/g to 5 mg/g depending on which type is being used (2). CBD binds to cellulose in a moderately wide range of pH's and a pH range of 3.5 – 9.5 were reported for *Clostridium cellulovorans* CBD's. Most studies however are performed at pH ~7 (15).

All of these factors should be taken into account to make an appropriate decision on which cellulose should be used for the purification of the target protein.

3.4 CBD as fusion tags

CBDs are found in various positions in expression vectors as affinity tags. The CBD domain can be positioned at the N-terminus or the C-terminus of the protein. This property of CBDs is particularly important for those biomolecules that can only tolerate foreign sequences at one end to retain activity (3). This study will focus on the pET expression vectors from Novagen (16). These vectors enable the user to generate gene fusions with a variety of CBDs for expression in bacteria. The commercially available vectors have been designed to add a CBD at either terminus of the target protein involved. CBDs from Family II and III can be used to orientate the target protein in different positions depending on the properties of the target protein. This relates to the potential effect of adding a new N- or C-terminal domain on the folding and/or activity of the target protein. These commercial vectors also incorporate protease cleavage sites to remove the CBD affinity tag from the fusion protein if necessary, which can be done while in solution or with the CBD fusion protein bound to cellulose. There are also provisions made to localize the fusion protein to the cytoplasm of the cell, or to add a leader peptide to facilitate transport of the protein to the periplasm in *E. coli*. Overexpression of these leader peptide proteins often result in non-specific leakage into culture medium, but the mechanism responsible for this process is not well understood. Overexpression of the fused protein to a CBD gene without the leader peptide may result in the formation of inclusion bodies in the cytoplasm, which will leave the target protein insoluble (2).

Four different CBDs are available which can be classified under the two families of interest. Three of these CBDs (CBD_{CenA}, CBD_{Cex} and CBD_{CenD}) fall under Family II, while CBD_{CloS} can be categorized under Family III. The cellulose binding properties of these four CBDs are almost identical.

The four CBDs can be described as follows (2):

- CBD_{CenA} : appropriate for production of hybrid proteins with an N-terminal CBD domain. A signal sequence is provided for export into the periplasm and potentially the culture supernatant.
- CBD_{Cex} : appropriate for production of hybrid proteins with a C-terminal CBD domain. A signal sequence is provided for export into the periplasm and potentially the culture supernatant.
- CBD_{CenD} : appropriate for production of hybrid proteins with a C-terminal CBD domain. This CBD is appropriate for production of hybrid proteins in hosts that N-glycosylate due to the lack of an N-glycosylation site in this particular CBD.
- CBD_{Clos} : appropriate for production of hybrid proteins with an N-terminal CBD domain. This vector is configured for cytoplasmic expression and will result in the formation of inclusion bodies, which can be an advantage with less stable proteins.

CBDs from *C. cellulovorans* (CBD_{Clos}) and *Cellulomonas fimi* (CBD_{CenA} and CBD_{Cex}) are expression vectors developed by Novagen and these CBD domains are well characterized. Each of these CBDs binds both amorphous and crystalline cellulose with high affinity. The properties of the target protein can also be influenced dramatically by the position of the fused sequences and/or its cellular localization. It is therefore very important to test the expression of the target protein in various CBD vectors to achieve optimum results. The different properties of these available vectors are summarized in table 3.1 and figure 3.3.

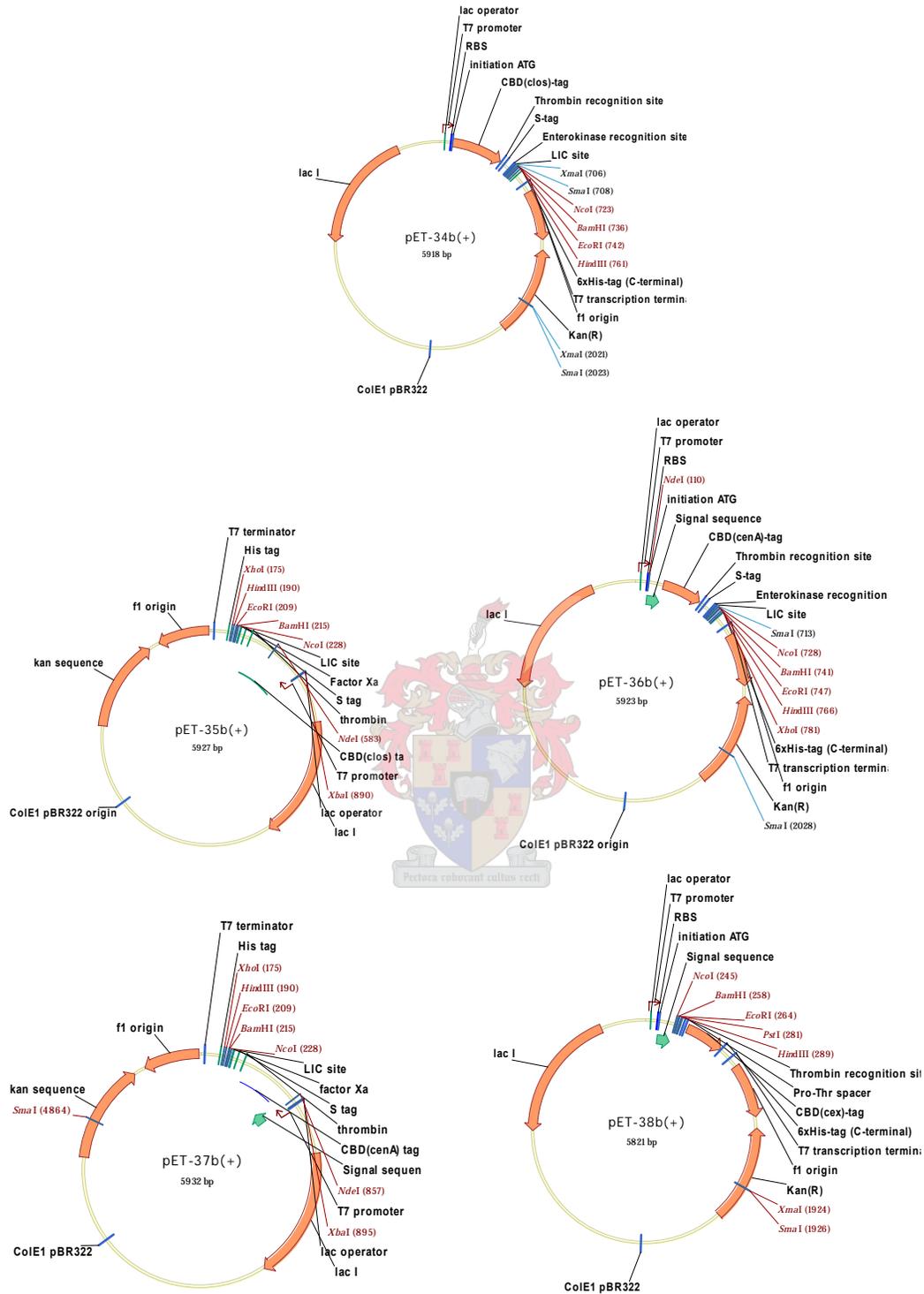


Figure 3.3 CBD expression vectors available from Novagen (16).

Table 3.1 pET CBD Vector features adapted form inNovations 7, Novagen (16).

Vector	CBD Type	CBD size	CBD location	Signal Sequence	Protease sites	S-Tag	C-Terminal His-Tag	T7lac Promoter	KanR	f1 ori
pET-34b(+)	Clos	17.0 kDa 158 aa	N-terminal	No	Tb, Ek	Yes	Optional	Yes	Yes	Yes
pET-35b(+)	Clos	17.0 kDa 158 aa	N-terminal	No	Tb, Ek	Yes	Optional	Yes	Yes	Yes
pET-36b(+)	CenA	11.7 kDa 114 aa	N-terminal	Yes	Tb, Ek	Yes	Optional	Yes	Yes	Yes
pET-37b(+)	CenA	11.7 kDa 114 aa	N-terminal	Yes	Tb, Ek	Yes	Optional	Yes	Yes	Yes
pET-38b(+)	Cex	10.8 kDa 107 aa	C-terminal	Yes	Tb	No	Yes	Yes	Yes	Yes

aa = amino acid, CBD = cellulose binding domain, kDa = kilodalton, Ek = enterokinase, Kan^R = kanamycin resistant, Tb = thrombin, Xa = Factor Xa.

It can also be desirable to produce fusion proteins intracellularly as insoluble aggregates or inclusion bodies. This strategy is used in the production of small peptides or proteins, which are prone to *in vivo* degradation when produced or secreted in soluble form. Recombinant CBD_{CloS} from *C. cellulovorans* promotes the formation of these inclusion bodies in *E. coli*. However, the homologous CBDs from *C. thermocellum* (CipA) or *C. cellulolyticum* (CipC), which are also members of Family III like *C. cellulovorans*, does not promote inclusion body formation.

In conclusion, the choice of CBD depends on the following 5 factors:

- Desired position of the CBD in the fusion.
- Whether the polypeptide is to be secreted or produced intracellularly.
- Expression host to be used.
- Localization of protease cleavage sites for downstream processing.
- Presence of detection tags or additional affinity tags in the fusion proteins.

The choice of a suitable CBD-tag for protein immobilization can be problematic. Various aspects need to be taken into consideration before making a choice on which CBD is most fit for the immobilization of the target protein involved. Properties of the different CBDs should be compared and the characteristics of the proteins to be immobilized also play an important role in this decision.

3.5 Immobilized fusion proteins as bioreactors

CBD is a versatile affinity tag because it contains the ability to construct fusion proteins attached to the N- or C-terminus of the target protein. The fusion protein can be expressed and purified via a cellulose matrix column. A restriction site can also be incorporated between the CBD and the target protein. This will allow the protein to be treated to release the target protein after affixing the fusion protein to the cellulose matrix. This is seen as an ideal way to purify small peptides that may be difficult to purify from crude extracts or to purify enzymes that are sensitive to chemical procedures used in other purification methods (15).

If the CBD affinity tag is not utilized for the purification of the target protein, it can also be used affixed to the cellulose column, where the fusion protein becomes an important tool to be used in a continuous operating bioreactor system. This allows development of a method to effectively combine a purification process with enzyme immobilization on cellulose as matrix. Various aspects affect the stability of the bioreactor and especially the fusion target protein plays an important role (15). Previous studies done by Shpigel *et al.* (5) have shown that CBD can be used for the efficient manufacture of a heparinase bioreactor. This bioreactor was used to produce heparin oligosaccharides for research and medical applications. This fused protein-cellulose complex was used for about 43 hours without the loss of activity due to the stability of the binding of the fusion protein to the cellulose. After three days the continuous bioreactor still retained about 40% of its initial activity (5).

In this particular study an attempt was made to use a chemo-enzymatic approach to synthesize coenzyme A analogues by utilizing CoaA, CoaD and CoaE (discussed in chapter 2) in solution or immobilizing them on a suitable matrix. These proteins can be utilized in column format as bioreactor system (figure 3.4) in the production of various analogues. The ideal would be to immobilize CoaA, CoaD and CoaE via a CBD affinity tag to cellulose in three different columns. A pantothenic acid derivative and ATP would be loaded on the CBD-CoaA column that will phosphorylate the substrate used. The eluate from this column will then be used to load a second column containing immobilized CoaD to adenylate the analogue, followed by loading of a column with immobilized CoaE to phosphorylate the dephospho-CoA analogue to result in the CoA-analogue in the presence of ADP. This particular method of chemo-enzymatic synthesis may eliminate tedious chemical synthesis of CoA analogues and side steps the problem of feedback inhibition of CoaA.

The development of a bioreactor system can play an important role in the method development of large-scale production of various CoA derivatives. However, before the bioreactor can be constructed in column format, the expression vectors

with the CBD-fused proteins need to be constructed. These CBD-fused proteins have to be expressed under soluble conditions. Therefore, various types of CBDs will have to be investigated to determine which of the CBDs suit the proteins from the biosynthetic pathway of CoA the best.

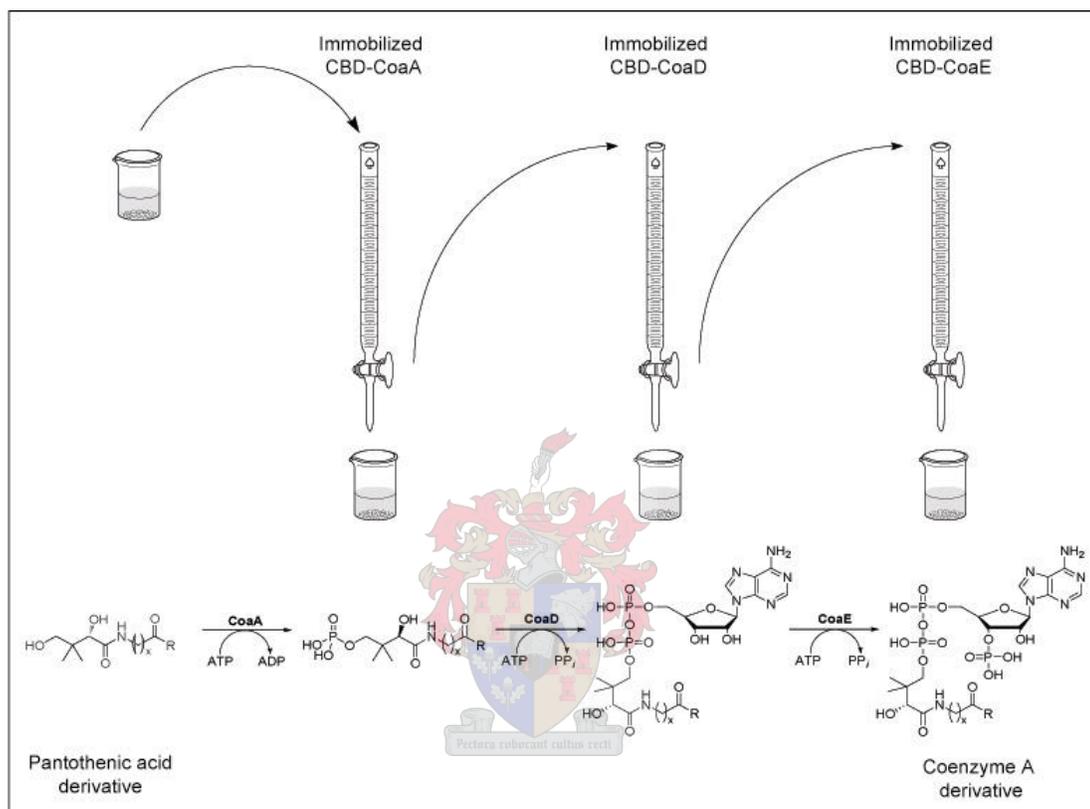


Figure 3.4 The biosynthesis of CoA analogues in column format using CBD immobilized enzymes. The reaction will take place in the presence of ATP and eluted product from the last column will be a mix of the CoA analogue, unreacted ATP and ADP.

3.6 Results and discussion

3.6.1 Expression, purification and immobilization of CBD_{CenA}-fusion proteins

The first attempt to produce soluble CBD-fusion proteins was based on a strategy to clone the CBD_{CenA}-tag form pET-36b(+) (figure 3.3) into expression vectors for *EcCoaA*, *EcCoaD* and *EcCoaE* already constructed. The next step is to optimize

the solubility of these CBD-fusion proteins via expression trials. Batch expression of these proteins was then used to immobilize the protein to cellulose.

Expression vectors for CBD_{CenA} fused enzymes

Different expression vectors are commercially available to construct fusion proteins of choice. The pET-CBD Fusion System by Novagen has several products based on vectors driven by the *T7lac* promoter for the expression of the CBD fusion proteins in *E. coli*. These vectors give one the choice to construct an expression vector with either an N-terminal or C-terminal CBD. However, the proteins involved in this study (*EcCoaA*, *EcCoaD* and *EcCoaE*) were already available in three pET-28a(+) vectors as 6xHis-tag fusions (pET-28a-*EcCoaA*, pET-28a-*EcCoaD* and pET-28a-*EcCoaE*). The His-tag was removed from each of these pET-28a(+) expression vectors and replaced with an N-terminal CBD_{CenA} from pET-36b(+) (figure 3.5), creating three new CBD expression vectors (pET-28a-CBD_{CenA}-*EcCoaA*, pET-28a-CBD_{CenA}-*EcCoaD* and pET-28a-CBD_{CenA}-*EcCoaE*). These vectors would encode for the CBD_{CenA}-fused proteins containing a signal sequence in front of the CBD tag (figure 3.5).

Protein transport in all systems is accomplished by a single mechanism. Each polypeptide that needs to be transported contains an amino acid sequence known as a signal or leader sequence. This amino acid sequence identifies the polypeptide to the appropriate transporting system (20). In this case the signal sequence resulted in periplasmic localization of the protein. The properties of the target protein influence the amount of fusion protein that is exported to the periplasm and whether it remains soluble once exported. The CenA leader sequence has been shown to be very useful for the export of fusion proteins to the periplasm in *E. coli*. (21). Due to the fact that *EcCoaA*, *EcCoaD* and *EcCoaE* expresses very well in pET-28a (as 6xHis-tag fusions) and is extremely soluble, it seemed as if it would not be a problem to obtain soluble CBD-fusion proteins in the medium or soluble cytoplasmic fractions.

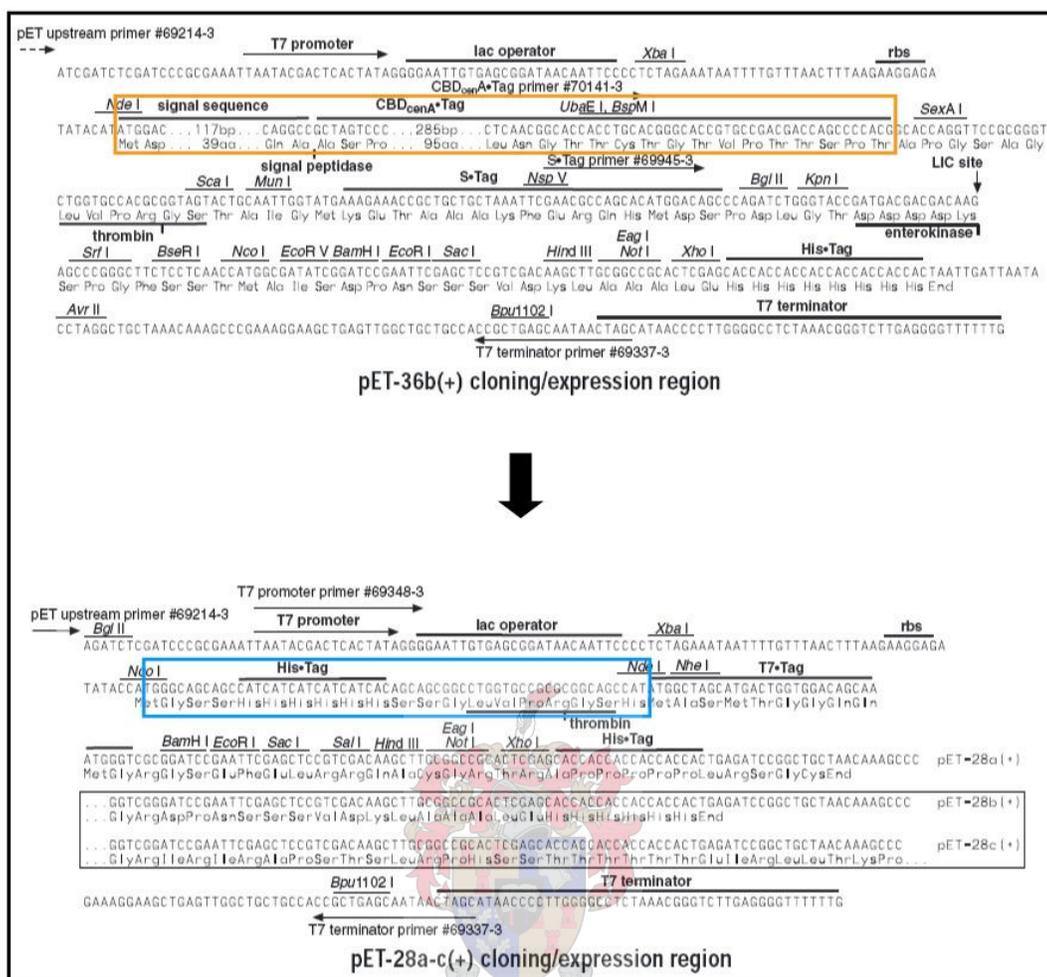


Figure 3.5 Expression regions for pET-28a(+) and pET-36b(+). The CBD_{CenA} fusion tag containing a signal sequence (indicated in orange) is used to replace the His-tag (indicated in blue) in pET-28a-*EcCoaA*, pET-28a-*EcCoaD* and pET-28a-*EcCoaE*, respectively. (Reproduced from Novagen pETvector Tables (www.novagen.com)).

Various expression trials were done with these three expression vectors (respectively for the expression of CBD_{CenA}-*EcCoaA*, CBD_{CenA}-*EcCoaD* and CBD_{CenA}-*EcCoaE*) to obtain optimum expression conditions for soluble CBD-fused proteins. Different temperatures (18–37 °C), IPTG concentrations (50–1000 μM), time of induction (3-18 hours) and different mediums were used to find optimum conditions for the soluble expression of these CBD proteins. Although expression was visible in the whole cell expression for all three proteins with both affinity tags, the general solubility of the CBD proteins were very low. This is clear from the cell free extracts figure 3.5, which shows the SDS-PAGE analysis of *EcCoaA*, *EcCoaD*

and *EcCoaE* with N-terminal His-tag fusions compared to the same proteins with CBD-tags. All these proteins were expressed under optimum expression conditions. The amount of protein in the medium due to export by the signal sequence was very little and not enough to pursue with further investigation.

Subsequently all three the plasmids were transformed into the Tuner(DE3) cell strain, a *lac* permease deletion mutant allowing uniform entry of IPTG into cells that provides strict concentration-dependant control of induction, to determine if this would not result in the expression of more soluble protein. It appeared to make a difference in only the CoaE expression, but soluble expression of this specific protein was still very low (results not shown).

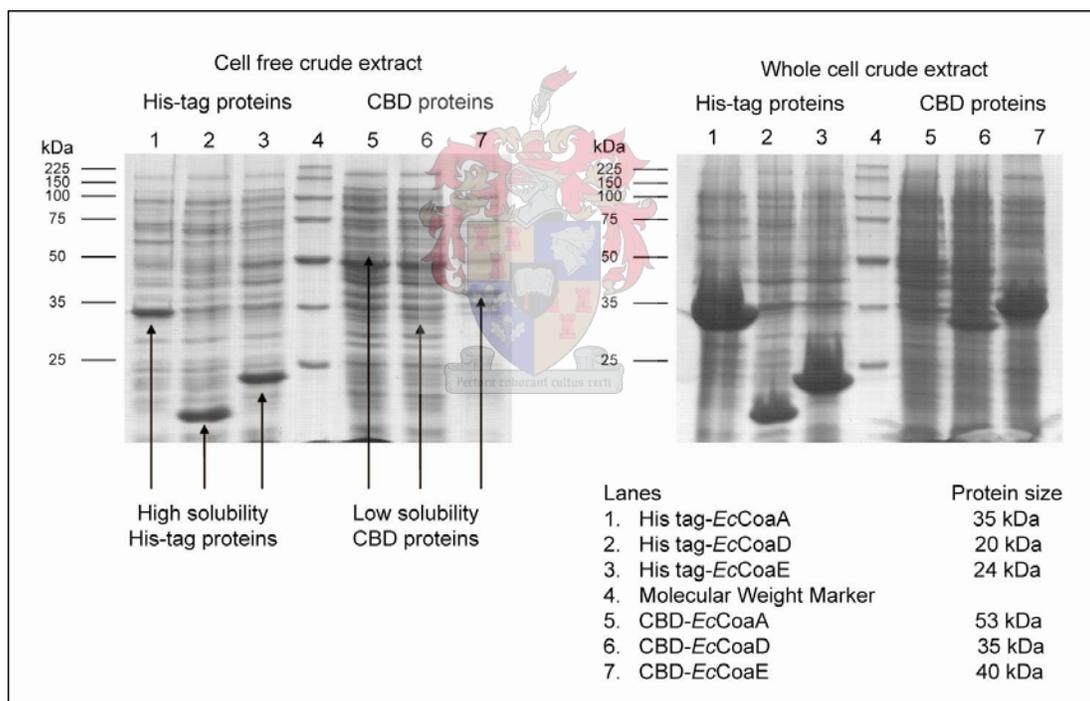


Figure 3.6 SDS-PAGE analysis of 6xHis-*EcCoaA*, -*EcCoaD* and -*EcCoaE* protein expression vs. CBD_{CenA}-*EcCoaA*, CBD_{CenA}-*EcCoaD* and CBD_{CenA}-*EcCoaE*. Expression for the CBD-tagged proteins was visible in the whole cell samples, but these proteins showed poor solubility in the cell free crude extracts.

Immobilization of CBD_{CenA}-fusion proteins

Although soluble expression was low, the binding capacity of the CBD-fusion proteins to cellulose was tested on a small scale to determine if purification of the proteins is possible via cellulose and to optimize elution conditions of the CBD-hybrid proteins. Samples are bound to cellulose, washed and then tested for elution by application of potential eluents. A simple assay was done which measured the amount of protein remaining in an aliquot of the cellulose matrix after treatment of the bound sample with candidate eluents. The potential eluents to remove CBD-fused proteins from cellulose were distilled water, 50% glycerol and 99% ethylene glycol.

The amount of protein removed from cellulose with the different eluents was determined for each protein via a Bradford assay. The results are summarized in table 3.2. From this data the conclusion was made that CBD_{CenA}-*EcCoaA* elutes better with 99% ethylene glycol, that CBD_{CenA}-*EcCoaD* prefers distilled water while CBD_{CenA}-*EcCoaE* also preferred elution with 99% ethylene glycol, showing almost no elution with distilled water. All the fractions were analyzed with SDS-PAGE gels along with the cellulose from each column to see if all the protein was removed with each of the different eluents. In all the samples, the enzymes were not totally removed from the cellulose. According to the intensity of the protein bands on the SDS-PAGE gels an estimated ~50% of the protein was still left after elution with any of the eluents (results not shown).

From these results it were clear that the enzymes can be immobilized on cellulose, but removal of all the immobilized protein is still not achieved. The type of solvent used to remove the immobilized enzyme also differed for CBD_{CenA}-*EcCoaA*, CBD_{CenA}-*EcCoaD* and CBD_{CenA}-*EcCoaE*. The results also showed that the purification of each of the CBD-fusion proteins via cellulose was less effective than purification of proteins via His-tag purifications. As a result we decided to construct a 6xHis-tag-CBD fusion-protein to purify the CoA biosynthetic proteins with (IMAC) Ni²⁺-affinity purification before using it in the bioreactor system.

Table 3.2 Elution results for CBD_{CenA}-*EcCoaA*, CBD_{CenA}-*EcCoaD* and CBD_{CenA}-*EcCoaE* with different eluents.

