

Myristoyl CoA:Protein *N*-Myristoyl Transferase: A Target for a Novel Antimalarial Drug

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A thesis submitted in fulfilment of the requirements for
the degree of Doctor of Philosophy and
the Diploma of Imperial College London

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June 2009

Abstract

Malaria, an illness caused by protozoan parasites of the genus *Plasmodium*, continues to be a key global health issue; around 40% of the world's population are at risk and more than one million people are killed each year according to the World Health Organisation (WHO). It is transmitted *via* bites of infected female mosquitoes (*Anopheles*) and its severest form, falciparum malaria, can lead to death if left untreated. Effective malarial treatment is complex due to drug resistance and socioeconomic issues in many of the most affected areas.

An enzyme from the parasite, myristoyl CoA:protein *N*-myristoyl transferase (NMT), has been identified as a potential target for antimalarial drugs. *N*-Myristoyl transferase, which catalyses the co-translational transfer of myristic acid to an N-terminal glycine of certain substrate proteins, has been shown to be essential for various pathogens. This thesis demonstrates the design, synthesis and analysis of potential inhibitors of *Plasmodium falciparum* NMT.

Approximately 50 inhibitors with systematic variations based on a benzothiazole scaffold have been synthesised. It is known that these benzothiazoles compete with binding of peptide substrate within the NMT enzyme binding cleft. Differences between the peptide binding pockets of *P. falciparum* and human NMTs were exploited to design effective and selective new antimalarial treatments. The level of inhibition was measured using SPA that monitors the transfer of ³H-labelled myristoyl CoA to the N-terminus of a polypeptide substrate. A plot of enzyme activity as a function of inhibitor concentration gave inhibition curves from which IC₅₀-values were derived.

In vitro tests resulted in four hits with improved activity in the low micromolar region against *P. falciparum* NMT compared to the lead compound. Nevertheless, the inhibitors were not exceptionally selective over *Homo sapiens* NMT with an IC₅₀ in the low micromolar region also. Selections of the most promising inhibitors have been tested *in vivo* and considerable reductions in parasitemia were noted.

Acknowledgements

First and foremost a big thanks to my supervisor Robin for all your support and guidance in the past years. Particularly throughout the writing up stage, I greatly appreciated all of your attention, help and patience. It was a pleasure to be a part of the group and I thoroughly enjoyed the project with all the ups and downs, thank you for this opportunity! Ed, you have been such a wonderful mentor as well. Thank you so much for all your help, patience and kindness. I learned so much over the past three years from new techniques to how a lab can be run successfully. Thank you both for your encouragement.

Will, you are a real asset to the group; you are smart, organized and helpful. Thank you for reading my drafts so thoroughly. Sasala, you have only been with us for a short period, but I have learned so much from you. Thank you for introducing and exciting me for molecular biology. Paul, you've introduced me to this project and got me enthused about developing improved inhibitors. Jone, thank you so much for all your help, especially in the last weeks. I hope I taught you everything I know and I am so excited to see how your progress brings the project forward. All the best! Thank you also to Debbie, Jim, Tony, David and Stuart for big contributions to the NMT project.

Thanks to all other present and past members of the Leatherbarrow/Tate research group, especially Nuria, Gillian and Took. I have never been in a lab, where team work has such high priority, everyone helps each other and work is so much fun. I enjoyed going to work every day, teaching and learning so much more, thanks to all of you. May be all the cakes, cookies, chocolates and other sweet indulgences were a big contributing factor to my happiness as well...

Thank you to Pete from the departmental NMR service for all his help with the carbon spectra. Thank you also to John Barton for fast mass spec analysis. I'd like to express my gratitude to the D Craig and A Spivey group for aldehyde samples for my library. Also, I'd like to acknowledge the K Brown group who have provided the FPLC machine and lent a helping hand with the old equipment.

I'd like to thank and acknowledge Marie Curie Fellowships for financial support.

Thank you also to all of my friends at Imperial and London, especially Natalia and Amy, to have made this journey so memorable, enjoyable and who stood by me through all the tough times. Diana, even though you weren't here with me, you are always in my heart.

To my family, especially mum, dad and my grandparents, thank you for your unconditional love and support. Jakob, I love you.

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1.5 Abbreviations

3D-QSAR	Three-dimensional quantitative structure–activity relationship
AA	Amino acid
Ac	Acetyl
ACN	Acetonitrile
ACT	Artemisinin-based combination therapy
ADP	Adenosine diphosphate
AIBN	<i>N,N</i> -Azobisisobutyronitrile
AIDS	Acquired immune deficiency syndrome
amp	Ampicillin
APS	Ammonium persulfate
ARF1	ADP-ribosylation factor 1
ARL	ARF-like
arom	Aromatic
ATR	Attenuated total reflection
aq	Aqueous
Boc	<i>tert</i> -Butyloxycarbonyl
Boc ₂ O	Di- <i>tert</i> -butyl dicarbonate
BSA	Bovine serum albumin
CaNMT	<i>Candida albicans</i> <i>N</i> -myristoyl transferase
CAP	Cytoskeleton associated protein
cDNA	Complementary deoxyribonucleic acid
CoA	Coenzyme A

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COSY	Correlation spectroscopy
cpm	Counts per minute
CV	Column volumes
DCE	Dichloroethane
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DEPT	Distortionless enhancement of polarisation transfer
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DIPEA	Diisopropylamine
DMF	Dimethylformamide
DMP	Dess-Martin periodane
DNA	Deoxyribonucleic acid
DTT	Dithiothriol
EC	Enzyme classification
EDTA	Ethylenediaminetetraacetic acid
e.g.	<i>exempli gratia</i>
EGTA	Ethyleneglycoltetraacetic acid
EI	Electronic ionisation
ELISA	Enzyme-linked immunosorbent assay
eq.	Equivalent(s)
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
Et	Ethyl
et al.	<i>et alii</i>
EtOAc	Ethyl acetate
FAB	Fast atom bombardment
FACS	Fluorescence-activated cell sorting
FL2-H	FL2-Height
FPLC	Fast protein liquid chromatography
FT-IR	Fourier transform infra-red
<i>g</i>	Gravity

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GF	Gel filtration
GTP	Guanosine Triphosphate
HASP	Hydrophilic acylated surface proteins
HBTU	2-(1-Benzotriazol-1-yl)-1,1,3,3-tetramethylmethylluroniumhexafluorophosphate
HE	Hydroethidine
HIV	Human immunodeficiency virus
HMQC	Heteronuclear multiple quantum coherence
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
HRP	Horseradish peroxidase
HsNMT	<i>Homo sapiens</i> N-myristoyl transferase
HTS	High-throughput screening
IC ₅₀	Concentration of a drug that is required for 50% inhibition <i>in vitro</i>
i.e.	<i>id est</i>
IEX	Ion exchange chromatography
IMAC	Immobilised metal affinity chromatography
IPT	Intermittent preventive treatment in pregnancy
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS	Indoor residual spraying of insecticide
ITN	Insecticide impregnated bed-nets
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
<i>J</i>	Coupling constant
LB	Lysogeny broth
LLIN	Long-lasting insecticidal nets
LmNMT	<i>Leishmania major</i> N-myristoyl transferase
MARCKS	Myristoylated alanine-rich C-kinase substrate
MeOH	Methanol
MEP	Molecular electrostatic potential

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MES	2-(<i>N</i> -Morpholino)ethanesulfonic acid
MOI	Mode of inhibition
MOPS	3-(<i>N</i> -Morpholino)propanesulfonic acid
M _r	Molecular weight
MWCO	Molecular weight cut off
<i>m/z</i>	Mass to charge ratio
NADPH	Nicotinamide adenine dinucleotide phosphate
NEt ₃	Triethylamine
NIMR	National Institute of Medical Research
NMR	Nuclear magnetic resonance
NMT	<i>N</i> -Myristoyl transferase
NTA	Nitrilotriacetic acid
OD	Optical density
p.	Page
p60 ^{src}	Tyrosine kinase of Rous sarcoma virus with M _r of 60 000
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDB	Protein Data Bank
PfNMT	<i>Plasmodium falciparum</i> <i>N</i> -myristoyl transferase
Pfp TFA	Pentafluorophenyl trifluoroacetate
pH	<i>Pondus hydrogenii</i>
pI	Isoelectric point
PIP ₂	Phosphatidylinositol 4,5-biphosphate
PMSF	Phenylmethylsulphonyl fluoride
ppm	Parts per million
PVT	Polyvinyl toluene
PyAOP	7-Azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	<i>O</i> -Benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate
<i>rac</i>	Racemic mixture
R _f	Relative front

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RNA	Ribonucleic acid
RP-HPLC	Reverse phase high performance liquid chromatography
rpm	Revolutions/rotations per minute
RT	Room temperature
SAR	Structure-activity relationship
ScNMT	<i>Saccharomyces cerevisiae</i> N-myristoyl transferase
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SIT	Sterile insect technology
SOC	Super optimal broth with catabolite repression
SPA	Scintillation proximity assay
spp.	Species
SPPS	Solid phase peptide synthesis
TBS	Tris buffered saline
TCEP	Tris(2-carboxyethyl)phosphine
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
t _R	Retention time
tRNA	Transfer ribonucleic acid
UK	United Kingdom
UNICEF	United Nations Children's Fund
UV	Ultraviolet
V:V	Volume per volume
vs.	<i>versus</i>
w/v	Weight per volume
WARN	World Antimalarial Resistance Network
WHO	World Health Organisation
X ⁻	Unknown anion
δ	Chemical shift

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Nomenclature and symbolism for amino acids and peptides were adopted from the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature 1972). The systematic nomenclature of benzothiazole derivatives starts counting on the N-atom and continues in clockwise direction (Figure I-1). The absolute and relative stereochemistry was defined as follows. Block-shaped bonds were used for racemates and wedge-shapes were used to define each stereocentre of single enantiomers.

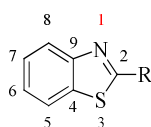


Figure I-1: Numbering of a benzothiazole

II INTRODUCTION

II.1 Malaria – An Introduction

II.1.1 Prologue

One of the leading causes of morbidity and mortality in the developing world is malaria. Around 3.3 billion people in 109 countries and territories throughout Africa, South East Asia, India, and South America were at risk at the end of 2006 (WHO 2008). This parasitic disease is caused by micro-organisms of the genus *Plasmodium* that are transmitted to human hosts *via* female mosquito (*Anopheles*) vectors. Malaria caused by *Plasmodium falciparum* is the most lethal with more than one million deaths each year (WHO and UNICEF 2005). Furthermore, due to synergy with other infections and illnesses such as HIV/AIDS, malaria contributes to an even higher death toll. Effective treatment is challenging due to the complexity of the life cycle, increasing drug resistance, mosquito-favouring changes in climate, and lack of socioeconomic development in the most affected areas. A comprehensive strategy to deal with malaria is indispensable, not only to diminish incidence but also to prevent re-emergence.

II.1.2 Global Distribution

WHO in conjunction with local authorities developed the “Global Malaria Control Strategy”. Funds, largely government subsidized, are being made available to malaria interventions which incorporate artemisinin-based combination therapy (ACT, (Bosman and Mendis 2007)), improvements of health infrastructure and prevention strategies, such as long-lasting insecticidal nets (LLIN), indoor residual

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spraying of insecticide (IRS) and intermittent preventive treatment in pregnancy (IPT) (WHO 2008). These ongoing efforts in disease management have reduced the number of reported regions that are at risk of malaria transmission from 140 countries and territories (1950s) to 107 endemic areas (Figure II-1) (WHO and UNICEF 2005). Persistent intervention strategies in countries at risk may promote further decline. Targets were set to “ensure a reduction in the burden of malaria of at least 50% by 2010 and by 75% by 2015”. This had been interpreted to mean a reduction in malaria morbidity as well as mortality (WHO 2008). World Antimalarial Resistance Network (WARN) has been initiated as a public resource to link open access global databases containing clinical, *in vitro*, molecular and pharmacological data to guide research into antimalarial treatment (Sibley, Barnes et al. 2008).

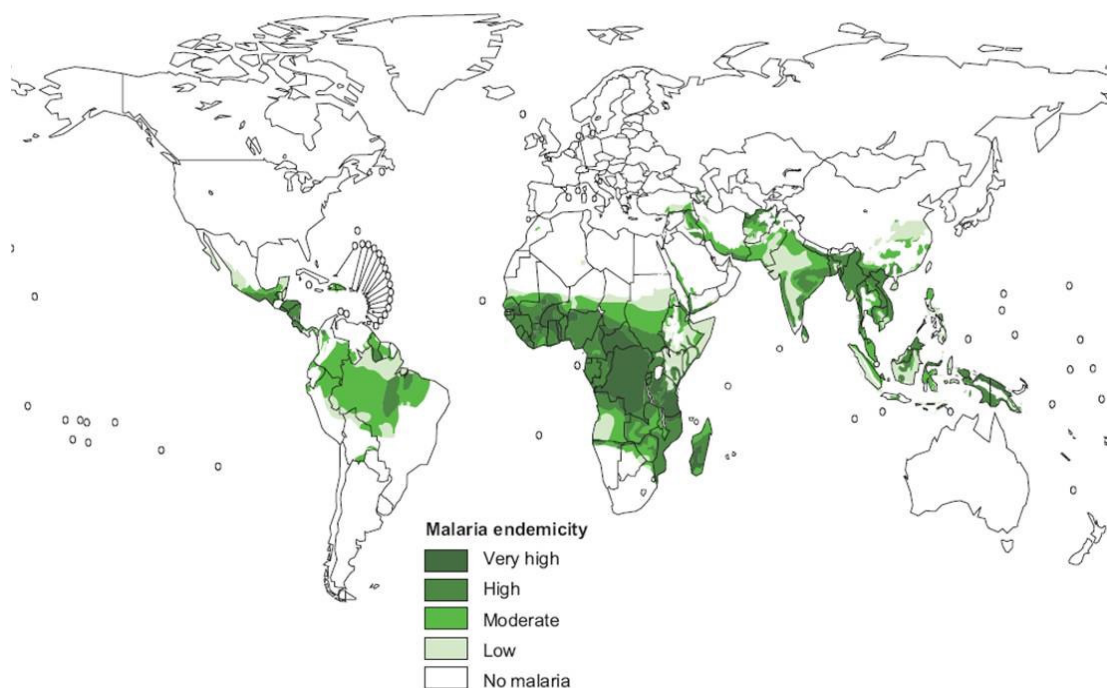


Figure II-1: Global distribution of malaria transmission risk

The Malaria Report 2005 states over three billion people are at risk of malaria transmission (WHO and UNICEF 2005). The figure shows, with different intensities of colour green, in which region of the world the risk is highest.

II.1.3 The Complex Life Cycle of the Parasite

The discovery of the parasitic agent of malaria was in 1880 when Laveran found *Plasmodium* to infect red blood cells in humans (Ross 1902; Laveran 1907). Decades later research groups established its transmission *via* mosquitoes of the genus *Anopheles* to humans (Bruce-Chwatt 1988). Figure II-2 shows a female mosquito (A) and infected red blood cells consumed by *Plasmodium* (B).

Anopheles gambiae is the most efficient malaria vector which lives exclusively on the African continent. The population is difficult to control; effective methods such as indoor residual spraying of insecticide (IRS), insecticide impregnated bed-nets (ITN) and sterile insect technology (SIT)¹ are being applied to reduce the possibility of transmission of malaria (Atkinson 2005; Baton and Ranford-Cartwright 2005; Catteruccia 2005; WHO and UNICEF 2005; WHO 2008).

There are over 100 known species of *Plasmodium*; however, only four appear to infect human hosts (*P. vivax*, *P. ovale*, *P. falciparum*, and *P. malariae*). A scheme of the life cycle is illustrated in Figure II-3. It is very complex and comprises an exogenous sexual phase in the mosquito vector (Baton and Ranford-Cartwright 2005) and an endogenous asexual phase in the vertebrate host.

The infectious stage begins with release of sporozoites in the salivary glands of the mosquito that are subsequently transmitted to the human host. After the exo-erythrocytic cycle (development in the liver) schizonts invade erythrocytes where they mature (erythrocytic cycle) and degrade iron and haemoglobin as a source of amino acids for their metabolism. This may lead to cell rupture, releasing waste of the parasite and cell debris that cause some clinical symptoms of malaria such as the periodical “chill and fever” (Wiesner, Ortmann et al. 2003). A more detailed description of the erythrocytic cycle is discussed in Chapter VI.1 (p. 170).

Falciparum malaria can be fatal due to sequestration. The parasite produces specific proteins that embed on the cell membrane of an infected erythrocyte. The infected red blood cells thus adhere to microvascular endothelium (cytoadherence) in capillaries and venules of vital organs such as the brain (Berendt, Ferguson et al.

¹ SIT is an alternative technology to chemical insecticides, whereby sterile male insects are released to suppress or eradicate entire populations through sterile mating. The paper by Atkinson 2005 summarises improved methods such as genetic engineering for sexing mosquitoes which was carried out by Catteruccia 2005.

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1994; Wiesner, Ortmann et al. 2003). They interfere with the microcirculatory flow thus reducing oxygen and substrate supplies (White and Ho 1992). The combination of cytoadherence and rosetting (erythrocyte adhesion) can cause severe malaria. It may result in anaemia, renal failure, placental dysfunction, spleen enlargement, cerebral malaria, coma and death if left untreated. Furthermore, the immune system of the host is weakened to other illnesses such as bacterial infections. However, there are genetic changes that have occurred over time to resist malaria naturally (Marquardt, Demaree et al. 2000). Sick cells, for example, have evolved due to low oxygen tension which results in sickling. Needle-like crystal formations of haemoglobin destroy the parasites mechanically. Nevertheless anaemia is fatal.

There are many opportunities for interception. Pre-erythrocytic vaccination examines the prevention of the invasion of hepatocytes by sporozoites. Erythrocytic vaccination targets surface proteins, responsible for binding to erythrocytes. Other strategies look into preventing fertilisation in the mosquito or blocking sequestration of parasite-infected erythrocytes (Waters 2006).

The inhibitor development of this project targets the erythrocytic cycle of *Plasmodium falciparum*. The potency of inhibitors can be tested by means of analysing reinvasion and maturation of parasites after incubation of infected erythrocytes for a complete life cycle of *P. falciparum* (Bowyer 2006). The parasite may reinvade red blood cells (Baton and Ranford-Cartwright 2005) to initiate the next phase in the malarial cycle or mature into gametocytes (Talman, Domarle et al. 2004) which are ingested by the mosquito where the life cycle closes.

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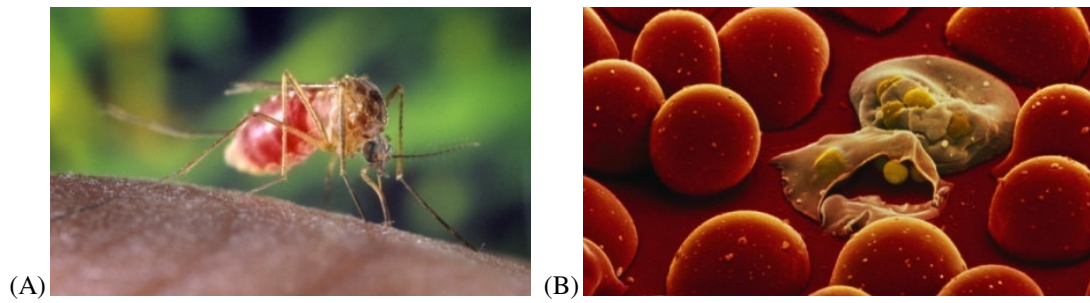


Figure II-2: Feeding female *Anopheles* and *Plasmodium* infected erythrocytes

When an infected female *Anopheles* takes a blood meal (A, (<http://sill-www.army.mil>, 04/2006)) the parasite gets transferred to the human host where it matures over several cycles, first in hepatocytes followed by erythrocytes (B, (www.nationalgeographic.com, 12/2008)), only to be taken up again by mosquitoes during feeding.

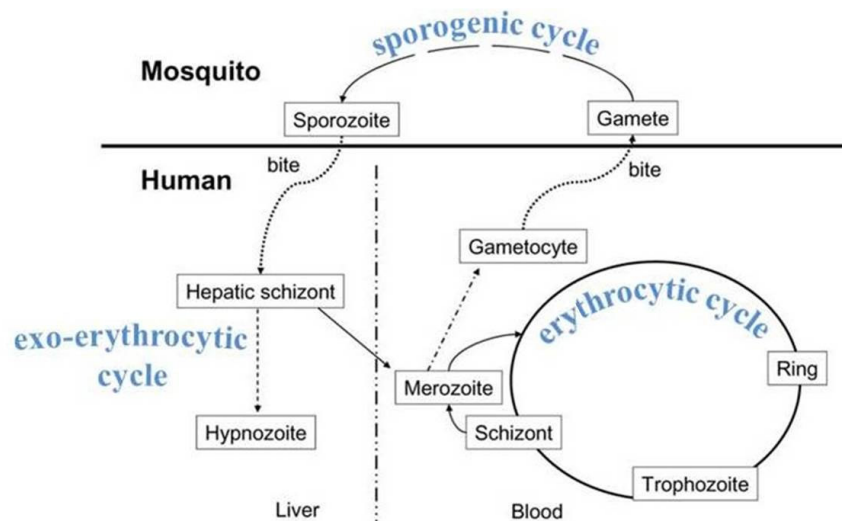


Figure II-3: Overview of the life cycles of *Plasmodium* spp.

An overview of the life cycles of *Plasmodium* spp. is displayed. It consists of the sporogonic cycle in the mosquito vector and the exo-erythrocytic and intra-erythrocytic cycle in the human host (adapted from (Schlitzer 2008)). The sporogonic cycle is the sexual life cycle of the parasite where the gametocytes mature to sporozoites. These are then passed on to the human host where the asexual development occurs.

II.1.4 Antimalarial Drugs

There are various prophylactic and therapeutic agents available against malaria (shown in Figure II-5), each targeting different pathways of *Plasmodium* spp. (Wiesner, Ortmann et al. 2003; Schlitzer 2008).

Figure II-5A shows a selection of quinolines which belong historically to the oldest antimalarial drugs and have been taken for many centuries. It is suggested, quinolines reside in food vacuoles of the parasite where they accumulate and inhibit haem polymerisation, which in turn leads to cell death due to toxicity (Warhurst 1981; Slater and Cerami 1992). Quinine **1**, extracted from chinchona bark, and its derivative chloroquine **2** have been employed in malaria eradication campaigns in the 1950s and 60s, yet effectiveness has declined rapidly and *Plasmodium falciparum* populations are widely resistant against **2** (see Figure II-4).

To combat chloroquine-resistant areas, antifolates were strategically employed (see Figure II-5B). The inexpensively produced drugs are competitive inhibitors that target the folate pathway of *Plasmodium* spp. (Hyde 2005), in particular dihydropteroate synthase (DHPS, (Triglia and Cowman 1994)) and dihydrofolate reductase (DHFR (Bzik, Li et al. 1987)). The products of the folate pathway are essential for DNA synthesis and the metabolism of certain amino acids. Combinations of DHPS and DHFR inhibitors act synergistically to increase efficacy against the malaria parasite with Fansidar[®], a combination of sulfadoxine **4** and pyrimethamine **5**, being very successful for decades. However, point mutations of *dhps* and *dhfr* genes in *Plasmodium falciparum* have led to increased resistance (Plowe, Kublin et al. 1998; Sibley, Hyde et al. 2001). Different combination therapies which are based on pyrimidines and triazin structures are currently under investigation such as dapsone **6** – chlorproguanil **7** and atovaquone **8** – proguanil **9** (Malarone[™]). Reviews for further reading include “Overview of the past and present antifolates with regards to affect *Plasmodium falciparum*” (Nzila 2006) as well as “Mechanisms of resistance of malaria parasites to antifolates” (Gregson and Plowe 2005).

Figure II-4 gives an outline of existing drug resistances in the malaria parasite revealing that traditional drugs such as chloroquine, sulfadoxine – pyrimethamine and mefloquine **3** (Lariam[®]) are no longer effective. At present, main treatments

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against malaria involve artemisinin **10** and its analogues shown in Figure II-5C (WHO and UNICEF 2005). **10** is a herbal natural product from the sweet wormwood (*Artemisia annua*) which was isolated in 1972 and since then numerous derivatives have been synthesised (Haynes 2001). The endoperoxide bridge in artemisinins is the active functionality. Currently prescribed are artemisinin derivatives in sequential combination, known as ACT, with assorted drugs such as pyronaridine **12** – artesunate **11** (PANDA, Pyramax[®]) and dihydroartemisinin **13** – piperaquine **14** (Eurartesim[®]) which are in Phase III (MMV 2006; MMV 2007; WHO 2008). ACT is aimed to delay or prevent emergence of resistance with multiple components. The active compounds target the same stage of the parasite but with individual modes of inhibition. For example, an artemisinin derivative affecting the mitochondrial electron transport combined with an antifolate reagent targeting the folate metabolism, both effective in the asexual blood stage, would be considered a good combination. (Baird 2005). Nevertheless, persistent increase of drug resistance makes ongoing inhibitor development indispensable.

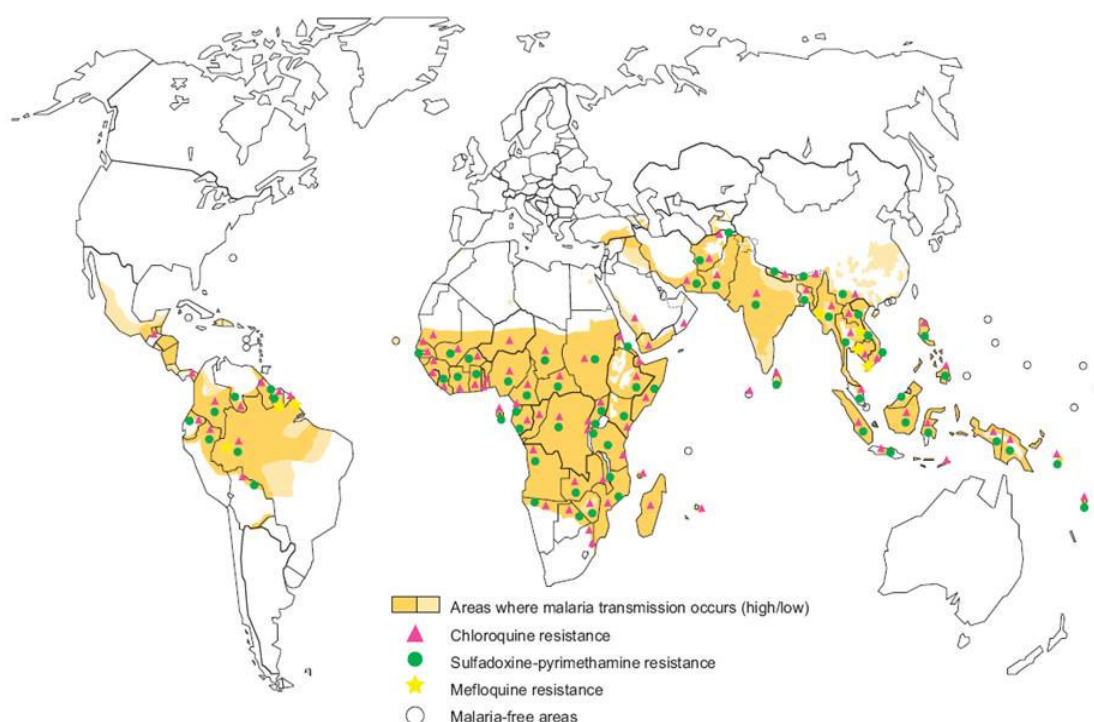


Figure II-4: Drug resistance in *P. falciparum* from studies in sentinel sites, up to 2004

Common prescribed medicine with active reagents such as chloroquine (▲), sulfadoxine-pyrimethamine (●), or mefloquine (★) are widely inactive against *Plasmodium falciparum* (WHO and UNICEF 2005).

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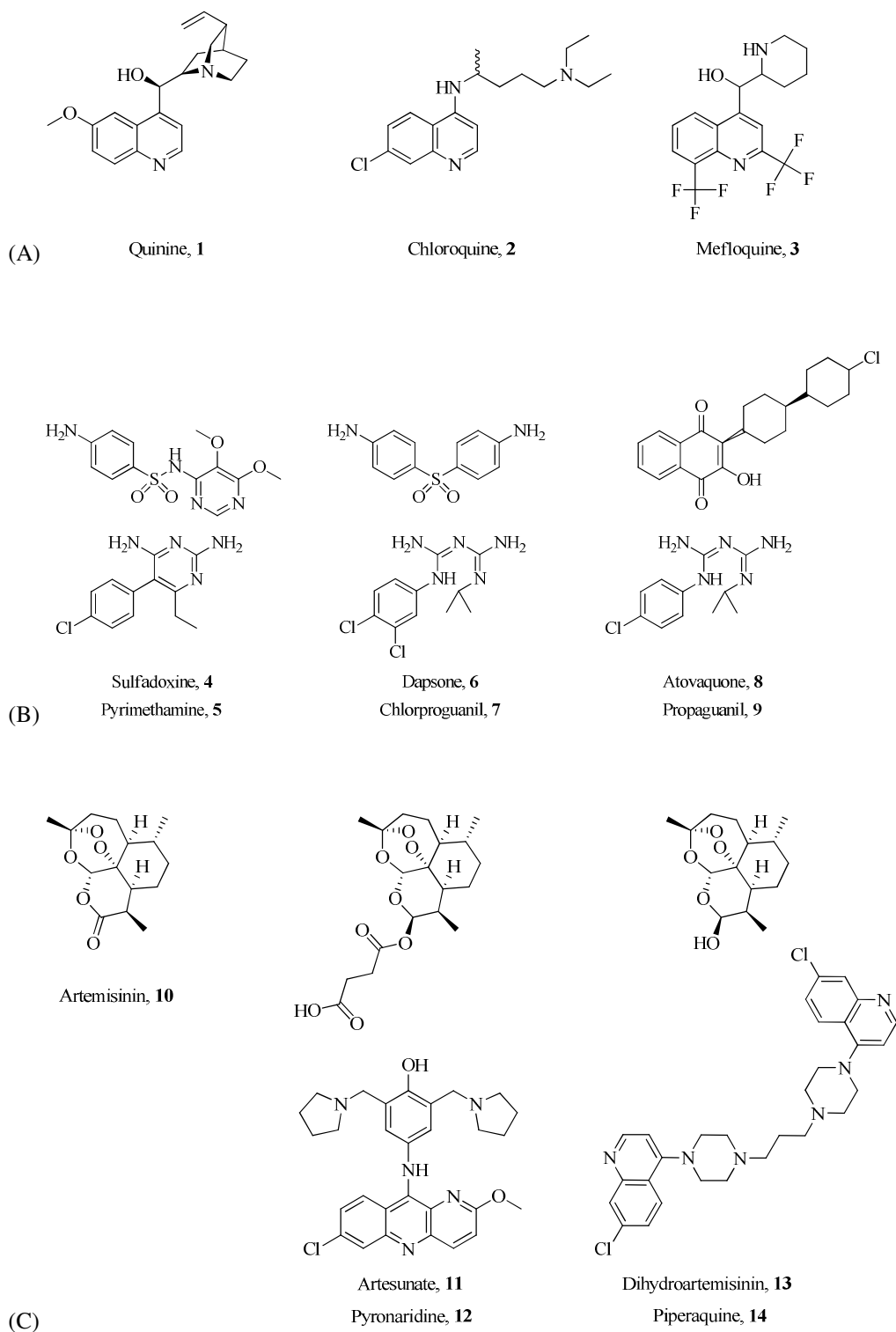


Figure II-5: Common active reagents in antimalarial drugs

There are several classes of antimalarials on the market. They can be classified under quinolines (A), antifolates (B) and artemisinins (C).

II.2 *Myristoyl CoA:Protein N-Myristoyl Transferase – An Overview*

II.2.1 Introduction

Myristoyl CoA:protein *N*-myristoyl transferase (NMT, EC 2.3.1.97)² is a monomeric enzyme which is ubiquitous in eukaryotes. It involves the irreversible co-translational transfer of myristate to an N-terminal glycine of particular peptides *via* formation of an amide bond (Wilcox 1987; Deichaite 1988).

The first isolation of NMT (from *Saccharomyces cerevisiae*) was carried out in 1987 (Towler, Adams et al. 1987) and since then the enzyme has been identified and characterised in organisms including fungi (*Candida albicans*, *Cryptococcus neoformans* (Wiegand, Carr et al. 1992; Lodge, Johnson et al. 1994)), parasitic protozoa (*Leishmania major*, *Trypanosoma brucei*, *Plasmodium falciparum* (Gunaratne, Sajid et al. 2000; Price, Menon et al. 2003)) and mammals (*Homo sapiens* (Duronio, Reed et al. 1992)). Given that between 0.5% and 1.7% of the proteome may undergo *N*-myristoylation depending on the organism (Maurer-Stroh, Eisenhaber et al. 2002; Boisson, Giglione et al. 2003; Boisson and Meinnel 2003), NMT is a key drug target. Experiments established that NMT is essential for the viability of certain protozoa such as yeast (Duronio, Towler et al. 1989) and other fungal micro-organisms (Weinberg, McWherter et al. 1995; Lodge, Jackson-Machelski et al. 1998). It has been suggested, NMT represents a potential drug target in the malarial intra-erythrocytic cycle (Gunaratne, Sajid et al. 2000; Gelb, Van Voorhis et al. 2003). Research is currently being carried out by Dr R Tewari (Institute of Genetics, University of Nottingham) in cooperation with Dr E W Tate (Department of Chemistry, Imperial College London) to verify whether NMT plays a crucial role in the survival of *Plasmodium falciparum* as well. It is already shown that inhibition of NMT in *Plasmodium falciparum* can lead to parasitic death (Bowyer 2006).

NMT is quite specific for myristoyl CoA, suggesting that the specific fatty acid moiety, chain length and consequently flexibility provide structural features for myristoylated proteins critical for correct orientation within the membrane and thus enabling interactions with essential substrates (James and Olsen 1990). Myristoyl

² 2 (transferase), 3 (transferase including acyltransferase), 1 (acyltransferases transferring groups other than amino-acyl groups), 97 (acyltransferases transferring groups other than amino-acyl groups)

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CoA (Figure II-6B) is biosynthesised from myristic acid (Figure II-6A) and CoA-SH through acyl CoA synthetase. Myristic acid is a rare 14-carbon saturated fatty acid (C14:0) and only represents 1–3% of fatty acids in eukaryotic systems (Khandwala and Kasper 1971). The activated thioester can then react easily with nucleophiles such as an amine moiety of N-terminal Gly peptides (Figure II-6C).

Potential substrates have been identified as ADP-ribosylation factor 1 (ARF1). ARF proteins, structurally and functionally conserved with an approximate size of 21 kDa, belong to the family of regulatory GTP-binding proteins and have been shown to play vital roles, such as aiding membrane trafficking (Boman and Kahn 1995). They can be divided into ARF and ARF-like (ARL) proteins, all of which may be myristoylated. The ARF family have been conserved throughout evolution and can be found in all eukaryotes, of which for example *Pfarf1* has been fully characterised (Stafford, Stockley et al. 1996). ARF is known to undergo N-terminal N-myristoylation in the presence of NMT (Kahn, Randazzo et al. 1992; Price 2005).

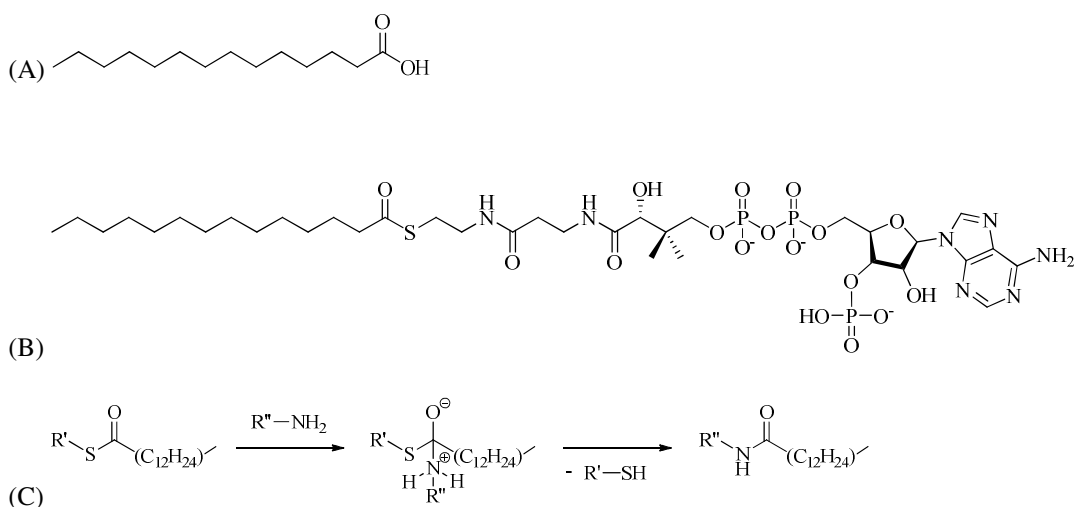


Figure II-6: From myristic acid to myristoylated substrates

Myristic acid (A), a rare saturated fatty acid, is generated during fatty acid synthesis. The reaction with coenzyme A creates myristoyl CoA (B). The thioester moiety is susceptible for nucleophiles such as 1° amines (C).

N-Myristoylation *in vivo* is an early co-translational event, although some targets, notably in the apoptotic pathway, are myristoylated post-translationally after cleavage by an endoprotease. NMT interacts with elongated polypeptides just after

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protein synthesis begins, but after removal of N-terminal methionine by methionine aminopeptidase. This was demonstrated by myristoylation of nascent polypeptides whilst still attached to tRNA (Wilcox 1987). Other experiments investigated the instance of *N*-myristoylation in a cell-free environment creating a translation-myristoylation system with the ability to synchronise protein synthesis by delayed addition of specific initiation inhibitors (Deichaite 1988).

Lipid modifications such as myristoylation are not only vital for attachment of proteins to the plasma membrane. Myristoylated proteins target a wide range of subcellular localisations throughout the cell such as lipid rafts (Resh 1999; Simons and Toomre 2000; Maurer-Stroh, Gouda et al. 2004). The impact of *N*-myristoylation will be explained in further detail later in this chapter.

N-Myristoylation has also been exploited for *in vitro* and *in vivo* bioorthogonal N-terminal labelling of recombinant proteins. Recently, Heal et al. described a method of labelling myristoyl CoA with an azide or alkyne tag to capture labelled myristoylated proteins with secondary tags using the Staudinger–Bertozzi reaction. This method could be used to further uncover the myristoylated proteome (Hang, Geutjes et al. 2007; Heal, Wickramasinghe et al. 2008; Heal, Wickramasinghe et al. 2008; Kostiuk, Corvi et al. 2008; Martin, Vilas et al. 2008).

II.2.2 Structure and Catalytic Mechanism

N-Myristoyl transferase has been well documented for *Saccharomyces cerevisiae*. Data on ScNMT range from isolation, expression and purification (Towler, Adams et al. 1987), to crystallisation studies (Figure II-7 (Bhatnagar, Fütterer et al. 1998; Farazi, Waksman et al. 2001; Wu, Tao et al. 2007)) and viability assays (Duronio, Towler et al. 1989).

