Characterization of prokaryotic pantothenate kinase enzymes and the development of type-specific inhibitors

by
Lizbé Koekemoer

Dissertation presented for the degree of Doctor of Chemistry at the University of Stellenbosch

Promoter: Prof Erick Strauss
Faculty of Natural Sciences
Department of Biochemistry

December 2011

Declaration

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2011

Abstract

Pantothenate kinase (PanK) enzymes catalyze the first reaction in the five step biosynthesis of the essential cofactor coenzyme A. Enzymes representing each of the three identified PanK types have been studied and characterized and these PanK types exhibits a unique diversity between different organisms, therefore highlighting them as potential drug targets. In this study the type III PanK of specifically pathogenic bacteria were characterized with the goal of developing type-specific inhibitors. Several questions about the activity of the *Mycobacterium tuberculosis* enzyme was answered, which addresses the contradicting results achieved in related PanK studies performed to date. Furthermore the first inhibitors, that are competitive to the pantothenate binding site, were designed, synthesized and tested against the *Pseudomonas aeruginosa* enzyme. This resulted in the discovery of the most potent inhibitors of the type III PanKs fto date.

Opsomming

Pantoteensuurkinase-ensiem (PanK) kataliseer die eerste stap in die vyf stap biosintese van die lewens belangrike en essensiële kofaktor, koënsiem A (KoA). Die meerderheid patogeniese bakterieë, waaronder die organisme wat tuberkulose veroorsaak, besit 'n unieke vorm van die PanK-ensiem. Gevolglik word hierdie ensieme as belangrike teikens vir die ontwikkeling van antibakteriële middels beskou. In hierdie studie is die aktiwiteit van die *Mycobacterium tuberculosis* ensiem gekarakteriseer wat verskeie teenstrydige bevindings oor hierdie ensiem beantwoord het. Verder is nuwe inhibitore vir die *Pseudomonas aeruginosa* ensiem ontwerp, gesintetiseer en getoets. Die beste inhibitore van hierdie tipe ensiem tot op hede is sodoende geïdentifiseer.

Acknowledgements

One of the main things people told me after I have worked for a while is that it is extremely difficult to quit your job and start to study again. I took the plunge in April 2008 and after three and a half years back I am wondering if the difficult part is not to start working again. Needless to say, there were many people involved in this adventure who I want to extend my gratitude to.

- Firstly I want to thank my supervisor, Prof Erick Strauss, for allowing me back into his lab after my washing powder stint. Thank you for the guidance, the freedom to explore, your endless well of ideas and all the opportunities you gave me over these last few years.
- All the Strauss Lab members over the years – starting with Leisl and Marianne in the "Girls Only lab" and ending today with a much more balanced group. Thank you for receiving me back with open arms and for all the good and bad times we shared together. I am probably the last person that can claim that I have worked together with all the Strauss' labrats to date.
- A special thank you goes out to Leisl Brand – even after all these years you still play an integral role in our lab. Thank you for all the prep work (which you probably can't even remember anymore) which build the foundation for my studies.
- On special request I also want to extend my gratitude to all my "minions" over the years, especially for all the valiant efforts to keep me sane during the write-up of this thesis.
- I also want to thank all my family and friends for supporting me and always being there for me. We had some good times and hopefully they will continue.
- Lastly to my Pappa God - for sustaining me, for bringing me back home, for being the reason I get up in the morning and for teaching me how to live life. I am looking forward to our next adventure!

Additional acknowledgements

- The University of Stellenbosch and Prof. E. Strauss for financial support and the opportunity to study at this institution
- The National Research Foundation (NRF) and the H.B. Thom trust for financial support
- Mrs. Elsa Malherbe and Dr. Marietjie Stander from the Central Analytical Facility of Stellenbosch University for NMR and LC-MS analyses

For the love of God and life

and to living the adventure

Table of content

List of abbreviations

^{13}C NMR	carbon nuclear magnetic resonance spectroscopy
^{19}F NMR	fluorine nuclear magnetic resonance spectroscopy
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
ACP	acyl carrier proteins
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AMP-PNP	5'-adenylyl-β,γ-imidodiphosphate (non-hydrolysable ATP analog)
Arg	arginine
ASKHA	acetate and sugar kinase/heat shock protein 70/actin superfamily
Asp	aspartate
Asn	asparagine
ATP	adenosine 5'-triphosphate
aq	aqueous
Ba	*Bacillus anthracis*
Baf	Bvg accessory protein
BCG	*M. bovis* bacille Calmette Guerin vaccine
BSA	Bovine serum albumin
CD	circular dichroism
CH_3COOH	acetic acid
CN^-	cyanide ion
COSY	Correlation spectroscopy

CTP	cytidine 5’-triphosphate
$CuCl_2$	copper (III) chloride
DCM	dichloromethane
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDC	*N*-(3-Dimethylaminopropyl)-*N*’-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
eq.	equivalent
ESI-MS	electron spray ionization mass spectrometry
EtOAc	ethyl acetate
Gly	glycine
GTP	guanosine 5’-triphosphate
h	hours
HCl	hydrochloric acid
HEPES	*N*-2-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
His	histidine
His_6-tag	6×histidine-tag
HIV	Human Immunodeficiency Virus
H_2O	water
HPLC	high performance liquid chromatography

Ile	isoleucine
IMAC	immobilized metal affinity chromatography
iPr_2NEt	*N,N*-diisopropylethylamine
IPTG	isopropyl-β-D-thiogalactoside
K^+	potassium ion
K_2CO_3	potassium carbonate
KCl	potassium chloride
K_i	inhibition constant
K_M	Michaelis-Menten constant
K_t	transfer constant
LB	Luria Bertani (media use for bacterial growth)
LC MS	liquid chromatography mass spectrometry
LDH	Lactate dehydrogenase
Leu	leucine
Lys	lysine
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MBP-tag	maltose binding protein-tag
MCR	multi-component reaction
MDGs	Millennium Development Goals
MDR-TB	multi-drug resistant TB
MeI	methyl iodide
MeOH	methanol

MES	2-(*N*-morpholino) ethanesulfonic acid
Me_2SO_4	dimethyl sulfate
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
Min	minute
Mj	*Methanocaldococcus jannaschii*
M_r	relative molecular mass
MS	mass spectrometry
Ms	*Mycobacterium smegmatis*
Mtb	*Mycobacterium tuberculosis*
Na_2SO_4	sodium sulfate
NaCl	sodium chloride / salt / brine
NADH	reduced nicotinamide adenine dinucleotide
NaH	sodium hydride
$NaHCO_3$	sodium bicarbonate
$NaIO_4$	sodium metaperiodate
NaOH	sodium hydroxide
NEt_3	triethylamine
NH_4^+	ammonium ion
NH_4Cl	ammonium chloride
NH_4CO_3	sodium carbonate
nr.	number

NTP	nucleoside 5'-triphosphate
Nus-tag	nut utilization substance-tag
-OCH_3	methoxy functional group
-OH	hydroxyl functional group
OsO_4	osmium tetroxide
PanK	Pantothenate kinase
P_{BAD}	*ara*BAD promoter
PCR	polymerase chain reaction
Pd	palladium
PDB	Protein database
PEP	phosphoenolpyruvate
Pf	*Pyrococcus furiosus*
Ph	*Pyrococcus horikoshii*
PK	pyruvate kinase
pK_a	acid dissociation constant
PMF	peptide mass fingerprinting
$POCl_3$	phosphoryl chloride
PoK	Pantoate kinase
PPS	Phosphopantoate syntethase
PPTase	Phosphopantetheinyl transferase
Pro	proline
PS	Pantothenate synthase

Rb^+	rubidium ion
RE	restriction enzyme
rpm	revolutions per minute
RT-PCR	real time polymerase chain reaction
s	seconds
SCID	Severely compromised immune deficient (mouse model)
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S_N2	nucleophilic substitution reaction
SOE PCR	splicing by overlap extension polymerase chain reaction
spp.	species
TAE	Tris-HCl/ acetate/ EDTA buffer
TB	tuberculosis
Temp	temperature
TEV	tobacco etch virus
Thr	threonine
$TiCl_4$	titanium (IV) chloride
TLC	thin layer chromatography
TMS-Cl	trimethylsilyl chloride
TRASH	transposon site hybridization
Trp	tryptophan
TTP	thymidine 5′-triphosphate
Tyr	tyrosine

U	units (enzyme concentration)
UN	United Nations
UTP	uridine 5’-triphosphate
w/w	mass fraction – weight / weight
WHO	World Health Organization
Val	valine
XDR-TB	extensively-drug resistant TB

Chapter 1:
Overview of thesis

1.1 Pantothenate kinase: Remaining unanswered questions

Pantothenate kinase (PanK) catalyses the first committed step in the biosynthesis of the universal and essential cofactor coenzyme A (CoA)[1]. Specifically, PanK catalyzes the ATP-dependent phosphorylation of pantothenic acid (Vitamin B_5) to form 4'-phospho-pantothenic acid as shown in Figure 1.1.

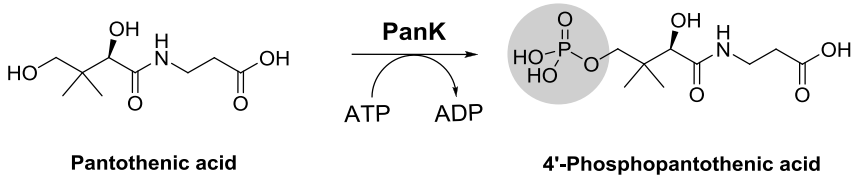


Figure 1.1: The ATP-dependent phosphorylation of pantothenic acid to form 4'-phosphopantothenic acid is catalyzed by the enzyme pantothenate kinase (PanK).

Enzymes representing each of the three identified PanK types have been studied and characterized in several studies conducted since the cloning and production of the *Escherichia coli* enzyme in 1992. However, significant gaps in our understanding of these kinases remain. This is especially true of type III PanKs, which were first characterized as recently as 2005, and represent an especially important target for antibacterial drug discovery. Summarized below are some of the most important questions regarding PanKs that still need to be addressed.

- In 12% of the bacterial species with sequenced genomes, which are predicted to have pantothenate kinase activity – as in the case of Archaea – no PanK-encoding gene can be identified by homology. This raises the question of whether significant variations of the three known types exist, or whether a fourth type remains to be discovered.[2]
- The genomes of both *Bacillus anthracis* and *Mycobacterium tuberculosis* encodes for two PanK enzymes representing different types: the former possesses Type II and III PanKs, while the latter has Type I and III enzymes. However, in both cases one of these PanK types is reported to lack demonstrable pantothenate kinase activity.[3,4]

If this is really the case, why did both organisms retain these genes and why are the proteins still expressed? Is there a possibility that these enzymes have another function, or that they are only activated under certain conditions (and what would these conditions be)?

- Type I and II PanKs are known for their substrate promiscuity,[5] and a range of pantothenate analogs have been shown to act as substrates and/or inhibitors of these enzymes. However, little is known about compounds that could act as alternative substrates and inhibitors of type III PanK enzymes, and the question remains as to the type or class of analog that could play such a role in its case.
- Type III PanKs have K_M-values for ATP which fall in the range of 2-10 mM.[6] Although these values can be explained using the available structures, from a physiological perspective such high values makes much less sense. What is the rationale for such low substrate specificity? Is ATP really the co-substrate, or are there other phosphoryl donors that still need to be investigated?
- The *coaX* homolog (the gene encoding for type III PanKs) from *Bordella pertussis* was first studied as an accessory protein in the regulation of pertussis toxin production by the BvgAS proteins.[7] In many instances *coaX* homologs are still annotated as Baf (Bvg accessory factor) or as putative transcriptional regulators. Are type III PanKs able to moonlight as both Baf proteins and PanKs, or was the activity observed in the *B. pertussis* study unique to the *coaX*-encoded protein from this organism?

1.2 Aims of this study

All the questions posed above cannot be answered in one study. In the studies described in this thesis we mainly focused on the type III PanKs from pathogenic bacteria with the aim to find answers relating to inhibitor identification (chapter 3) and enzyme activity (chapter 4). However, we also attempted to purify and characterize the first example of a type IV PanK from Archaea (chapter 5), to address the question in regards to the supposed "missing" PanK in this domain of life.

The outline of each chapter is given below.

1.2.1 Chapter 2

The importance of CoA and PanKs are placed into perspective in the context of the global drug discovery programs for neglected diseases. This is followed by a comprehensive outline of the PanKs in general, in which the PanK types are compared with each other and the gaps in knowledge are identified.

1.2.2 Chapter 3

This chapter describes the work done on the identification, synthesis and testing of potential inhibitors for the type III PanKs. Our knowledge of the active site of *Pseudomonas aeruginosa* type III PanK was used to devise a strategy for the rational design of inhibitors. This is followed by a survey of the molecules that meet the specified criteria and discussions on the synthetic routes that were followed to create them. Finally the kinetic characterization of the molecules that showed potential as alternative substrate and/or inhibitors is provided.

1.2.3 Chapter 4

In published studies CoaX (hypothetical PanK-III) from *M. tuberculosis* is reported to be inactive. Here we report the results of a comparative characterization of the putative PanK-III enzymes from *Mycobacteria* and several other pathogenic organisms, including the inactive *B. anthracis* BA2901 (hypothetical PanK-II), in an effort to explain the apparent contradictory results achieved in the PanK-related studies performed to date.

1.2.4 Chapter 5

In this chapter the work done towards the identification, heterologous expression, purification and kinetic characterization of the first type IV PanK from Archaea is described.

1.2.5 Chapter 6

In summary an overview is given of what is known of the type III PanKs at the conclusion of these studies. Future work to address the remaining knowledge gaps is also identified and discussed.

1.3 References

1. Strauss, E., Coenzyme A Biosynthesis and Enzymology, in *Comprehensive Natural Products II*, M. Lew and L. Hung-Wen, Editors. 2010, Elsevier: Oxford. p. 351-410.
2. Osterman, A. and Overbeek R., Missing genes in metabolic pathways: a comparative genomics approach. *Current Opinion in Chemical Biology*, 2003. **7** (2): p. 238-51.
3. Nicely, N.I., Parsonage D., Paige C., Newton G.L., Fahey R.C., Leonardi R., Jackowski S., Mallett T.C. and Claiborne A., Structure of the type III Pantothenate Kinase from *Bacillus anthracis* at 2.0 Å. Resolution: Implications for Coenzyme A-Dependent Redox Biology. *Biochemistry*, 2007. **46** (11): p. 3234-45.
4. Awasthy, D., Ambady A., Bhat J., Sheikh G., Ravishankar S., Subbulakshmi V., Mukherjee K., Sambandamurthy V. and Sharma U., Essentiality and functional analysis of type I and type III pantothenate kinases of *Mycobacterium tuberculosis. Microbiology*, 2010. **156**: p. 2691-701.
5. Strauss, E., de Villiers M. and Rootman I., Biocatalytic Production of Coenzyme A Analogues. *ChemCatChem*, 2010. **2** (8): p. 929-37.
6. Rowan, A.S., Nicely N.I., Cochrane N., Wlassoff W.A., Claiborne A. and Hamilton C.J., Nucleoside triphosphate mimicry: a sugar triazolyl nucleoside as an ATP-competitive inhibitor of *B. anthracis* pantothenate kinase. *Organic & Biomolecular Chemistry*, 2009. **7** (19): p. 4029-36.
7. DeShazer, D., Wood G.E. and Friedman R.L., Identification of a *Bordetella pertussis* regulatory factor required for transcription of the pertussis toxin operon in *Escherichia coli. Journal of Bacteriology*, 1995. **177** (13): p. 3801-7.

Chapter 2:
Introduction to CoA biosynthesis and PanKs

2.1 Tuberculosis and the need for new antimicrobial agents

Infectious diseases remain a leading cause of morbidity and mortality in third world and developing countries. As part of the ongoing battle against these diseases the sixth goal of the United Nations' Millennium Development Goals (MDGs) is to "Halt and begin to reverse the incidence of HIV/AIDS, malaria and other major diseases by 2015 (in comparison to 1990)".[1] One of the "other major diseases" listed is tuberculosis (TB).

The good news is that the worldwide number of TB incidences *per capita* is falling at an estimated 1% per year which satisfies both the MDGs and Stop TB Partnership goals for this disease. However, with the world's population growing at about 2% per year the total number of TB cases is still rising. In 2010 there was an estimated 9.8 million new infections which is the largest number of new cases in any year to date. This situation is aggravated by co-infection with the human immunodeficiency virus (HIV). HIV is seen as the most powerful known risk factor for susceptibility to infection by *Mycobaterium tuberculosis,* the causative agent of TB, and the progression from latent infection to active disease.[2] According to the World Health Organization (WHO) HIV is also the main reason for failure to meet TB control targets with Sub-Saharan Africa bearing the brunt of the HIV-fuelled TB epidemic.[3] In addition, years of misuse and abuse of known treatments, poor patient compliance and poor quality of available drugs led to the emergence of drug resistant pathogens.

This increase of drug resistance strains highlights the need to find new classes of antimicrobials. The last drug with a new mechanism of action approved for TB was rifampicin, discovered in 1963. Drug resistance to this compound and to other known TB treatments is already prevalent. Multi-drug resistant TB (MDR-TB) is resistant to rifampicin and isoniazid and extensively-drug resistant TB (XDR-TB) to these treatments as well as to fluoroquinolone and the second-line anti-TB injectable drugs amikacin, kanamycin and capreomycin.[4]

M. tuberculosis is not the only example of a drug-resistant bacterium in which current treatment are rendered ineffective. Other global examples include methicillin-resistant and multidrug-resistant *Staphylococcus aureus*, penicillin- and macrolide-resistant pneumococci, vancomycin-resistant enterococci and multidrug-resistant *P. aeruginosa*. Resistance to chloroquine and other firstline antimalarial agents are also increasing in the *Plasmodium* spp.[5]

The drug discovery program established in our group targets coenzyme A (CoA) biosynthesis in bacteria since CoA is an essential cofactor that has to be biosynthesized in the organism and is used in numerous metabolic processes. The goal of this program is to use rational design to develop compounds that specifically target this pathway, and will therefore have a mechanism of action different to those of the known antibiotics already available.

2.2 Coenzyme A – an essential cofactor

CoA is a ubiquitous cofactor in all living organisms with an estimated 9% of the enzymes reported in the BRENDA database utilizing it in one way or another.[6] The cofactor functions as an acyl group carrier and carbonyl-activating group for various processes in cells including Claisen condensations and amide-, ester- and thioester-forming reactions. It is also involved in fatty acid biosynthesis and in the breakdown and in the biosynthesis of nonribosomally synthesized peptides and polyketides.[7]

CoA is synthesized in five enzymatic steps from pantothenic acid (vitamin B_5). Bacteria, plants and fungi synthesizing pantothenic acid *de novo*, whereas animals and some microbes obtain the vitamin from their diets.[8] The universal biosynthesis pathway of CoA is shown in Figure 2.1.

Pantothenate kinase (PanK, CoaA) catalyses the first commited step of this pathway by phosphorylating pantothenic acid (**2.2**) to form 4'-phosphopantothenic acid (**2.3**). The second step involves formation of 4'-phosphopantothenoylcysteine (**2.4**) from **2.3** and L-cysteine. This reaction is catalyzed by phosphopantothenoylcysteine synthetase (PPCS, CoaB). Decarboxylation of **2.4** by the flavoenzyme phosphopantothenoylcysteine decarboxylase (PPCDC, CoaC) forms 4'-phosphopantetheine (**2.4**) as product. Phosphopantetheine

adenylyltransferase (PPAT, CoaD) catalyzes the adenylation of **2.5** to 3'-dephospho-Coenzyme A (**2.6**). The final step is the phosphorylation of the 3'-hydroxyl of the ribose moiety of **2.6** to form CoA (**2.1**) as catalyzed by the enzyme dephospho-Coenzyme A kinase (DPCK, CoaE).[7]

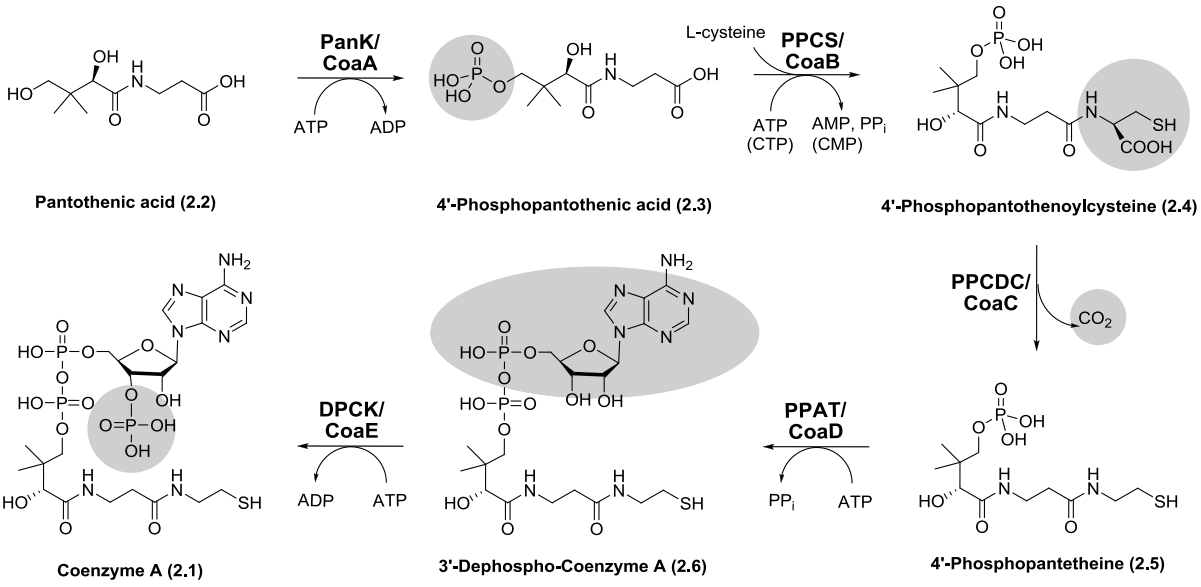


Figure 2.1: The five-step biosynthesis of CoA (**2.1**) from pantothenic acid (**2.2**).

Variations to this five-step pathway exist. For example Bacteria, with the exception of the streptococci and enterococci, do not produce distinct PPCS and PPCDC enzymes. Instead they contain a bifunctional CoaBC which catalyzes both steps.[6] A similar scenario is seen in mammals, although here PPAT and DPCK are combined to form the bifunctional protein CoA syntethase.[8]

2.3 CoA salvage pathway

In Figure 2.2 an alternative method for the biosynthesis of CoA is shown. The three-step CoA salvage pathway bypasses the PPCS and PPCDC enzymes by utilizing pantetheine (**2.7**) as substrate instead of pantothenic acid. The viability of the pathway was recently confirmed when pantethine (the disulfide of pantetheine) was shown to rescue *E. coli ΔcoaBC* knockout mutants, while the addition of pantothenic acid, dephospho-CoA (**2.6**) and CoA (**2.7**) to the growth media failed to do so. This also demonstrated that even though **2.6**

and **2.7** are the penultimate and final products of the pathway respectively, their polarity would appear to prevent them from entering the cells.[9]

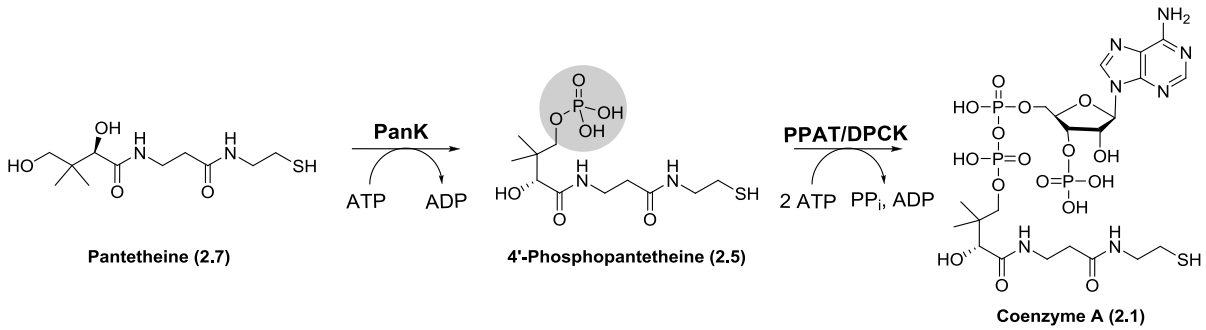


Figure 2.2: CoA salvage pathway converting pantetheine (**2.7**) to CoA (**2.1**).

The CoA salvage pathway is possible due to the substrate promiscuity of PanK (specifically type I and II PanKs as will be discussed later) that allows pantetheine to be accepted as an alternative substrate. Pantetheine (**2.7)** is phosphorylated to 4'-phosphopantetheine (**2.5**), which feeds into the normal CoA biosynthesis pathway. The alternate activity has been characterized for *E. coli* PanK-I and the kinetic parameters are given in Table 2.1 in comparison to pantothenic acid. These values translate into a specificity constant (k_{cat}/K_M) for pantetheine which is only about 6 times slower than that obtained for the natural substrate pantothenic acid.[10,11]

Table 2.1: Kinetic parameters for *E. coli* PanK-I with pantothenic acid and pantetheine.

Kinetic parameters	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}.mM^{-1}$)
Pantothenic acid (**2.2**)	27	0.55	20
Pantetheine (**2.7**)	91	0.32	3.5

2.4 CoA metabolism as drug target

The following three strategies have been identified to target CoA metabolism for drug development:

- Uptake and/or biosynthesis of pantothenic acid, the precursor to CoA
- CoA biosynthesis itself
- The CoA utilization processes

2.4.1 Uptake and/or biosynthesis of pantothenic acid

The first noticeable difference between CoA biosynthesis in pathogens and the human host is that the substrate, pantothenic acid, is biosynthesized *de novo* by the bacteria. However, metabolic labeling experiments in *E. coli*[12] and rat hearts[13,14] have shown that the supply of pantothenic acid does not control the rate of CoA biosynthesis, but rather the utilization thereof. Wild type *E. coli* produces 15 times more pantothenic acid than is required to maintain the intercellular CoA levels and the excess is excreted from the cells.[12,15] A controlled study showed that this overproduction and excretion of pantothenic acid by the intestinal flora of ruminants is sufficient to sustain the animals without further supplementation.[16] Furthermore, most organisms, regardless of their ability to synthesize pantothenic acid *de novo*, have various forms of transport systems that actively mediates the uptake of pantothenate. Of these the *E. coli* pantothenate permease (PanF) is the best characterized. It consists of a unidirectional Na^+-based symporter which is highly specific for pantothenate (K_t = 0.4 µM) and has a maximum velocity of 1.6 pmol/min per 10^8 cells.[17-20] Pantetheine, the substrate of the CoA salvage pathway, is also transported into the cells although no pantetheine-specific transporter has been identified.[6] Other transport systems include the H^+-coupled product of the *FEN2* gene in *Saccharomyces cerevisiae*, the "new permeability pathways (NPP)" in *Plasmodium falciparum*-infected erythrocytes[5] and Na^+-dependent multivitamin transporters in higher organisms.[6] Taking into account the relative abundance of pantothenic acid along with the excess in which it is produced and its facile transport, strategies that target the biosynthesis of this metabolite directly is unlikely to be successful in organisms such as *E. coli* and *Plasmodium falciparum*.

In contrast pantothenic acid biosynthesis is essential for the growth in *M. tuberculosis*[21] and the pathogen displays pantothenate mediated virulence.[22] In vaccine studies *ΔpanCD* knockout mutants were found to be auxotrophic for pantothenate with full virulence restored when the *panCD* wild type genes were reintegrated into the mutants.[22] It was also observed that the *ΔpanCD* mutant bacteria can persist for over eight months in the severely compromised immune deficient (SCID) mouse model.[22] Since no transport systems have been identified in *M. tuberculosis* to date, the pathogen may possess an alternative method of obtaining pantothenic acid; albeit at levels barely sufficient for persistence.

2.4.2 CoA biosynthesis

Two of the CoA biosynthetic enzymes are regulated by feedback inhibition – PanK and PPAT. The regulation of PanK will be discussed in detail later. *E. coli* PPAT is allosterically regulated by feedback inhibition of CoA and to a lesser extent by acetyl-CoA.[5,8] However, based on the high K_M-value for phosphopantetheine and the mediocre K_i-value for CoA, this regulation is only physiologically relevant when the levels of CoA are high and phosphopantetheine low.[6] In this study we focused on PanK as a drug target.

2.4.3 CoA utilization processes

CoA functions as the central acyl group carrier in central metabolism and constitutes the source of the 4'-phosphopantetheine moiety of the acyl carrier proteins (ACPs).[23] ACPs are central to fatty acid biosynthesis, an essential process in bacteria.[24] The 4'-phospho-pantetheine moiety is transferred from CoA by phosphopantetheinyl transferase (PPTase) enzymes to apo-ACP to convert it to its holo- (active) form. Thus modifications to the CoA molecule itself or decreases in the available CoA levels would affect the activation of the ACPs and subsequently affect all the ACP-dependent processes.

Other enzymes dependent on CoA for the acyl moiety include HMG-CoA reductase, 3-Hydroxyacyl-CoA dehydrogenase, 2-Enoyl-CoA reductase, Acyl-CoA oxidase, Acyl-CoA dehydrogenases, Benzoyl-CoA reductase, Stearoyl-CoA desaturase, 4-Chlorobenzoyl-CoA dehalogenase, Enoyl-CoA hydratase, 3-Hydroxybutyryl-CoA epimerase and Methylmalonyl-CoA mutase.[6]

2.5 Introduction to pantothenate kinases (PanK)

PanK catalyses the phosphorylation of pantothenic acid (**2.2**) to 4'-phosphopantothenic acid (**2.3**) as shown in Figure 2.3.

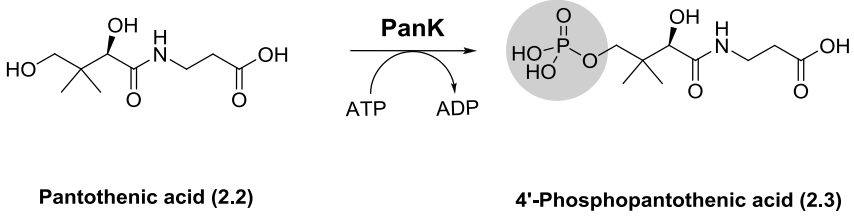


Figure 2.3: Pantothenate kinase (PanK) reaction: Phosphorylation of pantothenic acid (**2.2**) to 4'-phospho-pantothenic acid (**2.3**)

PanK is the best studied enzyme of the CoA biosynthetic pathway. The first prokaryotic PanK enzyme was produced and purified from *E. coli* as early as 1994.[25] In 1999, when the first eukaryotic example was purified from *Aspergillus nidulans,*[26] it was clear that the sequence of this enzyme had little similarity to the bacterial enzyme and that their primary structure and regulatory properties differed. This led to the classification of subsequently identified PanKs as either prokaryotic or eukaryotic based on origin and similarity. However, the phylogenetic split was shown not to be absolute when the enzyme from the Gram-positive bacteria *Staphylococcus aureus* was found to be more closely related to the eukaryotic than the prokaryotic enzyme.[27]

The existence of a third type of PanK was first proposed based on genome-wide analyses which revealed that bacteria, such as *Helicobacter pylori* and *P. aeruginosa*, possesses genes encoding homologs of the last four CoA biosynthetic enzymes, but had no PanK encoding genes.[28,29] This was true both when the prokaryotic or eukaryotic PanKs were used as search terms. A patent subsequently reported the discovery of a gene in the Gram-positive bacterium *Bacillus subtilis* which rescued a temperature-sensitive *E. coli coaA* mutant. This gene was distinct from the predicted PanK-encoding *coaA* gene, and was subsequently named *coaX.*[30] Furthermore, *B. subtilis coaA* deletion mutants remained viable, whereas double mutants in which both *coaA* and *coaX* were disrupted, failed to grow. In conclusion this strongly suggested that the *coaX* gene product has PanK activity. Homology searches of *coaX* indicated that most of the bacteria lacking PanKs indeed contain this homolog. First examples of this third type of PanKs were cloned, purified and characterized from *H. pylori* and *B. subtilis* in 2005.[31]

Today, PanKs are split into three distinct types based on sequence homology, enzyme structure, kinetic parameters and feedback inhibition. For ease of distinction they are labeled as type I (PanK-I), type II (PanK-II) and type III (PanK-III).[6] Moreover, type II PanKs – mainly eukaryotic – frequently occurs as different isoforms in the same organism. The term "PanK isoforms" implies that the same protein is expressed from different initiating exons (for example human PanK1α and PanK1β)[20] or that the same enzyme is expressed in different tissues or is found in different cellular locations (for example human PanK2 and PanK3 respectively are restricted to the mitochondria and the cytosol).[32] To distinguish PanK types and isoforms, the former use Roman numbers and the latter Latin numbers.

2.6 Distribution of the PanK types

All three known PanK types are found in bacteria, whereas eukaryotes only produce type II PanKs. The PanK-IIs identified and characterized from eukaryotic sources include fungi (*Aspergillus nidulans*), plants (*Arabidopsis thaliana* and spinach), insects (*Drosophila melanogaster*) and mammals (human, mouse and rat).[6] At the start of this study, no PanKs had been isolated or otherwise characterized from the archaea.

The distribution of the PanK types in the ≈ 280 genome sequenced bacterial species is shown in in Figure 2.3. It reveals that ≈ 80.5% species contain only a single PanK type, whereas ≈7.5% harbors more than one type. A further ≈ 12% of species lack a recognizable PanK, though they are predicted to possess PanK activity.[29] The considerable number of the latter bacterial species would appear to imply a greater variability in the sequences of the three established PanK types, or include the existence of a fourth PanK type.

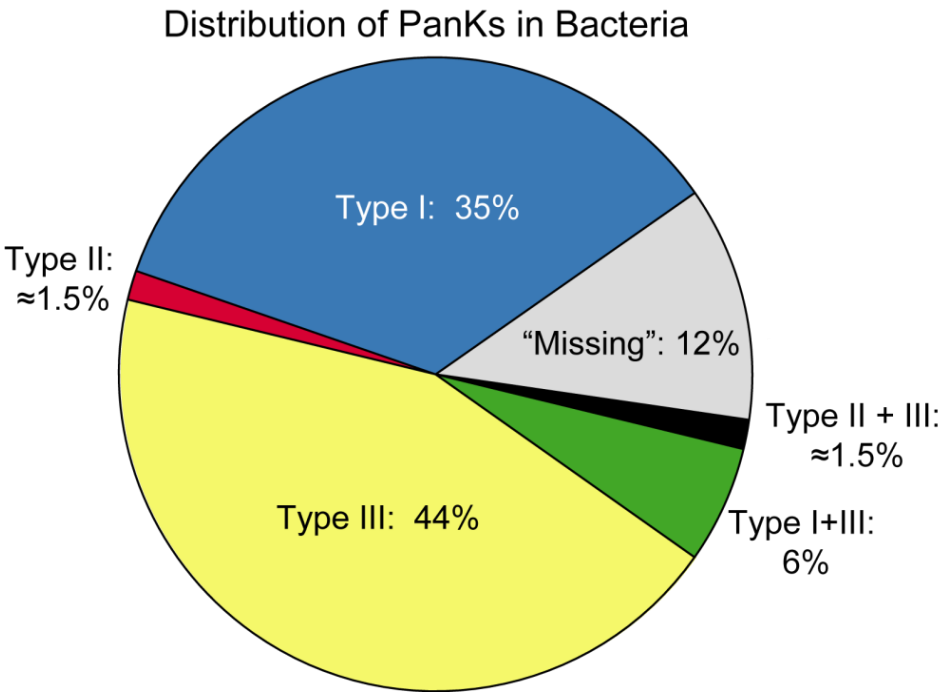


Figure 2.3: The distribution of the PanK in the ≈ 280 Bacterial species with sequenced genomes.

To survey the phylogenetic distribution of the PanK types in bacteria, the SEED genomic integration platform, with data from of than 400 complete or nearly complete sequenced bacterial genomes, was used.[33,34] Table 2.2 contains a summary of the results of such a survey conducted in all 13 major bacterial groups.

Table 2.2: Phylogenetic distribution of the PanK types in 13 major bacterial groups.

Bacterial Groups	**PanK type present**				
	Only Type I	Only Type II	Only Type III	Types I + III	Types II + III
Actinobacteria	√		√	√	
Aquificae			√		
Bacteroidetes/Chlorobi			√		
Chlamydiae					
Chloroflexi	√		√		
Cyanobacteria			√		
Deinococcus/Thermus			√		
Firmicutes	√	√	√	√	√
Fusobacteria			√		
Planctomycetes			√		
Proteobacteria					
α	√		√		
β			√		
δ/ε			√		
ϒ	√		√		
Unclassified			√		
Spirochaetes			√		
Thermotogae			√		

The results show that type III PanKs are found in the majority of the bacterial groups, and represent the only identified PanK in eight of the 13 groups. Type I PanKs are found in five bacterial groups and type II PanKs in only one bacterial group. To date, no PanK candidates have been identified in the Chlamydiae. Certain species of the Actinobacteria and Firmicutes also harbors another type along with the type III PanK. These species include the *Mycobacterium* spp. and *Streptomyces* spp. (Actinobacteria) which encodes both type I and III PanKs. Most of the *Bacilli* (Firmicutes), such as *B. subtilis,*[31] have combinations of type I and III PanKs, except *B. anthracis*,[35] *B. cereus* and the *Oceanobacillus* spp which have type II and III PanKs.

2.7 Comparison of PanK types

In the following sections the PanK types are reviewed and compared to reflect current data. To provide a brief overview of the major similarities and differences between the types their main characteristics, as described by their primary structure sequences, tertiary structures,

cofactor requirements, kinetic parameters, regulation and ability to utilize the alternative substrates *N*-substituted pantothenamides, are summarized in Table 2.3. Data obtained for the enzymes from *E. coli*[15] (type I), *P. aeruginosa*[36] (type III) and *A nidulans*[26] (the prototypical type II PanK) are used as representative examples of the PanK types. The atypical type II PanK from *S. aureus*[27] is also included to highlight the differences.