Enzymes	Protein removed by eluent (mg)		
	Distilled water	50% glycerol	99% ethylene glycol
CBD _{CenA} - <i>EcCoaA</i>	0.1283	0.3027	0.3326
CBD _{CenA} - <i>EcCoaD</i>	0.3367	0.1875	0.0440
CBD _{CenA} - <i>EcCoaE</i>	Under range*	0.2528	0.5251

*Protein concentration under Bradford assay standard concentration range

3.6.2 Construction of CBD_{Cex} expression vector.

Due to low solubility and the fact that the purification of the N-terminal CBD-fused proteins on cellulose was not as successful, the cloning and preparation of a C-terminal CBD fusion protein was attempted. A C-terminal CBD-tag would allow the *CoaA*, *CoaD* or *CoaE* to be folded first before the folding of the CBD-tag, whereas an N-terminal CBD-tag is folded first and determines how the folding of the rest of the protein is going to occur. Better folding of the fusion protein can lead to better solubility of the protein. Additionally, this expression vector would allow the protein to be purified via Ni²⁺-affinity purification prior to be immobilized on cellulose in the bioreactor system, because it contains a C-terminal 6×His-tag.

The pET-38b(+) vector (figure 3.3) from the pET expression vectors from Novagen was used in the construction of these new expression vectors for *EcCoaA*, *EcCoaD* and *EcCoaE*. The signal sequence of pET-38b(+) was removed and the *E. coli coaA*, *coaD* and *coaE* genes respectively were inserted before the C-terminal CBD_{Cex} domain (figure 3.7). The stop codons of the three different proteins were removed to allow the formation of the C-terminal CBD-tag. A short proline–threonine (PT) sequence separates the cloning region from the CBD_{Cex} domain and serves as a spacer to facilitate accessibility of the target protein domain. PT spacers are found in native cellulases separating the catalytic domain from the CBD domain. The pET-38b(+) vector has a 6xHis-tag sequence fused to the C-terminus of the CBD_{Cex} domain that enables purification of the fusion proteins via Ni²⁺-affinity purification.

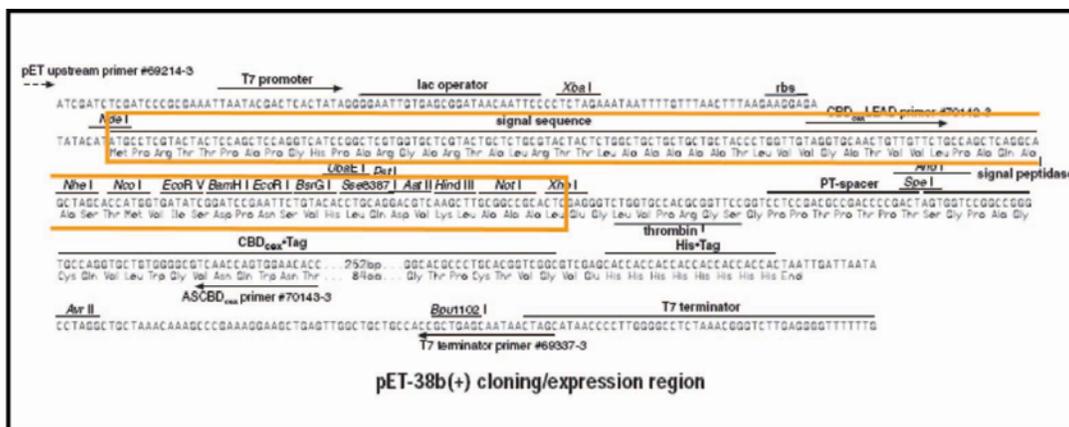


Figure 3.7 The expression region of pET38b(+). The signal sequence (indicated in orange) was removed and replaced with the *E. coli coaA*, *coaD* and *coaE* genes, respectively. (Reproduced from Novagen pETVector Tables (www.novagen.com)).

The cloning of the *E. coli coaD* and *coaA* genes into pET-38b(+) were done successfully, giving the plasmids pET-38b-*EcCoaA* and pET-38b-*EcCoaD*. However, the cloning of the *E. coli coaE* gene into pET-38b(+) was problematic and no successful results have been obtained yet. Expression trials were done for pET-38b-*EcCoaA* and pET-38b-*EcCoaD* under various conditions including different IPTG concentrations (50-1000 μ M), different induction times (3-18 hours) and different temperatures (37 and 30 $^{\circ}$ C). Very little soluble expression was visible. This was clear from the SDS-PAGE analysis in figure 3.8, which show expression trials done with different IPTG induction concentrations. Both these proteins were expressed in batch expression and purified by IMAC, but the purification unfortunately resulted in very little purified protein.

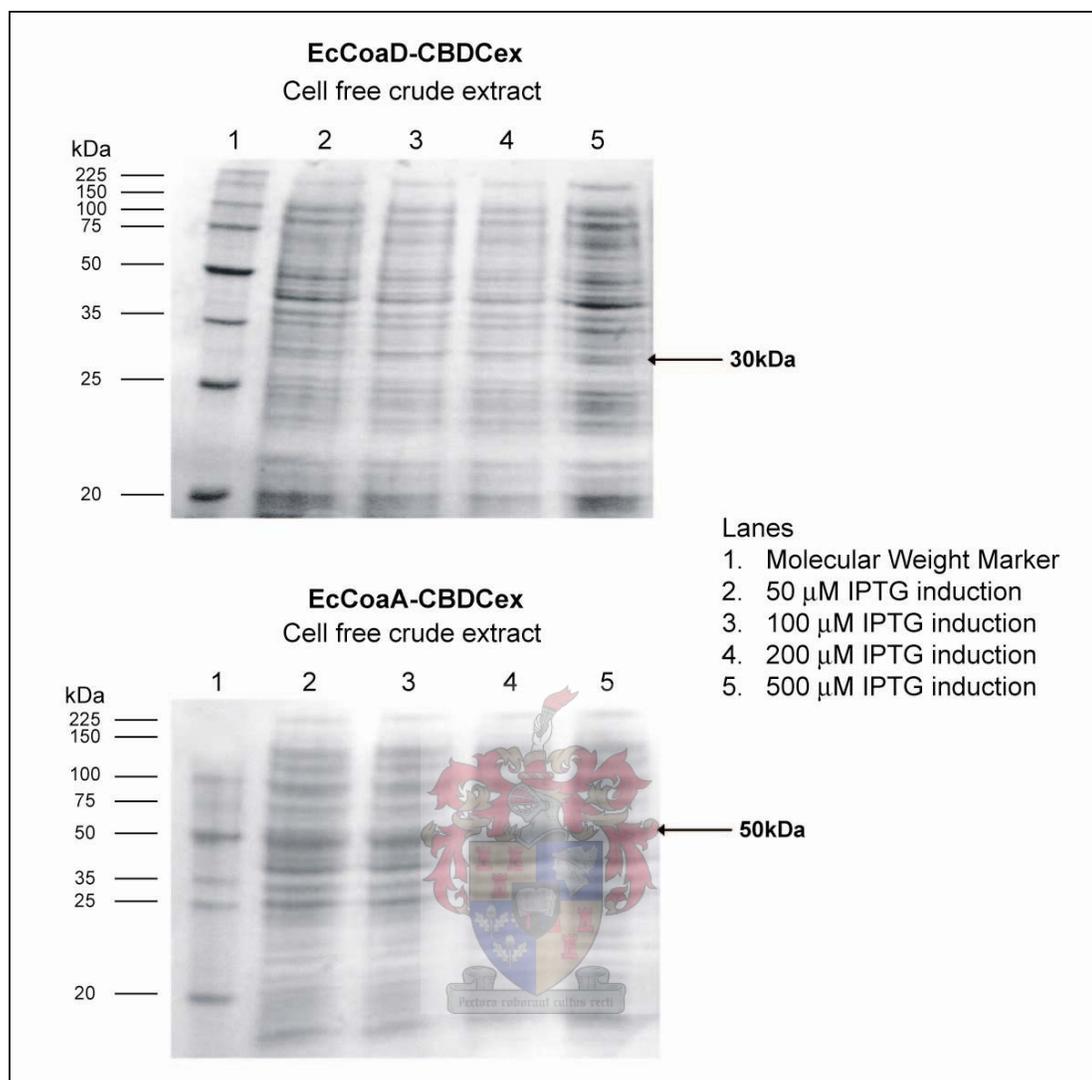


Figure 3.8 Expression trials for pET-38b-*EcCoaD* and pET-38b-*EcCoaA* for different IPTG concentrations at 37 °C.

Solubility of the CBD-fused target proteins plays a huge role in the effectiveness of the CBD column reactor and a third attempt to construct a more soluble CBD-fusion vector was undertaken.

3.6.3 CBD from CipA proteins

The CBD of the cellulosome-integrating protein CipA (CBD_{CipA}) from *C. thermocellum* belongs to family III. This specific CBD binds crystalline cellulose in a reversible manner. This cellulose-binding domain is attached to a cohesion domain via proline-threonine enriched linker (PT-linker) (9). This specific CBD has been used before for the immobilization of enzymes without any solubility issues (8, 22). Engel *et. al* used CBD_{CipA} to immobilize acetohydroxyacid synthase I from *E. coli* in a continuous flow reactor for the production of R-phenylacetyl carbinol. This bioreactor allowed purification and immobilization of the enzyme. The enzyme was produced with good solubility and was fully active as catalyst in R-phenylacetyl carbinol production (22).

In the study of Carrard *et al.* (8) a pCip14 plasmid was constructed encoding the CBD_{CipA}-cohesin hybrid and CelD, which was their choice of enzyme used in the immobilization technique. The expression vector used as basis for this new pCip14 plasmid was a plasmid from the pQE series (Qiagen, Chatsworth, CA) enabling fusion of the polypeptides with a 6×His-tag. However the pCip14 plasmid is not commercially available and was a kind gift from Pierre Béguin (Pasteur Institute, France). This plasmid was used in the cloning of a new CBD expression plasmid containing an N-terminal 6×His-tag, followed by the CBD_{CipA}-tag and a PT-linker in the expression vector pET-28a. The *E. coli* *coaA*, *coaD* and *coaE* genes enzymes were inserted into this new CBD vector (pET-28a-CBD_{CipA}; figure 3.6), which was used for the expression of the CBD fused enzymes.

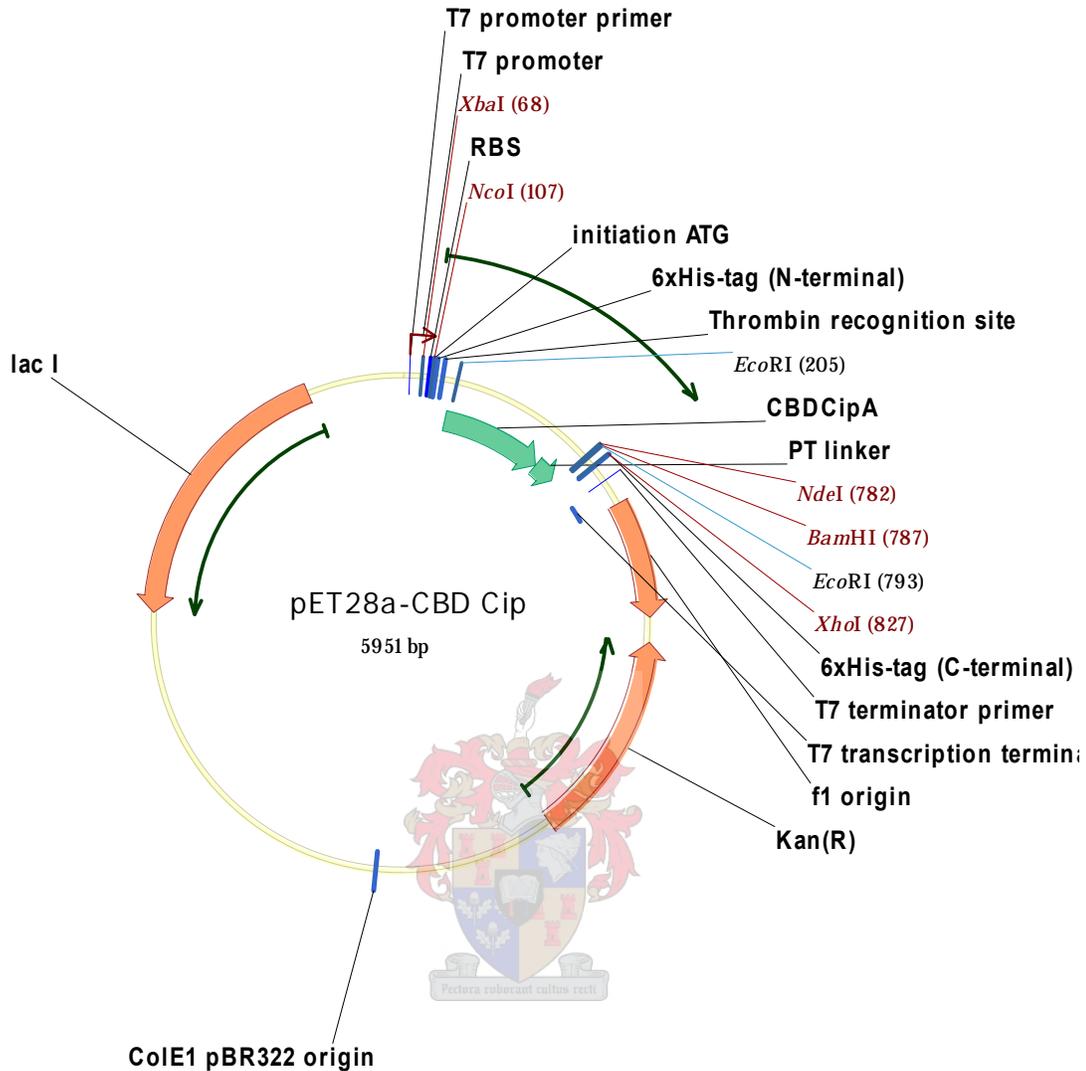


Figure 3.6 The new constructed pET28a-CBD_{CipA} expression vector. pET 28a(+) from Novagen was used as expression vector. CBD_{CipA} from pCIP14 was removed and inserted into pET28a(+) to construct the new CBD expression vector. The *coaA*, *coaD* and *coaE* genes can now be inserted into this vector.

Only one of the three genes was cloned first to check solubility of the fused protein before cloning all three expression vectors. The expression vector pET-28a-CBD_{CipA}-*EcCoaD* was successfully cloned. An expression trial was done with different IPTG concentrations at 37 °C to determine if the CBD_{CipA}-*EcCoaD* protein shows better solubility. As can be seen from the whole cell crude extract samples in figure 3.7, the protein is expressing well and shows good solubility in the cell free lanes with the best solubility at an IPTG concentration of 250 μM. The

construction of pET-28a-CBD_{CipA}-EcCoaA and pET-28a-CBD_{CipA}-EcCoaE is still underway, but the good solubility of CBD_{CipA}-EcCoaD shows promise for the solubility of CBD_{CipA}-EcCoaA and CBD_{CipA}-EcCoaE, and thus their subsequent use in immobilized form in column bioreactors.

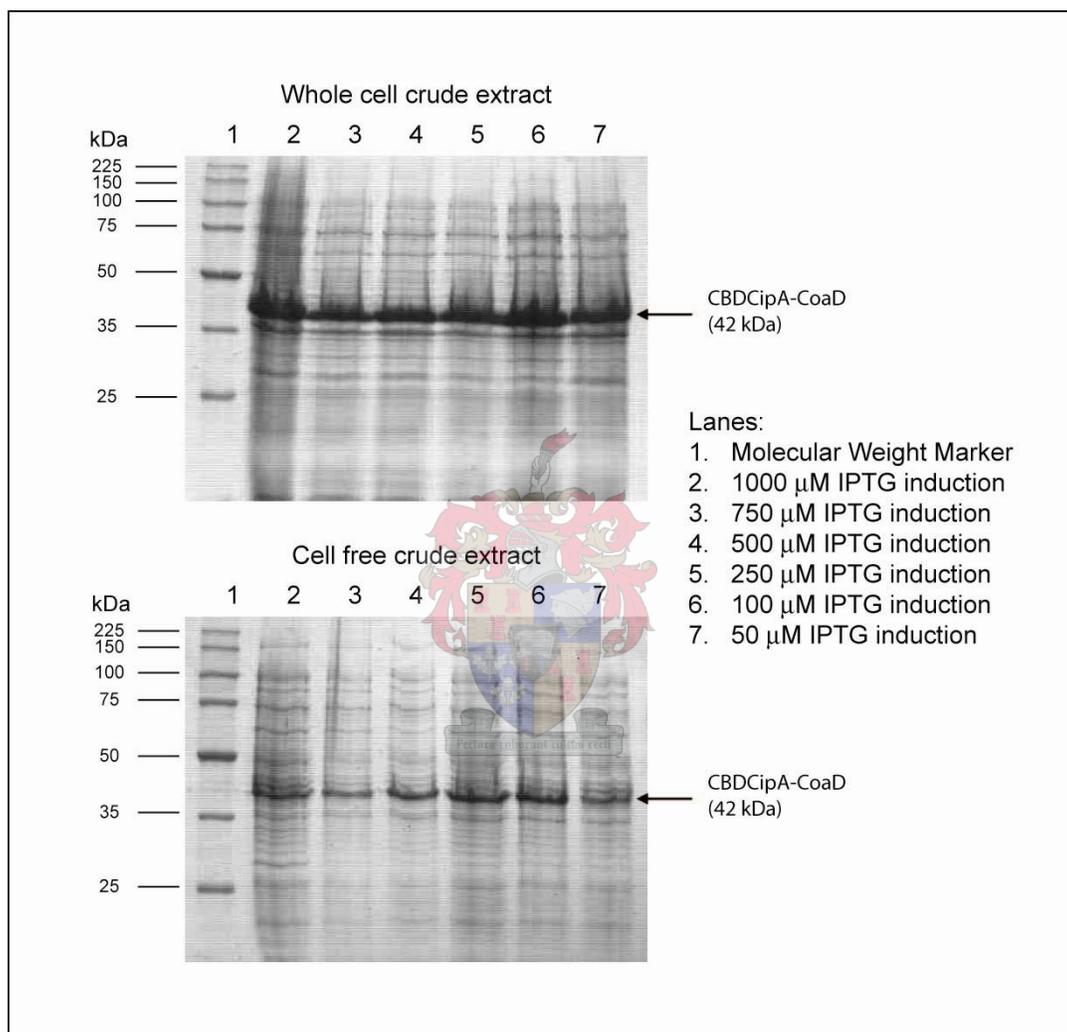


Figure 3.4 Expression trials of CBD_{CipA}-EcCoaD with various IPTG concentrations at 37 °C.

3.6 Conclusion

Coenzyme A is a very important cofactor in living systems and shows a lot of potential to be used in various biomedical studies by synthesizing CoA and its analogues for different purposes. An ideal method will use proteins from the biosynthetic pathway of CoA, which is immobilized on cellulose as matrix in a bioreactor system via a CBD tag. The convenience of operating reactions continuously is an advantage over a batch reaction, wherein enzymes are replenished periodically and products removed continuously to prevent inhibition of the reaction via negative feedback. The production of soluble CBD-fused enzymes however has shown to be a challenge, due to the fact that several criteria has to be met in choosing the most optimum CBD which will be compatible with the different enzymes involved. CBD_{CenA} from pET-36b(+) has shown to give low amounts of soluble enzymes when fused to *EcCoaA*, *EcCoaD* and *EcCoaE* in pET-28a as expression vector. The second attempt however, involving CBD_{Cex} form pET-38b, showed almost no solubility in the attempt to purify CBD-*EcCoaA* and CBD-*EcCoaD*.

A third attempt in producing soluble CBD-fusion proteins was attempted involving CBD_{CipA} from the cellulosome-integrating protein CipA. Previous studies have given positive results with this specific CBD and the expression vector for CBD_{CipA}-*EcCoaD* was constructed successfully. Expression trials of this protein have shown good expression as well as solubility and these results show promise for the solubility of CBD_{CipA}-*EcCoaA*, CBD_{CipA}-*SaCoaA* and CBD_{CipA}-*EcCoaE*.

Although all the CBD-fusion proteins required for the production of CoA derivatives via a continuous bioreactor system were not expressed and purified, the research already done on these CBD fusion proteins shows potential to in fact prepare these fusion proteins successfully.

3.7 Experimental Procedures

3.7.1 Materials and Methods

All chemicals were purchased from Aldrich or Sigma and were of the highest purity. Expression vectors and competent cell strains were purchased from Novagen. Primers were synthesized by Inqaba Biotechnology (Pretoria, South Africa). Sequencing of plasmids was done by automated DNA sequencing by Inqaba Biotechnology (Pretoria, South Africa).

All HiTrap™ Chelating Columns were from Sigma. Avicel® PH-101 was obtained from Fluka. A Quick Start Bradford Protein Assay Kit was purchased from Bio-rad and contained Bradford reagent and a Bovine Serum Albumin standard set. Large-scale centrifugation was done on a Heraeus Multifuge® 3S/3S-R. Centrifugation on a smaller scale was conducted on a Heraeus Biofuge pico centrifuge.

3.7.2 Optimum expression conditions for CBD_{CenA}-enzymes

Mrs. L.A. Brand did the cloning of pET28a-CBD_{CenA}-protein plasmids prior to this investigation.

Expression trials were done to determine optimum expression conditions as seen in section 3.7.5 for whole cell expression and cell free expression. From these expression trials the following parameters were determined for optimum expression:

- IPTG concentration for induction
- Temperature of induction
- Growth time after induction

CBD_{CenA}-EcCoaA

The plasmid pET28a-CBD_{CenA}-EcCoaA was transformed into *E. coli*. BL21-star(DE3) and grown in LB broth supplemented with 30 mg/litre kanamycin sulphate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 100 µM IPTG and grown overnight at 30 °C. The cells were harvested at 4500×g for 30 minutes and stored at -20 °C until used again.

CBD_{CenA}-EcCoaD

The plasmid pET28a-CBD_{CenA}-EcCoaD was transformed into *E. coli*. BL21-star(DE3) and grown in LB broth supplemented with 30 mg/litre kanamycin sulphate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 100 µM IPTG and grown overnight at 30 °C. The cells were harvested at 4500×g for 30 minutes and stored at -20 °C for further use.

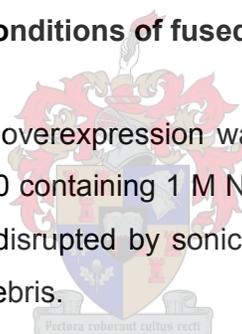
CBD_{CenA}-EcCoaE

The plasmid pET28a-CBD_{CenA}-EcCoaE was transformed into *E. coli*. Tuner(DE3) and grown in LB broth supplemented with 30 mg/litre kanamycin sulphate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 100 µM IPTG and grown overnight at 37 °C. The cells were harvested at 4500×g for 30 minutes and stored at -20 °C.

3.7.3 Binding and elution conditions of fused enzymes to cellulose

Sample preparation

The cell pellet obtained from overexpression was suspended in high salt elution buffer (10 mM Tris-HCl, pH 8.0 containing 1 M NaCl), suspending 1 g cell paste in 10 ml buffer. The cells were disrupted by sonication and centrifuged at 15000×g for 30 minutes to collect cell debris.



Preparation of Cellulose

Avicel™ cellulose was used and has a binding capacity of approximately 40 mg of target protein per gram cellulose (2). To ensure complete binding of the target protein 2-4 times the amount of cellulose required to bind the estimated amount of target protein was used. The 1 g cellulose was suspended in distilled water (2 times the cellulose volume), centrifuged for 5 minutes at 4500×g and the supernatant was decanted to remove fines. This process was repeated five times where after the cellulose was resuspended in 10 ml high salt elution buffer.

Column purification

Cellulose slurry from prepared cellulose was used to pack the columns and the cellulose was equilibrated with 5-10 ml high salt elution buffer (10 mM Tris-HCl, pH

8.0 containing 1 M NaCl). Crude extract (3.75 ml) from the prepared sample was loaded onto the resin which was washed with 5-10 ml high salt buffer followed by further washing with 5-10 ml low-salt elution buffer (10 mM Tris-HCl, pH 8.0 containing 50 mM NaCl). Enzymes were removed from the cellulose using various eluents. Each of the following eluents was tested separately: distilled water, 99% ethylene glycol and 50% glycerol, where 5 ml eluent was used each time to remove protein from cellulose. The amount of protein removed was determined using a Bradford concentration assay. The purity of the proteins was evaluated using SDS-PAGE analysis after samples were boiled at 95 °C (5 minutes) with an equal amount of SDS-PAGE loading buffer (150 mM Tris-HCl (pH 6.8), 2% SDS, 15% glycerol, 15% β -mercaptoethanol, 0.3 mM bromophenol Blue). The prepared samples were loaded onto a 12% SDS-PAGE gel that was stained with Coomassie Brilliant Blue R-250 dye.

Determining enzyme concentration with Bradford assay

The Quick Start Bradford protein assay is a simple and accurate procedure for determining the concentration of protein in solution. The assay involves the binding of Coomassie Brilliant Blue R-250 dye to proteins. When the dye binds to protein it is converted to a stable unprotonated blue form which was detected at 595 nm using a Multiskan Spectrum multiplate spectrophotometer (Thermo Labsystems).

Bovine Serum Albumin was used as standard and has a linear range of 125-1000 $\mu\text{g}\cdot\text{ml}^{-1}$. Each standard (5 μl) from the kit was transferred into a microtiter plate and 250 μl Bradford reagent (ambient temperature) was added to each standard. The same was done with the protein under investigation and a blank (distilled water) was also measured. The standards and samples were done in triplicate and the plate was incubated at room temperature for at least 5 minutes after addition of Bradford reagent before the absorbance was measured at 595 nm. From these measurements the protein concentration was determined using the Beer-Lambert law.

3.7.4 Cloning, overexpression and purification of CBD_{Cex}-fused enzymes

CBD_{Cex}-EcCoaA

The *coaA* gene was amplified by PCR using the *T7* promoter as forward primer (sequence: 5'-TAATACGACTCACTATAGGG-3') and the following sequence as reverse primer: 5'-CCCTGCAAATTACTCGAGTAGTCTGACCTC-3', introducing a *XhoI* site (underlined) which will remove the stop codon. The resulting purified PCR product coding for *coaA* (no stop) was cloned into *NdeI/XhoI*-digested pET-38b(+) expression vector from Novagen. The sequence of the resulting plasmid, pET-38b-*EcCoaA* was verified by automated DNA sequencing.