The ScNMT protein fold (pseudo two-fold symmetry) consists of a large saddle shaped β -sheet with several α -helices at its side (Bhatnagar, Fütterer et al. 1999). Similarities were observed in the crystal structure data of *Candida albicans* NMT (Weston, Camble et al. 1998) and *Leishmania major* NMT (Qiu, Hutchinson et al. To be published), with a mixed α/β fold and an internal two-fold symmetry. The N-terminal domain forms the myristoyl CoA binding site, whereas the C-terminal

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domain forms the majority of the peptide binding site and also interacts with the N-terminal domain (Bhatnagar, Futterer et al. 1999).

Studies on *S. cerevisiae* NMT resulted in identification of the Bi Bi reaction mechanism as the catalytic mode of action (Rudnick, McWherter et al. 1991; Bhatnagar, Jackson-Machelski et al. 1994; Yang, Shapiro et al. 2005), illustrated in Figure II-8. Myristoyl CoA enters the N-terminal domain of NMT and thereby induces a conformational change in the disordered Ab loop exposing the peptidic binding site. This allows the C-terminus to aid the polypeptide substrate in binding. At the time this mechanism was reported, NMT was the only enzyme where the C-terminus was known to exhibit a catalytic role (Bhatnagar, Futterer et al. 1999), although other enzymes have been reported since (Fuchs, Xiao et al. 2008). The N-terminal Gly of the substrate carries a positive charge due to the acidic environment in the cell, and deprotonation is achieved by the C-terminal carboxylate of the enzyme (Leu455) generating a nucleophilic amine.

The thioester moiety of myristoyl CoA is accommodated in an oxyanion hole formed by backbone amides Phe170 and Leu171. The hydrogen bond-carbonyl group interactions result in stronger polarisation facilitating nucleophilic attacks by the amine. Gly1 of the peptide substrate is aligned with the CoA thioester by hydrogen bonding with Asn169 and Thr205 in the C-terminal domain. In a nucleophilic addition-elimination reaction, the amine attacks the carbonyl of the thioester to form a tetrahedral intermediate that is stabilised by the oxyanion hole. Free CoA is then eliminated from the ternary complex, and the structure induced on binding is relaxed, resulting in the release of the myristoylated product (Bhatnagar, Futterer et al. 1999; Farazi, Waksman et al. 2001).

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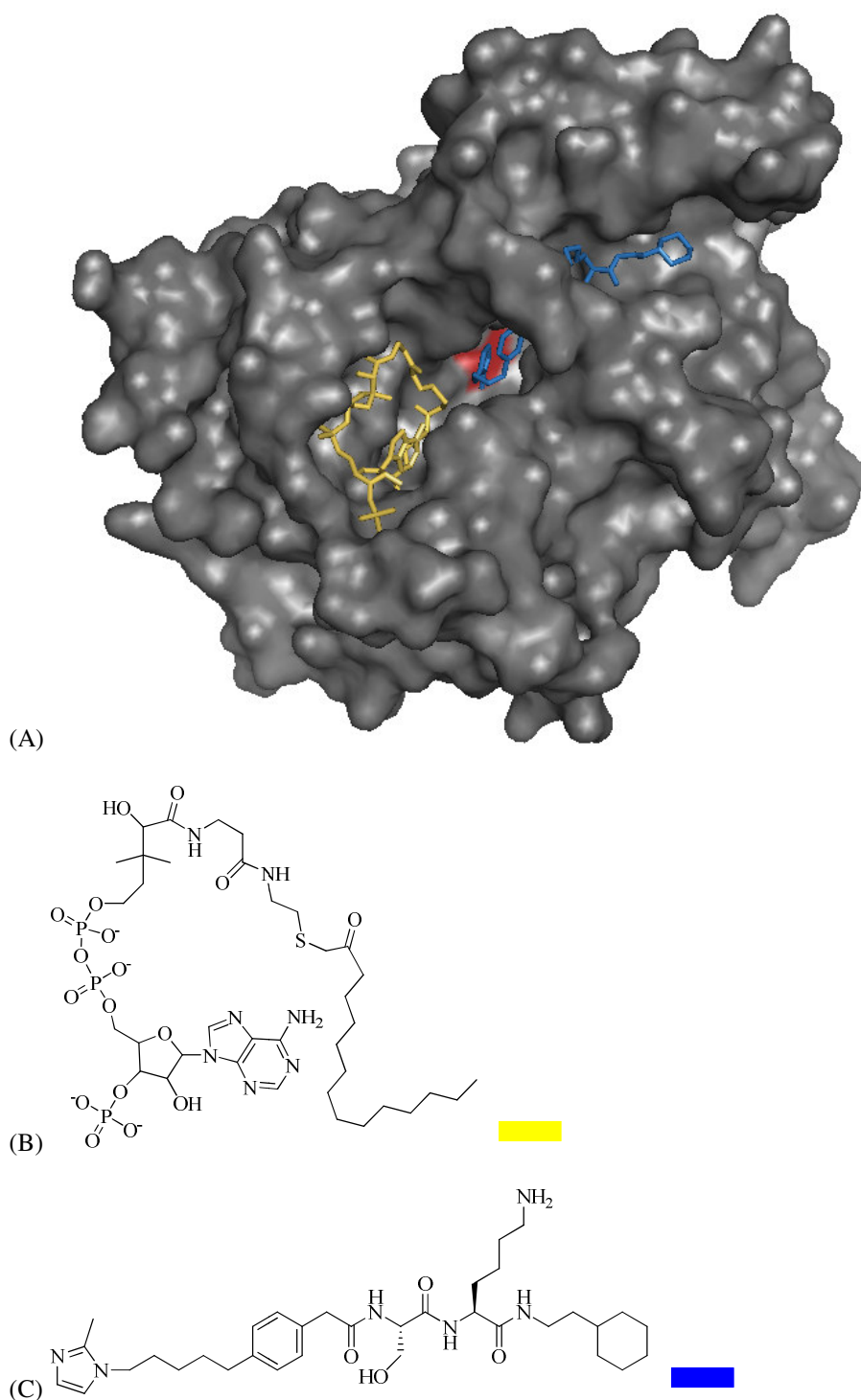


Figure II-7: Co-crystal structure of ScNMT

The ternary complex of ScNMT (A) is illustrated with bound *S*-(2-oxo)pentadecyl CoA (non-hydrolysable myristoyl CoA, B) and a peptide substrate (SC-58272, C) coloured in yellow and blue respectively (pdb file 2nmt, (Bhatnagar, Fütterer et al. 1998). The enzyme is shaded in grey. The C-terminus is highlighted in red and shows the interaction within the peptide binding pocket. The figure was generated in PyMOL™ (0.99rc6, DeLano Scientific LLC, <http://pymol.sourceforge.net/>) from published pdb files.

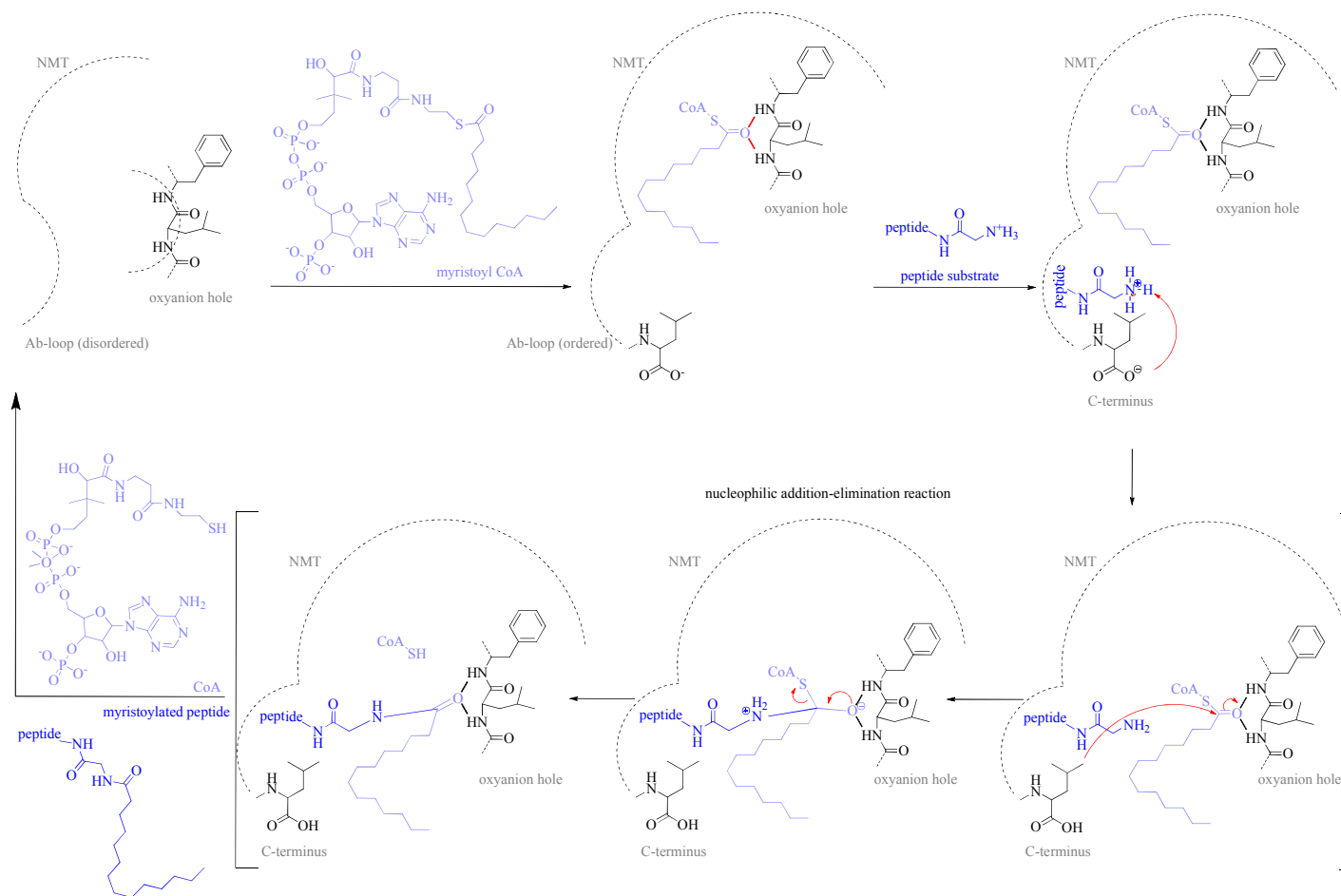


Figure II-8: The proposed Bi Bi reaction mechanism of *N*-myristoylation in detail

*The detailed mechanism of *N*-myristoylation is shown in this figure, adapted from (Bhatnagar, Futterer et al. 1999).*

II.2.3 Impact of *N*-Myristoylation

Myristoylation is a ubiquitous protein modification that has been subject of many reviews (Schultz, Henderson et al. 1988; Towler 1988; James and Olsen 1990; Boutin 1997; Farazi, Waksman et al. 2001; Nalivaeva and Turner 2001; Bowyer, Tate et al. 2008). The transfer of myristate to substrate proteins increases their affinity for membranes. Only N-terminal glycines of particular polypeptides are myristoylated. The second main type of protein fatty acylation is *S*-palmitoylation (Bhatnagar and Gordon 1997). Unlike myristoylation, palmitoylation is reversible. Palmitate (C16:0) is attached to the thiol moiety of variably located cysteine residues. *S*-Palmitoylated proteins can be found solely on the plasma membrane compared to a much wider distribution of *N*-myristoylated proteins, which are not only present on the plasma membrane but also in other subcellular compartments such as the cytosol, ER and nucleus (James and Olsen 1990).

The hydrophobic N-terminal myristate group of an *N*-myristoylated protein embeds in the phospholipid bilayer of the membrane forming an anchor that promotes reversible protein–membrane binding (Peitzsch and McLaughlin 1993; McIlhinney and McGlone 1996; Maurer-Stroh, Eisenhaber et al. 2002).

N-Myristoylation alone has been shown to be insufficient for persistent membrane-binding; secondary interactions such as protein–protein interactions, further lipidation, or attractive electrostatic forces are required to stabilise the protein–membrane interaction (Shahinian and Silviu 1995; Murray, Ben-Tal et al. 1997).

Figure II-9 displays various combinations of N-terminal lipid modifications to assist in the association of proteins to the membrane (McCabe and Berthiaume 1999; Resh 1999; Maurer-Stroh, Gouda et al. 2004). There are five major classes which have been defined to co-occur commonly with myristoyl anchors and thus contribute to subcellular localisation and membrane attachment: Class I – palmitoylatable cysteine, Class II – amino terminal cluster of positive charges, Class III – PIP₂-specific binding domain, Class IV – transmembrane segments, Class V – protein–protein interactions (Maurer-Stroh, Gouda et al. 2004).

This necessity for further membrane associations for stabilisation can be exploited as switches, as destabilisation may cause the release of proteins from the membrane (Nalivaeva and Turner 2001). For example, in an electrostatic switch protein

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phosphorylation adjacent to the membrane increases the local negative charge and thus repels the membrane, inducing dissociation, e.g. phosphorylation of Ser-residues in MARCKS (McLaughlin and Aderem 1995). Conformational switches are also regulated by ligand binding that can either uncover or bury the acyl chain, e.g. reversible membrane binding of ADP-ribosylation factor (Boman and Kahn 1995) or the calcium-myristoyl protein switch (Zozulya and Stryer 1992; Ames 1996).

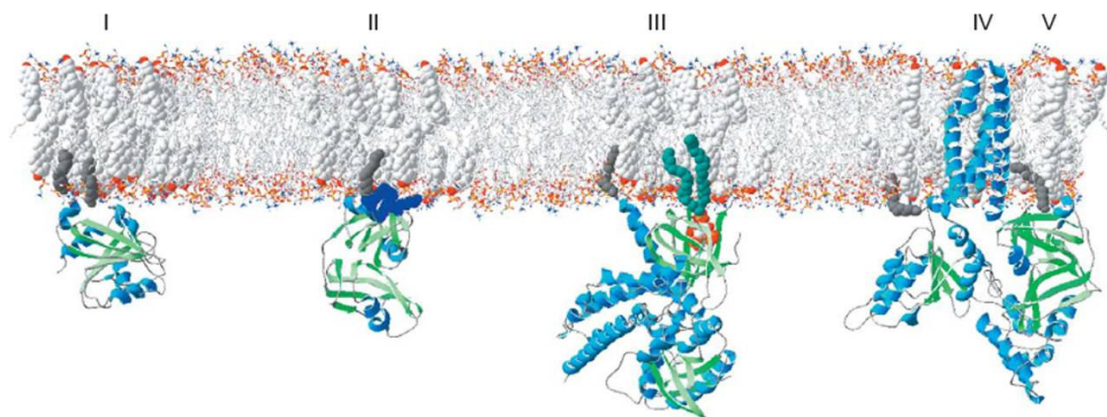


Figure II-9: Scheme of membrane-myristoylated protein interactions

This is a schematic representation of the membrane attachment of proteins with a myristoyl anchor (dark gray, space-filling atomic representation) in combination with different co-occurring membrane-attachment factors.

Class I, plus palmitoyl anchor (also dark gray, space-filling); Class II, plus cluster of positive charges (dark blue, space-filling); Class III, plus PIP_2 -specific binding domain (PIP_2 in space-filling, alkyl tails in cyan); Class IV, plus transmembrane segments; Class V, plus a domain for specific protein-membrane protein interactions. White space-filling molecules in the model membrane represent cholesterol and symbolise targeting to specialised compartments (Maurer-Stroh, Gouda et al. 2004).

II.2.4 Inhibitory Targets of N-Myristoyl Transferase

NMT belongs to the group of transferases which catalyse the irreversible transfer of a C14:0 saturated fatty acid to N-terminal Gly of assorted proteins. N-myristoyl transferase possesses two binding pockets and hence two potential targets for drug development, one for myristoyl CoA and one for peptide substrates. Efforts have gone into analysis of the N-myristoylome to investigate substrate sequence motifs (Maurer-Stroh, Eisenhaber et al. 2002; Maurer-Stroh, Eisenhaber et al. 2002; Heal,

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Wickramasinghe et al. 2008). The first direct analysis for a higher eukaryote was carried out in 2003 (Boisson, Giglione et al. 2003).

A phylogenetic analysis may give insight into similarities and divergences of NMT across species to aid rational drug design (Johnson, Bhatnagar et al. 1994). Figure II-10 illustrates both N- and C-terminal domains of NMT for various organisms, wherein three major separate clusters were identified: metazoan (*H. sapiens*), plants and fungi (*C. albicans*) ((Eisenhaber, Eisenhaber et al.) 2006). The N-terminal domain forms the myristoyl CoA binding site, whereas the C-terminal domain forms the majority of the peptide binding site and also interacts with the N-terminal domain (Bhatnagar, Futterer et al. 1999). Examination of *P. falciparum* NMT and *H. sapiens* NMT, highlighted by a black box, shows more similarities within the N-terminus (Figure II-10A) compared to the C-terminal (Figure II-10B) binding domain.

In conclusion, selective inhibition against the target parasitic NMT over human NMT may be achieved *via* the peptide binding site as it retains quite distinct differences compared to the myristoyl CoA binding domain that has many features in common (Rocque, McWherter et al. 1993).

II.2.4.1 N-Myristoyl Transferase in Humans and Parasites

II.2.4.1.1 N-Myristoyl Transferase in Humans

In the early 1990s, researchers isolated a single cDNA encoding for human NMT (Duronio, Reed et al. 1992; Glover, Hartman et al. 1997). It contains 416 AA and has found to be 44% identical to ScNMT. This enzyme was labelled HsNMT1 as there was biochemical evidence of several distinct NMTs *in vivo* (Boutin 1997). And indeed by the end of the decade a second human *N*-myristoyl transferase was discovered (Giang and Cravatt 1998); both myristoylate commonly-studied peptide substrates have comparable but distinguishable relative selectivity. HsNMT2 is 65 kDa in size whereas for HsNMT1 there are four splice isoforms ranging in size from 49 kDa to 68 kDa (Giang and Cravatt 1998). Comparative studies between HsNMT1 and HsNMT2 revealed only 76–77% sequence identity (Appendix, Figure VIII-1A), suggesting two divergent families of *N*-myristoyl transferases.

Specific inhibition of either human NMT may lead to selective myristoylation, which in turn may be beneficial for *in vivo* regulation and pharmaceutical targeting

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of NMT (Giang and Cravatt 1998; Maurer-Stroh, Eisenhaber et al. 2002). Crystallographic analysis of the human apoenzyme has been attempted but remain unpublished (3.00 Å, pdb file 1rtx (Yang To be published)).

Kinetic studies of human NMT have been carried out and it has been shown that the established Bi Bi reaction mechanism for ScNMT is retained in the *N*-myristoylation of peptides by human NMT (Rocque, McWherter et al. 1993). This information together with divergent properties of the peptide binding pocket among different eukaryotic systems makes NMT a potentially valuable drug target.

N-Myristoylation of p60^{src}, located to the inner surface of the membrane, has found to be essential as only myristoylation enables interactions with the membrane, enabling p60^{src} to carry out site-specific functions such as cell transformations (Glover, Goddard et al. 1988). Experiments with synthetic peptides based on the N-terminal sequence of p60^{src} established that the first nine residues were essential for enzyme specificity (Glover, Goddard et al. 1988). Inhibition assays against human NMT were carried out with peptide substrates based on the N-terminus of p60^{src} peptide: GSSKSKPKDP (10mer, (Giang and Cravatt 1998)) and GSSKSKPKDPSQRRRY (16mer, (McIlhinney, Patel et al. 1994)) for example.

II.2.4.1.2 *N*-Myristoyl Transferase in *Leishmania* spp.

N-Myristoyl transferase is also a target for protozoan parasitic diseases. Leishmaniasis, a tropical illness transmitted by sand flies, is caused by *Leishmania* spp. and has been under investigation by collaborators at York University, UK. They reported peptide substrates other than ARL-proteins (Price 2005) to be myristoylated: hydrophilic acylated surface proteins (HASP, (Denny, Gokool et al. 2000)). They are only expressed in infectious parasite stages and need to be myristoylated for exact localisation to the plasma membrane (Flinn, Rangara et al. 1994; Pimenta, Dasilva et al. 1994). Synthetic peptide substrates may be based on HASPA, HASPB or ARL (Panethymitaki, Bowyer et al. 2006). Recent developments in successful co-crystallisation of *Leishmania major* NMT (1.49 Å, pdb file 3h5z (Qiu, Hutchinson et al. To be published)) with myristoyl CoA and *Leishmania donovani* NMT with a myristoyl CoA analogue (unpublished data) open

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up possibilities of docking studies to analyse enzyme-inhibitor interactions, as already has been done for *Candida albicans* NMT (Ruge, Korting et al. 2005).

II.2.4.1.3 *N-Myristoyl Transferase in Trypanosoma spp.*

Sleeping sickness, caused by *Trypanosoma* spp., represents an additional target disease for drug development against *N*-myristoyl transferase. Collaborators at Dundee University, UK, are currently working at this at a large screening facility for drug discovery. The peptide substrates employed in assays may be based on the N-terminal sequence of CAP5.5, GCGGSKVK (Hertz-Fowler, Ersfeld et al. 2001; Panethymitaki, Bowyer et al. 2006).

II.2.4.1.4 *N-Myristoyl Transferase in Plasmodium spp.*

We have identified myristoyl CoA:protein *N*-myristoyl transferase as a potential target for novel antimalarial drugs against *Plasmodium falciparum*. The gene coding for PfNMT was cloned in 2000 (Gunaratne, Sajid et al. 2000). It is a single copy gene encoding a protein comprised of 410 AA; the corresponding mRNA is expressed in asexual blood stages making NMT a potential target in the intra-erythrocytic cycle. However, there is no co-crystal structure published up to date which would chiefly facilitate enhancement of potency and selectivity for PfNMT over human NMT.

The divergent peptide binding site may be exploited for drug development. Examples of peptide substrates are based on ARF proteins as they are readily myristoylated in *Plasmodium* spp.: GLYNSRLFNRLFQKK (15mer, (Stafford, Stockley et al. 1996)), GLYVSRLF (8mer, (Gunaratne, Sajid et al. 2000)) and GLYVSRLFNRLFQKK (15mer, (Bowyer, Gunaratne et al. 2007)). Key characteristics include an N-terminal Gly, large hydrophobic residues at position 2, neutral residues in 3 and 4 and Ser at position 5. Lys is also common in position 6, and basic residues are favourable in positions 7 and 8 (Rudnick, McWherter et al. 1991; Duronio, Reed et al. 1992; Sikorski, Devadas et al. 1997).

(B) Comparison of the C-terminus

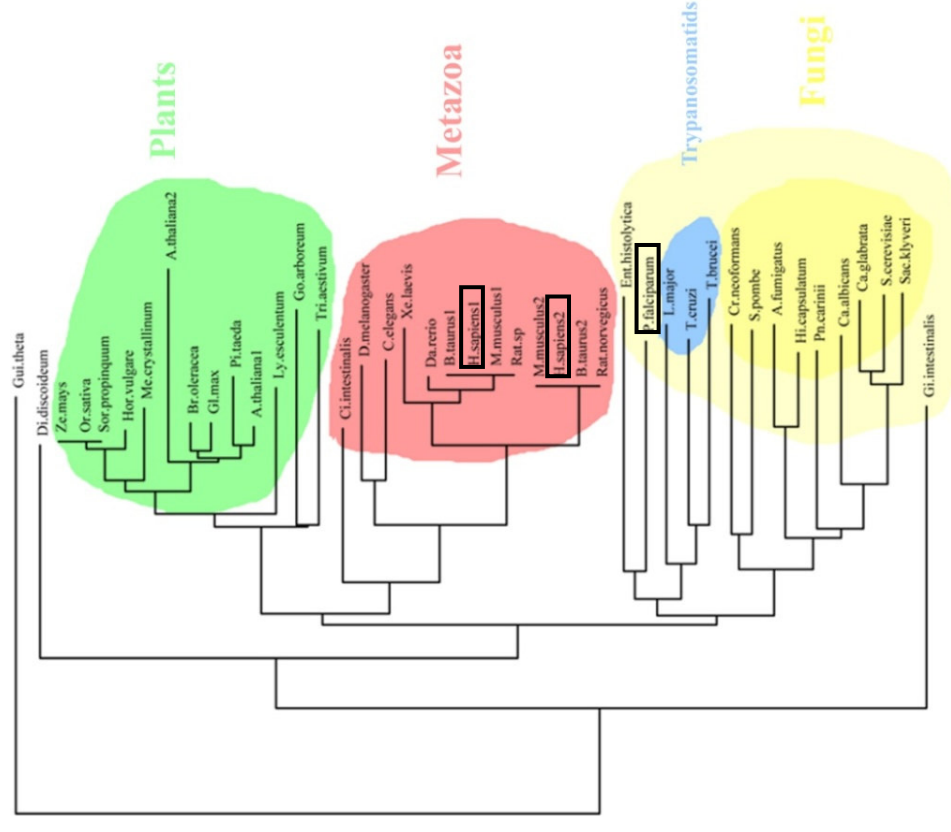
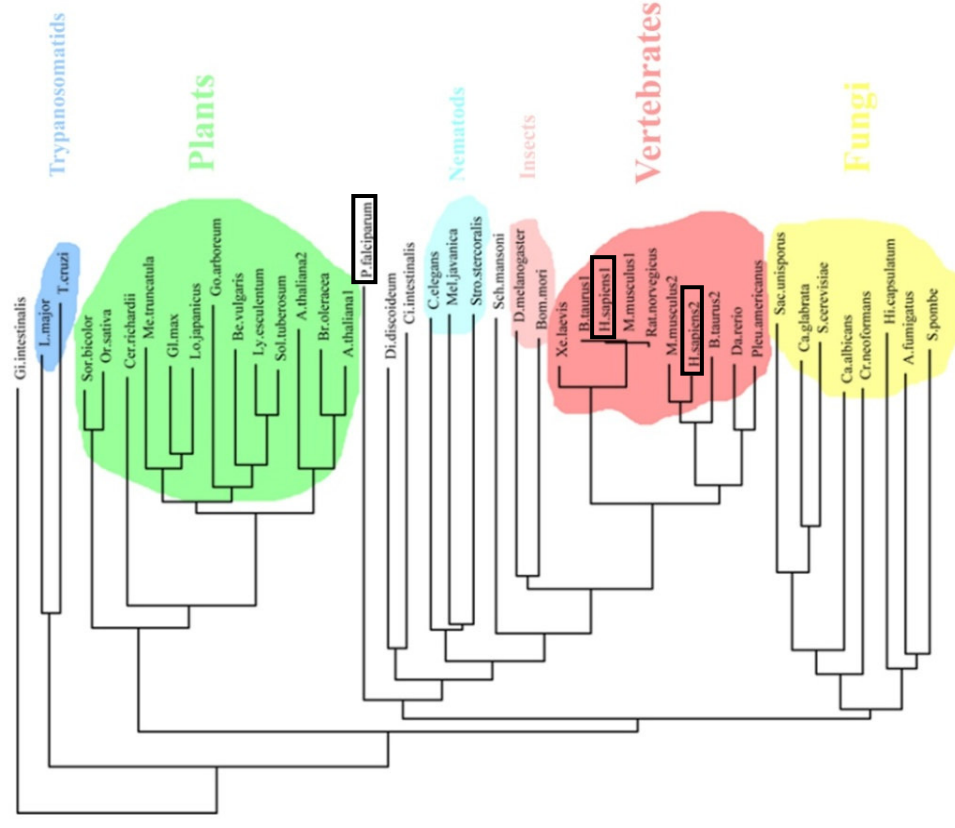


Figure II-10: Phylogenetic trees of NMT

A phylogenetic tree has been generated to compare NMT of different species (adapted from <http://mendel.imp.ac.at/myristate/SUPLPhylo.htm>, 09/2006). The N-terminus (A) as well as the C-terminus (B) have been closely examined. *P. falciparum* and *H. sapiens* are highlighted in a black box.

II.3 Rational Drug Design

II.3.1 Piggy-Back Approach

N-Myristoyl transferase is a key drug target and *N*-myristoylation is essential for various pathogens (Duronio, Towler et al. 1989; Weinberg, McWherter et al. 1995; Lodge, Jackson-Machelski et al. 1998). Attachment of the long hydrophobic myristate group to substrate proteins increases affinity for binding to the membrane, followed by secondary interactions with proteins to fully stabilise the embedded myristoylated protein anchor in the membrane, to then be able to interact with various proteins important for the function of the organism. Inhibition of *N*-myristoylation may have a harmful effect on the parasite. High similarities in homology studies of NMTs of organisms that have shown to require NMT for survival, propose a lethal effect on *Plasmodium* spp. Furthermore, apart from a highly conserved myristoyl CoA binding site across many organisms the peptidic binding cavity is quite divergent, in particular with respect to the human species, making it an ideal target site for drug development. Large research programmes developed selective inhibitors against *N*-myristoyl transferase for fungi (*Candida albicans* (Pfizer, unpublished work; (Rocque, McWherter et al. 1993; Yamazaki, Kaneko et al. 2005)) and parasitic protozoa (*Leishmania major*, *Trypanosoma brucei* (Panethymitaki, Bowyer et al. 2006)). To enable accelerated progress in drug development for malaria, the information of superior drug compounds for CaNMT is applied to the *Plasmodium falciparum* organism in the so-called piggy-back approach, whereby an established inhibitor for an enzyme is optimised for a new target organism (Gelb, Van Voorhis et al. 2003). This drug development aid is only applicable if the systems are homologous. Sequence alignment between NMT of *Candida albicans* and *Plasmodium falciparum* reveal moderately high similarities (Figure II-11). Amino acids that have been revealed as important for inhibition against CaNMT and thus may play a role in PfNMT are highlighted in Figure II-11. 12 out of 14 AA are identical, except F341 which is a Ser in PfNMT and Y351 which is a Phe in PfNMT. The latter change still enables CH- π stacking interactions, whereas serine may form hydrogen bonds with the inhibitor molecule.

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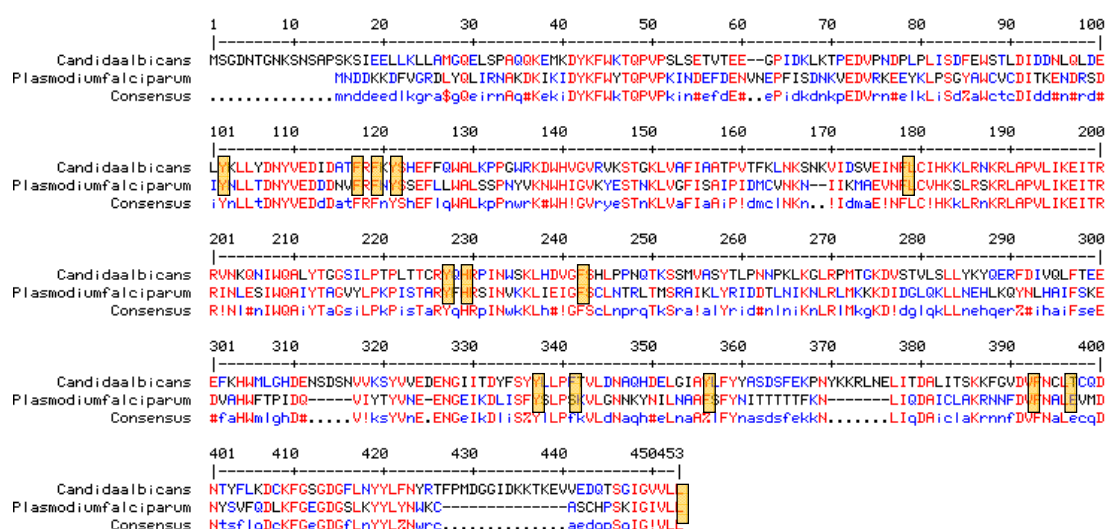


Figure II-11: Sequence alignments of CaNMT and PfNMT

The sequences of various NMT proteins have been aligned with the program MultAlign (Version 5.4.1.(Corpet 1988)). The colour code is as follows: Red = exact match, blue = AA of similar properties, black = no match, with a consensus level of 90% high and 50% low. The consensus symbols are as follows: ! = any of IV, \$ = any of LM, % = any of FY and # = any of NDQEBZ.

The highlighted amino acids are likely to undergo interactions with binding substrates in the active site. 12 out of 14 AA are identical, except F341 which is a Ser in PfNMT and Y351 which is a Phe in PfNMT.

II.3.2 Drug Development against CaNMT

High-throughput screening against *Candida albicans* NMT revealed numerous hits leading Roche to develop non-peptidic inhibitors based on a benzofuran scaffold (Figure II-12). **15** was a positive hit in a large HTS by Roche. Optimised further to **16**, it was more potent against CaNMT and ~5 000-fold selective over human NMT (Masubuchi, Kawasaki et al. 2001). Further development led to **17** (Ebiike, Masubuchi et al. 2002) and **18** (Kawasaki, Masubuchi et al. 2003) with an IC₅₀ (CaNMT) of 0.39 nM and 1 000 000-fold selectivity over human NMT. Crystallographic analysis revealed various binding with amino acids from the peptide binding pocket, enabling refinement for further drug development.

Crystallographic studies have been carried out within Roche, to gain more information about the conformation of the peptide binding pocket in CaNMT to increase the potency of inhibitors (Sogabe, Masubuchi et al. 2002). Figure II-13 illustrates three bound inhibitors (**19**, **20**, **21**) from the benzofuran series

II INTRODUCTION

co-crystallised with CaNMT. This knowledge enables 3D-QSAR, docking and MEP studies to analyse enzyme-inhibitor interactions in more depth (Hasegawa, Morikami et al. 2003; Purushottamachar and Kulkarni 2003).

Figure II-14 demonstrates key interactions of benzofuran-based inhibitor **22** with surrounding AA in the peptidic binding pocket of CaNMT (Hasegawa, Morikami et al. 2003). The first of four major interactions is a charge-charge interaction between the carboxylate of Leu451 and the 2° amine functionality. Hydrogen bonding occurs between the hydroxyl group of Tyr119 and the N-atom of the pyridine residue of the inhibitor, the 2° amine group of His227 and the benzofuran core, and the primary amine group of Asn192 interacts with both the oxygen of the ether moiety and the fluorine atom, whereby F-H the strongest hydrogen bond is and also orientated linearly. Surrounding Tyrosine-groups (Tyr225, Tyr354) interact with the π -system of the benzofuran core. Van der Waals forces such as hydrophobic interactions also fix the position of the inhibitor molecule in the binding cavity. Zihong Yu under the supervision of Prof R J Leatherbarrow (Department of Chemistry, Imperial College London) is currently optimising benzofuran inhibitors based on the published work by Roche with the aim of generating potent antimalarial compounds.

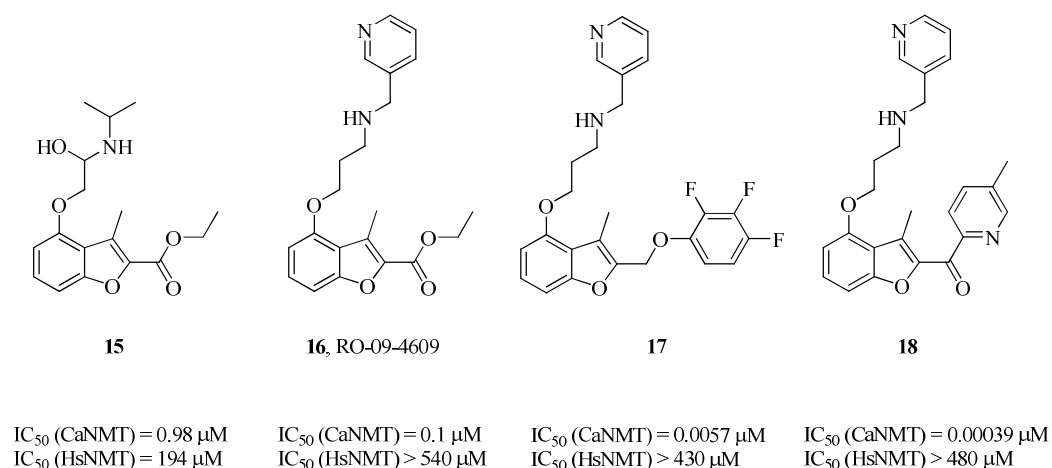


Figure II-12: Development of antifungal inhibitor based on benzofuran scaffold

The figure shows the development of non-peptidic inhibitors by Roche starting with a positive hit from high-throughput screening (**15**). Step-by-step progression of compound optimisation afforded an inhibitor (**18**) with an IC_{50} of 0.39 nM against CaNMT and 1 000 000 selectivity over human NMT (Masubuchi, Kawasaki et al. 2001; Ebiike, Masubuchi et al. 2002; Kawasaki, Masubuchi et al. 2003).

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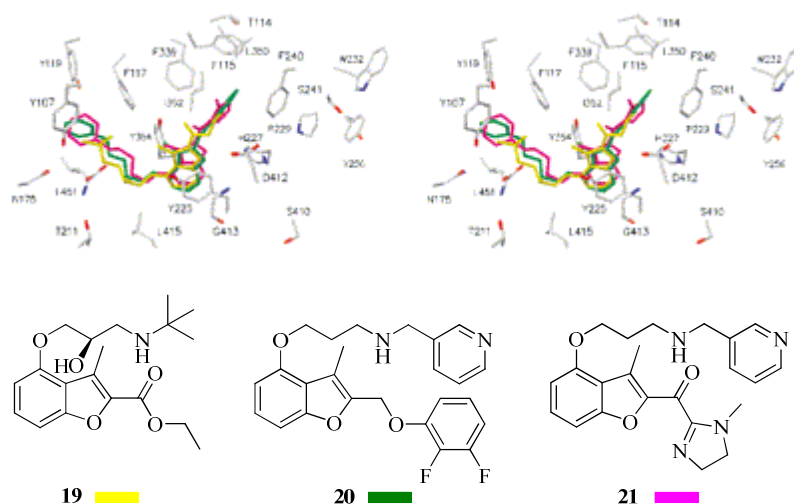


Figure II-13: Bound conformation of non-peptidic inhibitors in CaNMT

Crystallographic studies succeeded in the production co-crystals of benzofuran-based inhibitors with CaNMT (Sogabe, Masubuchi et al. 2002). Three inhibitors were separately co-crystallised with CaNMT to investigate the inhibitory effects of various R-groups with different chemical properties to investigate the peptide binding pocket more thoroughly.

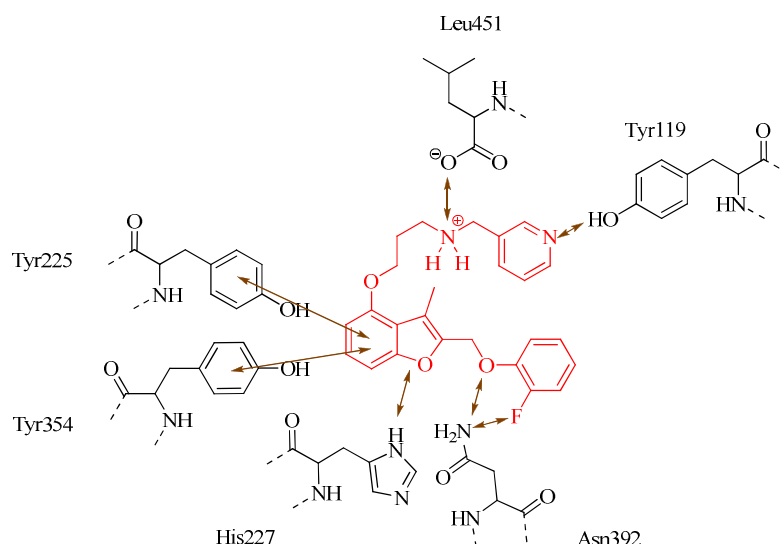


Figure II-14: Key interactions of active benzofuran-based inhibitor 22 and CaNMT

Detailed interactions of amino acids in the peptide binding pocket with inhibitor 22 were investigated, adapted from (Hasegawa, Morikami et al. 2003). Ionic interactions and H-bond donor/acceptor interactions can be seen clearly.