Table 2.3: General comparison between the three known PanK types

Attribute	PanK-I	PanK-II		PanK-III
Representative organism:	*E. coli*	*A. nidulans*	*S. aureus*	*P. aeruginosa*
Gene sequence:	*coaA*	*coaA*		*coaX*
Cofactor requirements:	Mg^{2+}	Mg^{2+}		Mg^{2+}, K^{+}, NH_4^{+}
Feedback inhibitor:	CoA	Acetyl-CoA	None	None
Pantothenamide analogs:	Substrates	Substrates		No effect
Structural fold:	P-loop kinase	ASKHA superfamily		ASKHA superfamily

2.7.1 Gene sequence and expression

Bacterial type I and II PanKs are both expressed by *coaA* gene. The sequence homology between the genes of the types is however low. Type III PanK is expressed by the *coaX* gene which bears no sequence homology to *coaA*.[31]

The first *coaA* gene was identified from *S. typhimurium,*[37] but most of what is known about *coaA* is based on the studies of the *E. coli* gene.[25] One of the ways in which CoA production is regulated in *E. coli* is by the gene expression of *coaA*.[8] The *coaA* promoter region has poor homology in comparison to consensus *E. coli* promoter sequences and at 0.565 the optimal codon usage frequency is low.[25] This translates into a low abundance of PanK-I relative to that of other *E. coli* proteins. However, this regulatory effect is secondary to the feedback inhibition of PanK by CoA. Overproduction of PanK-I in *E. coli* (containing multiple copies of the *coaA*-gene) leads to a 76-fold increase in specific activity of the kinase, but only increases the steady-state concentration of CoA ≈ 2.7-fold.[25]

Translation of *E. coli coaA* produces two isoforms of 36.4 kDa and 35.4 kDa respectively. The only known difference between these isoforms is that the smaller protein lacks the first eight *N*-terminal amino acids.[25] The reason and the significance thereof remain unknown.[7]

Eukaryotic PanK-II enzymes are encoded by *PANK*-genes. Some organisms have more than one *PANK*-gene encoding different PanK-II isoforms that exhibit tissue specific expression levels. In some cases these isoforms can be functionally distinguished as demonstrated by the human genetic disorder known as pantothenate kinase-associated neurodegeneration (PKAN) which has been linked to mutations in *PANK2.*[38-40]

Of the four known human isoforms, PanK1 is mostly expressed in the liver and kidneys and to a lesser extent in the heart and muscles. PanK2 expression is limited to the mitochondria of most tissue, PanK3 to the liver and PanK4 to the heart and skeletal muscles.[6] In some cases a single gene also gives rise to two isoforms being expressed, as in the case of *PANK1* in mice and humans. In the latter case different initiating exons lead to two distinct catalytically active isoforms – PanK1α and PanK1β.[20]

The *coaX* homolog from *B. pertussis* was the first to be studied, albeit as an accessory protein in pertussis toxin production via interaction with the two component transcriptional regulator BvgAS. This protein was therefore named Bvg accessory protein (Baf).[41,42] Baf does not have significant sequence homology to any of the known bacterial transcriptional regulators but has 28% identity and 49% similarity to PanK-III of *B. subtilis.*[43] Most *coaX* homologs are still annotated as Bvg accessory factors or as putative transcriptional regulators.

2.7.2 Gene essentiality

Since CoA is an essential cofactor in all organisms which must be produced *de novo* from pantothenic acid, a logical conclusion is that some of the genes encoding the biosynthetic enzymes should be essential (or conditionally essential).[6] In respect to the PanK-I encoding gene *coaA,* this has been shown to be the case in *E. coli, M. tuberculosis*[44] *and H. influenzae.*[45] These genes are listed in the database of essential genes (DEG; http://tubic.tju.edu.cn/deg/) which currently holds gene essentiality data for ten bacterial genomes and *S. cerevisiae*.[6]

The PanK-III encoding *coaX* genes of *B. anthracis*[46] and *B. pertussis*[41] are also labeled as essential. The apparent essentiality of the *M. tuberculosis coaA* and *B. anthracis coaX* genes are surprising since the genomes of both organisms encode two PanKs of different types. The essentiality data would thus only be valid if the other type present in these organisms

were not to function as a pantothenate kinase or if the functional pantothenate kinase encoded by *coaX* is only expressed under certain conditions, although this would be highly unexpected.

2.7.3 Structure classification and active site geometry

In general kinase enzymes are classified into families and fold groups based on structure, sequence and functional specificities. This classification system consists of 25 families of homologous proteins of which 22 falls into one of the 10 defined fold groups.[47,48] Type I PanKs belong to the Rossman fold group and form part of the P-loop kinase family. Other kinases in this family are the nucleotide and nucleoside kinases. Type II and III PanKs by contrast both belong to the ribonuclease H-like kinase group and are part of the acetate and sugar kinase/heat-shock protein 70/actin (ASKHA) superfamily.[31]

E. coli PanK-I binds pantothenic acid at the end of a large surface groove that stretches from the nucleotide binding domain on the one side and continues across the surface of the enzyme. ATP is bound in a groove formed by residues of the P-loop and connecting strands.[49] *M. tuberculosis* PanK-I is highly similar to *E. coli* PanK-I.[50] A few amino acid differences in the ATP binding domain allow the *M. tuberculosis* enzyme to accept both ATP and GTP as substrates as opposed to the *E. coli* enzyme that can only utilize ATP.[51]

Although the type II and III PanKs have the same conserved folds and key catalytic residues, there are significant differences in their ATP and pantothenate binding sites. Type II PanKs bind pantothenate in an open pocket, while type III PanKs have a fully enclosed binding pocket. On the other hand, ATP is tightly bound by PanK-II in a cavity that displays classical P-loop architecture combined with very specific interactions to the adenine moiety.[36] Analysis of *T. maritima* PanK-III bound to ATP indicates a low binding affinity for ATP. The nucleotide is bound in a solvent exposed cleft between the enzyme domains with few specific interactions to both the adenine and ribose moieties. This "loose" binding correlates with the high K_M-values for ATP of PanK-III enzymes.[52]

2.7.4 Available crystal structures

Several crystal structures of each PanK type are listed in the RCSB protein database (PDB: www.pdb.org). These include crystal structures for the PanK-I enzymes from *E. coli* (PDB: 1SQ5, 1ESN, 1ESM)[49,53] and *M. tuberculosis* (22 available structure).[50,54,55] For the

type II PanKs crystal structures have been obtained for human PanK1α (PDB: 2I7N)[32], human PanK3 (PDB: 2I7P)[32] and *S. aureus* PanK-II (PDB: 2EWS).[36] The PanK-IIIs with solved crystal structures include *T. maritima* PanK (PDB: 2GTD, 3BEX, 3BF1 and 3BF3),[52,56] *P. aeruginosa* PanK (PDB: 2F9T and 2F9W)[36] and *B. anthracis* PanK (PDB: 2H3G).[35]

2.7.5 Cofactor requirements

In common with most kinases, type I and II PanKs require Mg^{2+} for activation. The activity of the first isolated PanK-IIIs from *B. subtilis* and *H. pylori* were tested in buffer containing both $MgCl_2$ and KCl.[31] *P. aeruginosa* PanK-III was only slightly active when tested under these same conditions. Further studies established that PanK-IIIs require a monovalent cation, i.e. K^+, NH_4^+ or Rb^+, for activity and that *P. aeruginosa* PanK-III prefers NH_4^+. Although the activation by NH_4^+ has no effect on the enzyme's activity towards pantothenic acid, it affects both the K_M and the k_{cat} for ATP with an overall four- to five-fold increase in the specificity constant for this substrate.[36]

2.7.6 Reaction mechanism

a) Chemical mechanism

All PanKs are homodimers with each monomer containing a single nucleotide binding site. The pantothenate binding site of PanK-I is highly flexible such that the binding of each ligand causes a distinct conformational change in the protein.[49] PanK-I follows an ordered sequential mechanism with ATP binding occurring first.[7] ATP binding is highly cooperative and a Hill coefficient of 1.46 was determined for the *E. coli* enzyme. Pantothenic acid binding, by contrast, is not cooperative.[15]

The reaction mechanism for *A. nidulans* PanK-II was determined as an ordered bi-bi system.[26] An ordered mechanism was also observed with human PanK3 with ATP binding occurring first.[57] In contrast *S. aureus* PanK-II has two solvent-exposed openings to the active site and it is likely that ATP enters from one and pantothenic acid from the other direction. This would result in nonsequential binding.[36]

Little is known about the mechanism of the PanK-III enzymes except that sequential binding is expected with pantothenate binding before ATP. [40,51,54]

b) Kinetic mechanism

E. coli PanK-I has been proposed to follow a concerted mechanism with a dissociative transition state, despite strong evidence for an associative mechanism.[36,49] Crystal structures of PanK-ADP-pantothenic acid and PanK-AMP-PNP (AMP-PNP is a non-hydrolysable ATP analog) complexes show that there are many positively charged residues (Lys101 and Arg243) as well as Mg^{+2} present in the active site. These residues and ions support a kinetic mechanism with an associative transition state as the positive charges offset the multiple negative charges that develop during the reaction. In addition Asp127 interacts with the 1'-hydroxyl-group of pantothenic acid to increase its nucleophilicity which promotes an S_N2-like attack on the γ-phosphate of ATP.[49] Another prediction method used to determine the mechanism type is to measure the distance between the entering atom and the phosphorus-group undergoing substitution before the reaction begins. For distances below 4.9 Å the mechanism is likely to be associative while distances below 4.9 Å indicate a dissociative mechanism. These distances between the *γ*-phosphate in an AMP-PNP complex and the 4'-hydroxyl oxygen of pantothenate was determined to be 4.5 Å in *M. tuberculosis* PanK-I and 4.1 Å in the *E. coli* PanK-I.[55] These data thus also support an associative mechanism, although this conclusion would require further validation.

The PanK-III phosphorylation reaction is predicted to proceed via a dissociative mechanism with an active site Asp residue acting as catalytic base.[36,52,56] This hypothesis is based on the observation that in a PanK-AMP-PNP complex the metaphosphate leaving group is stabilized by electrostatic interactions between the γ-phosphate of ATP and the K^+/NH_4^+ and Mg^{2+} cations. These interactions for the phosphoryl transfer part are perceived to be of higher importance In the *P. aeruginosa* PanK-III active site than substrate activation for nucleophilic S_N2 attack and hence a dissociative mechanism was proposed.[49]

No information was found on the kinetic mechanism of the PanK-IIs.

2.7.7 Kinetic parameters

Broadly the largest difference in kinetic parameters between PanK types involves the K_M-values for ATP. For PanK-III enzymes it is in the mM range, usually 2-10 mM, with the lowest reported K_M-value being 510 µM for *B. anthracis* PanK-III and the highest 9.59 mM

for the *H. pylori* enzyme.[31,35,36,52,56] Type I and II PanKs typically have K_M-values for ATP of 100 – 200 µM, with the exception of *S. aureus* PanK-II with has a value of 34 µM.

Table 2.4 summarizes the kinetic parameter for representative examples of the PanK types, including data from the enzymes from *E. coli*[15] (type I), *A nidulans*[26] (the prototypical type II PanK), *S. aureus*[27] (the atypical type II PanK) and *P. aeruginosa*[36] (type III). The K_M-values for pantothenate is below 100 µM for all the types and the turnover number or k_{cat} is much smaller for type I PanKs than for other types.

Table 2.4: Comparison of the kinetic parameters from selective organisms for each PanK type.

Attribute	PanK-I	PanK-II		PanK-III
Representative organism:	*E. coli*	*A. nidulans*	*S. aureus*	*P. aeruginosa*
k_{cat}	0.30 ± 0.13 s^{-1}	1.95 s^{-1}	1.65 ± 0.09 s^{-1}	16 s^{-1}
K_M (Pantothenate)	36 ± 4 µM	60 µM	23 µM	20 µM
K_M (ATP)	136 ± 15 µM	145 µM	34 µM	3.18 mM

2.7.8 Regulation mechanism

E. coli PanK-I is inhibited both *in vivo* and *in vitro* by CoA and its thioesters.[15,58] Various kinetic and site-directed mutagenesis studies have shown that the feedback inhibition by CoA is competitive with respect to ATP (CoA binds in the ATP binding site) and uncompetitive towards pantothenic acid. This is seen by the 24-fold increase in the apparent K_M (K_M^{app}) of ATP in the presence of as little as 100 µM CoA.[45] Superimposition of the crystal structures of PanK-I in complex with either CoA or AMP-PNP shows that CoA and ATP bind in different orientations in the active site and that the adenine moieties are bound by different amino acid residues. The one similarity is that both use Lys101 to neutralize the charge on their respective phosphoryl groups (pyrophosphate moiety of CoA and β- and γ-phosphates of AMP-PNP). The competition for the same binding site by the respective phophoryl groups of the different molecules explains the competitive inhibition. The more potent inhibition exhibited by CoA relative to CoA thioesters is caused by a hydrophobic pocket of aromatic amino acids that binds the thiol group of CoA. The size of the pocket disfavors the bulkier thioester groups leading to poor binding of acetyl-CoA and hence to weaker feedback inhibition.[53] This explains why the inhibition by acetyl-CoA is only about 20% of that of the nonesterfied CoA.[45]

Mammalian PanK-IIs are regulated by CoA and its thioesters and plant PanK-IIs by malonyl-CoA. In general, acetyl-CoA is a more potent inhibitor of the eukaryotic enzymes[40] and CoA inhibition in mammals is noncompetitive towards pantothenic acid.[15] *A. nidulans* PanK-II is selectively and potently inhibited by acetyl-CoA and the inhibition is competitive with respect to ATP.[26] The stronger inhibition by acetyl-CoA is attributed to hydrogen bond formation between the thioester carbonyl oxygen and the main chain amide of Val. This bond stabilization is not possible with the shorter pantetheine chain of CoA. The acetyl-CoA binding pocket is also partially exposed to solvent which provides space for the binding of molecules with longer acyl chains.[32]

By contrast, *S. aureus* PanK-II is refractory to feedback inhibition by CoA and its thioesters.[59] The structural basis for this lack of feedback inhibition was identified by comparing the *S. aureus* enzyme to the human PanK3 structure and is attributed to mutations of two residues in the putative acetyl-CoA binding pocket. An Ala to Tyr mutation appears to sterically prevent the binding of an allosteric inhibitor, while exchange of Trp to Arg disrupts the hydrophobic thiol (CoA) or the acetyl group (thioester) binding pocket.[32]

A possible explanation for this lack of feedback inhibition is that *S. aureus* lacks glutathione which other bacteria use to maintain redox balance for subsequent protection from oxidative stress. Instead this pathogen relies on a CoA / CoA disulfide reductase system and the lack of feedback inhibition allows for the accumulation of millimolar levels of CoA for this purpose.

PanK-III is not feedback inhibited by CoA or acetyl-CoA.[31] The pantothenate binding pocket is enclosed on both ends which prevents the enzyme from accepting and binding the pantetheine moiety of CoA.[36,52]

2.7.9 Alternative substrates

a) *N*-substituted pantothenamides

The substrate specificity of *E. coli* PanK-I and *S. aureus* PanK-II has been well studied due to their use in biocatalysis.[60] Both enzymes are further known for their substrate promiscuity.[61,62] *E. coli* PanK-I accepts *N*-substituted pantothenamides as substrates with significant differences in its catalytic efficiency of natural substrate compared to the

analogs.[6] The *S. aureus* enzyme has low k_{cat} values for *N*-substituted pantothenamides and low K_M-values. The resulting specificity constants are only slightly lower than that for pantothenic acid. Additionally, *S. aureus* PanK-II phosphorylates a wider range of pantothenate analogs, such as the pantothenate thioesters, which *E. coli* PanK-I does not accept as substrates.[63]

N-substituted pantothenamides are not alternative substrates of type III enzymes.[31] The enclosed pantothenate binding pocket prevents both feedback inhibition and binding of the extended hydrophobic alkyl chains of the pantothenamide substrate analogs.[36,52] The narrow specificity of this enzyme has been highlighted by the fact that pantethine (the disulfide of pantetheine and substrate of the CoA salvage pathway) rescues *E. coli ΔcoaBC* knockout mutants, but not the same *P. aeruginosa* mutants.[9] *Treponema pallidum* PanK-III is similarly unable to phosphorylate pantetheine.[6]

b) Phosphoryl donors

Phosphoryl donors other than ATP are of potential interest due to the high ATP K_M-values for type III enzymes. As shown in Table 2.4, both type I and II PanKs have low K_M-values for ATP, clearly establishing this nucleotide as the main co-substrate. Some promiscuity has been noted for *M. tuberculosis* PanK-I. Its catalytic efficiency using either ATP or GTP is similar to that of *E. coli* using ATP, while GTP is clearly disfavored by *E. coli* PanK-I.[51] *A. nidulans* PanK-II prefers ATP, with GTP resulting in a drop of 30% in catalysis, whereas CTP and TTP reduce catalytic efficiency even further.[26]

This scenario is a little more complicated for the PanK-IIIs. The enzymes from *B. anthracis* and *P. aeruginosa* utilize a wide range of NTPs, some resulting in greater catalytic efficiency than ATP.[36,64] *H. pylori* utilizes CTP and GTP, but less efficiently than ATP and *B. subtilis* cannot utilize CTP, GTP or UTP.[31] The *H. pylori* and *B. subtilis* enzymes are unable to use PEP, acetyl- and carbamoyl-phosphate, phosphoserine and phosphothreonine as phosphoryl donors,[31] whereas *P. aeruginosa* PanK-III cannot utilize PEP, acetylphosphate, pyrophoshates, tripolyphosphates and polyphosphates (not defined).[36]

2.8 Drug development strategies utilizing PanKs

PanK is believed to catalyze the rate limiting step in CoA biosynthesis in most organisms, although pathway modeling studies still need to confirm this. *Plasmodium falciparum* constitutes an exception as 4'-phosphopantothenic acid is known to accumulate in its cells.[5] PanKs may be useful for drug development programs either by exploiting PanK as a gate-keeper to CoA biosynthesis and inhibiting down-stream enzymes and applications, or by targeting the enzyme itself.

2.8.1 PanK as gatekeeper

The promiscuity of type I and II PanKs may potentially be exploited to phosphorylate small molecule analogs of pantothenic acid that inhibit the down-stream enzymes in CoA biosynthesis or be converted to CoA antimetabolites.[61,65] *N*-pentylpantothenamide is an example of such an analog. It inhibits *E. coli* cell growth with a MIC of ≈ 2 µM. Initially the inhibition was ascribed to inhibition of PanK, but it was later shown to be due to the biosynthetic conversion of *N*-pentylpantothenamide to the corresponding CoA analog, ethyldethia-CoA, which acts as an antimetabolite of CoA in normal cell metabolism.[60,61]

More recent examples are the Michael acceptor-containing CoA analogs synthesized by van der Westhuyzen *et al.*[66] shown in Figure 2.4. The CoA antimetabolites, obtained by the conversion of the corresponding pantothenamides by the CoA biosynthetic enzymes, inhibit the CoA disulfide reductase enzyme of *S. aureus* requires to maintain the intracellular redox balance in this organism by converting CoA disulfide to CoA. The best K_i-value measured in this study was for the phenyl sulfone analog (R = SO_2Ph) which has a K_i ≈ 40 nM. This is 50-fold smaller than the K_M-value of the substrate, CoA disulfide, of ≈ 2.0 µM.

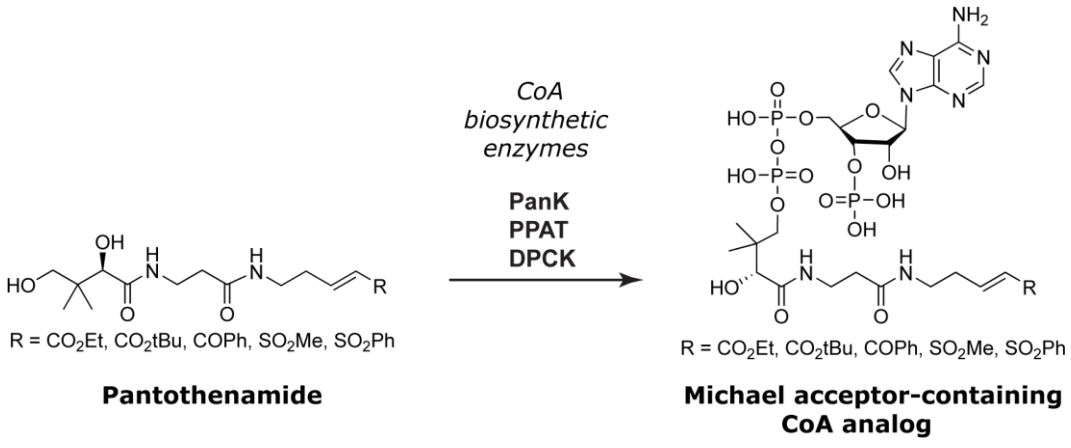


Figure 2.4: Michael acceptor pantothenamides transformed by the CoA biosynthetic enzymes to CoA analogs.

2.8.2 PanK as target

Although some PanK specific inhibitors have been identified, many molecules designed as inhibitors for the PanKs prove to be substrates that inhibit down-stream enzymes (for example the *N*-substituted pantothenamides and pantothenol).[67] An example of type specific inhibition is PanK-I from *M. tuberculosis*. This enzyme is essential for growth of the pathogen *in vitro,*[6] and has been targeted in extensive inhibitor discovery programs by both AstraZeneca and Novartis. Though several inhibitors of PanK-I were identified, none inhibited cell growth.[69] *M. tuberculosis* harbors two PanKs. The finding from the inhibition studies may suggest that the putative PanK-III (CoaX) functionally replaces PanK-I despite PanK-III being reported as inactive.[44] Were this true, the success of any drug discovery program would depend on the parallel inhibition of PanK-III. There is no record of substrate promiscuity for PanK-IIIs and no pantothenamides function as substrates or inhibitors thereof. The challenge thus remains to identify molecules that would inhibit PanK-III activity.

2.9 Known PanK-III inhibitors

To date the only known inhibitors for PanK-IIIs are nucleoside triphoshate mimetics of ATP.[64] In a recent study a library of compounds replacing the triphosphate sidechain of ATP by uncharged, methylene-triazole linked monosaccharide groups was created and screened using the PanK-III from *B. anthracis*. The removal of the phosphates disrupts the Mg^{2+} chelating ability of the nucleoside which in turn prevents binding to the enzyme. *B. anthracis,* the causative agent of anthrax, encodes both type II and III enzymes. PanK-III is the essential and active pantothenate kinase[35,46] while BA2901 (the putative PanK-II) was shown to be inactive in all *in vitro* studies using pantothenic acid as substrate. BA2901 also failed to rescue *E. coli coaA*(Ts) strain DV70 (transcription of the *coaA* gene in this strain is deactivated at 42°C) under conditions where an *S. aureus* construct, containing a functional PanK-II, restored growth.[35] Since the PanK-III seems to be the only functional pantothenate kinase, it is an essential target in this category A biodefense organism.

Most compounds in the library screen discussed above had very little effect on the enzyme, an exception being the mimetic shown in Figure 2.5. This compound competes with ATP binding *in vitro* with a K_i = 164 µM. This represents a three-fold decrease of the *B. anthracis* PanK-III K_M-value for ATP of 510 µM.

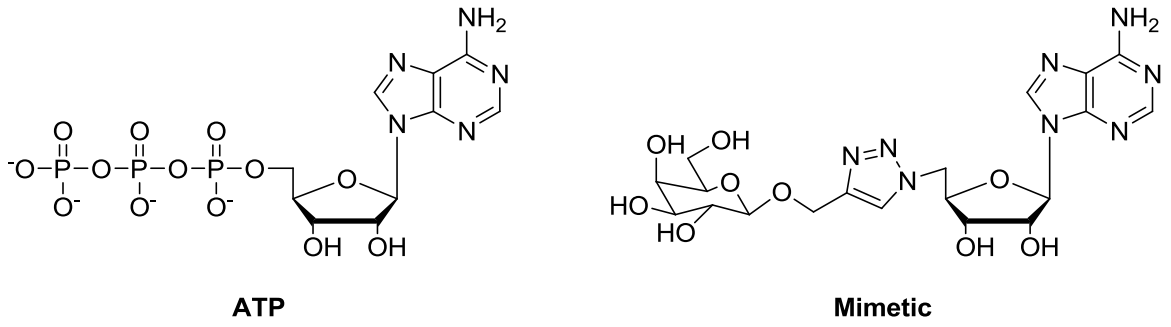


Figure 2.5: ATP and the nucleoside mimetic which gave the best inhibition in a screen for competitive inhibitors of *B. anthracis* PanK-III.

Although this study yielded good results, the reported K_i-value is still too high to be of pharmaceutical interest. Also no *in vivo* work was reported to demonstrate any inhibition of cell growth.

Another problem is that PanK-IIIs bind ATP very weakly in an open cavity with no interactions between the enzyme and the adenosine moiety. Since the question has been raised as to whether ATP is really the co-substrate of these enzymes[31,36] an ATP mimetic may not be the best lead to develop further.

In this study pantothenic acid analogs are evaluated and tested as a starting point in the development of potential competitive inhibitors for the PanK-IIIs.

2.10 Survey of pantothenic acid analogs

Pantothenic acid (D-3-(2,4-dihydroxy-3,3-dimethylbutyramido)propionic acid) (**1.2**), the natural substrate of PanK, can be divided into 2 moieties based on the biosynthesis thereof in bacteria (shown in Figure 2.6). It is the condensation product of pantoic acid (pantoyl moiety) and β-alanine (β-alanine moiety). Synthetically it is created in much the same way with a melting reaction of β-alanine with D-(-)-pantolactone (α-hydroxy-β,β-dimethyl-γ-butyrolactone) instead of pantoic acid.[70]

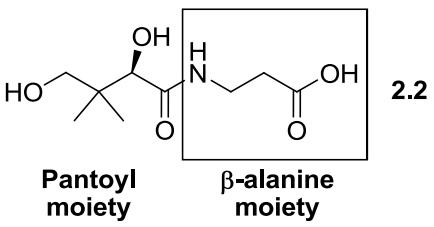


Figure 2.6: The moieties of pantothenic acid (**2.2**)

The structure determination of pantothenic acid in the 1940[71] was followed by a surge of activity to synthesize pantothenic acid analogs in an effort to produce molecules with similar biological activity as the original vitamin.[72] These compounds were mainly tested in growth assays on yeast and the *Lactobacillus* spp., although their effects on *Streptococcus haemolyticus* and strains of *Corynebacterium diptheriaeare* were also investigated.[5] Some of the analogs, such as hydroxypantothenic acid and pantothenol, were reported to promote growth. Many lacked activity but there were a few, like pantoyltaurine[5,73] that antagonize the growth-promoting activity of pantothenic acid. In general the most successful inhibitors were those in which the pantoyl moiety has remained unmodified. Examples of these compounds include pantoyltaurine, pantoyltauramide[74,75] and α-methyl pantothenic acid.[76]

Recent studies indicate that pantothenol inhibit growth in *P. falciparum* suggesting competitive inhibition of PanK as the reason.[77,78] This theory was disproved when both *M. tuberculosis* and *E. coli* PanK-Is were shown to accept this compound as substrate and phosphorylated it to 4'-phosphopantothenol. The phosphorylated product however, competitively inhibits the utilization of 4'-phosphopantothenate by CoaBC effectively blocking further CoA biosynthesis.[67]

Most molecules obtained by modifying the pantoyl moiety in earlier studies were either inactive or slightly stimulatory when tested on organisms requiring vitamin B_5 for growth.[79] These compounds include, but are not limited to, hydroxyl pantothenic acid,[80] 2'-aminopantothenic acid,[81,82] 4'-deoxypantothenic acid and 4'-deoxy-pantoyltaurine.[79] Slight inhibitory activity was observed for 2'-deoxypantothenic acid,[79] but this was reversed by the addition of pantothenic acid.[5,72,74,75] The only pantoyl-modified analog that was an effective antagonist of pantothenic acid was ω-methyl-pantothenic acid.[5,83] This compound was able to inhibit the growth of the lactic acid bacteria and to protect mice from *Streptococcus* infections when administered orally.[84] Other ω-methylpantothenic acid analogs were synthesized following the success of the original compound and although they all inhibit lactic acid bacteria, none were consistently more active than the parent compound.[85] We are not aware of any studies in which any of these compounds was tested on the pathogenic bacteria relevant to our study, i.e. *B. anthracis, H. pylori*, *M. tuberculosis and P. aeruginosa.*

To determine which of these compounds could be the most suitable for our analysis, we first studied the active site geometry of the *P. aeruginosa* PanK-III enzyme.

2.11 PanK-III active site

The crystal structure for *P. aeruginosa* PanK-III (*Pa* PanK-III) was solved in 2006 and is shown in Figure 2.7.[36] Pantothenic acid binds to a fully enclosed binding pocket with numerous amino acid residues participating in binding and stabilizing the vitamin.[40,54]

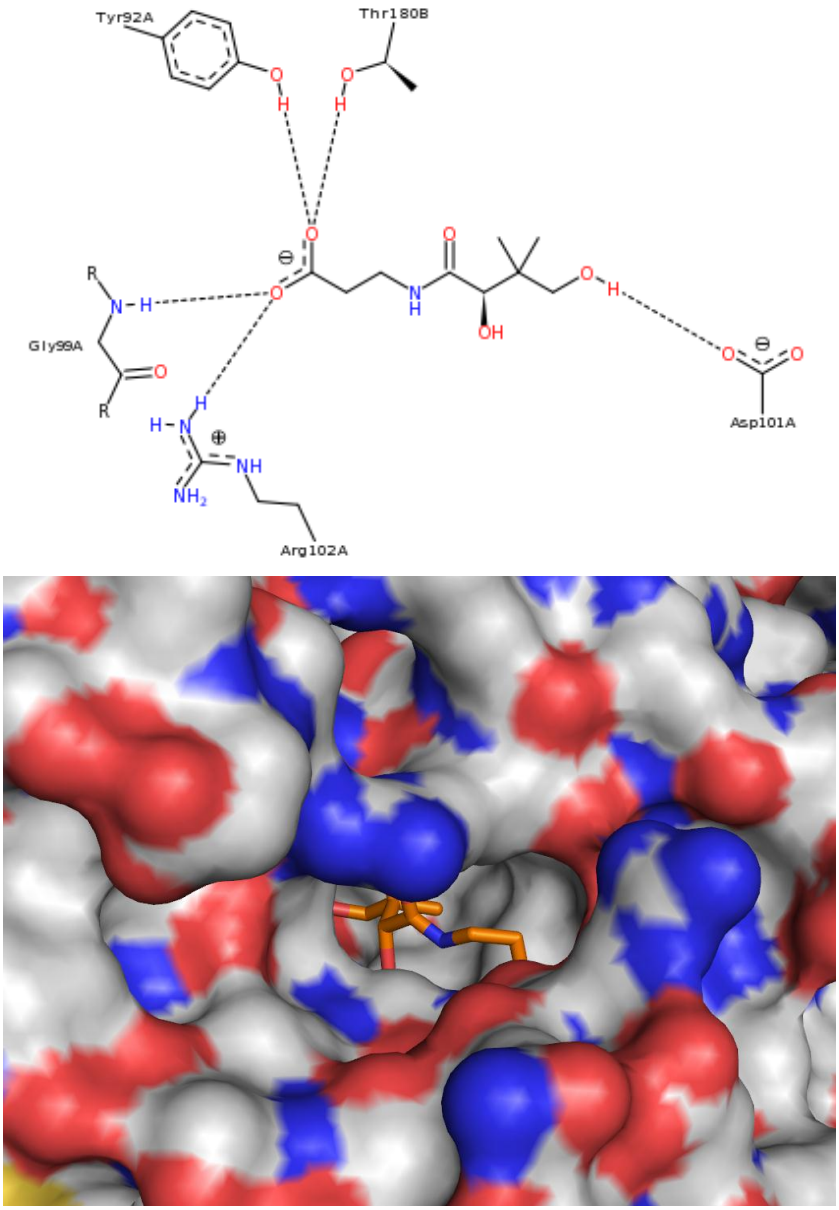


Figure 2.7: The crystal structure of *P. aeruginosa* PanK-III enzyme (PDB: 2F9W).[86] Top: Representation of pantothenic acid bound to the active site showing the interactions between the substrate and the active site amino acids. Bottom: Surface representation of the enzyme binding pantothenic acid (stick representation) with a solvent exposed opening to the side of the active site.

The tight binding pocket explains the inability of the PanK-IIIs to accept longer pantothenamides as alternative substrates. The pantothenic acid binding pocket is furthermore situated above the ATP-binding cleft and is only accessible by passing through the ATP binding site. This tight conformation makes it difficult to identify molecules that can access and bind to the pantothenic acid binding pocket.

In binding to PanK-II, the carboxyl group of the β-alanine moiety specifically interacts with Tyr92, Arg102, Thr180 and the backbone nitrogen of Gly99 to form H-bonds. Asp101 acts as a catalytic base and forms side chain hydrogen bonds to the 2'- and 4'-hydroxyl groups of the pantoyl moiety,[52] while His156 stabilizes the 4'-hydroxyl group via a water molecule. Further stabilization is obtained by van der Waals interactions between the 3'-geminal dimethyl groups with the side chains of Val55, Ile143 and Ile160. These interactions are shown in Figure 2.7 (top).

The crystal structure of *Pa* PanK-III was obtained with pantothenic acid bound in the active site (PDB: 2F9W).[86] As can be seen from the enzyme structure (Figure 2.7, bottom), there is a small opening to the side of the active site which exposes the $-CH_2$-groups of pantothenic acid (stick representation) to solvent. The conformation of the bound substrate also appears to force the flanking amine- and carboxyl groups into a "*cis*" conformation.

2.12 Conclusion

PanKs have been the focus of many inhibitor studies by pharmaceutical companies including AstraZeneca and Novartis.[69] Lead compounds have been identified that can inhibit type I and II enzymes. These compounds are however not effective on the type III enzymes. The only known inhibitors of the PanK-IIIs are ATP mimetics which has limited efficiency. The question thus remains as to the type or class of analog that could inhibit PanK-IIIs. A closer look at the active site of the *P. aeruginosa* PanK-III enzyme showed that there is a slight opening to the side of the active site which exposes the methylene-groups of pantothenic acid. It might therefore be possilbe that this opening could provide sufficient leeway to design type specific inhibitors for this PanK type.

2.13 References

1. United Nations (UN), *The millennium mevelopment goals report*, L. Jensen, Editor. 2010, United Nations Department of Economic and Social Affairs. p. 75.
2. Dye, C. and Williams B.G., The population dynamics and control of tuberculosis. *Science*, 2010. **328** (5980): p. 856-61.
3. World Health Organisation (WHO), *TB/HIV facts 2011*.
4. Koul, A., Arnoult E., Lounis N., Guillemont J. and Andries K., The challenge of new drug discovery for Tuberculosis. *Nature*, 2011. **469** (7331): p. 483-90.
5. Spry, C., Kirk K. and Saliba K.J., Coenzyme A biosynthesis: an antimicrobial drug target. *FEMS Microbiology Reviews*, 2008. **32** (1): p. 56-106.
6. Strauss, E., Coenzyme A biosynthesis and enzymology, in *Comprehensive Natural Products II*, M. Lew and L. Hung-Wen, Editors. 2010, Elsevier: Oxford. p. 351-410.
7. Begley, T.P., Kinsland C. and Strauss E., The biosynthesis of coenzyme A in bacteria. *Vitamins and Hormones (New York)*, 2001. **61**: p. 157-71.
8. Leonardi, R., Zhang Y.-M., Rock C.O. and Jackowski S., Coenzyme A: Back in action. *Progress in Lipid Research*, 2005. **44** (2-3): p. 125-53.
9. Balibar, C.J., Hollis-Symynkywicz M.F. and Tao J., Pantethine rescues phosphopantothenoylcysteine synthetase and phosphopantothenoylcysteine decarboxylase deficiency in *Escherichia coli* but not in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 2011: p. JB.00334-11.
10. Meier, J.L., Mercer A.C., Rivera H. and Burkart M.D., Synthesis and evaluation of bioorthogonal pantetheine analogues for *in vivo* Ppotein modification. *Journal of the American Chemical Society*, 2006. **128** (37): p. 12174-84.
11. Worthington, A.S. and Burkart M.D., One-pot chemo-enzymatic synthesis of reporter-modified proteins. *Organic & Biomolecular Chemistry*, 2006. **4** (1): p. 44-46.
12. Jackowski, S. and Rock C.O., Regulation of coenzyme A biosynthesis. *Journal of Bacteriology*, 1981. **148** (3): p. 926-32.
13. Robishaw, J.D., Berkich D. and Neely J.R., Rate-limiting step and control of coenzyme A synthesis in cardiac muscle. *Journal of Biological Chemistry*, 1982. **257** (18): p. 10967-72.

14. Robishaw, J.D. and Neely J.R., Pantothenate kinase and control of CoA synthesis in heart. *American Journal of Physiology*, 1984. **246** (4, Pt. 2): p. H532-H41.

15. Song, W.-J. and Jackowski S., Kinetics and regulation of pantothenate kinase from *Escherichia coli. Journal of Biological Chemistry*, 1994. **269** (43): p. 27051-8.

16. Finlayson, H.J. and Seeley R.C., The synthesis and absorption of pantothenic acid in the gastrointestinal tract of the adult sheep. *Journal of the Science of Food and Agriculture*, 1983. **34** (5): p. 427-32.

17. Nakamura, H. and Tamura Z., Pantothenate uptake in *Escherichia coli* K12. *Journal of Nutritional Science and Vitaminology*, 1973. **19** (5): p. 389-400.

18. Vallari, D.S. and Rock C.O., Pantothenate transport in *Escherichia coli. Journal of Bacteriology*, 1985. **162** (3): p. 1156-61.

19. Jackowski, S. and Alix J.H., Cloning, sequence, and expression of the pantothenate permease (*panF*) gene of *Escherichia coli. Journal of Bacteriology*, 1990. **172** (7): p. 3842-8.

20. Rock, C.O., Karim M.A., Zhang Y.-M. and Jackowski S., The murine pantothenate kinase (*PANK1*) gene encodes two differentially regulated pantothenate kinase isozymes. *Gene*, 2002. **291** (1-2): p. 35-43.

21. Zheng, R. and Blanchard J.S., Steady-State and pre-steady-state kinetic analysis of *Mycobacterium tuberculosis* pantothenate synthetase†. *Biochemistry*, 2001. **40** (43): p. 12904-12.

22. Sambandamurthy, V.K., Wang X., Chen B., Russell R.G., Derrick S., Collins F.M., Morris S.L. and Jacobs W.R., A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. *Nat Med*, 2002. **8** (10): p. 1171-74.

23. Mercer, A.C. and Burkart M.D., The ubiquitous carrier protein-a window to metabolite biosynthesis. *Natural Product Reports*, 2007. **24** (4): p. 750-73.

24. Lambalot, R.H. and Walsh C.T., Cloning, overproduction, and characterization of the *Escherichia coli* Holo-acyl carrier protein synthase. *Journal of Biological Chemistry*, 1995. **270** (42): p. 24658-61.

25. Song, W.J. and Jackowski S., Cloning, sequencing, and expression of the pantothenate kinase (*coaA*) gene of *Escherichia coli. Journal of Bacteriology*, 1992. **174** (20): p. 6411-17.

26. Calder, R.B., Williams R.S.B., Ramaswamy G., Rock C.O., Campbell E., Unkles S.E., Kinghorn J.R. and Jackowski S., Cloning and characterization of a eukaryotic pantothenate kinase gene (*panK*) from *Aspergillus nidulans. Journal of Biological Chemistry*, 1999. **274** (4): p. 2014-20.

27. Choudhry, A.E., Mandichak T.L., Broskey J.P., Egolf R.W., Kinsland C., Begley T.P., Seefeld M.A., Ku T.W., Brown J.R., Zalacain M. and Ratnam K., Inhibitors of pantothenate kinase: Novel antibiotics for staphylococcal infections. *Antimicrobial Agents and Chemotherapy*, 2003. **47** (6): p. 2051-55.

28. Gerdes, S.Y., Scholle M.D., D'Souza M., Bernal A., Baev M.V., Farrell M., Kurnasov O.V., Daugherty M.D., Mseeh F., Polanuyer B.M., Campbell J.W., Anantha S., Shatalin K.Y., Chowdhury S.A.K., Fonstein M.Y. and Osterman A.L., From genetic footprinting to antimicrobial drug targets: examples in cofactor biosynthetic pathways. *Journal of Bacteriology*, 2002. **184** (16): p. 4555-72.