The plasmid was transformed into *E. coli*. BL21-star(DE3). Expression trials were done to determine optimum expression conditions as seen in section 3.7.5 for whole cell expression and cell free expression. The plasmid was grown in LB broth supplemented with 30 mg/ml kanamycin sulphate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 50 µM IPTG and grown overnight at 37 °C. After the cells were harvested the cell paste was suspended in sonication buffer (5 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9; 10ml/1g cell paste) after which it was disrupted by sonication and centrifuged at 15 000×g for 30 minutes to collect cell debris. The crude extract was applied to a 1 ml HisTrap™ Chelating Column and purified on an ÄKTApurification system. Weakly bound proteins were removed by washing with sonication buffer followed by sonication buffer containing 75 mM imidazole. Increasing the imidazole concentration to 500 mM eluted the protein of interest and the elution was monitored at A₂₈₀.

CBD_{Cex}-EcCoaD

The *coaD* gene was amplified by PCR using the *T7* promoter as forward primer, and the following sequence as reverse primer: 5'-GGATCCAACGCTCGAGTAACTTCGCCATCAGCG-3', introducing an *XhoI* site (underlined) which will remove the stop codon. The resulting purified PCR product coding for *coaD* (no stop) was cloned into *NdeI/XhoI*-digested pET-38b(+) expression vector from Novagen. The sequence of the resulting plasmid, pET-38b-*EcCoaD* was verified by automated DNA sequencing.

The plasmid was transformed into *E. coli*. BL21-star(DE3). Expression trials were done to determine optimum expression conditions as seen in section 3.7.5 for whole cell expression and cell free expression. The plasmid was grown in LB broth supplemented with 30 mg/litre kanamycin sulfate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 500 µM IPTG and grown overnight at 30 °C. After the cells were harvested the cell paste was suspended in sonication buffer (5 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9; 10ml/1g cell paste) where after it was disrupted by sonication and centrifuged at 15 000×g for 30 minutes to collect cell debris. The crude extract was applied to a 1 ml HisTrapTM Chelating Column and purified on an ÄKTAprime purification system. Weakly bound proteins were removed by washing with sonication buffer followed by sonication buffer containing 75 mM imidazole. Increasing the imidazole concentration to 500 mM eluted the protein of interest and the elution was monitored at A₂₈₀.

CBD_{Cex}-EcCoaE

The *coaE* gene was amplified by PCR using the *T7* promoter from Inqaba as forward primer, and the following sequence as reverse primer: 5'-ATTCGGATC CGCACTCGAGTTTTTCCTGTGAG-3', introducing a *XhoI* site (underlined) which will remove the stop codon. Several attempts to clone the resulting purified PCR product, coding for *coaE* (no stop), into *NdeI/XhoI*-digested pET-38b(+) expression vector from Novagen was made, but with no success.

3.7.5 CBD_{CipA}-EcCoaD

Mrs. LA Brand did cloning of pET28a-CBD_{CipA}-EcCoaD. The plasmid was transformed into *E. coli*. BL21-star(DE3) and grown in 15 ml LB broth supplemented with 30 mg/litre kanamycin sulphate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with a range of IPTG concentrations (50 µM – 1000 µM) and grown overnight at 37 °C. The cells were harvested at 4500×g for 30 minutes.

Whole cell expression trial

The harvested cells were resuspended in 2 ml 10 mM Tris-HCl (pH 8) and 200 μ l of the cell suspension was transferred to a new Eppendorf tube. The suspension was centrifuged at 13 000 \times g for 2 minutes. The supernatant was removed after centrifugation (13 000 \times g for 2 minutes) and the pellet was again resuspended in 100 μ l 10 mM Tris-HCl (pH 8). Glass beads were used to disrupt cells and 50 μ l of the supernatant was boiled at 95 °C (5 minutes) with an equal amount of SDS-PAGE loading buffer (150 mM Tris-HCl (pH 6.8), 2% SDS, 15% glycerol, 15% β -mercaptoethanol, 0.3 mM bromophenol Blue). The prepared samples were loaded onto a 12% SDS-PAGE gel that was stained with Coomassie Brilliant Blue R-250 dye.

Cell free expression trial

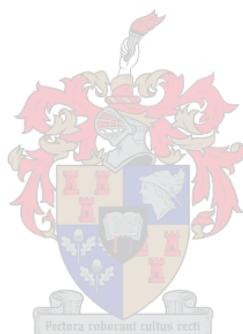
The harvested cells were resuspended in 2 ml 10 mM Tris-HCl (pH 8) and 200 μ l of the cell suspension was transferred to a new Eppendorf tube. The suspension was centrifuged at 13 000 \times g for 2 minutes. The supernatant was removed and the pellet was again resuspended in 50 μ l 10 mM Tris-HCl (pH 8). SDS-PAGE loading buffer (50 μ l; 150 mM Tris-HCl (pH 6.8), 2% SDS, 15% glycerol, 15% β -mercaptoethanol, 0.3 mM bromophenol Blue) was added and the samples were boiled for 5 minutes at 95°C. The prepared samples were loaded onto a 12% SDS-PAGE gel that was stained with Coomassie Brilliant Blue R-250 dye.

3.8 References

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Coenzyme A Analogues:

Chemo-enzymatic Synthesis of a Thioester CoA-synthon

4.1 Introduction

The numerous roles played by coenzyme A in metabolic pathways have been the subject of intensive research. Due to the importance and ubiquity of CoA, various substrates used in the production of CoA analogues are valuable as reagents in enzyme-catalyzed reactions and as probes of enzyme chemistry.

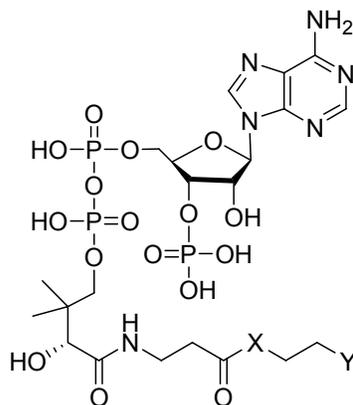
Methods to generate novel CoA analogues by means of either synthetic strategies or chemo-enzymatic synthesis have been reported by various investigators (1-6). An approach used by Drueckhammer *et al.* has been used to prepare a number of CoA analogues. This one-pot assembly of pantothenic acid derivatives into CoA analogues, by using purified recombinant CoA biosynthetic *E. coli* enzymes, has been used successfully in the preparation of these compounds (2-4, 7). These studies have been used as foundation to develop a new strategy for the production of CoA analogues.

4.1.1 Drueckhammer's strategy

CoA analogues have already been prepared by various different methods. One of these, the chemo-enzymatic synthesis of CoA analogues, relies on the action of the CoaD and CoaE enzymes on phosphopantetheine precursors to produce these analogues. However, in this method the various phosphopantetheine precursors each have to be prepared separately to prepare the corresponding CoA derivative. Preparation of these phosphorylated precursors can be tedious and time-consuming due to all the synthesis steps required.

To sidestep the problem of preparing different phosphopantetheine precursors, Drueckhammer *et al.* prepared an activated phosphopantetheine derivative from

which a general CoA analogue synthon was subsequently synthesized. This synthon had a thioester linkage in place of the amide bond nearest to the thiol group of CoA (figure 4.1).



Coenzyme A:
X = NH, Y = SH

CoA analogue synthon:
X = S, Y = CH₃

Figure 4.1 A general CoA analogue synthon used by Drueckhammer *et al.* (3).

The thioester was chosen because thioesters preferentially react with amines to undergo aminolysis even in the presence of other nucleophiles such as water. As a result, a variety of CoA analogues can be prepared from a single thioester precursor by treatment with excess amine to reform the amide bond present in CoA (2, 4). Drueckhammer's strategy is summarized in figure 4.2.

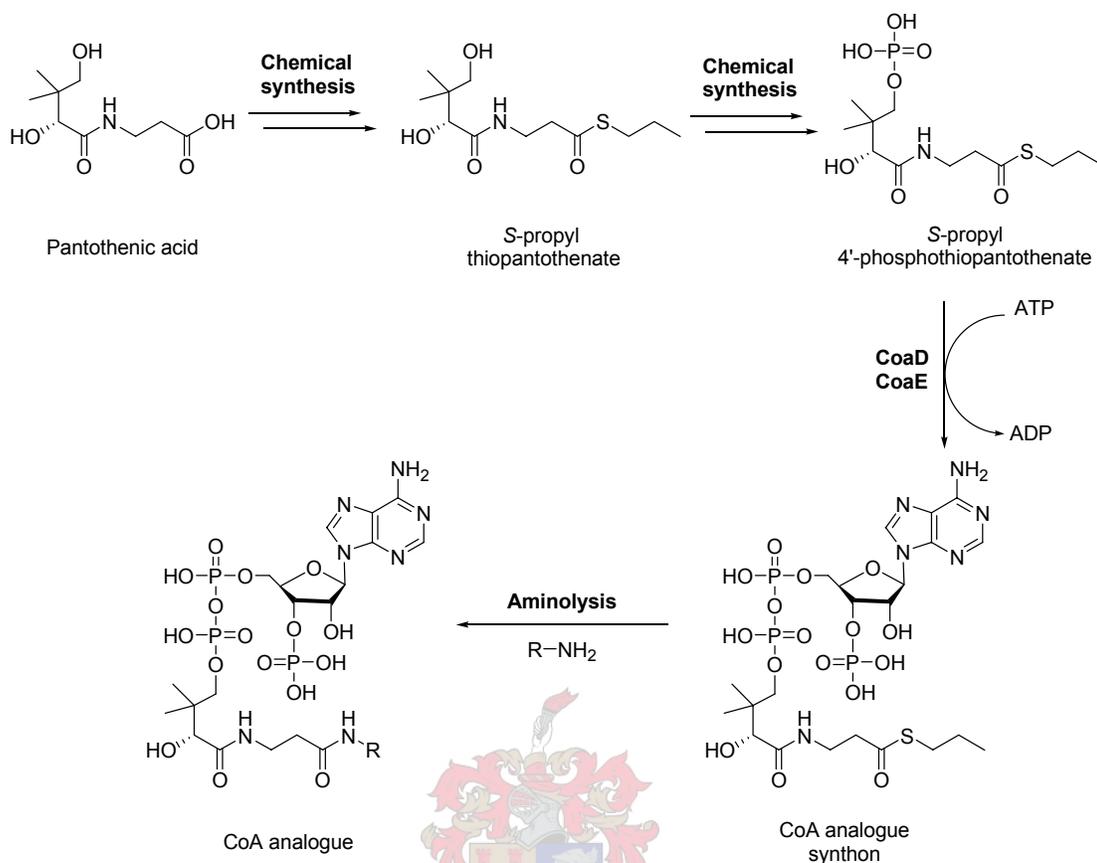


Figure 4.2 Strategy used by Drucekhammer *et al.* in the production of CoA analogues (2, 4).

However, this method has various disadvantages. The first of these is the synthesis of Drucekhammer's chosen activated phosphopantetheine derivative, the propyl thioester S-propyl 4'-phosphothiopantothenate, which is tedious and consists of various synthetic steps (figure 4.3). Drucekhammer chose the propyl thioester for his synthetic strategy because smaller thioesters, such as ethyl or methyl thioesters, could not be separated chromatographically from the methyl pantothenate precursor he used.

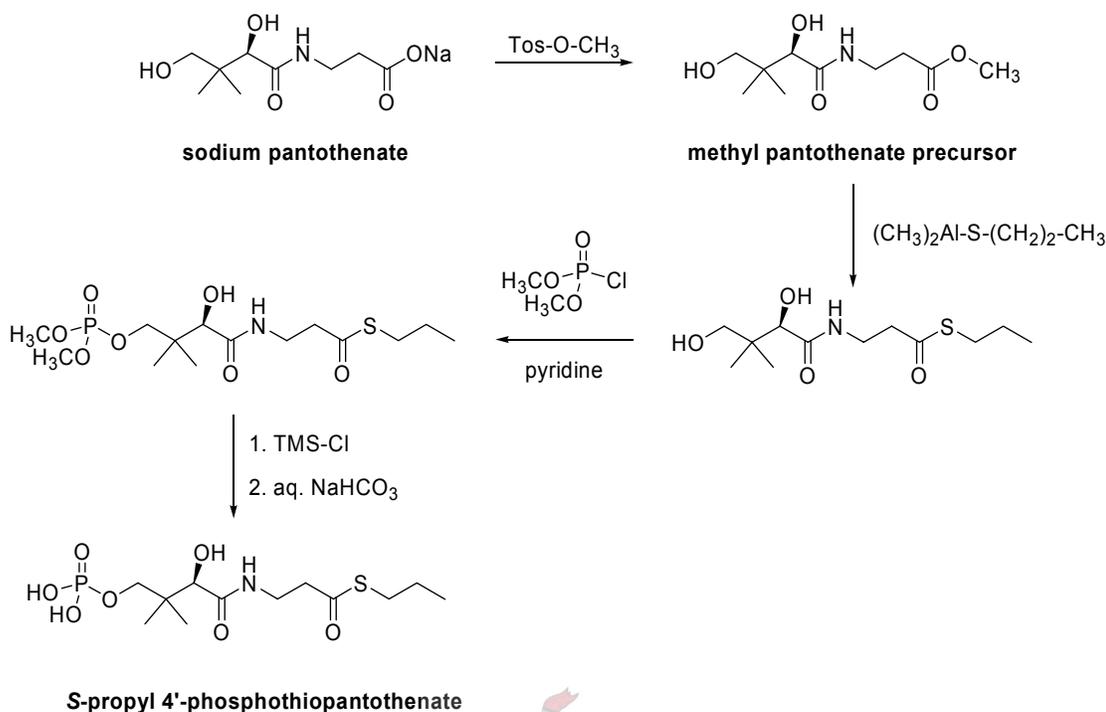
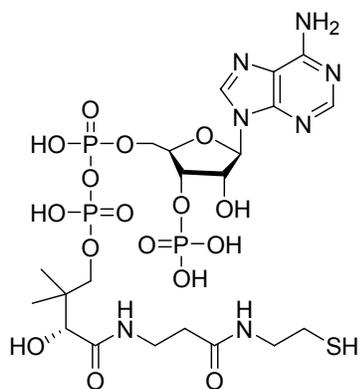


Figure 4.3 The synthesis of S-propyl 4'-phosphothiopantothenate used by Drueckhammer (2).

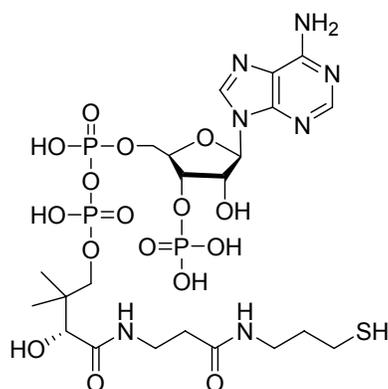
The second disadvantage is the high amine concentrations (~80-380 equivalents) and long reaction times (23 hours) that are used in the aminolysis reactions of the CoA thioester synthon (2). One of the reasons for this is the fact that propyl thioesters are not as activated towards aminolysis as other thioesters that are sterically less-hindered. In combination, this strategy produces CoA analogues in low yield, and necessitates extensive chromatographic purification.

4.1.2 Strategy in this study

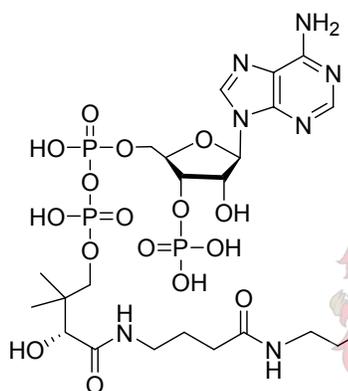
In this study, the strategy from Drueckhammer was adapted to produce CoA analogues that differ from each other in the length of their pantotheine moieties (figure 4.4).



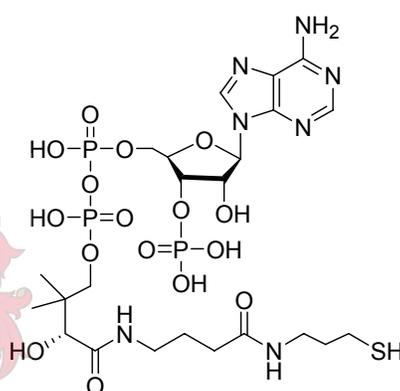
CoA (1.1)



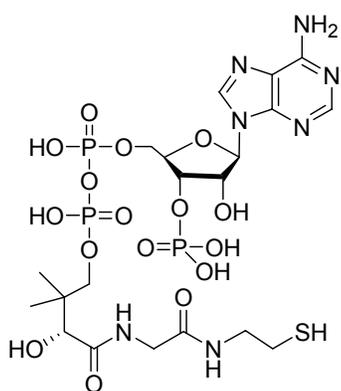
CoA with extended tether (4.1)



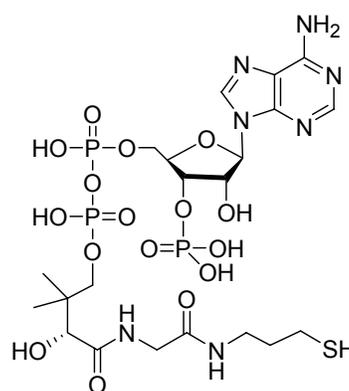
CoA with extended tether (4.2)



CoA with extended tether (4.3)



CoA with shortened tether (4.4)



Variation on CoA tether (4.5)

Figure 4.4 Different CoA analogues with different tether lengths.

This adapted strategy used a three-step enzymatic synthesis (figure 4.5) of the CoA thioester synthon from a pantetheine analogue, compared to the two-step enzymatic synthesis from phosphopantetheine used by Drueckhammer. The added enzymatic step uses CoaA in the phosphorylation of the pantetheine analogue, which eliminates time-consuming synthesis of the phosphopantetheine precursor. The use of CoaA to produce the phosphopantetheine precursor also ensures better yields of the substrate. Three ethyl thioesters were used instead of the propyl thioester utilized by Drueckhammer *et al.* (2). The ethyl thioesters were chosen to decrease the steric bulk around the carbonyl of the thioester to facilitate the aminolysis reaction (8).

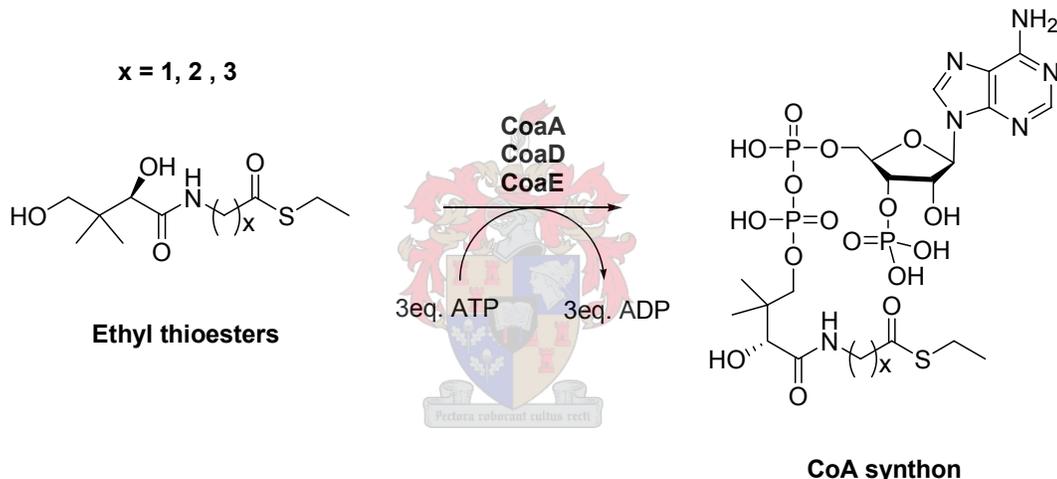


Figure 4.5 Preparation of CoA synthons using ethyl thioesters as substrates in a three-step enzymatic reaction.

The thiol-bearing amines cysteamine and homocysteamine were used in the aminolysis of three CoA analogue synthons (renamed ethyl pre-CoAs). These two amines first react with the ethyl pre-CoAs in a transthioesterification reaction, followed by an intramolecular N-S acyl shift to form the final CoA derivatives (figure 4.6). Since the primary transthioesterification effectively increases the amine concentration by allowing aminolysis to proceed intramolecularly, this adapted strategy requires considerably lower amine concentrations and shorter reaction times than those used by Drueckhammer.

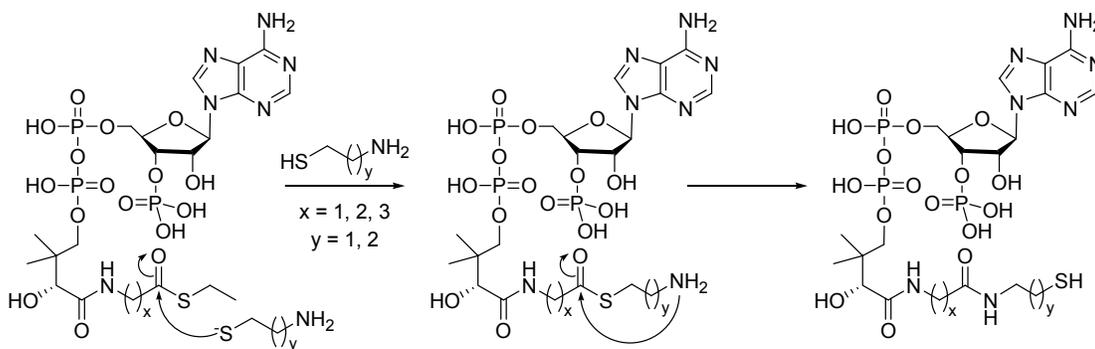


Figure 4.6 The aminolysis of pre-CoAs by either cysteamine or homocysteamine

In conclusion, the objectives of this chapter were the production of three different pantetheine analogues with different tether lengths. The activity of pantothenate kinase (CoaA) (from different prokaryotic sources), towards these substrates were investigated to evaluate the feasibility of these compounds to be phosphorylated by this enzyme. The various pantetheine analogues were used in the preparation, purification and characterization of three different CoA analogue synthons (pre-CoAs). These pre-CoAs were to be used in the aminolysis reactions with either cysteamine or homocysteamine in the production of CoA analogues with different tether lengths.

4.2 Results and discussion

4.2.1 Preparation of pantothenic acid ethyl thioesters

Three different ethyl thioesters were used in this study as substrates for the production of CoA analogues. The first thioester, *S*-ethyl thiopantothenate (**4.8**) is directly derived from pantothenic acid. The other two ethyl thioesters are derived from homopantothenic acid (**4.6**) and α -pantothenic acid (**4.7**), which differs from pantothenic acid by being either one methylene group longer or one methylene shorter than pantothenic acid itself. This resulted in the production of *S*-ethyl thiohomopantothenate (**4.9**) and *S*-ethyl thio- α -pantothenate (**4.10**), as shown in figure 4.7.

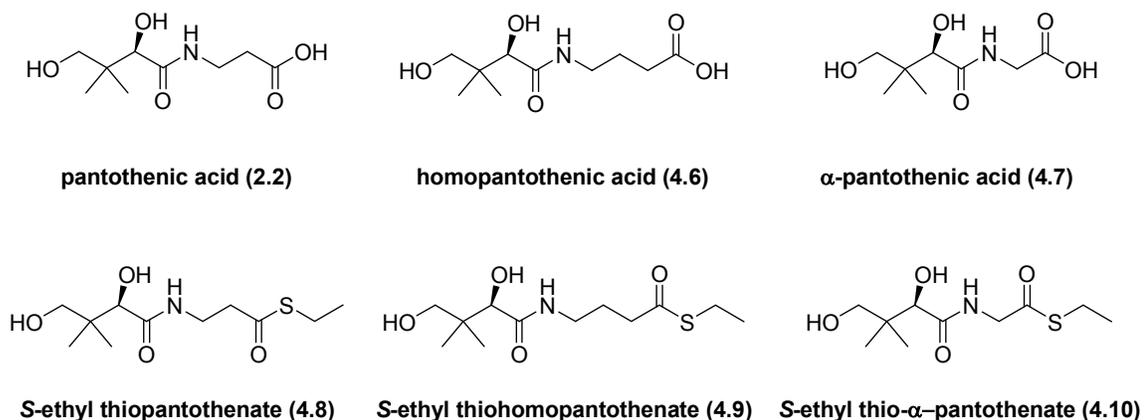


Figure 4.7 Ethyl thioesters derived from their pantothenic acid derivatives.

The pantothenic acid ethyl thioesters (**4.8 – 4.10**) were synthesized according to Yamada *et al.* (9) from the corresponding pantothenic acids (**2.2**, **4.6** and **4.7**) as shown in figure 4.7. The synthesis of the pantothenic acid derivatives were prepared based on a published work by McFall Desha and Fuerst (10) in combination with Ivey *et al.* (11) by reacting D-pantolactone with either γ -amino butyric acid (for **4.6**) or glycine (for **4.7**), depending on which acid was being prepared (figure 4.8; step 1). The thioesters were synthesized by treating the different pantothenic acid derivatives with diphenylphosphoryl azide in the presence of triethylamine to form a phosphoryl anhydride linkage with the carboxylate (figure 4.8; step 2). The diphenylphosphosphate was eliminated by the addition of ethanethiol resulting in the ethyl thioester as product.

To determine if the pantothenic acid ethyl thioesters can be phosphorylated by CoaA at position 4', the synthesized substrates were tested to see if they were accepted by pantothenate kinase (figure 4.9). Previous studies has shown that phosphorylation of substrates can be achieved with *Ec*CoaA (6). However, *Ec*CoaA is regulated by feedback inhibition which could be problematic later in the biosynthesis reaction of the CoA analogues. Therefore, *Sa*CoaA was also considered for the phosphorylation of substrates. Steady state kinetics was used to compare the activity of the CoaAs (*Sa*CoaA and *Ec*CoaA) with the purified substrates in comparison with pantothenic acid, which is the natural substrate in CoA biosynthesis. The phosphorylation of homopantothenic acid and α -pantothenic acid were also investigated in comparison to the ethyl thioesters.

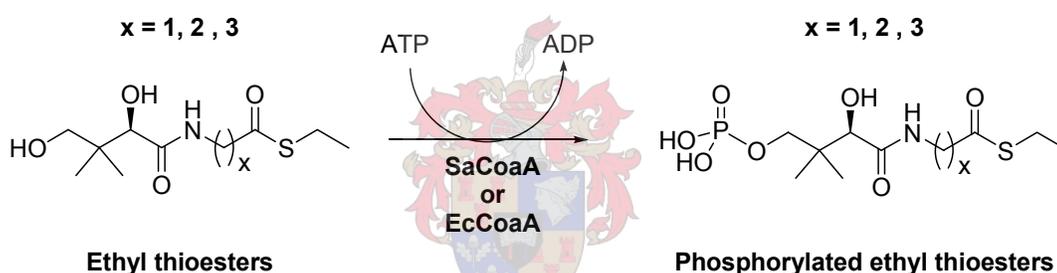


Figure 4.9 Phosphorylation of pantothenic acid ethyl thioesters by CoaA in the presence of ATP to yield the phosphorylated ethyl thioesters.