II.3.3 Benzothiazole Series

Drug design for the peptide binding site has led to peptidomimetic and non-peptidic inhibitors (Georgopapadakou 2002). Peptidomimetic inhibitor optimisation against CaNMT began from the derived N-terminal ARF sequence GLYASKLS; whereby the competitive octapeptide inhibitor ALYASKLS-NH₂ was developed. Mutagenesis experiments suggest that residues 1, 5 and 6 are valuable for binding (McWherter, Rocque et al. 1997). Further studies indicate, reduction of peptide bonds may increase *in vivo* activity (Devadas 1998). De-peptidisation, while maintaining the conformation of Ser and Lys at position 5 and 6, led to an effective non-peptidic inhibitor (**23**, SC-58272, Figure II-16) against *Candida albicans* NMT (IC₅₀ = 56 nM) (Bhatnagar, Fütterer et al. 1998).

Pfizer dedicated their antifungal research towards NMT inhibitors bearing a benzothiazole scaffold in search for broad-spectrum antifungal treatments. Intense developments including co-crystallisation trials yielded inhibitors specific towards *Candida albicans* (**24-30**, Figure II-16, Pfizer, unpublished work). The introduction of a dimethylamide group at C⁶-position of the benzothiazole ring, as well as an aromatic group attached at the free amine, enhance the binding properties in CaNMT significantly. The IC₅₀-value of the lead compound is in the low nanomolar region, IC₅₀ (CaNMT) = 13 nM (**29**, Figure II-16, Pfizer, unpublished work).

Figure II-17, Figure II-18 and Figure II-19 illustrate co-crystal structures of inhibitors in CaNMT. Figure II-17 demonstrates the penetration of the benzothiazole scaffold of **26** into the peptide binding pocket where it is able to form interactions with surrounding AA in contrast to peptidomimetic inhibitor **23**. Important interactions are indicated in Figure II-18 and include Asn392 for interactions with the oxygen atom of the dimethylamide, Leu451CO₂H for interactions with the amine and Thr211 for other polar interactions. The expansion of the compound with an aromatic group attached to the free amine seems to open up a new pocket for additional binding *via* the conformational change of Tyr210 (Figure II-19), reducing IC₅₀-values to the low nanomolar region.

Research and further development of benzothiazole-based antifungal inhibitors have been suspended as the lead compounds are unable to target NMT of fungal pathogens other than *Candida albicans* and *Candida tropicalis*. Pfizer has agreed to

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collaborate with Imperial College London to test compounds for activity in *Plasmodium falciparum* (Bowyer 2006; Bowyer, Gunaratne et al. 2007; Bowyer, Tate et al. 2008). These compounds will be referred to as “Pfizer series”. Initial screens using *in vitro* tests of enzyme activity as well as *in vivo* tests with *P. falciparum* infected erythrocytes with antifungal inhibitors have shown positive results (Bowyer 2006). A series of specific benzothiazole compounds was identified that inhibit PfNMT in the micromolar range, with an IC_{50} of $30 \pm 3 \mu M$ for the best compound (Figure II-15 (Bowyer, Gunaratne et al. 2007)).

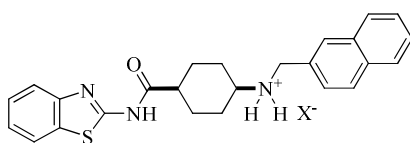


Figure II-15: Lead compound, 31

Out of the benzothiazole-based compounds UK-370,509 inhibited best against PfNMT with an IC_{50} of $30 \pm 3 \mu M$.

Recently, computer modelling has been performed utilising published crystal structure data of fungal NMTs to create 3D-models of PfNMT by homology modelling methods (Sheng, Ji et al. 2009). The homology score for CaNMT–PfNMT was calculated as 43.0% and ScNMT–PfNMT 46.8%. The 3D-model of PfNMT showed common features with the fungal NMTs such as a large β -sheet with several α -helices at the side and the internal pseudo two-fold symmetry.

The active site was found to have four characteristic pockets: two hydrophobic pockets, one hydrogen bonding pocket, one positively and one negatively charged hydrogen bonding pocket (Figure II-20A). Two key residues for activity were located; the first is Leu410, the C-terminal carboxylate, situated in the S4 negatively charged hydrogen bonding site and the second are Leu163 and Leu91 in the S2 hydrogen bonding pocket which form the oxyanion hole with their backbone amides to accommodate myristoyl CoA and subsequently polarise the thioester moiety.

Lead compound UK-370,509 (Figure II-20C) was docked into the active site using flexible molecular docking methods (Figure II-20B). The main interactions of the inhibitor with surrounding amino acids were hydrophobic and hydrogen bonding in

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nature. The oxygen of the amide was proposed to form two hydrogen bonds with Lys25 (S5 pocket) and Tyr211 (S4 pocket). The interaction with Lys25 is specific for PfNMT and not found in human NMT, thus is proposed to be vital for selectivity. Loss of this hydrogen bond was suggested to result in lower inhibition. A third hydrogen bond is suggested between Tyr107 (S2 pocket) and the secondary amine. Perhaps more controversially, the model proposes that the secondary amine does not interact with the C-terminus as initially suggested in Figure II-18 or Figure II-14. Hydrophobic interactions are thought to occur in the S1 and S3 pockets and involve the aromatic ring of the benzothiazole and the cyclohexyl unit. Proposed key residues are highlighted in Figure II-20B.

Japanese researchers have independently carried out further investigations to produce more potent and selective inhibitors against CaNMT. Employing solid phase synthesis, they developed a very potent inhibitor against CaNMT with an IC₅₀-value in the picomolar region (**30**, FTR1335, Figure II-16, (Yamazaki, Kaneko et al. 2005)).

II.4 **Project Aims**

The work presented in this thesis documents the development of effective inhibitors, whereby differences in the peptide binding pockets of PfNMT and human NMT were exploited to produce more selective and potent inhibitors, to lead to efficient novel antimalarial treatments.

- Synthesis of benzothiazole-based lead compound of the “Pfizer series”
- Optimisation of the lead compound for potency against PfNMT
- Improvement of inhibitors for selectivity against PfNMT over human NMT
- Test for activity of compounds in established *in vitro* and *in vivo* assays (with prior expression of PfNMT and human NMT for *in vitro* assay)

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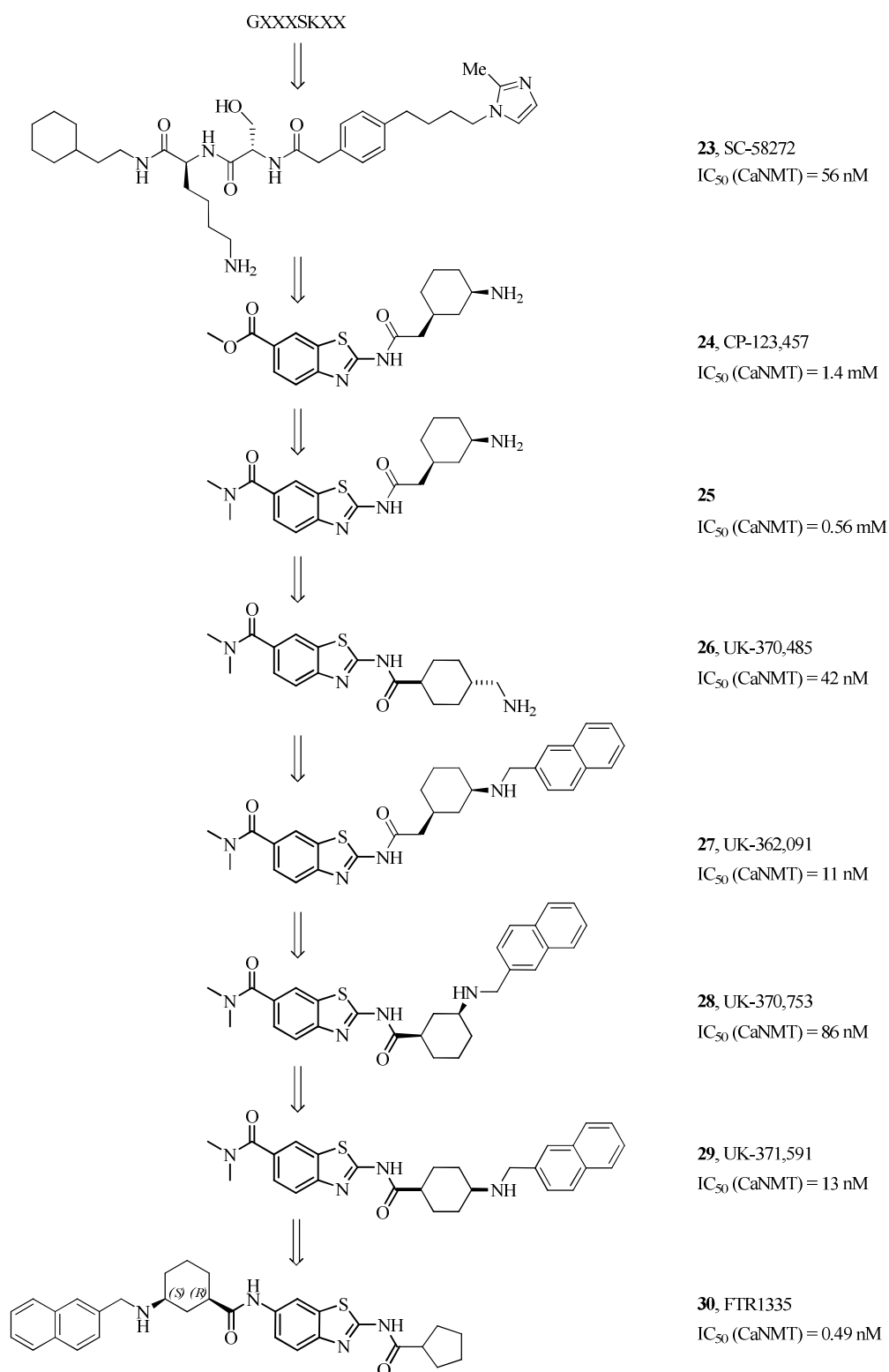


Figure II-16: Optimisation process of antifungal compounds

Another inhibitor development process is pictured. Starting from peptide-based inhibitors, Pfizer research developed into benzothiazole-based inhibitors (**23–30**) with an activity against CaNMT in the low nanomolar region. However, further research by Yamazaki *et al.* produced an even more potent inhibitor, **30** (Pfizer, unpublished results; (McWherter, Rocque *et al.* 1997; Bhatnagar, Fütterer *et al.* 1998; Yamazaki, Kaneko *et al.* 2005; Bowyer 2006)).¹¹²¹¹²

II INTRODUCTION

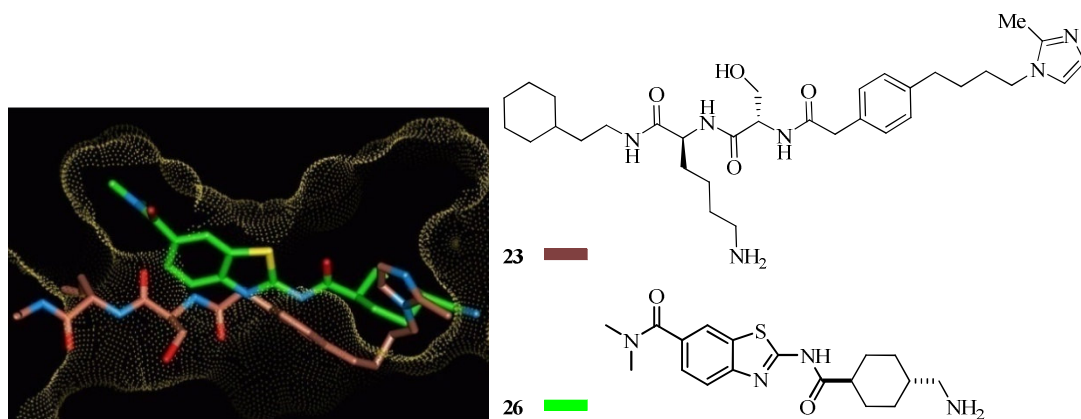


Figure II-17: Co-crystal structure of two inhibitors and CaNMT

A peptide mimetic (23, SC-58272, brown-coloured) and a non-peptidic molecule (21, UK-370,485, green-coloured) have been co-crystallised in CaNMT (Pfizer, unpublished data, undisclosed info).

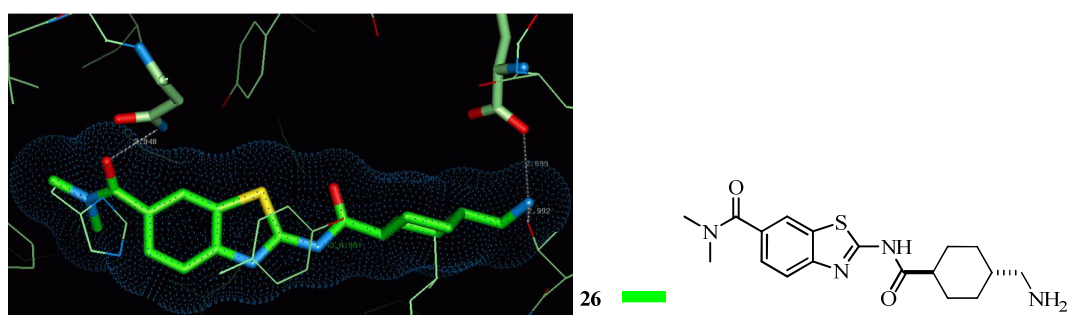


Figure II-18: In depth view of inhibitor binding interactions

A closer examination of interactions with surrounding amino acids from peptide binding pocket of CaNMT with non-peptidic compound (21, UK-370,485, green-coloured) can be seen (Pfizer, unpublished data, undisclosed information).

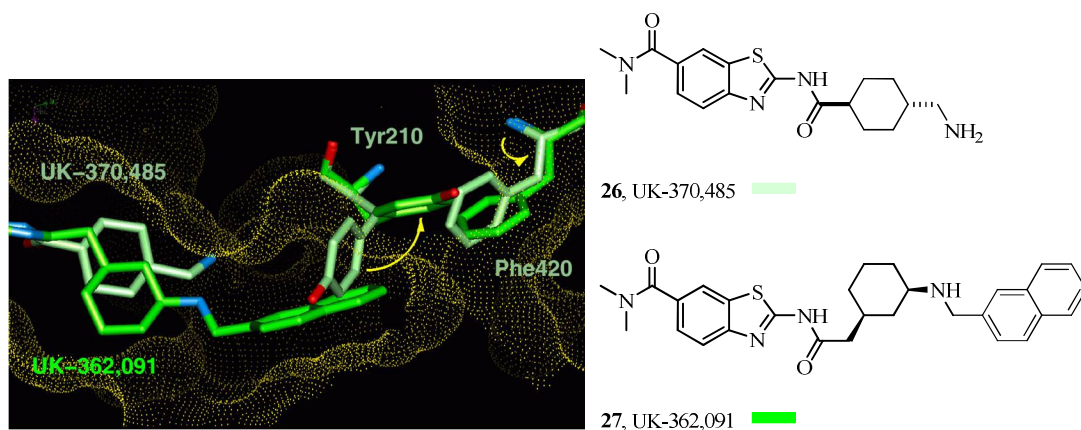


Figure II-19: Comparison of two different benzothiazole compounds

On comparison of two structurally closely related compounds (light green- and dark green-coloured) a change in the orientation of nearby amino acids of the peptide binding pocket was noticed (Pfizer, unpublished data, undisclosed information).

II INTRODUCTION

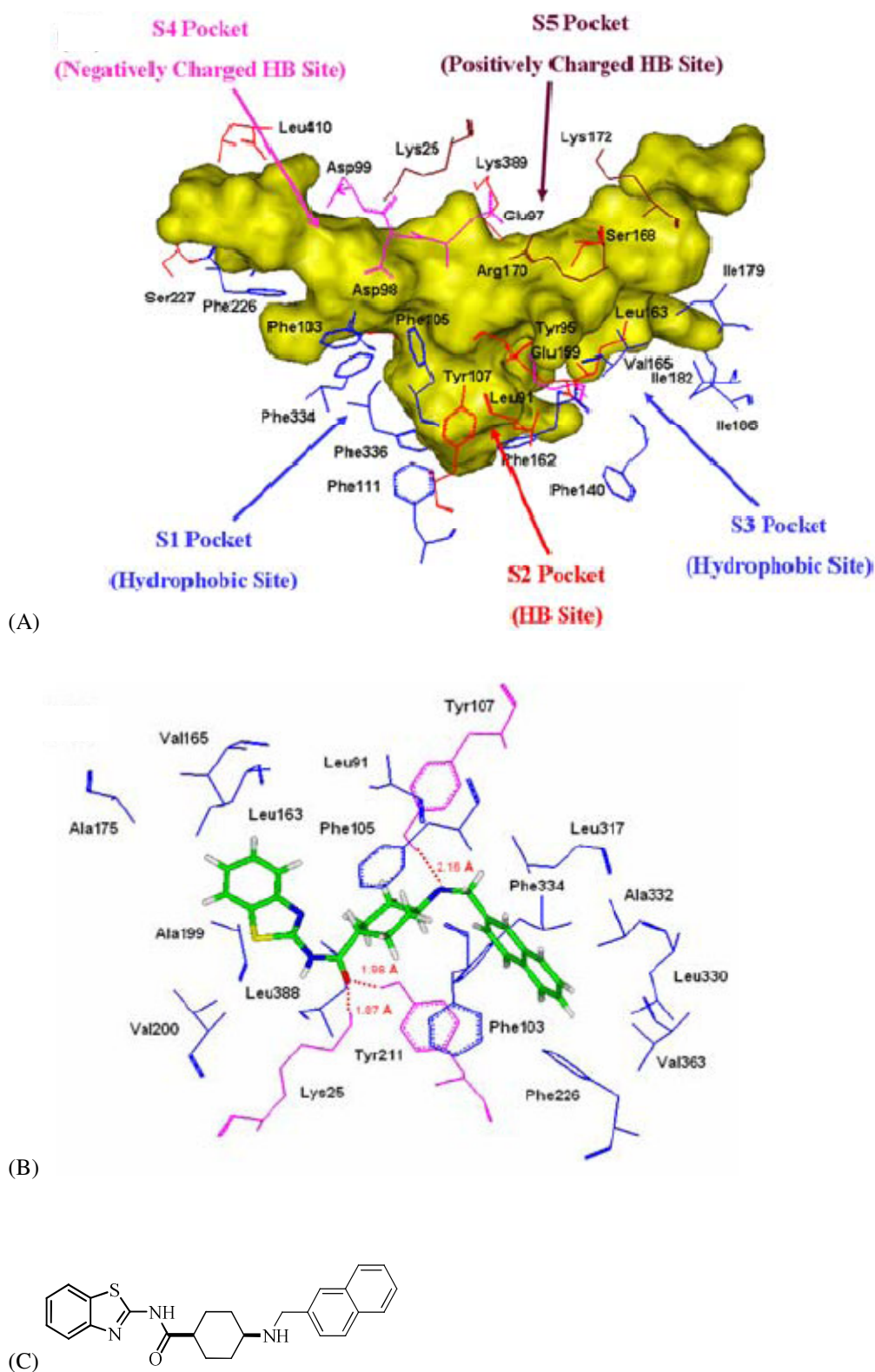


Figure II-20: Homology modelling for PfNMT

3D-Models of PfNMT were constructed on the basis of published data of fungal NMTs using homology modelling. The surface, showing the active site of PfNMT, is displayed in A. B shows the docking information of compound UK-370,509 (C) in the active site of PfNMT (Sheng, Ji et al. 2009).

III NMT EXPRESSION, ISOLATION & ANALYSIS

III.1 Introduction

The expression and purification procedures of *N*-myristoyl transferase for the *in vitro* assay from organisms *Plasmodium falciparum* (PfNMT), *Homo sapiens* (HsNMT1 and HsNMT2), *Candida albicans* (CaNMT) and *Leishmania major* (LmNMT) is described in this chapter. Various physical properties of these NMTs, exploited for efficient purification procedures, are listed in Table III-1.

Table III-1: Overview of physical properties for NMT of various organisms

The table lists molecular weight and formula for NMT of different species as well as the extinction coefficient, ϵ , which was predicted with ProtParam tool at a wavelength of 280 nm (<http://www.expasy.org/tools/protparam.html>). The isoelectric point, *pI*, was obtained through calculations with Vector NTI Advance™ software (Invitrogen).

Enzyme	N-terminal Tag	Molecular Formula	M _r [kDa]	Extinction Coefficient, ϵ , $\times 10^3$ [M ⁻¹ cm ⁻¹]	Reference (FASTA Sequence)	pI
PfNMT	His ₆ tag	C ₂₃₄₄ H ₃₆₁₀ N ₆₂₀ O ₆₇₈ S ₁₇	52	81	Table VIII-2	8.40
HsNMT1	His ₆ tag	C ₂₅₅₀ H ₃₉₇₉ N ₆₈₇ O ₇₃₈ S ₂₃ [*]	57 [*]	71 [*]	P30419	7.74 [*]
HsNMT2	His ₆ tag	C ₂₅₅₂ H ₄₀₀₉ N ₆₉₁ O ₇₅₀ S ₁₉ [*]	57 [*]	69 [*]	O60551	9.54 [*]
CaNMT	—	C ₂₃₆₂ H ₃₆₃₀ N ₅₉₈ O ₆₉₃ S ₁₂ [*]	52 [*]	78 [*]	P30418	6.06 [*]
LmNMT	His ₆ tag	C ₂₂₁₁ H ₃₃₆₇ N ₅₇₉ O ₆₂₂ S ₁₆ [*]	49 [*]	74 [*]	AAG38102	6.28 [*]

^{*}calculated from FASTA sequence

III.2 Material & Methods

III.2.1 Chemicals and Reagents

Solvents and reagents of the highest available standard were obtained from commercial sources (BDH, Fisher Scientific, GE Healthcare formerly known as Amersham Biosciences, HyClone, Invitrogen, Merck, Millipore, Sigma-Aldrich). *Escherichia coli* BL21(DE3) strains were obtained from Stratagene and *E. coli* DH5 α TM strains from Novagen.

III.2.1.1 Solutions – Media

Ultrapure laboratory grade water (filtered, deionised, sterilised) was obtained from in-house Elix[®] and MilliQ[®] water purification systems. All solutions were prepared in ultrapure laboratory grade water and filter sterilised through a 0.22 μ m GSWP filter (nitrocellulose membrane, Millipore).

Media were prepared, sterilised and stored according to the manufacturer and standard published protocols (Sambrook and Russel 2001). Media were handled in sterile conditions under an open flame. The composition of media is stated below.

LB: Lysogeny broth (Bertani 2004) (LB broth (Miller), Merck, Fisher Scientific) was prepared according to the manufacturer, sterilised by means of autoclaving and stored at RT. LB contains 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl in distilled water. LB-agar: Lysogeny broth-agar (LB-agar (Miller), Merck) was prepared according to the manufacturer, sterilised by means of autoclaving and stored at RT. It consists of LB supplemented with 1.2% (w/v) agar.

SOC medium: Super optimal broth with catabolite repression (Hanahan 1983) (SOC medium, Sigma) was used to maximise the transformation efficiency of competent cells by improving the molecular uptake whilst stabilising the cells. The solution contains 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄ and 20 mM glucose in distilled water. It was stored at 4 °C.

Antibiotic stock: Stocks of ampicillin (sodium salt, Sigma) were prepared on the day of handling in distilled water and stored at 4 °C. Ampicillin stock was used at a working concentration of 50 mg mL⁻¹. LB-amp: Lysogeny broth-ampicillin was prepared in a 1:1 000 dilution (1 mL 50 mg mL⁻¹ ampicillin stock in 1 L LB).

III.2.1.2 Bacterial Strains and Plasmids

Three expression vectors were used in the overexpression of various recombinant proteins, two from the pET vector series for *Homo sapiens* NMT and *Candida albicans* NMT and pTrc vector for *Plasmodium falciparum*, NMT (Table III-2). The pET system is often used for cloning and expression of recombinant proteins in *E. coli*. The plasmids carry a T7 RNA polymerase which can be induced by IPTG to selectively promote target gene expression. All vectors were stored in glycerol stocks at -20 °C.

The pET-11c vector is ampicillin resistant, encodes a T7 *lac* promoter, carries an N-terminal T7 tag sequence and a BamH1 cloning site (Table III-2A). The plasmid, a gift from Pfizer PLC, was utilised to overexpress CaNMT.

The pTrcHisA vector with PfNMT insert was obtained from Dr A A Holder (National Medical Research Institute, London, UK) and used for overexpression of *P. falciparum* NMT (Table III-2B). The insert is a synthetic gene, codon-optimised for optimum expression in *E. coli* (Bowyer, Gunaratne et al. 2007).

pET-15b vector is also ampicillin resistant, contains a T7 *lac* promoter, bears an N-terminal His₆ tag followed by a thrombin site and three cloning sites (Table III-2C). The plasmid was obtained from Dr S Elroy (University of Dundee, UK) and used for the overexpression of HsNMT1 and HsNMT2.

E. coli strains used for overproduction of proteins were DH5 α TM and BL21(DE3). A full genotype can be found in the Appendix (Table VIII-1).

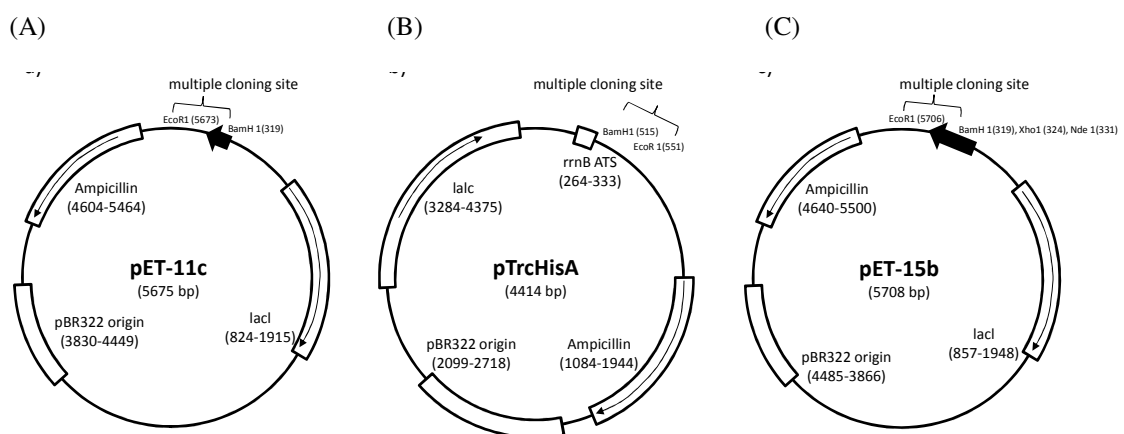
DH5 α TM was used for plasmid maintenance and BL21(DE3), the general purpose expression host, for overexpression of the recombinant proteins. They were stored at -80 °C.

III NMT EXPRESSION, ISOLATION & ANALYSIS

Table III-2: Plasmid vectors

The table lists primary expression vectors used in the course of this project along with relevant details, (A) pET-11c, (B) pTrcHisA, (C) pET-15b. Sequences were numbered by the pRB322 convention. (Source: Novagene, Vectorpedia, www.addgene.org, 14.07.2008)

Insert (Plasmid Name)	Vector System	Source of DNA	Comments
<i>Candida albicans</i> NMT (CaNMT)	pET-11c	a kind gift from Pfizer PLC, UK	No tag
<i>Plasmodium falciparum</i> NMT (PfNMT)	pTrcHisA	a kind gift from Dr A A Holder, National Institute for Medical Research, UK	N-terminal His ₆ tag
<i>Homo sapiens</i> NMT1 (HsNMT1)	pET-15b	a kind gift from Dr S McElroy, Drug Discovery Unit, University of Dundee, UK	N-terminal His ₆ tag
<i>Homo sapiens</i> NMT2 (HsNMT2)	pET-15b	a kind gift from Dr S McElroy, Drug Discovery Unit, University of Dundee, UK	N-terminal His ₆ tag



III.2.2 Methods and Equipment

Unless otherwise stated, all chromatography media and columns were purchased from GE Healthcare. Protein purification was carried out using FPLC on an Äkta System, Unicorn Version 4.11 (GE Healthcare) Software.

The pH of the buffered solutions was measured at room temperature with a pH meter (Hanna instruments 8519). Calibration was performed with ‘colour key’ buffer solution from BDH (red – pH 4.0 ± 0.02 at 20 °C, yellow – pH 7.0 ± 0.02 at 20 °C). The pH adjustments were made with a suitable acid or base, e.g. HCl, NaOH, NH₃. Buffered solutions used throughout the project are catalogued in Table III-4. Incubation was carried out at 37 °C (Innova 4400 Incubation Shaker or LTE IP60 incubator), 15 °C (Innova 4430 Incubation Shaker), or at 4 °C (Roto-Shake Genie, Scientific Industries, Inc.).

The centrifuge for speeds up to 20 000g was performed with Sorvall RC 5C Plus, Kendro Laboratory Products and SS-34 Rotor. For 4 000g the Centrifuge 5810 R, Eppendorff or Jouan BR4 or Jouan A14 were used.

OD measurements and quantitative analysis of enzymes were analysed on a Biowave II WPA UV detector at 600 nm and 280 nm respectively.

Sonication was performed with Bandelin Sonoplus at 30% power output.

III.2.2.1 Manipulation of Bacteria

III.2.2.1.1 Preparation of Agar-Amp Plates

Agar stock was melted carefully in the microwave with several 5 s heat pulses until clear. After it cooled down to ~37 °C, 50 mg mL⁻¹ freshly prepared ampicillin was added in a ratio of 1:500 (160 µL 50 mg mL⁻¹ ampicillin in 80 mL agar). 20 mL of antibiotic rich agar was poured in a Petri dish, cooled down to room temperature and stored upside down at 4 °C.

III.2.2.1.2 Growth of Subcultures

Bacteria were streaked on agar plates with the appropriate antibiotic selection, incubated overnight at 37 °C and stored at 4 °C. 50–100 mL LB-media were

inoculated with one colony from the agar plate. The liquid cultures were incubated overnight in a 1 L flask at 37 °C with shaking at 220 rpm.

III.2.2.1.3 Transformation of Bacteria

1 µL of DNA and 20 µL of cells were mixed and incubated on ice for 30 min. The cells were then heat shocked at 42 °C for 30 s, followed by a further incubation period on ice for 2 min. 250 µL of SOC medium was added after which the mixture was incubated in a shaker at 37 °C for 1 h. A portion of cell suspension was diluted 1:1 with fresh SOC medium (100 µL cells + 100 µL SOC) and then streaked on an agar-amp plate and incubated overnight at 37 °C.

III.2.2.2 Protein Manipulation

III.2.2.2.1 Polyacrylamide Gel Electrophoresis (PAGE) of Proteins and Staining

Protein samples were analysed by method of SDS-PAGE (X-Cell Sure Lock™, BioRad Power Pac Basic) which separates proteins according to size. For qualitative analysis of fractions after purification procedures, 5 µL was loaded in each well. 2–5 µg were loaded for quantitative analysis of pure protein. The protein was mixed with Sample Buffer (4 × NuPAGE LDS Sample Buffer, Invitrogen) and diluted with H₂O to a final volume of 20 µL. 1 µL β-mercaptoethanol was added. The samples were thoroughly mixed and boiled at 100 °C for 5 min prior to loading. 5 µL protein marker (SeeBlue® Pre-Stained Standard, Invitrogen) was loaded in the outer lanes and 21 µL samples in subsequent lanes. NuPAGE MOPS SDS Running Buffer (Invitrogen) or NuPAGE MES SDS Running Buffer (Invitrogen) was used with NuPAGE 12% Bis-Tris gels (precast, Invitrogen or for self-made see (Table III-3). Electrophoresis was carried out at 195 V.

For visualisation of proteins, gels were soaked in Coomassie Blue (Brilliant Blue G solution, Sigma) for 1 h and destained by soaking in Destaining Solution (25% (v/v) MeOH, 5% (v/v) CH₃COOH) overnight.

Table III-3: Recipe for two 12% Bis-Tris gels

The discontinuous SDS-PAGE method by Laemmli (Laemmli 1970; Sambrook and Russel 2001) exploits prior separation in a stacking gel (4%, top section of gel) before separation according to size in the resolving gel (12%, bottom section of gel). The composition of Bis-Tris gels is listed.

	Resolving Gel (12%) [mL]	Stacking Gel (4%) [mL]
30% Acrylamide	6	0.667
1.25 M Bis-Tris (pH 6.5)	4.300	1.428
H ₂ O	4.550	2.880
10% APS	0.150	0.050
TEMED	0.006	0.004

III.2.2.2.2 Immunoblotting

To visualise specific proteins, namely His₆-tagged proteins which can be visualised with anti-His HRP antibody plus its substrate by means of enhanced chemoluminescence (ECL), the gel was subjected to a Western Blot following electrophoresis.

Blotting filter paper (2 × 2.5 mm, Invitrogen) and nitrocellulose membrane (0.2 µm, GE Healthcare) were thoroughly soaked in Towbin Buffer (Table III-4D) for semi-dry blotting. They were then stacked on top of each other in the following order (from bottom to top): anode, blotting paper, membrane, gel, blotting paper, and cathode. A voltage of 20 V was applied over 27 min. After transfer of protein to the membrane, it was immersed in 10 mL 5% BSA in TBS-T (Table III-4D) at 4 °C overnight to block any non-specific binding sites. The membrane was then rinsed with TBS-T (10 mL, 3 × 5 min), incubated in a 1:4 000 antibody solution (10 mL TBS-T, 2.5 µL 4 mg mL⁻¹ Anti-His HRP (HisProbe™-HRP, Thermo Scientific)) at room temperature and washed again with TBS-T (10 mL, 3 × 10 min). His₆-tagged proteins were visualised by first covering the membrane with the detection solutions in a ratio of 1:40 (1 mL Solution A (ECL™ Plus substrate solution containing Tris Buffer) and 25 µL Solution B (acridan solution in dioxane and ethanol, both from ECL™ Plus Western Blotting Detection Reagents, GE Healthcare)), and placed it protein side up on the tray to expose it to light. Chemiluminescence was detected with Fujifilm LAS-3000.

III.2.2.2.3 Protein Concentration Determination

Protein concentration of purified enzyme was determined with Lambert-Beer Law (see Equation III-1). The length of the cuvette was 1 cm. Absorbance was measured at 280 nm. The extinction coefficient, ϵ , was predicted with ProtParam tool (<http://www.expasy.org/tools/protparam.html>).

Protein concentration of impure samples was determined with the Bradford Assay (Bradford 1976). It was carried out with BioRad Protein Assay according to the manufacturer. BSA was used as a protein model to generate a standard curve of absorbance at 595 nm against the concentration from which the absolute protein concentration was determined.

Equation III-1: Lambert-Beer Law

$$A = \epsilon cl$$

III.2.2.2.4 Buffer Exchange and Concentration

Buffer exchange and concentration were carried out in Vivaspin5 or 20 (5 000 MWCO, 5 or 20 mL capacity, Sartorius Stedim Biotech) as appropriate. Samples were subjected to five cycles of concentration and re-suspension to ensure complete buffer exchange.

Purified protein was dialysed into storage buffer (SPA buffer, Table III-4A), concentrated and stored at -20 °C.

With regards to the possibility of aggregation, in early stage purification steps dialysis was performed using dialysis cassettes. The protein was injected into the chamber of the dialysis cassette (Slide-A-Lyzer Dialysis Cassette, 3 500 MWCO, 3–12 mL capacity, Pierce) and dialysed into the appropriate buffer (3 × 2 L) for 1 h at 4 °C.

III.2.3 Preparation and Purification of PfNMT (Small Scale)

III.2.3.1 Overexpression of *Plasmodium falciparum* NMT

The construct PfNMT-pTrcHisA was transformed into BL21(DE3) expression cell lines. A single colony of transformed cells was used to inoculate 5 mL LB-amp in a 100 mL flask. After overnight incubation in a shaker at 37 °C, the cells from the

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cloudy starter culture were harvested by centrifugation (4 000g, 15 min, 25 °C). The supernatant was discarded whereas the pellet was re-suspended in 2.5 mL fresh LB-amp.

The cells were diluted further with fresh LB-amp (1:40 dilution, 2.5 mL cells per 90 mL LB) in a 1 L flask and incubated in a shaker at 37 °C to mid-log phase ($OD_{600\text{ nm}} = 0.6$). The culture was then split into falcon tubes (9 × 10 mL), appropriate amount of freshly prepared IPTG was added (see below) and incubated in a shaker at various temperatures (19 °C, 31 °C, 37 °C). 5 mL cells were harvested after 5 h and 19 h by centrifugation (4 000g, 30 min, 4 °C). The pellet was then re-suspended in PBS (HyQ PBS 0.0067 M, HyClone). After centrifugation (4 000g, 60 min, 4 °C) the pellet was snap frozen with N₂ (l) and stored at -80 °C.

Final Concentration of IPTG	Quantity Added to Culture
1.0 mM	10 µL of 1 M IPTG stock in 10 mL culture (1:1 000 dilution)
0.1 mM	10 µL of 0.1 M IPTG stock in 10 mL culture (1:1 000 dilution)
0.01 mM	10 µL of 0.01 M IPTG stock in 10 mL culture (1:1 000 dilution)

III.2.3.2 Sample Preparation

The pellet was thawed on ice in 1 mL of cold Lysis Buffer (Table III-4A). The solution was vortexed until smooth. After 30 min incubation on ice, Triton X-100™ (Sigma) was added to a final concentration of 1% (10 µL 10% stock in 1 mL). The mixture was sonicated (10 × 3 s at 30% power with 3 s rest on ice), centrifuged (17 000g, 10 min, 4 °C), snap frozen with N₂ (l) and stored at -20 °C.

III.2.4 Preparation and Purification of PfNMT (Large Scale)

III.2.4.1 Overexpression of *Plasmodium falciparum* NMT

The construct PfNMT-pTrcHisA was transformed into BL21(DE3) expression cell lines. A single colony of transformed cells was used to inoculate 25 mL LB-amp in a 100 mL flask. After overnight incubation in a shaker at 37 °C, the cells from the cloudy starter culture were harvested by centrifugation (4 000g, 15 min, 25 °C). The

supernatant was discarded whereas the pellet was re-suspended in 25 mL fresh LB-amp.

The cells were diluted further with fresh LB-amp (1:40 dilution, 25 mL cells per 1 L LB) and incubated in a shaker at 37 °C to mid-log phase ($OD_{600\text{ nm}} = 0.6$). Typically 4×1 L culture was grown in 2 L flasks. Protein expression was induced by freshly prepared IPTG (Sigma) with a final concentration of 0.1 mM (1:10 000 dilution, 100 μ L 1 M IPTG in 1 L LB-amp). Cells were harvested after incubation in a shaker for 5 h at 30 °C by centrifugation (4 000g, 30 min, 4 °C). The pellet was re-suspended in PBS (HyQ PBS 0.0067 M, HyClone). After centrifugation (4 000g, 60 min, 4 °C), the pellet was snap frozen with $N_2(l)$ and stored at -80 °C.