29. Osterman, A. and Overbeek R., Missing genes in metabolic pathways: a comparative genomics approach. *Current Opinion in Chemical Biology*, 2003. **7** (2): p. 238-51.

30. Yocum, R.R. and Patterson T.A., Microorganisms and assays for the identification of antibiotics acting on the pantothenate kinase encoded by the *coaX* gene. 2002, (Omnigene Bioproducts, Inc., USA). *Application: WO*. p. 128 pp.

31. Brand, L.A. and Strauss E., Characterization of a new pantothenate kinase isoform from *Helicobacter pylori. Journal of Biological Chemistry*, 2005. **280** (21): p. 20185-88.

32. Hong, B.S., Senisterra G., Rabeh W.M., Vedadi M., Leonardi R., Zhang Y.-M., Rock C.O., Jackowski S. and Park H.-W., Crystal structures of human pantothenate kinases: insights into allosteric regulation and mutations linked to a neurodegeneration disorder. *Journal of Biological Chemistry*, 2007. **282** (38): p. 27984-93.

33. Ye, Y., Osterman A., Overbeek R. and Godzik A., Automatic detection of subsystem/pathway variants in genome analysis. *Bioinformatics*, 2005. **21** Suppl 1: p. i478-i86.

34. Overbeek, R., Begley T., Butler R.M., Choudhuri J.V., Chuang H.Y., Cohoon M., de Crecy-Lagard V., Diaz N., Disz T., Edwards R., Fonstein M., Frank E.D., Gerdes S., Glass E.M., Goesmann A., Hanson A., Iwata-Reuyl D., Jensen R., Jamshidi N., Krause L.,

Kubal M., Larsen N., Linke B., McHardy A.C., Meyer F., Neuweger H., Olsen G., Olson R., Osterman A., Portnoy V., Pusch G.D., Rodionov D.A., Ruckert C., Steiner J., Stevens R., Thiele I., Vassieva O., Ye Y., Zagnitko O. and Vonstein V., The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Research*, 2005. **33** (17): p. 5691-702.

35. Nicely, N.I., Parsonage D., Paige C., Newton G.L., Fahey R.C., Leonardi R., Jackowski S., Mallett T.C. and Claiborne A., Structure of the type III pantothenate kinase from *Bacillus anthracis* at 2.0 Å. Resolution: implications for coenzyme A-dependent redox biology. *Biochemistry*, 2007. **46** (11): p. 3234-45.
36. Hong, B.S., Yun M.K., Zhang Y.-M., Chohnan S., Rock C.O., White S.W., Jackowski S., Park H.-W. and Leonardi R., Prokaryotic type II and type III pantothenate kinases: the same monomer fold creates dimers with distinct catalytic properties. *Structure*, 2006. **14** (8): p. 1251-61.
37. Dunn, S.D. and Snell E.E., Isolation of temperature-sensitive pantothenate kinase mutants of *Salmonella typhimurium* and mapping of the *coaA* gene. *Journal of Bacteriology*, 1979. **140** (3): p. 805-8.
38. Zhou, B., Westaway H.K., Levinson B., Johnson M.A., Gitschier J. and Hayflick S.J., A novel pantothenate kinase gene (*PANK2*) is defective in Hallervorden-Spatz syndrome. *Nature Genetics*, 2001. **28** (4): p. 345-49.
39. Wu, Z., Li C., Lv S. and Zhou B., Pantothenate Kinase-Associated Neurodegeneration: insights from a Drosophila model. *Human Molecular Genetics*, 2009.
40. Zhang, Y.-M., Rock C.O. and Jackowski S., Biochemical properties of human pantothenate kinase 2 isoforms and mutations linked to pantothenate kinase-associated neurodegeneration. *Journal of Biological Chemistry*, 2006. **281** (1): p. 107-14.
41. Wood, G.E. and Friedman R.L., The Bvg accessory factor (Baf) enhances pertussis toxin expression in *Escherichia coli* and is essential for *Bordetella pertussis* viability. *FEMS Microbiology Letters*, 2000. **193** (1): p. 25-30.
42. DeShazer, D., Wood G.E. and Friedman R.L., Identification of a *Bordetella pertussis* regulatory factor required for transcription of the pertussis toxin operon in *Escherichia coli*. *Journal of Bacteriology*, 1995. **177** (13): p. 3801-7.

43. Brand, L.A., Comparative characterisation and mutational analysis of type III pantothenate kinases, *M.Sc thesis (Biochemistry)*. 2006, Stellenbosch university: p. 121.

44. Awasthy, D., Ambady A., Bhat J., Sheikh G., Ravishankar S., Subbulakshmi V., Mukherjee K., Sambandamurthy V. and Sharma U., Essentiality and functional analysis of type I and type III pantothenate kinases of *Mycobacterium tuberculosis. Microbiology*, 2010. **156**: p. 2691-701.

45. Vallari, D.S., Jackowski S. and Rock C.O., Regulation of pantothenate kinase by coenzyme A and its thioesters. *Journal of Biological Chemistry*, 1987. **262** (6): p. 2468-71.

46. Paige, C., Reid S.D., Hanna P.C. and Claiborne A., The type III pantothenate kinase encoded by *coaX* is essential for growth of *Bacillus anthracis. J. Bacteriol.*, 2008. **190** (18): p. 6271-75.

47. Cheek, S., Zhang H. and Grishin N.V., Sequence and structure classification of kKinases. *Journal of Molecular Biology*, 2002. **320** (4): p. 855-81.

48. Cheek, S., Ginalski K., Zhang H. and Grishin N.V., A comprehensive update of the sequence and structure classification of kinases. *BMC Structural Biology*, 2005. **5**: p. No pp given.

49. Ivey, R.A., Zhang Y.-M., Virga K.G., Hevener K., Lee R.E., Rock C.O., Jackowski S. and Park H.-W., The structure of the pantothenate kinase-ADP-pantothenate ternary complex reveals the relationship between the binding sites for substrate, allosteric regulator, and antimetabolites. *Journal of Biological Chemistry*, 2004. **279** (34): p. 35622-29.

50. Das, S., Kumar P., Bhor V., Surolia A. and Vijayan M., Invariance and variability in bacterial PanK: a study based on the crystal structure of *Mycobacterium tuberculosis* PanK. *Acta Crystallographica, Section D: Biological Crystallography*, 2006. **D62** (6): p. 628-38.

51. Chetnani, B., Kumar P., Surolia A. and Vijayan M., *M. tuberculosis* pantothenate kinase: dual substrate specificity and unusual changes in ligand locations. *Journal of Molecular Biology*, 2010. **400** (2): p. 171-85.

52. Yang, K., Strauss E., Huerta C. and Zhang H., Structural basis for substrate binding and the catalytic mechanism of type III pantothenate kinase. *Biochemistry*, 2008. **47** (5): p. 1369-80.

53. Yun, M., Park C.-G., Kim J.-Y., Rock C.O., Jackowski S. and Park H.-W., Structural basis for the feedback regulation of *Escherichia coli* pantothenate kinase by coenzyme A. *Journal of Biological Chemistry*, 2000. **275** (36): p. 28093-99.

54. Das, S., Kumar P., Bhor V., Surolia A. and Vijayan M., Expression, purification, crystallization and preliminary X-ray crystallographic analysis of pantothenate kinase from *Mycobacterium tuberculosis. Acta Crystallographica Section F*, 2005. **61** (1): p. 65-67.

55. Chetnani, B., Das S., Kumar P., Surolia A. and Vijayan M., *Mycobacterium tuberculosis* pantothenate kinase: possible changes in location of ligands during enzyme action. *Acta Crystallographica Section D*, 2009. **65** (4): p. 312-25.

56. Yang, K., Eyobo Y., Brand L.A., Martynowski D., Tomchick D., Strauss E. and Zhang H., Crystal structure of a type III pantothenate kinase: insight into the mechanism of an essential coenzyme A biosynthetic enzyme universally distributed in bacteria. *Journal of Bacteriology*, 2006. **188** (15): p. 5532-40.

57. Leonardi, R., Zhang Y.-M., Yun M.-K., Zhou R., Zeng F.-Y., Lin W., Cui J., Chen T., Rock C.O., White S.W. and Jackowski S., Modulation of pantothenate kinase 3 activity by small molecules that interact with the substrate/allosteric regulatory domain. *Chemistry & biology*, 2010. **17** (8): p. 892-902.

58. Rock, C.O., Park H.-W. and Jackowski S., Role of feedback regulation of pantothenate kinase (CoaA) in control of coenzyme A levels in *Escherichia coli. Journal of Bacteriology*, 2003. **185** (11): p. 3410-15.

59. Leonardi, R., Chohnan S., Zhang Y.-M., Virga K.G., Lee R.E., Rock C.O. and Jackowski S., A pantothenate kinase from *Staphylococcus aureus* refractory to feedback regulation by coenzyme A. *Journal of Biological Chemistry*, 2005. **280** (5): p. 3314-22.

60. Strauss, E., de Villiers M. and Rootman I., Biocatalytic production of coenzyme A analogues. *ChemCatChem*, 2010. **2** (8): p. 929-37.

61. Strauss, E. and Begley T.P., The antibiotic activity of *N*-pentylpantothenamide results from its conversion to ethyldethia-coenzyme A, a coenzyme A antimetabolite. *Journal of Biological Chemistry*, 2002. **277** (50): p. 48205-09.

62. Virga, K.G., Zhang Y.-M., Leonardi R., Ivey R.A., Hevener K., Park H.-W., Jackowski S., Rock C.O. and Lee R.E., Structure-activity relationships and enzyme inhibition of pantothenamide-type pantothenate kinase inhibitors. *Bioorganic & Medicinal Chemistry*, 2006. **14** (4): p. 1007-20.

63. van Wyk, M. and Strauss E., One-pot preparation of coenzyme A analogues via an improved chemo-enzymatic synthesis of pre-CoA thioester synthons. *Chemical Communications*, 2007(4): p. 398-400.

64. Rowan, A.S., Nicely N.I., Cochrane N., Wlassoff W.A., Claiborne A. and Hamilton C.J., Nucleoside triphosphate mimicry: a sugar triazolyl nucleoside as an ATP-competitive inhibitor of *B. anthracis* pantothenate kinase. *Organic & Biomolecular Chemistry*, 2009. **7** (19): p. 4029-36.

65. van Wyk, M. and Strauss E., Development of a method for the parallel synthesis and purification of *N*-substituted pantothenamides, known inhibitors of coenzyme A bio-synthesis and utilization. *Organic & Biomolecular Chemistry*, 2008. **6** (23): p. 4348-55.

66. van der Westhuyzen, R. and Strauss E., Michael acceptor-containing coenzyme A analogues as inhibitors of the atypical coenzyme A disulfide reductase from *Staphylococcus aureus. J. Am. Chem. Soc.*, 2010. **132**: p. 12853-55.

67. Kumar, P., Chhibber M. and Surolia A., How pantothenol intervenes in coenzyme-A biosynthesis of *Mycobacterium tuberculosis. Biochemical and Biophysical Research Communications*, 2007. **361** (4): p. 903-09.

68. Sassetti, C.M., Boyd D.H. and Rubin E.J., Genes required for mycobacterial growth defined by high density mutagenesis. *Molecular Microbiology*, 2003. **48** (1): p. 77-84.

69. Keller, T.H. Lead Finding For TB Pantothenate Kinase: Lessons Learned. in *ICS-UNIDO workshop: "Advanced Design and Development of potential Drugs against Tuberculosis.* 2009.

70. Shimizu, S., Vitamins and related compounds: Microbial production, in *Biotechnology Set*. 2008, Wiley-VCH Verlag GmbH. p. 318-40.

71. Williams, R.J., Weinstock H.H., Jr., Rohrmann E., Truesdail J.H., Mitchell H.K. and Meyer C.E., Pantothenic acid. III. Analysis and determination of constituent groups. *Journal of the American Chemical Society*, 1939. **61**: p. 454-7.

72. Barnett, J.W. and Robinson F.A., Analogues of pantothenic acid: Attempted preparation of growth promoters. *Biochem. J.*, 1942. **36** (3-4): p. 357-0.

73. Snell, E.E., Growth inhibition by *N*-(α,γ-dihydroxy-β,β-dimethylbutyryl)taurine and its reversal by pantothenic acid. *Journal of Biological Chemistry*, 1941. **141**: p. 121-8.

74. Barnett, J.W. and Robinson F.A., Analogues of pantothenic acid: Preparation of growth inhibitors. *Biochem. J.*, 1942. **36** (3-4): p. 364-0.

75. McIlwain, H., Bacterial inhibition by metabolite analogues: Analogues of pantothenic acid. *Biochem. J.*, 1942. **36** (5-6): p. 417-0.

76. Pollack, M.A., Growth effects of α-methyl homologs of pantothenic acid and β-alanine. *Journal of the American Chemical Society*, 1943. **65** (7): p. 1335-39.

77. Spry, C., Chai C.L.L., Kirk K. and Saliba K.J., A class of pantothenic acid analogs inhibits *Plasmodium falciparum* pantothenate kinase and represses the proliferation of malaria parasites *Antimicrobial Agents and Chemotherapy*, 2005. **49** (11): p. 4649-57.

78. Saliba, K.J., Ferru I. and Kirk K., Provitamin B5 (Pantothenol) inhibits growth of the intraerythrocytic malaria parasite. *Antimicrob. Agents Chemother.*, 2005. **49** (2): p. 632-37.

79. Cheldelin, V.H. and Schink C.A., Pantothenic acid studies. I. Growth effect of pantoic acid analogs. *J. Am. Chem. Soc.*, 1947. **69**: p. 2625-8.

80. Mitchell, H.K., Snell E.E. and Williams R.J., Pantothenic acid. IX. The biological activity of hydroxypantothenic acid. *Journal of the American Chemical Society*, 1940. **62**: p. 1791-2.

81. Lipton, S.H. and Strong F.M., Synthesis of compounds related to pantothenic acid. *Journal of the American Chemical Society*, 1949. **71** (7): p. 2364-67.

82. Holly, F.W., Barnes R.A., Koniuszy F.R. and Folkers K., An amino analog of pantothenic acid. *Journal of the American Chemical Society*, 1948. **70** (9): p. 3088-90.

83. Drell, W. and Dunn M.S., Growth inhibition of lactic acid bacteria by *N*-(α,γ-dihydroxy-β,β-dimethylvaleryl)-β-alanine and its reversal by pantothenic acid. *Journal of the American Chemical Society*, 1946. **68** (9): p. 1868-68.

84. Drell, W. and Dunn M.S., Inhibition of lactic acid bacteria by analogs of pantothenic acid 1. *Journal of the American Chemical Society*, 1948. **70** (6): p. 2057-63.

85. Drell, W. and Dunn M.S., Inhibition of lactic acid bacteria by ω-substituted analogs of pantothenic acid. *J. Am. Chem. Soc.*, 1954. **76**: p. 2804-8.

86. Image from the RCSB protein database (PDB) (www.pdb.org); PDB ID 2F9W. Last accessed: 1 September 2011

Chapter 3:
Synthesis and characterization of PanK-III inhibitors

3.1 Introduction

PanK-III enzymes are widely distributed in Bacteria (see section 2.6) and are surprisingly common in bacterial pathogens, such as *M. tuberculosis*, *B. anthracis*, the category A biodefense organism *Fransicella tularensis* and *Neissera meningitis.* This fact, and because Type III PanKs are distinct from human PanK-IIs, make these enzymes excellent targets for drug development. However, to date the only known inhibitors for PanK-IIIs are ATP mimetics that were found to be mediocre competitive inhibitors of the *B. anthracis* enzyme (see section 2.9).[1] In this part of the study our goal was to identify and develop pantothenic acid analogs as inhibitors of the PanK-III enzyme from *P. aeruginosa*.

3.2 Strategy

The crystal structure of *P. aeruginosa* PanK-III (*Pa* PanK-III) shows that pantothenic acid binds to a fully enclosed binding pocket and that numerous charged amino acid residues are involved in the binding and stabilization of the vitamin (section 2.11).[2,3] The active site pocket further has a small opening to the surrounding solvent which could potentially be exploited for the rational design of inhibitors that would occupy this opening. For this study pantothenic acid analogs were designed, synthesized and tested as alternate substrates and/or inhibitors of *Pa* PanK-III to probe the most important binding interactions in its active site. Our goal was to interrogate these interactions in a manner that would allow the individual contributions of three distinct factors to be evaluated. These were:

- Factors influenced by steric considerations
- Factors influenced by electrostatic interactions
- Factors affecting catalysis

3.2.1 Probing factors influenced by steric considerations

Due to their enclosed pantothenate binding pockets, PanK-IIIs enzymes do not accept *N*-substituted pantothenamides as substrates. However, little is known about their ability to

accept and phosphorylate other pantothenic acid analogs. We were specifically interested in exploring the tolerance of the PanK-III active site for modifications that would increase the steric bulk of the β-alanine moiety of the pantothenic acid backbone, and to determine whether the opening on the side of the active site would accommodate such modifications.

3.2.2 Probing factors influenced by electrostatic interactions

The strong electrostatic interactions between the active site residues and the carboxyl group of the bound pantothenic acid constitute a unique feature of PanK-IIIs in comparison to other PanK types (refer to section 2.11). The addition or removal of electronegative substituents close to the carboxyl group could change the nature of these electrostatic interactions to such a degree as to increase affinity of substrate binding to the enzyme, or to destabilize the substrate-enzyme complex.

3.2.3 Probing factors involved in catalysis

A major drawback of using pantothenic acid analogs as inhibitors is that they can potentially also act as substrates of the PanK-III enzymes. In such a case they would be phosphorylated and effectively be removed as potential occupants of the active site. However, this problem can be overcome by preventing the phosphorylation of the 4'-hydroxyl group of pantothenic acid. This can be achieved by the derivatizing, reducing or substituting the 4'-hydroxyl group, or by introducing groups around the 4'-carbon that sterically hinder phosphate-group transfer. Alternatively, the active site residues involved in catalysis could potentially be restricted through novel interactions with the inhibitor.

3.4 Pantothenic acid analogs used in this study

The molecules shown in Figure 3.1 are proposed as suitable pantothenate analogs to individually probe the three factors discussed above. To probe the influence of steric effects the methyl derivatives **3.1 – 3.3** were synthesized, while α-pantothenic acid (**3.4**) and homo-pantothenic acid (**3.5**) were available in our laboratory.[4] Commercially available panto-thenol (**3.6**) and pantoyltaurine (**3.7**) were used to evaluate the electrostatic interactions between the substrate and enzyme, while the fluorinated methyl derivative (**3.8**) was synthesized to simultaneously test the effect of increased steric bulk and improved electrostatic interactions. To study the factors involved in catalysis, three molecules with

modifications in or close to the 4'-hydroxyl group were identified as analogs that could block or otherwise prevent catalysis – 4'-methoxypantothenic acid (**3.9**), ω-methylpantothenic acid (**3.10**) and 4'-deoxypantothenic acid (**3.11**). Molecules **3.9** and **3.11** were successfully synthesized, while the synthesis of **3.10** was abandoned due to various difficulties experienced (discussed in detail in section 3.5.2b).

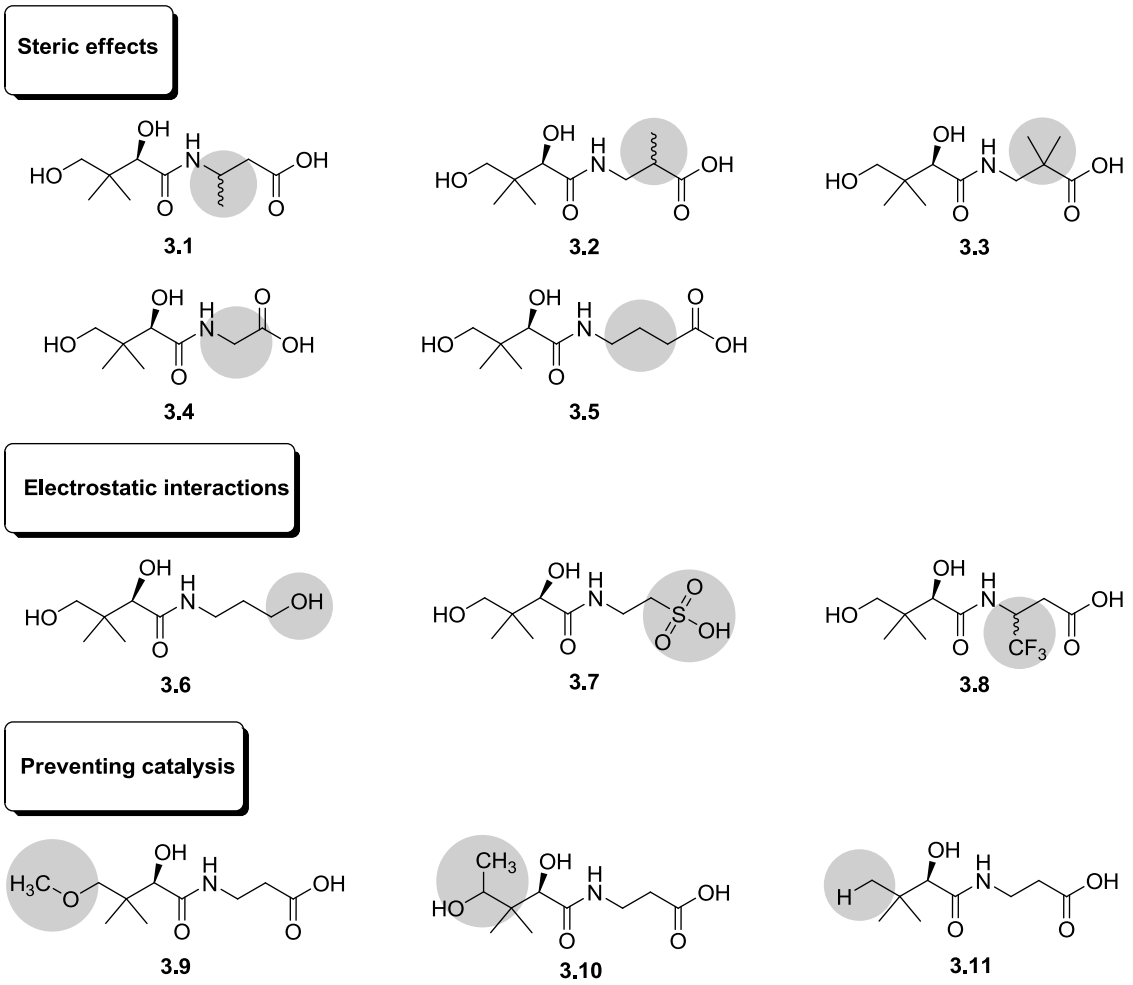
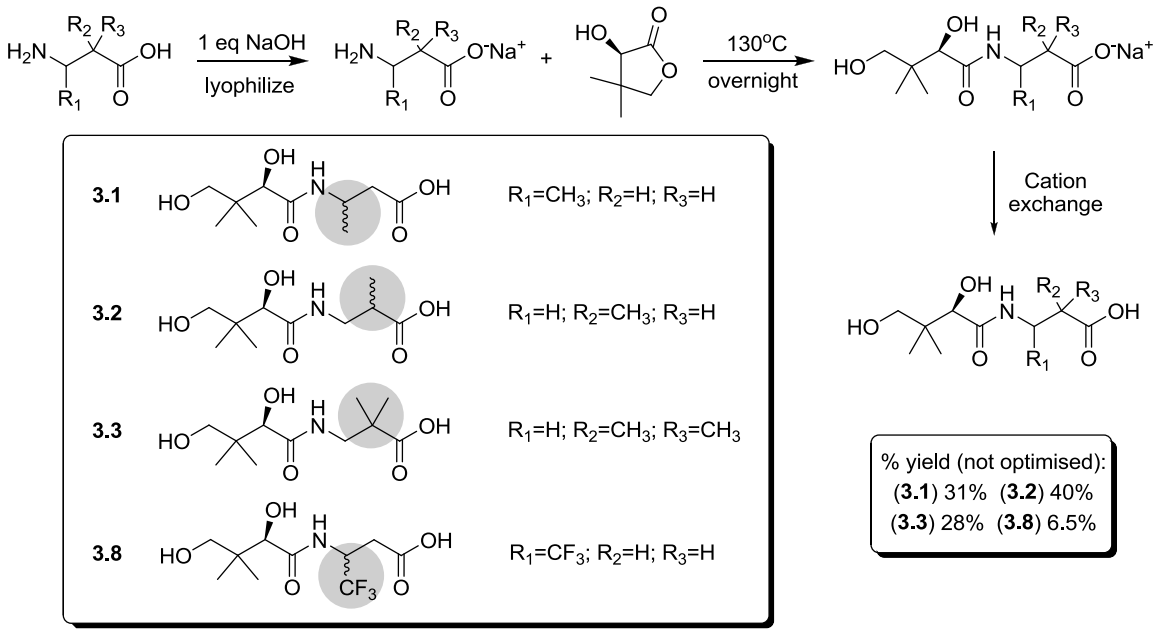


Figure 3.1: The molecules tested as alternative substrates and potential inhibitors of *P. aeruginosa* PanK-III.

3.5 Results

3.5.1 Synthesis of pantothenate analogs modified in the β-alanine moiety

All four molecules containing modifications in the β-alanine moiety were synthesized according to the general procedure shown in Scheme 3.1.



Scheme 3.1: General synthetic scheme for the preparation of methylated derivatives of pantothenic acid.

a) (*R,S*)-β-methylpantothenic acid (3.1)

In 2006 Meier *et al*. synthesized pantetheine analogs from D-pantolactone and various amines.[5] We based our first synthetic attempt of **3.1** on this procedure, in which a mixture of 3.0 eq. of pantolactone, 3.0 eq. NEt_3 and 1.0 eq. of amine was refluxed in methanol for 7 h. Although coupling was obtained, we experienced several problems with the purification of the product. An alternative method, which previously was used in our laboratory to synthesize homo- and α-pantothenic acid,[4] was therefore employed instead. Deprotonated 3-aminobutyric acid (1.1 eq.) was mixed with 1.0 eq. of pantolactone and melted together at 130°C under inert conditions overnight. This procedure allowed for the successful isolation of the desired product after removal of unreacted amine by cation exchange chromatography and unreacted pantolactone by flash chromatography. The final product was obtained in 31% yield.

b) (*R,S*)-α-methylpantothenic acid (3.2)

Pollack published a synthetic procedure for the preparation of **3.2** in 1943.[6] In the original synthesis, the deprotonated 3-aminoisobutyric acid was refluxed with pantolactone in a solvent mixture of 2:1 isopropanol: ethanol. This solvent mixture was used due to the low solubility of the compounds in organic solvents. After refluxing for 3 h, the mixture was

filtered hot to remove the undissolved starting materials, after which the product was crystalized from an isopropanol/petroleum ether mixture. Pollack reported that the resulting α-methylpantothenate sodium salt was extremely difficult to handle and analyze due to its hygroscopic nature, but that, based on elemental analysis, they believed they *essentially* had the correct compound. In contrast we were able to prepare α-methylpantothenic acid using the same procedure as described for β-methylpantothenic acid above, except that pantolactone was used in 1.1 eq. excess. In our hands α-methylpantothenic acid did not give any of the handling problems reported by Pollack and was obtained in 40% yield.

c) α,α-dimethylpantothenic acid (3.3)

This compound was synthesized in 28% yield from pantolactone and 3-amino-2,2-dimethyl-propanoic acid using the same melting reaction and column purifications as described for β-methylpantothenic acid above.

d) (*R,S*)-β-trifluoromethylpantothenic acid (3.8)

β-trifluoromethylpantothenic acid was synthesized from pantolactone and (*R,S*)-3-amino-4,4,4-trifluorobutyric acid using the same procedure as described above. The product was obtained in a very low yield (≈ 6.5%) presumably due to the lower nucleophilicity of the amine caused by the electron-withdrawing nature of the adjacent -CF_3 group.

3.5.2 Synthesis of pantothenate analogs modified in the pantoyl moiety

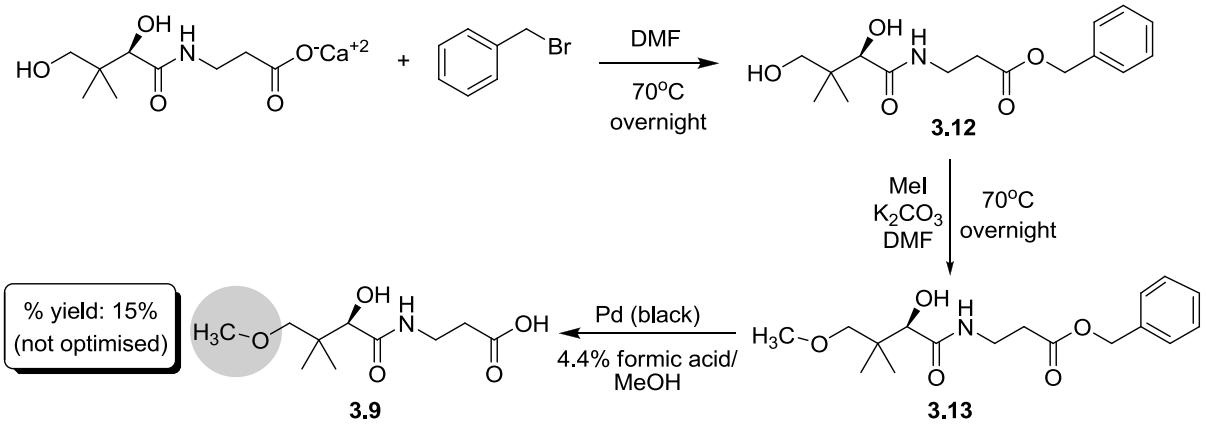
Different strategies were used to synthesize each of the molecules containing modifications in the pantoyl moiety. The synthesis of each molecule is discussed separately in the following sections.

a) 4'-methoxypantothenic acid (3.9)

The synthesis of **3.9** was undertaken as shown in Scheme 3.2. Pantothenic acid (**2.2**) was protected as the benzyl ester by the reaction of calcium pantothenate with benzylbromide according to an established procedure[7] to give **3.12** and was obtained in 57% yield.

The first attempts at the synthesis of **3.13** was based on a Haworth type methylation using dimethyl sulfate (Me_2SO_4) and a strong base to deprotonate the 4'-hydroxyl group.

Unfortunately the bases that were used in these attempts (NaH and NaOH) caused decomposition of **3.12** (based on TLC analysis). However, deprotonation with anhydrous K_2CO_3 and methyl iodide (MeI) as methylation agent resulted in the desired product **3.13**. Deprotection was done according to a standard published procedure using Pd (black) in 4.4% formic acid/methanol.[8] The product **3.9** was subsequently purified by flash chromatography and obtained in a final overall yield of 15%. To determine whether the correct hydroxyl group had been methylated, ^{1}H NMR and COSY analyses were performed. Both spectra are shown in Figure 3.2 together with the peak assignments.



Scheme 3.2: Synthesis of 4'-methoxypantothenic acid (3.9).

The three protons of the $-OCH_3$ group (labeled nr. 2 in the figure) are represented by the peaks found at 3.34 ppm, while the two protons of the 4'$-CH_2$-O- (nr. 3) are represented by a doublet of doublets at 3.29 ppm. In the proton spectrum, one of the peaks of this doublet of doublet overlaps with the $-OCH_3$ singlet. In the COSY spectrum it is clear that these peaks do not only overlap, but that they also couple with each other (shown in the block in the figure), indicating that the methyl group was introduced on the 4'-OH. On the other hand, the proton on the 2'-carbon (nr. 5) is shifted further downfield to 3.96 ppm. The COSY spectrum shows no interactions between this proton and any other proton, confirming that the 2'-hydroxyl group had not been methylated.

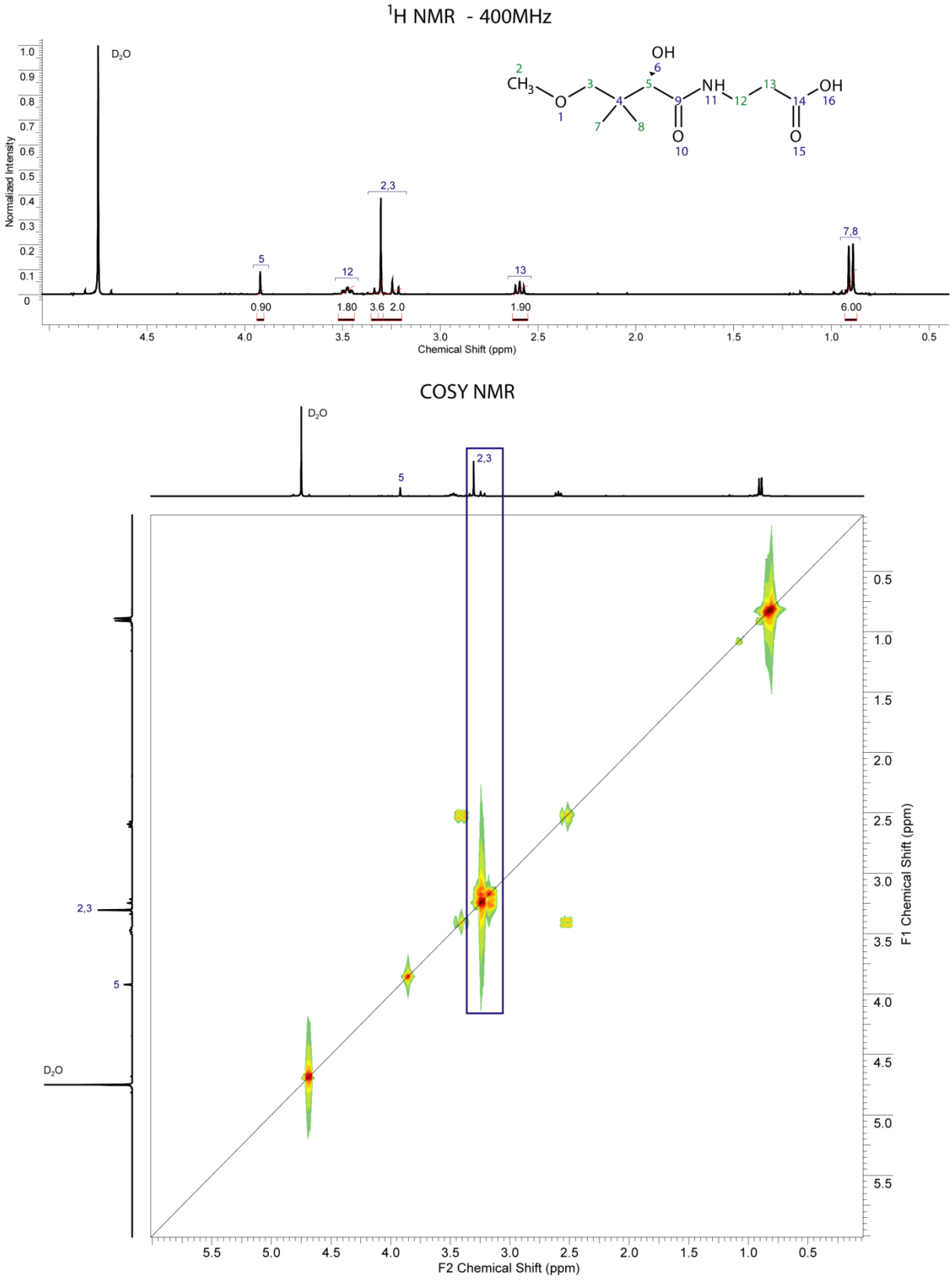
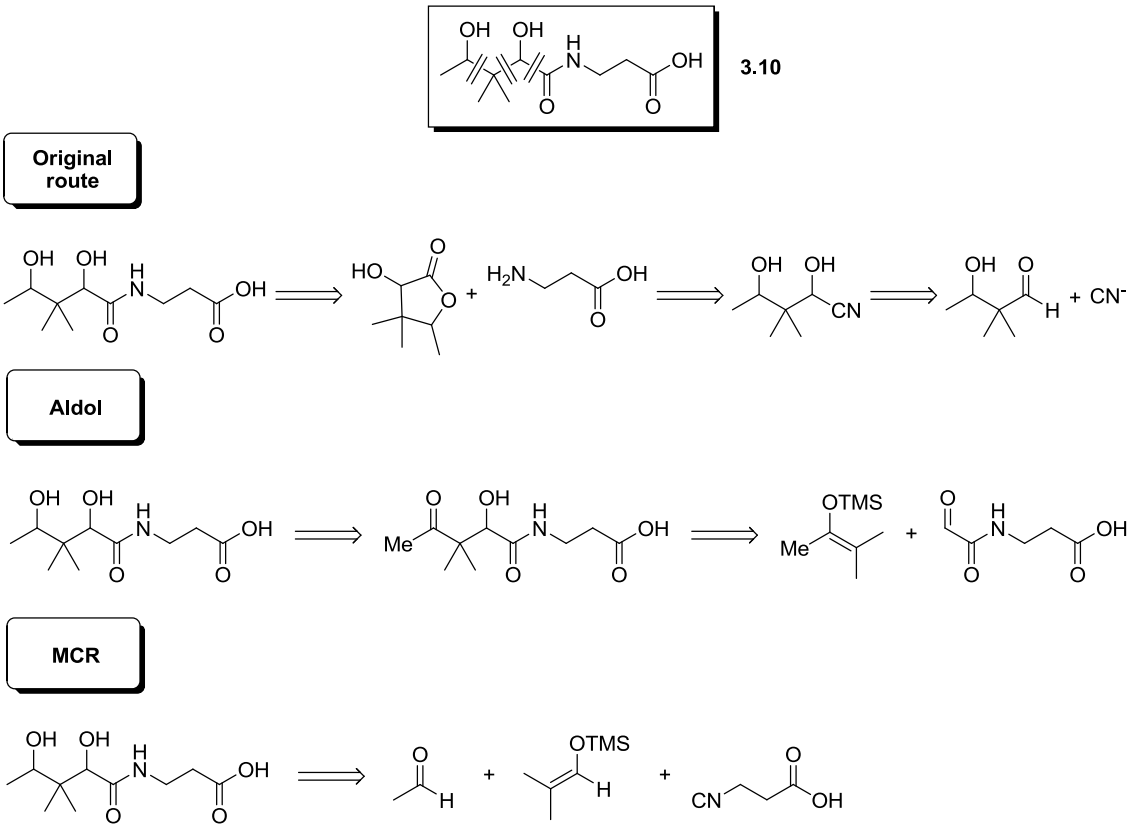


Figure 3.2: ^{1}H NMR and COSY spectra of 4'-methoxypantothenic acid

b) ω-methylpantothenic acid (3.10)

ω-Methylpantothenic acid (**3.10**) was the most promising inhibitory lead from the 1940s studies, in which the effects of pantothenic acid analogs on the lactic acid bacteria had been investigated. The chemical synthesis of this analog was first published in 1946.[9] In Scheme 3.3 a retrosynthetic analysis of the molecule is given. Three disconnections are possible in the pantoyl moiety, each of which leads to a different synthetic route: between 1'- and 2'-carbons (the original synthetic route), 2'- and 3'-carbons (aldol route) and the 3'- and 4'-carbons (multi-component reaction (MCR) route).



Scheme 3.3: Retrosynthetic analysis of ω-methylpantothenic acid (**3.10**) suggesting possible routes for its synthesis.