Assay method

To determine if phosphorylation of the substrates took place, we used a standard kinase assay based on the generation of ADP from ATP after phosphoryl transfer. In this assay the formation of ADP is coupled to NADH usage, which can be measured spectrophotometrically at 340nm (12). Specifically, the assay consisted of the ATP-dependent phosphorylation of pantothenic acid by pantothenate kinase to form 4'-phosphopantothenic acid and ADP (figure 4.10). ADP is recycled to ATP by a reaction with phosphoenol pyruvate (PEP), catalyzed by pyruvate kinase. This reaction produces pyruvate as product, which is reduced by lactate dehydrogenase to lactate in the presence of NADH to form NAD^+ . The

consumption of NADH is measured at 340 nm and this measurement can then be used to determine the activity of pantothenate kinase (CoaA) towards the substrate under investigation.

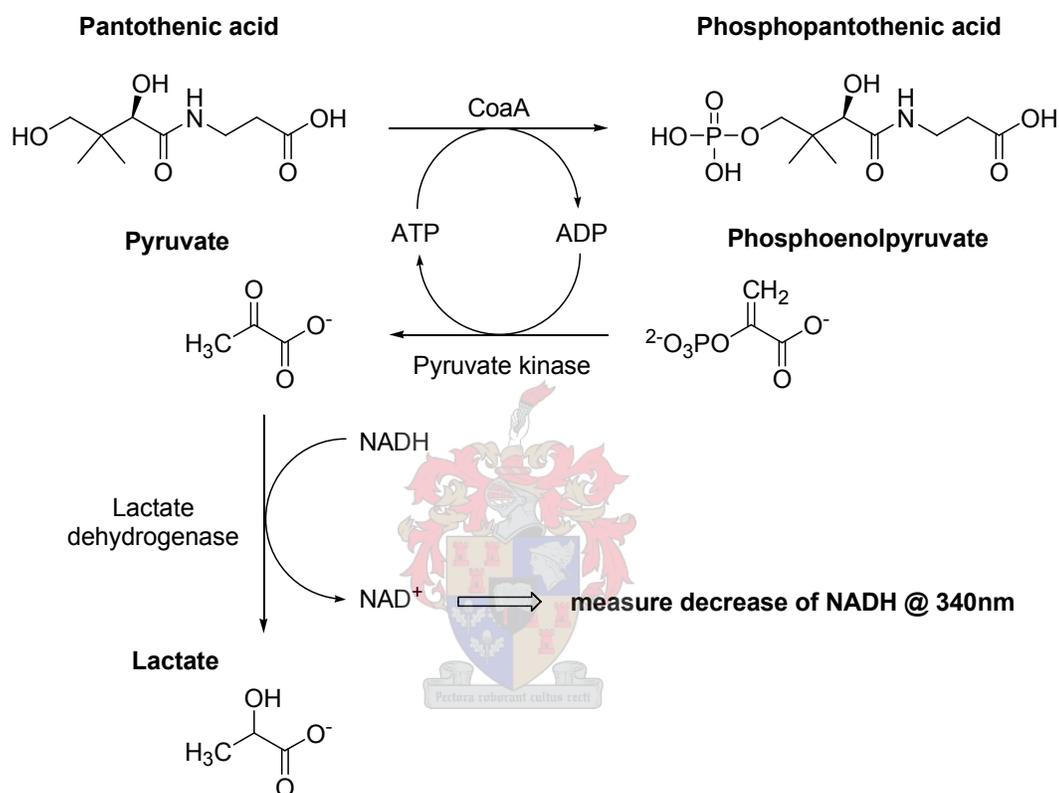


Figure 4.10 Phosphorylation assay measuring the generation of ADP through NADH utilization.

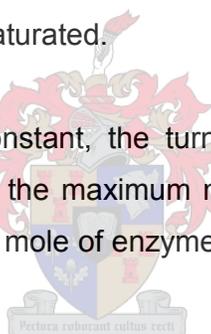
Steady state kinetic parameters

Steady state kinetic parameters for varying substrate concentrations were obtained by fitting the data points of the assay to the Michaelis-Menten equation, which is a mathematical equation expressing the hyperbolic relationship between initial rate and substrate concentration (14):

$$v^0 = \frac{V_{\max} [S]}{K_M + [S]}$$

where V_{\max} is the limiting value of the initial rate when all the active sites are occupied and K_M is the Michaelis constant. The Michaelis constant is numerically equal to the substrate concentration at which the initial rate is half of the maximum rate. This constant indicates the required substrate concentration at which all the active sites of the enzyme are saturated.

In addition to the Michaelis constant, the turnover number (k_{cat}) is a valuable catalytic constant that indicates the maximum number of moles of substrate that can be converted to product per mole of enzyme in unit time, where $[E_t]$ is the total concentration of enzyme.



$$k_{\text{cat}} = \frac{V_{\max}}{[E_t]}$$

The specificity constant (k_{cat}/K_M) is being defined as a measure of how efficiently an enzyme converts substrate to product at low substrate concentrations and this constant can be used to compare different substrates with different enzymes most efficiently (14).

Kinetic analysis

All three ethyl thioesters (4.8 - 4.10) as well as the pantothenic acid derivatives were analyzed as substrates for either *Ec*CoaA or *Sa*CoaA. These substrates were compared to pantothenic acid. Michaelis-Menten curves were used to interpret the results for all the substrates. From these Michaelis-Menten curve fits (figure 4.11 and 4.12), it was determined that *Sa*CoaA phosphorylated homopantothenic acid (4.6). *Ec*CoaA only showed activity towards α -pantothenic acid (4.7). The reaction of *Ec*CoaA with α -pantothenic acid had the lowest turnover rate (k_{cat}) in comparison to the turnover rate of *Ec*CoaA with pantothenic acid.

*Sa*CoaA phosphorylated all the pantothenic acid ethyl thioesters (figure 4.13), while *Ec*CoaA showed no activity towards these substrates. Although *Sa*CoaA showed relative high turnover rates (k_{cat}) for these thioesters, the K_M values were very high resulting in lower overall activity in comparison with pantothenic acid. These results are summarized in table 4.1 to compare the five different substrates.

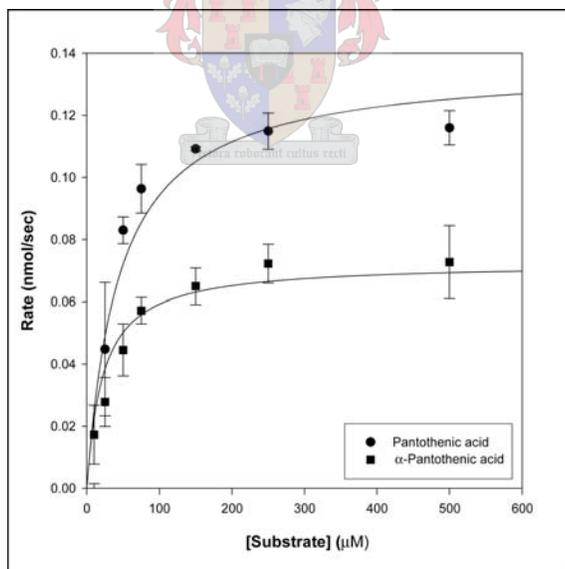


Figure 4.11 Activity of *Ec*CoaA with pantothenic acid and α -pantothenic acid. Initial rate plots of *Ec*CoaA against increasing substrate concentrations. Substrates used: pantothenic acid (●), α -pantothenic acid (■), respectively. Symbols show the average of three replicates, with error bars indicating the standard deviation. Curves show fits of the individual data sets to the Michaelis-Menten equation.

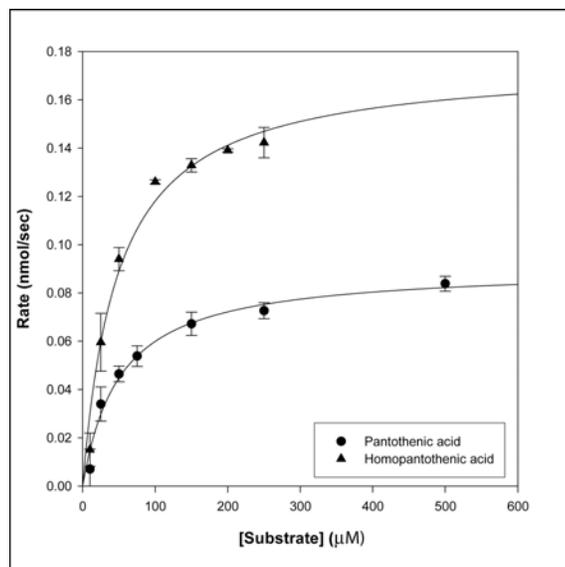


Figure 4.12 Activity of SaCooA with pantothenic acid and homopantothenic acid. Initial rate plots of *EcCooA* against increasing substrate concentrations. Substrates used: pantothenic acid (●), homopantothenic acid (▲), respectively. Symbols show the average of three replicates, with error bars indicating the standard deviation. Curves show fits of the individual data sets to the Michaelis-Menten equation.

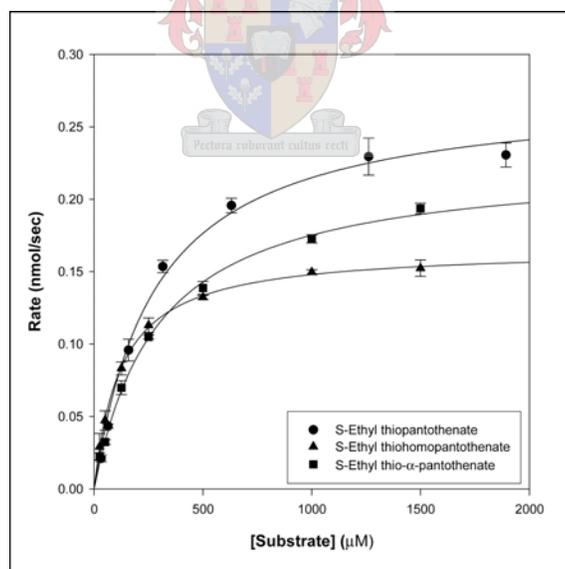


Figure 4.13 Activity of SaCooA with pantothenate thioesters. Initial rate plots of *EcCooA* against increasing substrate concentrations. Substrates used: S-ethyl thiopantothenate (●), S-ethyl thiohomopantothenate (▲) and S-ethyl thio- α -pantothenate (■), respectively. Symbols show the average of three replicates, with error bars indicating the standard deviation. Curves show fits of the individual data sets to the Michaelis-Menten equation.

Table 4.1 Kinetic parameters of *Ec*CoaA vs. *Sa*CoaA

Substrate	Enzyme	k_{cat}	K_M	k_{cat}/K_M
		(s^{-1})	(μM)	($mM^{-1} \cdot s^{-1}$)
pantothenic acid (2.2; $x = 2$)	<i>Ec</i> CoaA	1.75	44.7 ± 10.6	39.1 ± 11.2
	<i>Sa</i> CoaA	1.76	51.3 ± 6.9	34.3 ± 10.1
α -pantothenic acid (4.7; $x = 1$)	<i>Ec</i> CoaA	0.95	46 ± 7.3	20.6 ± 5.1
	<i>Sa</i> CoaA	NA*	NA*	NA*
homopantothenic acid (4.6; $x = 3$)	<i>Ec</i> CoaA	NA*	NA*	NA*
	<i>Sa</i> CoaA	3.41	48.7 ± 6.3	70 ± 21.4
S-ethyl thiopantothenate (4.8; $x = 2$)	<i>Ec</i> CoaA	NA*	NA*	NA*
	<i>Sa</i> CoaA	5.33	277.6 ± 21	19.2 ± 6
S-ethyl thio- α -pantothenate (4.10; $x = 1$)	<i>Ec</i> CoaA	NA*	NA*	NA*
	<i>Sa</i> CoaA	3.22	122.6 ± 7.2	26.3 ± 7
S-ethyl thiohomopantothenate (4.9; $x = 3$)	<i>Ec</i> CoaA	NA*	NA*	NA*
	<i>Sa</i> CoaA	4.40	293 ± 13.9	15 ± 5

Not Active ($k_{cat}/K_M \sim 9 \text{ mM}^{-1} \cdot \text{s}^{-1}$)

From this assay it was concluded that *Sa*CoaA showed wide specificity and high levels of activity. This enzyme also has the added advantage that it is not regulated by feedback inhibition. As a result *Sa*CoaA was used for the phosphorylation of the ethyl thioester precursors in the biosynthesis of the pre-CoAs.

4.2.3 Biosynthesis of pre-CoAs

To show that the modified biosynthetic pathway can be used to prepare CoA analogues, three pre-CoAs were synthesized from their pantothenate ethyl thioester precursors (figure 4.14). A method adapted from Nazi *et al.* (6) was used where *SaCoaA* phosphorylates the pantothenate thioester substrates, *EcCoaD* catalyzes the Mg^{2+} -dependant reversible transfer of the adenylyl group of ATP onto the phosphopantothenate substrate and finally *EcCoaE* phosphorylates the 3'-OH group of the adenylyl group.

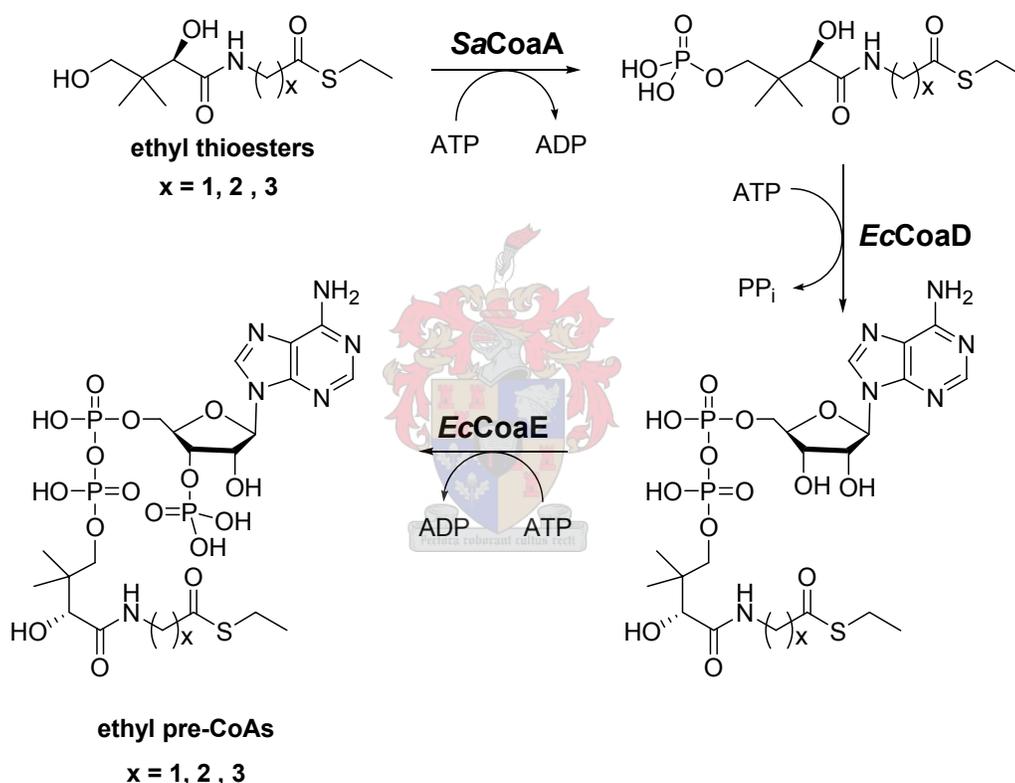


Figure 4.14 Biosynthesis of ethyl-pre-CoAs ($x = 1$ for ethyl pre- α -CoA (4.13); $x = 2$ for ethyl pre-CoA (4.11); $x = 3$ for ethyl pre-homo-CoA (4.12)).

The three pre-CoAs were biosynthesized and purified on C_{18} solid phase extraction cartridges. NMR analysis (figure 4.15) of the three pre-CoAs confirmed that these compounds were in fact prepared in moderate yields and high purity. These NMR spectra compared well with the spectrum of CoA obtained by D'Ordine *et al.* (15). The data is summarized in table 4.2.

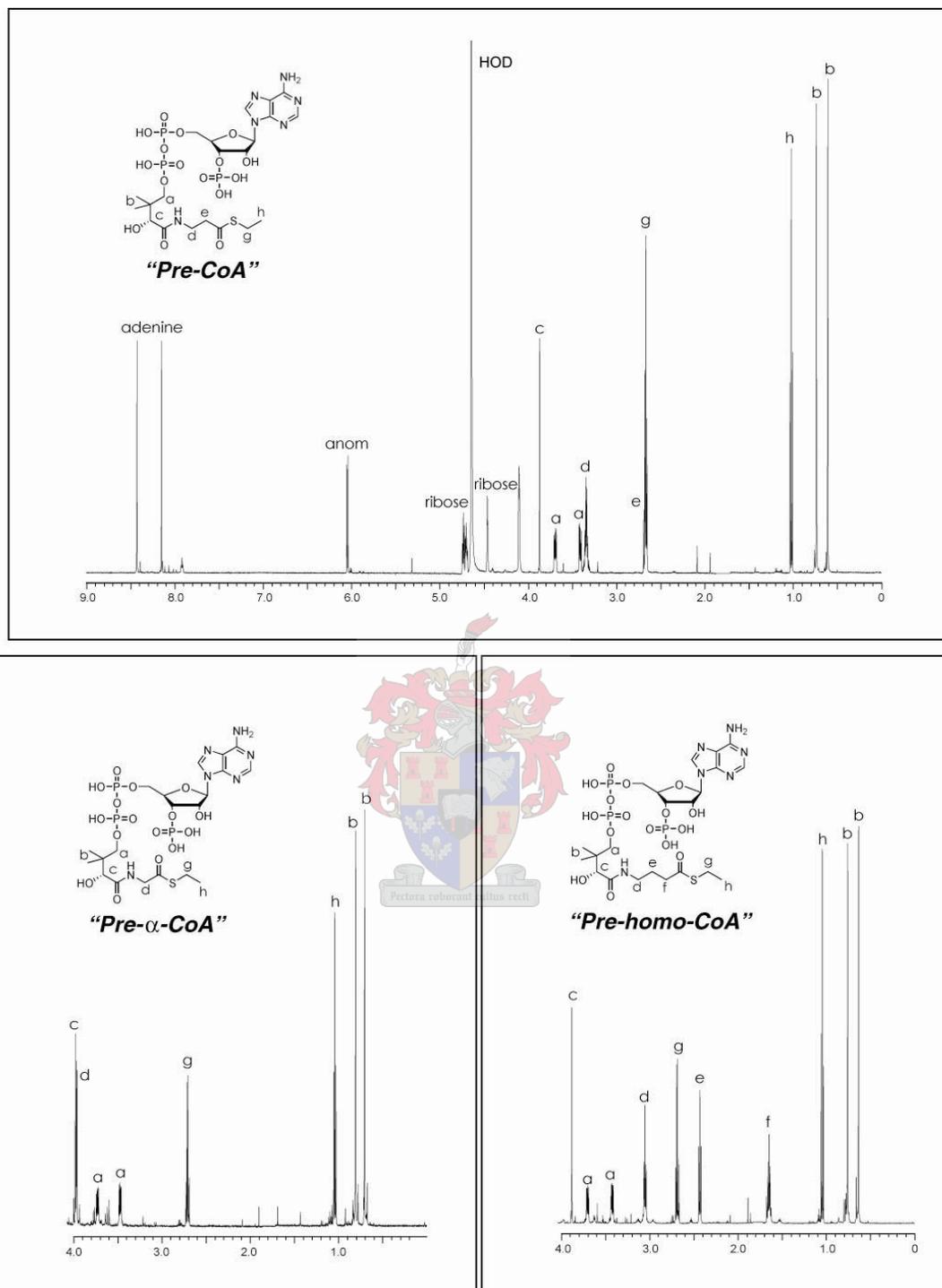


Figure 4.15 NMR verification of pre-CoAs prepared from pantothenic acid ethyl thioesters. Only the pantetheine signals are shown for the pre-homo-CoA (4.12) and pre- α -CoA (4.13) analogues.

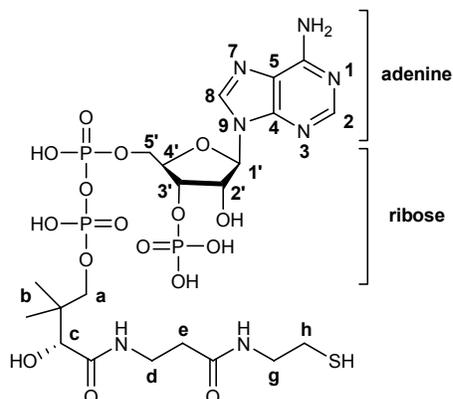


Table 4.2 NMR comparison between ethyl pre-CoAs vs. CoA (15). The numbering system used for the adenosine portion of CoA is standard and adapted from (15.) This system was also used in numbering the CoA derivatives. The numbering on the pantetheine portion of the molecules were changed and just shows numbering for carbons with hydrogens on them.

Assignments	CoA	Pre-CoA	Pre-homo-CoA	Pre- α -CoA
^1H	δ (ppm)	δ (ppm)	δ (ppm)	δ (ppm)
8	8.47 (s, 1H)	8.43 (s, 1H)	8.41 (s, 1H)	8.42 (s, 1H)
2	8.15 (s, 1H)	8.15 (s, 1H)	8.12 (s, 1H)	8.13 (s, 1H)
1'	6.10 (d, 1H)	6.05 (d, 1H)	6.03 (d, 1H)	6.04 (d, 1H)
3'	4.87 (t, 1H)	4.73 (t, 1H)	4.71 (t, 1H)	4.73 (t, 1H)
2'	4.85 (t, 1H)	4.70 (t, 1H)	4.67 (t, 1H)	4.70 (t, 1H)
4'	4.58 (s, 1H)	4.46 (s, 1H)	4.45 (s, 1H)	4.46 (s, 1H)
5'	4.23 (s, 2H)	4.10 (s, 2H)	4.10 (s, 2H)	4.11 (s, 2H)
C	3.94 (s, 1H)	3.87 (s, 1H)	3.88 (s, 1H)	3.98 (s, 1H)
A	3.80 (q, 1H)	3.69 (q, 1H)	3.70 (q, 1H)	3.73 (q, 1H)
	3.54 (q, 1H)	3.41 (q, 1H)	3.42 (q, 1H)	3.47 (q, 1H)
D	3.44 (t, 2H)	3.34 (m, 2H)	3.05 (t, 2H)	3.96 (d, 2H)
G	3.25 (t, 2H)	2.69 (t, 2H)	2.69 (q, 2H)	2.71 (q, 2H)
H	2.59 (t, 2H)	1.02 (t, 3H)	1.05 (t, 3H)	1.04 (t, 3H)
F	-	-	1.65 (m, 2H)	-
E	2.43 (t, 2H)	2.67 (s, 2H)	2.43 (t, 2H)	-
b' (pro-S)	0.71 (s, 3H)	0.61 (s, 3H)	0.63 (s, 3H)	0.71 (s, 3H)
b'' (pro-R)	0.84 (s, 3H)	0.74 (s, 3H)	0.76 (s, 3H)	0.81 (s, 3H)

In combination with the NMR analysis, ESI-MS analysis (table 4.3) confirmed that the ethyl pre-CoAs were biosynthesized utilizing *SaCoaA*, *EcCoaD* and *EcCoaE* from the substrates synthesized.

Table 4.3 ESI-MS results of ethyl pre-CoAs

CoA analogue synthon	[M+H] ⁺ calculated	[M+H] ⁺ found	[M-ADP] ⁺ found
Ethyl pre-CoA (4.11)	753.1	753.1	264.1
Ethyl pre-homo-CoA (4.12)	767.1	767.2	278.1
Ethyl pre- α -CoA (4.13)	739.1	739.2	250.1

4.2.4 Aminolysis of pre-CoAs

To obtain the final CoA derivatives, each of the ethyl pre-CoAs were subjected to an aminolysis reaction with excess amine. These reactions were done using purified ethyl pre-CoAs in the presence of 10 equivalents of homocysteamine or cysteamine. Although an excess amount of amine is present in the reaction, a considerable lower amount of amine was used in comparison to reactions done by Drueckhammer *et al.* where amounts of up to ~80-380 equivalents were used (2, 7). Because thiol-bearing amines are used, the reaction proceeds through a primary transthioesterification step, followed by an intramolecular N-S acyl shift to form the CoA analogue as shown in figure 4.16.

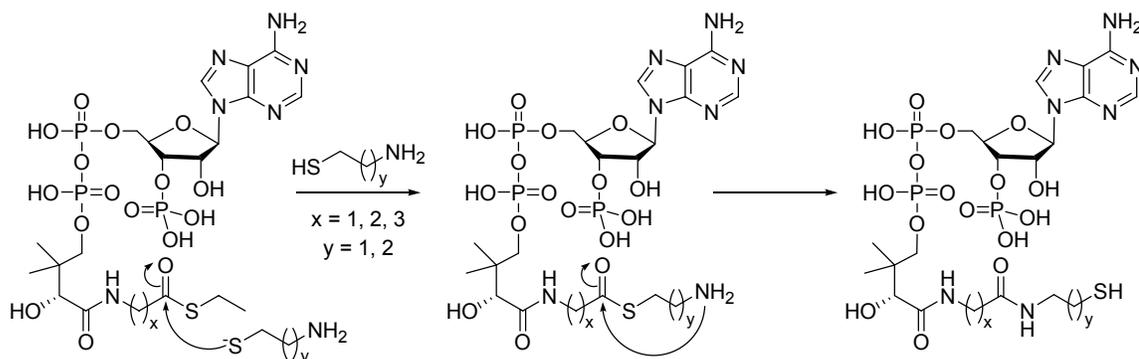


Figure 4.16 Aminolysis of pre-CoAs with either cysteamine ($y = 1$) or homocysteamine ($y = 2$).

Preparation of homocysteamine.HCl

While cysteamine is commercially available homocysteamine had to be synthesized in a simple three-step synthesis as summarized in figure 4.17.