III.2.4.2 Purification of *Plasmodium falciparum* NMT

The pellet was thawed on ice. 20 mL of cold Lysis Buffer (see Table III-4A) was added as well as a protease inhibitor cocktail tablet (complete, EDTA-free, Roche). The solution was vortexed at regular intervals until smooth. Lysozyme (from hen egg white, Fluka) was added to a final concentration of 1 mg mL⁻¹ (30 mg in 30 mL). After 30 min incubation on ice, Triton X-100™ (Sigma) was added to a final concentration of 1% (3.2 mL 10% stock for 32 mL). The mixture was sonicated (8 $\times$ 30 s at 30% power with 30 s rest on ice). DNAaseI (Roche) was added to the viscous solution and incubated on ice for further 30 min. The now smooth mixture was centrifuged (18 000g, 45 min, 4 °C), the lysate (soluble fraction) filtered to remove debris (insoluble fraction) and subjected to three purification steps.

III.2.4.2.1 Immobilised Metal Affinity Chromatography (IMAC)

The His₆-tagged protein was captured by immobilised Ni²⁺ (HisTrap™ HP, 1 mL, pre-packed Ni Sepharose™ High Performance, dynamic binding capacity 40 mg His₆-tagged protein / mL medium) according to the protocol of the manufacturer using an ÄKTA_{FPLC} system (flow rate, 1 mL min⁻¹; pressure limit, 0.7 MPa). The lysate was loaded *via* a 50 mL superloop on IMAC Buffer A (see Table III-4A) equilibrated column. After 20 CV column wash, PfNMT was eluted by increasing the concentration of imidazole with IMAC Buffer B (see Table III-4A) from 20 mM to 1 M. The gradient employed was 0–50% in 10 CV, 50–100% in 5 CV, and 100%

for 2 CV. The fractions were analysed by SPA and SDS-PAGE, whereby active fractions were pooled, concentrated, filtered to remove debris and further purified.

III.2.4.2.2 Size Exclusion Chromatography (SEC)

The column (HiPrep™ 16/60 Sephacryl™ S-100) was used according to the protocol of the manufacturer on an ÄKTA_{FPLC} system (flow rate, 0.5 mL min⁻¹; pressure limit, 0.5 MPa). Using a 2 mL loop, the concentrated fractions were loaded on the column. It was previously equilibrated with SEC Buffer (see Table III-4A). The fractions were analysed first by SPA, followed by analysis of the active fractions by SDS-PAGE. They were then pooled, concentrated, dialysed into IEX Buffer A (see Table III-4A), filtered to remove debris and further purified.

III.2.4.2.3 Ion Exchange Chromatography (IEX)

The final step of cation exchange chromatography was carried out using a Resource™ S column (6 mL, methyl sulfonate strong cation) according to the protocol of the manufacturer on an ÄKTA_{FPLC} system (flow rate, 1.0 mL min⁻¹; pressure limit, 1.5 MPa). Using a 2 mL loop, the concentrated fractions were loaded on the column. It was previously equilibrated with IEX Buffer A (see Table III-4A). After 20 CV column wash, PfNMT was eluted by increasing the concentration of salt with IEX Buffer B (see Table III-4A) from 25 mM to 1 M. The gradient employed was 0–15% in 20 CV, 15–60% in 10 CV, 60–100% in 15 CV, and 100% for 2 CV. The fractions were analysed first by SPA, followed by analysis of the active fractions by SDS-PAGE. They were then pooled, concentrated, dialysed into SPA Buffer (see Table III-4A), filtered to remove debris, aliquoted and stored at -20 °C.

III.2.5 Preparation and Purification of *Homo sapiens* NMT1 and NMT2

III.2.5.1 Overexpression of *Homo sapiens* NMT1 and NMT2

Before the construct HsNMT1-pET-15b/HsNMT2-pET-15b was transformed into BL21(DE3) expression cell lines, the DNA was bulked up in a Miniprep. The plasmid HsNMT1-pET-15b/HsNMT2-pET-15b was transformed into DH5 α ™ expression cell lines.

1 μ L of DNA and 20 μ L of cells were mixed and incubated on ice for 30 min. The cells were then heat-shocked at 40 °C for 30 s, followed by a further incubation

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period on ice for 2 min. 500 μ L of SOC medium was added after which the mixture was incubated in a shaker at 37 °C for 1 h. A portion of cell suspension was diluted 1:3 with fresh SOC medium (50 μ L cells + 100 μ L SOC) and then streaked on an agar-amp plate and incubated overnight at 37 °C.

A single colony of transformed cells was used to inoculate 10 mL LB-amp. After overnight incubation in a shaker at 37 °C, the cells from the cloudy starter culture were harvested by centrifugation (4 000g, 15 min, 25 °C). The supernatant was discarded whereas the pellet was re-suspended in 10 mL fresh LB-amp. The bench protocol QIAprep Spin Miniprep Kit (Qiagen) was followed to isolate the plasmid DNA from the culture.

1 μ L of amplified pure DNA and 20 μ L of BL21(*DE3*) cells were mixed and incubated on ice for 30 min. The cells were then heat-shocked at 42 °C for 30 s, followed by a further incubation period on ice for 2 min. 250 μ L of SOC medium was added after which the mixture was incubated in a shaker at 37 °C for 1 h. A portion of cell suspension was diluted 1:1 with fresh SOC medium (100 μ L cells + 100 μ L SOC) and then streaked on an agar-amp plate and incubated overnight at 37 °C.

A single colony of transformed cells was used to inoculate 10 mL LB-amp. After overnight incubation in a shaker at 37 °C, the cells from the cloudy starter culture were harvested by centrifugation (4 000g, 20 min, 25 °C). The supernatant was discarded whereas the pellet was re-suspended in 10 mL fresh LB-amp.

The cells were diluted further with fresh LB-amp (1:100 dilution, 10 mL cells per 1 L LB) and incubated in a shaker at 37 °C to mid-log phase ($OD_{600\text{ nm}} = 0.6$). Protein expression was induced by freshly prepared IPTG (Sigma) with a final concentration of 0.05 mM (1:20 000 dilution, 50 μ L 1 M IPTG in 1 L LB-amp). Cells were harvested after incubation in a shaker for 19 h at 15 °C by centrifugation (4 000g, 30 min, 4 °C). The pellet was re-suspended in PBS (HyQ PBS 0.0067 M, HyClone). After centrifugation (4 000g, 60 min, 4 °C), the pellet was snap frozen with $N_2(l)$ and stored at -80 °C.

III.2.5.2 Purification of *Homo sapiens* NMT1 and NMT2

The pellet was thawed on ice in 20 mL of cold Lysis Buffer (see Table III-4B). The solution was vortexed at regular intervals until smooth. The mixture was sonicated (8×30 s at 30% power with 30 s rest on ice). DNAaseI (Roche) was added to the viscous solution and incubated on ice for further 30 min. The now smooth mixture was centrifuged (15 000g, 30 min, 4 °C), the lysate (soluble fraction) filtered to remove debris (insoluble fraction) and subjected to two purification steps.

III.2.5.2.1 Immobilised Metal Affinity Chromatography (IMAC)

The His₆-tagged protein was captured by immobilised Ni²⁺ (Ni-NTA agarose resin, Qiagen, dynamic binding capacity 5–10 mg His₆-tagged protein mL⁻¹ medium) according to the protocol of the manufacturer. 10 mL Ni-beads (50% suspension, in nitrilotriacetic acid and ethanol, 5 mL beads) were centrifuged (2 000g, 5 min, 4 °C) and washed 3×20 mL with IMAC Buffer A (see Table III-4B) before they were incubated with crude lysate for 45 min on a roller at 4 °C. The bead suspension was then transferred in a fritted polypropylene tube (5 mL, Qiagen) which has been cleaned with IMAC Buffer A. The beads were washed with 20 CV IMAC Buffer B (see Table III-4B) followed by 20 CV IMAC Buffer C (see Table III-4B). The target protein was eluted with 3 CV IMAC Buffer D (see Table III-4B). The sample was concentrated without further analysis, dialysed in IEX Buffer A (see Table III-4B), filtered to remove debris and further purified.

III.2.5.2.2 Ion exchange chromatography (IEX)

Anion exchange chromatography was carried out using a HiTrap™ Q HP (5 mL, pre-packed Q Sepharose™ High Performance, quaternary ammonium strong anion) according to the protocol of the manufacturer on an ÄKTA_{FPLC} system (flow rate, 3.0 mL min⁻¹; pressure limit, 0.8 MPa). Using a 50 mL superloop, the lysate was loaded on the column. It was previously equilibrated with IEX Buffer A (see Table III-4B). After 10 CV column wash HsNMT was eluted by increasing the concentration of salt with IEX Buffer B (see Table III-4B) to 500 mM. The gradient employed was 0–100% in 40 CV and 100% for 2 CV. The fractions were analysed by SPA and SDS-PAGE, whereby active fractions were pooled, concentrated, dialysed into SPA Buffer (see Table III-4A), filtered to remove debris, aliquoted and stored at -20 °C.

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Table III-4: Buffered solutions

A list of all the buffered solutions employed in the expression and purification of (A) PfNMT, (B) HsNMT1 and HsNMT2, (C) other buffered systems is displayed.

	Name of Buffers	Components
(A)	Lysis Buffer	20 mM Na ₃ PO ₄ pH 7.5, 500 mM NaCl, 20 mM imidazole, 5 mM DTT
	IMAC Buffer A	20 mM Na ₃ PO ₄ pH 7.5, 500 mM NaCl, 20 mM imidazole, 5 mM DTT
	IMAC Buffer B	20 mM Na ₃ PO ₄ pH 7.5, 500 mM NaCl, 1 M imidazole, 5 mM DTT
	SEC Buffer	100 mM Na ₃ PO ₄ pH 7.0, 50 mM NaCl, 5 mM DTT
	IEX Buffer A	50 mM Na ₃ PO ₄ pH 6.5, 25 mM NaCl
	IEX Buffer B	50 mM Na ₃ PO ₄ pH 6.5, 1 M NaCl
	SPA Buffer	30 mM Tris/HCl pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA
(B)	Lysis Buffer	50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.02 mM EDTA, 0.02 mM EGTA, 1% Triton X-100, 0.06% β-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF
	IMAC Buffer A	50 mM Tris/HCl pH 7.5, 500 mM NaCl, 0.06% β-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF
	IMAC Buffer B	50 mM Tris/HCl pH 7.5, 500 mM NaCl, 0.02 mM EDTA, 0.02 mM EGTA, 20 mM imidazole, 0.06% β-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF
	IMAC Buffer C	50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.02 mM EDTA, 0.02 mM EGTA, 0.06% β-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF
	IMAC Buffer D	50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.02 mM EDTA, 0.02 mM EGTA, 300 mM imidazole, 0.06% β-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF
	IEX Buffer A	50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF
	IEX Buffer B	50 mM Tris/HCl pH 7.5, 500 mM NaCl, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF
(C)	Towbin Buffer	12.5% 25 × Tris-Glycine Transfer Buffer (Novex®, Invitrogen), 10% MeOH
	TBS-T Buffer	100 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20

III.3 Results

III.3.1 *Plasmodium falciparum* NMT

III.3.1.1 Optimised Overexpression of Recombinant *Plasmodium falciparum* NMT

Previously-developed expression and purification procedures for *Plasmodium falciparum* NMT required optimisation due to unsatisfactory yields of 0.4 mg L⁻¹ (Bowyer 2006; Bowyer, Gunaratne et al. 2007). Invariable factors such as constructs, vectors, cell lines and growth media were already well researched by former lab member Dr P W Bowyer (Bowyer 2006). To improve expression in *Escherichia coli* by reducing the number of steps required for purification and maximising the yield of soluble, active protein, an optimum for IPTG, temperature and expression times were investigated.

The employed vector system was pTrcHisA, which contained a T7lac promoter with neighbouring cDNA encoding for His₆-tagged PfNMT. In the presence of lactose analogue IPTG, the *lac* repressor no longer prevents RNA polymerase from binding; a process of transcription of the gene to mRNA followed by translation to the encoded protein is induced. Three different concentrations of IPTG (1.0 mM, 0.1 mM and 0.01 mM) were employed to explore optimum expression conditions (Table III-5).

The second factor investigated was temperature. Whilst 37 °C might be optimal for *E. coli* cell growth, it may result in the production of recombinant protein as aggregates called inclusion bodies. To increase the proportion of soluble protein, temperature is often lowered to e.g. 30 °C (Schein and Noteborn 1989). Further improvements may be achieved by means of prolonged induction at even lower temperatures (15 °C – 20 °C) to increase yields of soluble protein and obtain correct folding. Protein expression at three different temperatures (19 °C, 31 °C, 37 °C) was examined (Table III-5).

Two time points (5 h and 19 h) were chosen to test the influence of different levels of IPTG and incubation temperature to determine the optimum expression conditions for PfNMT in *E. coli* cells (Table III-5).

Table III-5: Optimisation procedure for the overexpression of PfNMT

Combinations of various IPTG concentrations, incubation temperatures and expression times were examined to find improved reaction conditions that yield maximised soluble protein.

	Variation Sets	Original Expression Conditions (Bowyer, Gunaratne et al. 2007)
Concentration of IPTG [μ M]	1 0000 100 10	100
Incubation Temperature [$^{\circ}$ C]	37 31 19	31
Expression Time [h]	5 19	5

The PfNMT construct (donated by Dr A A Holder, National Institute of Medical Research, UK) was transformed into BL21(*DE3*) expression cell lines using standard procedures. After the culture has reached $OD_{600\text{ nm}} = 0.6$, it was induced with three levels of IPTG (1.0 mM, 0.1 mM and 0.01 mM) and incubated at three temperatures (19 $^{\circ}$ C, 31 $^{\circ}$ C, 37 $^{\circ}$ C). The samples were then either incubated for 5 h or 19 h expression time. Standard Lysis Buffer was used to partition the protein into soluble and insoluble fractions. It also contained a non-ionic detergent to solubilise cell walls and membrane components to release cellular proteins. Additionally, lysozyme and DNAase were necessary to maximise the amount of target protein in the soluble fractions as well as sonication procedures. It was furthermore found that DTT needs to be present at all times to prevent precipitation of the target protein.

The experiment was carried out in duplicate. The soluble fractions were loaded on SDS-gels and stained with Coomassie Blue for comparative studies. Figure III-1A illustrates soluble fractions after 5 h incubation time, whereas isolated proteins from 19 h incubation period are shown in Figure III-1B. The direct comparison to enriched PfNMT confirms the expression of PfNMT (Figure III-1C).

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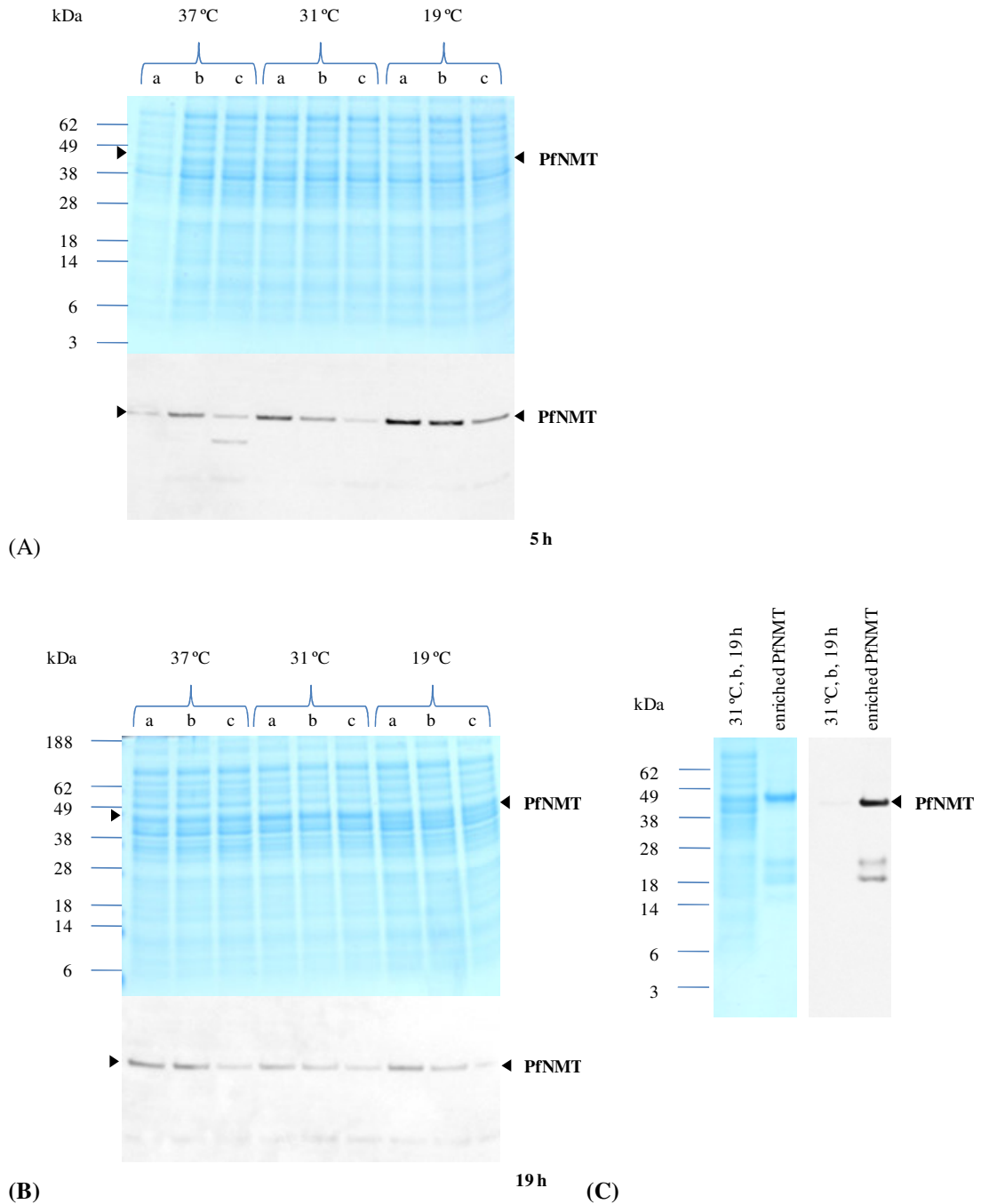


Figure III-1: Optimisation of expression conditions for PfNMT

10 µg samples of protein were loaded per lane, separated by SDS-PAGE and visualised by Coomassie Blue stain (top) or Anti-His Western Blot (bottom). The protein expression after 5 h incubation time (A) was compared to 19 h expression period (B). Other variables included temperature (19 °C, 31 °C, 37 °C) and IPTG concentration ((a) 1.0 mM IPTG, (b) 0.1 mM IPTG, (c) 0.01 mM IPTG). A direct comparison of the soluble fractions with enriched PfNMT as a control, confirms expression of PfNMT (C).

The assessment of the level of overexpressed recombinant PfNMT was carried out with ImageJ (Figure III-2). The intensity of PfNMT bands in the Coomassie-stained SDS-PAGE gel were first normalised to the total amount of protein in the lane, followed by the intensity of the stain to enable direct comparison between all reaction conditions. Values were normalised to the maximum average.

The results are presented in Figure III-3; a bar graph with the relative amount of PfNMT and error bars representing standard deviation. Nine expression conditions were compared for an incubation period of 5 h and 19 h.

It was found that increased incubation time resulted in a higher production of PfNMT over time. No significant effects were found for different levels of IPTG in the experiment. A slight increase of PfNMT production was noted when IPTG was reduced from 1.0 mM to 0.1 mM for each temperature at 5 h incubation time (Table III-6A). However this trend was not followed with further decrease in IPTG to 0.01 mM. Conversely, it seemed that a low amount of IPTG (0.01 mM) was favoured with longer incubation times (Table III-6B).

The expression of PfNMT was also largely unaffected by the three chosen incubation temperatures (Table III-6). However, degradation was noticed at 37 °C. Protein production at 31 and 19 °C was satisfactory.

Optimisation trials of PfNMT expression suggested higher production of target enzyme at long expression times (19 h) and ambient temperatures (19–31 °C).

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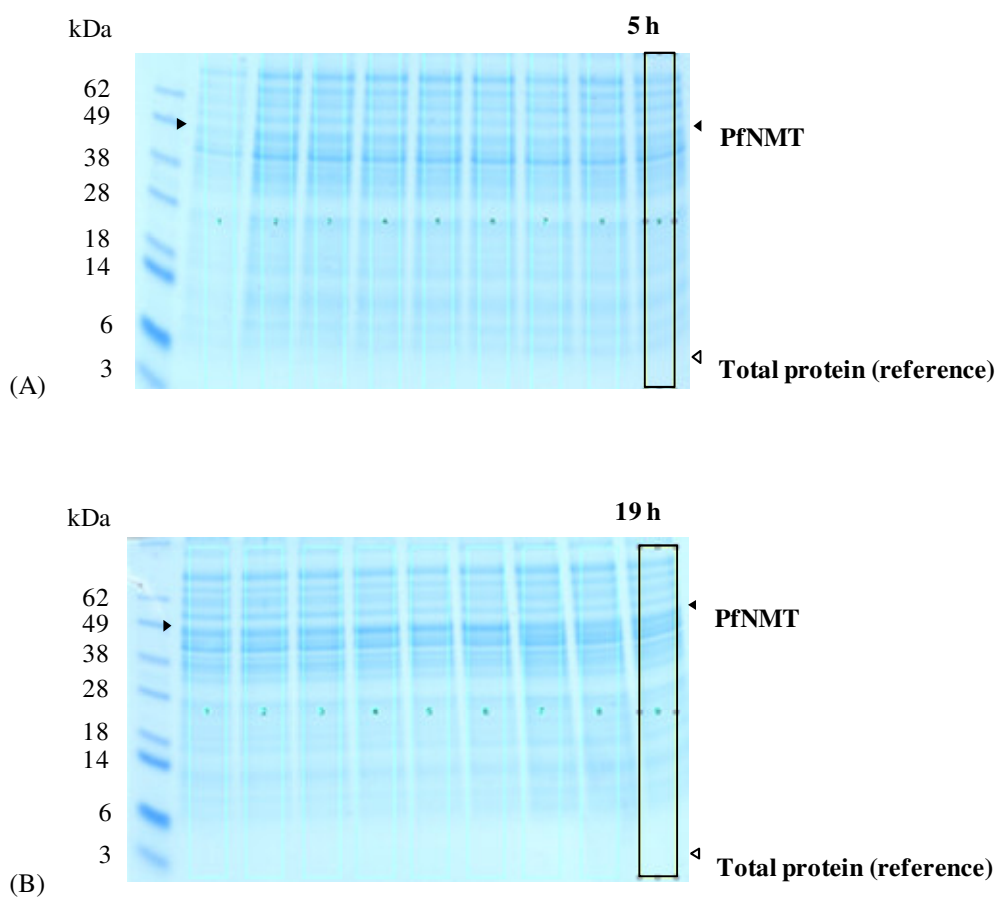


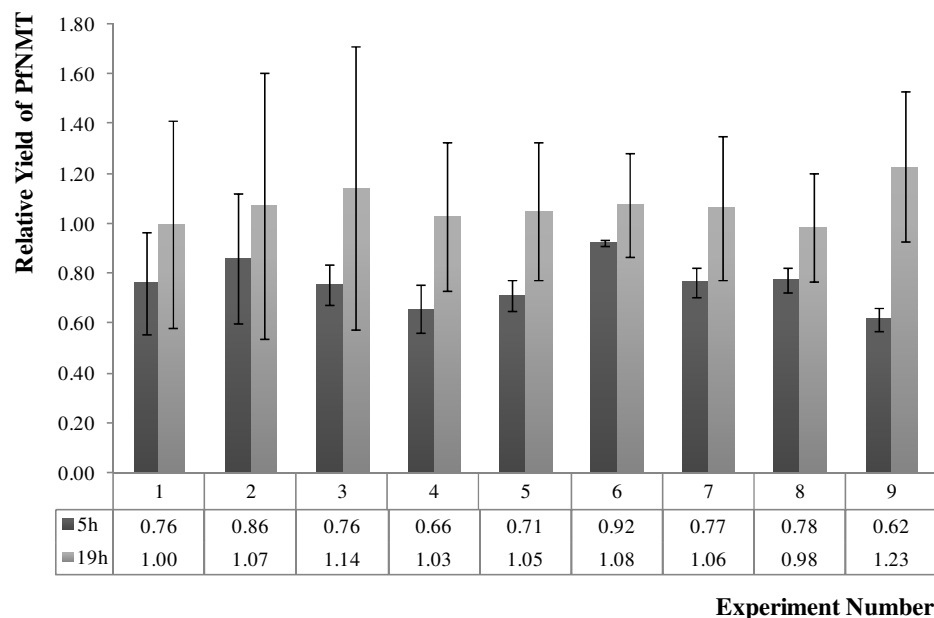
Figure III-2: Analysis of PfNMT with ImageJ

Intensity of protein bands was analysed with ImageJ Software (1.40g). The intensity of the PfNMT band (black triangle) was normalised to the loading of total protein (black rectangle) as well as the colour intensity of the gel to enable direct comparison between all conditions (A, B, Lane 2-10).

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Figure III-3: Evaluation of expression levels in various conditions

The intensity of normalised PfNMT bands was evaluated according to concentration of IPTG, incubation temperature and time. The bar graph compares nine different conditions with respect to incubation time of 5 h and 19 h. The values of each column are listed in the data table.



Legend:

1 – 1.0 mM IPTG, 37 °C	4 – 1.0 mM IPTG, 31 °C	7 – 1.0 mM IPTG, 19 °C
2 – 0.1 mM IPTG, 37 °C	5 – 0.1 mM IPTG, 31 °C	8 – 0.1 mM IPTG, 19 °C
3 – 0.01 mM IPTG, 37 °C	6 – 0.01 mM IPTG, 31 °C	9 – 0.01 mM IPTG, 19 °C

Table III-6: Comparison of average expressed PfNMT

This table lists the average amounts of expressed PfNMT from *E. coli* with regards to the incubation time 5 h (A) and 19 h (B). Data was taken from Figure III-3.

(A) 5 h		1.0 mM IPTG	0.1 mM IPTG	0.01 mM IPTG
	37 °C	0.76	0.86	0.76
	31 °C	0.66	0.71	0.92
	19 °C	0.77	0.78	0.62
(B) 19 h		1.0 mM IPTG	0.1 mM IPTG	0.01 mM IPTG
	37 °C	1.00	1.07	1.14
	31 °C	1.03	1.05	1.08
	19 °C	1.06	0.98	1.23

III.3.1.2 The Optimisation of Purification Methods for PfNMT

For isolation of the target protein from a mixture of proteins, a single chromatographic step is rarely sufficient. Separate, sequential steps of different modes of separation are necessary. A purification protocol for PfNMT has previously been described; however, it involves a time-consuming procedure yielding low amounts of active protein, 0.4 mg L^{-1} (Bowyer, Gunaratne et al. 2007). It is a three-step procedure that includes immobilised metal affinity chromatography (IMAC, Figure III-4A), followed by size exclusion chromatography (SEC, Figure III-4B) and concludes with cation exchange chromatography (IEC, Figure III-4C).

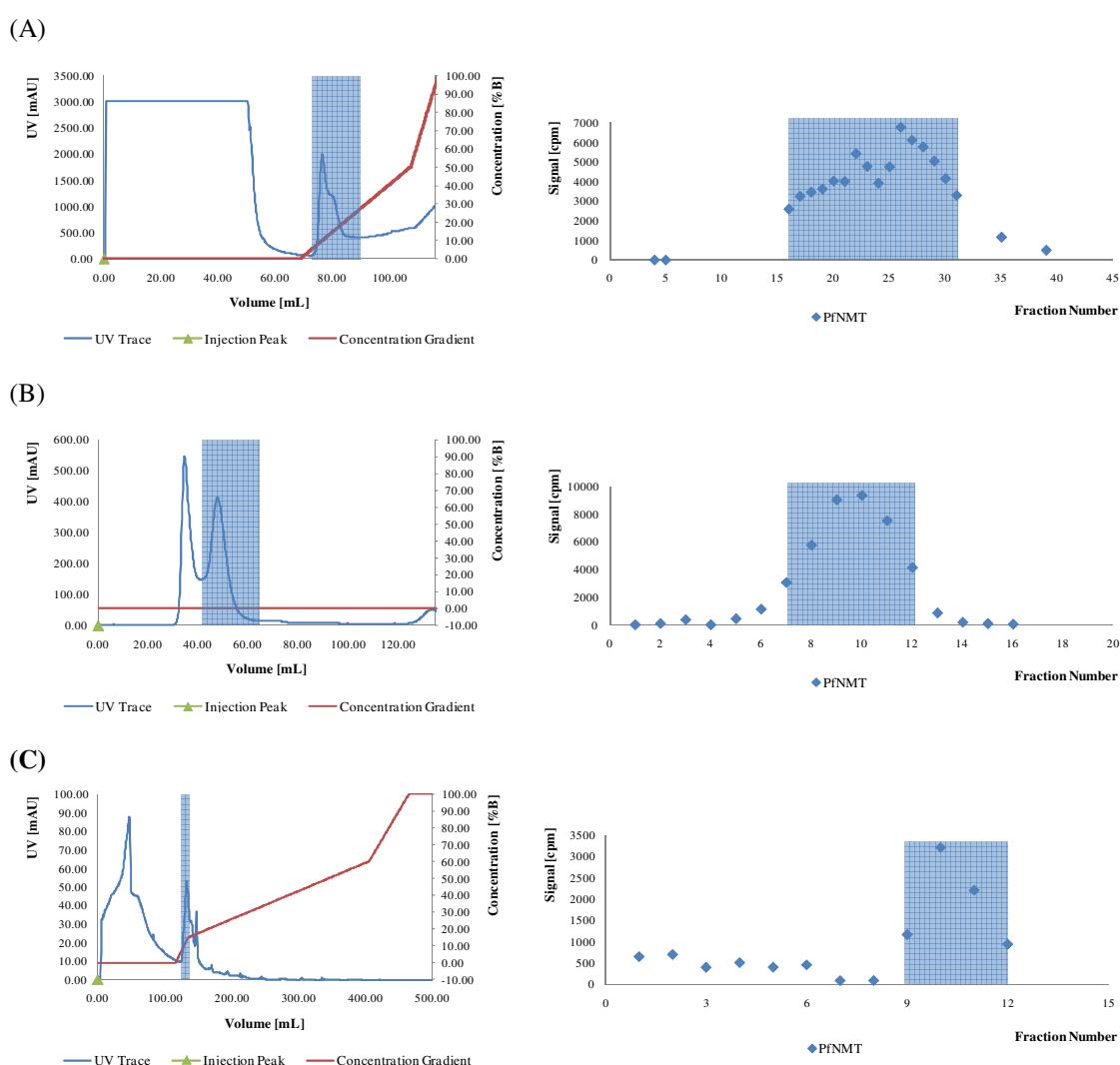


Figure III-4: Purification of PfNMT as optimised by Dr P W Bowyer

The previously established three-step purification procedure for PfNMT is illustrated (Bowyer, Gunaratne et al. 2007). The target protein was enriched using a HisTrap column (A), a size exclusion column (B) and lastly a cation exchange column (C). Enzyme activity was tested in a scintillation

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proximity assay (SPA) and is shown next to the chromatogram. Active fractions are highlighted in blue. The fraction number correlates to solvent volume, i.e. 0 mL – fraction number 0.

Optimisations were carried out to minimise the number of essential purification steps in an attempt to obtain larger amounts of active PfNMT. In particular, it was felt that size exclusion chromatography reduced overall yields greatly and so should be avoided if possible. The column has a small volume capacity; hence, the volume of the impure sample fractions needs to be concentrated significantly prior to loading. This may have caused aggregation of soluble protein (Bowyer 2006). Thus in a revised procedure the first stage remained the same as previous but a reverse order of Step 2 and Step 3 was investigated as well as different affinity columns to exploit chemical properties of the target and unwanted proteins (Figure III-5). Activity was analysed with the scintillation proximity assay (SPA) and SDS-PAGE.

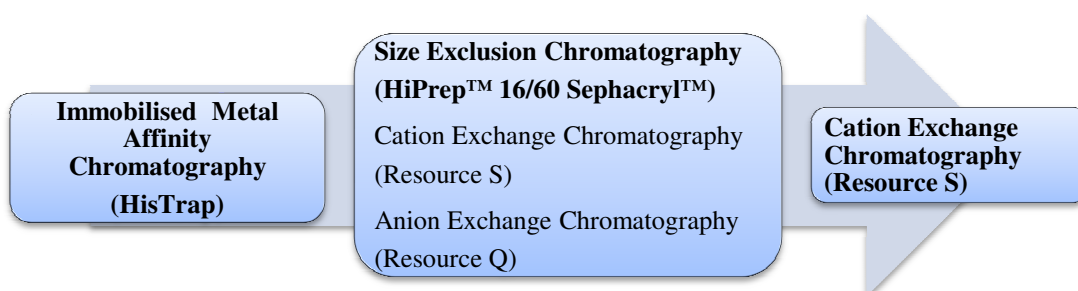


Figure III-5: Optimisation of the purification procedure for PfNMT

A scheme of different chromatographic columns for the second purification step in an effort to eliminate a third step and thus maximise yield is pictured. The original conditions are highlighted in bold (Bowyer, Gunaratne et al. 2007).

For the first stage of purification, the soluble protein fraction (lysate) was loaded onto a HisTrap column whereby immobilised Ni^{2+} -ions trapped His₆-tagged proteins (Figure III-6A). Bound material was eluted with imidazole-based buffer. Active fractions, tested in the SPA (Chapter V, p. 121), were combined and subjected to further chromatographic steps. The flowthrough was subjected to the column a second time (Figure III-6B). Results indicate the binding capacity of 1 mL HisTrap column was sufficient for His₆-labelled PfNMT from a 2 L culture (Figure III-6C).

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The batch purification was followed by ion exchange chromatography, where the pI of PfNMT is an important factor in deciding which column type is appropriate (Table III-1). The lysate was either purified on a cation exchange column at pH 6.5 (Figure III-7) or on an anion exchange column at pH 7.5 (Figure III-8). As expected, most of the target protein, with a calculated pI of 8.40, bound to the cation exchange column (Figure III-7B). PfNMT was eluted with increasing salt gradient. The anion exchange chromatography resulted in PfNMT in the flowthrough as well as bound to the column (Figure III-8C). Unbound target protein was pure and did not have to be subjected to a third purification step, unlike bound PfNMT. The final chromatographic procedure carried out was size exclusion (Figure III-8B).

As listed in Table III-7, initial batch purification with a HisTrap column removed roughly 99% of total protein. IMAC purified sample was subjected to either cation or anion exchange chromatography, of which cation IEX resulted in the highest amount of enriched PfNMT with 1.0 mg L⁻¹ total yield. Purification with the latter system yielded less protein, 0.7 mg L⁻¹ total yield, due to a necessary third purification step.

Table III-7: Analysis of yields of PfNMT after each purification step

The total amount of protein for each step is listed in the table. It was calculated after each purification step according to the absorption at 280 nm. The value for the crude was estimated.

Total Protein After Expression	Total Protein After IMAC	Total PfNMT After Cation IEX	Total PfNMT After Anion IEX(and SEC)
612 mg	5.1 mg	0.95 mg	0.95 mg
(for 2 L culture)	(for 2 L culture)	(yield: 1.0 mg L ⁻¹)	(yield: 0.7 mg L ⁻¹)
Crude	~72% pure*	>95% pure*	>95% pure*

* purity estimated from intensity of protein band in Coomassie-stained SDS-PAGE gels (with ImageJ)

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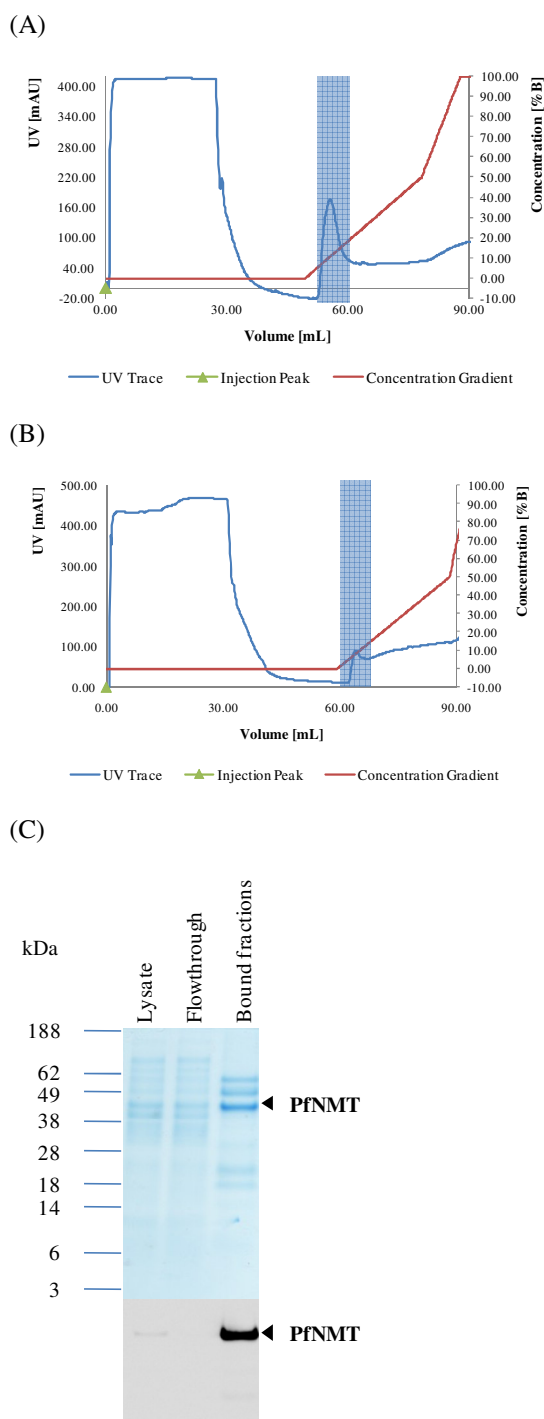
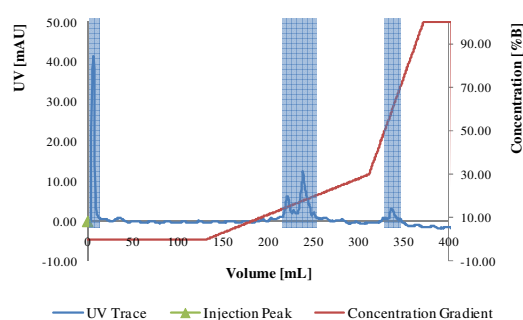


Figure III-6: Step-by-step analysis of PfNMT purification - Step 1

This figure show details of the IMAC purification of crude lysate. Batch purification of the lysate was carried out with a HisTrap column. Chromatogram (A) shows the purification of PfNMT and (B) additional purification attempts of the flowthrough. Activity of the enzyme was tested with the SPA (Chapter V, p. 121). Active enzyme fractions are highlighted in blue. The SDS-PAGE gel (C) was used to analyse the protein composition after each step. 5 μ g samples of protein were loaded per lane and the gel was developed with Coomassie Blue stain (top) or anti-His Western Blot (bottom).