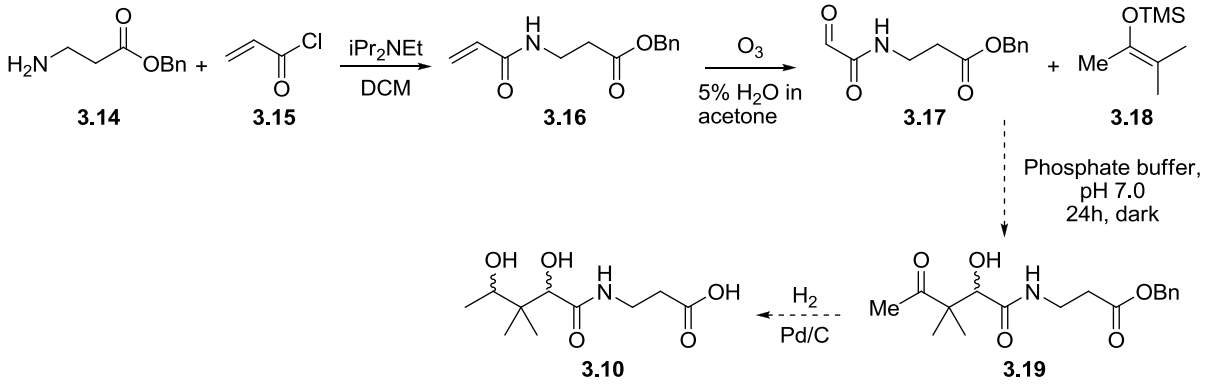
Originally, ω-methylpantothenic acid was synthesized by the condensation of β-alanine and α-hydroxy-β,β-dimethyl-γ-valerolactone. The lactone was obtained from hydrolysis of the cyanohydrin synthesized from α,α-dimethyl-β-hydroxybutyraldehyde and CN^-.[9,10] Due to the use of CN^- and the extensive extraction and purifications steps of the original procedure, other protocols were investigated to identify a simpler and safer synthetic route.

Based on the other disconnects two additional synthetic strategies were proposed. The first strategy relies on an aldol condensation. A synthetic procedure, based on the methods of Evans *et al.*[11] and Alam *et al.*[12], was devised in which methylbutane acts as donor by trapping its enol as the TMS ether. The glyoxalamide acts as acceptor to form the new C–C bond between the 2'- and 3'-carbons of the pantoyl moiety.

The second strategy was based on a study by Pellissier *et al.* [13] and involves the formation of two new C–C bonds. The bond between the 3'- and 4'-carbons is formed first by an aldol condensation between acetaldehyde as acceptor and methylpropanal as donor, while the bond between the 1'- and 2'-carbons is formed by nucleophilic attack of an isocyanide on the aldehyde product that results from this aldol reaction. The overall reaction is a multi-component reaction (MCR) that bears similarity to the Passerini and Ugi reactions.

Strategy 1: Aldol

A synthetic procedure based on the aldol condensation is given in Scheme 3.4.



Scheme 3.4: Proposed synthesis of **3.10** based on an aldol condensation (Strategy 1).

The acrylamide **3.16** was prepared by reaction of **3.14** in its free amine form (prepared by extraction from the commercially available β-alanine benzyl ester tosylate salt) with acryloylchloride (**3.15**) according to the procedure of Cho *et al.*[14] with *N,N*-diisopropyl-ethylamine (iPr_2NEt) as base. The product **3.16** was obtained in 57% yield.

There are two possible methodologies to oxidatively cleave **3.16** to form **3.17**. The first method, the Lemieux-Johnson oxidation, is a two-step reaction in which the double bond is first dihydroxylated with OsO_4 before cleavage of the vicinal diol with $NaIO_4$.[15,16] Alternatively, ozonolysis followed by reductive cleavage can be used, although a drawback of this procedure is that the resulting peroxide intermediates can cause further unwanted

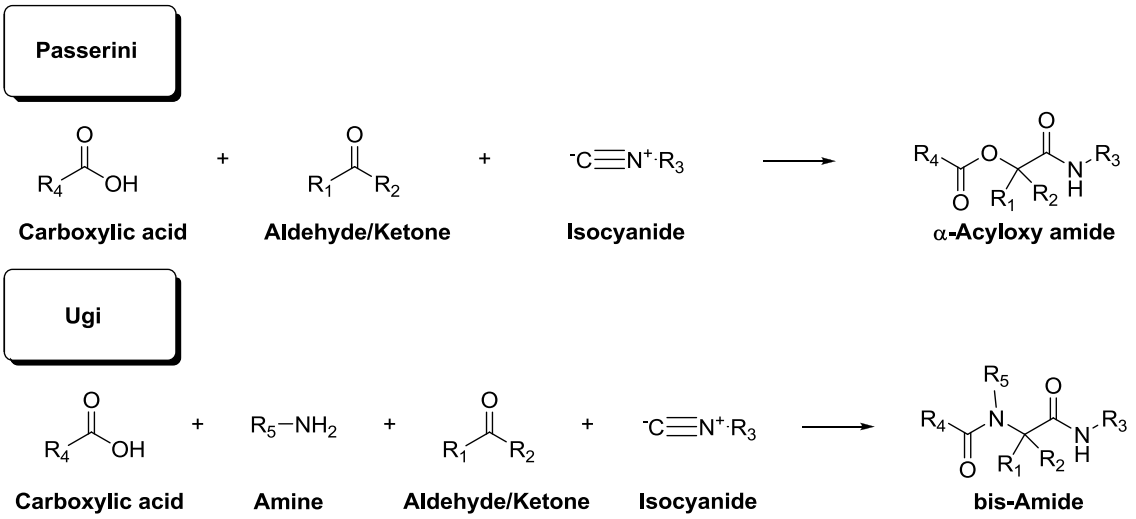
oxidations.[17] Many reducing agents have been identified and are used for the reductive cleavage reaction; these include acetic anhydride/NEt_3,[18] Me_2S,[19] Zn/CH_3COOH[20], $P(OMe)_3$,[21] Pt/H_2, BH_3 and $LiAlH_4$.[17] We chose to focus our efforts on employing this procedure, because of the toxicity of OsO_4 in the first method, and difficulty in obtaining the reagent.

A recent study by Schiaffo *et al.* used ozonolysis to oxidatively cleave a variety of alkenes at 0°C (in comparison to the normal −80°C) in a 5% H_2O/acetone solvent system.[17] The attractiveness of this method lies in milder reaction conditions and a reducing agent not being required. We oxidatively cleaved **3.16** using this method with Sudan red III (solvent red 23) as indicator.[22] Based on TLC analysis, all the starting reagent in the reaction was consumed, which was confirmed by the disappearance of the alkene peaks in ^{1}H NMR analysis. Unfortunately, product formation could not be conclusively confirmed because the aldehyde peak of the observed product did not yield the expected integration values relative to the other proton peaks. MS analysis of the product was also inconclusive.

In the procedure of Evans *et al*. a glyoxylate ester and TMS enol ether is coupled using $Sc(OTf)_2$ as catalyst under extreme dry and inert conditions (in a glove box) at −78°C.[11] In the Alam *et al*. protocol Mukaiyama couplings are executed with the same type of reactants by stirring the compounds together in sodium phosphate buffer, pH 7.0, at room temperature in the dark for 24 h.[12] For the coupling of **3.17** to **3.18** the latter method was used with the minor adjustment of dissolving **3.17** in acetonitrile as it is insoluble in H_2O. Unfortunately the coupling reaction was unsuccessful. This could be due to bad quality starting material as the formation of **3.17** could not be conclusively confirmed with either ^{1}H NMR and MS. Alternatively the procedure of Alam *et al.* could also be ineffective for this specific reaction and a better result might have been obtained with the $Sc(OTf)_2$ catalyst. Due to the problems experienced this procedure was abandoned.

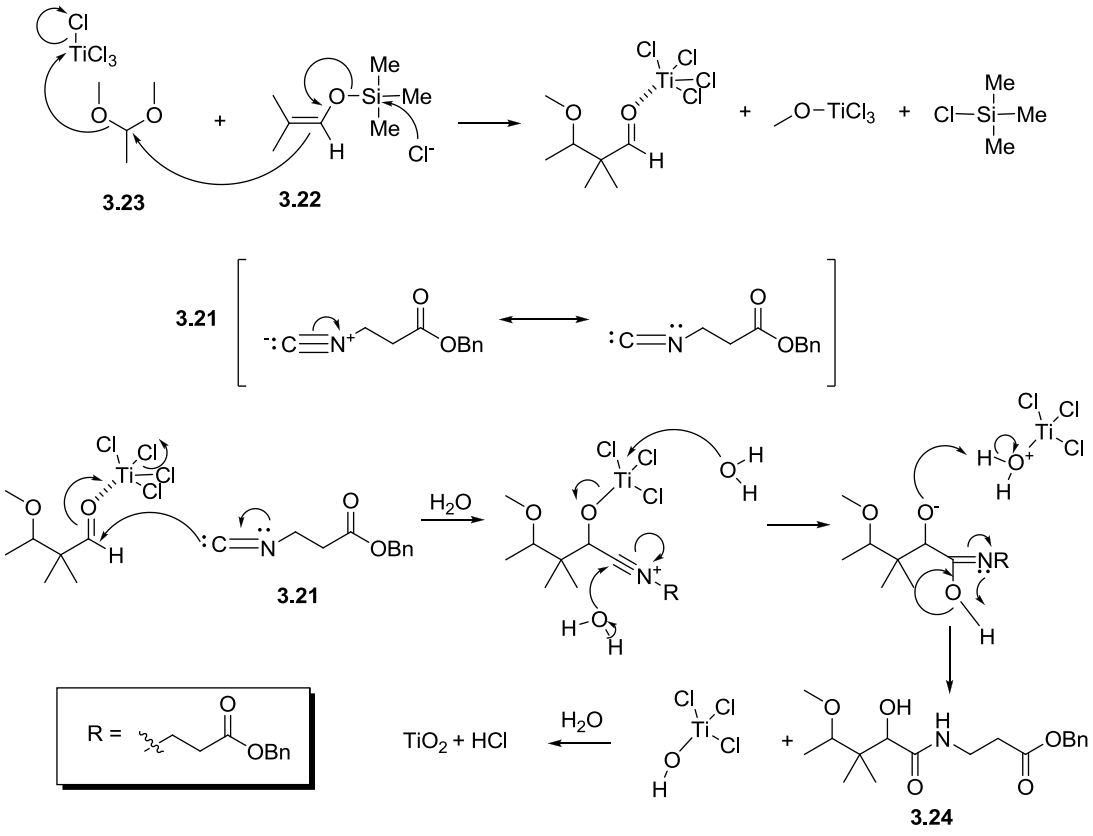
Strategy 2: MCR

Strategy 2 is based on a multi-component reaction (MCR) that makes use of an isocyanide as one of the reactants. Isocyanides (isonitriles, carbylamines) are organic molecules in which bonding occurs through reaction with the N- instead of the C-atom (in comparison to cyanide CN^-, where carbon exclusively acts as the nucleophile).[23] Isocyanides are known for their applications in the Passerini and Ugi MCRs, shown schematically in Scheme 3.5.



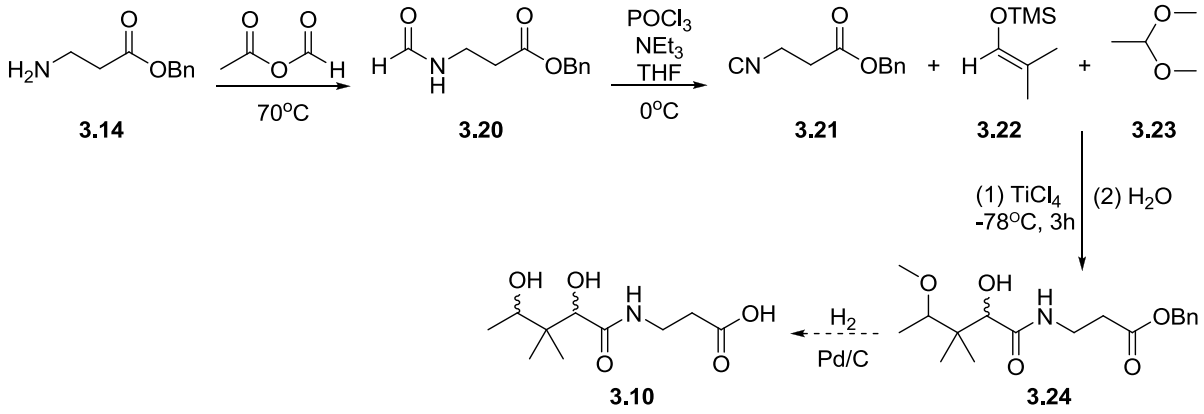
Scheme 3.5: Generalized scheme of the Passerini and the Ugi reactions

The Passerini reaction is a three component reaction utilizing an oxo-compound, an isocyanide and a nucleophile and the four component Ugi reaction requires a Schiff base/ enamine, a nucleophile and an isocyanide. Of the two, the Ugi reaction is more versatile in terms of the library sizes that can be assembled and in the range of scaffolds that can be used. The reaction of Strategy 2 is a three component MCR, but unlike the Ugi and Passerini reactions only C–C bonds are formed. The proposed mechanism is shown in Scheme 3.6.



Scheme 3.6: Proposed mechanism of the MCR

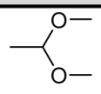
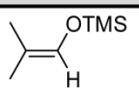
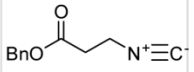
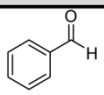
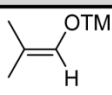
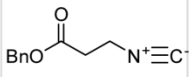
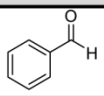
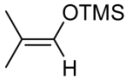
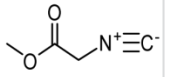
The synthetic route to **3.10** based on this MCR strategy is given in Scheme 3.7. Isocyanide **3.21** was synthesized from **3.14** according to a modified procedure of Elders *et al*.[24] β-Alanine benzyl ester (**3.14**) was formylated by addition of acetic formic anhydride in a single portion, followed by stirring at 70°C for 2 h. For purification Elders *et al*. only removed the volatiles *in vacuo,* but in our hands we were not satisfied that the CH_3COOH produced as side product was completely removed. An aqueous work-up step was therefore introduced in which the reaction mixture was dissolved in DCM and the organic layer subsequently washed with saturated $NaHCO_3$ and H_2O before the DCM was removed *in vacuo* to obtain the product. The changes in the purification procedure were sufficient to remove the CH_3COOH and increased the recovery of pure **3.21** from 18.9% to 63.9% (in comparison to purification with flash chromatography).



Scheme 3.7: Proposed synthesis of **3.10** based on an MCR (Strategy 2). The dehydration of **3.20** to **3.21** was done by the slow addition of the dehydration agent, $POCl_3$, to the solution at -78°C. The reactions stirred for an additional 3 h at 0°C. During this time the color changed from yellow to dark brown.

The procedure of Pellissier *et al.* was used as a starting point in the synthesis of **3.24.**[13] In their method, the reaction was performed at −60°C for 3h under inert conditions with a reagent ratio of 1.0 eq. acetal: 1.1 eq. TMS enol ether: 1.2 eq. isocyanide. The catalyst, $TiCl_4$, was diluted to 1.0 M using dry DCM and was added last to the reaction mixture via a dropping funnel. All our attempts to reproduce this MCR, including several variations of this original procedure, are summarized in Table 3.1.

Table 3:1: Summary of the variations made to the MCR.

				$TiCl_4$	Temp	Time	Comments
Variation 1							
Ideal eq	**1**	**1.1**	**1.2**	**1.2**	**-60°C**	**3h**	
Addition order	2	3	1	4			
Entry 1	2.8	1.6	3.3	6.0	-60°C	3h	As in literature
Entry 2	0.94	0.55	0.19	1.0	-60°C	3h	Isocyanide limiting
Entry 3	0.94	0.55	0.16	2.5	-78°C	3h	Lower temperature
Entry 4	0.94	0.55	0.24	2.5	-78°C	3h	Change work-up
Variation 2							
				$TiCl_4$	Temp	Time	Comments
Addition order	1	2	4	3			
Entry 5	1.0	0.55	0.27	1.5	-78°C	3h	Change order of addition
Entry 6	0.50	0.27	0.37	2.5	-78°C	4h	Change molar ratios
Entry 7	0.50	0.27	0.37	2.5	0°C	4h	Increase temperature
Variation 3							
				$TiCl_4$	Temp	Time	Comments
Addition order	1	2	4	3			
Entry 8	0.50	0.55	0.55	0.60	25°C	2h	Add catalyst neat

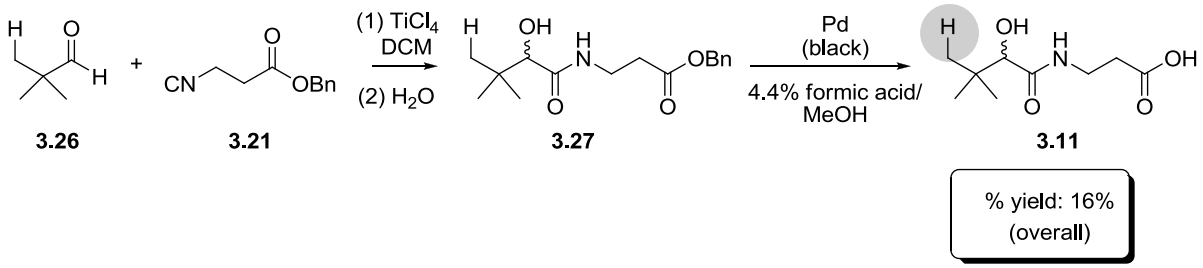
Entry 1 reflects the literature procedure, except that the amount of catalyst was increased. In Entries 2 to 4 the ratio of reactants was changed to render the isocyanide the limiting reagent; the same addition order was used as before. These changes were made mainly due to difficulties in the preparation of the isocyanide. In Entry 3, the temperature at which the reaction occurred was lowered further to −78°C from −60°C. The work-up was changed for Entry 4 by replacing diethyl ether with DCM. However, none of these modifications led to the formation of the desired product.

Based on the proposed mechanism shown in Scheme 3.5 the catalyst is required early in the mechanism, while the isocyanide is only involved later. In Variation 2 the sequence of addition was therefore changed - $TiCl_4$ was first added to a mixture of the acetal and TMS enol ether before addition of the isocyanide. The second change we made was to replace the acetal with benzaldehyde, since aldehydes are more reactive than acetals and benzyaldehyde is easier to detect by TLC, which makes the reaction easier to follow. Unfortunately again none of these reactions (Entries 5-7 in Table 3.1) were successful, even with increased reaction times and elevated temperatures.

The last variation attempted is shown in Entry 8. This reaction was done with a commercially available isocyanide. Several other changes were made to the procedure which included increasing the temperature at which the additions were made to 0°C, increasing the reaction temperature to 25°C, shortening the reaction time to 2 h and adding the catalyst in undiluted form. A product that was formed under these conditions was isolated, but ^{1}H NMR analysis showed that the benzaldehyde coupled directly to the isocyanide without involvement of the TMS enol ether. Due to these continued failures to prepare ω-methylpantothenic acid **3.10**, its synthesis was abandoned.

c) 4'-deoxypantothenic acid (3.11):

Our finding that an aldehyde couples directly to the isocyanide (result of Entry 8, Table 3.1 above) proved advantageous in our attempts at the synthesis of 4'-deoxypantothenic acid (**3.11**). A proposed synthesis that makes use of this reaction is shown in Scheme 3.8.



3.26 **3.21** **3.27** **3.11**

Scheme 3.8: Synthesis of 4'-dehydroxypantothenic acid (**3.11**).

Isocyanide **3.21** was coupled to pivaldehyde (**3.26**) according to the adapted methodology summarized in Variation 3 in Table 3.1. The catalyst was added neat to a solution of **3.21**

and **3.26** in DCM at 0°C, after which the reaction was stirred at 25°C for 2 h. The work-up was performed with DCM as organic solvent. A 17% overall yield was obtained for the benzyl protected product **3.27** (based on the formylation of **3.14** as the first step). 4'-Deoxypantothenic acid (**3.11**) was formed in 16% overall yield after deprotection with Pd (black) in 4.4% formic acid/methanol.[8]

The drawback of the methodology is that a racemic mixture was obtained due to lack of steric control on the formation of the new chiral center. PanK enzymes require the 2'-OH group of its substrates to be in the *R*-configuration, and the consequence of a racemic mixture is only one of the enantiomers would be an alternative substrate/inhibitor. In general steric control on the formation of new stereocenters in MCRs are challenging[25] and only two options have been identified for stereoselective synthesis under certain conditions – the use of chiral starting compounds[23] and the use of chiral Lewis acid catalysts.[26] Since the starting materials for our MCR are achiral, the use of a chiral catalyst is the only viable option. Based on the activity results obtained for this compound with *Pa* PanK-III (discussed in next section), the asymmetric synthesis was not investigated further.

3.5.3 Activity screening of pantothenate analogs as substrates/inhibitors

All the pantothenate analogs (Figure 3.1) were first screened to determine whether they are substrates or inhibitors (or both) of *Pa* PanK-III. For activity determination a coupled enzymatic assay was used which links the formation of ADP to the consumption of NADH.[27] This assay is extensively used to characterize PanK activity and coupling enzymes were added in excess as to not be rate-limiting. To test whether the compounds were substrates, reaction mixtures containing 500 µM of a particular analog were assayed by monitoring the decrease in absorbance of NADH followed at 340 nm. The absorbance profiles were compared to that of a blank (in which substrate was replaced with water) and a positive control (in which pantothenic acid was added as substrate). In cases where the absorbance profile showed a noticeable decrease in absorbance at 340 nm over time, the compound was considered to act as an alternate substrate and its activity was characterized further.

To determine if the analogs function as inhibitors of the normal PanK-catalyzed reaction, reaction mixtures containing equal amounts of pantothenic acid and a particular analog were assayed. Any reaction mixture which showed reduced activity compared to a positive

control reaction (containing only pantothenic acid), was deemed to contain a compound that acted as an inhibitor that could be characterized further. The results of this initial screen are given in Table 3.2.

Table 3.2: Results from initial screen for substrates and inhibitors

Compound	Substrate	Inhibitor
(*R,S*)-β-methylpantothenic acid (**3.1**)	√	√
(*R,S*)-α-methylpantothenic acid (**3.2**)	×	√
α,α-dimethylpantothenic acid (**3.3**)	×	×
α-pantothenic acid (**3.4**)	×	*Not tested*
homo-pantothenic acid (**3.5**)	×	*Not tested*
panthenol (**3.6**)	×	×
(*R,S*)-pantoyltaurine (**3.7**)	×	×
(*R,S*)-β-trifluoromethylpantothenic acid (**3.8**)	×	√
4'-methoxypantothenic acid (**3.9**)	×	×
4'-deoxypantothenic acid (**3.11**)	×	×

The results show that the substrate specificity of PanK-III is even more stringent than originally anticipated, since only one analog, β-methylpantothenic acid, acted as an alternate substrate. Moreover, only three analogs were found to act as inhibitors: α- and β-methylpantothenic acid, and β-trifluoromethylpantothenic acid.

3.5.4 Characterization of alternate substrate

The kinetic characterization of β-methylpantothenic acid was done using the same assay as described above in the presence of a final concentration of 5.0 mM ATP. The initial rates were measured and plotted against the concentrations of the respective substrates used (Figure 3.3). The kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation (1) and are given in Table 3.3 along with previously reported values for pantothenic acid for comparison purposes.[3]

$$v = \frac{V_{max} \times [S]}{K_M + [S]} \qquad (1)$$

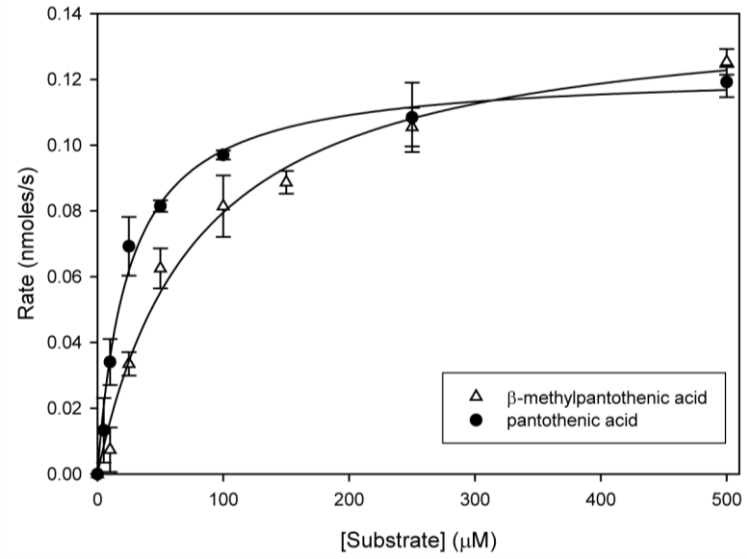


Figure 3.3: The steady state kinetics of *P. aeruginosa* PanK-III with pantothenic acid (•) and β-methylpantothenic acid (Δ) as substrates. Each point represents the average of three readings and the bars the standard error. Solid lines represent the best-fit of the data to the Michaelis-Menten equation.

Table 3.3: Kinetic parameters of the PanK-III substrates.

Kinetic parameters	V_{max}	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}.mM^{-1}$)
Pantothenic acid	0.123 ± 0.003	24.5 ± 2.7	0.793 ± 0.022	32.3 ± 8.2
Pantothenic acid (published)	not reported	20	0.27	13.3
(*R,S*)-β-Methylpantothenic acid	0.118 ± 0.004	77.4 ± 7.5	0.102 ± 0.003	1.32 ± 0.44

3.5.5 Inhibitor characterization

For the inhibitor characterization, the initial rates were measured over a range of pantothenic acid concentrations in the presence of increasing concentrations of inhibitor. The initial rates were measured and plotted against the concentrations of the substrates used and fitted to the Michaelis-Menten equation to obtain the apparent K_M-values (K_M^{app}). This data was subsequently used to determine the type of inhibition and the respective K_i-values.

Both α-methylpantothenic acid **3.2** and β-trifluoromethylpantothenic acid **3.8** were found to act as competitive inhibitors based on the observed increase in the K_M^{app}-values with increasing inhibitor concentration (Figure 3.4A and B). Moreover, a plot of K_M^{app} against inhibitor concentration clearly gave straight lines in both cases, indicating a directly proportional relationship between K_M^{app} and K_M/K_i as expected for competitive inhibitors.

The kinetic parameters, V_{max}, K_M and K_i, for these two inhibitors were therefore determined by non-linear regression analysis by fitting the data to the equation describing single substrate/single inhibitor competitive inhibition (Equation 2).

$$v = \frac{V_{max}[S]}{[S] + K_M(1 + \frac{[I]}{K_i})} \qquad (2)$$

However, the Michaelis-Menten fits suggest that β-methylpantothenic acid **3.1** shows classical uncompetitive behavior as shown in Figure 3.4C. The apparent V_{max} decreases as the concentration of inhibitor increases, while the K_M^{app} values stay nearly constant. For uncompetitive inhibition a plot of $^1/V_{max}$ against the inhibitor concentration should result in a straight line. For β-methylpantothenic acid only the lowest three inhibitor concentrations appear to follow this trend (a linear regression fit through these points and the control without inhibitor yields a $R^2 = 0.999$). In contrast, a plot of K_M^{app}-values against the inhibitor concentrations gives a straight line for the inhibitor concentrations higher than 150 µM ($R^2 = 0.985$), suggesting that competitive inhibition predominates at higher inhibitor concentrations. The inhibition modality of β-methylpantothenic acid therefore seems to be dependent on inhibitor concentration, and may in fact be influenced by this analog's dual ability to act as both substrate and inhibitor.

The kinetic parameters for β-methylpantothenic acid were determined by fitting the high inhibitor concentrations data to equation 2 (to obtain the parameters for competitive inhibition) while low concentrations data were fitted to the equation describing single substrate/single inhibitor uncompetitive inhibition (Equation 3).

$$v = \frac{V_{max}}{1 + \frac{[I]}{K_i} + \frac{K_M}{[S]}} \qquad (3)$$

Legend for Figure 3.4 (next page):
Characterization of the inhibition of *Pa* PanK-III by various pantothenic acid analogs.
Panel A: Inhibitor: α-methylpantothenic acid, Panel B: Inhibitor: β-trifluoromethylpantothenic acid, Panel C: Inhibitor: β-methylpantothenic acid.
Left: Michaelis-Menten fits to the plots of initial rates over a range of pantothenic acid concentrations in the presence of increasing concentrations of inhibitor. Each point represents the average of three readings and the bars the standard error.
Right (top three graphs): Plots of the K_M^{app}-values vs. inhibitor concentration for competitive inhibition. Right (bottom graph): Plot of $1/V_{max}$ vs. inhibitor concentration for uncompetitive inhibition

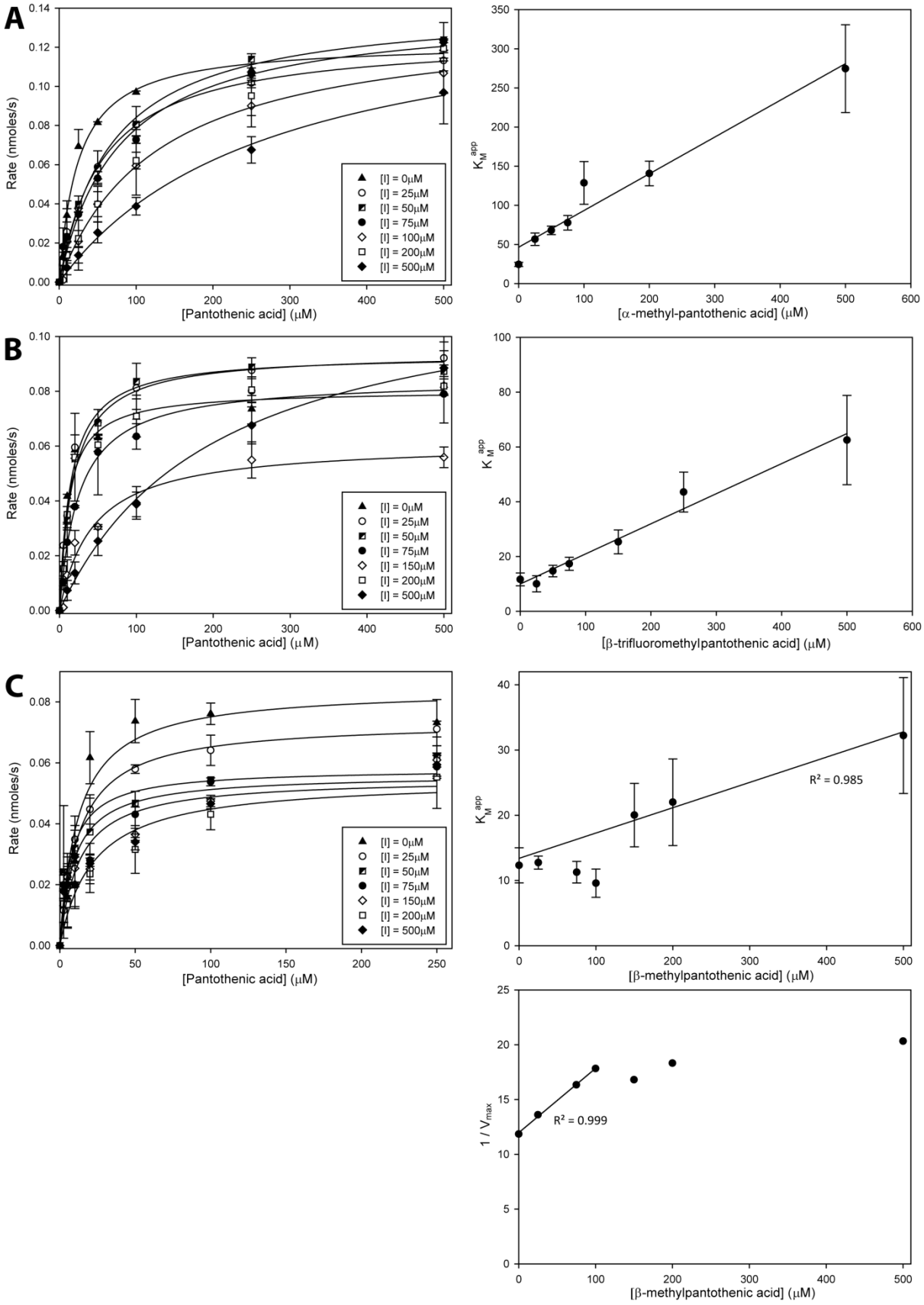


Figure 3.4: Characterization of the inhibition of *Pa* PanK-III by various pantothenic acid analogs. Legend supplied on previous page.

The kinetic parameters obtained in this manner for all the analogs are summarized in Table 3.4. Interestingly the K_i-values obtained for β-methylpantothenic acid for the high inhibitor concentrations was lower than the inhibitor concentrations where competitive inhibition was observed. Although this effect is not clearly understood at present, it might largely be due to the analog's dual ability to act as substrate and inhibitor.

Table 3.4: Summary of inhibition data

Kinetic parameters	Type of inhibition	V_{max}	K_M (μM)	K_i (μM)
(*R,S*)-α-methylpantothenic acid	competitive	0.132 ± 0.001	39.4 ± 4.7	96.1 ± 17.7
(*R,S*)-β-trifluoromethylpantothenic acid	competitive	0.0947 ± 0.053	8.69 ± 3.31	73.4 ± 42.5
(*R,S*)-β-methylpantothenic acid	uncompetitive (low concentrations)	0.0781 ± 0.0050	13.1 ± 2.2	358 ± 79
(*R,S*)-β-methylpantothenic acid	competitive (high concentrations)	0.0655 ± 0.0030	6.56 ± 1.80	74.6 ± 30.3
Best ATP mimetic	competitive			164

3.6 Discussion

3.6.1 Synthesis of pantothenate analogs modified in the β-alanine moiety

The synthesis of the compounds containing modified β-alanine moieties was done by the nucleophilic ring-opening of pantolactone by the relevant amines. These reactions were initially performed according to the method of Meier *et al*.[5] in which a three-fold excess of pantolactone relative to amine was used. However, we found that since both the desired analogs and pantolactone were water-soluble, organic extraction could not successfully be used for removal of the unreacted starting material. In addition, the removal of excess NEt_3 by cation exchange chromatography resulted in some of the product reverting back to pantolactone due to acid-catalyzed lactonization. In contrast, the melting reaction that was used instead made use of only two starting materials – pantolactone and the amine – in equal amounts. This greatly simplified the purification of the products, successfully achieved using flash chromatography on silica gel as final purification step.

However, a drawback of these melting reactions were that the overall yields were found to be low, ranging between 6.5% for β-trifluoromethylpantothenic acid to 40% for α-methylpanthothenic acid. Attempts to increase yields by increasing the amount of pantolactone from 1.0 to 1.1 eq. only gave a slightly increased yield in the case of α-methyl-

panthothenic acid. For α,α-dimethylpantothenic acid the low yield may be due to the steric bulk of the geminal dimethyl-groups on the amine which interferes with the nucleophilic ring-opening reaction. Similarly, the highly electronegative F-substituents would decrease the nucleophilicity of the amine group of 3-amino-4,4,4-trifluorobutyric acid, which in turn would hinder the formation of the amide bond in the case of β-trifluoromethylpantothenic acid. This analog also proved to be especially difficult to purify due to problems with removal of the solvents, and required repeated dissolution and lyophilization cycles to be isolated. We postulate that the electronegative F's form strong interactions with the solvents molecules (methanol and ethylacetate) which made them difficult to evaporate.

None of these syntheses were optimized further beyond these initial synthesis attempts as sufficient amounts of pure products were obtained to undertake the *in vitro* activity screens.

3.6.2 Synthesis of pantothenate analogs modified in the pantoyl moiety

For the modification of the 4'-OH of pantothenate we exploited the fact that the primary hydroxyl group is more reactive than the secondary 2'-OH. This difference in reactivity is further influenced by the steric hindrance of the latter by adjacent geminal dimethyl-groups. Selective methylation of the 4'-OH could therefore be performed as confirmed by COSY NMR analysis. However, benzyl pantothenate (**3.12**) proved to be less stable than anticipated and the molecule was found to decompose with the addition of bases such as NaH and NaOH, but the synthesis was successful when anhydrous carbonate was used as base instead.

The synthesis of ω-methylpantothenic acid proved more difficult. The first reported synthesis of this analog used CN^- and required extensive extraction and purification steps. In an attempt to design a safer and less complicated procedure, aldol condensations and multi-component reactions (MCRs) were investigated to introduce the key carbon-carbon bonds. Unfortunately, the aldol condensation failed to give products regardless of the strategy used, mainly due to the failure of the aldehyde acceptors to react with the TMS enol ether donors, which were both commercially obtained and synthesized before use. Currently it remains unclear why these reactions failed, as examples of similar reactions are well documented. In the case of the MCR reaction, the isocyanide was found to couple directly to the aldehyde without any participation of the TMS enol ether. Although this

finding made it impossible to prepare ω-methylpantothenic acid according to this route, the reaction was subsequently exploited in the successful synthesis of for 4'-deoxypantothenic acid (**3.11**).

3.6.3 *In vitro* assays

P. aeruginosa PanK-III was used in all the *in vitro* activity studies as a crystal structure is available (PDB: 2F9T and 2F9W) and its kinetic parameters are the most representative of this type of PanK. For *B. anthracis* PanK-III by contrast a crystal structure is also available, but it exhibits an uncommonly "low" K_M-value for ATP.

Pa PanK-III showed a surprisingly high selectivity for its substrate. Of the 11 pantothenate analogs tested, only β-methylpantothenic acid **3.1** was characterized as an alternative substrate for the enzyme. The kinetic characterization was done with a coupled enzymatic assay that links the amount of ADP produced to the consumption of NADH.[27] A good correlation was obtained for the parameters determined using this assay and published kinetic parameters determined by radioactive assay with D-[1-^{14}C] pantothenic acid.[3] The kinetic parameters indicate that β-methylpantothenic acid had a K_M-value of only about three times higher than pantothenic acid, but its specificity constant was ≈ 25 times lower (1.32 $s^{-1}.mM^{-1}$ compared to the 32.3 $s^{-1}.mM^{-1}$ of pantothenic acid). The differences in both parameters suggest that the introduced methyl group not only influences the binding of the analog, but also the enzyme's ability to phosphorylate its 4'-OH.

Only three compounds were identified as PanK-III inhibitors: α-methylpantothenic acid **3.2**, β-methylpantothenic acid **3.1** and β-trifluoromethylpantothenic acid **3.8**. Surprisingly, none of the analogs with modified pantoyl moieties – including 4'-deoxypantothenic acid, in which the only modification is the exchange of an OH-group for a hydrogen – was observed to be inhibitors. This suggests that active site interactions with the 4'-OH group of the substrate is not only important for catalysis, but also for binding.

There are three types of inhibition - competitive, uncompetitive and noncompetitive. In competitive inhibition the inhibitor competes with the natural substrate (S) for binding to the free enzyme (E). Uncompetitive inhibitors bind to the substrate bound enzyme (ES) binary complex and not to the free enzyme (E), while noncompetitive inhibitors bind both the free enzyme (E) and the ES binary complex.[29] The results show that

α-methylpantothenic acid and β-trifluoromethylpantothenic acid both function as competitive inhibitors, while β-methylpantothenic acid shows a mixed behavior, acting as uncompetitive inhibitor at low inhibitor concentrations, but as a competitive inhibitor at high concentrations.