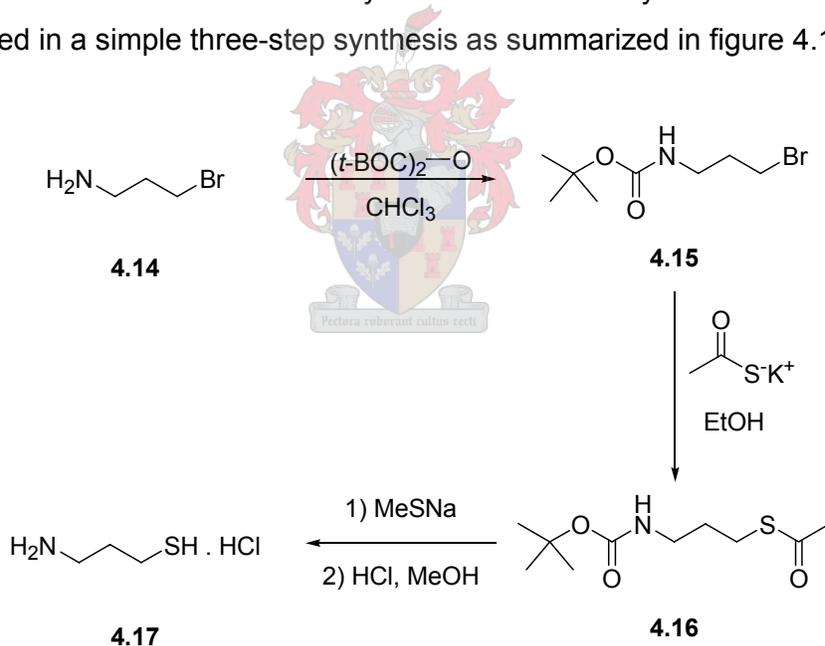


Figure 4.17 Synthesis of homocysteamine.HCl from 3-amino propylbromide

A *t*-BOC group was used to protect the primary amine of 3-amino 1-bromopropane, as described by Tarbell *et al.* (16). Potassium thioacetate was used to thioacetylate **4.15** (17). Treatment of an ethanolic solution of the thioacetate **4.16** with sodium thiomethoxide affords the desired thiol (18). The deprotection of the amine can be achieved by addition of HCl to the solution,

which will remove the *t*-BOC group resulting in the hydrochloric acid salt of homocysteamine. The product was confirmed with NMR, which showed a 2:1 ratio of homocysteamine hydrochloride and its corresponding disulfide.

Preparation of final CoA analogues

The reaction mixtures of the three ethyl pre-CoAs with cysteamine and homocysteamine respectively were analyzed by LC-MS. The samples were analyzed in positive and negative mode to identify the desired CoA analogues. These results are summarized in table 4.4 and the corresponding chromatograms of each reaction mix are shown in figure 4.18.

Table 4.4 LC-MS identification of CoA analogues.

CoA analogue	Retention time (minutes)	Calculated Molecular Mass	Found	
			[M+H] ⁺	[M-H] ⁻
CoA 1.1	5.15	767.1	768	766
CoA analogue 4.1	7.68	781.1	782	780
CoA analogue 4.2	6.97	781.1	782	780
CoA analogue 4.3	7.81	795.1	796	794
CoA analogue 4.4	4.10	753.1	754	752
CoA analogue 4.5	6.28	767.1	768	766

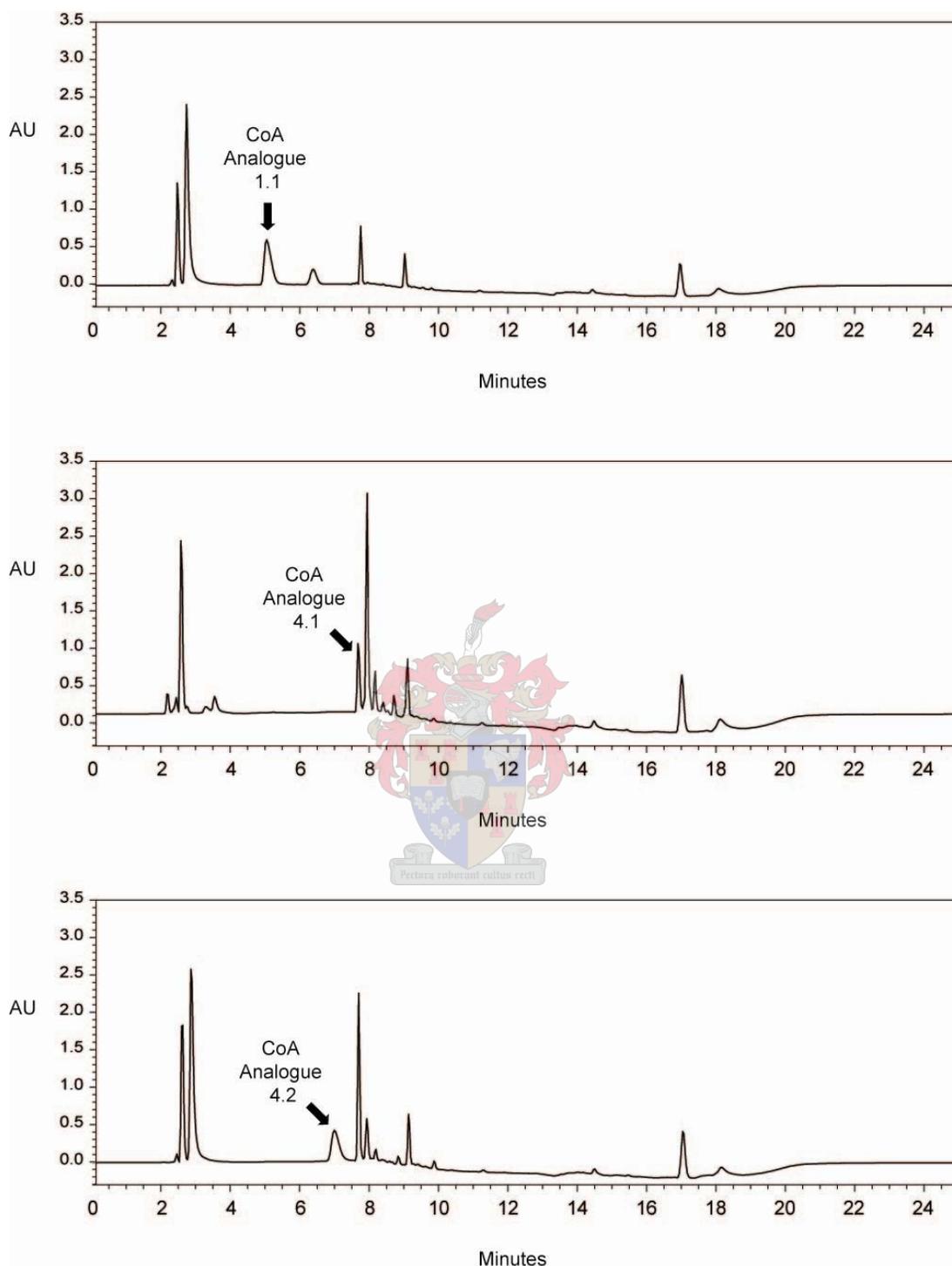


Figure 4.18 (a) Chromatograms of the aminolysis of ethyl pre-CoAs with cysteamine or homocysteamine (visualized @ 220 nm). Peak identification was done via MS analysis. The arrows indicate the formation of the desired products.

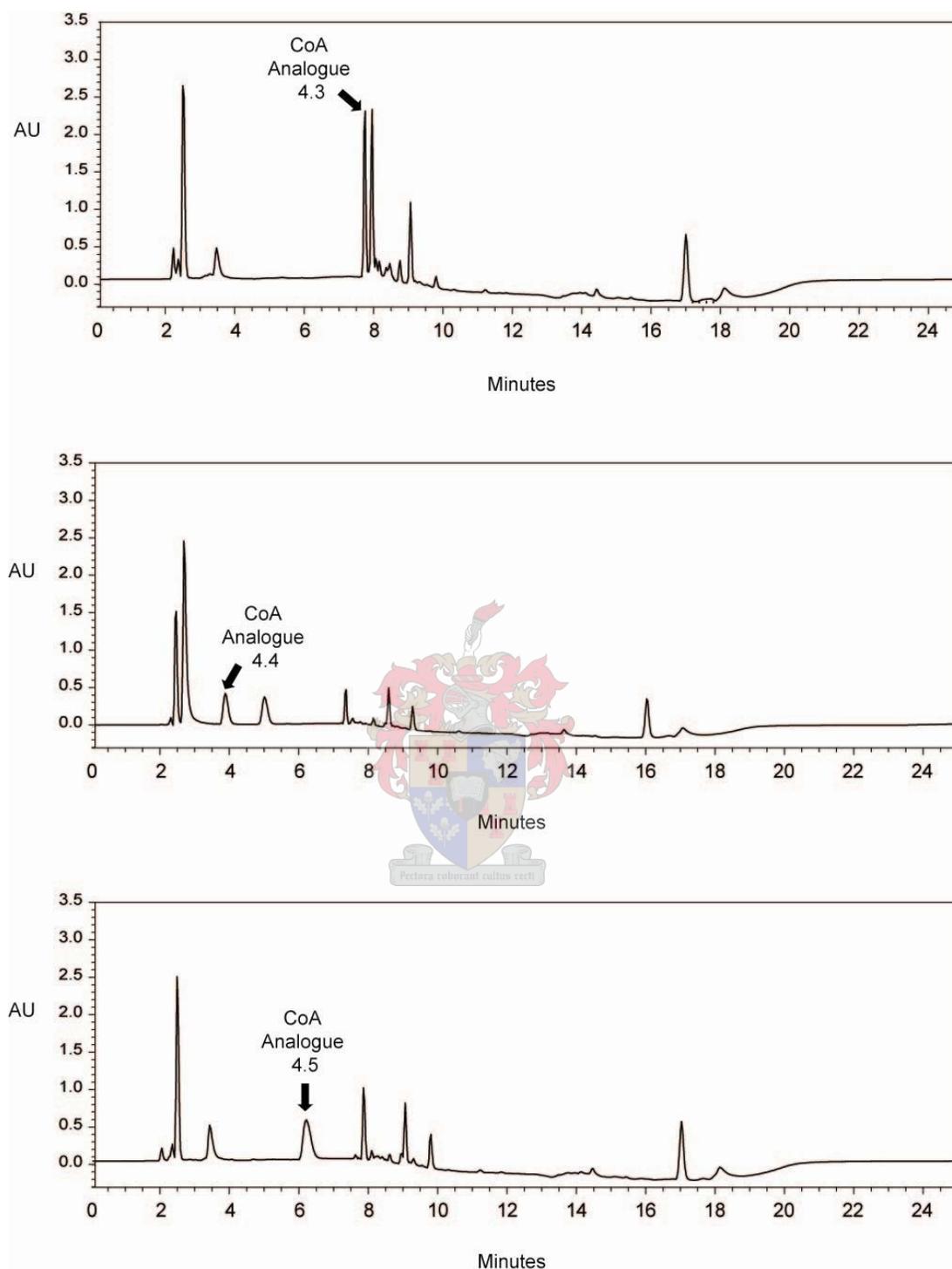
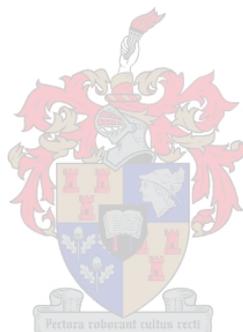


Figure 4.18 (b) Chromatograms of the aminolysis of ethyl pre-CoAs with cysteamine or homocysteamine (visualized @ 220 nm). Peak identification was done via MS analysis. The arrows indicate the formation of the desired products.

Our results show that all the expected CoA analogues were prepared by aminolysis of the pre-CoAs. Furthermore, no remaining ethyl pre-CoA peaks were visible. This indicates that all the ethyl pre-CoAs were utilized in the reaction. However, several other peaks were also visible in the HPLC chromatograms. While the peaks visible at 2.53 minutes were due to the HEPES reaction buffer present in the reaction mixture, other peaks were not identified. These peaks most probably represent disulfide products forming between cysteamine, homocysteamine and/or DTT and the desired products, or could be due to the degradation of the pre-CoA precursor. While these reactions clearly may be optimized for product formation, we decided to rather focus our attention on the role of the thioester in the aminolysis reaction, and whether aminolysis should follow the enzymatic transformation as outlined above, or perhaps precede it. These attempts are outlined in the following chapter.



4.3 Conclusion

In this study we aimed to present a modification of the CoA biosynthetic pathway to produce analogues of CoA with differing tether lengths in their pantetheine moieties. The first step in the study was the kinetic analysis of the phosphorylation of pantothenate thioester substrates by CoaA enzymes from different sources, since the synthetic phosphorylation of similar substrates showed to be tedious in previous studies. In our results SaCoaA showed the most promise in accepting a variety of substrates.

Three general CoA thioester synthons, or ethyl pre-CoAs, were prepared as intermediates to CoA analogue production. These pre-CoAs were biosynthesized, purified and characterized by NMR and ESI-MS. The ethyl pre-CoA intermediates were utilized in an aminolysis reaction with either cysteamine or homocysteamine. Aminolysis of the pre-CoAs were achieved and the CoA analogues were identified by LC-MS. However, our analysis showed the formation of various side products, most probably mixed disulfides or products of degradation.

To eliminate other reactions interfering with the aminolysis reaction the possibility of using a more reactive thioester was considered. For such a reaction to be optimized different thioesters with better leaving group characteristics than ethyl thiolate have to be explored. We decided to focus on thiophenol esters and to also explore the possibility of achieving aminolysis with a variety of other amines that do not bear a sulfhydryl group. This results of this investigation is detailed in chapter 5.

4.4 Experimental Procedures

4.4.1 Materials and Methods

All chemicals and resins were purchased from Sigma-Aldrich (Aldrich, Sigma or Fluka) or Acros Organics and were of the highest purity. C₁₈ solid phase extraction cartridges (Supelclean LC18), HisTrapTM Chelating and Desalting Columns (Amersham Biosciences) were from Supelco (Sigma). A Quick Start Bradford Protein Assay Kit was purchased from Bio-rad and contained Bradford reagent and a Bovine Serum Albumin standard set. Pyruvate kinase and Lactate dehydrogenase was purchased from Roche Applied Sciences. Large-scale centrifugation was done on a Heraeus Multifuge® 3S/3S-R. Centrifugation on a smaller scale was conducted on a Heraeus Biofuge pico centrifuge. Enzyme assays were performed on a Multiskan Spectrum multiplate spectrophotometer (ThermoLabsystems). All curve-fitting analyses were performed using SigmaPlot 9.0 (Systat software).

¹H-NMR was performed on a Varian INOVA 300MHz instrument and CoA analogues were analyzed on a Varian INOVA 600MHz instrument at the Central Analytical Facility of the University of Stellenbosch. ESI-MS analyses were performed on a Waters Micromass Q-TOF Ultima API mass spectrometer. LC-MS: HPLC analysis were performed on a WatersTM 2690 Separations Module with a Waters® 996 Photodiode Array Detector using a Gemini 5 µm C18 110Å column (250 × 4.6 mm) from Phenomenex, followed by Waters Quattro Micromass mass spectrometer analyses. All MS analyses were done by Dr. M Stander at the Central Analytical Facility of the University of Stellenbosch.

4.4.2 Synthesis of homopantothenic acid

4-Amino butyric acid (0.950 g; 9.216 mmol) was dissolved in 9.216 ml sodium hydroxide (1 M) and the excess water was evaporated *in vacuo*. The resulting white salt and 1.00 g D-pantolactone (7.68 mmol) were melted together and refluxed under argon gas for 17 hours at 130 °C giving a colourless sticky oil. The sodium 4-amino butyrate was exchanged to the free acid over pre-washed

Amberlite IR-120 (H⁺) eluting with deionized water. The unreacted pantolactone was extracted with 3 × 20 ml dichloromethane and the aqueous layer was lyophilized resulting in a colorless oil as product (1.76 g; 99%). ¹H NMR (300 MHz, D₂O): δ 0.91 (s, 3H), δ 0.95 (s, 3H), δ 1.83 (m, 2H), δ 2.38 (m, 2H), δ 3.28 (t, 2H), δ 3.42-3.53 (m, 2H), δ 4.00 (s, 1H).

4.4.3 Synthesis of α-pantothenic acid

Sodium glycerate was synthesized by dissolving L-glycine (0.404 g; 5.380 mmol) in 5.380 ml sodium hydroxide (1 M). The excess water was evaporated *in vacuo* and 0.500 g pantolactone was added to the resulting salt. The salts were melted together and refluxed at 130 °C under argon for 17 hours, resulting in a brown, sticky oil. The sodium glycerate was exchanged to the free acid over prewashed Amberlite IR-120 (H⁺) eluting with deionized water. The unreacted pantolactone in the reaction mix was extracted with 5 × 20 ml dichloromethane and the aqueous layer was lyophilized to give an orange oil (0.715 g, 65%). ¹H NMR (300 MHz, D₂O): δ 0.97 (s, 3H), δ 0.95 (s, 3H), δ 3.43-3.59 (m, 2H), δ 4.04 (s, 2H), δ 4.10 (s, 1H).

4.4.4 Synthesis of S-ethyl thiopantothenate

Sodium pantothenate (0.650 g; 2.695 mmol) was exchanged to the free acid over Amberlite IR-120 (H⁺) and lyophilized. The resulting syrup was dissolved in DMF (3.247 ml) and diphenylphosphoryl azide (0.9558 ml; 5.39 mmol) and ethanethiol (0.24 ml; 3.25 mmol) were added. After cooling to 0 °C triethylamine (0.748 ml) was added and the solution was stirred for 10 minutes at 0 °C, and then at room temperature for 2 hours. Ethyl acetate was added (33 ml) and the solution was washed with 1 M HCl (2 × 10 ml), 1 M NaHCO₃ (2 × 10 ml) and saturated NaCl (1 × 10 ml). The solution was dried over Na₂SO₄ and the solvent was removed *in vacuo*. The product was purified by flash chromatography (silica gel; ethyl acetate/hexane 2:1 to 4:1) to give S-ethyl thiopantothenate as a yellow oil (0.780 g; 32 %). ¹H NMR (300 MHz, CDCl₃): δ 0.87 (s, 3H), δ 0.98 (s, 3H), δ 1.22 (t, 3H), δ 2.78 (t, 2H), δ 4.30 (q, 2H), δ 3.48 (d, 2H), δ 3.57 (m, 2H), δ 3.99 (s, 1H).

4.4.5 Synthesis of S-ethyl thiohomopantothenate

4-Amino butyric acid (0.475 g; 4.608 mmol) was dissolved in 4.608 ml sodium hydroxide (1M) and the excess water was evaporated *in vacuo*. The resulting white salt and 0.500 g pantolactone (3.84 mmol) were melted together and refluxed under argon gas for 17 hours at 130 °C giving a colourless sticky oil. The sodium 4-amino butyrate was exchanged to the free acid over prewashed Amberlite IR-120 (H⁺) eluting with deionized water and lyophilized. The resulting yellow oil was dissolved in 4.5 ml DMF and diphenylphosphoryl azide (1.620 ml; 7.5 mmol) and ethanethiol (0.335 ml; 4.52 mmol) were added. After cooling to 0 °C triethylamine (1 ml) was added and the solution was stirred for 10 minutes at 0 °C, and then at room temperature for 2 hours. Ethyl acetate was added (40 ml) and the solution was washed with 1 M HCl (2 × 10 ml), 1 M NaHCO₃ (2 × 10 ml) and saturated NaCl (1 × 10 ml). The solution was dried over Na₂SO₄ and the solvent was removed *in vacuo*. The product was purified by flash chromatography (silica gel; ethyl acetate/hexane 3:1 to 4:1) to give S-ethyl thiohomopantothenate as a yellow oil (0.520 g; 50 %). ¹H NMR (300 MHz, CDCl₃): δ 0.88 (s, 3H), δ 0.99 (s, 3H), δ 1.21 (s, 3H), δ 1.86 (s, 2H), δ 2.57 (t, 2H), δ 2.85 (q, 2H), δ 3.30 (m, 2H), δ 3.53-3.444 (m, 2H), δ 4.00 (s, 1H).

4.4.6 Synthesis of S-ethyl thio-α-pantothenate

Sodium glycerate was synthesized by dissolving L-glycine (0.346 g; 4.608 mmol) in 4.608 ml sodium hydroxide (1 M). The excess water was evaporated *in vacuo* and 0.500 g pantolactone (3.84 mmol) was added to the resulting salt. The salts were melted together and refluxed at 130 °C under argon for 17 hours, resulting in a brown sticky oil. The sodium glycerate was exchanged to the free acid over prewashed Amberlite IR-120 (H⁺) eluting with deionized water and lyophilized. The resulting yellow oil was dissolved in 3.76 ml DMF and diphenylphosphoryl azide (1.939 ml; 9 mmol) and ethanethiol (0.402 ml; 5.424 mmol) were added. After cooling to 0 °C triethylamine (0.748 ml) was added and the solution was stirred for 10 minutes at 0 °C, and then at room temperature for 2 hours. Ethyl acetate was added (40 ml) and the solution was washed with 1 M HCl (2 × 10 ml), 1 M NaHCO₃ (2 × 10 ml) and saturated NaCl (1 × 10 ml). The solution was dried over

Na₂SO₄ and the solvent was removed *in vacuo*. The product was purified by flash chromatography (silica gel; ethyl acetate/hexane 3:1 to 4:1) to give S-ethyl thio- α -pantothenate as a yellow oil (0.322 g; 44 %). ¹H NMR (300 MHz, CDCl₃): δ 0.94 (s, 3H), δ 1.03 (s, 3H), δ 1.23 (s, 3H), δ 2.91 (q, 2H), δ 3.52 (d, 2H), δ 4.10 (s, 1H), δ 4.23-4.21 (m, 2H).

4.4.7 Synthesis of homocysteamine. HCl

Synthesis of 3-[(*tert*-Butoxy)carbonyl]amino-1-propane (4.15)

The synthesis of the **4.15** was done as described in Tarbell *et al.* (16).

Synthesis of 3-[(*tert*-Butoxy)carbonyl]amino-1-(acetylthio)propane (4.16)

A solution of **4.15** (1.50 g; 6.3 mmol) in ethanol (65 ml) was treated with potassium thioacetate (2.16 g; 18.9 mmol) under argon and was then stirred at ~75 °C for 17 hours. Water (50 ml) was added to the reaction mixture. The residue was extracted with diethyl ether (2 \times 100 ml) and the organic layers were washed with saturated aqueous NaCl solution (60 ml). The organic layer was dried over NaSO₄. The solvent was removed *in vacuo* to give crude product of 920 mg.

Deprotection of 4.16 to yield homocysteamine (4.17)

The crude product containing **4.16** (920 mg; 4.81 mmol) was dissolved in 60 ml methanol. The solution was treated with 6 ml sodium thiomethoxide (1 equivalent; 1 M solution in methanol). The reaction mixture was stirred for 2 hours at room temperature under argon. HCl in methanol (final concentration of 1 M in total volume of 100 ml) was added and the reaction was stirred for another 90 minutes. The solvent was evaporated forming a white precipitate, which was dissolved in water and lyophilized. This process was repeated twice forming homocysteamine.HCl, which is very hygroscopic (955 mg; >100%). The NMR data showed a 2:1 ratio of homocysteamine.HCl and the homocysteamine disulfide as products. Homocysteamine.HCl: ¹H NMR (400 MHz, CDCl₃): δ 1.82 (m, 2H), δ 2.46 (t, 2H), δ 3.01 (t, 2H). Homocysteamine disulfide: ¹H NMR (400 MHz, CDCl₃): δ 2.00 (m, 2H), δ 2.66 (t, 2H), δ 3.01 (t, 2H).

4.4.8 Overexpression and purification of enzymes

EcCoaA

The expression plasmid used for the overexpression of *EcCoaA*, pET28a-*EcCoaA*, was cloned by Dr. E. Strauss for a previous study (12).

The plasmid was transformed into the *E. coli*. BL21-star(DE3) expression strain (Novagen) and grown in LB broth supplemented with 30 mg/litre kanamycin sulfate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 800 µM IPTG and grown overnight at 37 °C. After the cells were harvested the cell paste was suspended in sonication buffer (5 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9; 10ml/1g cell paste). The resuspended cells were disrupted by sonication and centrifuged at 15 000×g for 30 minutes to collect cell debris. The crude extract was applied to a 1 ml HisTrap™ Chelating Column and purified on an ÄKTAprime purification system (Amersham Biosciences). Weakly bound proteins were removed by washing with sonication buffer, followed by washing with sonication buffer containing 75 mM imidazole. The protein of interest was eluted by increasing the imidazole concentration to 500 mM and the elution was monitored at A₂₈₀.

The collected protein was desalted using a HiTrap™ Desalting Column on a ÄKTAprime purification system and the protein concentration was determined as 4.9 mg.ml⁻¹ using a Bradford Assay.

SaCoaA

The *SaCoaA* expression (pET28a-*SaCoaA*) plasmid was a gift from Cynthia Kinsland (Department of Chemistry and Chemical Biology, Cornell University). The *SaCoaA* protein was purified according to published methods, using BL21-star(DE3) (Novagen) as expression strain (19).

The plasmid was transformed into the *E. coli*. BL21-star(DE3) (Novagen) expression strain and grown in LB broth supplemented with 30 mg/litre kanamycin sulphate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 500 µM IPTG and

grown for 3 hours at 37 °C. After the cells were harvested the cell paste was suspended in sonication buffer (5 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9; 10ml/1g cell paste). The cells were disrupted by sonication and centrifuged at 15 000×g for 30 minutes to collect cell debris. The crude extract was applied to a 1 ml HisTrap™ Chelating Column and purified on an ÄKTAprime purification system (Amersham Biosciences). Weakly bound proteins were removed by washing with sonication buffer, followed by washing with sonication buffer containing 75 mM imidazole. The protein of interest was eluted by increasing the imidazole concentration to 500 mM and the elution was monitored at A₂₈₀.

The collected protein was desalted using a HiTrap™ Desalting Column on a ÄKTAprime purification system and the protein concentration was determined as 5 mg.ml⁻¹ using the Bradford Assay.

EcCoaD

The *EcCoaD* expression plasmid (pET28a-*EcCoaD*) was constructed by Dr. E. Strauss for a previous study (12).

The plasmid was transformed into the *E. coli*. BL21-star(DE3) (Novagen) expression strain and grown in LB broth supplemented with 30 mg/litre kanamycin sulphate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 1000 µM IPTG and grown overnight at 37 °C. After the cells were harvested the cell paste was suspended in sonication buffer (5mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9; 10ml/1g cell paste). Resuspended cells were disrupted by sonication and centrifuged at 15 000×g for 30 minutes to collect cell debris. The crude extract was applied to a 1 ml HisTrap™ Chelating Column and purified on an ÄKTAprime purification system (Amersham Biosciences). Weakly bound proteins were removed by washing with sonication buffer, followed by washing with sonication buffer containing 75 mM imidazole. Increasing the imidazole concentration to 500 mM eluted the protein of interest and the elution was monitored at A₂₈₀.

The collected protein was desalted using a HiTrap™ Desalting Column on a ÄKTApurification system and the protein concentration was determined as 4 mg.ml⁻¹ using the Bradford Assay.

EcCoaE

The *EcCoaE* expression plasmid (pET28a-*EcCoaE*) was constructed by Dr. E. Strauss for a previous study (12).

The plasmid was transformed into the *E. coli*. BL21-star(DE3) (Novagen) expression strain and grown in LB broth supplemented with 30 mg/litre kanamycin sulphate at 37 °C to OD₆₀₀ ~0,6. The cells were induced with 1000 µM IPTG and grown overnight at 37 °C. After the cells were harvested the cell paste was suspended in sonication buffer (5 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9; 10ml/1g cell paste). The resuspended cells were disrupted by sonication and centrifuged at 15 000×g for 30 minutes to collect cell debris. The crude extract was applied to a 1 ml HisTrap™ Chelating Column and purified on an ÄKTApurification system (Amersham Biosciences). Weakly bound proteins were removed by washing with sonication buffer, followed by washing with sonication buffer containing 75 mM imidazole. The protein of interest was eluted by increasing the imidazole concentration to 500 mM and the elution was monitored at A₂₈₀.