III NMT EXPRESSION, ISOLATION & ANALYSIS

(A)



(B)

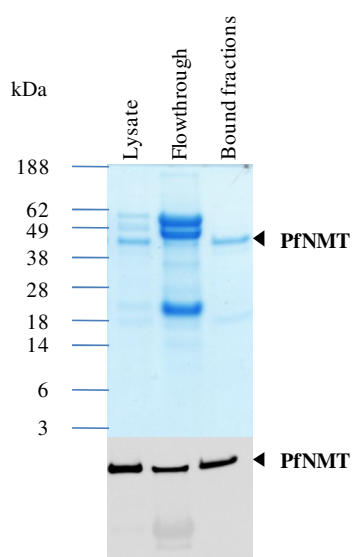
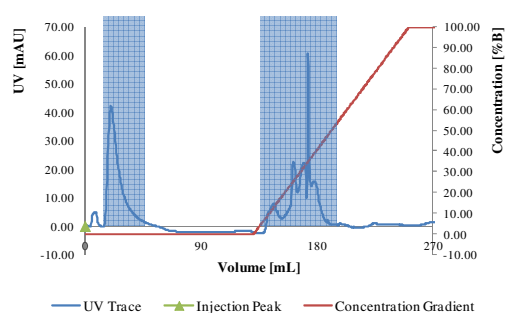


Figure III-7: Step-by-step analysis of PfNMT purification - Step 2a (cation exchange)

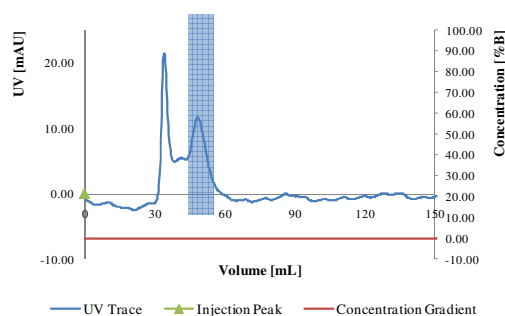
This figure shows ion-exchange chromatography of the IMAC-purified material. With pH of 6.5, it was loaded on to a cation exchange column (A). The activity of the enzyme was tested with the SPA (Chapter V, p. 121). Active enzyme fractions are highlighted in blue. The SDS-PAGE gel (B) was used to analyse the protein composition after each step. 5 µg samples of protein were loaded per lane and the gel was developed with Coomassie Blue stain (top) or anti-His Western Blot (bottom).

III NMT EXPRESSION, ISOLATION & ANALYSIS

(A)



(B)



(C)

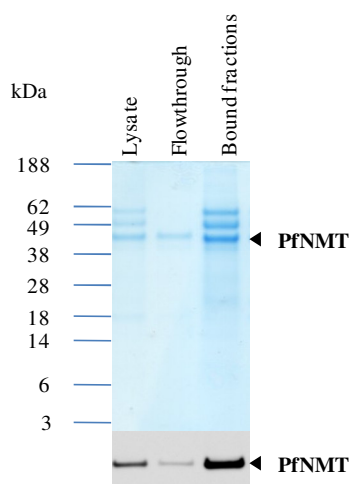


Figure III-8: Step-by-step analysis of PfNMT purification - Step 2b (anion exchange)

This figure shows ion-exchange chromatography of the IMAC-purified material. With pH of 7.5, it was loaded on an anion exchange column (A). PfNMT that bound to the column was subsequently enriched on a size exclusion column (B). The activity of the enzyme was tested with the SPA (Chapter V, p. 121). Active enzyme fractions are highlighted in blue. The SDS-PAGE gel (C) was used to analyse the protein composition after each step. 5 μ g samples of protein were loaded per lane and the gel was developed with Coomassie Blue stain (top) or anti-His Western Blot (bottom).

III.3.2 *Homo sapiens* NMT

Isoforms of two distinct families of *Homo sapiens* NMT, HsNMT1 and HsNMT2, were expressed and enriched for *in vitro* assays to determine specificity of the inhibitors that are ideally selective for PfNMT over the human analogue.

Initially, His₆-tagged HsNMT1 and HsNMT2 plasmids in a pET-15b vector system, (kind donations from Dr S McElroy (Drug Discovery Unit, University of Dundee, UK)) were amplified and transformed into DH5 α TM expression cell lines before insertion into *E. coli* BL21(DE3) cells.

Protein purification was carried out according to methods established by Dr S McElroy which involved initial batch purifications with an immobilised Ni²⁺ column followed by an anion exchange column. Fractions of the final purification step were analysed by SPA to determine NMT activity (Chapter V, p. 121), from which active fractions were pooled to use in the inhibition assays. Figure III-9 demonstrates the IEX step for HsNMT2 (A) and SPA analysis (B). Figure III-10 shows Coomassie-stained SDS-PAGE gels of all purification stages for the enrichment of HsNMT1 and HsNMT2.

The yield of HsNMT1, which has an approximate size of 56 kDa, was 1.5 mg L⁻¹ using a 3 L *E. coli* culture (Figure III-10A). Under equivalent conditions, HsNMT2 yielded 0.6 mg L⁻¹ from *E. coli* (Figure III-10B). The approximate size was also 56 kDa. The His₆ tag was still present for both human NMTs as can be seen in the anti-His Western blot (Figure III-10).

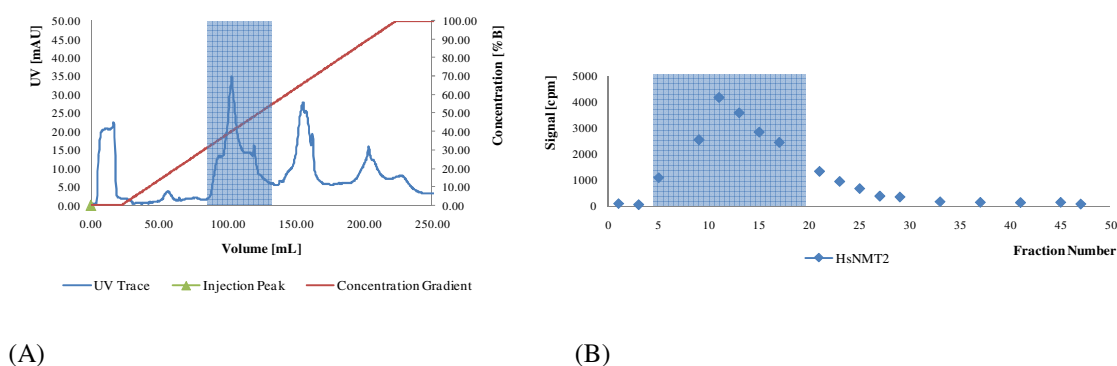


Figure III-9: Human NMT protein purification

The chromatogram displays the 2nd purification step (A), the anion exchange chromatography of HsNMT2. The blue highlighted area represents active enzyme which was determined in the SPA (B).

III NMT EXPRESSION, ISOLATION & ANALYSIS

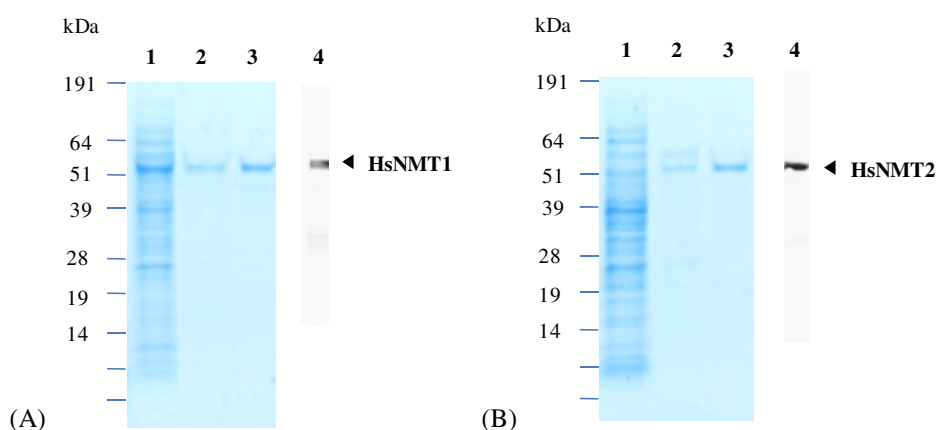


Figure III-10: SDS-PAGE analysis of purification stages of HsNMT1 and HsNMT2

The SDS-PAGE gels show the purification procedure of HsNMT1 (A) and HsNMT2 (B) expressed from *E. coli* in two steps. Legend: (1) Lysate prior to any purification, (2) eluent after IMAC, (3) enriched enzyme after IEX, (4) Western Blot with Anti-His stain. Lane 1-3 were visualised with Coomassie stain. 5 μ g samples of protein were loaded per lane.

III.3.3 *Candida albicans* NMT

Dr P W Bowyer kindly donated purified *Candida albicans* N-myristoyl transferase (Figure III-11). The construct used to overexpress CaNMT in *E. coli* was pET11c. Colonies, grown on amp-agar, were transformed into BL21(DE3) expression cell lines using standard procedures. Conditions for overexpression were 5 h incubation period at 37 °C, followed by harvest, cell lysis and purification over an anion exchange column (Bowyer 2006).

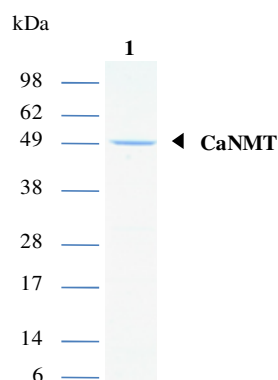


Figure III-11: SDS-PAGE analysis of enriched CaNMT

The Coomassie-stained SDS-PAGE gel verifies enriched CaNMT, 5 μ g sample of protein was loaded.

III.3.4 *Leishmania major* NMT

Purified *N*-myristoyl transferase of *Leishmania major* (Figure III-12) was kindly donated by Prof D F Smith (Department of Biology, University of York, UK). Colonies of LmNMT plasmid pET15b in BL21(*DE3*) were grown on kanamycin-agar, overexpressed in LB medium and subsequently purified (Rein 2008).

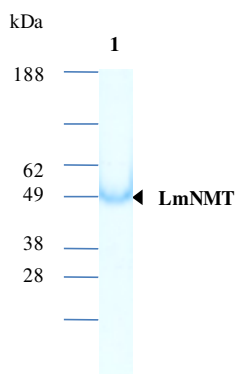


Figure III-12: SDS-Page analysis of enriched LmNMT

The Coomassie-stained SDS-PAGE gel confirmed enriched LmNMT, 5 µg protein was loaded.

III.4 Conclusion and Discussion

The main objective of this chapter was to express and purify *Plasmodium falciparum* *N*-myristoyl transferase for the analysis of potency of synthesised potential antimalarials. A published protocol was followed but was found to yield low amounts of protein (Bowyer, Gunaratne et al. 2007). Small-scale experiments were set up to increase the level of recombinant protein and to reduce the number of purification steps involved.

Expression of soluble protein is dependent on various factors; incubation period, temperature and concentration of expression inducer IPTG were varied. Small-scale experiments showed that overexpression of PfNMT was greatly amplified when the expression time was increased from original 5 h to 19 h incubation period. A temperature of 31 °C and 0.1 mM of IPTG were found to work well in combination with 19 h incubation period to produce large amounts of PfNMT.

III NMT EXPRESSION, ISOLATION & ANALYSIS

Not only could the level of expression of recombinant protein be increased, importantly the three-step purification procedure was reduced to a two-step enrichment by elimination of gel filtration chromatography which accounted for a large loss in protein yield. The initial purification step was metal chelate affinity chromatography (1 mL, HisTrap column) because the protein was His₆-tagged. This largely removed proteases, non-protein material and untagged endogenous proteins from bacteria out of the mixture and also reduced the volume for subsequent steps. The second purification step was carried out with cation exchange chromatography (Resource S) with yields of enriched PfNMT, 1.0 mg L⁻¹. Purification trials with an anion instead of cation exchange column yielded similar amounts; however, a third step, gel filtration, was necessary.

Expression protocols from *E. coli* as well as purification procedures were significantly improved to enhance yields for PfNMT from 0.4 mg L⁻¹ to 1.0 mg L⁻¹.

Not only was the analysis of potency of inhibitors against PfNMT important, but also the activity against human NMT. HsNMT1 and HsNMT2 were expressed and enriched to test PfNMT inhibitors for potency and selectivity against the human enzyme. Yields of 1.5 mg L⁻¹ HsNMT1 and 0.6 mg L⁻¹ HsNMT2 were attained.

For additional *in vitro* testing of potential inhibitors against other organisms, stocks of *N*-myristoyl transferase of *Candida albicans* and *Leishmania major* were used from Dr P W Bowyer and Prof D F Smith respectively (Bowyer 2006; Rein 2008).

IV SYNTHESIS OF ANTIMALARIAL AGENTS

IV.1 Introduction

A small collection of the best inhibitors against CaNMT, the “Pfizer series”, was supplied to our research group and tested for activity against *Plasmodium falciparum* NMT (Bowyer 2006). The most active compounds showed potency in the low micromolar region; UK-370,509 was selected as the lead inhibitor with 30 μM activity *in vitro* against PfNMT. The structure of UK-370,509 can be segmented into three main parts (Figure IV-1). The benzothiazole scaffold (Section C) was to remain constant throughout the studies in this thesis. Variations in structure could be achieved from the C⁶-position of the benzothiazole unit and from reactions with the N-terminus of the aminobenzothiazole. Building block B, an amino acid analogue, was attached *via* the formation of an amide bond. Unit A was connected to B *via* the formation of a 2° amine bond.

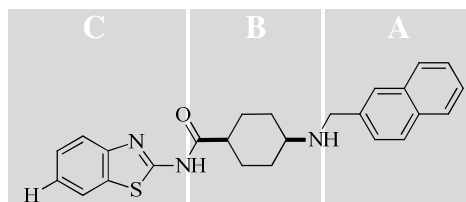


Figure IV-1: Segmentation of the lead compound into three main parts (A, B and C)

IV SYNTHESIS OF ANTIMALARIAL AGENTS

As seen below, there is some information that the C⁶-position may be crucial for the selectivity of *N*-myristoyl transferase across organisms. Small-sized substituents such as a fluorine-atom or hydrogen in the C⁶-position showed greater activity against PfNMT than larger substituents such as pyridine or dimethylamide (Figure IV-2). Activity against human NMT was 10-fold lower for UK-370,509 and UK-370,713. However, a pyridine group in that position greatly increased activity against human NMT for UK-370,710; the compound inhibited human NMT four times stronger than PfNMT (Figure IV-2). The substituent presumably underwent interactions with surrounding amino acids in the peptidic binding pocket of human NMT, such as hydrogen bonding, favouring tighter binding of the inhibitor. Due to time constraints, Section A and B were the main focus of this library.

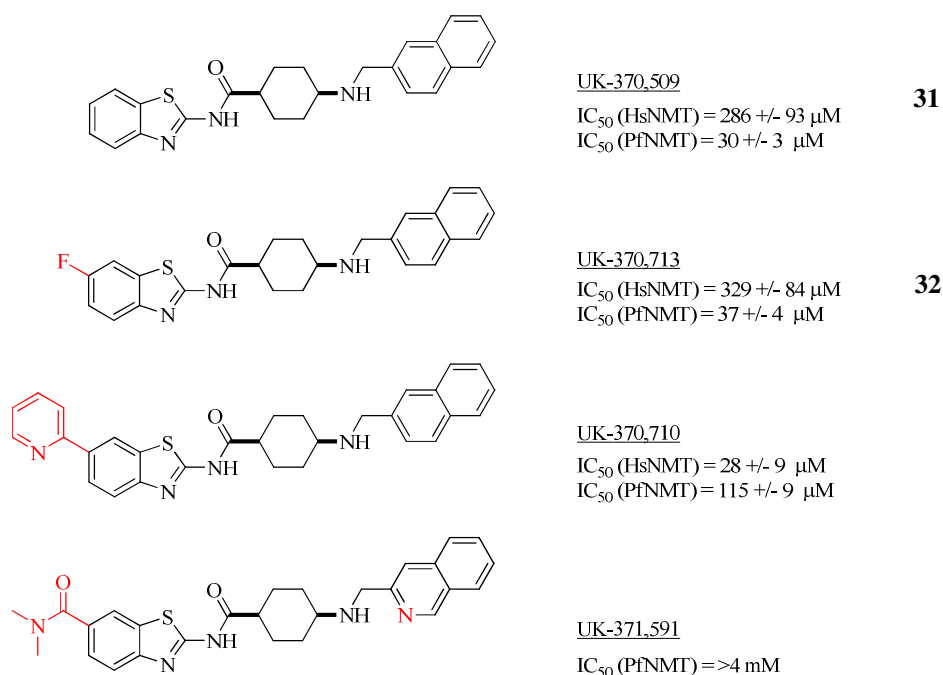


Figure IV-2: Comparison of selectivity of inhibitors with different building blocks D

The compounds were tested for potency against PfNMT and for selectivity over human NMT in an *in vitro* assay (Bowyer 2006). The lead compound was compared to analogues with differing units D, colour-coded in red.

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Further investigations examined the orientation of substituents of building block B. Structures investigated by Pfizer consisted of two types: disubstituted cyclohexane and the open-chain equivalent. There is some evidence that indicated rigidity of the ring structure is essential for potency (Pfizer, unpublished data). Variations to the cyclohexane were achieved by the positioning of the amine and carboxylic acid functionalities; either in 1,3- or 1,4-position and *trans* or *cis* orientated (Figure IV-3). There are gaps in the “Pfizer series”, but Figure IV-3 proposes the significance of the orientation of substituents with regards to activity against PfNMT. The additional CH₂-group, to distance the peptide bond from the amine bond, did not seem to increase activity for UK-362,091 compared to UK-370,753. In fact, the activity was reduced 10-fold. Perhaps the additional CH₂-group has to change the orientation of the 2° amine moiety in the peptidic binding pocket.

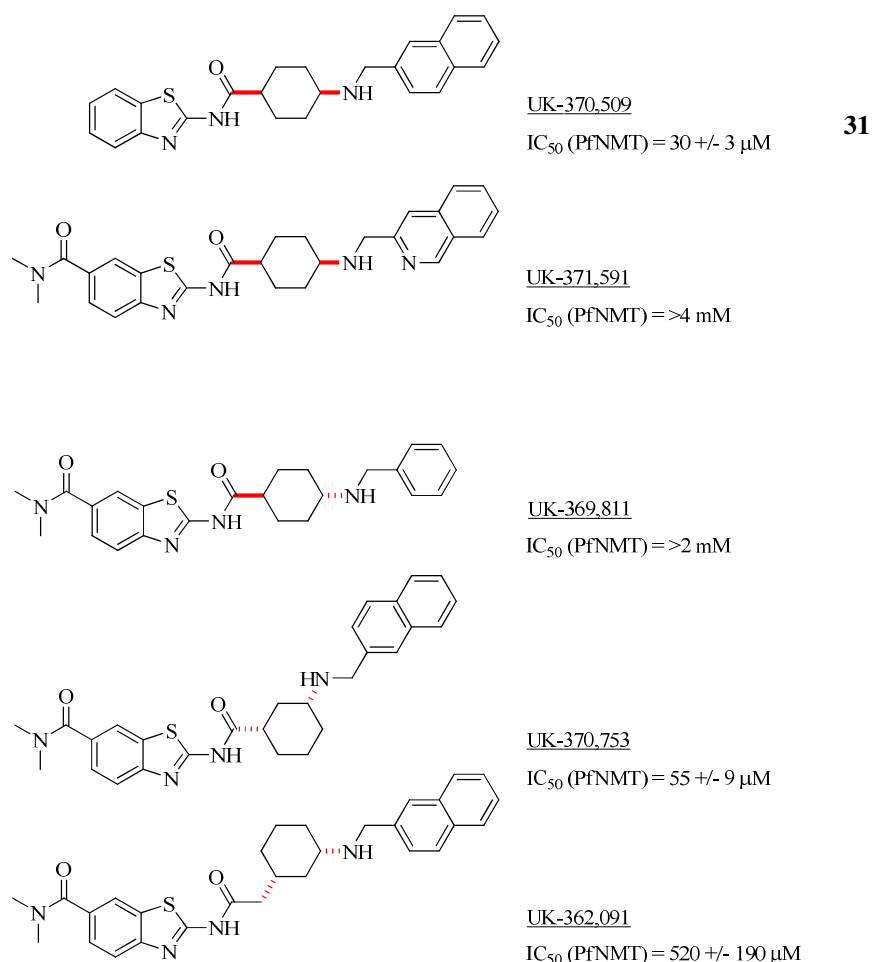


Figure IV-3: Comparison of IC₅₀ data against PfNMT with varying building block B

IC₅₀ data of selected compounds from the “Pfizer series” with different orientations of substituents, colour-coded in red (Bowyer 2006).

IV SYNTHESIS OF ANTIMALARIAL AGENTS

There were a number of different building blocks A in the “Pfizer series” with characteristics such as aromaticity, the inclusion of heteroatoms and halogens (Figure IV-4). Results demonstrated an increase of activity against PfNMT with additional heteroatoms and halogens in the aromatic ring system (UK-372,699 vs. UK-370,753) but their orientation was essential for significant activity (UK-370,627 vs. UK-370,624).

This chapter describes the synthesis of potential antimalarial compounds with variations in building blocks A and B, to discover high potency against *Plasmodium falciparum* NMT and in addition good selectivity over human NMT.

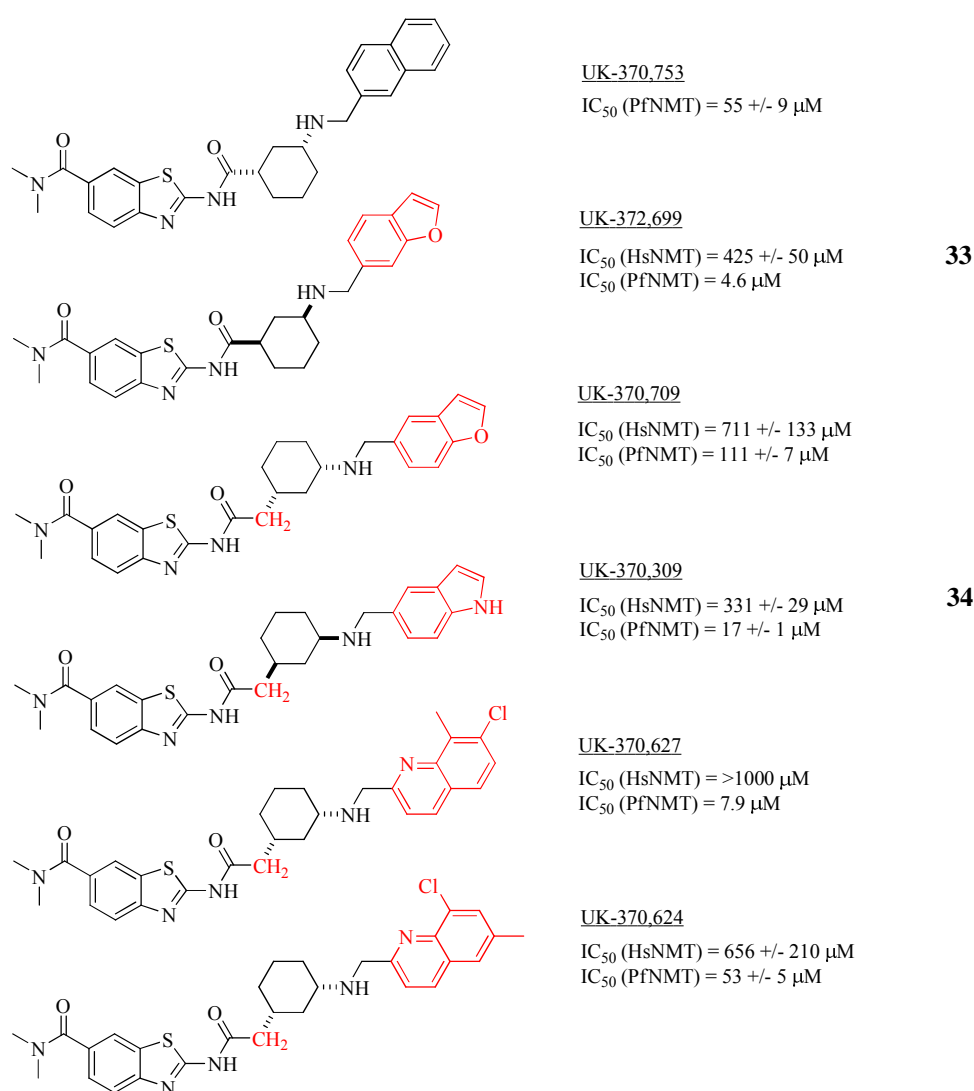


Figure IV-4: Comparison of IC_{50} data against PfNMT with varying building block A

IC_{50} data of selected compounds from the “Pfizer series” with differing units A were analysed and compared to UK-370,753 which is nearly as potent as lead compound UK-370,509 (Bowyer 2006). Changes from UK-370,753 are colour-coded in red.

IV.2 Material and Methods

IV.2.1 Chemicals and Reagents

Solvents and reagents were obtained from commercial sources (Sigma-Aldrich, NovaBiochem, Neomps, J. T. Bruker, Merck, BDH). Ultrapure laboratory grade water was obtained from in-house Elix[®] and MilliQ[®] millipore water purification systems.

IV.2.2 Methods and Equipment

Unless otherwise stated, all reactions were carried out in anhydrous solvents and in an inert atmosphere of nitrogen with standard Schlenk techniques. Elevated temperatures were maintained in thermostat-controlled oil baths. Organic solutions were concentrated *in vacuo* using a rotary evaporator (Heidolph Rotavapor R110) with a diaphragm vacuum pump (Büchi Vac[®] V-500). Lyophilisation was performed on a Christ Alpha1-4 freeze drier.

pH was measured with a Hanna Instrument 8519 digital pH meter, which was calibrated with BDH colour key buffer solutions at pH 4 and pH 7. Centrifugation was performed with a Jouan BR4 i centrifuge at 5 000 rpm for 5 min.

Reactions were followed by means of TLC where the reaction mixture was spotted on aluminium-coated TLC-plates (Merck, Si₆₀, F₂₅₄) and visualised under UV-light (BDH UV spectrometer), using acidic *p*-anisaldehyde followed by heating as required.

Flash column chromatography was carried out on BDH silica gel (Si₆₀, 33–70 µm). An alternative method of compound purification and analysis was RP-HPLC. Compound purification was performed on a preparative HPLC from Gilson (column specifications: ACE, 5 µM, C18, 250 × 21.2 mm, ACT) and compound analysis with an analytical HPLC from Gilson (column specifications: ACE, 5 µM, C18, 250 × 4.6 mm, ACT). UV detection was set at 220 nm (Gilson 155 UV/Vis detector, Anachem). The solvent system employed a gradient of ACN (+ 0.1% TFA) in H₂O (+ 0.1% TFA). The purity of compounds was determined by NMR, IR, accurate mass spectroscopy, TLC and HPLC.

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For structural characterisation of synthesised compounds, one-dimensional ^1H - and ^{13}C -NMR spectra, as well as two-dimensional NMR spectra were recorded on Bruker AV spectrometer at 300, 400, 500 MHz or 75, 100, 125 MHz respectively. NMR spectroscopy at 500 and 125 MHz were carried out by P R Haycock and D Sheppard at the departmental NMR Service (Imperial College London). The internal references were either CDCl_3 ($\delta_{\text{H}} = 7.26$ ppm, $\delta_{\text{C}} = 77.23$ ppm), DMSO-d_6 ($\delta_{\text{H}} = 2.50$ ppm, $\delta_{\text{C}} = 39.51$ ppm), MeOH-d_4 ($\delta_{\text{H}} = 3.31$ ppm, $\delta_{\text{C}} = 49.10$ ppm) or DMF-d_7 ($\delta_{\text{H}} = 8.03$ ppm). The average temperature upon recording was 25 °C (300 K). DEPT135, COSY and HMQC-NMR spectroscopy were used as a guide to assign signals in ^1H and ^{13}C -spectra. Chemical shifts, δ , are reported in units of ppm and coupling constants, J , in Hz.

Compounds were also analysed with a Fourier transform infra-red (FT-IR) spectrophotometer. Crystalline material was deposited on the diamond of the Universal ATR accessory of a PerkinElmer Spectrum 100 FT-IR spectrophotometer. The IR spectra were then analysed with Spectrum Express, Version 1.01.00 (PerkinElmer). Abbreviations in assignments are as follows: *s* – strong, *m* – medium, *w* – weak, *br* – broad, *sh* – sharp.

Mass analysis was executed on an AUTOSPEC P673 spectrometer by J Barton at the departmental Mass Spectrometry Service (Imperial College London). Unless otherwise stated, nominal and accurate mass analysis using electronic ionisation (EI) was obtained.

Three melting point apparatus have been employed for the determination of melting points of compounds: Griffin and Gallenkamp (Sanyo). OptiMelt (Automated Melting Point System) from Stanford Research Systems was also used with a temperature accuracy of ± 0.3 °C (up to 100 °C) and ± 0.5 °C (up to 250 °C).

IV.2.3 Common Synthetic Protocols

Below is a list of general procedures that were carried out in this project. Detailed synthesis for each compound as well as a complete characterisation is outlined in the Appendix (VIII.1, p. 193).

IV.2.3.1 General Method for the Boc-Protection of Primary Amines

To a stirred solution of amine (1.0 eq.) and NEt_3 (6.0 eq.) in MeOH, with a final amine concentration of 1 M, was added dropwise di-*tert*-butyl dicarbonate in methanol (2.1 eq.) after which the reaction mixture was heated at 60 °C. After 18 h at 60 °C the reaction mixture was filtered and all volatiles removed *in vacuo*. The residual yellow oil was dissolved in 2% NaHCO_3 (aq) (50 mL) and washed with hexane (3×50 mL). The aqueous phase was acidified with cold 1 M HCl (aq) to pH 2–3 upon which precipitation occurred. The aqueous phase was then extracted with EtOAc (3×50 mL), the organic phases combined, dried (MgSO_4), filtered and the solvent removed *in vacuo* to yield the boc-protected product (Ho, Michael Crider et al. 2001).

IV.2.3.2 General Method for Boc-Deprotection

The Boc-group was cleaved in TFA– H_2O –TIPS (90:5:5, V:V:V, 20 eq. TFA). After 1 h no further evolution of gas was observed. Cold Et_2O was added until precipitation occurred (approximately 1:10, V:V, reaction mixture: Et_2O). Precipitate was collected by means of centrifugation, washed with cold Et_2O (3×2 mL), and dried *in vacuo* to yield the desired product as a TFA salt (Yoshida, Hayakawa et al. 2005).

IV.2.3.3 General Method for Peptide Bond Formation

To a stirred solution of carboxylic acid (1.0 eq.) in DMF, with a final concentration of 0.5 M, were added amine (1.1 eq.), PyBOP (1.1 eq.) and DIPEA (2.0 eq.) at 0 °C. It was warmed to RT after 10 min on ice. After a couple of hours reaction time, the reaction mixture was diluted with DCM (90 mL), washed with 1 M HCl (aq) (3×100 mL), H_2O (3×100 mL), brine (1×100 mL), dried (Na_2SO_4), filtered and

all volatiles removed *in vacuo*. The crude was either purified by precipitation or purified over silica (Li 2001).

IV.2.3.4 General Method for Reductive Amination

To a stirred solution of the 1° amine TFA salt (1.0 eq.), anhydrous NEt_3 (2.0–2.2 eq.) and activated molecular sieves in anhydrous DMF, with a final amine concentration of 0.2 M, was added the aldehyde (1.0–2.0 eq.). After 1 h $\text{NaBH}(\text{OAc})_3$ (2.0–4.0 eq.) was added. After overnight reaction, the reaction mixture was quenched with 2% NaHCO_3 (aq) (10 mL) and washed with DCM (3×10 mL). The combined organic phases were extracted with H_2O (3×30 mL), brine (1×20 mL), dried (MgSO_4), filtered, and the solvent removed *in vacuo* to give the crude. Further purification was performed on HPLC to yield the desired product as a TFA salt (Abdel-Magid, Carson et al. 1996; Yamazaki, Kaneko et al. 2005).

IV.2.3.5 General Method for the Acetylation of 2° Amines

First, the free base was generated upon the addition of NEt_3 (1.0 eq.) to the 2° amine (1.0 eq.) under stirring. Acetylating reagent was added and stirred for 1 h. All volatiles were removed *in vacuo* to give the crude product. Further purification was performed on HPLC to yield the desired product.

IV.2.3.6 General Method for Alkylation of 2° Amines

To a stirred solution of 2° amine TFA salt (1.0 eq) and NEt_3 (2.2 eq.) in DCM, with a final amine concentration of 0.1 M, was added halide alkylation reagent (1.0 eq.). The reaction mixture was diluted with DCM (90 mL), washed with 1 M HCl (aq) (3×100 mL), H_2O (3×100 mL), brine (1×100 mL), dried (Na_2SO_4), filtered and all volatiles were removed *in vacuo*. The crude product was either purified over silica or with preparative HPLC.

IV.3 Results

IV.3.1 Synthesis of the Lead Compound

The first task was to re-synthesise UK-370,509 from the “Pfizer series”, the lead compound for the current studies (Compound **31**) (Figure II-15). Retrosynthetic analysis discriminated between two major bonds, highlighted in Figure IV-5A. The amide bond can be created with standard peptide coupling conditions. Synthesis is very straightforward between a primary amine and an activated carboxylic acid in a basic environment (Figure IV-5B). The 2° amine bond, however, can be synthesised in several ways. Alkylation of a primary amine is not a preferred option as the reaction occurs quickly and uncontrollably; dialkylation is difficult to prevent. A better route is reductive amination, whereby the primary amine reacts with an aldehyde to form the intermediate hemiaminal which dehydrates to form an imine. The unstable species is readily reduced by metal hydrides to a secondary amine species (Figure IV-5C).

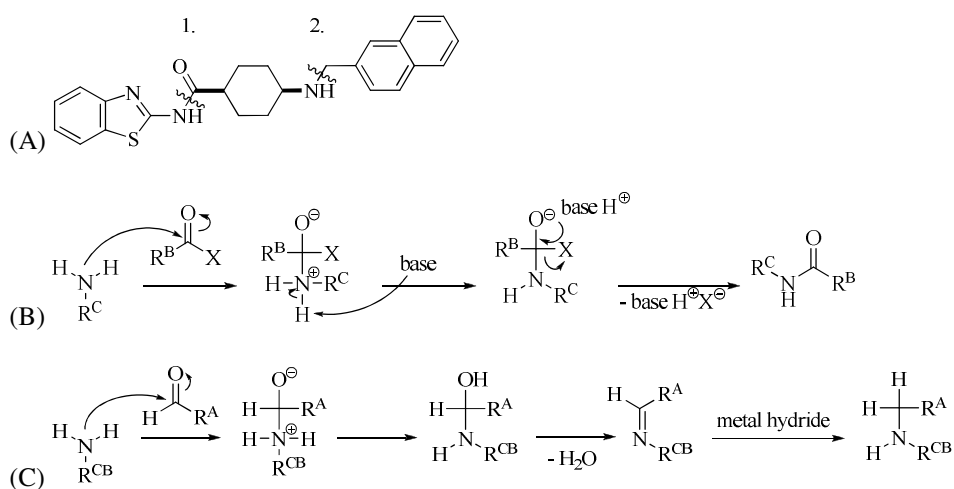


Figure IV-5: Analysis of the lead compound

The retrosynthetic analysis of UK-370,509 (A) revealed two linkages: a peptide bond (1.) and an amine bond (2.). The peptidic coupling occurs between the lone pair on the nitrogen that attacks the activated carboxylic acid to form a stable amide bond (B). Reductive amination is a two-step process whereby an imine is formed and subsequently reduced with a metal hydride reagent to form a secondary amine (C).

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The solution phase synthesis of **35** was successful. Figure IV-6 outlines the four-step synthesis. Initially, the N-terminus of building block B, *cis*-1,4-aminocyclohexanecarboxylic acid, was Boc-protected. The reaction was performed with Boc anhydride under standard Boc-protecting methods with good yields of 70%. The peptidic coupling step between the N-terminus of 2-aminobenzothiazole and the C-terminus of **36** was carried out with PyBOP as the activating reagent and gave **37** in a 90% yield. Optimisation trials of amide bond formation for building up the library will be discussed in IV.3.2.2 (p. 93). Prior to the final reductive amination step, the N-terminus was deprotected under strong acidic conditions. The resulting salt was reacted with aldehyde to form an imine *in situ*, which was then reduced to a secondary amine, which gave **35** in a 60% yield. Again, optimisation procedures of each individual step are discussed in the following subchapters. On the whole, the four-step synthetic route of the lead compound was quite successful and resulted in an overall yield of 40%.

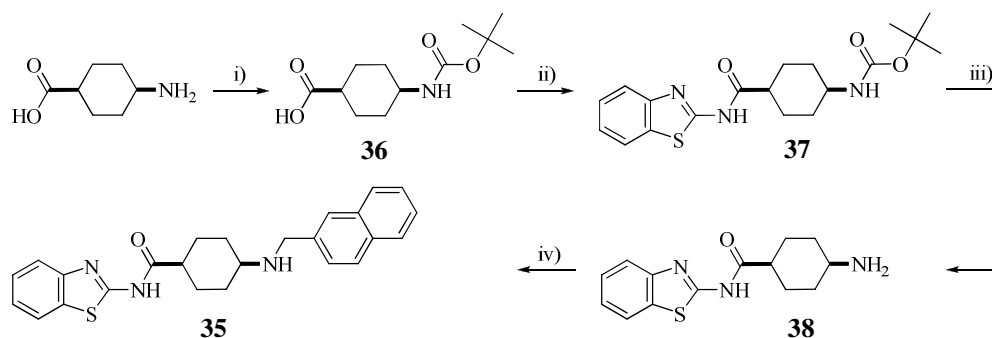


Figure IV-6: Synthetic route to lead compound 35

Reagents and reaction conditions: (i) Boc_2O , NEt_3 , MeOH , 60°C , 12 h, 70% yield; (ii) 2-aminobenzothiazole, PyBOP, DIPEA, DMF-DCM , 0°C , 12 h, 90% yield; (iii) $\text{TFA-TIPS-H}_2\text{O}$ (18:1:1, V:V:V), 79% yield; (iv) 2-naphthaldehyde, $\text{NaBH}(\text{OAc})_3$, NEt_3 , DCE , 12 h, 60% yield.

The reaction was followed by NMR spectroscopy. Figure IV-7 displays NMR data of each intermediate of the synthetic route to synthesise **35**. Key protons were labelled as follows: a – $\text{C}(\text{CH}_3)_3$, b – CHNH , c – CHNH , d – CHCOO . The first major shift is the CHCOOH proton (d) in the peptidic coupling step; it shifts quite far upfield from 3.4 ppm to 2.8 ppm. The aromatic benzothiazole protons are very well resolved as two doublets and two triplets in the area of 7–8 ppm. The chemical shift of the methyl protons of the *tert*-butyl group (a) typically at 1.3 ppm were no

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longer detected after Boc-deprotection of **37**. Instead, protons of the free amine (b) appeared at 7.8 ppm, coupling with the adjacent proton c. Deprotection of the amine caused a chemical shift downfield for proton c from 2.4 ppm to 3.2 ppm. The final step of reductive amination with naphthaldehyde introduced various aromatic protons in the area of 7–8 ppm as well as an additional aliphatic CH₂-group at 3.8 ppm. The remaining C_{cyclohexyl}H₂-protons were present between 1 and 2 ppm. Proton c shifted back upfield to 2.5 ppm.

The synthesis of **35** was successful and was verified with spectroscopic methods including NMR, IR and mass analysis to compare to data from UK-370,509; **35** was identical to UK-370,509.