Inhibitor analysis showed that α-methylpantothenic acid and β-trifluoromethylpantothenic acid have very similar K_i-values, in the range of 80-90 µM. Importantly, when the methyl group of β-methylpantothenic acid was substituted for CF_3, the observed K_i-value decreased by three-fold. This strongly suggests that the electronegative nature of the F's increases the potency of the inhibitor, and that that factors that influence electrostatic interactions therefore have greater potential for inhibitor development than those that depend on steric effect. Based on this result, it would be interesting to determine whether this same lowering in K_i would be observed if α-trifluoromethylpantothenic acid was tested as inhibitor. Since the measured K_i-value for α-methylpantothenic acid is already below ≈ 100 µM, a further reduction could lower the K_i-value to ≈ 30 µM, which would be an important step closer to PanK-III inhibitors with K_i-values in the nM range.

3.7 Conclusion

In this part of the study several pantothenic acid analogs were tested as PanK-III inhibitors. Although the K_i-values obtained for the active inhibitors are all in the µM range, these values are an important improvement on the inhibition characteristics of the best hit obtained from the study of ATP mimetics as PanK-III inhibitors.[1] It is important to note, however, that in this study all compounds were tested as mixtures of the stereoisomers (in regards to the stereochemistry of the introduced methyl groups). It may therefore be possible that of the two stereoisomers tested in each case (the 2'-OH of all three active inhibitors had the required R-configuration), only one acts as an inhibitor. The true K_i-values may therefore be half or less of those reported in this study, assuming that the stereoisomers were present in equal amounts (this assumption was not confirmed with polarimetry). With this said, only two compounds have K_i-values low enough to justify resolution of the stereoisomers and testing of this proposal, namely α-methylpantothenic acid **3.2** and β-rifluoromethylpantothenic acid **3.8**. Such resolution studies will be attempted in future work.

Other future work will also include redesigning the synthesis of ω-methylpantothenic acid and, based on the initial success of the fluorinated analog **3.8**, the synthesis of other selected fluorinated compounds.

3.8 Experimental

3.8.1 Synthesis

a) (*R,S*) -β-methyl-pantothenic acid (3.1)

(*R,S*) -3-aminobutyric acid (222.7 mg, 2.16 mmol) was dissolved in 4.0 M NaOH (540 µl, 2.16 mmol) and lyophilized. Pantolactone (246.6 mg, 1.89 mmol) was added to the flask and stirred at 130°C overnight under inert atmosphere. The resulting black, sticky substance was dissolved in water and purified by ion exchange chromatography with weakly acidic Amberlite IRC-86 resin (≈ 1.0 g). The eluent was lyophilized and purified with flash chromatography (silica, 5:1:1:1 ethylacetate: methanol: acetonitrile: H_2O). R_f = 0.38; Product: Yellow oil, very hygroscopic; Yield: 128.2 mg, 0.67 mmol, 31%; ^{1}H NMR (300 MHz, D_2O, 25°C): δ = 0.88 (d, J = 1.8 Hz, 3H, -CH_3) 0.91 (d, J = 3.18 Hz, 3H, -CH_3) 1.21 (dd, J = 6.75 Hz, J = 2.27 Hz, 3H, -CH_3) 2.58 (m, J = 2.02 Hz, 2H, -CH_2) 3.37 (dd, J = 36.5 Hz, J = 11.2 Hz, 2H, -CH_2-O) 3.95 (d, J = 5.02 Hz, 1H, -CH-O) 4.29 ppm (m, J = 6.45 Hz, 1H, -CH-N); ^{13}C NMR (300 MHz, D_2O, 25°C): δ = 14.93, 19.76, 21.12, 39.22, 40.24, 42.22, 42.26, 69.08, 76.48, 77.60 ppm; MS–ESI: *m/z* $[M+1]^+$ calculated for $C_{10}H_{20}NO_5$: 234.13, found: 234.24; $[M-1]^-$ calculated for $C_{10}H_{18}NO_5$: 232.12; found: 232.09

b) (*R,S*) -α-methyl-pantothenic acid (3.2)

(*R,S*)-3-amino-isobutyric acid (47.9 mg, 0.465 mmol) was dissolved in 1.3 M NaOH (357 µl, 0.465 mmol) and lyophilized. Pantolactone (80.2 mg, 0.616 mmol) was added to the flask and stirred at 130°C overnight under inert atmosphere. The resulting black, sticky substance was dissolved in water and purified by ion exchange chromatography with weakly acidic Amberlite IRC-86 resin (≈ 1.0 g). The eluent was lyophilized and purified with flash chromatography (silica, 5:1:1:1 ethylacetate: methanol: acetonitrile: H_2O). R_f = 0.38; Product: Pinkish yellow oil; Yield: 23.8 mg, 0.186 mmol, 40%; ^{1}H NMR (400 MHz, D_2O, 25°C): δ = 0.90 (s, 3H, -CH_3) 0.93 (s, 3H, -CH_3) 1.17 (d, J = 7.12 Hz, 3H, -CH_3) 2.74 (q, J = 7.21 Hz, 1H, -CH-) 3.38 (m, J = 7.88 Hz, 2H, - CH_2) 3.39 (dd, J = 48.2 Hz, J = 11.5 Hz, 2H, -CH_2-N)

3.99 ppm (d, J = 2.37 Hz, 1H, - CH-O); ^{13}C NMR (400 MHz, D_2O, 25°C): δ = 14.93, 19.77, 21.12, 39.22, 40.24, 40.28, 42.22, 42.26, 69.08, 76.48 ppm; MS–ESI: *m/z* $[M+1]^+$ calculated for $C_{10}H_{20}NO_5$: 234.13, found: 234.14; $[M-1]^-$ calculated for $C_{10}H_{18}NO_5$: 232.12; found: 232.09

c) α,α-dimethyl-pantothenic acid (3.3)

3-amino- 2,2-dimetyl propanoic acid hydrochloric salt (75.1 mg, 0.49 mmol) was dissolved in 2 eq. 1.0 M NaOH (978 µl, 0.98 mmol) and lyophilized. Pantolactone (57.9 mg, 0.45 mmol) was added to the flask and stirred at 130°C overnight under inert atmosphere. The resulting black, sticky substance was dissolved in water and purified by ion exchange chromatography with weakly acidic Amberlite IRC-86 resin (≈ 1.0 g). The eluent was lyophilized and purified with flash chromatography (silica, 5:1:1:1 ethylacetate: methanol: acetonitrile: H_2O). Product: white powder; R_f = 0.33; Yield: 33.7 mg, 0.137 mmol, 28%; ^{1}H NMR (400 MHz, D_2O, 25°C): δ = 0.91 (s, 3H, -CH_3) 0.94 (s, 3H, -CH_3) 1.21 (s, 6H, 2 × -CH_3) 3.39 (dd, J = 47.52 Hz, J = 11.9 7Hz, 2H, -CH_2-) 3.41 (s, 2H, - CH_2) 4.03 ppm (s, 1H, -CH-O); ^{13}C NMR (400 MHz, D_2O, 25°C): δ = 19.80, 21.09, 23.16, 23.20, 39.22, 43.67, 47.29, 69.05, 76.47, 175.72 ppm; MS–ESI: *m/z* $[M+1]^+$ calculated for $C_{11}H_{21}NO_5$: 248.15, found: 248; $[M-1]^-$ calculated for $C_{11}H_{19}NO_5$: 246.13; found: 246

d) (*R,S*) - β-trifluoromethyl pantothenic acid (3.8)

(*R,S*) -3-amino-4,4,4-trifluorobutyric acid (54.2 mg, 0.35 mmol) was dissolved in 1.3 M NaOH (265 µl, 0.35 mmol) and lyophilized. Pantolactone (64.1 mg, 0.49 mmol) was added to the flask and stirred at 130°C overnight under inert atmosphere. The resulting residue was dissolved in water and purified by ion exchange chromatography with weakly acidic Amberlite IRC-86 resin (≈ 1.0 g). The eluent was lyophilized and purified with flash chromatography (silica, 5:1:1:1 ethylacetate: methanol: acetonitrile: H_2O). R_f = 0.38 Product: yellow oil. Yield: 6.5 mg, 0.023 mmol, 6.5%; ^{1}H NMR (400 MHz, D_2O, 25°C): δ = 0.90 (d, J = 2.95 Hz, 3H, -CH_3) 0.94 (d, J = 3.37 Hz, 3H, -CH_3) 2.74 (dm, J = 80.37 Hz, 2H, -CH_2) 3.38 (qd, J = 43.53 Hz, J = 11.01 Hz, J = 2.27 Hz, 2H, - CH_2) 4.06 (d, J = 9.29 Hz, 1H, -CH-O) 5.02 ppm (broad s, 1H, - CH-CF_3); ^{19}F NMR (400 MHz, D_2O, 25°C): δ = -75.00 ppm (d, J = 7.62 Hz, 3F); *m/z* $[M+1]^+$ calculated for $C_{10}H_{17}F_3NO_5$: 288.10, found: 288; $[M-1]^-$ calculated for $C_{10}H_{15}F_3NO_5$: 286.09, found: 286

e) Pantothenate benzyl ester (3.12)

Benzyl bromide (500 µl, 4.02 mmol) was added to calcium pantothenate (1.00 g, 4,26 mmol) dissolved in 15 ml dry DMF and heated at 70°C overnight. DMF was removed *in vacuo*, ethylacetate (100 ml) added to the residue and the organic layer washed with aq NaCl (3 × 20 ml) before drying over Na_2SO_4. After the solvent was removed *in vacuo,* **3.12** was purified with flash chromatography (silica gel; ethylacetate:hexane, gradient from 2:1 to 4:1). The product was confirmed by ^{1}H NMR and MS. Product: yellow oil; R_f = 0.23; Yield: 704.2 mg, 2.27 mmol, 56.6%.

f) 4'-methoxy pantothenate benzyl ester (3.13)

To **3.12** (100 mg, 0.32 mmol) dissolved in 1.6 M dry DMF (final concentration = 0.2 M) was added 0.55 eq. K_2CO_3 (24.5 mg, 0.18 mmol) and 1.1 eq. MeI (53 µl, 0.35 mmol) and heated at 70°C overnight. DMF was removed *in vacuo*, ethylacetate (25 ml) added, the organic layer washed with H_2O (3 ×5.0 ml) and dried over Na_2SO_4. After the solvent was removed the product was purified with flash chromatography (silica gel; ethylacetate:hexane, 3:1). Product: yellow oil; R_f = 0.45; Yield:39 mg, 0.12 mmol, 38%; ^{1}H NMR (400 MHz, $CDCl_3$, 25°C): δ = 0.90 (s, 3H, -CH_3) 0.96 (d, J = 3.21 Hz, 6H, 2 × -CH_3) 2.59 (t, J = 6.32 Hz, 2H, -CH_2) 3.19 (dd, J = 38.22 Hz,J = 8.94 Hz, 2H, -CH_2) 3.33(3, 3H, - CH_3) 3.54 (m, 2H, -CH_2-N) 3.94 (d, J = 3.79 Hz, 1H, -CH-O) 4.21 (d, J = 4.36 Hz, 2H, unknown) 5.13, (s, 2H, -CH_2) 7.13 (broad s, 1H, -NH) 7.35 ppm (m, 5H, benzyl ring); ^{13}C NMR (400 MHz, $CDCl_3$, 25°C): δ = 20.59, 22.09, 34.53, 34.75, 38.57, 59.57, 66.82, 78.79, 82.66, 128.56, 128.64, 128.89, 172.33, 172.40 ppm; Methylation was also confirmed by COSY (400 MHz, $CDCl_3$, 25°C); *m/z* $[M+1]^+$ calculated for $C_{17}H_{26}NO_5$: 324.18; found: 324.5

g) 4'-methoxy pantothenic acid (3.9)

Compound **3.13** (34 mg, 0.11 mmol) was dissolved in 4.4% formic acid/methanol (2.0 ml) and stirred at room temperature. Pd (black) (20 mg) was added and the reaction and stirred until all starting material was consumed (based on TLC analysis, 3:1 ethylacetate: hexane, R_f = 0.54). The Pd (black) was removed by filtration through a cotton wool plug and washed with methanol (10 ml). The solvent was removed *in vacuo*, the residue dissolved in 1.0 M HCl (10 ml) and heated to reflux for 24 h. The reaction mixture was cooled to room temperature and lyophilized and purified with flash chromatography (silica, 5:1:1:1 ethylacetate: methanol: acetonitrile acetonitrile: H_2O). R_f = 0.40; Product: yellow oil; Yield:

11.68 mg, 0.05 mmol, 45% yield for step, 15% overall yield; ^{1}H NMR (300 MHz, D_2O, 25°C): δ = 0.93 (s, 3H,-CH_3) 0.95 (s, 3H, -CH_3), 2.61 (t, J = 6.53 Hz, 2H, -CH_2) 3.29 (dd, J = 26.99 Hz, J = 9.69 Hz, 2H,-CH_2-O) 3.34 (s, 3H, -O-CH_3) 3.49 (td, J = 6.39 Hz, J = 2.09 Hz, 2H, -CH_2-N) 3.96 ppm (s, 1H,O-CH-N); ^{13}C NMR (300 MHz, D_2O, 25°C): δ = 20.57, 21.50, 34.34, 35.44, 38.76, 59.34, 76.41, 79.70, 175.50, 176.97 ppm; Methylation was confirmed by COSY (300 MHz, D_2O, 25 °C); *m/z* $[M+1]^+$ calculated for $C_{10}H_{20}NO_5$: 234.14, found: 234; $[M-1]^-$ calculated for $C_{10}H_{18}NO_5$: 232.12, found: 232

h) β-alanine benzyl ester hydrochloric salt (3.14)

To β-alanine (4.15 g, 47 mmol) was added benzyl alcohol (25 ml) and TMS-Cl (10 ml). After stirring at 100°C for 3 h the mixture was poured in diethyl ether (500 ml) and left at 4.0°C overnight. The resulting white crystals were filtered, washed with cold diethyl ether and oven dried. The product was confirmed by ^{1}H NMR and MS. Yield: 8.95 g (2:1 mixture of **3.14** and β-alanine).

i) *N*-acryloyl β-alanine benzyl ester (3.16)

To **3.14** (366 mg, 2.04 mmol) dissolved in DCM (10 ml) was added iPr_2NEt (365 µl, 2.13 mmol) and acryloylchloride (**3.15**) (182 µl, 2.24 mmol). After stirring at 0°C for 4 the reaction was quenched by the addition of 1.0 M HCl (5.0 ml). The organic layer was washed with saturated $NaHCO_3$ (2 × 5.0 ml) and the solvent removed *in vacuo*. The product was purified with flash chromatography (silica, 3:1 ethylacetate:hexane); R_f = 0.47; Product: light yellow oil; Yield: 273 mg, 1.17 mmol, 57.4%; ^{1}H NMR (400 MHz, $CDCl_3$, 25°C): δ = 2.62 (t, J = 5.81 Hz, 2H,-CH_2) 3.60 (q, J = 6.12, 2H, -CH_2) 5.14 (s, 2H, -CH_2) 5.62 (d, J = 10.03 Hz, 1H, =CH_2) 6.00 (dd, J = 16.97 Hz, J = 10.18 Hz, 1H, =CH-) 6.15 (broad s, 1H, -NH) 6.23 (d, J=18.42 Hz 1H, =CH_2), 7.35 ppm (m, 5H, benzyl ring); ^{13}C NMR (400 MHz, $CDCl_3$, 25°C): δ = 33.96, 35.08, 66.46, 126.31, 128.13, 128.31, 128.57, 130.85, 165.90, 172.16 ppm

j) Oxidative cleavage product 3.17

N-acryloyl β-alanine benzyl ester (**3.16**) (137 mg, 0.59 mmol) was dissolved in 7.5 ml 5.0% H_2O/acetone and Sudan Red III added until the mixture was slightly pink. The flask was cooled to 0°C and O_3 bubbled through until the solution turned colorless. The reaction was purged by bubbling O_2 through the solution for 1 min after which H_2O was added (25 ml), the aqueous layer extracted with DCM (2 × 25 ml) and dried over Na_2SO_4. Based on NMR

analysis ≈ 20% of **3.16** was converted to the desired product **3.17**. Yield: not calculated; ^{1}H NMR (400 MHz, $CDCl_3$, 25°C) only given as indication since characterization results are dubious: δ = 2.64 (m, 2H, -CH_2) 3.56 (m, 2H, -CH_2) 5.14 (s, 2H, -CH_2) 7.35 (m, 5H, benzyl ring) 9.27 ppm (s, theoretically 1H, H-CO); MS: analysis unsuccessful.

k) TMS silyl enol (3.18)

To 3-methyl-2-butanone (500 µL, 4.67 mmol) dissolved in DMF (12.5 ml) was added NEt_3 (2.2 ml, 15.77 mmol) and TMS-Cl (1.2 ml, 9.46 mmol). The reaction was stirred at 130°C overnight; hexane (50 ml) added and the organic layer washed with saturated $NaHCO_3$ (3 × 2 5 ml) and brine (1 × 25 ml). After drying overnight on anhydrous K_2CO_3, the solvent was removed *in vacuo* to give **3.18** as a dark brown oil. The product was confirmed by ^{1}H NMR; Yield: 87 mg, 0.3 mmol, 1.3%.

l) ω-methyl pantothenic acid precursor 3.19

To **3.17** (119 mg, 0.51 mmol) was dissolved in acetonitrile (500 µl) and added to **3.18** (87 mg, 0.3 mmol) in sodium phosphate buffer, pH 7.0 (2.5 ml, 25 mM). The reaction was stirred at room temperature for 36 h in the dark, the aqueous layer extracted with ethylacetate (5 × 5 ml) and purified with flash chromatography (silica, 3:1 ethylacetate: hexane). The isolated products were not the desired product.

m) *N*-formyl β-alanine benzyl ester (3.20)

β-alanine benzyl ester tosylate salt (1.0 g, 2.8 mmol) was dissolved in H_2O and $NaHCO_3$. NaOH was added until the pH ≈ 10.5 and **3.14** extracted from the aqueous solution with DCM (4 × 25 ml). The solvent was removed *in vacuo* with a recovery efficiency of 75%. To the colorless oil (437 mg, 2.44 mmol) was added acetic formic anhydride which was prepared by stirring acetic anhydride (2.5 ml) with formic acid (1.1 ml) for 2.5 h at 55°C. The reaction was stirred at 70°C for a further 2 h, the volatiles removed *in vacuo* and redissolved in DCM (25 ml). The organic layer was washed consecutively with saturated $NaHCO_3$ (2 × 25 ml) and H_2O (2 × 25 ml) before removing the solvent *in vacuo* to give **3.20**. Product: yellow oil, Yield: 323 mg, 1.56 mmol, 63.9% (calculated from after the recovery step). R_f = 0.36 (3:1 ethylacetate: hexane). The product was confirmed by ^{1}H NMR and MS.

n) *N*-isocyanide β-alanine benzyl ester (3.21)

To a solution of **3.20** (323 mg, 1.56 mmol) was added NEt_3 (1.7 ml) in dry THF (6 ml) at -78°C under inert atmosphere. $POCl_3$ (300 µl) in dry THF (6 ml) was added slowly while stirring continuously. After the addition was completed the reaction was stirred for an additional 3 h at 0°C. Over this period the color changed from yellow to dark orange to brown. The reaction was quenched by the addition of cold H_2O (50 ml) and the aqueous layer extracted with dietyl ether (3 × 30 ml). The combined organic layers were washed with H_2O (3 × 30 ml), dried over $MgSO_4$ and the solvent removed *in vacuo.* Product: black, foul smelling oil. The product was used as is without further purification. The product was confirmed by ^1H NMR and MS. Yield: ≈ 289.1 mg, ≈ 1.5 mmol, ≈ 97%.

o) 4'-deoxy pantothenic acid benzyl ester (3.27)

A solution of **3.22** (≈ 289.1 mg, ≈ 1.5 mmol) in dry DCM (10 ml) was cooled to 0°C under inert conditions. To the solution neat $TiCl_4$ was added and the reaction mixed thoroughly before addition of pivaldehyde (**3.26)** (200 µl, 1.84 mmol). The reaction was warmed to 25°C and stirred for a further 2 h before it was quenched by pouring into saturated K_2CO_3 (50 ml). The mixture was filtered through celite and the aqueous layer extracted with DCM (3 × 30 ml). The combined organic layers were washed with H_2O (3 × 30 ml), dried on $MgSO_4$ and the solvent removed *in vacuo* before purification with flash chromatography (silica, 1:1 ethylacetate: hexane). R_f = 0.25, Product: very light yellow, almost colorless oil; Yield: 123 mg, 0.42 mmol, 17%; ^1H NMR (400 MHz, $CDCl_3$, 25°C): δ = 0.94 (s, 9H, 3 × $-CH_3$) 2.59 (t, J = 6.12 Hz, 2H, $-CH_2$) 3.54 (q, J = 12.93 Hz, J = 6.67 Hz, 2H, $-CH_2$) 3.64 (d, J = 5.04 Hz, 1H, -O-CH) 5.13 (s, 2H, CH_2) 6.68 (broad s, 1H, -NH) 7.34 ppm (m, 5H, benzyl group); *m/z* $[M+1]^+$ calculated for $C_{16}H_{24}NO_4$: 294.17, found: 294.2; $[M-1]^-$ calculated for $C_{16}H_{22}NO_4$: 292.15; found: 292.2

p) 4'-deoxy pantothenic acid (3.11)

To **3.27** (m = 123 mg, 0.42 mol) in 20 ml 4.4% formic acid/methanol was added 200 mg Pd (black) and the reaction stirred overnight at room temperature. The Pd (black) was removed by filtration through a cotton wool plug and washed with methanol (3 ×1 0 ml). The solvent was removed *in vacuo*, the residue dissolved in 5.0 M HCl (20 ml) and refluxed for 24 h. The reaction mixture was cooled to room temperature and lyophilized. The resulting yellow oil was dissolved in a minimal amount of water and separated from the

insoluble residue. NMR confirmed that the water fraction, which contained the majority of the mass, was the desired product. Product: dark yellow oil. Yield: 116 mg, 0.40 mmol, 95% for step, 16% overall. ^{1}H NMR (400 MHz, D_2O, 25°C): δ = 0.94 (s, 9H, 3×-CH_3) 2.61 (t, J = 6.14 Hz, 2H, -CH_2) 3.48 (q, J = 12.09 Hz, J = 6.67 Hz, 2H, -CH_2) 3.76 ppm (s, 1H, -O-CH); ^{13}C NMR (400 MHz, D_2O, 25°C): δ = 25.90, 34.20, 34.65, 35.3, 79.88, 175.86, 176.74 ppm; *m/z* $[M+1]^+$ calculated for $C_9H_{18}NO_4$: 204.12, found: 204.1; $[M-1]^-$ calculated for $C_9H_{16}NO_4$: 202.11; found: 202.1

3.8.2 Activity determination

All reactions were performed in triplicate at 25°C in 96-well plates and the decrease of NADH measured with the Thermo Varioskan™ spectrophotometer at 340 nm over a period of 5 min. Each 300 µl contained 100 mM HEPES, pH 7.5, 1.0 mM $MgCl_2$, 60 mM NH_4Cl_2, 2.0 mM PEP, 0.5 mM NADH, 5.0 mM ATP, 2.75 U lactate dehydrogenase (LDH), 2.0 U pyruvate kinase (PK) and enzyme. The reactions were initiated with the addition of 500 µM substrate. To test whether a compound was an inhibitor, 500 µM pantothenic acid was added to each reaction.

3.8.3 Kinetic characterization

The assays were done as above. For the characterization as substrates, a range of increasing substrate concentrations was used. In the inhibitor studies a range of pantothenic acid concentrations were tested in the presence of a set concentration of inhibitor. All graphs were plotted and kinetic parameters determined using the SigmaPlot vs. 11 statistical package.

3.9 References

1. Rowan, A.S., Nicely N.I., Cochrane N., Wlassoff W.A., Claiborne A. and Hamilton C.J., Nucleoside triphosphate mimicry: a sugar triazolyl nucleoside as an ATP-competitive inhibitor of *B. anthracis* pantothenate kinase. *Organic & Biomolecular Chemistry*, 2009. **7** (19): p. 4029-36.
2. Yang, K., Strauss E., Huerta C. and Zhang H., Structural basis for substrate binding and the catalytic mechanism of type III pantothenate kinase. *Biochemistry*, 2008. **47** (5): p. 1369-80.
3. Hong, B.S., Yun M.K., Zhang Y.-M., Chohnan S., Rock C.O., White S.W., Jackowski S., Park H.-W. and Leonardi R., Prokaryotic type II and type III pantothenate kinases: The same monomer fold creates dimers with distinct catalytic properties. *Structure*, 2006. **14** (8): p. 1251-61.
4. van Wyk, M. and Strauss E., Development of a method for the parallel synthesis and purification of *N*-substituted pantothenamides, known inhibitors of coenzyme A biosynthesis and utilization. *Organic & Biomolecular Chemistry*, 2008. **6** (23): p. 4348-55.
5. Meier, J.L., Mercer A.C., Rivera H. and Burkart M.D., Synthesis and evaluation of bioorthogonal pantetheine analogues for *in vivo* protein modification. *Journal of the American Chemical Society*, 2006. **128** (37): p. 12174-84.
6. Pollack, M.A., Growth effects of α-methyl homologs of pantothenic acid and β-alanine. *Journal of the American Chemical Society*, 1943. **65** (7): p. 1335-39.
7. Strauss, E., *Thiols, radicals and antibiotics: mechanistic studies in coenzyme A biosynthesis,* PhD thesis (Department of Chemistry and Chemical Biology). 2003, Cornell University: Ithaca, NY. p. 182 pp.
8. de Villiers, J., Koekemoer L. and Strauss E., 3-Fluoroaspartate and pyruvoyl-dependant aspartate decarboxylase: exploiting the unique characteristics of fluorine to probe reactivity and binding. *Chemistry – A European Journal*, 2010. **16** (33): p. 10030-41.
9. Drell, W. and Dunn M.S., Growth inhibition of lactic acid bacteria by *N*-(α,γ-dihydroxy-β,β-dimethylvaleryl)-β-alanine and its reversal by pantothenic acid. *Journal of the American Chemical Society*, 1946. **68** (9): p. 1868-68.

10. Stiller, E.T., Harris S.A., Finkelstein J., Keresztesy J.C. and Folkers K., Pantothenic acid. VIII. The total synthesis of pure pantothenic acid. *Journal of the American Chemical Society*, 1940. **62**: p. 1785-90.

11. Evans, D.A., Wu J., Masse C.E. and MacMillan D.W.C., A general method for the enantioselective synthesis of pantolactone derivatives. *Organic Letters*, 2002. **4** (20): p. 3379-82.

12. Alam, J., Keller T.H. and Loh T.-P., Functionalization of peptides and proteins by Mukaiyama aldol reaction. *Journal of the American Chemical Society*, 2010. **132** (28): p. 9546-48.

13. Pellissier, H. and Gil G., Réaction des isonitriles IV. synthèse de γ-alkoxy α-hydroxyamides par double condensation d'énxysilane et du tert-butylisonitrile aux acétals. *Tetrahedron*, 1989. **45** (11): p. 3415-22.

14. Cho, S.W. and Romo D., Total Synthesis of (-)-Belactosin C and derivatives via double diastereoselective tandem Mukaiyama aldol lactonizations. *Organic Letters*, 2007. **9** (8): p. 1537-40.

15. Pappo, R., Allen J.D.S., Lemieux R.U. and Johnson W.S., Notes - Osmium tetroxide-catalyzed periodate oxidation of olefinic bonds. *The Journal of Organic Chemistry*, 1956. **21** (4): p. 478-79.

16. de Fatima, A. and Pilli R.A., Enantioselective approach to the asymmetric synthesis of (6R)-hydroxymethyl-5,6-dihydro-2H-pyran-2-one. A formal synthesis of (R)-argenti-lactone and total synthesis of (R)-goniothalamin. *ARKIVOC*: p. 118-26.

17. Schiaffo, C.E. and Dussault P.H., Ozonolysis in solvent/water mixtures: Direct conversion of alkenes to aldehydes and ketones. *The Journal of Organic Chemistry*, 2008. **73** (12): p. 4688-90.

18. Claus, R.E. and Schreiber S.L., Ozonolytic cleavage of cyclohexene to terminally differentiated products. Methyl 6-oxohexanoate, 6,6-dimethoxyhexanal, and methyl 6,6-dimethoxyhexanoate. *Org. Synth.*, 1986. **64**: p. no pp. given.

19. Pappas, J.J., Keaveney W.P., Gancher E. and Berger M., A new and convenient method for converting olefins to aldehydes. *Tetrahedron Letters*, 1966. **7** (36): p. 4273-78.

20. Templeton, J.F., Ling Y., Kumar V.P.S. and LaBella F.S., Synthesis and structure-activity relationships of 14-β-hydroxy-5α-pregnanes: Pregnanes that bind to the cardiac glycoside receptor. *Steroids*, 1993. **58**: p. 518-23.
21. Knowles, W. and Thompson Q., A convenient method for reduction of hydroperoxide ozonation products. *The Journal of Organic Chemistry*, 1960. **25** (6): p. 1031-33.
22. Veysoglu, T., Mitscher L.A. and Swayze J.K., A convenient method for the control of selective ozonizations of olefins. *Synthesis*, 1980: p. 807-10.
23. Dömling, A., Recent developments in isocyanide based multicomponent reactions in applied chemistry. *Chemical Reviews*, 2006. **106** (1): p. 17-89.
24. Elders, N., Schmitz R.F., de Kanter F.J.J., Ruijter E., Groen M.B. and Orru R.V.A., A Resource-efficient and highly flexible procedure for a three-component synthesis of 2-imidazolines. *The Journal of Organic Chemistry*, 2007. **72** (16): p. 6135-42.
25. Dömling, A. and Ugi I., Multicomponent reactions with isocyanides. *Angewandte Chemie International Edition*, 2000. **39** (18): p. 3168-210.
26. Denmark, S.E. and Fan Y., Catalytic, enantioselective α-Additions of isocyanides: Lewis base catalyzed Passerini-type reactions. *The Journal of Organic Chemistry*, 2005. **70** (24): p. 9667-76.
27. Strauss, E. and Begley T.P., The antibiotic activity of *N*-pentylpantothenamide results from its conversion to ethyldethia-coenzyme A, a coenzyme A antimetabolite. *Journal of Biological Chemistry*, 2002. **277** (50): p. 48205-09.
28. Yan, X., Akinnusi T.O., Larsen A.T. and Auclair K., Synthesis of 4'-aminopantetheine and derivatives to probe aminoglycoside *N*-6'-acetyltransferase. *Organic & Biomolecular Chemistry*, 2011. **9** (5): p. 1538-46.
29. Copeland, R.A., *Enzymes: A Practical Introduction to Structure, Mechanism and Data analysis*. 2nd ed. 2000, Hoboken, NJ, 2005: John Wiley & Sons Inc. 396.

Chapter 4:
M. tuberculosis CoaX

4.1 Introduction

The genome of *M. tuberculosis* (*Mtb*) encodes both PanK-I and PanK-III enzymes. The PanK-I (the *coaA* gene product) was identified as essential for growth *in vitro*,[1] and has been targeted in extensive inhibitor discovery programs by AstraZeneca and Novartis. On the other hand, its PanK-III (the *coaX* gene product) was reported to be inactive based on various genetic complementation and *in vitro* activity studies. In fact, it has been suggested that this enzyme may have a different native activity altogether.[2] While these findings are in agreement with the apparent essentiality of the PanK-I enzyme in *Mtb*, they do not help to explain why *Mtb* maintains an apparently inactive second PanK, while other organisms such as *Bacillus subtilis* (also has type I and III PanKs) both enzymes are active.

In this part of the study the evidence for and against *Mtb* CoaX – the organism's putative PanK-III enzyme – as a functional pantothenate kinase were examined. We also examined the methodologies used to study this enzyme as well as the reported problems related to working with *Mtb* CoaX – all relevant in explaining the contradictory results achieved to date. Based on these analyses, new experiments were executed to provide additional insight into the apparent inactivity of this enzyme. Finally, this chapter concludes with the results of an investigation into another organism that has an inactive second PanK homolog: *B. anthracis* and its putative type II PanK.[3] Taken together, these studies suggest that we do not have a complete understanding of the catalytic diversity of PanK enzymes as yet, and that the apparent redundancy of multiple PanK enzymes may have a functional role in certain cases.

4.2 Evidence supporting *Mtb* CoaX having PanK activity

4.2.1 PanK-I inhibitors do not translate into cell growth inhibitors

In a study by Novartis several promising leads were identified as *in vitro* inhibitors for of *Mtb*'s PanK-I, but none of these compounds translated into cell growth inhibitors that showed MIC < 20 µM.[4] A range of hypotheses were presented to explain this finding, including the potential inability of the inhibitors to cross the cell membranes, their rapid elimination by efflux pumps, compound insolubility and target insensitivity. However, none of these provided a conclusive answer. Another alternative explanation for the lack of cell growth inhibition by PanK-I inhibitors is that *Mtb*'s putative PanK-III might functionally replace PanK-I under conditions where the latter enzyme is inhibited, or not present. PanK-III enzymes are structurally distinct from PanK-I enzymes (see section 2.7.3) and would therefore not be affected by PanK-I inhibitors. They would thus remain functional in conditions where the type I PanK is inhibited.

4.2.2 CoaX shows sequence and structural similarity with other PanK-IIIs

To evaluate whether mycobacterial CoaX proteins have all the structural elements and conserved catalytic residues present in other PanK-III enzymes, we performed a multiple sequence alignment of the *Mtb* and *Mycobacterium smegmatis* (*Ms*) CoaX proteins with known and characterized PanK-IIIs (Figure 4.1).

The mycobacterial proteins show high sequence similarity to the other PanK-IIIs of which crystal structures have been determined. They are predicted to contain all equivalent secondary structure elements of these enzymes which include the relevant motifs that are conserved among members of the ASKHA superfamily.[5-7] They further contain all the motifs unique to PanK-IIIs, all three highly conserved Asp residues necessary for catalysis as well as all the residues involved in ATP and pantothenic acid binding.[8-10]

The six identifiable PanK-III motifs (three belonging to all ASKHA superfamily members, as well as the three motifs that are specific to PanK-IIIs) are underlined in Figure 4.1. The conserved sequences of these motifs are given in Table 4.1. The secondary structure elements are indicated at the top of the alignment with arrows representing β-strands and cylinders representing α-helices.

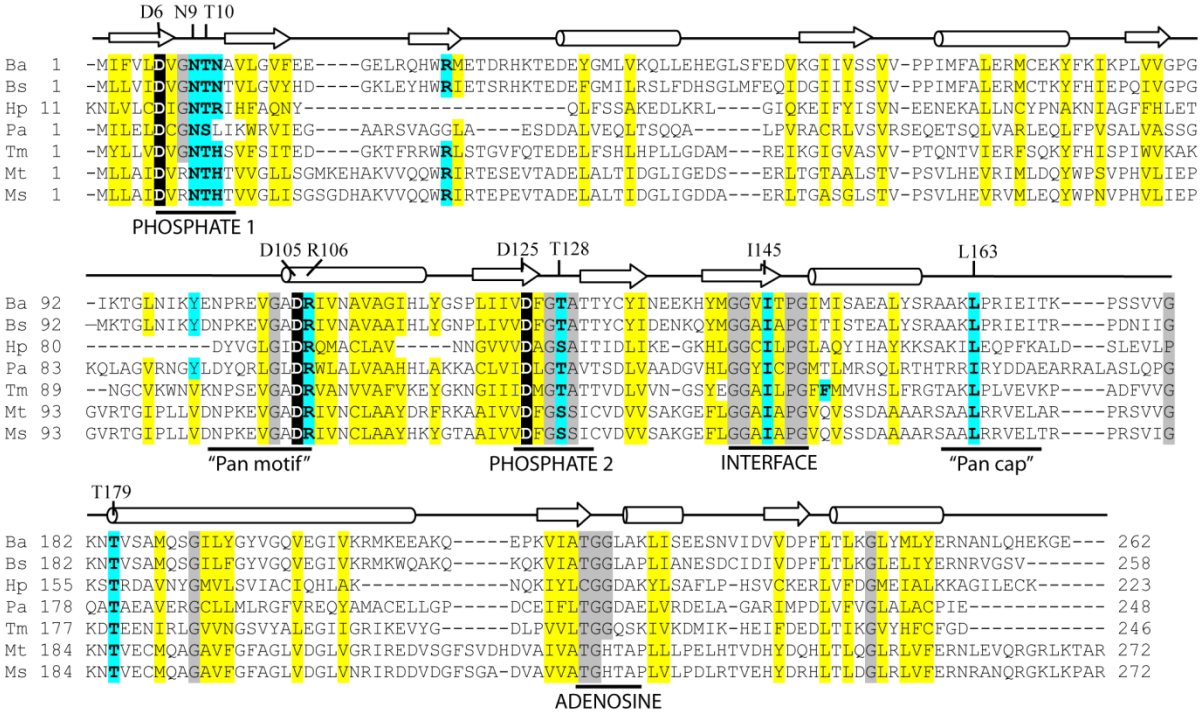


Figure 4.1: Multiple sequence alignment of selected PanK-III sequences including those with available 3D structures and the putative *Mtb* and *Ms* proteins. Secondary structure elements are indicated at the top with arrows representing β-strands and cylinders representing α-helices. The conserved motifs within the ASKHA superfamily (PHOSPHATE 1, PHOSPHATE 2 and ADENOSINE) and those specific for PanK-III ("pan motif", "pan cap" and INTERFACE) are underlined. Residues that interact with bound substrates are shaded in blue, and indicated on top of the secondary structure cartoon (using *Tm*PanK-III numbering). The generally conserved hydrophobic or neutral amino acid residues are shaded in yellow, while conserved small residues (G, P, S, A, T) are shaded in gray. The alignment was done according to the methodology outlined by Yang *et al.*[8]

Ba – Bacillus anthracis; Bs - Bacillus subtilis; Hp – Helicobacter pylori; Pa – Pseudomonas aeruginosa; Tm – Thermotoga maritima; Mt – Mycobacterium tuberculosis; Ms – Mycobacterium smegmatis.

Table 4.1: The characteristic motifs of the ASKHA superfamily and PanK-IIIs with their conserved residues. No information on the conserved residue pattern was found for the "pan cap" motif.