The collected protein was desalted using a HiTrap™ Desalting Column on a ÄKTApurification system and the protein concentration was determined as 2.9 mg.ml⁻¹ using the Bradford Assay.

4.4.9 Determining enzyme concentration with Bradford assay

The Quick Start Bradford protein assay is a simple and accurate procedure for determining the concentration of protein in solution. The assay involves the binding of Coomassie Brilliant Blue R-250 dye to proteins. When the dye binds to protein it is converted to a stable unprotonated blue form which where detected at

595 nm using a Multiskan Spectrum multiplate spectrophotometer (Thermo Lab systems).

Bovine Serum Albumin was used as standard and has a linear range of 125-1000 $\mu\text{g}\cdot\text{ml}^{-1}$. Each standard (5 μl) from the kit was transferred into a microtiter plate and 250 μl Bradford reagent (ambient temperature) was added to each standard. The same was done with the protein under investigation and a blank (distilled water) was also measured. The standards and samples were done in triplicate and the plate was incubated at room temperature for at least 5 minutes after addition of Bradford reagent before the absorbance was measured at 595 nm. From these measurements the protein concentration was determined using the Beer-Lambert law.

4.4.10 Steady state kinetics - Phosphorylation Assay

The kinetic analyses of the phosphorylation reaction by CoaA were performed based on the assay used by Strauss (12) and measures the consumption of NADH at A_{360} using an extinction coefficient of $6220 \text{ M}^{-1}\cdot\text{cm}^{-1}$ for NADH. A master reaction mix was prepared and contained a reaction buffer consisting of 50 mM Tris-HCl, 10 mM MgCl_2 and 20 mM KCl with a final pH of 7.6. The mixture also had the following reagents added to it in constant concentrations: 1.5 mM ATP, 0.5 mM NADH and 0.5 mM PEP. The following enzymes were also added to this master mix: 3 units pyruvate kinase, 3 units lactate dehydrogenase and 3 μg *EcCoaA* or 1.5 μg *SaCoaA* depending on which enzyme was assayed. All substrates were varied between concentrations of 0-1.5 mM and assayed in triplicate in 96-well flat-bottomed microtiter plates. The reaction was initiated at 25 °C by the addition of the substrate in a final reaction volume of 300 μl and was monitored at A_{340} on a Multiskan Spectrum multiplate spectrophotometer (Thermo Lab systems).

4.4.11 Biosynthesis and purification of ethyl pre-CoAs (4.11 – 4.13)

A 1500 μl reaction mixture contained 5 mM ethyl thioester, 16.5 mM ATP, 20 mM KCl and 10 mM MgCl_2 in 50 mM Tris-HCl buffer (pH 7.6). The reaction was

initiated by addition of biosynthetic enzymes (145 μg *SaCooA*, 252 μg *EcCooD* and 252 μg *EcCooE*) and incubated for 3 hours at 37 °C. The reaction was stopped by transferring the reaction to 95 °C for 5 minutes and the precipitated protein was removed by centrifugation (13 000 \times g for 5 minutes). C_{18} solid phase extraction cartridges (500mg/3ml) were used to purify ethyl pre-CoAs. Prior to loading the sample, the cartridge was conditioned with ~4ml methanol and equilibrated with ~4 ml 200 mM NH_4OAc (pH 6). The supernatant of the reaction mix was loaded onto the cartridge, followed by washing with ~4 ml 200 mM NH_4OAc (pH 6), ~4 ml 20 mM NH_4OAc (pH 6) and finally the product was eluted with ~4 ml 20% acetonitrile. The chromatography was monitored at A_{235} , A_{254} and A_{280} using a Multiskan Spectrum multiplate spectrophotometer (Thermo Labsystems). Corning UV detection 96-well microtiter plates were used for product detection at 235, 254 and 280 nm. The product-containing fractions were combined and lyophilized, dissolved in water and lyophilized again. This was repeated until a constant weight of product was achieved. The yields are summarized in table 4.5. ^1H NMR was done on a 600 MHz NMR in D_2O (section 4.2.3 – table 4.2). ESI-MS-results discussed in section 4.2.3 – table 4.3.

Table 4.5 Yields obtained for ethyl pre-CoAs

Product	Mass (mg)	% Yield
Ethyl pre-CoA (4.11)	4.5	80
Ethyl pre-homo-CoA (4.12)	4.1	72
Ethyl pre- α -CoA (4.13)	2.3	42

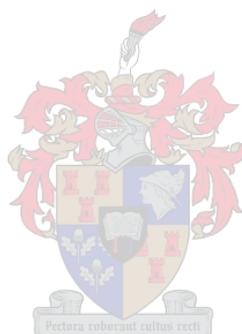
4.4.12 Aminolysis of pre-CoAs to form CoA analogues (1.1, 4.1 – 4.5)

Aminolysis of pre-CoAs were done in a final volume of 250 μl for each CoA analogue. Either cysteamine or homocysteamine.HCl (10 equivalents; 50 mM final concentration) was added to 5 mM ethyl pre-CoA. The reaction was done in 200 mM HEPES (pH 8) containing 6 mM DTT. The reaction mix was incubated at 50 °C for 1 hour. Samples were diluted (1:1) in 10 mM NH_4OAc , pH 6 for LC-MS analysis.

The following HPLC gradient was used to analyze the reactions:

Time (minutes)	Flow rate (ml/min)	% Solvent A 10mM NH ₄ OAc, pH 6	% Solvent B Acetonitrile
0.01	1.00	95	5
3.00	1.00	95	5
6.00	1.00	60	40
10.00	1.00	60	40
11.00	1.00	40	60
15.00	1.00	40	60
16.00	1.00	20	80
20.00	1.00	20	80

Peaks were detected by a PDA detector at 254 nm. MS detection was done in negative and positive mode.

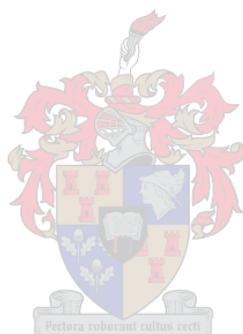


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Coenzyme A Analogues: Optimization of aminolysis reaction

5.1 Introduction

In chapter 4 it was shown that three ethyl thioester-CoA synthons (ethyl pre-CoAs) were constructed, which upon aminolysis resulted in CoA analogues of different tether lengths. It was hypothesized that these compounds would undergo aminolysis in the presence of excess amine (10 equivalents) to form the desired CoA derivatives. In previous studies by Drueckhammer *et al.* (1-3) such aminolysis reactions were used to prepare CoA derivatives, but these required with long reaction times (23 hours) and with large amounts of amine present (~80-380 equivalents) in aqueous solution (3). In this study we wanted to utilize the thioester substrates to achieve aminolysis in shorter reaction times with less amine present. The ethyl pre-CoAs were reactive enough to allow aminolysis resulting in the elimination of ethanethiol. However, the aminolysis of these compounds had to be optimized to ensure total aminolysis of the CoA analogue synthon, with either cysteamine or homocysteamine, and to eliminate the formation of side-products.

5.1.1 Other thiol ester possibilities

One way to optimize reactivity of the ethyl pre-CoA thioester is to change the ethanethiolate functional group to a functional group that displayed better leaving group characteristics. Methanethiolate was considered due to its smaller size (figure 5.1), but it was decided to focus the study on thiophenolate since it has been used in numerous other studies that relies on the aminolysis of thioesters.

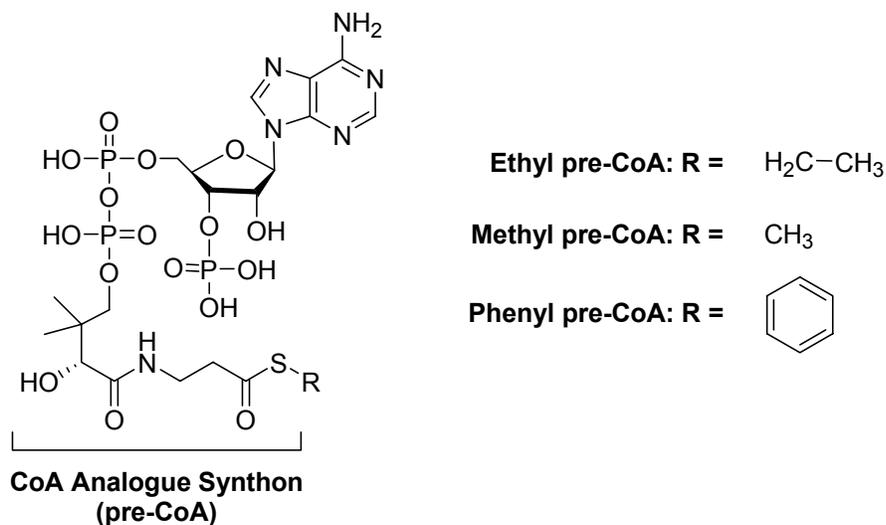
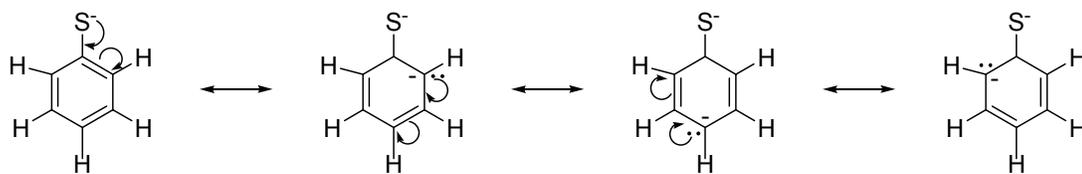


Figure 5.1 Different thioester CoA synthons used in this study.

Although thiophenolate is more bulky in the thiophenyl pre-CoA than ethanethiolate or methanethiolate, the aromatic ring makes this functional group more reactive. The negative charge on the thiophenolate is stabilized by electron delocalization into the aromatic ring:



5.1.2 Other applications of thiophenol

Previous studies have shown that the characteristics of thiophenol can be used in different applications. The use of thiophenol in the generation of more reactive thioesters has already been shown to be successful in the formation of peptide bonds, for example (4). In such a reaction, the addition of thiophenol to a native chemical ligation reaction resulted in a more reactive phenyl thioester through thiol exchange. A peptide- α -phenyl thioester reacted with a second unprotected peptide containing an N-terminal cysteine side chain. This resulted in a thioester-linked intermediate as the initial covalent product. A rapid intramolecular rearrangement reaction occurs forming the amide bond, which is thermodynamically favoured (figure 5.2).

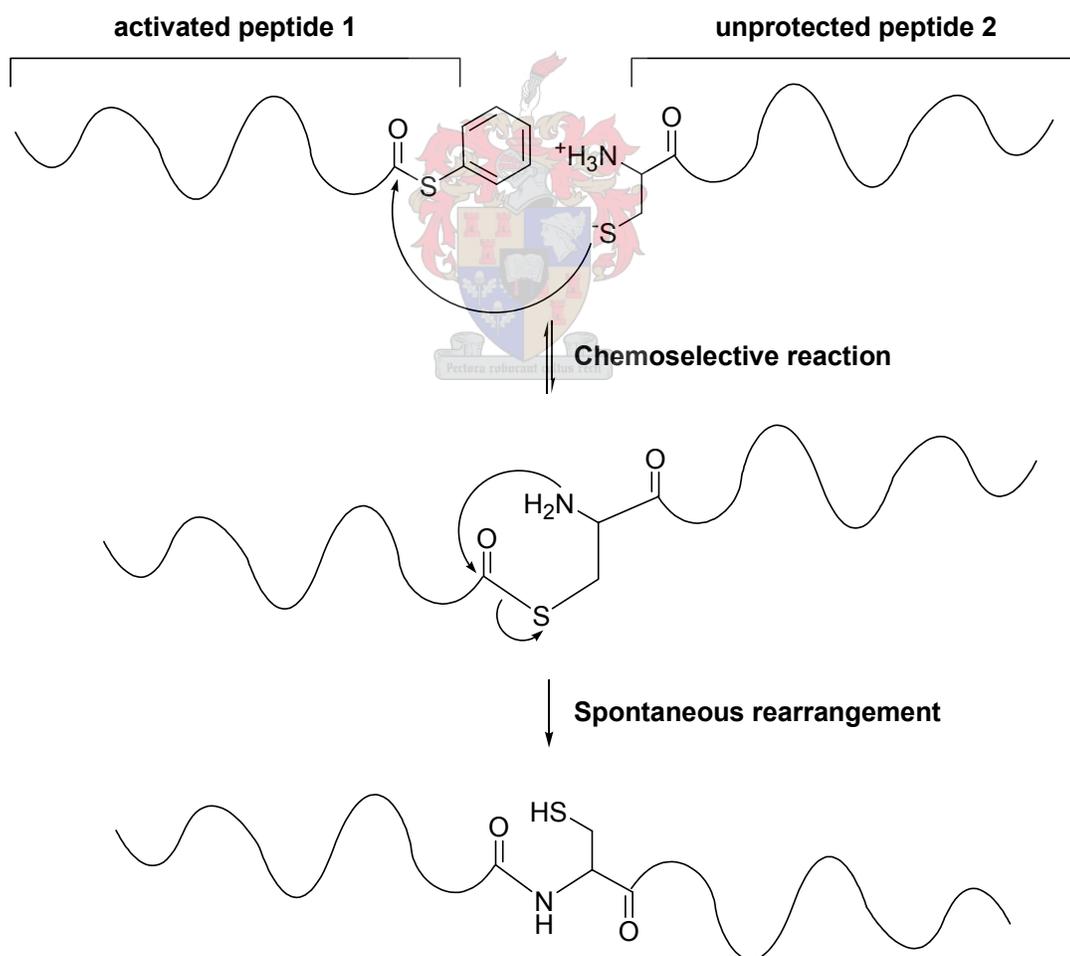


Figure 5.2 Mechanism of the aminolysis reaction of a thiophenol activated peptide (4).

Wehofsky *et al.* (5) also used various thioesters for the *in situ* preparation of protease-specific reactants for peptide formation. An acyl methyl thioester was prepared as standard precursor to be used as uniform parent substrate. This methyl thioester was then changed to a different thioester with aromatic properties by spontaneous transthioesterification. These activated compounds were used directly as reactants for protease mediated peptide-coupling reactions (5).

Thiophenol activation was also used in non-ribosomal peptide cyclase reactions. A new chemo-enzymatic route to cyclic compounds through activity-based thioesterase domain enzyme acylation has been developed. This was achieved by using reactive thioesters as leaving groups. This method was used in the biochemical characterization of fengycin, mycosubtilin and syringomycin peptide cyclases, which are not active with *N*-acetylcysteamine thioesters. It was shown that the reactivity of the peptides was determined more by the reactivity of the leaving group than the cofactor recognition. Peptides with thiophenol and other aromatic leaving groups showed much more activity. These compounds were used to characterize new non-ribosomal peptide cyclases (6).

5.1.3 Thiophenol in CoA analogue production

From the examples in section 5.1.2 it is clear that thiophenolate is a good leaving group that favours amide bond formation. The ethyl pre-CoAs used in chapter 4 allowed aminolysis, but various side products formed after aminolysis with cysteamine or homocysteamine. The strategy in chapter 4 (strategy 1) was expanded, by investigating the possibility to use the phenyl pre-CoA synthon for general aminolysis with various primary amines. The aims set out in this chapter were to prepare phenyl pre-CoA as intermediate to test if this synthon allows aminolysis to occur more readily than ethyl pre-CoA (figure 5.3; strategy 1).

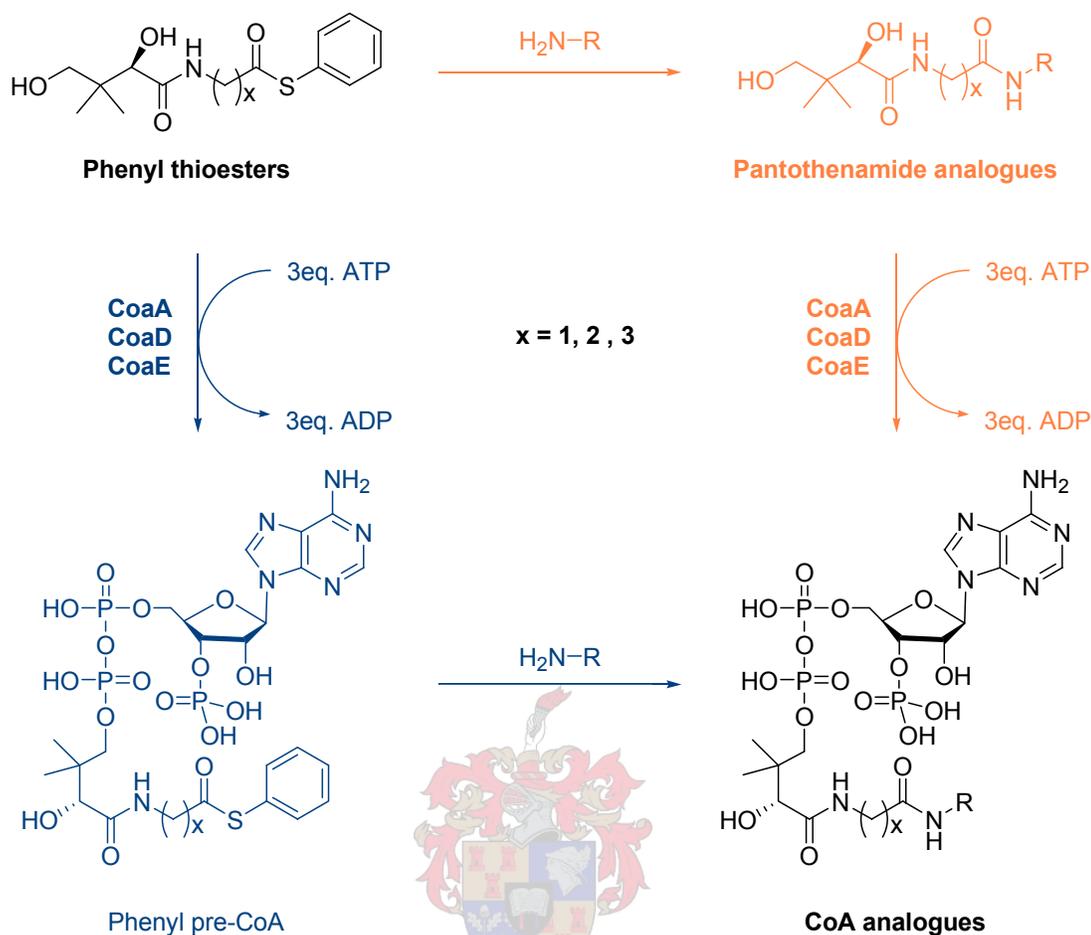


Figure 5.3 Strategy 1: The production of phenyl pre-CoAs followed by aminolysis.

Strategy 2: The production of pantothenamide analogues by aminolysis followed by the biosynthesis of CoA analogues.

In search of a simple method for CoA analogue production, an alternative method to develop these analogues was also investigated. In this second strategy aminolysis of the phenyl thioester precursor was performed prior to biocatalytic transformation to the CoA analogue. The resulting pantothenamide analogues are then treated with *Sa*CoaA, *Ec*CoaD and *Ec*CoaE to prepare the CoA analogues of choice (figure 5.3; strategy 2). Due to the reactivity of the thiophenol thioester substrates, and the reduced steric bulk of the substrate, the aminolysis of this system should proceed without difficulty. Various studies have shown that CoaA has the ability to accept various pantothenamide substrates (7-9). A recent study by Virga *et al.* (8) prepared a wide range of pantothenamide analogues as substrates/inhibitors to investigate their antibacterial activity. This study has shown

that a wide variety of pantothenamide substrates were accepted by SaCoaA, which makes this second strategy of CoA analogue preparation viable.

In this study various substrate precursors were synthesized, purified and characterized. These substrates were used in phenyl pre-CoA production followed by aminolysis, which was analyzed by HPLC. In the second strategy, the pantothenamide intermediates were prepared and used in the biosynthesis of different CoA analogues. These analogues were analyzed by LC-MS.

5.2 Results and discussion

5.2.1 Synthesis of pantothenic acid phenyl thioesters

The pantothenic acid phenyl thioesters (figure 5.4) were synthesized by the same procedure as the ethyl thioesters. However, a study of Yamada *et al.* has shown that better yields are obtained for phenyl thioesters if diethyl cyanophosphonate (DECP) is used rather than diphenylphosphoryl azide (DPPA) (10). Therefore, DECP was used in the synthesis of three pantothenic acid phenyl thioesters with different tether lengths (S-phenyl thiopantothenate **5.1**, S-phenyl thiohomopantothenate **5.2** and S-phenyl thio- α -pantothenate **5.3**). These substrates were synthesized, purified and characterized successfully in good to moderate yields.

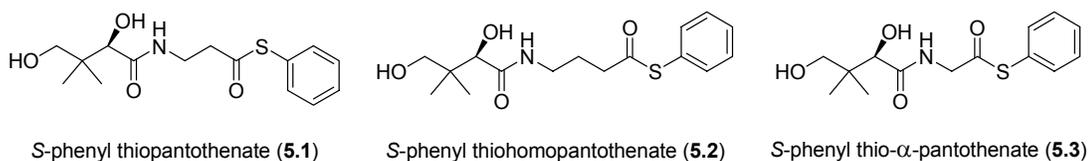


Figure 5.4 Pantothenic acid phenyl thioesters.

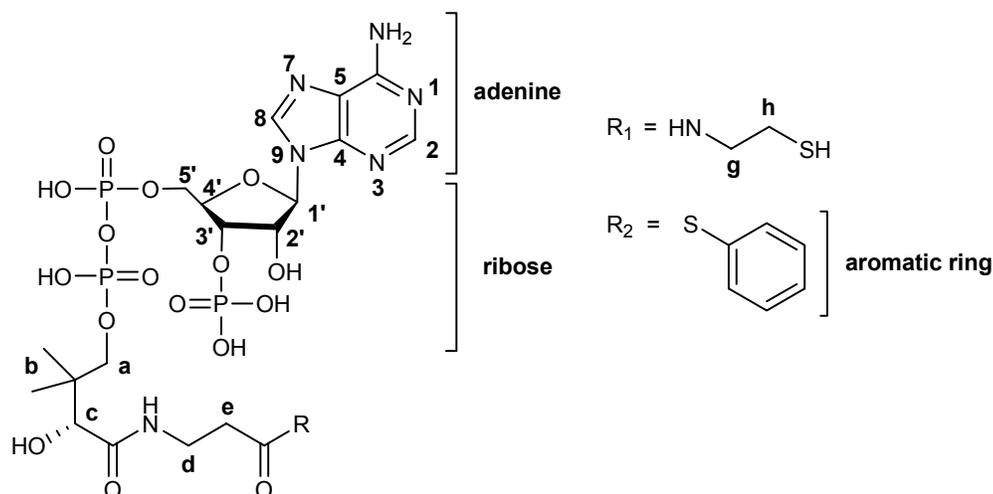


Table 5.1 NMR comparison between CoA (R_1) (11) vs. phenyl pre-CoA (R_2). The same numbering system was used as in table 4.2.

Assignments	CoA	Phenyl-pre-CoA
^1H	δ (ppm)	δ (ppm)
8	8.47 (s, 1H)	8.64 (s, 1H)
2	8.15 (s, 1H)	8.34(s, 1H)
aromatic ring	-	7.51 - 7.37 (m, 5H)
1'	6.10 (d, 1H)	6.17 (d, 1H)
3'	4.87 (t, 1H)	4.77 (t, 1H)
2'	4.85 (t, 1H)	4.58 (t, 1H)
4'	4.58 (s, 1H)	4.44 (t, 1H)
5'	4.23 (s, 2H)	4.23 (m, 2H)
c	3.94 (s, 1H)	4.10 (s, 1H)
a	3.80 (q, 1H)	3.94 (q, 1H)
	3.54 (q, 1H)	3.67 (q, 1H)
d	3.44 (t, 2H)	3.58 (t, 2H)
g	3.25 (t, 2H)	-
h	2.59 (t, 2H)	-
e	2.43 (t, 2H)	3.00 (t, 2H)
b' (pro-S)	0.71 (s, 3H)	0.87 (s, 3H)
b'' (pro-R)	0.84 (s, 3H)	0.99 (s, 3H)

5.2.3 Aminolysis of phenyl pre-CoA

To determine if the phenyl pre-CoA is more reactive than ethyl pre-CoA, aminolysis of phenyl pre-CoA was tested with 10 equivalents pentylamine in HEPES (pH 8) to produce ethyldethia-CoA (**5.1**) (figure 5.6). The reaction was monitored by HPLC at 254 nm (figure 5.7). Aminolysis did occur, but total conversion of the phenyl pre-CoA to the CoA analogue did not take place. The peaks were identified by comparison with the following standards: purified phenyl pre-CoA (14 minutes), ethyldethia-CoA (13 minutes) (7) and thiophenol (23 minutes). No other identified peaks were visible on the chromatogram at 254 nm. This indicated that no side products formed.

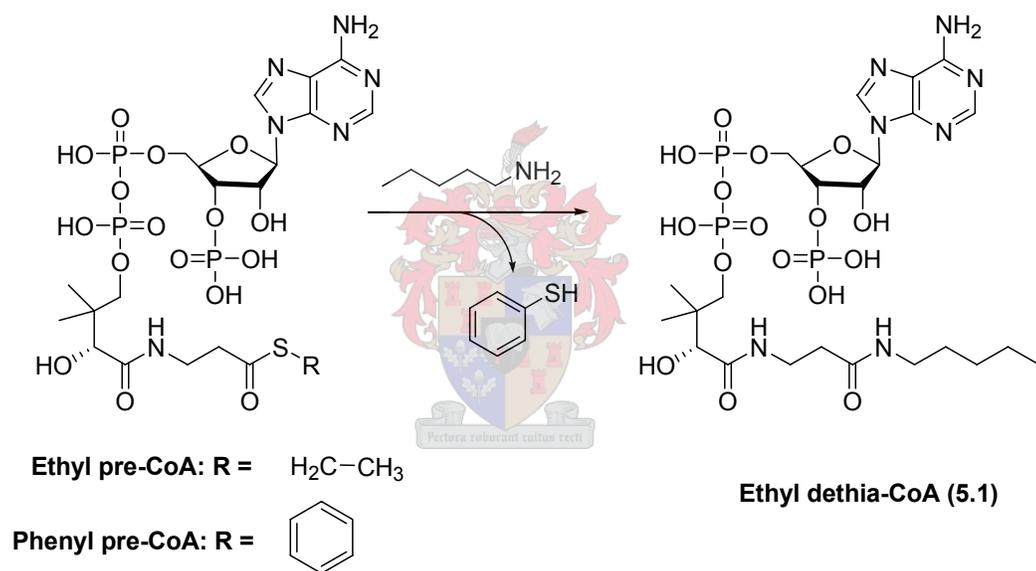


Figure 5.6 Aminolysis of pre-CoAs with pentylamine to produce ethyldethiaCoA.