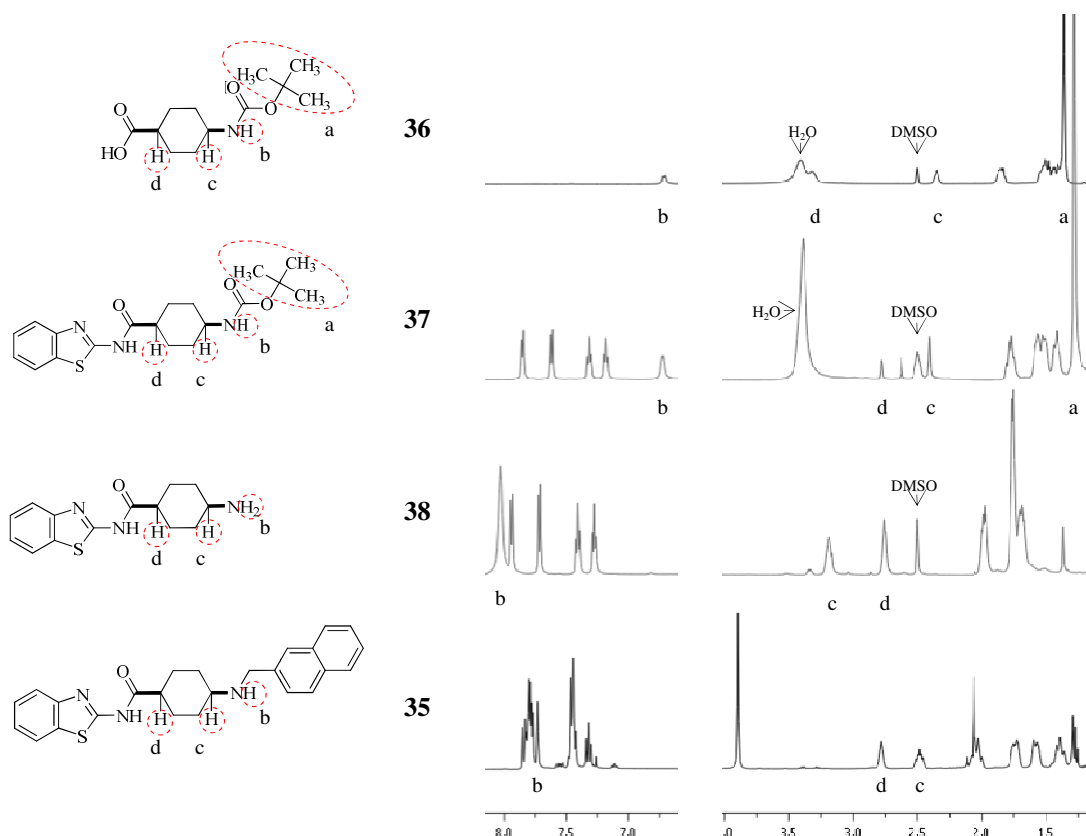


Figure IV-7: NMR analysis of the intermediates in the synthetic route towards the lead compound

*NMR data of the intermediates were analysed to compare the CH proton shifts between different steps of the synthetic route. The top three NMR spectra were taken in DMSO-*d*₆ and were referenced to 2.50 ppm. The bottom spectrum of **35** was taken in CDCl₃ and referenced to 7.24 ppm.*

IV.3.2 The Design of Building Block B

Utilised building blocks B were amino acid analogues (Figure IV-8). Unnatural amino acids such as aminocyclohexylcarboxylic acids were examined with a focus on *cis*-1,4- and *cis*-1,3-aminocyclohexylcarboxylic acid (Figure IV-8A and B). To investigate the importance of building block A with regards to inhibition of PfNMT, compound R^{CB} was synthesised as a control. For this case, cyclohexylcarboxylic acid and cyclohexylacetic acid (Figure IV-8D and E), derivatives of aminocyclohexylcarboxylic acid that lack amino functionalities for further coupling reactions, were employed. Another option for building block B was proline as L-proline is a natural amino acid and thus readily available for purchase (Figure IV-8C). Five-membered rings have not (to our knowledge) been investigated by Pfizer, but data from other research groups suggest that size of block B is not important for activity of compounds; rather it seems to act as a spacer for substituents, which in turn interacts with amino acids in the target binding domain (Yamazaki, Kaneko et al. 2005). Also, both substituents (N- and C-terminus) have a completely different orientation compared to the disubstituted cyclohexyl building blocks. The dimension of the peptide binding site could be explored by variations in position of the two substituents R^A and R^C.

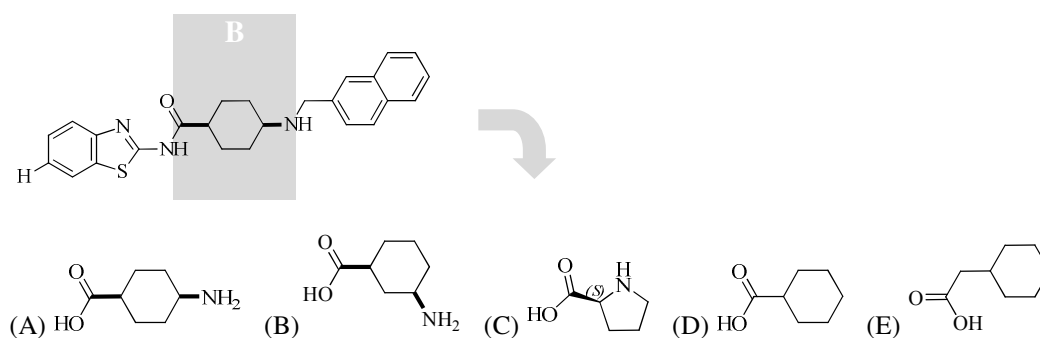


Figure IV-8: Proposed building blocks B

A selection of cyclohexyl- and cyclopentyl analogues was studied as building block B, namely *cis*-1,4-aminocyclohexylcarboxylic acid, *cis*-1,3-aminocyclohexylcarboxylic acid, L-proline, cyclohexylcarboxylic acid and cyclohexylacetic acid (from left to right).

The proposed scheme to make lead compound analogues is pictured in Figure IV-9. The N-terminus of building block B was protected (Step 1) prior to the coupling step

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of the carboxylic acid functionality and the amine of component C (Step 2). Once the protecting group of the N-terminus of unit B was removed (Step 3), building block A was attached in a reductive amination step (Step 4a) for 1° amines or *via* alkylation for 2° amines (Step 4b). Each individual step is discussed in the following subchapters.

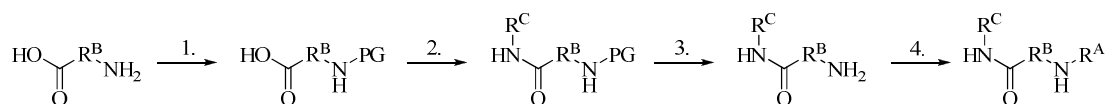


Figure IV-9: Proposed synthetic reaction scheme

Unprotected amine functionalities of building block were protected (Step 1) prior to coupling to building block C under standard peptide coupling conditions (Step 2). After deprotection of the N-terminal protecting group (Step 3), the amine was coupled to building block A (Step 4). Reductive amination was employed with 1° amines; however, alkylation was performed on 2° amines.

IV.3.2.1 Step 1: N-Terminal Protecting Groups

Any free amine groups that were not desired to react with the C-terminus of the carboxylic acid moiety of unit B were protected prior to peptide bond formation between R^C and R^B. The *tert*-butoxycarbonyl (Boc) group was chosen because of its stability towards catalytic hydrogenolysis and most basic and nucleophilic reagents (Greene and Wuts 1999). The reaction mechanism is displayed in Figure IV-10.

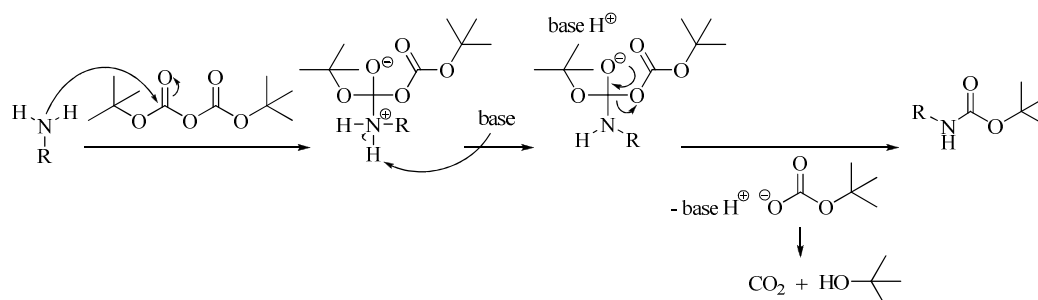


Figure IV-10: Mechanism of Boc-protection

Boc-protected amines are generated under aqueous conditions with base and Boc anhydride.

Boc-protection was performed under standard conditions (IV.2.3.1, p. 86). The generation of Boc-protected amines was achieved with a base and Boc anhydride

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under aqueous conditions in moderate yields of 60% for **36** (Castonguay, Lherbet et al. 2002). Boc-protected *cis*-1,3-aminocyclohexanecarboxylic acid and L-proline were commercially available.

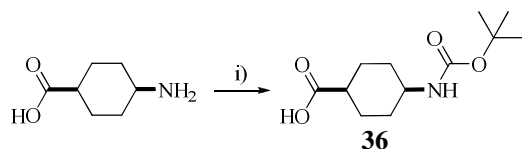


Figure IV-11: Boc-protection (formation of 36)

Reagents and reaction conditions: (i) Boc anhydride, NEt₃, MeOH, 60 °C, 12 h, 60% yield (Castonguay, Lherbet et al. 2002)

IV.3.2.2 Step 2: Amide Bond Formation

Peptide bond formation between the N-terminus of aminobenzothiazoles and the C-terminus of cyclohexyl derivatives was aided by activating reagents to create an activated ester. A much more electropositive carbon atom favours the nucleophilic attack of the nitrogen atom. There are various ways for the conversion of carboxylic acids into activated esters, of which some were examined (Reviews: (Albericio and Carpino 1997; Han 2004; Katritzky, Suzuki et al. 2004; Montalbetti and Falque 2005; Valeur and Bradley 2009)). The carboxylic acid was converted either into acyl halides, mixed anhydrides or esters. All activated reagents were made in a separate step, followed by the immediate treatment with the amine species to form a peptide.

IV.3.2.2.1 Acyl Halides

Central reaction partners to form amide bonds are functional groups that exhibit strong electron-withdrawing effects, such as halogens. The conversion of carboxylic esters to acyl halides may be achieved with reagents such as thionyl chloride or oxalyl chloride in a nucleophilic addition elimination reaction (Montalbetti and Falque 2005). The carbonyl group of the acyl halide is more susceptible towards nucleophiles due to the electron-withdrawing effect of the electronegative halogen. The formation of the peptide bond proceeds *via* the nucleophilic attack of the amine at the carbonyl (displayed in Figure IV-12). The reaction with acyl chloride and aminobenzothiazole, Figure IV-13, yielded **39** in moderate yields of 78% (Yoshida,

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Hayakawa et al. 2005). Peptide bond formation with acyl chlorides or other acyl halides was not carried out with more building blocks B; these were not readily available commercially. Although acyl halides are one of the easiest to produce activated esters with, there are drawbacks due to easy hydrolysis and racemisation *via* ketene formation (Montalbetti and Falque 2005). For this reason, other activating reagents were investigated.

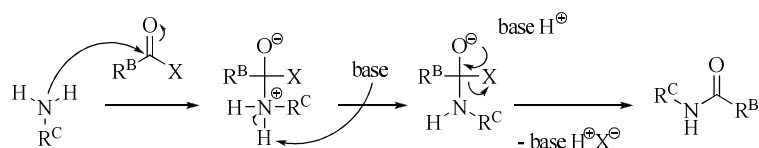


Figure IV-12: Reactions with acyl halides

A peptide bond can be formed easily when a nucleophilic amine reacts with an activated ester such as an acyl halide ($X = \text{halogen}$) (Montalbetti and Falque 2005).

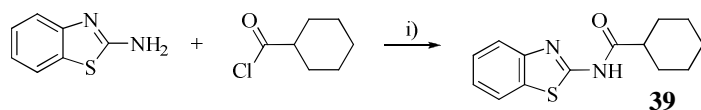


Figure IV-13: Synthesis of 39

Reagents and reaction conditions: (i) 4-pyrrolidine-pyridine, DMF, RT, 12 h, 78% yield (Yoshida, Hayakawa et al. 2005).

IV.3.2.2.2 Esters

An alternative halogenic activating reagent was pentafluorophenol (Kisfaludy, Löw et al. 1973; Carey, Bordas et al. 1997; Kaplan and McGrath 1997; Rajappan and Hosmane 1998; Montalbetti and Falque 2005). It absorbs strongly in the UV region and the reaction can thus be readily tracked by TLC. The suggested reaction mechanism with pentafluorophenyl trifluoroacetate (Pfp TFA) is outlined in Figure IV-14. A mild base deprotonates the carboxylic acid to form a carboxylate ion, which then reacts with the carbonyl atom of Pfp TFA in a nucleophilic addition-elimination step. The mixed anhydride intermediate has two electrophilic carbonyls. If the pentafluorophenyl anion attacks C¹, an activated ester is formed to readily react with amine species, resulting in a peptide bond. If the pentafluorophenyl anion attacks C², which is much more electrophilic due to the electron-withdrawing

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character of the CF_3 -substituent, Pfp TFA and the carboxylate species are re-generated and the reaction will start again until starting material has been consumed.

The reaction was performed under basic conditions with Pfp TFA and NEt_3 and monitored by TLC (McReynolds, Bhat et al. 2002). The starting material was consumed within 1 h after which the amine was added. However, the outcome of this reaction was unsuccessful with yields of less than 10% of **37**. Different reaction conditions to improve the reactivity of the ester, by the addition of HOBt to form an even more activated ester, were also unsuccessful (Albericio 2000). Presumably the activating reagent pentafluorophenyl trifluoroacetate had already decayed.

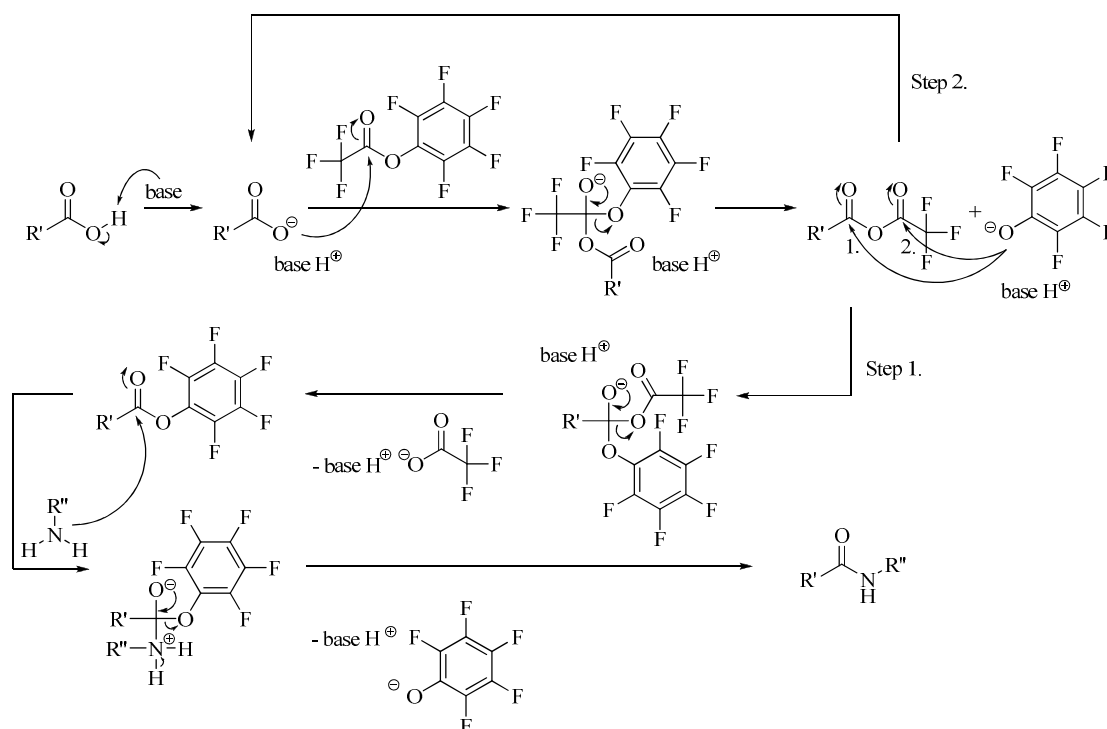


Figure IV-14: Activation with pentafluorophenyl trifluoroacetate

The proposed mechanism of activation with Pfp trifluoroacetate comprises of the electrophilic attack of the carboxylic anion to the activating reagent. After several steps the amine reacts with the pentafluorophenyl-activated carbonyl species to form the amide product.

The following coupling reactions were carried out with HOBt and HBTU, of which the latter belongs to the family of guanidinium peptide coupling reagents. For a long time HBTU was assumed to be a uronium salt (Figure IV-15A); however, crystal

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studies have shown that its true structure is the guanidinium salt (Figure IV-15B). It is less active compared to the uronium species due to a greater amount of energy required to break the C-O bond compared to the C-N bond (Carpino, Imazumi et al. 2002). Hence, they require a longer pre-activation time. Furthermore, guanidinium salts are much more attractive reagents than carbodiimides because the active esters are more stable and may suppress racemisation (Marder and Albericio 2003).

The mechanism of the HBTU/HOBt reaction is proposed in Figure IV-16. The carboxylate reacts with the guanidinium salt to form an intermediate ester that reacts rapidly with the benzotriazole derivative HOBt. The added amine can then readily react with the activated ester *via* peptide bond formation (Li and Xu 2000; Montalbetti and Falque 2005; Valeur and Bradley 2009).

The reaction of **36**, the Boc-protected *cis*-1,4-aminocyclohexylcarboxylic acid, with 2-aminobenzothiazole was carried out under standard peptide coupling procedure in basic conditions with HBTU, HOBt and NEt₃. It gave **37** in 22% yield, higher than with the previous Pfp TFA activation method. However, activation of cyclohexylacetic acid with HBTU/HOBt followed by the reaction with 2-aminobenzothiazole resulted in a very low amount of **40**, only 2% (Figure IV-17).



Figure IV-15: Proposed structures of HBTU

The uronium salt (A) was thought to be the structure of HBTU until crystallographic studies revealed it is a guanidinium salt (B) (Carpino, Imazumi et al. 2002).

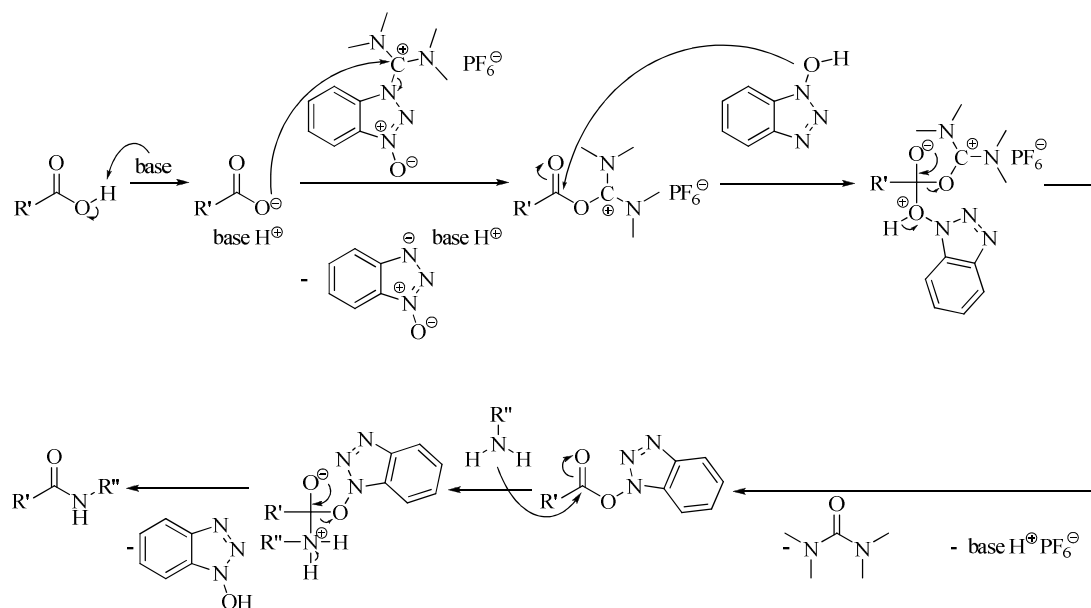


Figure IV-16: HBTU/HOBt activation of carboxylic acids

The reaction mechanism of HBTU/HOBt activation is illustrated. First, an ester is formed with HBTU under basic conditions. The ester is then attacked by HOBt to form a more reactive species. The amine attacks the carbonyl and readily forms a peptide bond (Li and Xu 2000; Montalbetti and Falque 2005; Valeur and Bradley 2009).

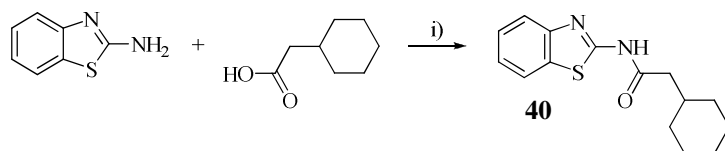


Figure IV-17: Synthesis of 40

Reagents and reaction conditions: (i) HBTU, HOBt, DIPEA, DMF–DCM, RT, 12 h, 2% yield.

Alternative coupling reagents were investigated and included phosphonium salts such as PyBOP and PyAOP, which are usually 1-hydroxybenzotriazole-based, just like the uronium and guanidinium salts. They are often used in combination with HOBt or HOAt (Figure IV-18) and are very common in solid phase peptide synthesis (SPPS) (Katritzky, Suzuki et al. 2004). Furthermore, PyBOP causes little racemisation.

The reaction mechanism with PyBOP is illustrated in Figure IV-19 and is very similar to the previous mechanism for HBTU/HOBt activation (Li and Xu 2000; Montalbetti and Falque 2005; Valeur and Bradley 2009). The carboxylate reacts with

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PyBOP in basic conditions to form an intermediate activated ester. HOBt then attacks the ester to form an even more reactive ester. The amine species can then react readily with the activated ester to form a peptide bond.

PyBOP is commonly used in *N*-acylations; it is able to activate the carboxylic acid without combining it with HOBt (Li 2001). Reactions were carried out under basic conditions with PyBOP and DIPEA at 0 °C. High yields of 90% (Table IV-1), 66% (Figure IV-20A) and 86% (Figure IV-20B) for similar building block Bs were obtained and simple work-up methods such as precipitation in cold diethyl ether to eliminate by-products of PyBOP easily, made carboxylic activation with PyBOP a desirable method.

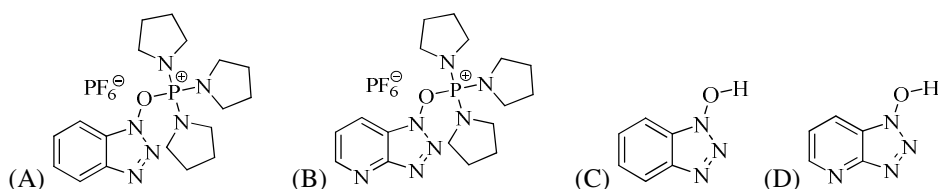


Figure IV-18: 1-Hydroxybenzotriazole-based activating reagents

Various activating reagents are illustrated: PyBOP (A), PyAOP (B) and their respective coupling partners HOBt (C) and HOAt (D).

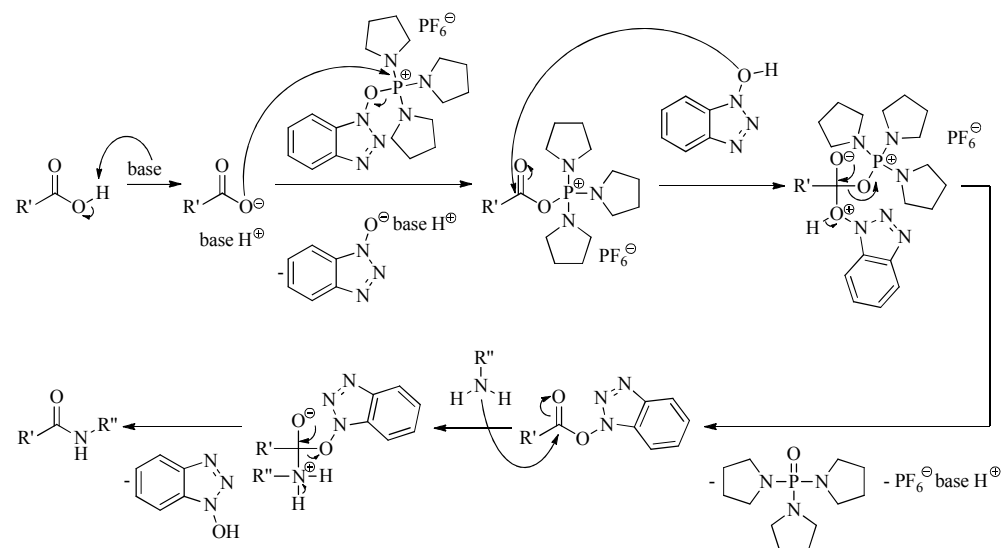
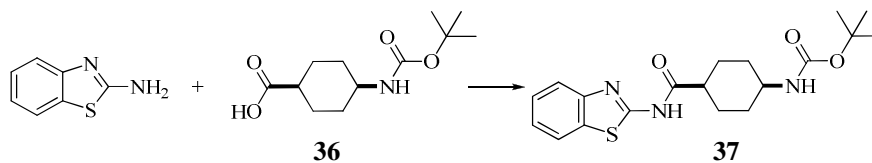


Figure IV-19: Activation of carboxylic acids with PyBOP

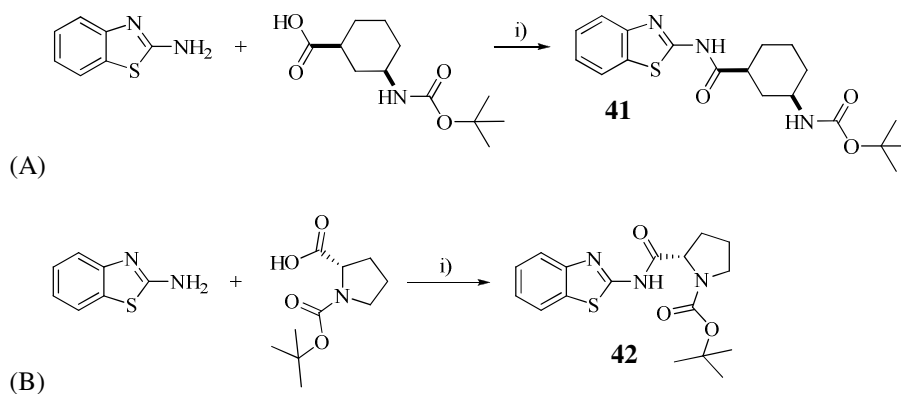
The reaction mechanism of PyBOP/HOBt activation is illustrated. First, an ester is formed with PyBOP under basic conditions. The ester is then attacked by HOBt to form a more reactive species. The amine attacks the carbonyl and readily forms a peptide bond (Valeur and Bradley 2009).

Table IV-1: Optimisation of peptide bond formation

The activation of compound **36** was optimised using various activating reagents as. The outcome (% yield of **37**) is compared to demonstrate the most favourable reaction condition for further peptide coupling steps. The reaction that produced the highest yield is highlighted in grey.



Activating Reagents	Reaction Conditions	Yield
Halides: Pentafluorophenol	1.2 eq. Pfp TFA, 2.0 eq. NEt ₃ , 1.0 eq. carboxylic acid, 0.9 eq. amine, RT	7%
Guanidinium salt: HBTU	1.5 eq. HBTU, 1.0 eq. HOBt, 2.0 eq. DIPEA, 1.0 eq. carboxylic acid, 0.9 eq. amine, RT	22%
Phosphonium salt: PyBOP	1.1 eq. PyBOP, 2.0 eq. DIPEA, 1.0 eq. carboxylic acid, 1.1 eq. amine, 0 °C	90%

**Figure IV-20: PyBOP-aided peptide bond formation (synthesis of **41** and **42**)**

(A) Reagents and reaction conditions: (i) PyBOP, DIPEA, DMF–DCM, 0 °C, 12 h, 66% yield; (B) reagents and reaction conditions: (i) PyBOP, DIPEA, DMF–DCM, 0 °C, 4 h, 86% yield.

The reaction with Boc-L-proline, compound **42**, gave rise to some interesting NMR data. Analysis of **42** showed a mixture of two slowly exchanging species, probably rotamers. NMR spectra were recorded at two different temperatures, 298 K and 323 K. Spectra are usually taken at 298 K, whereby the hydrogen atom at the

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stereocentre (CHN-Boc) appears as an apparent doublet. However upon increase of temperature, the apparent doublet merges to a singlet. The system has more energy and hence the substituents rotate much faster around the σ -bond. Hence at 323 K, a broad singlet at 4.5 ppm (CHN-Boc) and a broad doublet at 3.50 ppm ($\text{CH}_2\text{N-Boc}$) were noted which became an apparent doublet (CHN-Boc) and an apparent quadruplet ($\text{CH}_2\text{N-Boc}$) when cooled down to 298 K (Figure IV-21).

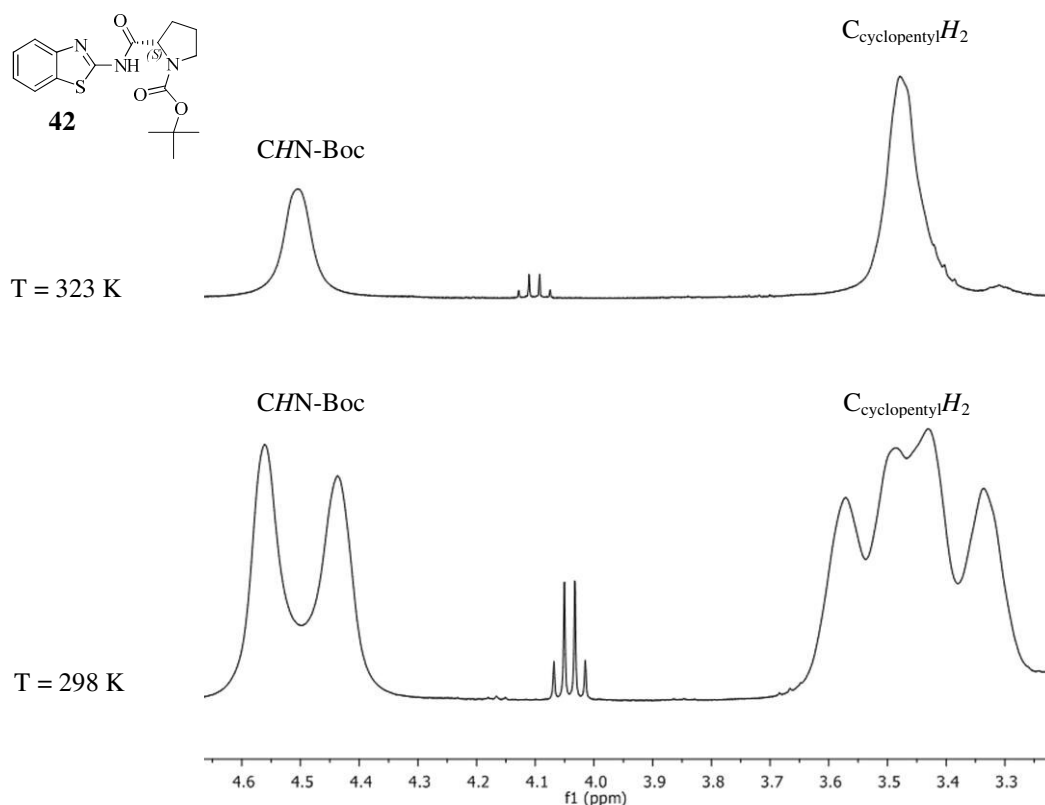


Figure IV-21: Comparison of NMR data of compound 42 at different temperatures

Spectra were taken at two different temperatures, 298 K and 323 K. The isomers of compound **42** were distinguishable at lower temperatures. The singlet at 4.5 ppm split into an apparent doublet once the temperature was reduced from 323 K to 298 K. The NMR spectra were taken in CDCl_3 with the reference peak at 7.24 ppm. Note solvent peaks of ethyl acetate at 4.12 ppm (q).

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Simple amide bonds coexist as *cis-trans* isomers in solution (Dugave 2006). The preferred orientation is the *trans*-conformation due to less steric hindrance of the amide hydrogen, H^a , with C^a (Wedemeyer, Welker et al. 2002). The *cis-trans* equilibrium seems to be coupled to the overall molecular conformation. The transition state involves not only changes in torsion angles about the X-Pro peptide bond but is also affected by numerous single bonds within the molecule (Figure IV-22) (Grathwohl and Wüthrich 1981).

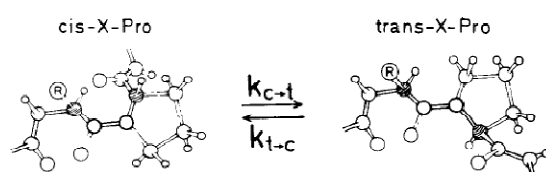


Figure IV-22: *cis-trans* Isomerisation of proline

The peptide fragment -X-Pro- isomerises from *cis*- to *trans*-conformation. *R* denotes the side chain of *X*, $k_{c \rightarrow t}$ and $k_{t \rightarrow c}$ the *cis-trans* and *trans-cis* interconversion rates respectively (Grathwohl and Wüthrich 1981).

IV.3.2.3 Step 3: The Removal of *N*-Terminal Protecting Groups

The *tert*-butoxycarbonyl group is acid labile and can be cleaved with strong acids *via* an E_1 -mechanism (Figure IV-23, (Kocienski 1994)). Scavengers may be employed to prevent alkylation of the substrate by the stable 3° carbocation. Soft nucleophiles such as thiophenols or thioethers are suitable. The Boc-protection group was removed with TFA employing a standard decoupling procedure (IV.2.3.2, p. 86). Compounds were precipitated with cold diethyl ether to generally yield >80% as the TFA salt (Figure IV-24).

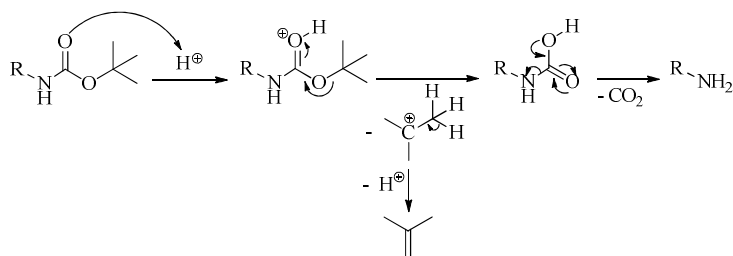


Figure IV-23: Mechanism of Boc-deprotection

Tert-butyl carbamates are cleaved under anhydrous acidic conditions to form the free amine (Kocienski 1994).

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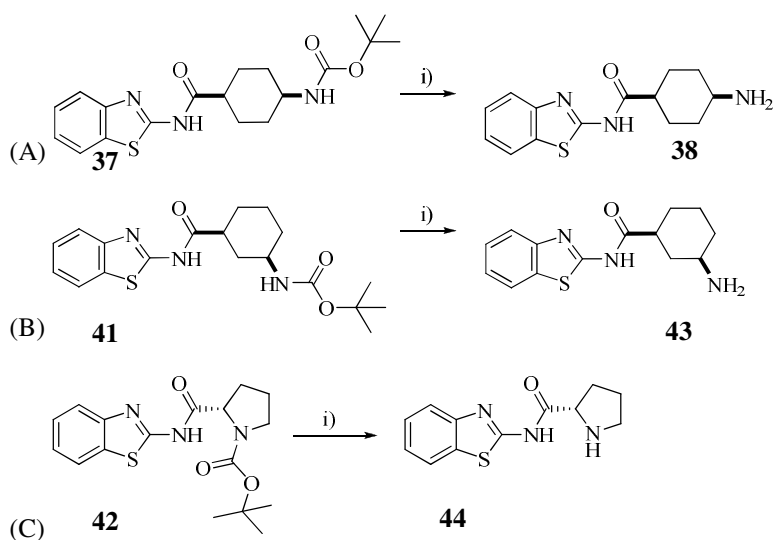


Figure IV-24: Boc-deprotection (Formation of **38**, **43** and **44**)

(A) 79% yield, (B) 99% yield, (C) 84% yield. Reagents and reaction conditions: (i) TFA–H₂O–TIPS (90:5:5, V:V:V), RT, 1 h.

IV.3.2.4 Step 4a: Reductive Amination

After the deprotection step, combining the 1° amine with building block A was the last step for the library of modified lead compound analogues. A complete list for diverse units A is in Section IV.3.3.1 (p. 105). Synthesis was achieved during the reaction between a 1° amine and an aldehyde in the presence of a reducing agent. The mechanism is outlined in Figure IV-25. The N-terminus reacts with the carbonyl to form an unstable intermediate. The hemiaminal dehydrates to form an imine (also called Schiff's base), which is then readily reduced by alkyl hydride reducing agents, where an H-nucleophile attacks the C-atom of the imine to form an amine (Abdel-Magid, Carson et al. 1996). Optimisation of this procedure is discussed in Section IV.3.3.2 (p. 108).

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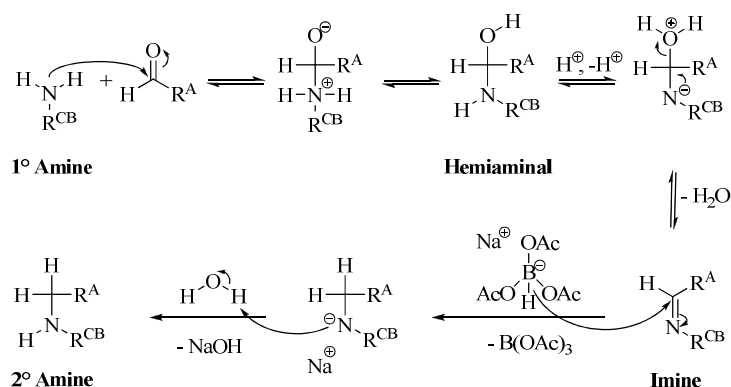


Figure IV-25: Mechanism of reductive amination

The reductive amination consists of two steps. The reversible formation of the imine results in the reaction of an amine with a carbonyl to form an intermediate hemiaminal. After the elimination of water, the imine reacts in an irreversible step with the added reducing agent such as NaBH(OAc)₃ to form the product, an amine (Abdel-Magid, Carson et al. 1996).

IV.3.2.5 Step 4b: N-Alkylation

After deprotection, yielding 2° amines, the R^A unit was attached as the last step. A common method for transforming secondary amines into tertiary amines is *via* alkylation with alkyl halides. In Figure IV-26, the mechanism of alkylation is outlined. This straightforward synthesis involves the nucleophilic attack of the 2° amine towards the C^α-atom of the alkyl halide. The formed *in situ* tetrahedral intermediate is deprotonated by addition of base to yield an alkylated amine.

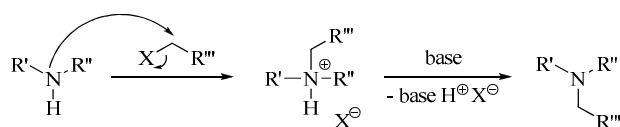


Figure IV-26: Mechanism of alkylation

Secondary amines can be alkylated with alkyl halides. The tetrahedral intermediate is treated with base to yield alkylated amines.