Motif	Conserved sequences
PHOSPHATE 1	*ffxf* **D***x***G***s(***T/S***)xxhhxhxpxp*
PHOSPHATE 2	*hff* **D***x***G***tsthpxtxf*
ADENOSINE	*pphhhs* **G***sxh*
Pan motif	*h* **G***h* **D***R*
INTERFACE	**G***gx* **I***x* **PG**

Legend: one letter code = type of amino acid (hydrophobic, polar etc.); letters in brackets = specific amino acids that can by encoded by the one letter code. *h = hydrophobic (VLIFWY), f = partly hydrophobic (VLIFWYM CGATKHR), t = tiny (GSAT), s = small (GSATNDVCP), p = tiny and polar (GSATNDQEKHR), x = any amino acid*

The three characteristic motifs of the ASKHA superfamily are PHOSPHATE 1, PHOSPHATE 2 and the ADENOSINE motif. These three motifs are involved in recognition and binding of ATP, as well as in the coordination of a divalent metal ion (such as Mg^{2+}) for phosphotransferase activity. These motifs also contain two of the three highly conserved Asp residues. Mutagenesis studies have shown that a conserved mutation of any of these Asp residues to either Asn or Glu reduces activity to less than 6%, suggesting a critical role for these residues in catalysis. Asp6 (using *Tm*PanK-III numbering as indicated at the top of Figure 4.1) is found in the PHOSPHATE 1 motif and coordinates the Mg^{2+} which interacts with the β- and γ-phosphates of ATP. The second residue, Asp125, forms part of the PHOSPHATE 2 motif and coordinates $Mg^{2+}/K^{+}/NH_4^{+}$ through an H_2O molecule.[9-12]

The three motifs specific to the PanK-IIIs are the "Pan motif",[12] "Pan cap"[8,9] and INTERFACE.[12] The "Pan motif" incorporates the third important Asp residue which is located in close proximity to the phosphoryl acceptor group. Asp105 has been proposed that act as a catalytic base since it forms side chain hydrogen bonds with the 2'- and 4'-hydroxyl groups of pantothenic acid.[12] The other highly conserved residues in the pantothenic acid binding pocket include Asn9, Arg106, Ile145, Leu163 and Thr179.[9] Ile145 is found in the INTERFACE motif and forms a Van der Waals interaction with the bound pantothenic acid. The two conserved small glycine residues in INTERFACE, Gly142 and Gly143, create space for the substrate in the active site. Lastly, the conserved Pro147 and Gly148 plays a role in the protein dimerization.[12]

Based on this analysis, and the fact that it apparently has all the conserved residues and binding motifs required for PanK activity, *Mtb* CoaX should function as a pantothenate kinase.

4.2.3 *Mtb*'s *coaX* gene is expressed in actively growing *Mtb*

One of the theories for the lack of activity of the CoaX was that the *coaX*-gene is not expressed. However, when the gene expression was analyzed by RT-PCR in actively growing *M. tuberculosis* bacteria, the results indicated that both the *coaA*- and *coaX*-genes are transcribed,[2] indicating that this gene was expressed in mycobacteria.

4.3 Evidence against *Mtb* CoaX having PanK activity

4.3.1 Mycobacterial *coaX* genes are not essential

In a 2003 study Sassetti *et al.* set out to identify the genes required for optimal growth of *Mycobacteria*,[1] which included *Mtb* strain H37Rv, *M. bovis* and the closely related vaccine strain *M. bovis BCG* Pasteur. The study was done using transposon site hybridization (TraSH), in which a gene was labeled as essential for optimal growth only if it had an insertion: genomic probe ratio of less than 0.2 and if the results could be reproduced in both *Mtb* and *M. bovis*. Mutants were only considered to be reproducibly attenuated after the libraries were analyzed twice, and if the average of the resulting ratios were significantly different from 1.0 with $P < 0.05$ (determined by student t-test). In this study the gene encoding for PanK-I (*coaA*, Rv1092c) was identified as essential for optimal growth, while the gene encoding the putative PanK-III (*coaX*, Rv3600c) was not. This result has since been confirmed with directed gene knockout and genetic silencing experiments.[2]

4.3.2 *Mtb coaX* does not functionally complement *coaA* mutations

In vivo functional complementation is often used to demonstrate that a protein has a particular enzyme activity. In the case of PanK activity, this complementation is usually done using *E. coli* strains that have a temperature sensitive mutation in their PanK-I-encoding *coaA* genes (such as DV62). At temperatures of 42°C and higher the PanK-I enzyme encoded by the mutant *coaA* gene of these strains is deactivated and subsequently the bacteria do not grow at this temperature. However, normal growth occurs at 30°C which allows for a positive control test to be performed at this temperature.[13]

In a study by AstraZeneca scientists, Awasthy *et al*. tested the ability of *Mtb coaA* and *Mtb coaX* to rescue *E. coli* DV62 grown at elevated temperatures.[2] Cells transformed with plasmids containing either the *coaA*- or *coaX*-genes were plated on rich solid media (LB agar) and incubated at 30°C (positive controls) or 42°C (experiments) for 24 h. At 30°C, the cultures transformed with either the *coaA*- or *coaX*-containing plasmids grew as expected, but at 42°C those complemented with *coaX* failed to rescue the growth of the *E. coli* DV62 cells. To ascertain if this result was not merely due to a lack of expression the CoaX protein, or to its expression in an insoluble (and therefore inactive form), a Western blot analysis was performed using anti-CoaX antibodies. Analysis of the cytosolic fractions of the cell

lysate indicated that sufficient amounts of the protein were expressed and lack of expression could therefore not explain the negative complementation result.

4.3.3 *Mtb* CoaX does not show PanK activity *in vitro*

In the same study by Awasthy *et al*.[2] the *Mtb* CoaX protein was purified and assayed for *in vitro* PanK activity using the PK/LDH-dependent coupled enzymatic assay[14] described in Chapter 3 (section 3.5.4). This test also indicated that *Mtb* CoaX does not have PanK activity.

4.4 Reevaluating the evidence: Results from our studies

4.4.1 Reevaluation of the functional complementation studies

To determine whether the negative functional complementation results of the reported studies could be dependent on the conditions under which the tests were conducted, we prepared constructs of *coaX* genes from several organisms. We repeated the functional complementation experiments with pBAD-DEST49 constructs in the *E. coli* DV64 mutant strain on solid rich medium. Among these were the *coaX*s from *H. pylori* and *B. subtilis*, as the PanK-IIIs encoded by these genes have confirmed PanK activity[5] and have been used in functional complementation studies before on both solid and in liquid media.[15] The constructs were prepared using Gateway™ pBAD-DEST49 as acceptor vector, because this plasmid places the gene in question under control of a tightly-controlled arabinose-inducible *ara*BAD (P_{BAD}) promoter. This is in contrast to the T7 promoters of the expression plasmids, pDEST and pET, which allow a degree of leaky expression. In addition, the tight regulation of P_{BAD} by *ara*C is useful for the expression of potential toxic or essential enzymes.[16]

We subsequently repeated the functional complementation experiments using the *E. coli* DV64 mutant strain and the PanK-I-encoding *E. coli coaA* as positive control. The results of this experiment, which was also conducted on solid LB agar medium, are shown in Figure 4.2.

Both the positive control and the cultures containing the plasmid with the *B. subtilis coaX* gene grew as expected, while the constructs containing the *Mtb* and *H. pylori coaX* genes failed to grow. This result was in agreement with previous reports of the *Mtb* studies, but is contradictory to the fact that the activity of *H. pylori* PanK-III (encoded by its *coaX* gene) has

been confirmed in several *in vitro* assays. This finding shows that complementation studies performed on solid LB agar medium may not necessarily give accurate results.

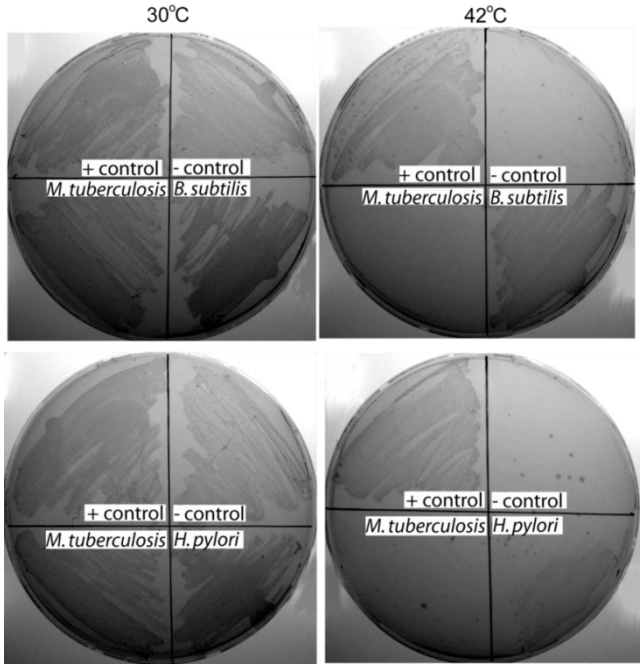


Figure 4.2: Functional complementation studies done on solid LB media. The relevant genes were cloned into the complementation vector, pBAD-DEST52 and transformed into *E. coli DV62*. At 30°C all cultures grew as expected, but at 42 °C only *B. subtilis coaX* and the positive control (*E. coli coaA*) rescued the mutant strain.

In previous studies conducted in our laboratory we achieved more reliable results in functional complementation studies when these were conducted using a liquid minimal medium (2% E-salts, 0.2% glycerol, with relevant supplements for growth, induction and selection) instead of solid media. We therefore repeated the experiment under these conditions by inoculating the media with starter cultures of the *E. coli* DV64 strains containing the relevant constructs. Cell growth was subsequently followed for 30 h by monitoring the increase in optical density (OD_{600}) at 600 nm. All experiments were done in triplicate. The absorbance plots obtained from one of the repeats of this experiment are shown in Figure 4.3.

The results show that under these conditions, *Mtb coaX* rescued the *E. coli* DV64 strain in one out of every three repeats of the experiment. *H. pylori coaX* again failed to complement in all experiments, and this was observed even with increased incubation times. *B. subtilis coaX* did provide functional rescue, but at a slower rate than would normally be expected for an enzyme with confirmed pantothenate kinase activity.[5]

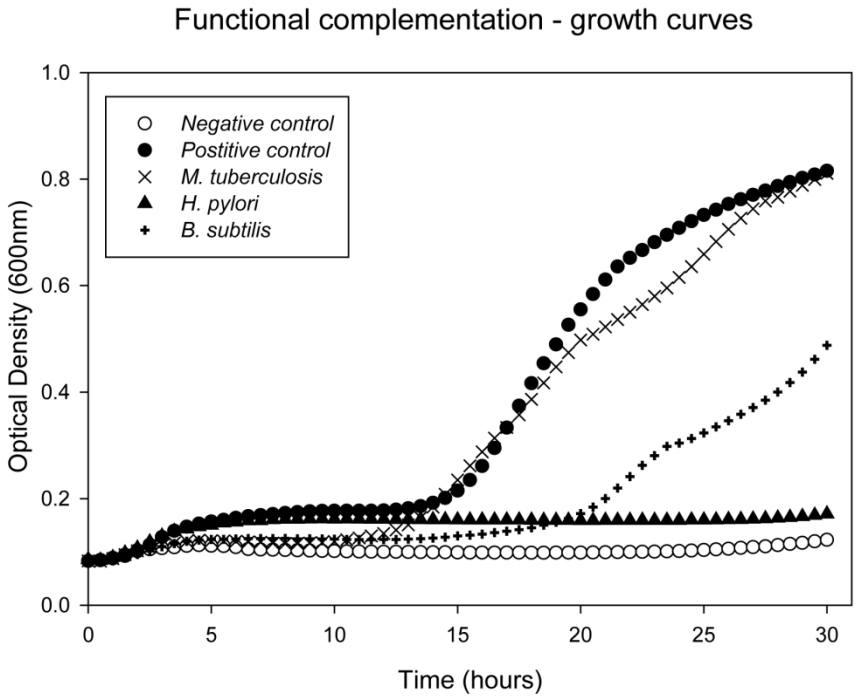


Figure 4.3: Functional complementation studies done in liquid minimal media clearly shows complementation of the temperature sensitive *E. coli* DV62 strain by *Mtb coaX*. However, constructs with genes encoding proteins with known PanK activity either failed to complement (*H. pylori*) or did so at a slower rate than expected (*B. subtilis*). Each point represents the average of three readings.

4.4.2 Constructing expression vectors containing Mycobacterial *coaX* genes

Based on the ambiguous results obtained in the cell growth studies, we set out to determine the *in vitro* activity of the *coaX* gene product. We decided to include the homologous *coaX*-gene from *M. smegmatis* (*Ms* CoaX) in this study, since the proteins obtained from this organism are highly similar to their *Mtb* homologs, but are often easier to handle and purify.

The first attempt to amplify the *coaX* gene from genomic DNA failed for both homologs. We postulated that the reason for this might be due to secondary effects caused by the fact that the mycobacterial genomes are known to be unusually rich in GC nucleotides.[17] This problem was subsequently solved either by extension of the primers at the 3' side, or by the addition of DMSO to the amplification mixture to disrupt the formation of secondary structures.[18]

Mtb coaX was cloned into pET28a(+) and pPROEX using the *Nde*I and *Xho*I restriction sites. Both pET28a(+) and pPROEX plasmids are used for the heterologous expression of proteins as *N*-terminal His_6-tag fusions, but they differ in the cleavage sites between the tag and target protein. Novagen's pET28a(+) contains a thrombin cleavage site, while Invitrogen's

pPROEX has a TEV-cleavage site. It has been postulated that the differences in residues that surround the His_6-tag may affect the strength of the interactions between the tag and the imidazole (a structural analog of histidine) used during the immobilized metal affinity chromatography (IMAC) purification of the target protein.

Furthermore, the heterologous expression of *Mtb* proteins in *E. coli* is known to be problematic, often giving low expression levels or leading to the expression of insoluble protein in inclusion bodies. Such difficulties in protein synthesis and folding may be due to the differences in codon usage between the organisms.[19] Eleven scarce codons, i.e. codons that are not generally used by *E. coli* for expression of its native proteins,[20] were identified in the *Mtb coaX*-gene: 7 × CGG (Arg), 3 × GGA (Gly) and 1 × CCA (Pro). This gives a ≈ 4% scarce codon incorporation. To circumvent this as a possible cause of poor soluble expression, an optimized version of the *coaX*-gene (i.e. a version in which the scarce codons were replaced with codons more regularly used by *E. coli*) was synthesized by GenScript USA INC and cloned into pET20b(+) for expression of the CoaX protein with a *C*-terminal His_6-tag fusion.

The amplified *Ms coaX* was only cloned into pET28a(+) with *Nde*I and *Not*I restriction enzymes. *Not*I was used instead of X*ho*I since the gene has an internal *Xho*I site.

4.4.3 Expression and purification of the recombinant CoaX proteins

a) *Mtb* CoaX

The native *Mtb coaX* gene contained in the pET28a(+) and pPROEX plasmids was expressed with an *N*-terminal His_6-tag by induction with 1.0 mM isopropyl-β-D-thiogalactoside (IPTG) at 37°C overnight. The resulting proteins were subsequently purified by IMAC using Ni^{2+} as the bound metal ion. The success of expression and purification was evaluated by SDS PAGE analysis of the proteins obtained during various stages of the process (Figure 4.4). This shows that both plasmid constructs are expressed and that the resulting protein could be successfully purified using IMAC.

The optimized gene cloned into a pET20b(+) construct was similarly expressed (albeit with a *C*-terminal instead of an *N*-terminal His_6-tag) following the same procedures as described above. However, there were no noticeable differences in the amount or the purity of the

protein obtained in this case compared to the protein expressed from the genes that were not codon optimized (results not shown).

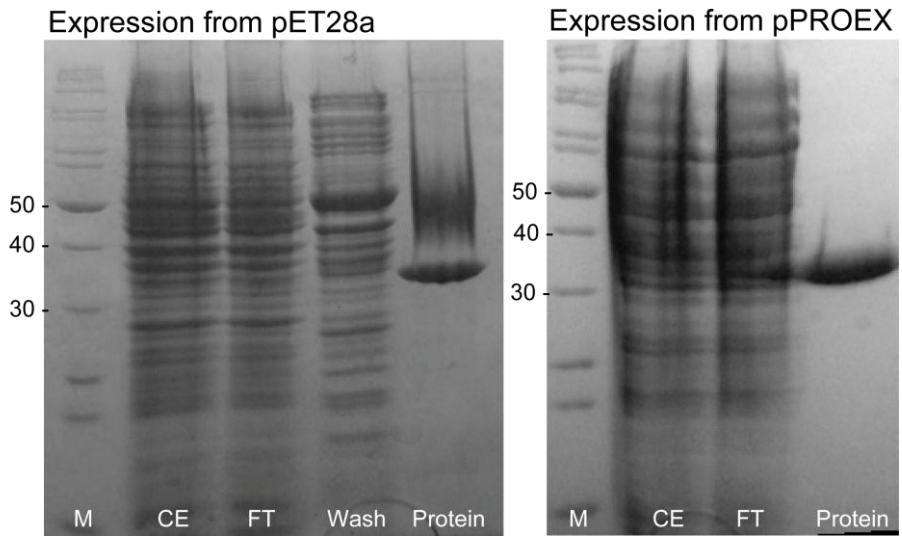


Figure 4.4: Expression and purification of *Mtb* CoaX from pET28a(+) and pPROEX. The amount of pure protein shown in the lane marked "Protein" is normalized and is not an indication of the amount expressed or purified. M = PageRuler marker (Fermentas), CE = crude extract, FT = flow through (proteins that are not retained by IMAC, Wash = non-specifically bound proteins, Protein = *Mtb* CoaX.

The proteins from all three expression constructs were found to be unstable and precipitated during buffer exchange by gel filtration using desalting columns. This suggested that the CoaX protein was highly sensitive to buffer conditions or to possible contaminants present on the desalting columns. Since the use of new desalting columns did not solve the problem, we concentrated our investigation on the use of various purification conditions to stabilize the protein and prevent precipitation. The first set of experiments focused on optimizing the IMAC purification and subsequent gel filtration by evaluating various the buffer systems. The conditions tested are summarized in Table 4.2.

Three different buffers are normally used for the purification of His_6-tagged proteins by IMAC: binding buffer, which is used for cell lysis and to load the sample onto the column; elution buffer, which is used to elute the protein from the column and which therefore contains high concentrations of imidazole, and gel filtration buffer, which is used to exchange the purified protein to buffers containing no imidazole and lower concentrations of salts. We evaluated several different systems that make up these three buffers to determine whether it influenced protein stability. The Tris-buffer system (entry 1) is a standard system for protein purification. Alternatively, a phosphate-based system with a

lower pH is often used if the target protein is not stable in Tris-buffer. If problems with stability persist, the last system (entry 3), in which 10% glycerol is added to the binding and elution buffers to further stabilize the target protein, is used. The concentration of imidazole can also be decreased, since the removal of tightly-bound imidazole during gel filtration could be an important cause of the protein precipitation. Unfortunately, none of these systems improved protein stability, and coaX was found to precipitate in all cases upon buffer exchange by gel filtration.

Table 4.2: Different buffer systems used for IMAC purification of *Mtb* CoaX.

System	Binding buffer	Elution buffer	Gel filtration buffer
1 - Tris-based:	20 mM Tris-HCl	20 mM Tris-HCl	25 mM Tris-HCl
	500 mM NaCl	500 mM NaCl	5.0 mM $MgCl_2$
	50 mM imidazole	500 mM imidazole	
	0.05% NaN_3	0.05% NaN_3	
	pH 7.9	pH 7.9	pH 8.0
2 - Phosphate-based:	20 mM phosphate	20 mM phosphate	25 mM phosphate
	500 mM NaCl	500 mM NaCl	5.0 mM $MgCl_2$
		500 mM imidazole	
	pH 7.4	pH 7.4	pH 7.4
3 - Tris/Glycerol-based:	20 mM Tris-HCl	20 mM Tris-HCl	
	500mM NaCl	150 mM NaCl	
	10% glycerol	400 mM Imidazole	
		10% glycerol	
	pH 8.0	pH 8.0	

As an alternative to the buffer exchange by gel filtration, dialysis experiments were performed in which the concentration of NaCl and imidazole was gradually decreased. Regardless of the buffering system used, buffer exchange was only successful if the concentration of NaCl and imidazole were not reduced below 250 µM respectively.

b) *Ms* CoaX

Ms CoaX was expressed at 20°C overnight after inducing with 1.0 mM IPTG. It was purified using phosphate-based buffers (refer to entry 2 in Table 4.1), but the same precipitation issues were experienced. The enzyme-containing fractions were therefore not desalted or dialyzed.

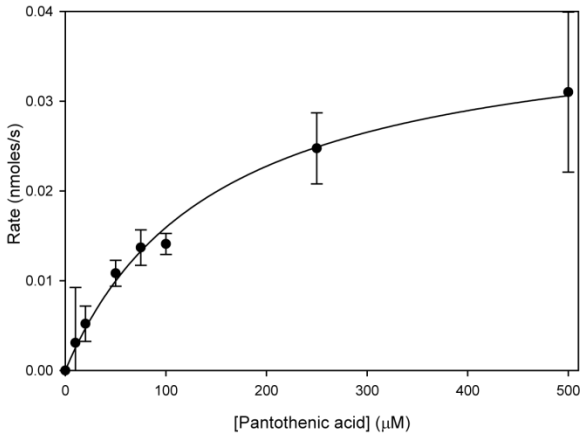
4.4.4 *In vitro* activity of *Mtb* and *Ms* CoaX proteins

To determine whether CoaX proteins display pantothenate kinase activity, the coupled enzymatic assay described in Chapter 3 (section 3.5.4), was used.[27] The protein fractions were tested in the salt- and imidazole-containing buffers used for protein purification.

Initial activity determinations failed to show any PanK activity for the *Mtb* CoaX containing fractions. The *Ms* CoaX homolog by contrast did prove active. This protein was therefore characterized further using the described coupled enzymatic assay.[2] The initial rates were determined and plotted against the substrate concentration (Figure 4.5). The kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation (1).

$$v = \frac{V_{max} \times [S]}{K_M + [S]} \qquad (1)$$

The kinetic parameters obtained in this manner are summarized in the table given in Figure 4.5 (right). The parameters for *P. aeruginosa* PanK-III with pantothenic acid is also shown for comparison.



Kinetic parameters	Pantothenate	
Organism	*M. smegmatis*	*P. aeruginosa*
V_{max} (nmol.min^{-1}.mg^{-1})	0.0398 ± 0.0044	0.123 ± 0.003
K_M (mM)	149 ± 37	24.5 ± 2.7
k_{cat} (s^{-1})	0.285 ± 0.031	0.793 ± 0.022
k_{cat}/K_M (s^{-1}.mM^{-1})	2.04 ± 1.21	32.3 ± 8.2

Figure 4.5: Left: Steady state kinetics of *M. smegmatis* CoaX with pantothenic acid. Each point represents the average of three readings, while the error bars denote the standard deviation. Solid lines represent the best-fit of the data to the Michaelis-Menten equation.
Right: Table summarizing the kinetic parameters for *M. smegmatis* CoaX and *P. aeruginosa* PanK-III.

4.4.5 Alternate Phosphoryl donors

One of the main characteristics of PanK-III enzymes is their unusually high K_M-values for ATP, which seems to indicate that this is not the main phophoryl donor of these enzymes. To test whether this might be the reason for the apparent inactivity of *Mtb* CoaX, i.e. that it

requires a different activated phosphate for activity, UTP, CTP, GTP and PEP (at a final concentration of 1.5 mM) were tested as alternative phosphoryl donors for the phosphorylation of pantothenate using the PanK-IIIs from *B. subtilis, H. pylori* [5] and *Mtb* (L.A. Brand, unpublished data). The reaction mixture with PEP was analyzed by a two enzyme coupled assay, while the rest of the reaction mixtures were analyzed by ESI-MS for the presence of 4'-phosphopantothenate. The results were compared with mixtures containing ATP as well as to control reactions with *E. coli* PanK-I. Only CTP and GTP could substitute for ATP in the case of *H. pylori* PanK-III, albeit to a lesser extent. None of the other potential phosphoryl donor was active in the reactions catalyzed by *B. subtilis* PanK-III and *Mtb* CoaX.

4.4.6 Modeling

The multiple sequence alignment shown in Figure 4.1 indicated that the *Mtb* and *Ms* CoaXs bear high sequence similarity to known PanK-III enzymes, and contained the conserved motifs and residues required for activity. However, closer examination shows that the mycobacterial protein sequences diverges from the normal PHOSPHATE 1 and ADENOSINE binding motifs as shown in Figure 4.6.

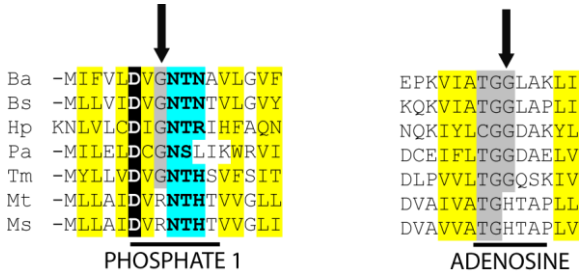


Figure 4.6: PHOSPHATE 1 and ADENOSINE motifs showing the Gly to Arg and Gly to His mutations in the mycobacterial CoaX proteins.

In PHOSPHATE 1 the highly conserved signature residue, Gly8, is replaced by Asp, while the Gly229 in the ADENOSINE motif is replaced by His. To evaluate the effect of these substitutions on the protein structure, we modeled the *Mtb* enzyme based on the structure of *T. maritima* PanK-III (*Tm*PanK-III) with pantothenic acid and ADP bound. A comparison of the *Mtb* CoaX model and the *Tm*PanK-III structure clearly shows that in the case of the *Mtb* protein the substitutions give rise to an additional negative charge involving the His and Arg residues. These negative charges lead to H-bonding which closes the ATP binding site.

To disrupt the H-bonds, mutagenesis studies were subsequently performed to create *Mtb* CoaX H229G in which the His at position 229 was mutationally replaced by Gly to resemble the CoaX from non-mycobacterial proteins. The effective removal of one of the charged residues should prevent H-bond formation and allow ATP to enter into the active site. However, this was found not to be the case, as this protein still failed to show activity. Due to the instability of the protein, circular dichroism (CD) studies could not be performed to verify that the folding of the protein was not compromised by the mutational change.

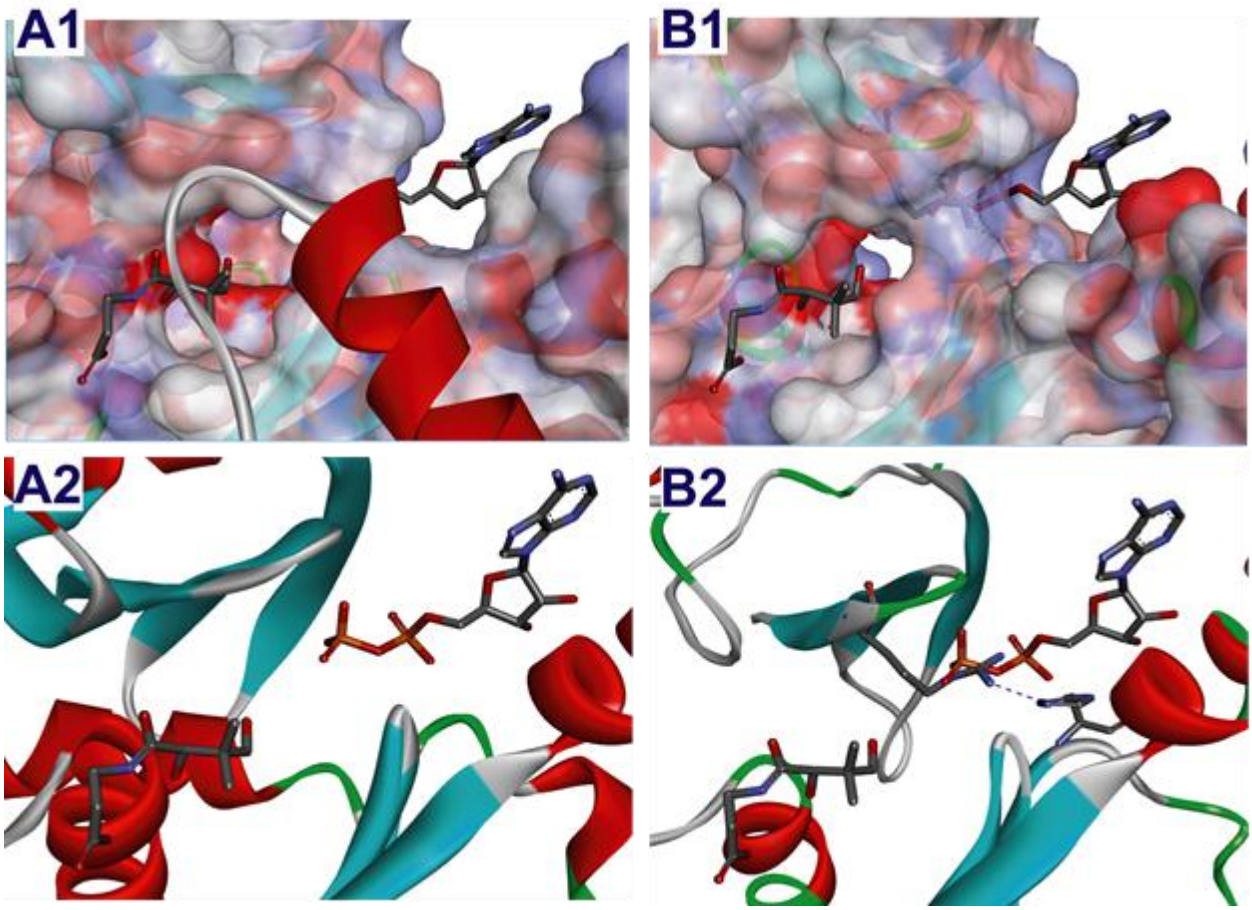


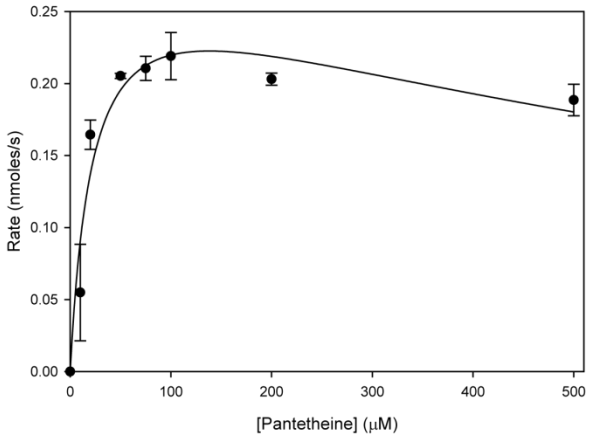
Figure 4.7: Comparison of the structures of *Tm*PanK-III and a model of the *Mtb* enzyme illustrating the consequence of the mutation of the conserved Gly residues to Arg and His respectively.
Panel A: The structure of *Tm*PanK-III bound to pantothenic acid and ADP. A1, Structure showing the solvent accessible surface of the protein. The red ribbon represents the helix and adjacent loop of the "Pan cap" from the adjacent monomer. A2, Ribbon structure showing the binding of pantothenic acid and ADP in the active site. For clarity the helix loop of the "Pan cap" was omitted.
Panel B: The modeled structure of *Mtb* CoaX based on that of *Tm*PanK-III. B1, Structure showing the solvent accessible surface of the protein (the "Pan cap" was omitted for clarity). The salt bridge that forms between the His and Arg residues clearly prevents ADP from binding in the same way it does in the *Tm*PanK-III structure. B2, Ribbon structure of the model highlighting the H-bonding that is formed and its clash with the phosphate groups of ADP.

4.4.7 *B. anthracis* BA2901

The only other bacterial PanK reported inactive to date is the hypothetical PanK-II from *B. anthracis* (*Ba*PanK-II).[12] The protein, BA2901, was unable to rescue an *E. coli* DV70 temperature deactivated *coaA* mutant at 42°C and no *in vitro* activity was observed with pantothenic acid as substrate. Since PanK-IIs are normally known to have the ability to phosphorylate alternative substrates (section 2.7.9), we wanted to test the enzyme with another known PanK substrate before also dismissing its potential as a pantothenate kinase. Pantetheine (the substrate of the CoA salvage pathway) was therefore tested as an alternative substrate, and *Ba* PanK-II was found to be active with this substrate.

BA2901 was assayed using the PK/LDH-dependent coupled enzymatic assay[14] described in Chapter 3 (section 3.5.4). The initial rates were determined at various substrate concentrations and plotted against the concentrations of the substrate used (Figure 4.8). The plots were fitted with the equation for substrate inhibition 2 (graph on right).

$$v = \frac{V_{max}}{1 + \frac{K_M}{[S]} + \frac{[S]}{K_i})} \qquad (2)$$



Kinetic parameters	Pantetheine	
Organism	*B. anthracis*	*E.coli*
V_{max} (nmol.min^{-1}.mg^{-1})	0.296 ± 0.029	
K_M (μM)	22.7 ± 5.8	91
k_{cat} (s^{-1})	0.889 ± 0.086	0.32
k_{cat}/K_M (s^{-1}.mM^{-1})	39.1 ± 1.1	3.53
K_i (μM)	839 ± 312	

Figure 4.8: The steady state kinetics of *B. anthracis* BA2901 with pantetheine. Each point represents the average of three readings and the bars the standard error. Left: Solid line represents the best-fit of the data to the Michaelis-Menten equation. Right: Solid line represents the best-fit of the data to the Substrate inhibition equation.

The kinetic parameters obtained are summarized in the table in Figure 4.8 (right). For comparison the parameters for *E. coli* PanK-II with pantetheine are provided. The enzyme was found to be subject to substrate inhibition at high concentrations, as demonstrated by the activity profile.

4.7 Discussion

4.7.1 Functional complementation

The design of functional complementation studies needs to be done with care as the results can otherwise be ambiguous and misleading. This is highlighted by the contradictory results shown in Figure 4.2 and 4.3 where, the construct containing *H. pylori coaX* failed to rescue the temperature sensitive *E. coli* mutants on both solid and liquid media. The pantothenate kinase activity of *H. pylori* PanK-III has been confirmed *in vitro* and one could therefore expect a construct containing its *coaX* gene to rescue the *E. coli* mutant strain. Moreover, the complementation studies with the *B. subtilis coaX* construct worked much better on solid than in liquid media, although in the latter growth occurred at a slower rate than with the cultures containing the constructs for the positive control (*Ec coaA*) and *Mtb coaX.*

Our studies reveal that, contrary to the result of Awasthy *et al.* in 2010, *Mtb coaX* does complement the *E. coli* DV64 strain in liquid media. However, this result was not reproducible, and showed positive complementation on average only in one of every three repeats of the experiment. The reason for this is currently unknown.

4.7.2 Purification, gel filtration and dialysis

Mtb CoaX enzymes were successfully expressed from three different constructs and purified by immobilized metal affinity chromatography (IMAC) with Ni^{2+}. However, the resulting proteins were all unstable and precipitated during buffer exchange by gel filtration or dialysis. The three constructs were used to investigate the link between gel filtration induced degradation and tight binding of the imidazole to the 6xhistidine-tag. While it has been suggested that the amino acid residues surrounding the 6xhistidine-tags may influence the strength of the interactions with imidazole, and that this may subsequently destabilize the enzyme when the imidazole is removed during gel filtration, we found no noticeable difference in stability of proteins expressed from either the pET28 or pPROEX constructs.

Buffer exchange procedures by dialysis were more successful than those by gel filtration, although the NaCl and imidazole concentrations could only be reduced to half the initial values. No further stabilization was observed by changing either the buffer (Tris-HCl, phosphate, MES and HEPES), or by varying the pH of the buffers over a range of 6.5 to 8.5.

4.7.3 Determination of *in vitro* enzymatic activity

All *Mtb* CoaX enzyme variants produced as part of this analysis proved inactive *in vitro*, regardless of the buffer used for purification and gel filtration/dialysis as well as the phosphoryl donors tested. In contrast the *M. smegmatis* CoaX homolog was indeed observed to be active. Compared to *P. aeruginosa* PanK-III, the activity levels of *Ms* CoaX were low as exemplified by a ≈ 15 fold lower specificity constant than that of *Pa* PanK-III. Interestingly this is largely due to a lower binding affinity: the K_M for pantothenic acid *Ms* CoaX is about five times higher, while the k_{cat}-value is about a third than that of *Pa* PanK-III.

The central problem encountered for *Ms* CoaX however is its instability. Even without gel filtration or dialysis, the enzyme precipitated and rapidly lost activity after purification. This raises the question of whether the determined parameters truly reflect the enzyme native activity or whether the slower observed rates are due to compromised enzyme quality. The purification strategy would therefore need to be revisited to produce stable enzyme for future kinetic studies.

Another possible reason for the decreased activity observed with *Ms* CoaX, and a possible explanation for the lack of activity with *Mtb* CoaX, is the two substitutions naturally occurring in the PHOSPHATE 1 and ADENOSINE motifs of the active site. In both cases a small uncharged residue, Gly, is replaced by a larger charged amino acid, Asp and His respectively. Referring back to the model of the *Mtb* active site in Figure 4.7(B2), it is clear that the two point mutations introduce H-bonding in the active site of the enzyme that partly obstructs the ATP binding site by clashing with the terminal phosphate-group of the bound ADP. These interactions may prevent ATP from binding in the active site and hence prevent the phosphoryl transfer reaction.

The inactivity of the *Mtb* CoaX H229G mutant suggests that the additional Arg may still block the binding of ATP. It could be of interest to prepare the double mutant in which both the His and Asp are replaced by Gly as seen in other active enzymes.

Finally the bacterial PanK-III candidate from *B. anthracis,* BA2901, previously reported as inactive, has been demonstrated to be a pantetheine kinase rather than a pantothenate kinase. Compared to *E. coli* PanK-I utilizing the same substrate, the K_M-value of BA2901 is four-fold lower and its catalytic efficiency ≈ 10-fold higher. This enzyme appears to be more

effective than *E. coli* PanK-I in phosphorylating pantetheine and, subject to further characterization of the enzyme, may be a good candidate enzyme to use in biocatalytic studies. However, the enzyme is subject to substrate inhibition at high pantetheine concentration, for which a K_i-value ≈ 800 µM was measured.

4.8 Conclusion

Available information would appear to indicate that *Mtb* CoaX is a pantothenate kinase. This could however not be confirmed as the results obtained in the *in vivo* studies were ambiguous and the *in vitro* results are questionable due to purification and stability issues. Activity was confirmed for the homologous *Ms* CoaX, although this enzyme is less active than other characterized PanK-IIIs.

Two major factors that may account for the loss or reduced enzyme activity include the overall instability of both *Mtb* and *Ms* CoaX and two substitutions in the PHOSPHATE 1 and ADENOSINE motifs. These substitutions are not specific to our test strains, as multiple sequence alignment of various virulent *Mtb* strains and other Mycobacterial CoaXs showed these mutations to be common. The same replacements across various species imply that this is not a random occurrence. Rather, it is possible that the H-bond introduced by these mutations has a specific function, such as acting as gatekeeping residues that open under certain conditions.

Lastly, the only other putative bacterial PanK reported as inactive, BA2901 from *B. anthracis*, was found to be active when tested with pantetheine as substrate, the precursor of the CoA salvage pathway. Although this is an important breakthrough in our understanding of the *B. anthracis* PanK enzymes, *Mtb* CoaX is unlikely to behave in a similar manner due to the enclosed active site of the PanK-IIIs (section 2.7.3) and previous reports have indicated that both the *Treponema pallidum*[21] and *P. aeruginosa*[22] PanK-IIIs are unable to phosphorylate pantetheine. However, this does support the hypothesis that this enzyme may have an alternative metabolic function and that inactivity should not be assumed prematurely.

4.9 Experimental

M. tuberculosis H37Rv genomic DNA was a gift from Rob Warren from Medical Biochemistry at Tygerberg campus, University of Stellenbosch.