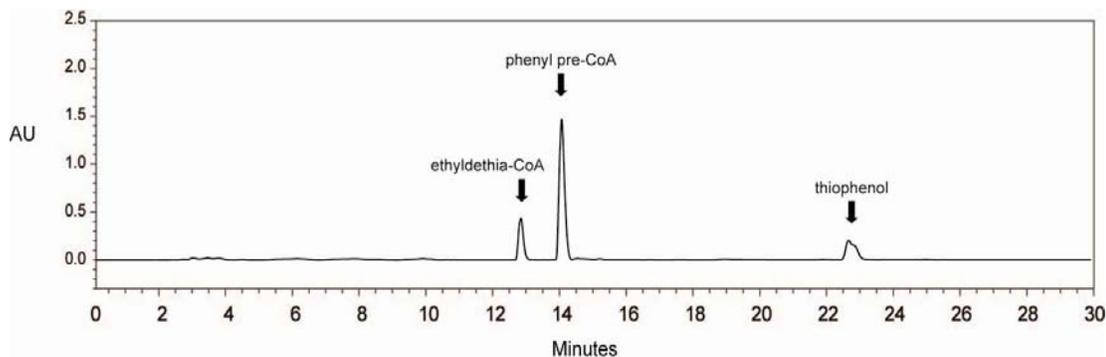


Figure 5.7 Chromatogram of the aminolysis of phenyl pre-CoA with pentylamine at 254 nm.

Pantothenamide analogues with different tether lengths

The aminolysis of the phenyl thioesters, with different tether lengths, with either cysteamine or homocysteamine produces pantothenamides with different tether lengths. The success of these reactions was investigated by using only *S*-phenyl thiopantothenate and cysteamine to determine if pantetheine, the corresponding pantothenamide analogue, is formed. If aminolysis indeed did occur, the same reaction will be viable for all three phenyl thioesters with either, cysteamine or homocysteamine. The reaction was monitored using HPLC (figure 5.9) and visible peaks were compared to the following standards: pantetheine and purified *S*-phenyl thiopantothenate. It is clear from these chromatograms at 214 nm that all the substrate was consumed (18.5 minutes) and that the pantetheine product formed. The chromatogram shows the product at 14 minutes with the formation thiophenol, which elutes at 23 minutes. However, another unidentified peak is visible at 14.5 minutes, which could be disulfide formation between two pantetheine molecules. The HEPES reaction buffer is also visible 2.5 minutes.

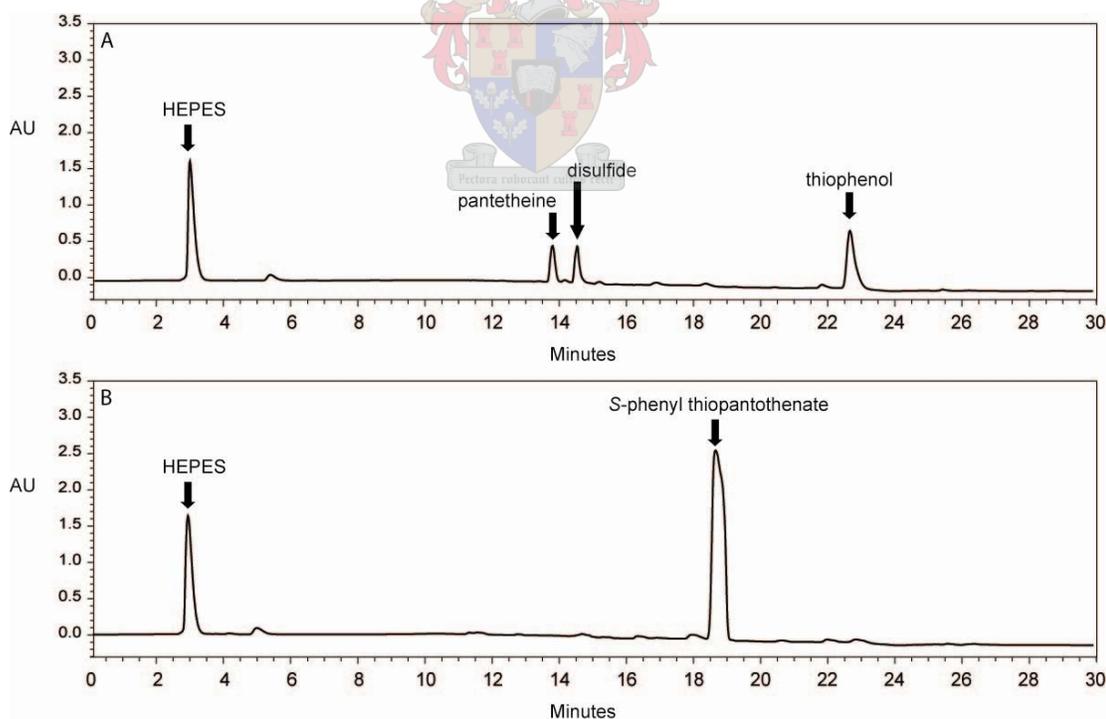


Figure 5.9 Chromatogram of the aminolysis of *S*-phenyl thiopantothenate with cysteamine in comparison to *S*-phenyl thiopantothenate at 214 nm.

Pantothenamide intermediates with different amide functionalities

An analysis was done to determine if aminolysis with a primary amine without a thiol functional group is viable. The aminolysis reaction of *S*-phenyl thiopantothenate with pentylamine was analyzed by HPLC (figure 5.10).

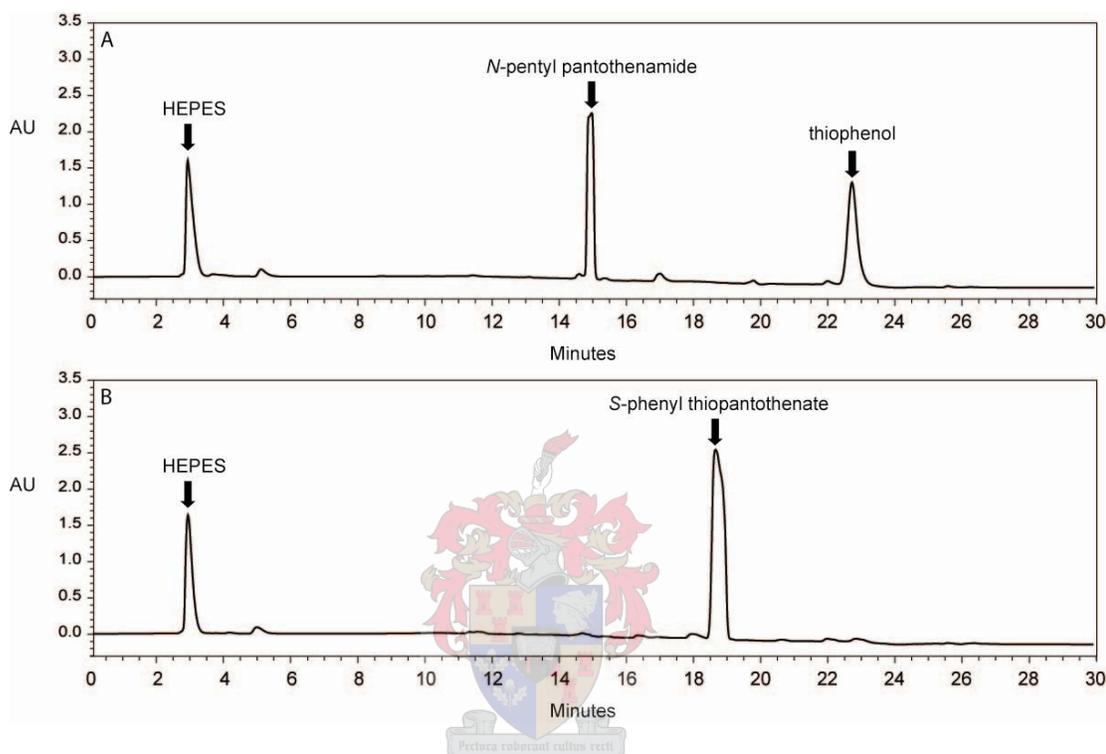


Figure 5.10 Chromatogram of the aminolysis of *S*-phenyl thiopantothenate with pentylamine in comparison to *S*-phenyl thiopantothenate at 214 nm.

The chromatogram of the reaction mix showed total disappearance of the *S*-phenyl thiopantothenate at 18.5 minutes. The appearance of *N*-pentylpantothenamide at 15.5 minutes with the formation thiophenol at 23 minutes is clearly visible. The product was identified by injecting a *N*-pentylpantothenamide standard (7). This confirmed complete aminolysis, forming the pantothenamide intermediate to be used in the biosynthesis of the corresponding CoA derivatives. The HEPES reaction buffer is visible 2.5 minutes.

Previous studies have shown that *N*-pentylpantothenamide can be utilized in the production of the ethyldethia-CoA (7-9). The ideal would be to utilize different amines with different tether lengths of thioester derivatives, which will provide a

method to synthesize various CoA analogues. However, it is unclear if all these pantothenamide intermediates will be accepted by the enzymes involved to produce the corresponding CoA derivative.

5.2.5 Biosynthesis of CoA analogues from pantothenamide intermediates

Since the aminolysis of the phenyl thioesters with different types of amines were successful, these reactions were used to prepare the different pantothenamide intermediates to be used in the biosynthesis of the corresponding CoA analogues (figure 3.5; strategy 2).

CoA analogues with different tether lengths

The three different phenyl thioester substrates (figure 5.4) were used to prepare pantothenamide intermediates of different tether lengths, after aminolysis with cysteamine or homocysteamine. These intermediates were then used in the biosynthesis of CoA and analogues of CoA from chapter 4 (1.1, 4.1 – 4.5; figure 5.11).

These reactions were analyzed by LC-MS, but no product peaks for these six CoA analogues were identified (data not shown). This was a surprising result, because the pantetheine analogues should be readily accepted by the enzymes. In combination with our results in chapter 4 it is clear that the thiol-bearing amines were not only involved in the aminolysis of the thioesters. The formation of unidentified side-products in chapter 4 indicates that there were other reactions competing with the aminolysis reaction. Further investigation into these side-products will have to be done to determine the optimum conditions to prepare these CoA analogues with different tether lengths.

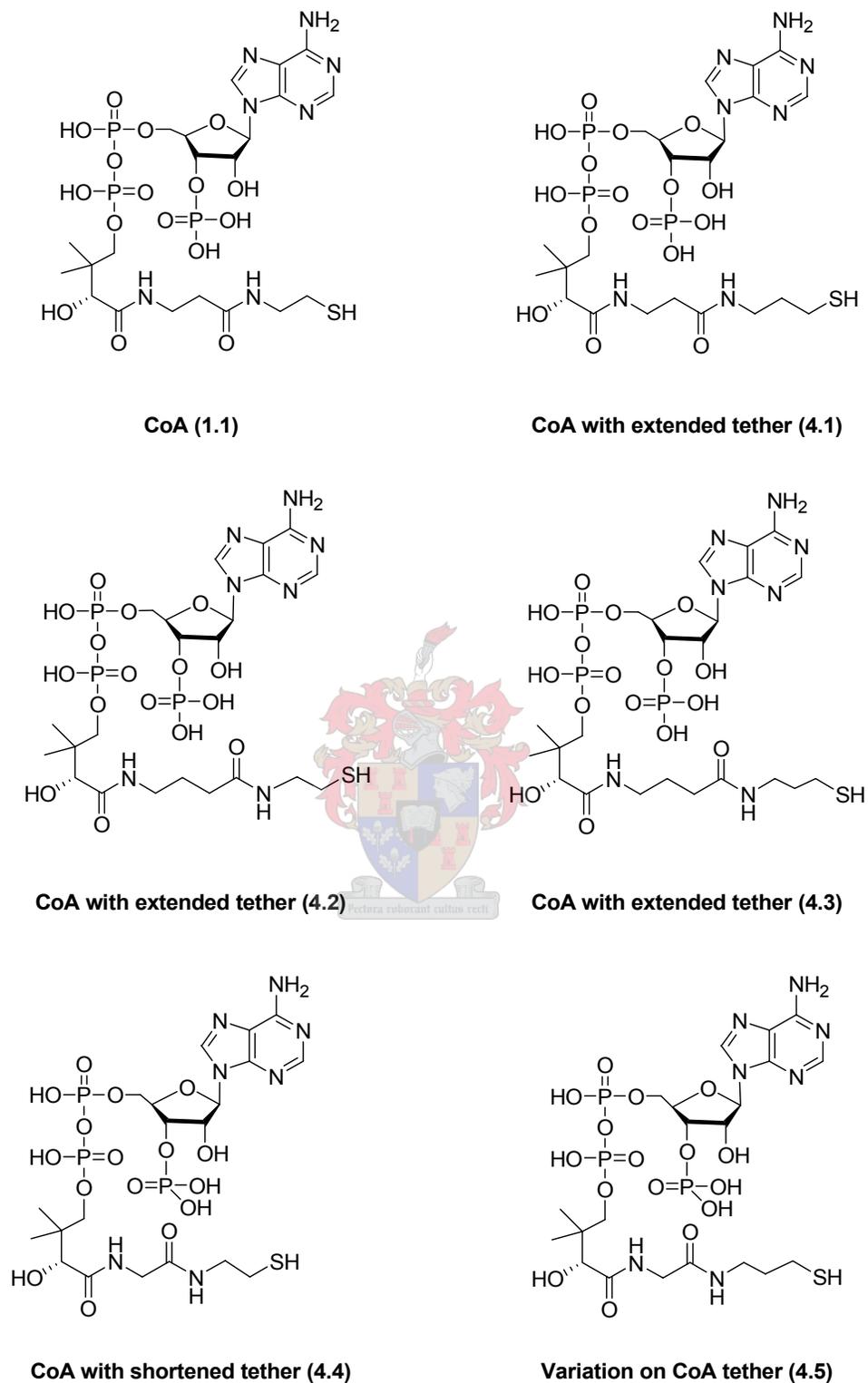


Figure 5.11 CoA analogues with different tether lengths.

CoA analogue with different amide functionalities

To expand the strategy in the production of CoA analogue with various primary amines, *S*-phenyl thiopantothenate was used in three different aminolysis reactions. Pentylamine, ethanolamine and benzylamine were used in the production of CoA analogues with different functional groups (figure 5.12).

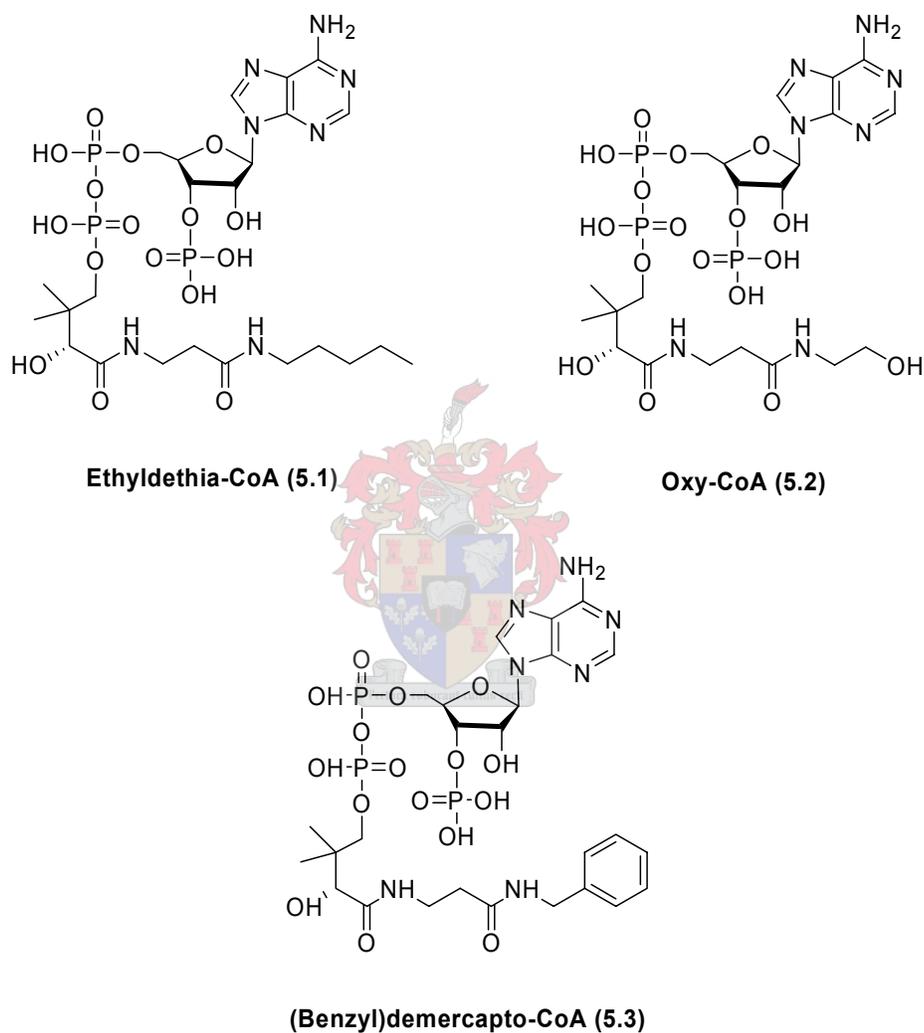
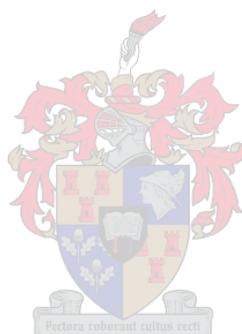


Figure 5.12 CoA analogues prepared in strategy 2 with different functional groups.

These reactions were also analyzed with LC-MS (figure 5.13). Peaks for each product were identified in positive and negative mode in the MS analyses (table 5.2). No other peaks were visible in the chromatograms at 220 nm, except a peak at 2.3 minutes due to the HEPES reaction buffer. This indicates that the preparation of these compounds was very successful with strategy 2.

Table 5.2 LC-MS results of CoA derivatives with different tether lengths.

CoA analogue	Retention time (minutes)	Calculated Molecular Mass	Found	
			[M+H] ⁺	[M-H] ⁻
CoA analogue 5.1	8.31	777.2	778	776
CoA analogue 5.2	2.83	751.5	752	750
CoA analogue 5.3	8.09	797.5	798	796



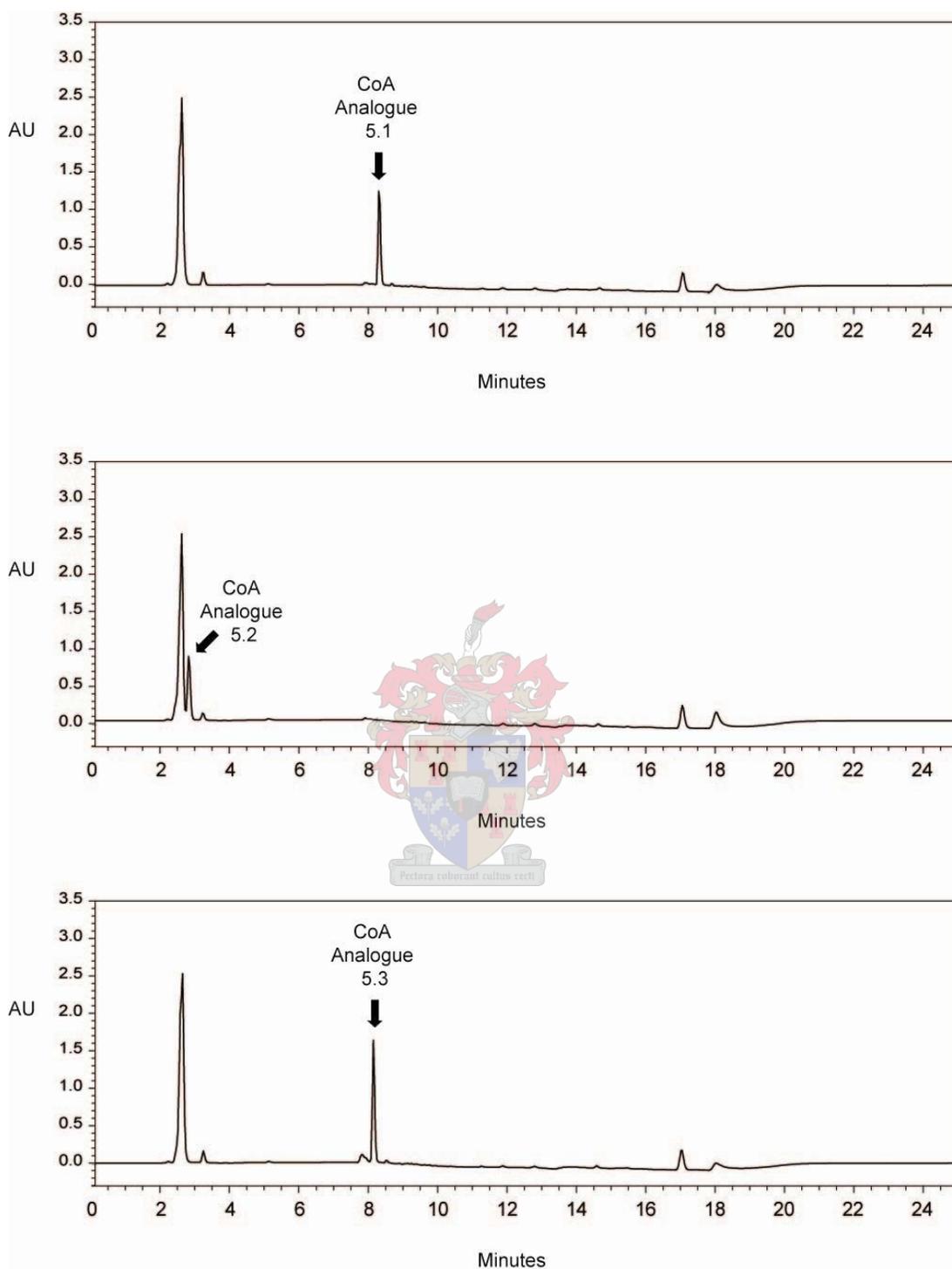


Figure 5.13 LC-MS results at 220 nm. The arrows indicate the CoA products for each reaction.

5.3 Conclusion

This chapter has shown that active thioester substrates, based on pantothenic acid can be synthesized in an easy chemical synthesis with good yields. These substrates can be used in the production of phenyl pre-CoA, but with lower yields than the biosynthesis of ethyl pre-CoAs. The aminolysis of the phenyl pre-CoA pentylamine did occur, but there was not a complete conversion of the pre-CoA to the corresponding CoA product.

A different route was explored where the phenyl thioesters were exposed to various types of amines to form pantothenamide intermediates. These intermediates were used to produce different CoA analogues with different tether lengths. Although the aminolysis of *S*-phenyl thiopantothenate was successful, the biosynthesis reactions did not produce any products. In combination with results in chapter 4, where various side-products were formed in an attempt to prepare these compounds (4.1 – 4.5), it is clear that the reaction conditions still need to be optimized.

However, the second strategy aimed at the production of analogues with functional groups other than a thiol-bearing amide proved extremely successful. This was exemplified by the aminolysis of *S*-phenyl thiopantothenate with pentylamine. The strategy was subsequently expanded to include the production of three different CoA analogues with either a pentylamide, ethanolamide or benzylamide functional group. While these reactions still need to be optimized for large-scale production, the methodology we have developed and outlined in this chapter should prove a simple and general way to prepare a large variety of CoA analogues. Attempts at expanding the scope of this study in this direction are currently underway in our laboratory.

5.4 Experimental Procedures

5.4.1 Materials and Methods

All chemicals and resins were purchased from Sigma-Aldrich (Aldrich, Sigma or Fluka) or Acros Organics and were of the highest purity. Homocysteamine.HCl was prepared as seen in chapter 4. C₁₈ solid phase extraction cartridges (Supelclean LC18), HisTrap™ Chelating and Desalting Columns (Amersham Biosciences) were from Supelco (Sigma). A Quick Start Bradford Protein Assay Kit was purchased from Bio-rad and contained Bradford reagent and a Bovine Serum Albumin standard set. Large-scale centrifugation was done on a Heraeus Multifuge® 3S/3S-R. Centrifugation on a smaller scale was conducted on a Heraeus Biofuge pico centrifuge. Vivaspin 6 Centrifugal Concentrators (MWCO 10,000) from Sigma-Aldrich was used for individual sample preparations to remove enzymes from reaction mixes. Fraction scans were performed on a Multiskan Spectrum multiplate spectrophotometer (ThermoLabsystems).

¹H-NMR was performed on a Varian INOVA 400MHz instrument and pre-CoAs were analyzed on a Varian INOVA 600MHz instrument at the Central Analytical Facility of the University of Stellenbosch. ESI-MS analyses were performed on a Waters Micromass Q-TOF Ultima API mass spectrometer. LC-MS: HPLC analysis were performed on a Waters™ 2690 Separations Module with a Waters® 996 Photodiode Array Detector using a Gemini 5 μm C18 110Å column (250 × 4.6 mm) from Phenomenex, followed by Waters Quattro Micromass mass spectrometer analyses. All MS analyses were done by Dr. M Stander at the Central Analytical Facility of the University of Stellenbosch. Pantetheine and ethyl dethia-CoA HPLC standards were provided by Dr E. Strauss from a previous study (7).

5.4.2 Synthesis of S-phenyl thiopantothenate

Sodium pantothenate (1.0 g; 4.15 mmol) was exchanged to the free acid over Amberlite IR-120 (H+) and lyophilized. The resulting syrup was dissolved in DMF (5 ml), diphenylphosphoryl azide (1.470 ml; 8.3 mmol) and thiophenol (0.513 ml; 5 mmol) were added. After cooling to 0 °C, triethylamine (1.150 ml) was added and

the solution was stirred for 10 minutes at 0 °C, followed by stirring for 2 hours at room temperature. Ethyl acetate was added (50 ml) and the solution was washed with 1 M HCl (2 × 10 ml), 1 M NaHCO₃ (2 × 10 ml) and saturated NaCl (1 × 10 ml). The solution was dried over Na₂SO₄ and the solvent was removed *in vacuo*. The product was purified by flash chromatography (silica gel; ethyl acetate/hexane 2:1 to 4:1) to give S-phenyl thiopantothenate as an orange oil (0.550 g; 42 %). ¹H NMR (400 MHz, CDCl₃): δ 0.93 (s, 3H), δ 01.02 (s, 3H), δ 2.90 (d, 1H), δ 2.976 (d, 1H), δ 2.95 (t, 2H), δ 3.53 (d, 2H), δ 4.04 (s, 1H), δ 7.46 (s, 5H).

5.4.3 Synthesis of S-Phenyl thiohomopantothenate

4-Amino butyric acid (0.500 g; 4.8 mmol) was dissolved in 4.8 ml sodium hydroxide (1 M) and the excess water was evaporated *in vacuo*. The resulting white salt and 0.5 g D-pantolactone (7.68 mmol) were melted together and refluxed under argon gas for 17 hours at 130 °C giving a colorless sticky oil. The sodium 4-amino butyrate was exchanged to the free acid over prewashed Amberlite IR-120 (H⁺) eluting with deionized water. The unreacted pantolactone was extracted with 3 × 20 ml dichloromethane and the aqueous layer was lyophilized resulting in a colorless oil as product.