Figure IV-27 gives an overview of synthesised compounds **45**, **46** and **47**, ready to be tested for activity against PfNMT *in vitro*. Compound **45** is a direct analogue of lead compound **94** with aminobenzothiazole as unit R^C and methylnaphthalene as building block R^A. Both components are joined by (*S*)-proline. The alkylation

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reaction with bromomethylnaphthalene gave a relatively low yield of 28% yield. For synthesis of **46**, the reaction was observed over time at two different temperatures, RT and 0 °C. TLC analysis of the reaction mixture showed multiple spots suggesting degradation or polymerisation over time for both reaction trials. But by-product formation was much greater at elevated temperatures. Starting material and alkyl halide was also still present after 24 h reaction time. Overall, changes in temperature to favour product formation and different reaction times still led to low yields of 15%. Hence reductive amination for **47** was attempted with **44** and 1-benzofuran-5-carbaldehyde. The reaction was more successful with 77% yield of compound **47**.

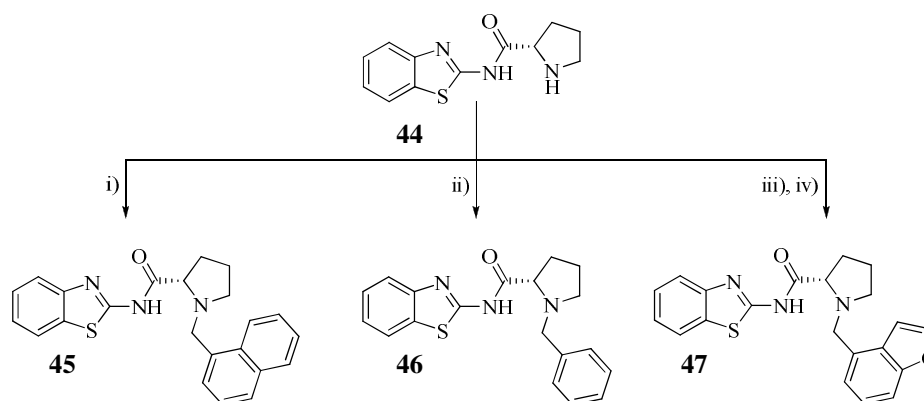


Figure IV-27: Synthesis of lead compound analogues (45, 46, 47) with proline as building block B

Reagents and reaction conditions: (i) bromomethylnaphthalene, NEt_3 , DCM, RT, 18 h, 28% yield; (ii) benzylbromide, NEt_3 , DCM, RT, 18 h, 14% yield; (iii) 1-benzofuran-5-carbaldehyde, acetic acid, DMF–DCM, molecular sieves, RT, 1h; (iv) $NaBH(OAc)_3$, RT, 18 h, 77% yield.

IV.3.3 Building a Library with Variations in Building Block A

The main focus was centred on building block A. As mentioned previously (Figure II-19), the addition of unit A may cause a conformational change in the surrounding amino acids of the peptidic binding pocket, thus extending the cavity. As a result tighter interactions with even more AA in the target binding site are possible, creating a better inhibitor. Studies indicated large-sized aromatic groups as well as the addition of heteroatoms may play an important role upon binding (IV.1, p. 80). Hence a collection was synthesised to systematically investigate several aspects of binding that included size, aromatic character, electron withdrawing and donating

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properties, as well as H-bond donor and acceptor groups (Figure IV-28). A complete discussion regarding the choice of unit A is outlined in Section V.3.3.1 (p. 143).

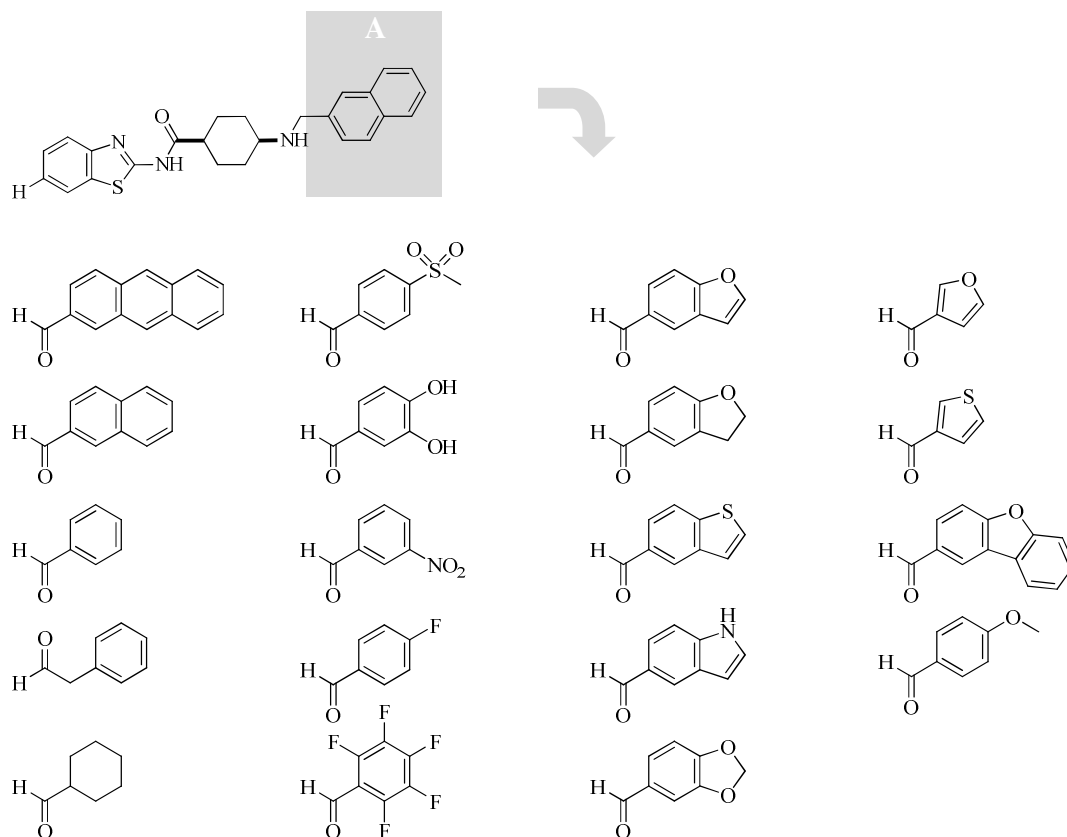


Figure IV-28: Complete list of building blocks A

A list of aldehydes for variations of the lead compound is shown. Variations of building block A were subject to availability.

IV.3.3.1 Building Up a Small-Sized Library

Parallel synthesis was carried out to start a small-sized library. First an excess of aldehyde, 2.0 eq. was employed to investigate the rate of reaction for various building blocks A (Table IV-2A, Table IV-3A). The other reagents were 2.0 eq. NEt₃, 2–4 eq. NaBH(OAc)₃ and molecular sieves in DMF in a two-step procedure with 1 h imine formation and overnight reaction time for reduction, followed by aqueous work-up.

Unfortunately, overalkylation products were noticed. The ratio of monosubstituted product vs. disubstituted by-product was dependent on ring size as well as electronic properties of the aromatic system. The yield of compound **48** with an aliphatic unit A

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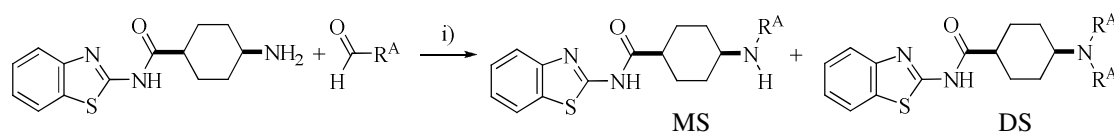
was much smaller compared to the yield of compound **49** with an aromatic system as unit A (1% vs. 16%). A large amount of disubstituted by-product was noticed. Generally, the formation of monoalkylated species was unfavoured under these reaction conditions due to the competitive side reaction of dialkylation.

To minimise overalkylation, the amount of aldehyde was lowered from 2.0 eq. (Table IV-2A, Table IV-3A) to 1.5 eq. (Table IV-2B, Table IV-3B) and 1.0 eq. (Table IV-2C). As a result disubstitution diminished, driving the product reaction to completion, resulting in a higher yield. The yield of compound **48** increased greatly from 1% with 2.0 eq. aldehyde to 29% with 1.5 eq. aldehyde. Simultaneously the amount of formed disubstituted by-product, **50**, dropped from 63% to <1%. This was also noted with compound **51**, with an increase in yield from 3% to 17% and a significant decrease for the disubstituted by-product **52** from 78% to <1%.

Overall, there was a drop in by-product formation once the aldehyde substrate was reduced; however the yield of the product was not greatly increased. Usually moderate yields of 20% were monitored with exception of higher yields around 50% for some compounds. Thus the reaction was studied in detail to improve yields and reduce by-product formation.

Table IV-2: Generation of a small-sized library with *cis*-1,4-cyclohexyl building block B

The library was generated in a two-step-synthesis under the following reaction conditions: (i) 1.0 eq. amine, 1.0–2.0 eq. aldehyde, 2.0–2.2 eq. NEt_3 , 2.0–4.0 eq. $NaBH(OAc)_3$, 0.2 M in DMF and molecular sieves (IV.2.3.4, p. 87). A more detailed procedure for each individual compound can be found in the Appendix (VIII.1, p. 193).



(A) 2.0 eq. aldehyde

Compound Number	R^A	Yield [%]	
		MS	DS
49/53		16	5

Compound Number	R^A	Yield [%]	
		MS	DS
61/62		21	17

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Compound Number	R ^A	Yield [%]		Compound Number	R ^A	Yield [%].	
		MS	DS			MS	DS
54/55		31	41	63		11	n.i.
56/57		25	7	64		48	n.i.
58		5	n.i.	51/52		3	78
59/60		7	23	65/66		10	13
48/50		1	63	67		2	n.i.

Note: MS – monosubstituted; DS – disubstituted; n.i. – not isolated

(B) 1.5 eq. aldehyde

Compound Number	R ^A	Yield[%]		Compound Number	R ^A	Yield[%]	
		MS	DS			MS	DS
68		3	n.i.	67		2	n.i.
69		17	n.i.	71		26	n.i.
70		3	n.i.	72		19	n.i.
48		29	n.i.	51		17	n.i.

Note: MS – monosubstituted; DS – disubstituted; n.i. – not isolated

(C) 1.0 eq. aldehyde

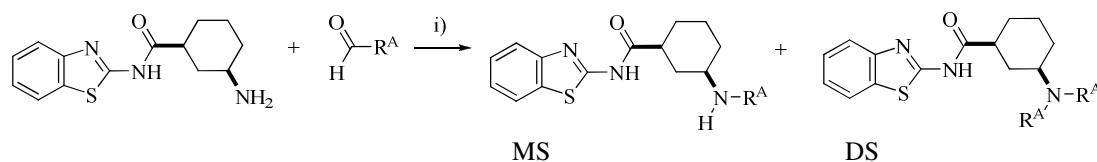
Compound Number	R ^A	Yield[%]		Compound Number	R ^A	Yield[%]	
		MS	DS			MS	DS
35		67	n.i.	73		51	n.i.

Note: MS – monosubstituted; DS – disubstituted; n.i. – not isolated

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Table IV-3: Generation of a small-sized library with *cis*-1,3-cyclohexyl building block B

The library was generated in a two-step synthesis under the following reaction conditions: (i) 1.0 eq. amine, 1.5–2.0 eq. aldehyde, 2.0–2.2 eq. NEt_3 , 2.0–4.0 eq. $NaBH(OAc)_3$, 0.2 M in DMF and molecular sieves (IV.2.3.4, p. 87). A more detailed procedure for each individual compound can be found in the Appendix (VIII.1, p. 193).



(A) 2.0 eq. aldehyde

Compound Number	R^A	Yield [%]	
		MS	DS
74/75		10	14
76/77		45	32
78/79		3	36

(B) 1.5 eq. aldehyde

Compound Number	R^A	Yield [%]	
		MS	DS
80		15	n.i.
81		13	n.i.
82		28	n.i.

Note: MS – monosubstituted; DS – disubstituted; n.i. – not isolated

IV.3.3.2 Optimisation of the Reductive Amination Step

There are several comprehensive reviews on reductive amination (Gomez, Peters et al. 2002; Abdel-Magid and Mehrman 2006) with Abdel-Magid et al. employing sodium triacetoxyborohydride, for various aliphatic and aromatic ketones and aldehydes, as the general reducing agent which is much milder compared to the commonly used $NaBH_4$. Furthermore, it gave consistently higher yields and fewer side products. This method however had limitations with aromatic, unsaturated ketones and sterically hindered ketones (Abdel-Magid, Carson et al. 1996).

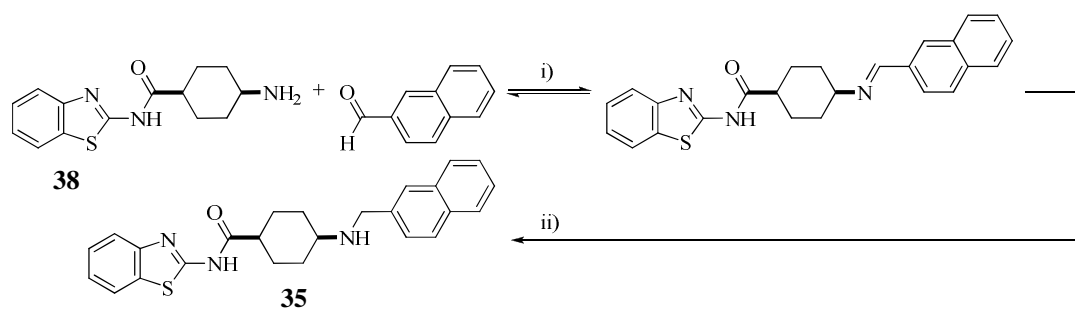
Acetic acid may be added as a catalyst during reductive amination of ketones, but it is not essential for aldehydes. Under the weakly acidic to neutral reaction conditions, the imine is protonated to form an iminium ion, which is subsequently reduced to the amine product (Abdel-Magid, Carson et al. 1996). However, the pH needs to be carefully monitored for selective reduction of the imine over the aldehyde. At pH

values in the range 3–4, aldehydes and ketones are reduced effectively to the respective alcohol. The rate of reaction will decrease by a more neutral environment. At pH of 6–7, other species such as more basic imines will be reduced selectively. Also, if reaction conditions are too acidic, the 1° amine substrate is protonated and thus cannot act as a good nucleophile for the attack on the carbonyl species. Methanol may be used as a proton source (Borch, Bernstein et al. 1971).

The reductive alkylation of primary amines to yield secondary amines may result in overalkylation. This side reaction has been observed and reported extensively in literature, especially regarding sterically unhindered aldehydes. The problem of dialkylation can be overcome by a stepwise procedure of first forming the imine, followed by the reduction to the amine with reducing agent. In addition, by using less than 1 eq. of the carbonyl species relative to the amine species, overalkylation may be reduced (Abdel-Magid, Carson et al. 1996; Szardenings, S. et al. 1996; Yamazaki, Kaneko et al. 2005).

Indirect reductive amination can be summed up in an initial step of formation of intermediate imine followed by reduction to the amine species in a final step. The reaction times differ largely and are dependent on the substrate. Hence the reaction was performed in deuterated solvent (CDCl₃), to observe imine formation at various time points using NMR spectroscopy. The alternative of the isolation of imines would be too difficult as imines are quite unstable. General conditions to synthesise **35** were used as according to the literature (Abdel-Magid, Carson et al. 1996).

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Reagents and reaction conditions: (i) 1.5 eq. aldehyde, 2.2 eq. NEt_3 , 0.03 M in CDCl_3 ; (ii) 3.2 eq. $\text{NaBH}(\text{OAc})_3$, aqueous work-up.

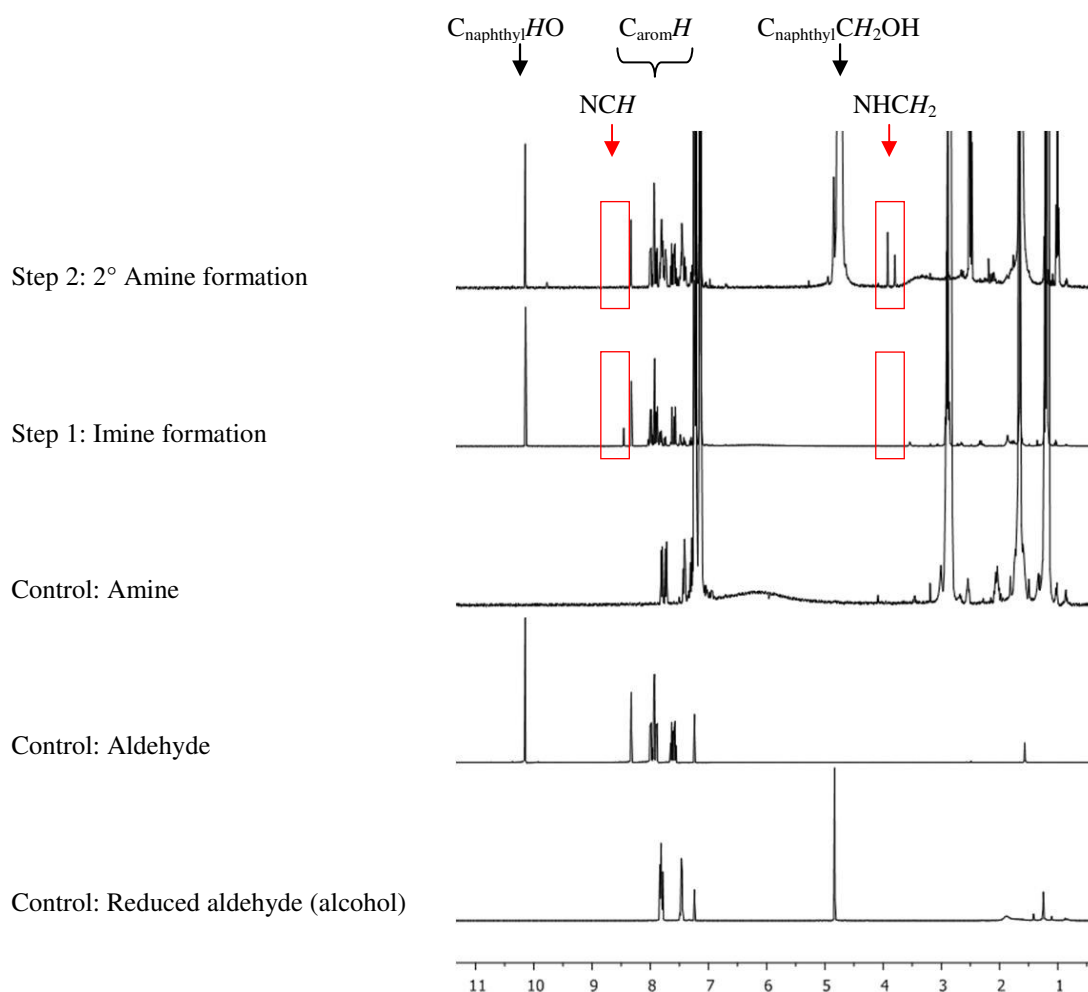


Figure IV-29: Complete NMR analysis for reductive amination to yield 35

Formation of **35** was investigated with NMR spectroscopy. A series of NMR spectra were taken in CDCl_3 ($\delta_{\text{H}} = 7.26 \text{ ppm}$) that show reduced aldehyde (2-naphthylmethanol), aldehyde (2-naphthalene), amine, the reaction mixture after 24 h reaction time prior to the addition of reducing agent and after the addition of reducing agent followed by aqueous work-up (from bottom to top). Red boxes and red arrows point out characteristic proton shifts for reductive amination: first the imine peak at 8.5 ppm and second the aliphatic NCH_2 protons after reduction at 3.8 ppm. The peaks of the base NEt_3 are characteristic at 2.56 (q) and 1.03 (t) ppm. The bottom two spectra have been included as a control and the key spectral regions are blank as expected.

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NMR data are pictured in Figure IV-29, which shows a summary of each reaction step including controls of only aldehyde and amine for comparative purposes. Red boxes point out key characteristic spectral features for each step; first the imine proton that has a unique peak at 8.5 ppm upon addition of aldehyde and base to the amine (Step 1) and second the pair of aliphatic protons of the 2° amine at 4.8 ppm which appear after the addition of reducing agent and aqueous work-up (Step 2). The imine proton disappears during the second step of reductive amination.

The reaction at various time points, to investigate the formation of the imine intermediate product is shown in Figure IV-30. The top NMR segment shows the aromatic region of 7–10 ppm to look at the imine proton more closely. The intensity of the imine proton at 8.5 ppm increases over time, whereas the intensity of the aldehyde proton decreases. However there is no continuous amplification of the peak of the imine proton noted at the observed time points of 12–40 h (Table IV-4). The intensity of the peak at 12 h is similar in intensity to the peak at 24 h.

This can also be noted elsewhere for other hydrogen atoms. The bottom NMR section illustrates the aliphatic hydrogen shifts: 3.0 ppm to 3.5 ppm for $CHNH_{(2)}$, 2.5 ppm to 2.7 ppm for $CHCONH$ and 2.0 ppm to 2.3 ppm for CH_2CHN . This was expected as the environment changes for these particular protons upon addition of the aldehyde species. It was furthermore noted that the downfield shifts were also complete after 12 h reaction time.

Table IV-4: Progression of imine formation

The table lists the ratio of the imine proton vs. the aldehyde proton with regards to Figure IV-30. The integral was calculated with respect to the aromatic hydrogen from the amine substrate.

Reaction Time	10 min	12 h	21 h	24 h	40 h
NCH	0.2	0.6	0.7	0.7	0.7
CHO	5.9	4.4	3.8	3.5	2.9

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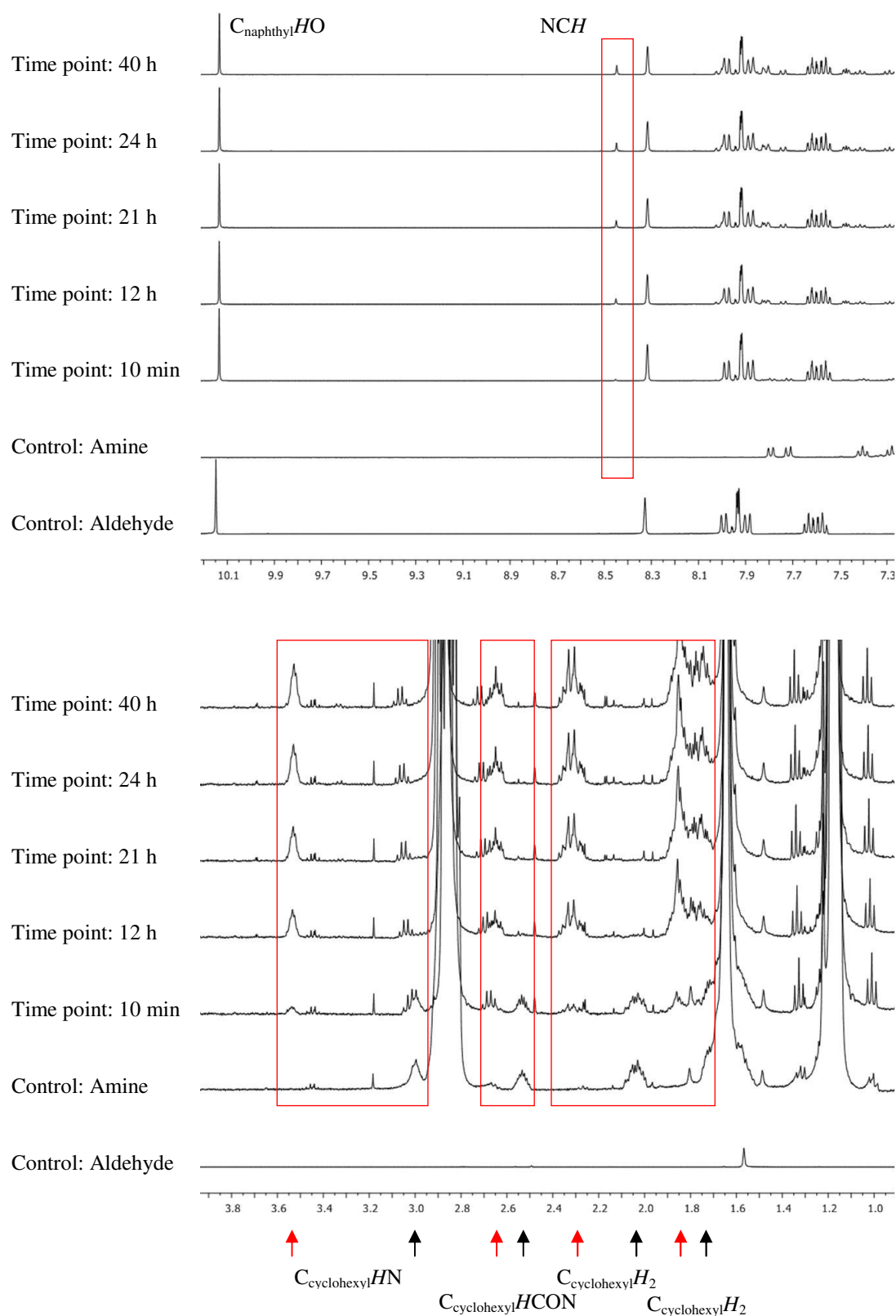


Figure IV-30: Detailed time analysis of imine formation

The first step of reductive amination, imine formation, for compound 35 was studied at various time points with NMR spectroscopy. The displayed series is a more detailed study of Figure IV-29. Two sections of the spectra are displayed (from bottom to top: control aldehyde, control amine, 10 min, 12 h, 21 h, 24 h, 40 h reaction time). The characteristic protons are highlighted in red boxes. The top

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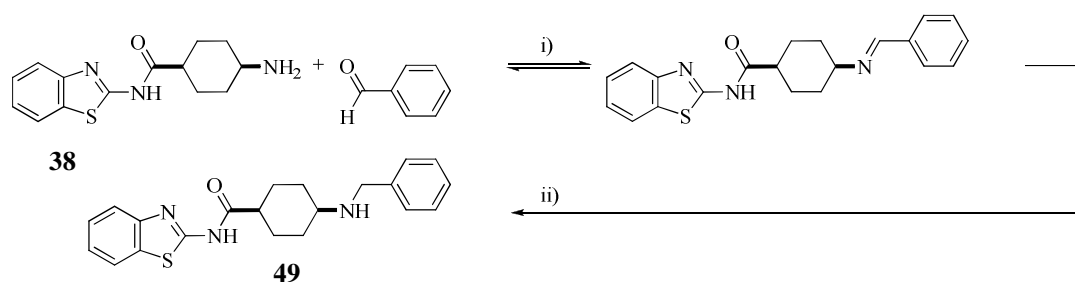
spectra point out the imine proton with a unique peak at 8.3 ppm. The bottom spectra show the shift of the aliphatic protons where the original peak is pointed out with a black arrow and the shifted peak of the same proton with a red arrow.

According to results from the previous NMR spectroscopic analysis, imine formation was complete after at least 12 h but the speed of imine formation was still unknown as the imine hydrogen was already present at 10 min and complete imine formation was already noted at the next time point of 12 h. The time period in between 10 min and 12 h was subject to the next NMR series in line to investigate this matter further (Figure IV-31). Foremost, a different deuterated solvent was employed as several problems arose in trial number one with CDCl_3 . The amine species were sparingly soluble and the deuterated solvent also seemed to be contaminated (7.2 ppm, dd; Figure IV-29).

Hence the next set of NMR spectroscopic experiments was carried out in DMF-d_7 . It greatly improved solubility and all reagents were completely dissolved. The reaction was carried out in two steps; reaction conditions for the imine formation were as follows: 1.2 eq. aldehyde, 2.2 eq. NEt_3 at 1 mM in DMF-d_7 . An NMR was taken every three hours for the first day to determine the time point for complete imine formation under the particular reaction conditions. The second step was to investigate the reduction with $\text{NaBH}(\text{OAc})_3$. This was carried out with stepwise addition of reducing agent to determine how many equivalents were needed for full reduction of the amine product and remaining starting material of the aldehyde.

The formation of the intermediate imine is documented in Figure IV-31 over various time points. Table IV-5 displays the ratio between imine formation and aldehyde consumption; the integral of NCH increases whereas CHO decreases. The aldehyde proton did not diminish completely as a slight excess of 1.2 eq. was used in the reaction. The values in the table indicate complete conversion to the imine after an overnight reaction. The tertiary aliphatic protons also shift downfield as documented earlier. This step was completed overnight as well. In combination with the information of the previous NMR experiment, complete imine formation occurred within 12 h.

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Reagents and reaction conditions: (i) 1.2 eq. aldehyde, 2.2 eq. NEt_3 , 0.001 M in DMF-d_7 ; (ii) 2×3 eq. $\text{NaBH}(\text{OAc})_3$, aqueous work-up.

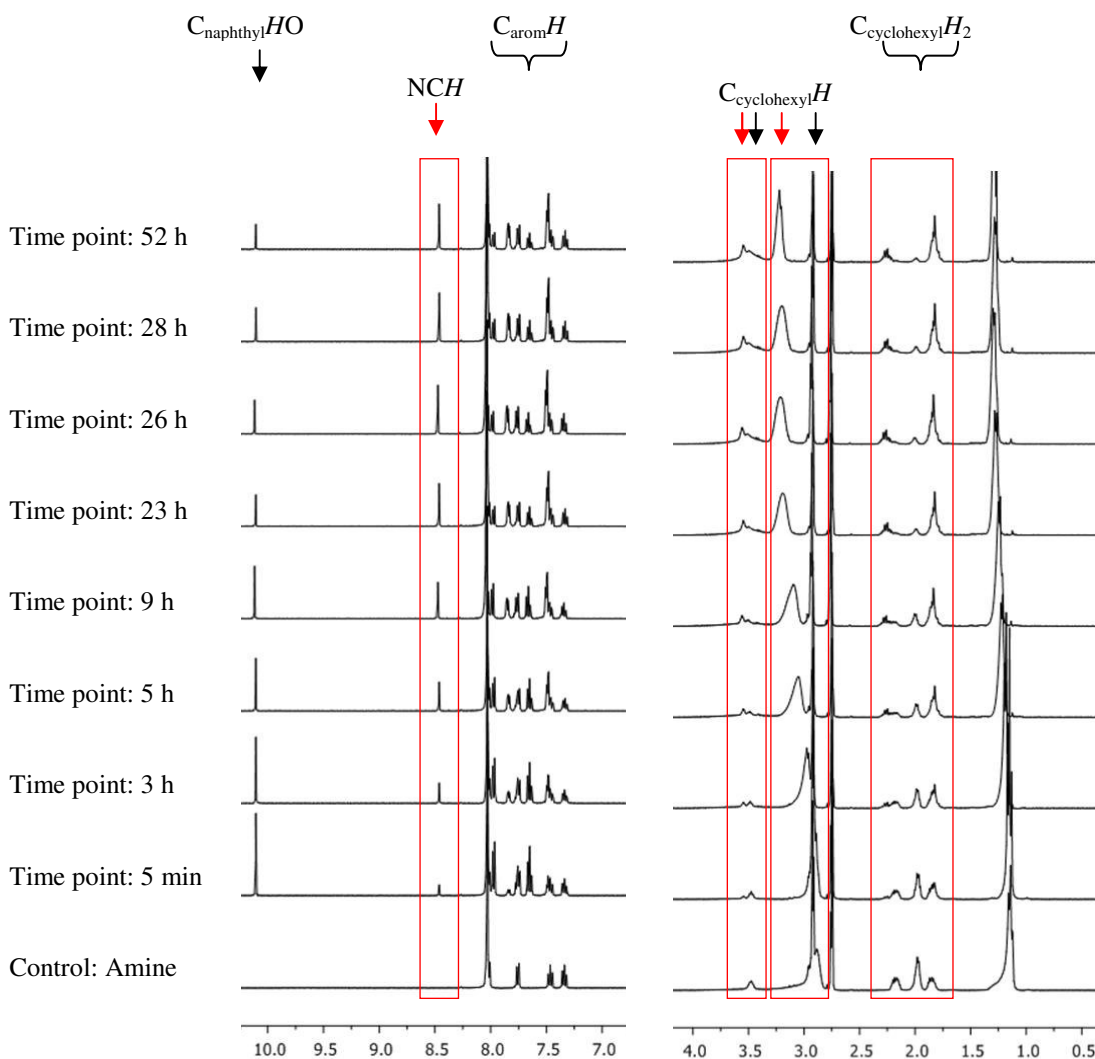


Figure IV-31: NMR analysis of imine formation over time for compound 49

The first step of reductive amination, imine formation, for compound **49** was studied at various time points with NMR (5 min, 3 h, 5 h, 9 h, 23 h, 26 h, 28 h, 52 h from 2nd bottom to top). The bottom spectrum of the amine was added for analysis as a control. Synthesis as carried out in DMF-d_7 ($\delta_{\text{H}} = 8.03$ ppm). Protons of NEt_3 can be found at 1.2 and 2.6 ppm. The characteristic protons are highlighted in red boxes and pointed out with red arrows. The characteristic peak for the imine proton was at 8.3 ppm. The aliphatic protons of the starting material are pointed out with a black arrow and the shifted peaks of the same proton of the imine intermediate with a red arrow.

Table IV-5: Progression of imine formation

The table lists the ratio of the imine proton vs. the aldehyde proton with regards to Figure IV-31. The integral was calculated with respect to the aromatic hydrogen from the amine substrate.

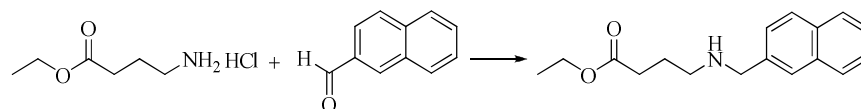
Reaction Time	5 min	3 h	5 h	9 h	23 h	26 h	28 h	52 h
NCH	0.17	0.17	0.55	0.59	0.82	0.83	0.80	0.94
CHO	1.0	0.83	0.67	0.56	0.40	0.17	0.36	0.34

For the second step of the reaction, the reduction of the imine, 3 eq. of reducing agent in DMF- d_7 was added to the reaction mixture. NMR spectroscopic analysis showed that the imine proton was completely gone but to reduce all of aldehyde substrate a second batch of 3 eq. $\text{NaBH}(\text{OAc})_3$ had to be added (data not shown).

To study other parts of reductive amination more closely, a model reaction was devised and carried out in deuterated solvent at 0.1 M concentration of the aldehyde to follow the reaction by NMR. The first aspect was the influence of the amount of aldehyde substrate on the reaction time of the initial imine formation which was not looked at in the previous two NMR experiments. Table IV-6 suggests that addition of base to the amine salt was essential for imine formation as it breaks it up. Also, the reaction time was greatly reduced from 24 h to just 3 h with a slight excess of aldehyde. Imine formation was nearly complete within 24 h using 1.0 eq. aldehyde but when the amount of aldehyde substrate was increased to 1.5 eq., full imine formation was observed after 3 h. The order of addition was also investigated by NMR spectroscopy but there was no noticeable difference in reaction time between later additions of reducing agent or a one-pot synthesis (data not shown).

Table IV-6: Model reaction to study reductive amination

Various reaction conditions were explored such as the amount of aldehyde, base, reaction time and order of addition to develop the speed of formation of the intermediate imine and subsequent reduction to the amine species.

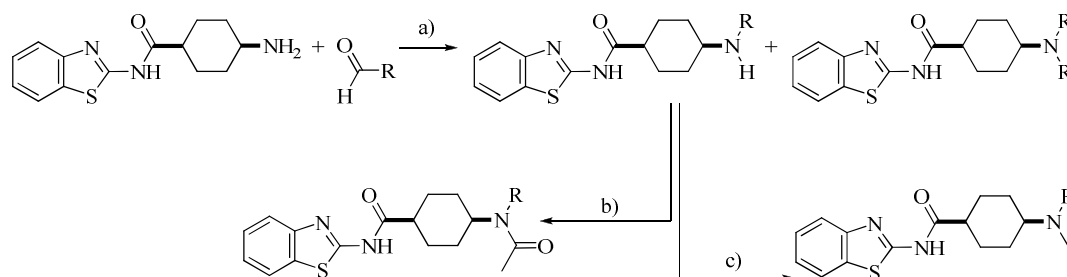


Amine	Aldehyde	Base	Reaction Time	Imine Proton*
1.0 eq.	-	1.2 eq.		0%
1.0 eq.	1.0 eq.	-	24 h	0%
1.0 eq.	1.0 eq.	1.2 eq.	3 h / 24 h	83% / 92%
1.0 eq.	1.5 eq.	1.2 eq.	3 h	97%

* yields based on integral area of peak in NMR

IV.3.4 Investigation of the A-B Linkage

Crystallographic studies, as seen in Figure II-14, indicated that the C-terminus of NMT, which partially forms the peptide binding site, may interact with secondary amine moieties (Hasegawa, Morikami et al. 2003). This was examined more closely for this compound series. The 2° amine functionality was modified for three selected units A (naphthyl, benzyl and benzofuran) and *in vitro* assays were performed to examine the contribution of this amine (Figure IV-32).

**Figure IV-32: Modification of the A-B Linkage**

Variations of secondary amine moieties can be attained either through overalkylation during the reductive amination step (a), acetylation (b), or alkylation (c).

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Modifications of the secondary amine bond that were looked into include dialkylated species, the by-product of the reductive amination step (Figure IV-32a), acetylation of the 2° amine (Figure IV-32b) and methylation (Figure IV-32c). As pointed out earlier, reductive amination of a primary amine can easily result in overalkylation. The dialkylated by-product was isolated for testing as well as the secondary amine product, which was then modified further.

Acetylation of secondary amines was achieved with acetic anhydride under solvent free conditions (IV.2.3.5, p. 87). A moderate yield of 61% was obtained after purification procedure for **83**. Acetylation of compounds **84** and **85** also resulted in yields of 74% and 14% respectively (Figure IV-33).

Methylation was carried out with **35** (IV.2.3.6, p. 87); however, a dimethylated species was isolated. According to mass spectroscopy, the 2° amine that was methylated did not result in a quaternary amine. The second CH₃-group could be attached to the nitrogen of the amide bond (Figure IV-34).

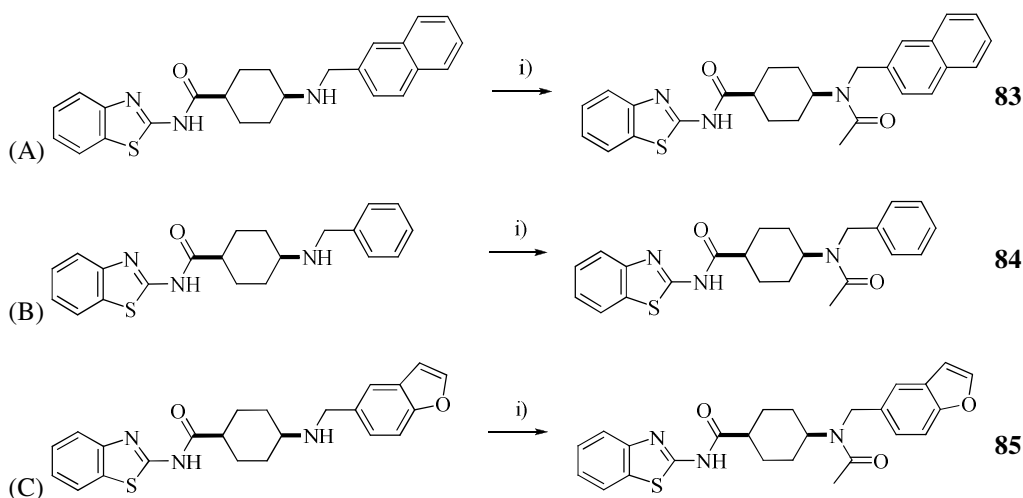


Figure IV-33: Synthesis of compounds 83, 84 and 85

Reagents and reaction conditions: (i) 1.0 eq. 2° amine, 1.0 eq. NEt_3 , 0.2 M in Ac_2O , 2 h, RT.