M. smegmatis culture was a gift from Prof Daan Steenkamp from the Medical School, University of Cape Town.

The *B. anthracis* BA2901 encoding plasmid construct was a gift from A.L. Claiborne from Wake Forest University School of Medicine, Winston-Salem, North Carolina.

4.9.1 Genomic DNA isolation

A culture of *M. smegmatis* was inoculated from freezer stocks into 7H9 media (0.52 $g.l^{-1}$ Middlebrook 7H9 broth base, 0.44% glycerol and 0.1% Tween 80 – mixed according to manufacturer's instructions) and grown for 36 h at 37°C. Genomic DNA was isolated from the culture using the the Gram-positive procedure purification of the Wizard™ Genomic DNA Purification kit from Promega (cat # A1120). After the ethanol wash step, the DNA pellet was aspirated at 65°C overnight before rehydration with the rehydration solution.

4.9.2 Amplification and cloning

a) General procedures

All primers were synthesized by Inqaba Biotech. Amplification was done using *pfu* enzyme (Fermentas) in the provided buffer with 0.4 mM dNTP mix, 1.0 mM forward primer, 1.0 mM reverse primer, 1.0 µl genomic DNA /plasmid template, varied $MgCl_2$ concentrations (2.0 mM, 2.5 mM, 3.0 mM and 4.0 mM) and water to a final volume of 25 µl. The PCR program started with an incubation step of 2 min at 94˚C before the cycle started with a denaturation step at 94˚C for 30 s, an annealing step at 60˚C for 30 s and a polymerization step at 68˚C for 1.5 min. This cycle was repeated 30 times followed by an extended polymerization step of 6 min at 68˚C and the program ended with a hold step at 4.0˚C. The reaction mixtures were analyzed by gel electrophoresis on 1.0% agarose gels and the bands visualized by staining with SYBR®gold on the Darkreader. Product bands (in comparison to New England biolabs 2-log ladder marker) were purified from the gel with the GeneJET™ Gel Extraction kit (Fermentas).

The PCR products and plasmids were digested with the relevant Fastdigest enzymes (Fermentas). A double digestion mix typically consists of 2.0 µl Fastdigest buffer, 16 µl PCR

product/plasmid, 1.0 µl of each enzyme in a final volume of 20 µl. The digestion mixures were incubated at 37˚C for 1 h before resolving the entire mixture on 1.0% agarose gels. The target DNA fragments were from the gels (as described above) and the concentration determined with Quant-iT™ dsDNA HS dye (Fermentas) with the Qubit™ fluorometer.

Based on the concentration different ratios of insert: plasmids were combined in a volume of 5.0 µl with 5.0 µl Quick ligase buffer with 0.5 µl Quick ligase enzyme (New England Biolabs). The ligation mixtures were incubated at 37˚C for 1 h before the entire reaction mixture was transformed into *E. coli* Mach1 chemical competent cell, plated on Luria Bertani (LB) agar enriched with the relevant antibiotic and incubated overnight at 37˚C.

Any colonies obtained the next day were screened by mixing a smear of each colony into 25 µl Epilyse solution 1 (30 mM Tris-HCl, pH 8.0, 5.0 mM Na_2EDTA, 50 mM NaCl, 20% sucrose, 0.047 $\mu g.ml^{-1}$ lysozyme and 0.047 $\mu g.ml^{-1}$ RNAse). After 10 µl Epilyse solution 2 (1 × TAE buffer, 2% SDS, 5% sucrose and 2 $mg.ml^{-1}$ bromophenol blue) was added, the mixtures were analyzed on 1.0% agarose gels by comparing the samples to the undigested parent plasmid. DNA-samples larger than the parent plasmid were subjected to further screening. This involved a digest check of the purified target plasmids (purified from overnight cultures with the Zippy™ Plasmid Miniprep kit) with selected enzymes to screen base on the size patterns obtained. If the band sizes were correct, the plasmids were sent to Inqaba Biotech for sequencing with T7 primers.

b) Mtb coaX

pET28a(+)-coaX

(Amplification and cloning of Mtb coaX was performed by Mrs LA Brand.)

The c*oaX*-gene was amplified from genomic DNA. The forward primers introduced an *Nde*I restriction sites and the reverse primers *Xho*I restriction sites (underlined) and was cloned into pET28a(+). The following primer set was used:

forward primer: 5’ – CGGTGTGGGATACATATG CTGCTGGCGATTGACGTCCGC– 3’

reverse primer: 5’ –CGGCGCGCTGACGTCGATGCCGG CCTCGAGTCTGGG – 3’.

pPROEX-coaX

The *coaX*-gene was subcloned from the pET28a(+)-*coaX* construct into pPROEX using the *Nde*I and *Xho*I RE sites.

pENTR4N-coaX

The *coaX*-gene was subcloned from the pET28a(+)-*coaX* construct into pENTR4N using the *Nde*I and *Xho*I RE sites.

pBAD EXP49-coaX

(Cloning was performed by Ms Ndivhuwo Muneri.)

This expression vector was constructed by making use of the Gateway™ LR reaction system. This system allows the formation of expression clones by adding together an entry plasmid (pENTR) containing the gene sequence and a destination plasmid (pDEST) together with LR clonase. Both plasmids contain defined *att*-sites. During the LR reaction the fragments of DNA between the *att*-sites get exchanged. This allows the formation of an expression vector containing the target DNA sequence.

For the LR reaction 1.0 µl pBAD DEST49, 0.5 µl pENTR4N-*coaX,* 0.5 µl LR clonase buffer and 0.5 µl LR clonase was mixed together and incubated at 25˚C overnight. The reactions were terminated by addition of 1.0 µl Proteinase K and incubation for 10 min at 37˚C. The entire LR-mixture was transformed into *E.coli* Mach1 chemical competent cells and plated on LB plates enriched with 100 $mg.l^{-1}$ ampicillin. Starter cultures were made of the colonies obtained and after incubation at 37°C overnight the plasmid was extracted and digested with Fastdigest *BsrG*I (Fermentas). *BsrG*I only digests within the *att*-sites of the expression clones if the LR exchange occurred correctly and thus a unique pattern is observed when the digestion mixture is resolved on a 1.0% agarose gel.

c) Ms coaX

Ms coaX was amplified from genomic DNA by two rounds of amplification. The first round amplified a region wider than the gene and the second round was targeted amplification of the target *coaX*-gene. In the second set of primers the forward primer introduced an *Nde*I restriction site and the reverse primer a *Not*I site (underlined) for cloning into pET28a(+).

The following primer sets were used:

First set: forward primer: 5' – AACGACCAGAACC CGAACTGCCCTGG – 3'
reverse primer: 5' – CTCCCACCCGTGCACGACGTTCGACAAG – 3'

Second set: forward primer: 5' – CCGTGGCGC CGAGAATCCCATATGCTGCTCGCG - 3'
reverse primer: 5' – CGTCGACCTGGTGCGGCCGC TCTTCTGATCAG – 3'

All amplification mixtures were supplemented with 5% DMSO. The *coaX*-gene was cloned into pET28a(+).

d) Mutagenesis of *Mtb coaX* H229G

(Amplification and cloning of Mtb coaX H229G was performed by Mr J. Albert Abrie.)

The mutation was introduced with SOE PCR using pET28(a)-*Mtb coaX* plasmid as template.

First set: forward primer: T7 forward primer
reverse primer: 5' – GGGCGCGGTA CCCCCGGTAGCCACG– 3'

Second set: forward primer: 5' – CGTGGCTACCGGGGGTACCGCGCCC– 3'
reverse primer: T7 reverse primer

Third set: forward primer: T7 forward primer
reverse primer: T7 reverse primer

The mutated gene was cloned into pET28a(+) and expressed as described above from pET28(a)-*Mtb coaX*.

4.9.3 Protein expression and purification

a) *Mtb* CoaX

Protein was produced in LB media (500 ml), supplemented with 30 mg/L kanamycin, and inoculated with plasmid containing *E. coli* BL21(DE3) starter culture. The culture was grown until midlog phase was reached and induced with a final concentration of 1.0 mM isopropyl-β-D-thiogalactoside (IPTG) and grown further at 37°C overnight.

The harvested pellet was resuspended in a volume of 10 × the pellet weight of binding buffer (various buffers used) and cooled to < 10°C. Cells were disrupted by sonication and the cell debris collected by centrifugation at 25 000 rpm for 20 min at 10°C. To prepare the supernatant for purification it was filtered using CAMEO 25 AS acetate filters with a pore size of 0.45 micron before injection into the ÄKTA*prime*-system. The His_6-tagged protein was loaded to a 1.0 ml Amersham Biosciences HiTrap Chelating HP column preloaded with Ni^{2+}. After a wash step (15% elution buffer, 85% binding buffer) to remove any non-specifically bound proteins from the column, the target protein was eluted by stepwise increasing the concentration of imidazole (the functional group of histidine) in the buffer. The elution was monitored by UV absorption at 280 nm.

b) Ms CoaX

Expression was induced at midlog phase with a final concentration of 1.0 mM IPTG at 20°C overnight. The protein was purified as above using Phosphate buffers (Table 4.2, entry 2). To stabilize the enzyme for freezing and thawing, glycerol was added to 10% of the final volume. The enzyme was used "as is" in further applications without further purification by gel filtration due to instability problems.

c) Mtb CoaX H229G

The protein was purified as above using Tris- based buffer at pH 8.0 supplemented with 10% glycerol (Table 4.2, entry 3). Due to precipitation problems, the enzyme containing fractions were not desalted and used "as is" in further applications.

4.9.4 Dialysis of *Mtb* CoaX

All samples used for dialysis were purified using an ÄKTA*prime* liquid chromatography system with Tris-based elution buffer (Table 4.2, entry 1). The dialysis set-up shown in Figure 4.9 consisted of the lid of an Eppendorf tube in which 100 µl of enzyme sample was pipetted before covering with SnakeSkin dialysis tubing (Thermo Scientific, Pierce). The dialysis tubing was secured with parafilm and a dialysis clamp before suspending it in a beaker containing the relevant buffer (> 250× sample volume). Dialysis was performed at 4.0°C with continual stirring and the buffer changed every 2.0 h.

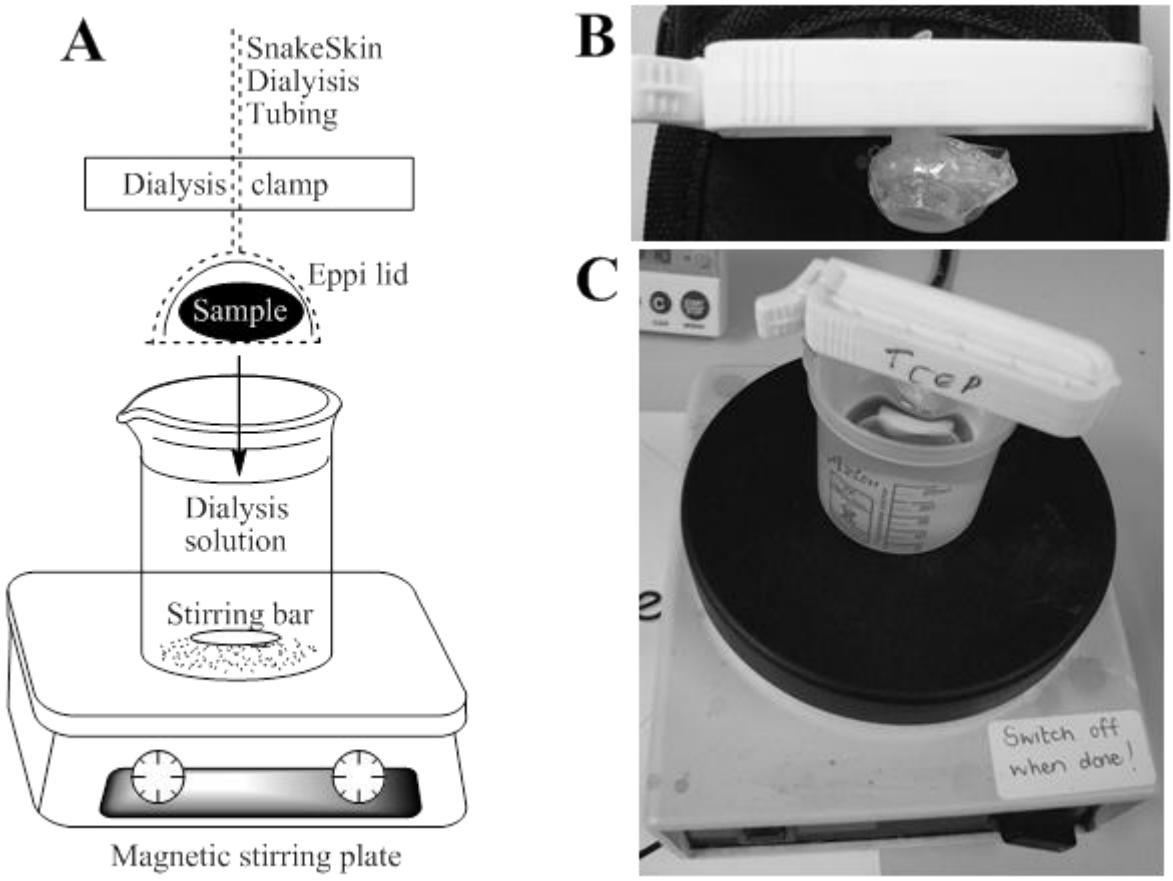


Figure 4.9: Panel A – schematic representation of dialysis set-up. Panel B – Photo of Eppi lid containing the sample, covered with SnakeSkin Dialysis tubing and secured with a dialysis clip. Panel C – Photo of final set-up.

4.9.5 Determination of protein purity and concentration

The proteins were visualized by SDS PAGE. All samples of the appropriate volume were mixed with an equal volume of 2 × SDS PAGE loading buffer (0.125 M Tris-HCl, pH 6.8, 4.0% SDS, 30% glycerol, 1.5% β-mercaptoethanol and 0.02% bromophenol blue), boiled at 95°C for 5.0 min, loaded on the 12% gel and ran in SDS PAGE running buffer (0.1% SDS, 0.025 M Tris-HCl and 0.192 M glycine). After completion the gel was stained with Coomassie blue stain (45 ml methanol, 40 ml H_2O, 10 ml CH_3COOH and 250 mg Coomassie Brilliant Blue) and destained with Destaining solution (45% methanol, 45% H_2O, 10% CH_3COOH).

The protein concentrations were determined using the Bradford method in comparison to bovine serum albumin (BSA) standards.

4.9.6 Functional complementation

The relevant pBAD EXP49 constructs were transformed into chemical competent *E. coli* DV62 cells. Starter cultures were made in the morning on the day of the experiment from a single colony in 5 ml LB media supplemented with 100 mg/L ampicillin.

a) Solid LB media

The cultures were streaked unto either minimal media plates (2.0% E-salts, 0.2% glycerol, 0.01% thiamine, 0.1% methionine, 0.2% arabinose, 10 mM β−alanine, 100 $mg.ml^{-1}$ ampicillin and 1.5% agar) or rich LB plates (LB agar supplemented with 100 $mg.ml^{-1}$ Ampicillin). The plates were sealed with parafilm and incubated at the relevant temperatures (positive control: 30°C; experiment: 42°C) for 24 h.

b) Liquid minimal media

Complementation was done in Corning 24-well flat bottom cell culture plates using a Thermo Varioskan™ spectrophotometer. To initiate the experiment, 10 ml minimal media (2.0% E-salts, 0.2% glycerol, 0.01% thiamine, 0.1% methionine, 0.2% arabinose, 10 mM β−alanine and 100 mg/L ampicillin) was inoculated with 10 µl starter culture. The plate was incubated at 42°C with shaking and the increase in optical density followed at 600 nm by taking a reading every 30 min over a period of 30 h.

4.9.7 Activity test

PanK activity was measured by a continuous enzymatic coupled assay which links the formation of ADP to the consumption of NADH.[14] All reactions were performed at 37°C in a Thermo Varioskan™ spectrophotometer. Each 300 µl contained 100 mM HEPES, pH 7.5, 1.0 mM $MgCl_2$, 60 mM NH_4Cl_2, 2.0 mM PEP, 0.5 mM NADH, 0.5 mM pantothenic acid, 10.0 mM ATP, 2.75 U LDH, 2.0 U PK, and enzyme. The reactions were initiated with the addition of the enzyme and the decrease of NADH measured at 340 nm over a period of 5 min.

4.9.8 Kinetic characterization

The kinetic characterization of *B. anthracis* BA2901 was undertaken as described in Chapter 3 for *P. aeruginosa* PanK-III with β-methylpantothenic acid.

4.10 References

1. Sassetti, C.M., Boyd D.H. and Rubin E.J., Genes required for mycobacterial growth defined by high density mutagenesis. *Molecular Microbiology*, 2003. **48** (1): p. 77-84.
2. Awasthy, D., Ambady A., Bhat J., Sheikh G., Ravishankar S., Subbulakshmi V., Mukherjee K., Sambandamurthy V. and Sharma U., Essentiality and functional analysis of type I and type III pantothenate kinases of *Mycobacterium tuberculosis. Microbiology (Reading, U. K.)*, 2010. **156** (Copyright (C) 2011 American Chemical Society (ACS). All Rights Reserved.): p. 2691-701.
3. Paige, C., Reid S.D., Hanna P.C. and Claiborne A., The type III pantothenate kinase encoded by *coaX* is essential for growth of *Bacillus anthracis. J. Bacteriol.*, 2008. **190** (18): p. 6271-75.
4. Keller, T.H. Lead finding for TB pantothenate kinase: Lessons learned. in *ICS-UNIDO workshop: "Advanced Design and Development of potential Drugs against Tuberculosis.* 2009.
5. Brand, L.A. and Strauss E., Characterization of a new pantothenate kinase isoform from *Helicobacter pylori. Journal of Biological Chemistry*, 2005. **280** (21): p.20185-88.
6. Cheek, S., Ginalski K., Zhang H. and Grishin N.V., A comprehensive update of the sequence and structure classification of kinases. *BMC Structural Biology*, 2005. **5**: p. No pp given.
7. Cheek, S., Zhang H. and Grishin N.V., Sequence and structure classification of kinases. *Journal of Molecular Biology*, 2002. **320** (4): p. 855-81.
8. Yang, K., Eyobo Y., Brand L.A., Martynowski D., Tomchick D., Strauss E. and Zhang H., Crystal structure of a type III pantothenate kinase: insight into the mechanism of an essential coenzyme A biosynthetic enzyme universally distributed in bacteria. *Journal of Bacteriology*, 2006. **188** (15): p. 5532-40.
9. Yang, K., Strauss E., Huerta C. and Zhang H., Structural basis for substrate binding and the catalytic mechanism of type III pantothenate kinase. *Biochemistry*, 2008. **47** (5): p. 1369-80.
10. Hong, B.S., Yun M.K., Zhang Y.-M., Chohnan S., Rock C.O., White S.W., Jackowski S., Park H.-W. and Leonardi R., Prokaryotic type II and type III pantothenate kinases:

The same monomer fold creates dimers with distinct catalytic properties. *Structure*, 2006. **14** (8): p. 1251-61.

11. Bork, P., Sander C. and Valencia A., An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proc. Natl. Acad. Sci. U. S. A.*, 1992. **89**: p. 7290-4.
12. Nicely, N.I., Parsonage D., Paige C., Newton G.L., Fahey R.C., Leonardi R., Jackowski S., Mallett T.C. and Claiborne A., Structure of the type III Pantothenate Kinase from *Bacillus anthracis* at 2.0 Å. Resolution: Implications for Coenzyme A-Dependent Redox Biology. *Biochemistry*, 2007. **46** (11): p. 3234-45.
13. Vallari, D.S. and Rock C.O., Isolation and characterization of temperature-sensitive pantothenate kinase (*coaA*) mutants of *Escherichia coli. Journal of Bacteriology*, 1987. **169** (12): p. 5795-800.
14. Strauss, E. and Begley T.P., The antibiotic activity of *N*-pentylpantothenamide results from its conversion to ethyldethia-coenzyme A, a coenzyme A antimetabolite. *Journal of Biological Chemistry*, 2002. **277** (50): p. 48205-09.
15. Brand, L.A., Comparative characterisation and mutational analysis of type III pantothenate kinases, *M.Sc thesis (Biochemistry)*. 2006, Stellenbosch university: p. 121.
16. Guzman, L.-M., Belin D., Carson M.J. and Beckwith J., Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *Journal of Bacteriology*, 1995. **177** (14): p. 4121-30.
17. Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G., Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 1998. **393**: p. 537-44.
18. Jensen, M.A., Fukushima M. and Davis R.W., DMSO and betaine greatly improve amplification of GC-Rich constructs in *de novo* synthesis. *PLoS ONE*, 2010. **5** (6): p. e11024.

19. Bellinzoni, M. and Riccardi G., Techniques and Applications: The heterologous expression of *Mycobacterium tuberculosis* genes is an uphill road. *Trends in Microbiology*, 2003. **11** (8): p. 351-58.

20. Novy, R., Drott, D., Yaeger, K., and Mierendorf, R, Overcoming the codon bias of *E. coli* for enhanced protein expression. *inNovations*, 2001. **12**: p. 1-3.

21. Strauss, E., Coenzyme A biosynthesis and enzymology, in *Comprehensive Natural Products II*, M. Lew and L. Hung-Wen, Editors. 2010, Elsevier: Oxford. p. 351-410.

22. Balibar, C.J., Hollis-Symynkywicz M.F. and Tao J., Pantethine rescues phosphopantothenoylcysteine synthetase and phosphopantothenoylcysteine decarboxylase deficiency in *Escherichia coli* but not in *Pseudomonas aeruginosa. J. Bacteriol.*, 2011: p. JB.00334-11.

Chapter 5:
Expression and characterization of the putative Archaeal PanK-IV (COG1829)

5.1 Background

At the beginning of this study no PanK enzymes had been identified, isolated or otherwise characterized from any of the Archaea. Homology searches of all available Archaeal genomes, using sequences of any one of the three known PanK types as search terms, failed to identify potential candidates of putative PanK-encoding genes. By contrast candidates for the four remaining CoA biosynthetic enzymes could be identified. The presence of these four enzymes indicates that the Archaea should be able to biosynthesize 4'-phosphopantothenate, either through phosphorylation of pantothenic acid by an as yet uncharacterized type of PanK, or by a novel mechanism. To identify the "missing" Archaeal CoA biosynthesis genes, Ulrich Genchel (from Technische Universitat, München) reconstructed the CoA biosynthesis pathway using of phylogenetic profiles and chromosomal proximity methods.[1] This identified strong candidates for two of the "missing proteins" in the Archaeal pathway – COG1829 for the PanK and COG1701 for the pantothenate synthase (PS). COG1829 was found to be unrelated to any of the three characterized PanK types. The aim of this chapter was to isolate and characterize the first example of COG1829 as a putative Archaeal PanK-IV.

5.2 Results – Identification, isolation and purification

5.2.1 Functional complementation studies

To determine whether the COG1829 candidates displayed pantothenate kinase activity as predicted by the Genchel study, functional complementation studies were done. Candidate genes from *Pyrococcus furiosus* (*Pf*) and *Pyrococcus horikoshii* (*Ph*) were cloned into the pBAD-DEST49 vector, transformed into *E. coli* DV62 and the cultures grown in liquid minimal media at 42°C following the same procedure as outlined in section 4.4.1. The gene products from both extremophile strains were able to rescue the *E. coli* mutant strain at 42°C (refer to Figure 5.1). Based on the positive result obtained, we set out to isolate and characterize these enzymes *in vitro*.

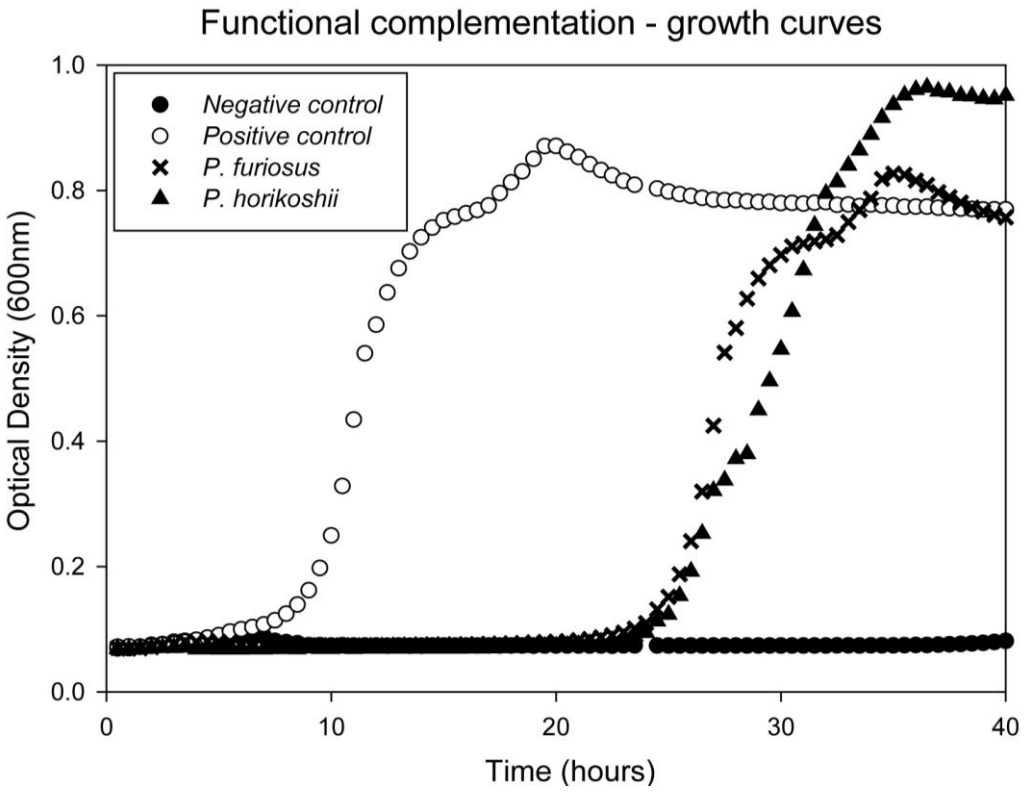


Figure 5.1: Functional complementation studies done in liquid minimal media clearly shows complementation of the temperature sensitive *E. coli* DV62 strain by *Pf* and *Ph* candidate *COG1829s*.

5.2.2 Purification of the candidate COG1829 from *Pyrococcus furiosus*

At the onset of this project the putative *Pf* PanK-encoding gene had already been cloned into pET28a(+), pDEST544 and pDEST566. Expression was obtained from the pET28a(+) construct, from which the target protein was expressed as an *N*-terminal His_6-tag fusion, but the enzyme was found to aggregate in inclusion bodies. Attempts to solubilize and purify the protein from the inclusion bodies failed to deliver active protein.

The solubility of target proteins can often be increased by introduction of *N*-terminal fusions with highly soluble and relatively large protein partners. Maltose binding protein (MBP) is a 43 kDa fusion tag that is encoded by pDEST566, while nut utilization substance (Nus) protein is 54 kDa tag encoded by pDEST544. The constructs in these destination vectors were designed in such a way that a His_6-tag precedes the fusion partner, and a tobacco etch virus (TEV)-cleavage site is introduced between the tag and the target gene. Soluble protein was only observed for the pEXP566-TEV-construct which produce the smaller His_6-MBP-fusion (as appose to the larger His_6-Nus-fusion). However, purification of this protein by IMAC

failed because the Ni^{2+} appeared to be chelated and stripped from the column by the protein during the injection step. Affinity purification with amylose resin of the His_6-MBP-fusion was also unsuccessful.

In light of the problems experienced with IMAC purification, alternative purification methods were investigated for the MBP-tagged protein. Ion exchange chromatography was attempted first, but the enzyme obtained in this manner was neither pure nor active. Next, purification by heat denaturing of the contaminating *E. coli* proteins were investigated. Since the optimum temperatures of the organisms used in this study is ≈ 100°C, it was expected that the target extremophile kinase would remain soluble and in an active form, while the *E. coli* proteins would precipitate and be removed by centrifugation. However, prior to heating, the MBP-tag would need to be cleaved from the target protein to prevent it from negatively affecting the heat stability of the latter.

The pellet obtained from the expression of the MBP-tagged COG1829 candidate enzyme was lysed by sonication, and the lysate was subsequently treated with TEV protease in a ratio of 1:100 w/w of TEV protease: total protein in cell lysate. The protein concentration in the cell lysate was determined by the Bradford method in comparison to BSA standards. After the digestion step, the fractions were heated to 90°C, the denatured *E. coli* proteins pelleted by centrifugation and the soluble fraction analyzed by SDS PAGE in comparison to a control experiment in which the lysate was not treated with TEV protease. In Figure 5.2 a 12% SDS-polyacrylamide gel is shown with the purified protein bands clearly visible.

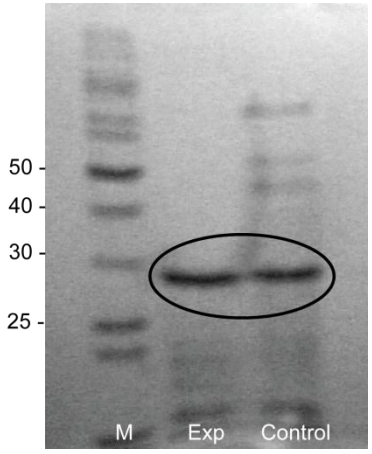


Figure 5.2: Fractions obtained after heat purification of TEV digested (Exp) and undigested (Control) MBP-tagged *Pf* COG1829; M = PageRule™ unstained protein marker (Fermentas).

Although good purification was obtained with this method, there were three disconcerting factors: (1) the protein purified in this manner was smaller than the expected 35 kDa of the target protein, (2) the purified protein displayed no noticeable activity in the LDH/PK coupled PanK assay (as described in section 3.5.4)[2] and (3) the same bonds were observed with the negative control (boiled lysate undigested by TEV protease). While the lack of activity could possibly be due to the LDH/PK assay conditions not being optimal for the determination of the activity of thermophilic enzymes (a shortcoming addressed in section 5.3), the apparent decreased size of the target protein warranted further investigation.

5.2.3 Characterization of the candidate COG1829 from *Pyrococcus furiosus*

To determine whether the smaller size of the target protein obtained through the heat denaturing purification was due to degradation, the resulting protein band was analyzed by various mass spectrometry techniques. However, neither ESI-MS, LC-MS, capillary MS nor MALDI-TOF were able to provide the size of the protein. The 12% SDS-polyacrylamide gel from Figure 5.2 demonstrates that the purified samples contained other contaminating proteins. To eliminate interference from these sample impurities with the MS analysis and to avoid extensive buffer exchange steps, the sample was purified by and extracted from the SDS-polyacrylamide gel. The 12% gel was negatively stained with $CuCl_2$[3] and the protein extracted by the gel extraction method A described by Cohen *et al.*[4] The protein obtained by this method was analyzed by MS, but still the mass could not be determined.

The problems in determining the size of the protein was interpreted to occur because the protein candidate was too large for the instrumentation. To obtain smaller fragments peptide mass fingerprinting (PMF) was used. This involves the digestion of the target enzyme to produce smaller fragments followed by the quantification of each band by MS. To do this analysis in house, COG1829 was extracted from the SDS-polyacrylamide gel and digested using clostripain as described by Shevchenko *et al*.[5,6] Clostripain was chosen as it is predicted to cut COG1829 only 15 times compared to the 34 sites for trypsin. The fewer and bigger bands are easier to visualize with SDS PAGE before analysis of the fragments by MS. After this in house analysis failed to produce usable results, the protein was sent to the Centre of Proteomic and Genomic Research, Faculty of Health Sciences, University of Cape Town for PMF analysis by digestion with trypsin followed by MS analysis. This analysis

identified the protein as D-ribose binding protein from *E. coli* (the top 5 hits all indicated this protein or mutants thereof) with 100% confidence in identity assignment.

5.2.4 Further attempts to solubilize the candidate COG1829

As heat precipitation could not be used for protein purification, extensive new expression trials were undertaken to produce native COG1829 protein or various fusion proteins thereof. The fusions tags tested included *N*- or *C*-terminal His_6-, *N*-terminal His_6-MBP-TEV and *N*-terminal thioredoxin. The proteins were produced using either *E. coli* BL21(DE3), *E. coli* C41 or C43 cell strains (for toxic protein expression) at various temperatures and cells were induced using increasing concentrations of IPTG.

To further increase translation efficiency, co-expressions were performed with either the pRARE (Novagen) or pLysS (Lucigen) plasmids, which express the tRNAs of certain scarce codons (discussed in section 4.4.2). None of these conditions yielded sufficient soluble, active protein for purification by IMAC.

5.2.5 *Methanocaldococcus jannaschii* COG1829 candidate

As all attempts to produce and purify the *P. furiosus* COG1829 protein failed, we set out to purify the same protein from a different Archaon. The gene encoding COG1829 from *Methanocaldococcus jannaschii* was subcloned into pET28a using *Nde*I and *Bam*HI restriction sites. Extensive expression trials in *E. coli* BL21(DE3) at various temperatures and induction with increasing IPTG concentration did not lead to clear bands of soluble protein being observed by SDS PAGE. To identify whether any protein could be purified, batch expressions were done with the best results obtained from the expression trials and purification attempted by IMAC with Ni^{2+}. However no protein was purified in this manner, leading us to conclude that the soluble expression was unsuccessful. Based on an article published at this time (discussed in section 5.4.4), this study was abandoned.

5.3 Results - Assay development

5.3.1 Survey of available assay methods

To determine the kinetic parameters of the extremophile PanKs an assay is required that can be performed at the predicted high optimal temperatures of these enzymes. (Although it is presumed that the COG1829 candidate will function optimally at temperatures in excess

of 90°C, the optimal temperature will need to be determined first before full kinetic characterization is undertaken). The continuous PanK assay that is most often used, the coupled LDH/PK assay described earlier,[2,7] makes use of coupling enzymes which would denature at these high temperatures. We therefore investigated other, mainly discontinuous assays, which could be employed at elevated temperatures.

Other established PanK assays include HPLC based and radioactivity assays. However, currently it would be difficult to separate and detect pantothenate (the substrate) and 4'-phosphopantohenate (the reaction product) by HPLC. Assays with radioactive ^{14}C-pantothenic acid are widely used,[8-12] as are methods that assay ATP consumption by following the time course with ^{31}P NMR.[13-15] These assays are known for their sensitivity and are preferable for accurate quantification. However, for routine work we were interested in developing an assay that is easier to set up and cheaper to use. Two other options were therefore investigated. In the first assay, the concentration of ADP remaining after completion of the enzymatic reaction is quantified using the LDH/PK coupling enzymes normally used for the continuous assay.[16] In the second method, Luciferase is used to quantify the concentration of the remaining ATP.[17,18] We adapted the assay with the coupling enzymes to quantify the [ADP].

5.3.2 ADP quantification

In the LDH/PK coupled assay (Figure 5.3) one molecule of pantothenic acid reacts with one molecule of ATP to form one molecule of ADP. This ADP molecule is recycled by PK generating one molecule of pyruvate which in turn is consumed by LDH while one molecule of NADH and one molecule lactate are generated. Thus for every molecule of ADP produced in the phosphorylation reaction, one molecule of NADH is consumed.

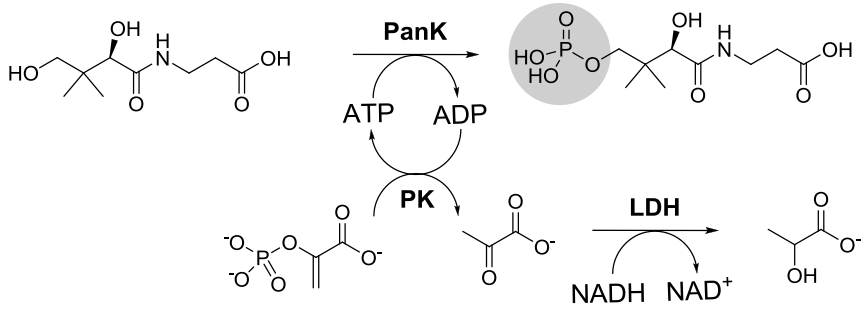


Figure 5.3: Coupled enzymatic assay used to quantify the [ADP] produced by the PanK reaction.

The assay was split into two parts. First the enzymatic reactions are conducted at the required high temperature for a predetermined time. After the enzymatic reaction is terminated, the assay mix, containing the coupling enzymes and reagents, are added to the reaction mixture and the absorbance of NADH measured at 340 nm. Using a standard curve, the amount of remaining NADH can be determined and correlated to the amount of substrate consumed in the reaction.

Our first course of action was to test the stability of our system at different incubation temperatures. Solutions were made in Tris-buffer containing pantothenic acid and varying concentrations of ADP. After incubation for 20 min at either 37°C or 90°C the assay mixture (PEP, PK, LDH and NADH) was added and the absorbance measured at 340 nm at 25°C. The solutions were stable at the higher temperatures and excellent linear regressions were obtained as can be seen in Figure 5.4 for the ADP standard curve. R^2-values of 0.996 were obtained for linear regression fits on both data sets.

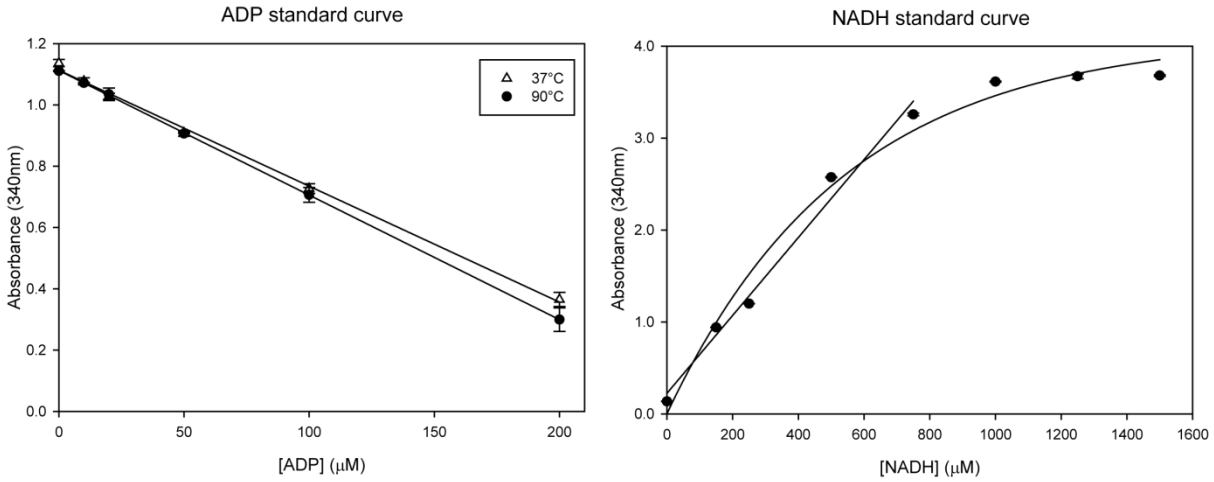


Figure 5.4: Standard curves for ADP and NADH

For the NADH standard curve solutions of different concentrations were made in H_2O and measured as is. As shown in the graph in Figure 5.4, the NADH curve is only approximately linear to a concentration of 600 µM. A final concentration of 500 µM NADH was used in all following assays as this value is within the linear range.