The resulting syrup was dissolved in DMF (3.8 ml) followed by the addition of diethyl cyanophosphonate (1.14 ml; 7.6 mmol) and thiophenol (0.493 ml; 4.8 mmol). After cooling to 0 °C, triethylamine (1.059 ml) was added and the solution was stirred for 10 minutes at 0 °C followed by stirring for 2 hours at room temperature. Ethyl acetate was added (50 ml) and the solution was washed with 5% citric acid (3 × 10 ml), 1 M NaHCO₃ (3 × 10 ml) and saturated NaCl (2 × 10 ml). The solution was dried over Na₂SO₄ and the solvent was removed *in vacuo*. The product was purified by flash chromatography (silica gel; ethyl acetate/hexane 2:1 to 4:1) to give S-phenyl thiohomopantothenate as an orange oil (0.761 g; 61 %). ¹H NMR (400 MHz, CDCl₃): δ 0.93 (s, 3H), δ 01.03 (s, 3H), δ 1.95 (m, 2H), δ 2.73 (t, 2H), δ 3.70 (m, 2H), δ 3.52 (q, 2H), δ 4.04 (s, 1H), δ 7.41 (s, 5H).

5.4.4 Synthesis of S-Phenyl thio- α -pantothenate

Sodium glycerate was synthesized by dissolving L-glycine (0.360 g; 4.8 mmol) in 4.8 ml sodium hydroxide (1 M). The excess water was evaporated *in vacuo* and 0.500 g pantolactone were added to the resulting salt. The salts were melted together and refluxed at 130 °C under argon for 17 hours, resulting in a brown, sticky oil. The sodium glycerate was exchanged to the free acid over prewashed Amberlite IR-120 (H⁺) eluting with deionized water. The unreacted pantolactone in the reaction mix was extracted with 3 × 20 ml dichloromethane and the aqueous layer was lyophilized to give an orange oil.

The resulting syrup was dissolved in DMF (3.8 ml) followed by the addition of diethyl cyanophosphonate (1.14 ml; 7.6 mmol) and thiophenol (0.493 ml; 4.8 mmol). After cooling to 0 °C, triethylamine (1.059 ml) was added and the solution was stirred for 10 minutes at 0 °C followed by stirring for 2 hours at room temperature. Ethyl acetate was added (50 ml) and the solution was washed with 5% citric acid (3 × 10 ml), 1 M NaHCO₃ (3 × 10 ml) and saturated NaCl (2 × 10 ml). The solution was dried over Na₂SO₄ and the solvent was removed *in vacuo*. The product was purified by flash chromatography (silica gel; ethyl acetate/hexane 2:1 to 4:1) to give S-phenyl thiohomopantothenate as a orange oil (0.226 g; 20 %). ¹H NMR (400 MHz, CDCl₃): δ 0.98 (s, 3H), δ 01.06 (s, 3H), δ 2.882-3.957 (d, 2H), δ 3.55 (q, 2H), δ 4.13 (s, 1H), δ 7.42 (s, 5H).

5.4.5 Biosynthesis of phenyl pre-CoA

A 1500 μ l reaction mixture contained 5 mM S-phenyl thiopantothenate, 16.5 mM ATP, 20 mM KCl and 10 mM MgCl₂ in 50 mM Tris-HCl buffer (pH 7.6). The reaction was initiated by addition of biosynthetic enzymes (145 μ g SaCoaA, 252 μ g EcCoaD and 252 μ g EcCoaE) and incubated for 90 minutes at 37 °C. The reaction was stopped by transferring the reaction mix to a Vivaspin 6 Centrifugal Concentrator (MWCO 10,000), which was centrifuged for 30 minutes at 13 000 \times g.

A C₁₈ solid phase extraction cartridge (500mg/3ml) was used to purify the phenyl pre-CoA. Prior to loading the sample, the cartridge was conditioned with ~4ml

methanol and equilibrated with ~4 ml 200 mM NH₄OAc (pH 6). The supernatant of the reaction mix was loaded onto the cartridge, followed by washing with ~4 ml 200 mM NH₄OAc (pH 6), ~4 ml 20 mM NH₄OAc (pH 6) and finally the product was eluted with ~4 ml 20% acetonitrile. The chromatography was monitored at A235, A254 and A280 using a Multiskan Spectrum multiplate spectrophotometer (Thermo LabSystems). Corning UV detection 96-well microtiter plates were used for product detection at 235, 254 and 280 nm. The product-containing fractions were combined and lyophilized, dissolved in water and lyophilized again. This was repeated until a constant weight of product was achieved (Yield 2.7 mg: 45 %). ¹H NMR was done on a 600 MHz NMR in D₂O (section 5.2.2).

5.4.6 Aminolysis of pre-CoAs

Aminolysis of pre-CoAs were done in 200 mM HEPES (pH 8). The reaction mix with final volume of 200 µl contained 5 mM pre-CoA and 50 mM amine. The reaction mix was incubated for 60 minutes at 50 °C. Analysis was done via HPLC by injecting 10 µl of the undiluted reaction mix and extracting the resulting chromatogram at a wavelength of 214 nm.

The following HPLC gradient was used to analyze the reactions:

Time (minutes)	Flow rate (ml/min)	% Solvent A 10mM NH ₄ OAc, pH 6	% Solvent B Acetonitrile
0.01	1.00	95	5
5.00	1.00	80	20
10.00	1.00	80	20
11.00	1.00	60	40
15.00	1.00	60	40
16.00	1.00	40	60
20.00	1.00	40	60
21.00	1.00	20	80
25.00	1.00	20	80

5.4.7 Aminolysis of S-phenyl thiopantothenate

The aminolysis reaction was done in 200 mM HEPES (pH 8), containing 20 mM S-phenyl thiopantothenate in a final volume of 500 μ l. The reaction was initialized by addition of 10 equivalents amine (final concentration 100 mM). The reaction was incubated at 50 °C for 1 hour. Analysis was done via HPLC by injecting 10 μ l of the undiluted reaction mix and extracting the resulting chromatogram at a wavelength of 214 nm.

The following HPLC gradient was used to analyze the reactions:

Time (minutes)	Flow rate (ml/min)	% Solvent A 10mM NH ₄ OAc, pH 6	% Solvent B Acetonitrile
0.01	1.00	95	5
5.00	1.00	80	20
10.00	1.00	80	20
11.00	1.00	60	40
15.00	1.00	60	40
16.00	1.00	40	60
20.00	1.00	40	60
21.00	1.00	20	80
25.00	1.00	20	80

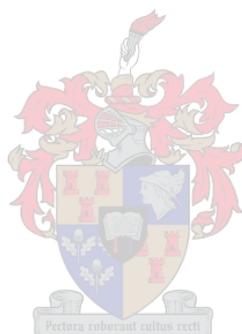
5.4.8 Biosynthesis of CoA derivatives from pantothenamide intermediates

The aminolysis reaction was done in a final reaction volume of 150 μ l. The reaction was done in 200 mM HEPES (pH 8) containing 10 mM S-phenyl thiopantothenate and 100 mM amine. The mix was incubated at 50 °C for 1 hour.

The finished aminolysis reaction mix was then used in the biosynthesis of the CoA analogue. To this mix (final volume of 500 μ l) 16.5 mM ATP and 10 mM MgCl₂ was added. The reaction was initiated by addition of 51 μ g of the following enzymes: *SaCoaA*, *EcCoaD* and *EcCoaE*. This final reaction mix was incubated for 2 hours at 37 °C. The reaction mix was diluted (1:1) in 10 mM NH₄OAc, pH 6 for LC-MS analysis.

The following HPLC gradient was used to analyze the reactions:

Time (minutes)	Flow rate (ml/min)	% Solvent A 10mM NH₄OAc, pH 6	% Solvent B Acetonitrile
0.01	1.00	95	5
5.00	1.00	80	20
10.00	1.00	80	20
11.00	1.00	60	40
15.00	1.00	60	40
16.00	1.00	40	60
20.00	1.00	40	60
21.00	1.00	20	80
25.00	1.00	20	80



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Chapter 6

Conclusion

6.1 Importance of CoA analogue production

Research involving CoA has always been popular due to its biochemical centrality. Derivatives of CoA as inhibitors and mechanistic probes of CoA utilizing enzymes are fairly well developed. These derivatives have been highly valuable in the elucidation of mechanistic details. It is expected that new and existing analogues of CoA will be valuable in the study of the enzymes that use CoA and CoA esters as substrates. The development of new antibiotics designed as inhibitors of CoA utilizing enzymes (such as CoA antivitamins or antimetabolites) would be very beneficial. The production of CoA analogues with different tether lengths will also be used in the production of unsaturated fatty acids, using these specific analogues in combination with a biocatalyst.

6.2 Cellulose binding domains

An ideal method to produce CoA analogues will use proteins from the biosynthetic pathway of CoA, which is immobilized on cellulose in a bioreactor system. Various CBDs were available for protein immobilization. CBD_{CenA} from pET-36b(+) (1) has shown to give low amounts of soluble enzymes when fused to *EcCoaA*, *EcCoaD* and *EcCoaE* in pET-28a as expression vector. CBD_{Cex} from pET-38b (1), showed almost no solubility in the attempt to purify CBD-*EcCoaA* and CBD-*EcCoaD*. CBD_{CipA} from cellulosome-integrating protein CipA (CBD_{CipA}) of *C. thermocellum* was used to construct CBD_{CipA}-*EcCoaD* (2). Expression trials of this protein have shown good expression as well as solubility. These results show promise for the solubility of CBD_{CipA}-*EcCoaA*, CBD_{CipA}-*SaCoaA* and CBD_{CipA}-*EcCoaE*.

6.3 CoA analogue production

Two different strategies were investigated to produce CoA analogues (figure 6.1). The first strategy successfully produced CoA analogues with different tether lengths. However, various unidentified side-products formed in the reactions. The identical production of a second type of CoA synthon (phenyl pre-CoA) from pantothenic acid phenyl thioesters was also investigated as a means to increase reactivity of the thioester substrates. However, the corresponding phenyl pre-CoA intermediate did not deliver better aminolysis results than the ethyl pre-CoAs, which lead us to investigate a second strategy.

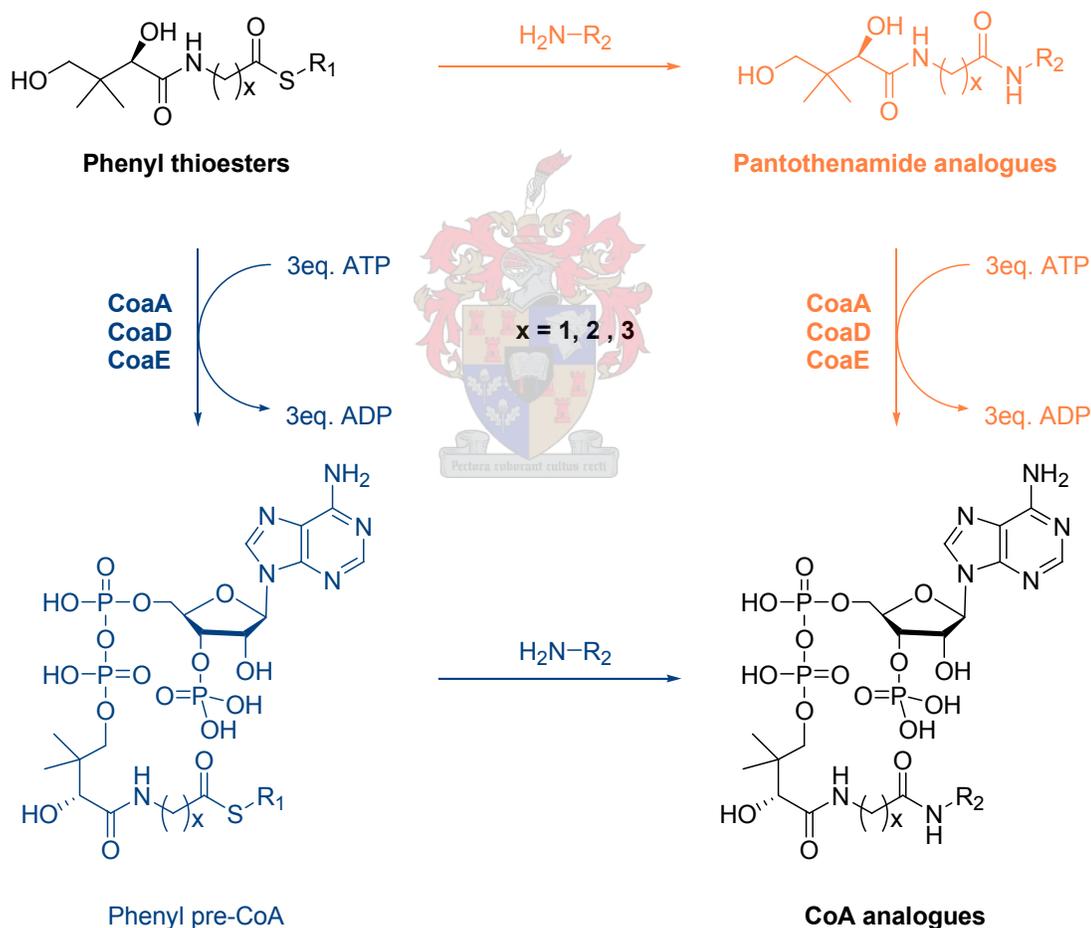


Figure 6.1 Different strategies used in CoA analogue production (strategy 1: blue and strategy 2: orange).

The phenyl thioesters were used as substrates in strategy 2. Although the aminolysis reaction was successful, the production of CoA analogues with different tether lengths from the corresponding pantothenamide intermediates was not successful. This method still needs to be optimized. It seems as if strategy 1, utilizing ethyl pre-CoAs followed by aminolysis produces these compounds. However, the reaction conditions will require some more optimization. The phenyl thioesters work very well in the production of CoA analogues with different amide functionalities by utilizing strategy 2.

In general, both methods resulted in the chemo-enzymatic synthesis of CoA analogues with much lower reaction times and amine concentrations as shown by other investigations (3, 4). Different types of amines were viable for this method and can be utilized in the production of different types of CoA analogues. However, these reactions still need to be optimized for large-scale production. The possibility to use other primary amines and secondary amines will also be explored in future work.

6.4 Future research

6.4.1 Construction of bioreactor

Due to the fact that the CBD_{CipA}-fused proteins can be expressed with good solubility, these enzymes can now be immobilized on cellulose as matrix. The binding capacity of the proteins to cellulose will be determined. The immobilized proteins will then be used in combination with pantothenic acid analogues in the production of the corresponding CoA analogues in a bioreactor system as shown in figure 6.2.

This process will then be optimized for the large scale, continuous production of these analogues to be used in fatty acid biosynthesis and antimetabolite production.

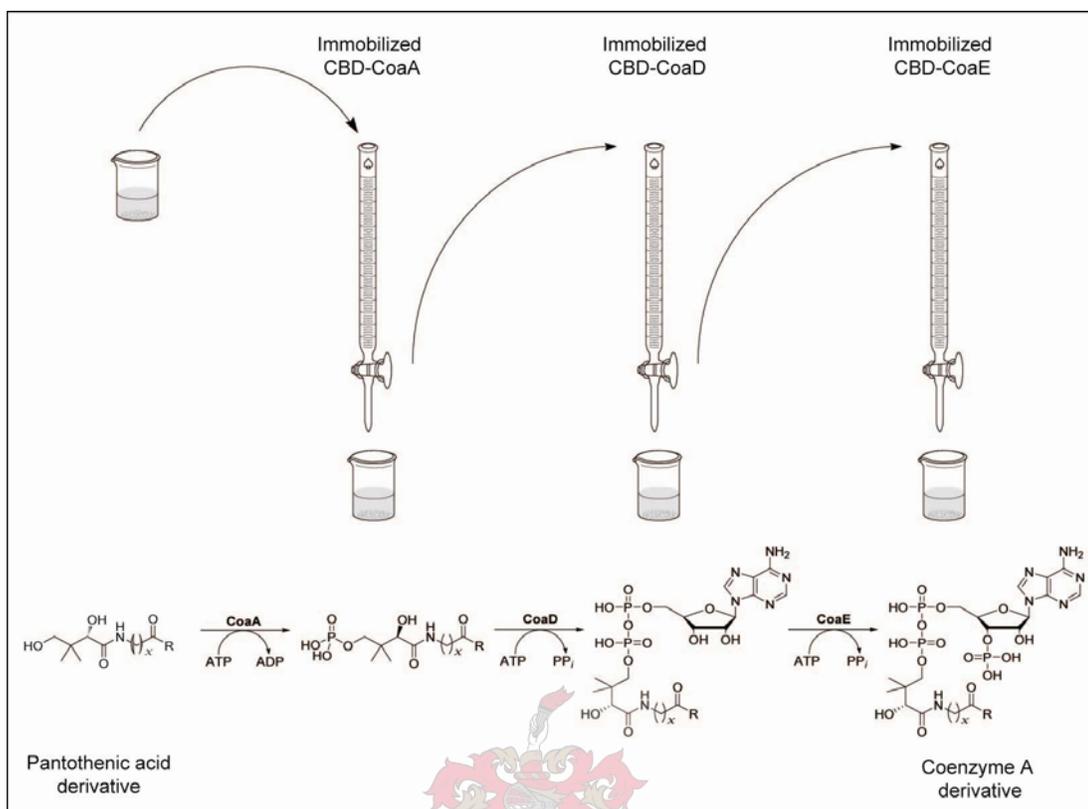


Figure 6.2 Bioreactor system for CoA analogue production.

6.4.2 Biocatalysis of fatty acids

Unsaturated fatty acids are important for normal cell function. The production of unsaturated fatty acids can be achieved by using CoA analogues in combination with a biocatalyst. The biocatalyst will be based on a soluble desaturase enzyme such as the acyl-ACP Δ^9 desaturase from the castor oil plant as described in chapter 1 (5). This desaturase enzyme will be used in combination with the different tether length CoA analogues (4.1 – 4.2) to result in a transfer of the modified phosphopantetheine groups to ACP. It is important to keep the terminal thiol group of the different tether length CoAs intact. The modified phosphopantetheine tethers should still allow esterification of fatty acids due to the thiol group, which reacts with the acid. The modified acyl-ACP will act as a substrate for the castor acyl-ACP Δ^9 desaturase after esterification. This will result in regioselectivity, depending on the nature of the modification of CoA structure. This specific strategy to change the position of desaturation is summarized in

figure 6.3. If this method is successful it will result in a range of different desaturated fatty acids while using the same soluble desaturase enzyme.

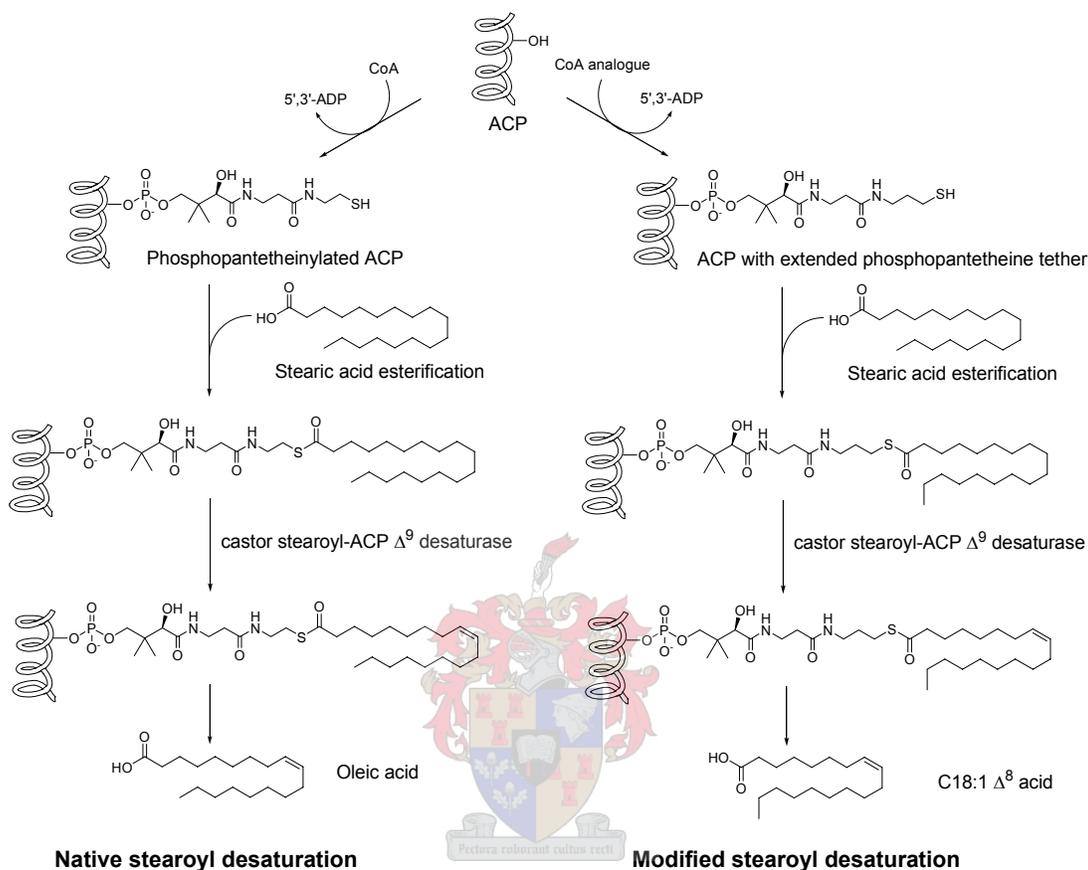


Figure 6.3 Modification of the position of desaturation in saturated fatty acids

6.4.3 High throughput synthesis of CoA analogues

A high-throughput method can be developed for the production of various CoA analogues. The first step will be to determine if aminolysis of S-phenyl thiopantothenate can be achieved with various primary and secondary amines. It was already shown in this thesis that aminolysis can be achieved with four different types of amines, utilizing short reaction times and mild reaction conditions. If a wide range of pantothenamide analogues can be produced these analogues can be used in the production various CoA analogues (figure 6.4).

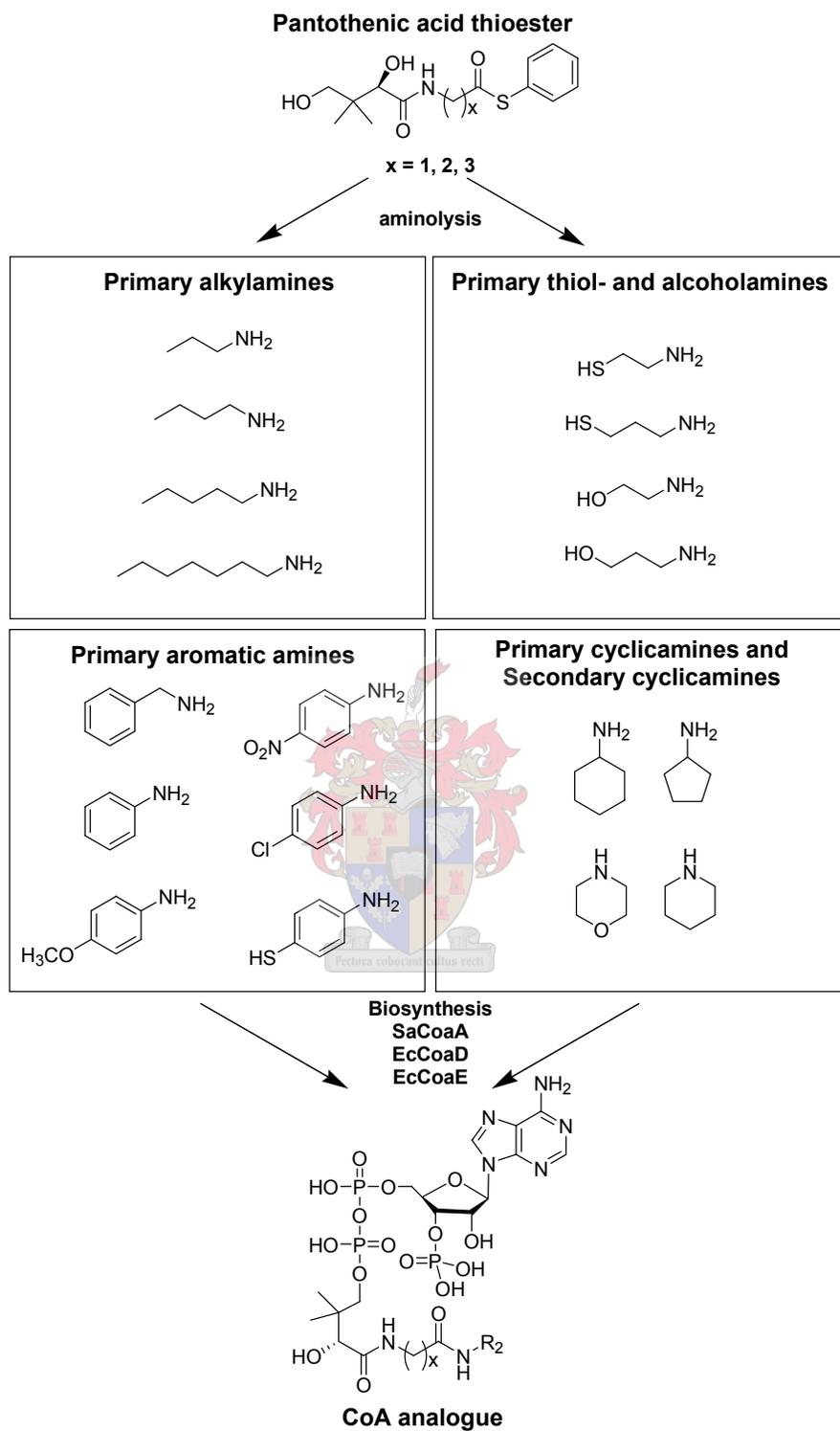


Figure 6.4 Production of a wide variety of pantothenamide intermediates for CoA analogue production.

A study by Virga *et al.* has shown that different pantothenate kinases accept a wide variety of pantothenamide analogues as substrates (6). Therefore, if CoaD and CoaE accepts these phosphopantothenamide products a high through-put screening method can be developed, utilizing chemo-enzymatic synthesis in the production of different CoA analogues. The production of these analogues can be optimized on large-scale to be used in different applications like non-ribosomal peptide synthesis and as antimetabolites.

6.5 Final remarks

The biosynthesis of CoA has been the focus of a range of studies in the production of different CoA analogues. These analogues can be used in various applications. This pathway provided the foundation in this particular study, where it was shown that different types of CoA analogues can be produced by a simple chemo-enzymatic method. It is expected that this method will be valuable in the production of both new and existing CoA analogues that will continue to be very valuable in future research.



6.5 References

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