Another aspect that had to be established was the degree of denaturation of PanK at the reactions at elevated temperatures. Because the extremophile PanKs are believed to be heat-stable, the enzyme reaction cannot be terminated by heat denaturation, while stopping the enzyme reaction in any other way may influence the performance of the

coupling enzymes. Various enzyme denaturing techniques were investigated, including additives (1 M NaOH (base), 1 M HCl (acid), detergents Tween and Triton, ethanol), flash cooling in ice water and removing the enzyme by syringe filter. Addition of acid and base were found to be problematic as the pH had to be neutralized prior to the addition of the coupling enzymes. Tween appear not to affect the enzyme. Removing the protein by syringe filter, flash cooling in ice water, and the addition of Triton and ethanol all proved useful and resulted in comparable results. For assays in Eppendorf tubes, flash cooling in ice water was used to stop the enzymatic reaction, while reactions in multiwell plates were stopped by the adding ≈ 5% ethanol.

The LDH/PK coupled assay was successfully modified and used to quantify the concentration of ADP in the reaction mixtures and was used in all further screening work. All assays were executed at 90°C since, *E. coli* PanK-I was observed to be marginally active at 60°C.

5.4 Discussion

5.4.1 COG1829 as a candidate PanK

The candidate enzymes from *Pyrococcus furiosus* and *Pyrococcus horikoshii* were both able to rescue the *E. coli* mutant strain at 42°C, albeit at a slower rate than the *E. coli* PanK-I positive control. The slower growth rate was not a concern as the optimum growth temperatures of the extremophile, and subsequently the optimum activity temperature of the enzymes, are much higher than the assay temperature. This result therefore supported the prediction of COG1829 being a PanK. However, as we were unable to produce and purify a candidate COG1829 as described above, this interference could not be confirmed *in vitro*.

New information published in 2009 (see below), indicated that COG1829 is not a PanK as originally postulated. This study was therefore suspended.

5.4.2 Assay development

An initial screen indicated that the PK/LDH coupled enzymatic assay is not suitable to quantify *Pf* COG1829 activity as it is affected by the required high optimum temperatures. Variations of the techniques were investigated resulting in a discontinuous version of the above assay being employed in all further application.

5.4.3 Alternative pathway in Archaea

A report from 2009 reported that PanK activity could not be detected in cell free extracts of the hyperthermophilic *Thermococcus kodakaraenis*.[19] The study also aimed at characterizing the first example of an archaeal PanK. The approach was, however, different in that an expression plasmid designed for extremophiles was constructed. The candidate genes for both PS (COG1701, TK1686) and PanK (COG1829, TK2141) were cloned into the specialized plasmid and subsequently overexpressed and the enzymes purified to homogeneity. Enzyme activity tests indicated that *T. kodakareanis* displays very little PS and PanK activity. Instead these are two novel enzymes, pantoate kinase (PoK) and phosphopantoate synthetase (PPS).

The kinetic parameters measured for the PanK alternative PoK, are listed in Table 5.1. The enzyme has high K_M-values for both pantoate and panthothenic acid, but its overall catalytic efficiency is higher with pantoate as substrate.

Table 5.1: Kinetic parameters for PoK

Kinetic parameters	V_{max} (nmol.min^{-1}.mg^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}.mM^{-1})
Pantothenic acid	390 ± 10	1 300 ± 100	0.21 ± 0.01	0.17
Pantoate	2 870 ± 60	1 200 ± 10	1.56 ± 0.03	1.3
ATP	2 710 ± 20	470 ± 10	1.48 ± 0.01	3.1

In the classical CoA biosynthesis pathway (Figure 5.5), pantoate is condensed with β-alanine to form pantothenic acid by the enzyme PS, followed by phosphorylation to 4'-phosphopantothenate by PanK. In *T. kodakareanis* pantoate is first phosphorylated by PoK to 4'-phosphopantoate before the condensation reaction with β-alanine to yield the same end product.

Homologs of the genes encoding the novel PoK and PPS are widely distributed among various Archaeal species suggesting that this is the route used in these organisms to produce 4'-phosphopantothenic acid.[19]

However, this pathway is not universal or absolute in Archaea as a PoK gene was not identified in the *Thermoplasmatales* order. A BLAST search on the complete genome

sequence of *Picrophilus torridus* identified the PTO0232 gene as a distant homolog of *E. coli coaA*. This gene was therefore amplified, cloned, over-expressed from a pET28a(+) construct, purified with IMAC and kinetically characterized. The Archaeal PanK-I has an extremely high K_M-value for pantothenic acid (621 µM vs. 36 µM of *E. coli* PanK-I), while the K_M-values' for ATP is more or less the same as that of the *E. coli* enzyme. The other major difference is that the *P. torridus* PanK-I, like *S. aureus* PanK-II, is refractory to feedback inhibition by CoA. Based on these differences it was suggested that Archaeal PanK-I constitute a new subtype of type I PanKs.[20]

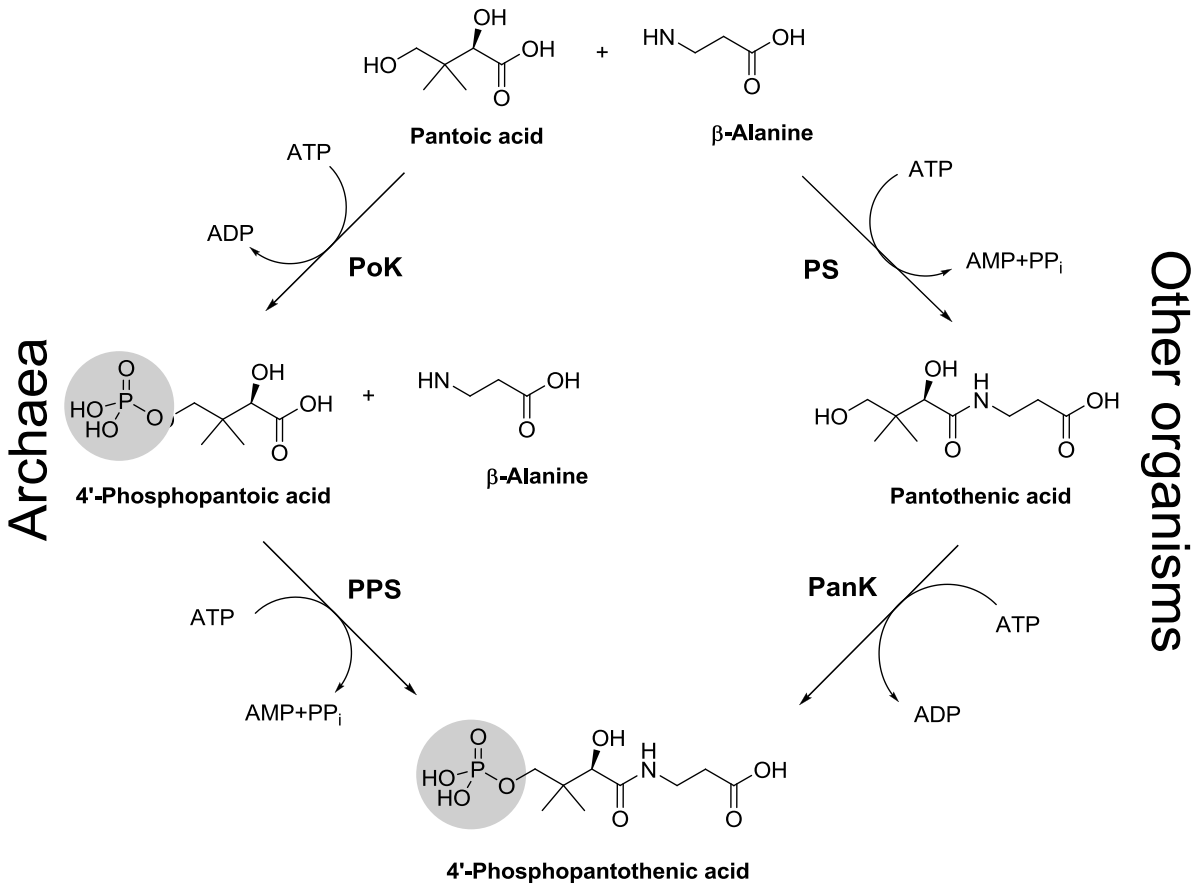


Figure 5.5: General pathway for the production of 4'-Phosphopantothenic acid in pro- and eukaryotes (right) and the alternative pathway in the Archaea (left).

5.5 Conclusion

Research by ourselves and other groups indicate conclusively that Archaea do not harbor a Type IV PanK as had previously been though. Instead Archaea have novel PoK and PPS enzymes that provide an alternative route for the biosynthesis of 4'-phosphopantothenate from β-alanine and pantoic acid. The first of example of an archaeal Type I PanK has been characterized from *P. torridus* apart of this thesis. The enzyme exhibits some unique characteristics and may constitute the first characterized example of a new subtype of the type I PanK enzymes.

Our efforts to isolate and characterize an Archaeal PanK/PoK candidate enzyme proved unsuccessful. Background activity of *E. coli* PanK-I and co-purification of other *E. coli* proteins complicated purification strategies and activity determinations. Nonetheless we were able to successfully develop a new discontinuous assay for thermophilic kinase enzymes. This may prove useful in future studies.

5.6 Experimental

Three strains of Archaea were identified and used in this study - *Pyrococcus furiosus*, ATCC 43587, *Pyrococcus horikoshi,* ATCC 700860, and *Methanocaldococcus janaschii.*

Plasmid MJ0969, a pT7-7 construct containing the *Methanocaldococcus janaschii* candidate gene, was a gift from Robert White, Virginia Tech, US.

5.6.1 Revival of extremophiles, DNA isolation and amplification

a) Growing and stocking of organisms

(Growing pure cultures was undertaken by Mrs Leisl Brand.)

A 15 ml starter culture was prepared in pyrococcus medium enriched with 0.83 g/l Na_2S (normally 0.5 g/l) and purged with 100% N_2. After incubation at 95°C for ± 24 h, the culture was used to inoculate 50 ml pyrococcus media, enriched with the same concentration Na_2S, under inert atmosphere. This culture was grown overnight at 95°C, harvested by centrifugation, the supernatant decanted and the pellet resuspended in 3.0 ml TE buffer, divided into aliquots and stored at -80°C.

b) Amplification for cloning into pET28a(+)

(DNA was isolated and amplified by Mrs Leisl Brand.)

Genomic DNA was extracted from cultures of the organisms and used as template for the amplification of candidate genes. The forward and reverse primers introduced *Nde*I and *Xho*I restriction sites (underlined) respectively for the cloning into pET28a(+). The following primer sets were used:

Pyrococcus furiosus: forward primer: 5' – GGAGGTGAGCATATGCTAATTAGAGC – 3'
reverse primer: 5' – CTAG AAGTATAGTTCTCGAGAAAAGCAAATC – 3'

Pyrococcus horikoshii: forward primer: 5'-GGTAAGGCATATGCTAATTCGAGCG -3'
reverse primer: 5'-TAT AGTTAATAACTCGAGGATATCTCTG-3'.

c) Amplification for cloning into pET22b(+)

The COG1829 candidate gene was subcloned from the pET28a(+) construct. The reverse primer introduced an *Xho*I-site (underlined) and removed the stop codon.

Pyrococcus furiosus: T7 forward primer: 5'-TAATACGACTCACTATAGGG-3'
reverse primer: 5'-GGGGGGGGCTCGAGCTCATTTGGTATCCACC-3'

The amplification utilized Dream*Taq* (Fermentas) polymerase and the PCR reaction mix contain of 1 × Dream*Taq*-buffer, 0.4 mM dNTP mix, 1.0 mM forward primer, 1.0 mM reverse primer, 1.0 µg genomic DNA, various $MgCl_2$ concentrations (1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM and 3.5 mM), 2.5 U Dream*Taq* and H_2O to a final volume of 25 µl.

The PCR program started with an incubation step of 2 min at 94˚C before the cycle was initiated with a denaturation step at 94˚C for 15 s, an annealing step at 55˚C for 30 s and a polymerization step 72˚C for 1.5 min. This cycle was repeated 30 times and the program ended with a hold step at 4.0˚C. Reaction mixtures were analyzed by gel electrophoresis on a 1.0% agarose gel and the bands visualized by staining with SYBR®gold. Product bands were obtained at 735 bp in all instances and the bands were purified from the gel with the Fermentas GeneJET™ Gel Extraction kit.

5.6.2 Cloning

The same general procedures were used for cloning and expression trials as described in Section 4.9.2.

a) *Pyrococcus furiosus* and *Pyrococcus horikoshii*

(Cloning was performed by Mrs Leisl Brand.)

The COG1829 candidate genes and plasmids, pET28a(+) and pENTR4N, were digested with *NdeI* and *XhoI.* The genes were cloned into the respective plasmids using the Quick ligase method. The only difference in the procedure involved the DNA being incubated at 60°C for 5 min (to break any unproductive bonding in the DNA) before the Quick ligase buffer and ligase were added to the ligation mixture.

Both genes were subcloned into pBAD DEST49, pBAD544 and pBAD566 using LR clonase and the relevant pENTR4N constructs.

b) *Pyrococcus furiosus*: Subcloning into pET22b(+)

The PCR product (section 5.7.1c) was digested with *NdeI* and *XhoI* and cloned into digested pET22b(+) with Quick ligase to yield the construct without a stop codon before the C-terminal His_6-tag sequence.

The COG1829 candidate gene was digested from the pET28a-construct with *NdeI* and *XhoI* and subcloned into digested pET22b(+) with Quick ligase to yield the construct for native expression.

c) *Methanocalcococcus janaschii*: Subcloning into pET28a(+)

The COG1829 candidate-gene was digested from the pT7-7 construct using 2 × Fermentas Tango buffer, 20 U *Nde*I (New England Biolabs), 20 U *Bam*HI (Fermentas) in a final reaction volume of 20 µl. After 2.5 h of incubation at 37 °C, the digestion mixture was resolved on 1.0% agarose gel and purified from the gel with the Fermentas GeneJET™ Gel Extraction kit. The candidate gene was cloned into pET28a(+) using the Quick ligation method.

5.6.3 Functional complementation studies

The relevant pBAD EXP49 constructs were transformed into chemical competent *E. coli* DV62 cells. Starter cultures were prepared in the morning on the day of the experiment from a single colony in 5.0 ml LB media supplemented with 100 mg/L ampicillin. Comple-

mentation was done in Corning 24-well flat bottom cell culture plates using the Thermo Varioskan™ spectrophotometer. To initiate the experiment, 10 ml minimal media (2.0% E-salts, 0.2% glycerol, 0.01% thiamine, 0.1% methionine, 0.2% arabinose, 10 mM β–alanine and 100 mg/L ampicillin) was inoculated with 10 µl starter culture. The plate was incubated at 42°C with shaking and the increase in optical density was followed at 600 nm by taking a reading every 30 min over a period of 30 h.

5.6.4 Expression of the putative COG1829 *Pyrococcus furiosus* candidate

a) MBP-tagged protein from pEXP566-TEV construct

LB media (500 ml), supplemented with 100 mg/L ampicillin, was inoculated with plasmid containing *E. coli* BL21(DE3) starter culture. The culture was grown to midlog phase and induced with a final concentration of 0.2 mM isopropyl-β-D-thiogalactoside (IPTG). Growing continued at 37°C overnight.

b) Thioredoxin-tagged protein from pBAD EXP49 construct

LB media (500 ml), supplemented with 100 mg/L ampicillin, was inoculated with plasmid containing *E. coli* BL21(DE3) starter culture. The culture was grown until midlog phase, induced with a final concentration = 0.2% L-arabinose and grown at 37°C overnight.

5.6.5 Purification strategies

a) TEV cleavage of MBP-tagged protein

The cell pellet, obtained from the pEXP566-TEV construct expression, was lysed using glass beads and vortexed for 10 min in TEV buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA). Novagen Bugbuster® protein extraction reagent was also used, but didn't lyse the cells sufficiently. TEV protease (24.8 µg) was added to the cell lysate containing 2.48 mg protein (quantified by Bradford) and the reaction incubated at 4.0°C overnight.

b) Purification by heat denaturation of *E. coli* proteins

Aliquots (100 µl in 1.5 ml Eppendorf tubes) of the TEV cleaved reaction mixture were warmed in a heating block for a predetermined time at a predetermined temperature. The denatured protein was pelleted by centrifugation at 13 000 rpm for 20 min at 4.0°C. The supernatant was removed and used in further applications.

c) Purification from 12% SDS-polyacrylamide gels

The protein purification from a 12% SDS-polyacrylamide gel was based on the procedures described by Bricker *et al*.[3] and Cohen *et al*.[4] Protein samples were concentrated by centrifugation (Nanosep, centrifugal device, 10 K cut-off) and resolved on a 12% SDS-polyacrylamide gel. The gel was reversible stained with 0.3 M $CuCl_2$ for 5 min before rinsing with distilled water to remove the excess $CuCl_2$. The protein band was excised from the gel, 1 ml destaining solution (0.1 MTris-HCl, pH 9.0, 0.25 M EDTA) was added and followed by agitation for 10 min. After removing solvent, the gel slice was rinsed with distilled H_2O to remove traces of destaining solution and crushed. Extraction buffer (40 µl, 1:3:2 (v/v/v) formic acid: H_2O: isopropanol) was added to the crushed gel piece and incubated for 4 h at room temperature with continued agitation. The gel debris were pelleted by centrifugation at 8 000 rpm for 20 min at room temperature, the supernatant removed and the gel debris washed with extraction buffer (40 µl). The protein containing supernatants were combined and the buffer exchanged as described in the next section.

d) Buffer exchange strategies

The buffer was exchanged using Bio-Gel P-6 DG (Bio-Rad) and micro Bio-Spin chromatography columns (Bio-Rad). The gel was hydrated with either H_2O or buffer (10 mM Tris-HCl, pH 7.2) for 4 h at room temperature. Gel (800 µl) was added to each column. Excess liquid was allowed to drain by gravity and the columns were centrifuged at 6 000 rpm for 1.0 min. Protein sample (75 µl) was added to each column and eluted by centrifugation at 2 000 rpm for 4.0 min. Protein in the elution fraction was confirmed by 12% SDS PAGE analysis.

5.6.6 In-gel clostripain digest

The 12% SDS-polyacrylamide gel was reversibly stained and destained as described above. The protein containing bands were excised from the gel and cut into smaller pieces. The gel pieces were covered with acetonitrile and incubated at room temperature for 10 min to dehydrate and shrink the pieces. After removal of the acetonitrile, the pieces were rehydrated in 150 µl buffer 1 (100 mM NH_4CO_3, 10 mM DTT) for 1 h at 56 °C. The samples were cooled, the buffer replaced by 150 µl buffer 2 (100 mM NH_4CO_3, 10 mM iodoacetamide buffer) and incubated for 45 min at room temperature in the dark with occasional vortexing. After the solution was removed, the gel pieces were washed with 100

mM NH_4CO_3 (150 µl) and dehydrated again with acetonitrile (150 µl). This step was repeated twice before removing the acetonitrile and air drying the pellet. After the gel pieces were sufficiently dry, 100 µl digestion buffer (1% Trition X-100, 10% acetonitrile, 1 mM DTT, 10 mM $CaCl_2$, 100 mM Tris-HCl, pH 8.0) and clostripain (10 µl) was added. The digestion mix was incubated for 45 min in ice water, the buffer replaced with 50 mM NH_4CO_3 (20 µl) and incubated overnight at 37°C. The gel pieces were pellet by centrifugation at 13 000 rpm for 1 min and washed by incubating at room temperature for 10 min with 20 mM NH_4CO_3 (100 µl). The cleaved protein peptides were extracted from the gel by incubating the gel pieces in a 5% formic acid, 50% acetonitrile solution (100 µl) for 20 min at room temperature before pelleting the debris by centrifugation at 13 000 rpm for 1 min. The extraction step was repeated twice. All the supernatants were combined, dried and analyzed by 12% SDS PAGE.

5.6.7 Expression trials

Plasmids were transformed into *E. coli* strains: BL21(DE3), C41 or C43 as required. LB medium (1.425 ml), supplemented with 100 mg/L ampicillin, was inoculated with 30 µl starter culture. The culture was grown at 37°C with vigorous shaking until midlog phase was reached and induced with 45 µl of a predetermined concentration of IPTG and incubated overnight. To increase the translation efficiency and solubility of the target protein, the following parameters were varied at which expression occurs after induction:

1. IPTG concentration: 0.00 mM, 0.25 mM, 0.50 mM or 1.00 mM
2. Expression temperature: 15˚C, 20˚C, 25˚C, 30˚C or 37˚C
3. Co-expression with plasmids that encodes for the scarce codons - pRARE and pRARE2 plasmids for expression from BL21(DE3) and pLysS for expression from C41 and C43.

The cultures were harvested by centrifugation at 13 000 rpm for 20 min at 4.0°C and the pellet resuspended in 200 µl elution buffer (20 mM Tris-HCl, pH 8.0). The suspended pellet was analyzed as is to determine the total protein content of the cell lysate. To evaluate the amount of soluble expression, glass beads (equal to half the volume of the suspended pellet) was added to a 100 µl aliquot. The pellet was manually lysed by vortexing for 10 min, the cell debris pelleted by centrifugation at 13 000 rpm for 2 min and the protein containing supernatant analyzed by 12% SDS PAGE.

5.6.8 Assay development

a) ADP and NADH standard curves

For ADP standerd curves the reaction mixtures (5.0 mM Tris-HCl, pH 7.6, 2 mM KCl, 1.0 mM $MgCl_2$, 0.5 mM pantothenic acid, varying ADP concentrations and H_2O instead of enzyme) were incubated at either 37°C or 90°C for 20 min. After incubation the assay mixture (5 mM Tris-HCl, pH 7.6, 2.0 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM PEP, 0.3 mM NADH, 2.75 U LDH and 2.0 U PK) were added to the reaction mixtures and incubated at 25°C for 5 min before the absorbance was measured at 340 nm and 25°C in a Thermo Varioskan™ spectrophotometer.

For the NADH standard curve solutions of different NADH concentrations were made in H_2O and measured as is.

b) Terminating the enzymatic reaction

Reaction mixtures (150 µl) were incubated 60 °C for 5 min before the reaction was terminated using one of the following methods. The additives (10 µl) - 1.0 M NaOH, 1.0 M HCl, Tween, Triton or Tween (all neat) - were added directly to the reaction and the eppi was placed in ice water for flash cooling. The flash cooling reaction was left in the ice water for an additional 5 min while the reactions containing additives were incubated for the additional 5 min at 60°C. All reactions were quantified and compared to a positive control (incubate for 10 min at 60°C) and a negative control (no enzyme).

5.7 References

1. Genschel, U., Coenzyme A Biosynthesis: Reconstruction of the pathway in Archaea and an evolutionary scenario based on comparative genomics. *Mol Biol Evol*, 2004. **21** (7): p. 1242-51.
2. Strauss, E. and Begley T.P., The antibiotic activity of *N*-pentylpantothenamide results from its conversion to ethyldethia-coenzyme A, a coenzyme A antimetabolite. *Journal of Biological Chemistry*, 2002. **277** (50): p. 48205-09.
3. Bricker, T.M., Green-Church K.B., Limbaugh P.A. and Frankel L.K., Documentation of negatively stained polyacrylamide gels. *Analytical Biochemistry*, 2000. **278**: p. 237-39.
4. Cohen, S.L. and Chait B.T., Mass spectrometry of whole proteins eluted from sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. *Analytical Biochemistry*, 1997. **247**: p. 257-67.
5. Shevchenko, A., Wilm M., Vorm O. and Mann M., Mass apectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical Chemistry*, 1996. **68** (5): p. 850-58.
6. Fernandez, J. and Mische S.M., Enzymatic digestion of proteins on PVDF membranes, in *Current Protocols in Protein Science*. 2001, John Wiley & Sons, Inc.
7. Kiianitsa, K., Solinger J.A. and Heyer W.-D., NADH-coupled microplate photometric assay for kinetic studies of ATP-hydrolyzing enzymes with low and high specific activities. *Analytical Biochemistry*, 2003. **321** (2): p. 266-71.
8. Hong, B.S., Yun M.K., Zhang Y.-M., Chohnan S., Rock C.O., White S.W., Jackowski S., Park H.-W. and Leonardi R., Prokaryotic type II and type III pantothenate kinases: The same monomer fold creates dimers with distinct catalytic properties. *Structure*, 2006. **14** (8): p. 1251-61.
9. Song, W.-J. and Jackowski S., Kinetics and regulation of pantothenate kinase from *Escherichia coli*. *Journal of Biological Chemistry*, 1994. **269** (43): p. 27051-8.
10. Leonardi, R., Chohnan S., Zhang Y.-M., Virga K.G., Lee R.E., Rock C.O. and Jackowski S., A pantothenate kinase from *Staphylococcus aureus* refractory to feedback regulation by coenzyme A. *Journal of Biological Chemistry*, 2005. **280** (5): p. 3314-22.

11. Rock, C.O., Park H.-W. and Jackowski S., Role of feedback regulation of pantothenate kinase (CoaA) in control of coenzyme A levels in *Escherichia coli. Journal of Bacteriology*, 2003. **185** (11): p. 3410-15.

12. Vallari, D.S., Jackowski S. and Rock C.O., Regulation of pantothenate kinase by coenzyme A and its thioesters. *Journal of Biological Chemistry*, 1987. **262** (6): p. 2468-71.

13. Jaffe, E.K. and Cohn M., ^{31}P nuclear magnetic resonance spectra of the thiophosphate analogs of adenine nucleotides; effects of pH and Mg^{2+} binding. *Biochemistry*, 1978. **17** (4): p. 652-57.

14. Mulquiney, P.J., Bubb W.A. and Kuchel P.W., Model of 2,3-bisphosphoglycerate metabolism in the human erythrocyte based on detailed enzyme kinetic equations: *in vivo* kinetic characterization of 2,3-bisphosphoglycerate synthase/phosphatase using ^{13}C and ^{31}P NMR. *Biochem. J.*, 1999. **342** (3): p. 567-80.

15. Le Rumeur, E., Le Tallec N., Kernec F. and de Certaines J.D., Kinetics of ATP to ADP β-phosphoryl conversion in contracting skeletal muscle by *in vivo* ^{31}P NMR magnetization transfer. *NMR in Biomedicine*, 1997. **10** (2): p. 67-72.

16. Ward, D.E., Kengen S.W.M., van der Oost J. and de Vos W.M., Purification and characterization of the alanine aminotransferase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its role in alanine production. *J. Bacteriol.*, 2000. **182** (9): p. 2559-66.

17. Chappelle, E.W., Picciolo G.L. and Deming J.W., Determination of bacterial content in fluids, *Methods in Enzymology*, A.D. Marlene, Editor. 1978, Academic Press. p. 65-72.

18. Belinga, H.F., Steghens J.P. and Collombel C., Firefly luciferase purification using polyethylene glycol and Dyematrex Orange A. *J. Chromatogr., A*, 1995. **695**: p. 33-40.

19. Yokooji, Y., Tomita H., Atomi H. and Imanaka T., Pantoate kinase and phosphopantothenate synthetase, two novel enzymes necessary for CoA biosynthesis in the Archaea. *Journal of Biological Chemistry*, 2009. **284** (41): p. 28137-45.

20. Takagi, M., Tamaki H., Miyamoto Y., Leonardi R., Hanada S., Jackowski S. and Chohnan S., Pantothenate kinase from the thermoacidophilic archaeon *Picrophilus torridus. J. Bacteriol.*, 2010. **192** (1): p. 233-41.

Chapter 6:
Conclusion and Future work

6.1 PanKs: Remaining unanswered questions revisited

PanK is the best studied enzyme of the CoA biosynthetic pathway. Several gaps in our understanding of these kinases however still remain, especially in regards to the type III PanKs. In Chapter 1 several lingering questions regarding the PanKs were listed. The studies described in this thesis were designed to answer some of these by:

- Identifying alternative substrates and inhibitors of the type III PanKs from pathogenic bacteria.
- Investigating the enzyme activity of two "inactive" PanKs: the putative PanK-III from *M. tuberculosis* and PanK-II from *B. anthracis*.
- Attempting to purify and characterize the first example of a type IV PanK from the Archaea.

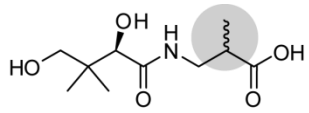
6.1.1 Identification of alternative substrates and inhibitors of PanK-IIIs

At the beginning of the study the only known inhibitor for the PanK-IIIs was the ATP mimetic by Rowan *et al.*,[1] a relatively poor competitive inhibitor of *B. anthracis* PanK-III. In our study 11 pantothenic acid analogs were designed, synthesized and tested as alternate substrates and/or inhibitors of *P. aeruginosa* PanK-III.

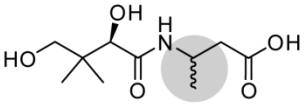
Pa PanK-III showed a surprisingly selectivity for its substrate as only one of the 11 pantothenate analogs tested, β-methylpantothenic acid, was characterized as an alternative substrate for the enzyme and only three, α- and β-methylpantothenic acid and β-trifluoromethylpantothenic acid, were identified as PanK-III inhibitors (Figure 6.1).

Inhibitor analysis showed that α-methylpantothenic acid and β-trifluoromethylpantothenic acid have very similar K_i-values, in the range of 80-90 μM, and that both are competitive inhibitors of pantothenate, the natural substrate. β-Methylpantothenic acid displayed a mixed competitive profile with uncompetitive inhibition towards pantothenic acid at low concentrations and competitive inhibition at high concentrations. The reason for this dual behaviour may be due to β-methylpantothenic acid also acting as a substrate of *Pa* PanK-III.

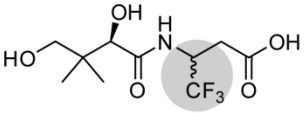
Kinetic parameters	Type of inhibition	K_i (μM)
(*R,S*)-α-methylpantothenic acid	competitive	96.1 ± 17.7
(*R,S*)-β-trifluoromethylpantothenic acid	competitive	73.4 ± 42.5
(*R,S*)-β-methylpantothenic acid	uncompetitive (low concentrations)	358 ± 79
(*R,S*)-β-methylpantothenic acid	competitive (high concentrations)	74.6 ± 30.3
Best ATP mimetic	competitive	164



α-methylpantothenic acid



β-methylpantothenic acid



β,β-trifluoromethylpantothenic acid

Figure 6.1: Three compounds identified as inhibitors of *P. aeruginosa* PanK-III and their K_i-values.

6.1.2 Investigating the activity of *M. tuberculosis* CoaX

Even though all sequence and structural evidence concurs that *Mtb* CoaX could be a functional pantothenate kinase, we were unable to verify the activity *in vitro*. This could be due to the instability of the purified protein in buffers with NaCl and imidazole concentrations below 250 μM. Another possible explanation for the apparent lack of activity relates to the two point mutations observed in the PHOSPHATE 1 and ADENOSINE motifs of mycobacterial CoaX proteins, in which the small uncharged residue, Gly, was replaced by larger charged amino acids, namely Asp and His respectively. These exchanges result in the introduction of a H-bonds in the active site of the enzyme that blocks the entrance of the active site, and therefore possibly ATP binding.

Importantly, the homologous *Ms* CoaX was purified and shown to have PanK activity. While the kinetic parameters of *Ms* CoaX show the enzyme to be catalytically less efficient than other known PanK-III enzymes, this finding strongly suggests that *Mtb* CoaX should also display pantothenate kinase activity under suitable physical conditions.

6.1.3 *B. anthracis* BA2901 is a pantetheine kinase

The putative *B. anthracis* PanK-II, BA2901, was the only other putative bacterial PanK that was reported to be inactive at the start of this study. We demonstrated that although the enzyme is inactive towards pantothenic acid as substrate, it is active towards pantetheine, the substrate of the CoA salvage pathway. While other PanK-I and PanK-II enzymes are known to have low substrate specificity and accept both pantothenate and pantetheine as substrates, BA2901 is the first known example of a kinase that exclusively accepts

pantetheine. The specific reason for this specificity, and particular its molecular basis currently remains unclear. Ideally the crystal structure of this enzyme would be able to address this. However, since *B. anthracis* has a PanK-III enzyme that does not accept pantetheine as substrate, the specificity of BA2901 could allow for the operation of a functional CoA salvage pathway in this organism. Both the fact that BA2901's K_M-value for pantetheine is a quarter of that of *E. coli* PanK-I,[2] and that its catalytic efficiency is ≈ 10 times higher, support this hypothesis. Importantly, *B. anthracis* is one of the few Gram-positive bacteria (together with *S. aureus*) that relies on high levels of CoA to maintain its intracellular redox balance.

6.1.4 Origin of 4'-phosphopantothenic acid in Archaea

The Archaea do not harbor a type IV PanK as previously speculated. Instead they have novel PoK and PPS enzymes which provide an alternative route for the biosynthesis of 4'-phosphopantothenate from β-alanine and pantoic acid. The first of example of an Archaeal type I PanK from *P. torridus* has also been characterized and suggested to represent a new subtype of the type I enzymes.

6.2 Future work

6.2.1 Further studies with the identified PanK-III inhibitors

One possible explanation for the high K_i-values determined for the newly identified PanK-III inhibitors could be the methyl substituents in the β-alanine moiety of pantothenic acid gives rise to a new stereocenter. In the inhibition activity tests, reaction mixtures contained both stereoisomers. Both α-methylpantothenic acid and β,β-trifluoromethylpantothenic acid inhibit *P. aeruginosa* PanK-III with K_i-values in the range of 80-90 μM. The true K_i-values may therefore be half or less of those determined in this study, depending on the stoichiometry of the stereoisomers. Pure enantiomers may be investigated in future to determine the true K_i.

Currently inhibition data of our inhibitors is limited to one enzyme, *Pa* PanK-III. Future studies will expand the data to other PanK-IIIs, including the enzymes from *Actinobacter baumannii, Bacillus anthracis, H. pylori* and *Treponema pallidum*, as well as representative PanK-I and PanK-II enzymes.

6.2.2 Synthesis of other analogs as potential inhibitors

Our inhibitor characterization studies revealed that exchange of the methyl group of β-methylpantothenic acid with the -CF_3-group resulted in a three-fold decrease in the observed K_i-value. Similar substitution of the methyl group of α-methylpantothenic acid may therefore possible result in a K_i of ≈ 30 µM, a third of the K_i for α-methylpantothenic acid. Future work could therefore include the synthetic preparation and kinetic characterization of α-trifluoromethylpantothenic acid, shown in Figure 6.2, as inhibitor.

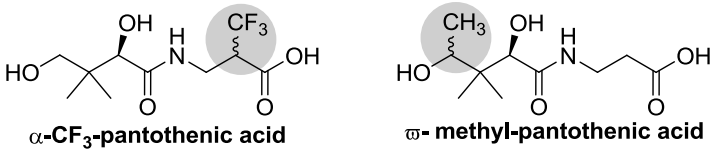
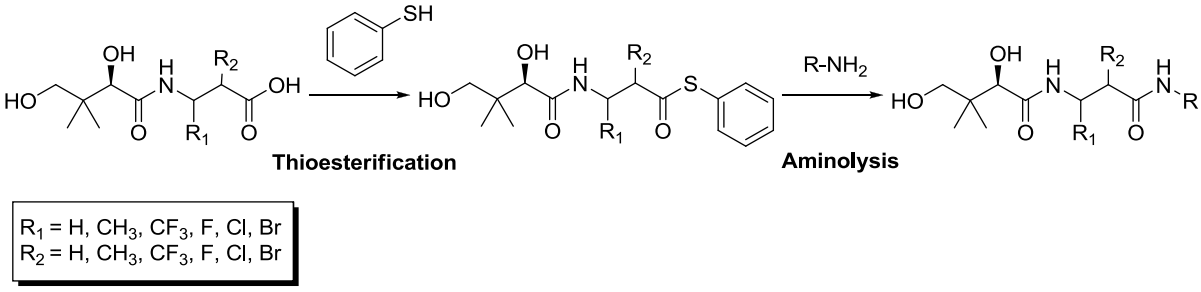


Figure 6.2: Structure of α-trifluoromethyl pantothenic acid and ω-methyl pantothenic acid

Other areas of research may further include redesigning and executing the synthesis of ω-methylpantothenic acid, also shown in Figure 6.2.

6.2.3 Utilizing synthesized scaffolds in pantothenamide synthesis

Van Wyk *et al*. developed a method for the parallel synthesis of *N*-substituted panthothenamides from pantothenic acid as starting compound.[3] The various pantothenic acid analogs synthesized in this study; including α- and β-substituted pantothenic acid analogs and 4'-deoxy- and 4'-methoxypantothenic acid could be used as starting materials to expand the current library of compounds in a similar manner. A schematic representation of such a parallel synthesis is shown in Scheme 6.1.



Scheme 6.1: Synthetic scheme for the preparation of *N*-substituted pantothenamides from pantothenic acid analogs modified on the β-alanine moiety.

In the parallel synthesis the pantothenic acid analogs are converted to the corresponding thioester by thioesterification, followed by transformation into the final pantothenamide library through aminolysis. The new library of compounds could be used in screening experiments to identify inhibitors for relevant PanK-I- and PanK-II-containing organism, such as *E. coli*, *Plasmodium falciparum* and *Staphylococcus aureus*.

6.2.4 *M. tuberculosis* and *M. smegmatis* CoaX mutagenesis studies

The *Mtb* CoaX H229G mutant was prepared to establish whether the disruption of the interaction of His and Arg (discussed in section 4.4.6) would restore activity to *Mtb* CoaX. This variant was, however, found to remain inactive. The instability of the purified protein makes it unclear whether the inactivity is due to the protein preparation, or because exchange of the one residue is insufficient to unblock the active site. Future work may include extensive mutagenesis studies to create and test the constructs containing single mutations – *Mtb* CoaX R8G, *Ms* CoaX R8G and *Ms* CoaX H228G - and the double mutants – *Mtb* CoaX R8G, H229G and *Ms* CoaX R8G, H228G. The *Ms* CoaX homologs should be included because the activity of this protein has been confirmed, and the data obtained with this enzyme can be used to test the disruption of the salt bridge theory if the problems with *Mtb* CoaX persist.

6.2.5 Addressing the other PanK related unanswered questions

There are two remaining questions raised in Chapter 1 that have not been addressed in this study and which deserves further investigation:

- Determination of the identity of the true phosphoryl donor of type III PanKs, or elucidation for the basis of the high K_M for ATP that these proteins exhibit.
- Investigation of the possible link between Baf (Bvg accessory factor) proteins and type III PanKs, or whether the activity observed for the *B. pertussis coaX*-encoded protein is unique to this organism.

6.3 References

1. Rowan, A.S., Nicely N.I., Cochrane N., Wlassoff W.A., Claiborne A. and Hamilton C.J., Nucleoside triphosphate mimicry: a sugar triazolyl nucleoside as an ATP-competitive inhibitor of *B. anthracis* pantothenate kinase. *Organic & Biomolecular Chemistry*, 2009. **7** (19): p. 4029-36.
2. Meier, J.L., Mercer A.C., Rivera H., Jr. and Burkart M.D., Synthesis and evaluation of bioorthogonal pantetheine analogues for *in vivo* protein modification. *Journal of the American Chemical Society*, 2006. **128** (37): p. 12174-84.
3. van Wyk, M. and Strauss E., Development of a method for the parallel synthesis and purification of *N*-substituted pantothenamides, known inhibitors of coenzyme A biosynthesis and utilization. *Organic & Biomolecular Chemistry*, 2008. **6** (23): p. 4348-55.