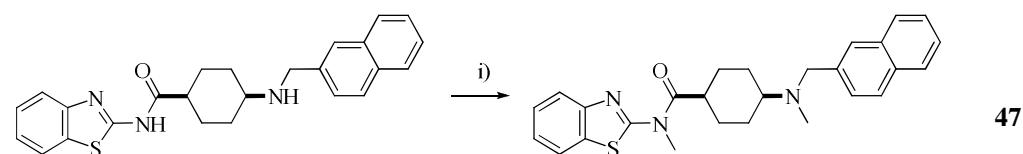


Figure IV-34: Synthesis of compound 86

Reagents and reaction conditions: (i) 1.0 eq. 2° amine, 2.0 eq. ICH₃, 3.0 eq. K₂CO₃, 0.1 M in DMF, no hv, 18 h.

IV.4 Conclusion and Discussion

A chemical library was established with analogues based on UK-370,509. The lead compound from the “Pfizer series” was successfully re-synthesised in a four-step route with an overall yield of 40%. It began with Boc-protection of the amine moiety of *cis*-1,4-aminocyclohexylcarboxylic acid, building block B, with good yields of 70%. The C-terminus was coupled to 2-aminobenzothiazole in a peptidic coupling step with excellent yields of 90%. After Boc-deprotection in strong acidic conditions, the N-terminus was reacted with an aldehyde, building block A, to form a secondary amine bond under reductive amination conditions with moderate yield of 60%. Compound **35** was verified with spectroscopic methods including NMR, IR and mass analysis.

Each step of the synthesis was systematically optimised to facilitate synthesis of numerous lead compound variants for the library. The formation of the amide bond connecting the C-terminus of building blocks B with the N-terminus of 2-aminobenzothiazole was examined with three different units B; *cis*-1,4- and *cis*-1,3-aminocyclohexylcarboxylic acid, as well as proline. Out of various peptide coupling conditions such as acyl halides, HBTU/HOBt and PyBOP, the last gave by far the best results of 70–90% yield.

The focal point of this library was building block A to improve potency and selectivity against *Plasmodium falciparum* NMT with respect to human NMT. A large range of aldehyde derivatives was used to explore several characteristics of the target binding site. Examples included variations in size, aromaticity, electron withdrawing and donating properties, as well as H-donor and -acceptor groups. Parallel synthesis was carried out on a small scale, 0.1 mmol. Aldehydes were attached in a reductive amination step to 1° amine functionalities of R^{CB}. For 2° amines, the N-terminus was alkylated with halide substrates. However, it was found that the yield greatly increased when 2° amines were also subjected to the reductive amination protocol. The reaction mechanism of reductive amination of secondary amines is similar to that of primary amines (Figure IV-25); however, it involves an iminium ion as the reaction intermediate.

Several issues arose, one being limited solubility. Initially the reactions were carried out in DCM for more facile work-up. However, it was found that reactions worked

better in DMF–DCM. Solubility of the starting material, reagents and product were greatly enhanced. To eliminate water from the reaction, anhydrous solvents and reagents, as well as pre-activated molecular sieves were employed to increase yields.

Reductive amination was carried out in two steps with 2.0 eq. aldehyde to form the imine followed by subsequent reduction with reducing agent after 1 h and aqueous work-up. Average yields were relatively low, typically 15%, although yields of 50% were also observed. A competitive side reaction was observed with higher yields of disubstituted by-product. It was found that an excess of aldehyde favoured overalkylation. The ratio of monosubstituted product vs. disubstituted by-product was dependent on the properties of the aldehyde such as size and aromaticity. Disubstituted by-products were reduced by 1/10th by using less aldehyde in the reaction mixture, typically 1.0 eq. The average yield was increased to 60%.

To improve yields even further, the two-step reaction was analysed thoroughly with NMR spectroscopy. It was found that after 1 h reaction time, imine formation was not completed. Only about 20% conversion was observed after a period of 3 h. That would support the observed average yields above. NMR studies indicated that imine formation was complete after overnight reaction with reduction occurring spontaneously. Hence further reactions for next generation libraries should employ 1.0 eq aldehyde to react with the amine for 24 h before addition of reducing agent.

Further research could go into the selectivity of C⁶-substituents against *N*-myristoyl transferase for different species. It could be attached *via* ring formation of the benzothiazole. It was examined in a Master thesis project (Tomlinson 2007). After initial solubility issues of the product after ring formation, it was decided to switch from combinatorial solution chemistry to solid phase assisted synthesis to build a small scale library as it is a faster method to prepare a bigger number of compounds simultaneously. The C⁶-position was substituted with a carboxylic acid (Matsuoka 1997) that could in turn be attached to resins, for example rink amide resin. Unfortunately, the attempt to exploit solid phase assisted synthesis has not yet been achieved. Types of resins, most favourable reagents, coupling hours and cleavage cocktails need to be investigated further. In summary, the lead compound from the “Pfizer series” had been re-synthesised successfully in high yields. The four-step synthetic route was optimised for the generation of a small-sized library of analogues

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which could then be tested *in vitro* for potency against PfNMT and selectivity over human NMT (Chapter V).

V *IN VITRO* EVALUATION OF INHIBITORS

V.1 Introduction

The potential of synthesised antimalarial candidates was explored initially *in vitro* prior to testing in *in vivo* systems (Chapter VI). The combination may aid to evaluate inhibitory modes against PfNMT and outline structure-activity relationships (SAR) to guide further improvement for antimalarial drug design.

There are two main formats of assays for NMT activity that have been reported in the literature: continuous and discontinuous. The latter method has generally employed radioactively labelled substrates. The label is incorporated into the product making detection simple due to the extremely sensitive tag, usually tritium. Kinetic analysis of the reaction requires multiple sample extractions and product isolation of radioactive material, which can be done in various ways; ELISA (Takamune 2002), HPLC (Towler and Glaser 1986; Glover, Goddard et al. 1988; McIlhinney and McGlone 1989), IEX (Paige, Chafine et al. 1989), selective adsorption on acidic alumina (Wagner and Retey 1990) and phosphocellulose paper binding (King and Sharma 1991; Panethymitaki, Bowyer et al. 2006). These assays have the advantages of high sensitivity and hence the need for only small amounts of reaction material; this outweighs the disadvantages of needing to take samples of product over the reaction time course and the inherent hazards associated with radioactive assays.

Continuous assays allow for automated measurements to study the progress of the whole reaction, which results in an increased number of data points. Fluorescent tags are an alternative to radioactive manipulation (Pennise, Georgopapadakou et al.

2002). This assay format is generally more cost effective and easier to handle, yet the relative insensitivity requires large amounts of substrate and a bulky fluorophore may prevent substrate interactions. Similar problems of high dose requirements of reagents also occur with reported enzyme linked assays where, for example, CoA formation was coupled to the NAD⁺/NADH system and pyruvate dehydrogenase whereby NADH formation was continuously detected spectrophotometrically (Boisson and Meinel 2003). Continuous assays would, in principle, be valuable for kinetic studies; however, the sensitivity limitations and consequently higher concentration of substrates and enzyme make this format impractical for our studies.

The method employed in this project was based on a published medium-throughput discontinuous assay (French, Zhuang et al. 2004). In this publication, [³H]-labelled myristoyl CoA was reacted with biotinylated peptide in the presence of HsNMT1 to form tritiated myristoylated biotinylated peptide which was then bound to streptavidin-coated 96-well plates. Reactants were washed off and wells filled with scintillant fluid to allow the amount of isolated product to be measured. This method was adapted by using streptavidin-coated scintillation beads instead (Bowyer, Gunaratne et al. 2007). Washing steps were eliminated and handling of radioactive material was reduced.

V.1.1 The Scintillation Proximity Assay

The inhibitory properties of the compounds were analysed in the SPA, as outlined in Figure V-1, against *Plasmodium falciparum* N-myristoyl transferase (PfNMT) for potency and two *Homo sapiens* NMTs (HsNMT1 and HsNMT2) for selectivity. [³H]-Labelled myristoyl CoA and biotinylated peptide were incubated in the presence of NMT to form radiolabelled myristoylated biotinylated peptide. The inhibitor will interfere with N-myristoylation of the peptide substrate which was based on the N-terminus of PfARF1; GLYVSRLFNRLFQKK(biotin) (Stafford, Stockley et al. 1996; Gunaratne, Sajid et al. 2000; Bowyer, Gunaratne et al. 2007). A mixture of unreacted peptide and N-myristoylated peptide were subsequently captured by streptavidin-coated scintillation beads due to the high affinity between biotin and streptavidin. The tetrameric protein has four biotin-binding sites, of which each forms multiple H-bonds and van der Waals interactions with biotin (Figure

V-2). Upon binding, the surface polypeptide loops arrange themselves to bury biotin in the protein interior resulting in strong bonds (Weber, Ohlendorf et al. 1989).

The close proximity of the tritium label in the captured species to the polymer beads containing scintillant fluid enables energy transfer of the β^- -decay of tritium (Equation V-1). The energy released, with an approximate travel path of 1.5 μm , is transferred to the scintillant molecules, which in turn causes excitation of the fluorophore. Upon relaxation, energy is emitted in the form of light, which is detected by the scintillation counter and converted into electrical signals. Only tritium labels within close range to the scintillant exhibit an effect; this makes physical separation between unbound and bound material redundant, minimising time and handling of radioactive material.

Read-out values were used to interpret and quantify *N*-myristoylation. High counts indicate a great level of *N*-myristoylation and reduced signals suggest substantial levels of inhibition. A plot of enzyme activity as a function of inhibitor concentration gives an inhibition curve from which an IC_{50} was derived. The IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro*. The data was used to rank the inhibitors from which structure-activity relationships were deduced.

The plot was performed with GraFit, using non-linear regression analysis involving a 3 parameter fit to the data (Equation V-2B). Regression analysis is a technique to calculate the best fit curve in minimising the deviation of data from the curve (Leatherbarrow 2007).

V.1.2 Kinetic Analysis

Inhibitors were not designed for the myristoyl CoA binding site, hence concentration of myristoyl CoA substrate in the assay should be at a saturating level, well above K_m . Due to the Bi Bi reaction mechanism, a saturated myristate binding pocket will lead to an accessible peptide binding site for either peptide substrate or inhibitor; evaluating the level of inhibition of *N*-myristoylation of the compounds.

The K_m is the substrate concentration at which the rate of reaction is half its maximum value. Kinetic parameters (K_m and V_{max}) were derived according to the Michaelis Menten model (Equation V-3, Figure V-3) with GraFit.

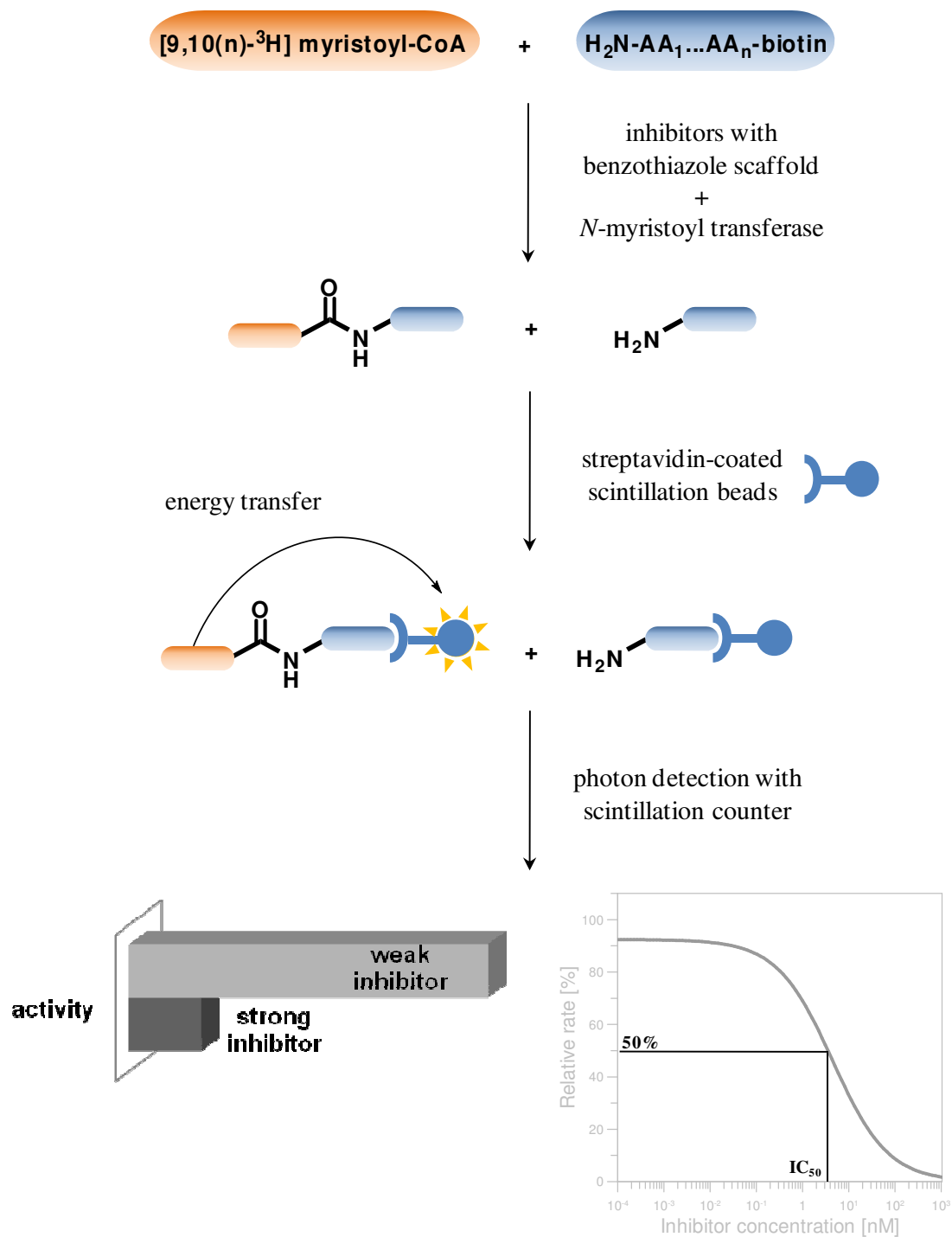


Figure V-1: SPA to test inhibitors against NMT

SPA was used to quantify inhibitory properties against NMT. Tritium-labelled myristate and biotinylated peptide substrate were incubated in the presence of NMT as well as the inhibitors. Both unmyristoylated and myristoylated, biotinylated peptide were captured with streptavidin-coated scintillation beads. Only the radioactive myristoylated biotinylated peptide produced signals, which were interpreted by the scintillation counter and transformed into electrical pulses. Ratios of signal to inhibitor concentration were used to evaluate relative IC₅₀-values.

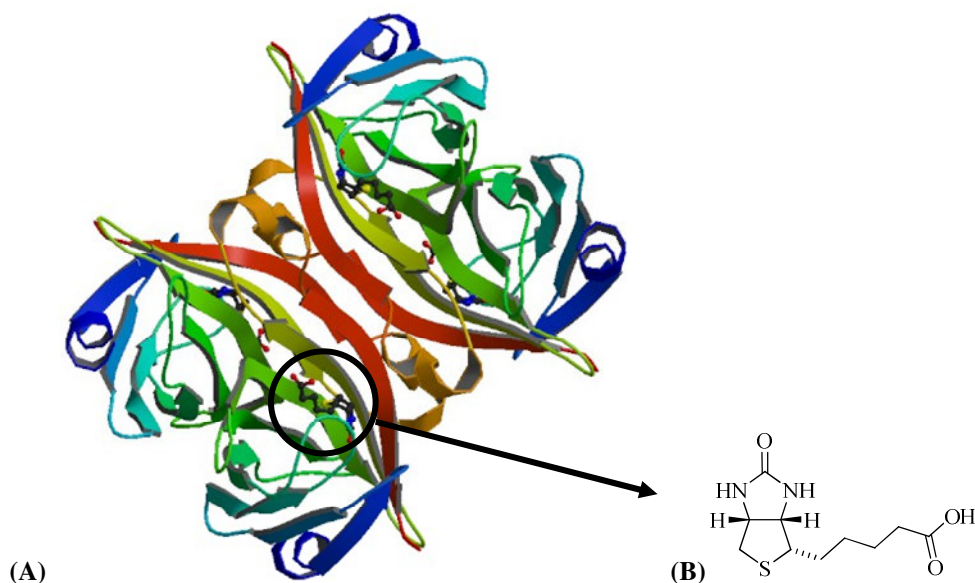


Figure V-2: Assumed biological unit of a saturated streptavidin-biotin complex

(A) This crystal structure shows the biological unit of tetrameric streptavidin with four coordinated biotin molecules (pdb file 1stp, (Weber, Ohlendorf et al. 1989)); (B) Biotin.



Equation V-1: Beta minus decay

Radioactive decay of tritium is referred to as beta minus decay (β^-) whereby neutrons are converted to protons forming stable nuclei of helium. Furthermore beta particles similar to electrons as well as antineutrinos are emitted.

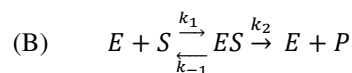
$$(A) \quad y = \frac{\text{range}}{1 + \left(\frac{x}{IC_{50}}\right)^s} + \text{background} \quad (B) \quad y = \frac{100\%}{1 + \left(\frac{x}{IC_{50}}\right)^s}$$

Equation V-2: Equations for best fit curve

The data is either fitted to a 4 parameter (A) or a 3 parameter equation (B) if background corrected. A graph is plotted from this data (rate of reaction vs. substrate concentration) from which an IC_{50} value can be determined.

(Legend: y is the response, x is the concentration of substrate, range is the maximum y range, s is the slope factor).

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



$$(C) \quad K_m = \frac{k_{-1} + k_2}{k_1}$$

$$(D) \quad \frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

Equation V-3: Michaelis Menten kinetics

The basic Michaelis Menten enzyme kinetic equation (A) to calculate V_{max} and K_m of the substrates is derived from the enzymatic reaction (B) by substituting constants for K_m , the Michaelis constant (C). For straightforward graphical interpretation values are plotted in a double reciprocal (Lineweaver-Burk) plot (D).

(Legend: v is the rate of reaction where all enzymes are unbound, V_{max} is the maximum rate where the enzyme is completely saturated with substrate, $[S]$ is the substrate concentration, K_m is the Michaelis constant)

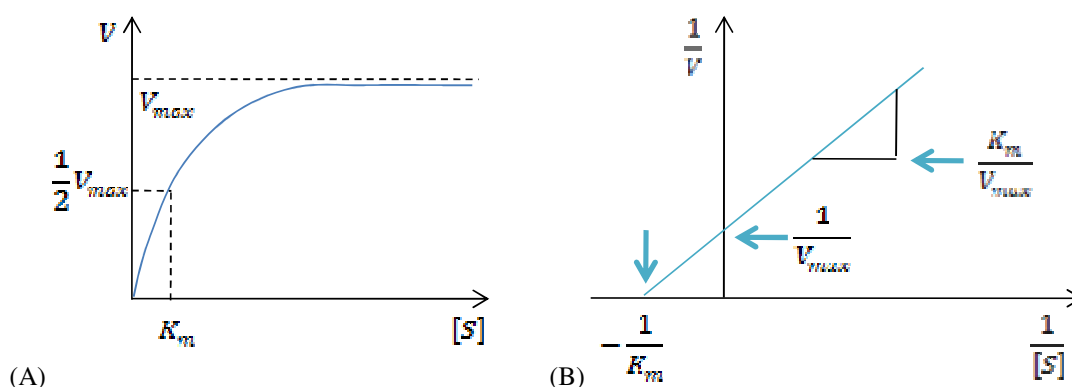


Figure V-3: Graphical evaluation of enzyme inhibition

The graph illustrates an enzyme saturation curve with plotted data of rate of reaction vs. substrate concentration (A). The double reciprocal conversion leads to the Lineweaver-Burk plot (B) from which kinetic constants can easily be determined.

V.2 Material & Methods

V.2.1 Chemicals and Reagents

Solvents and reagents of the highest available purity were obtained from commercial sources (BDH, GE Healthcare [formerly known as Amersham Biosciences], Sigma-Aldrich). Ultrapure laboratory grade water was obtained from in-house Elix[®] and MilliQ[®] millipore water purification systems. All solutions were prepared in ultrapure laboratory grade water.

³H-[9,10]-Myristoyl CoA and PVT scintillant beads were supplied by GE Healthcare. Unlabelled myristoyl CoA was purchased from Sigma. The peptide substrate was synthesised by Dr P W Bowyer (Bowyer 2006). Confirmed active candidates assayed for comparative purposes were supplied by Pfizer PLC. The myristoyl CoA analogue *S*-(2-oxo)pentadecyl CoA, named non-hydrolysable myristoyl CoA, was used as a positive control and was synthesised by Dr E W Tate. The triazole-based compounds **87**, **88**, **89**, **90**, **91**, **92**, **93** were made by J Sudlow as part of a Master thesis project (Figure V-4, (Sudlow 2008)). The synthesis of all other inhibitors is described in Chapter IV.

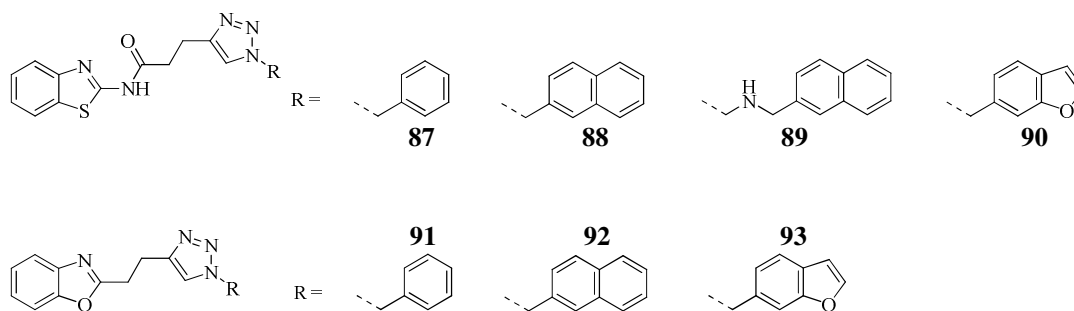


Figure V-4: Triazole-based inhibitors

V.2.2 Methods and Equipment

Buffered solutions are catalogued in Table V-1. The pH was measured at room temperature with a pH meter (Hanna instruments 8519). Calibration was performed with ‘colour key’ buffer solution from BDH (red – pH 4.0 ± 0.02 at 20 °C, yellow – pH 7.0 ± 0.02 at 20 °C). Adjustments of the pH were made with a suitable acid or base, e.g. HCl, NaOH, NH₃.

Plates for the SPA were standard opaque-coloured 96-well plates (Perkin Elmer) with separate thin film seals from Nunc, Apogent. A procedure of heating (Digital Heatblock, VWR) in combination with vortexing (Vortex-Genie2, Scientific Industries) was employed to improve solubility of inhibitors. Plates were incubated in an oven, set at 37 °C (IP60, LTE). Signal counting was performed with a Plate Chameleon multilabel reader (Hidex Oy, Finland) and analysis carried out with MikroWin software (MikroWin 2000, Version 4.33, Mikrotek Laborsysteme GmbH). Kinetic data such as K_m -determination and IC_{50} -values were evaluated with GraFit (Leatherbarrow 2007).

V.2.2.1 General SPA Procedure

The reactions were carried out in standard opaque 96-well plates. 25 μ L Buffer or inhibitor, 25 μ L NMT solution and 25 μ L 125 nM [^{3} H]-myristoyl CoA at a specific activity of 8 Ci mmol $^{-1}$ were incubated for 5 min at 37 °C. To the reaction mixture was added 25 μ L 250 nM peptide solution and it was further incubated for precisely 30 min at 37 °C. The reaction was quenched by the addition of 100 μ L Bead-Stop Solution (Table V-1E). The plate was then sealed with a new film because of condensation during the incubation step, which led to reductions in signal. Measurements were performed the following day on a Plate Chameleon multilabel reader (Hidex Oy, Finland) to ensure complete settlements of the beads.

V.2.2.1.1 Assay Substrates

The components of the SPA buffer (30 mM Tris/HCl at pH 7.4, 0.5 mM EDTA and EGTA) were adopted from literature (King and Sharma 1991). DTT as well as Triton X-100 was added additionally (Table V-1A). Instead of 2.5 mM DTT and 1% Triton X-100 (Bowyer, Gunaratne et al. 2007), 1.25 mM DTT and 0.1% Triton X-100 were sufficient. Aliquots of 20 $\times$ Tris/HCl, EDTA, EGTA and 1 M DTT (800 $\times$) were stored separately at -20 °C. A 10% Triton X-100 solution (100 $\times$) was stored at RT.

The Stop Solution (Table V-1C) was prepared fresh as storing the mixture at RT resulted in precipitation, most likely due to MgCl $_2$. The Bead Solution (Table V-1D) was stored at 4 °C.

Stocks of [^3H]-myristoyl CoA at 3.51 μM at a specific activity of 8 Ci mmol^{-1} were prepared with dilutions of 3.51 μM cold myristoyl CoA and 3.51 μM [^3H]-labelled myristoyl CoA at a specific activity of 32 Ci mmol^{-1} . Finally, the mixture was diluted further with 4% DMSO Standard SPA Buffer for a final concentration of 125nM at a specific activity of 8 Ci mmol^{-1} . The specific activity for inhibition studies was always 8 Ci mmol^{-1} unless otherwise stated. Unlabelled myristoyl CoA was stored in 200 μM stocks in ethanol–0.01 M NaOAc pH 5.6 buffer (1:1, V:V) at 4 °C. [^3H]-Myristoyl CoA was stored at -20 °C.

DMSO peptide stocks (GLYVSRLFNRLFQKK(biotin)-NH₂) were prepared in 4% DMSO Standard SPA Buffer at a top concentration of 200 μM and were stored at 4 °C.

In these SPA studies, 73 ng (1.40 μmol) PfNMT, 3 ng (0.06 μmol) CaMNT, 6 ng (0.11 μmol) HsNMT1, 83 ng (1.46 μmol) HsNMT2 and 300 ng (6.12 μmol) LmNMT were employed. Aliquots of NMT were kept in storage buffer (Table V-1F) at -20 °C. To prevent copious freeze-thaw cycles, thawed enzymes were used once. NMT was thawed on ice and prepared in cold 4% DMSO Standard SPA Buffer just before use.

Table V-1: List of buffered solutions for the *in vitro* assay

Name of Buffer	Components
(A) Standard SPA Buffer	30 mM Tris $\times$ HCl (adjusted to pH 7.4 with HCl), 0.5 mM EGTA, 0.5 mM EDTA, 1.25 mM DTT, 0.1% Triton X-100
(B) 4% SPA Buffer	4% DMSO in Standard SPA Buffer (A)
(C) Stop Solution	0.2 M H ₃ PO ₄ (buffered to pH 4.0 with NaOH), 750 mM MgCl ₂ , 0.05% (w/v) BSA, 0.01% (w/v) NaN ₃
(D) Bead Solution	50 mg mL ⁻¹ PVT scintillant beads in PBS/0.05% NaN ₃
(E) Bead-Stop Solution	2% Bead Solution (D) in Stop Solution (C)
(F) Storage Buffer	20% glycerol in Standard SPA Buffer (no DTT, no Triton X-100)

V.2.2.1.2 Controls

Several interplate controls were included in each SPA experiment to ensure optimal enzyme activity, such as an enzyme dilution series and enzymatic assays over time.

The dilution series was carried out with a dilution factor of 1.5 at five different concentrations. The performance of the enzyme was examined over time at 1, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 90 min.

Other controls include no enzyme, which served as the background control. This value was subtracted from every signal throughout the course of the assay.

V.2.2.1.3 *Inhibitors*

A collection of inhibitors, 100 mM, 25 mM and 10 mM, were prepared in dry DMSO and stored at -20 °C. Stocks were diluted 25-fold with 50 °C warmed-up Standard SPA Buffer (Table V-1A) for *in vitro* tests. Furthermore, inhibitors were incubated at 50 °C in a heating block for 1 h to enhance solubility.

V.2.2.1.4 *IC₅₀-Determinations*

All inhibition assays were carried out in triplicate. The percentage activity was calculated from uninhibited reaction vs. inhibited reaction. Thus a negative control with no inhibitor was included on every plate, as well as a strong inhibitor of known activity for a positive control. Non-hydrolysable myristoyl CoA was chosen to ensure that the efficacy of batches of enzymes were comparable.

As an initial evaluation of an unknown compound, “single point” assays of potential inhibitors were performed in triplicate at a single concentration (100 µM) against various NMT enzymes in order to save time and material. All inhibitors that showed inhibition in the single point assay at 100 µM against recombinant NMT enzymes were assessed more thoroughly. IC₅₀-values were determined over a range of 12 concentration points. The top three concentration points were 1 000, 250 and 100 µM. In well numbers 4–12, dilutions were carried out from 100 µM with a dilution factor of 1.5 typically.

V.2.2.1.5 *K_m-Determinations*

The assay was carried out according to V.2.2.1 (p. 128). Different concentrations of tritiated myristoyl CoA (500–2 nM) were prepared in 2-fold dilution steps to investigate the myristate binding pocket. The final concentration of biotinylated peptide was kept constant at 250 nM. The inhibitors were added at a concentration around the respective IC₅₀-value. The approximate amount of PfNMT in each well was 73 ng (1.40 µmol) and 3 ng (0.06 µmol) for CaNMT.

V.3 Results

V.3.1 Kinetic Analysis of Substrates

V.3.1.1 Mode of Inhibition

To evaluate the mode of inhibition (MOI) of the drug molecules, the effect on IC_{50} was examined using different concentrations of one substrate at a constant value of the second substrate. This shows which binding pocket (peptide or myristate) is blocked by the inhibitor and also gives insight into the degree of competitiveness. The IC_{50} -value of one inhibitor was compared for varying concentrations of peptide and myristoyl CoA. Conclusions about competitive binding for either substrate can be drawn from the IC_{50} -shift.

The myristate binding pocket was investigated by changing concentration of myristoyl CoA and subsequent analysis of the inhibitory effect of two structurally different compounds. Non-hydrolysable myristoyl CoA (Figure V-5A) was thought to be competitive against myristoyl CoA (Figure V-5B) due to its high structural similarity. Compound **54** (Figure V-5C) on the other hand was hypothesised to target the peptidic binding domain. By variations in the concentration of myristoyl CoA, the IC_{50} of competitive inhibitors will vary accordingly. Yet IC_{50} -values of uncompetitive inhibitors will remain fairly constant.

The MOI studies were performed with inhibitor concentration producing 50% inhibition *in vitro* to clearly see effects of changing substrate concentration. The IC_{50} of **54** was calculated as $19.9 \pm 2.7 \mu\text{M}$; hence 20 μM of inhibitor **54** was the working concentration in the assay. Non-hydrolysable myristoyl CoA was a much more potent inhibitor with an IC_{50} of $3.4 \pm 0.8 \text{ nM}$; 10 nM of non-hydrolysable myristoyl CoA was used in the SPA. The final concentration of myristoyl CoA substrate at a specific activity of 8 Ci mmol^{-1} varied from 250 nM to 2 nM over 12 concentration points with a dilution factor of 2.

Non-hydrolysable myristoyl CoA closely resembles myristoyl CoA, the main substrate, from which the myristic acid group is transferred to N-terminal Gly of substrate peptides during N-myristoylation. Non-hydrolysable myristoyl CoA bears an additional CH_2 -group in between the thioester, splitting the functional group to ketone and thioether moieties (Figure V-5A and B). The lone pair of electrons of the amine from the N-terminus of the peptide will not attack the ketone and thus

N-myristoylation of the peptide will not occur. Figure V-6A displays the kinetic evaluation; non-hydrolysable myristoyl CoA is a competitive inhibitor against myristoyl CoA and thus targets the myristoyl CoA binding pocket. The inhibitor slowed down the rate of reaction at low concentrations of myristoyl CoA. However an increase of substrate may saturate the apoenzyme so that the inhibitor has no access to free binding pockets. The values of the rate of reaction reached similar values to the control reaction upon addition of large amounts of myristoyl CoA. At 500 nM substrate concentration, the fitted blue curve nearly corresponded with the grey line. It could be said that competitive inhibition was overcome at 50-fold concentration of the substrate. $V_{\max}$ -values were equivalent.

On the other hand, compound **54**, which represents the investigated compound series, is an inhibitor that targets the peptidic binding pocket. Figure V-6B suggests **54** is a non-competitive inhibitor for myristoyl CoA. Increase of substrate concentration did not affect the rate of inhibition. A flattening of the curve indicates $V_{\max}$ may never be reached, even at higher inhibitor concentrations. Compound **54** does not target the myristate binding pocket but most likely the peptide binding domain.

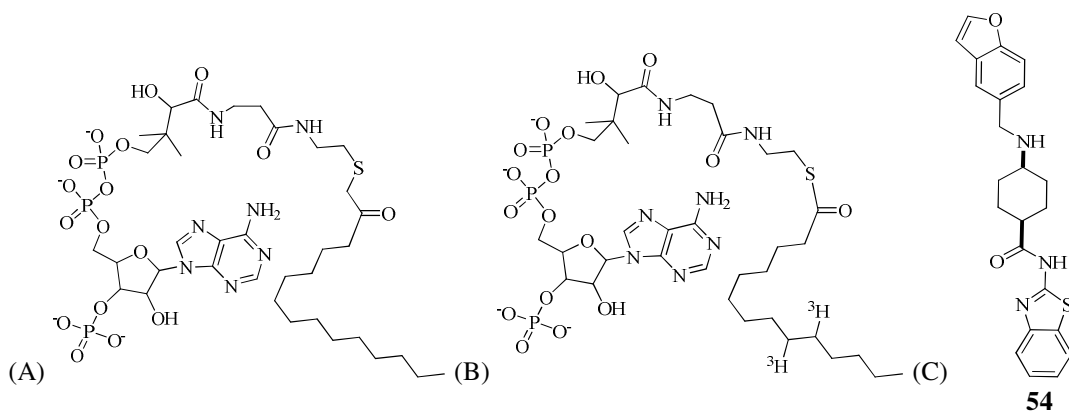


Figure V-5: MOI study

Shifts in IC_{50} for different inhibitors were examined to determine mode of inhibition for potential antimalarials. MOI of a known inhibitor, non-hydrolysable myristoyl CoA (A), that is competitive against myristoyl CoA (B, tritiated substrate) in the myristoyl CoA binding pocket was compared to MOI of an unknown inhibitor **54** (C) which should solitarily target the peptidic binding pocket.

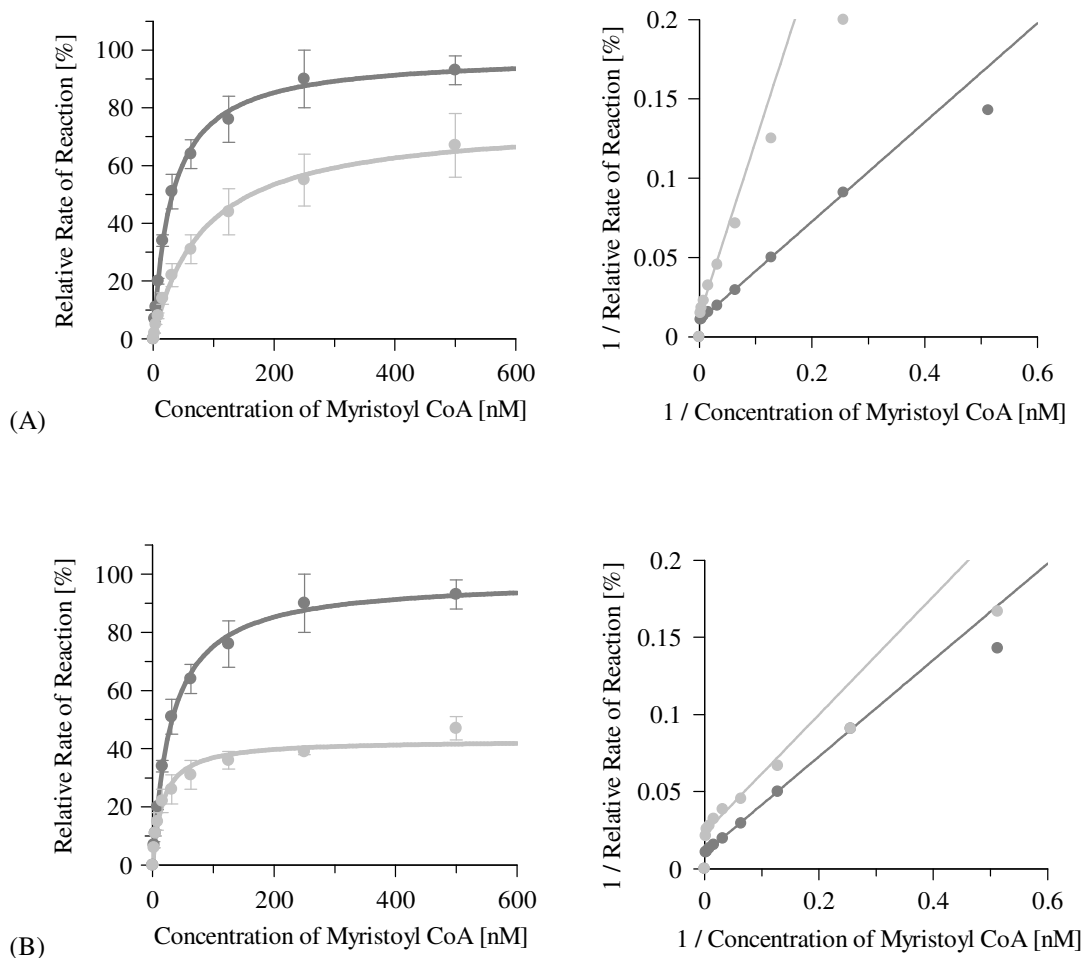


Figure V-6: Influence of myristoyl CoA on enzyme kinetics of inhibitors

The rate of reaction was measured without any inhibitor (dark grey-coloured) and compared to the rate of reaction with inhibitor (light grey-coloured) while changing concentration of one substrate, myristoyl CoA, and keeping the second substrate concentration, that of the peptide, constant. Two inhibitors were evaluated: Non-hydrolysable myristoyl CoA (A) and **54** (B). The small-sized plot is a double reciprocal (Lineweaver-Burk) plot.

V.3.1.2 K_m -Determination

Figure V-7 shows the graphical evaluation of kinetic parameters for myristoyl CoA in PfNMT (A) and CaNMT (B). The K_m for myristoyl CoA for the malarial enzyme was calculated to be 14.3 ± 0.4 nM and for fungal NMT 9.9 ± 1.1 nM. The rate of reaction seemed to be faster for CaNMT but overall K_m -values for myristoyl CoA in PfNMT and CaNMT are similar. This was expected, as the myristoyl CoA binding site is highly conserved throughout species. A final working concentration of 250 nM [^3H]-myristoyl CoA, which is well above K_m , was employed in the SPA. The experimentally determined K_m -value of 14 nM for myristoyl CoA in PfNMT was comparable to literature data (12 nM, (Bowyer 2006)).

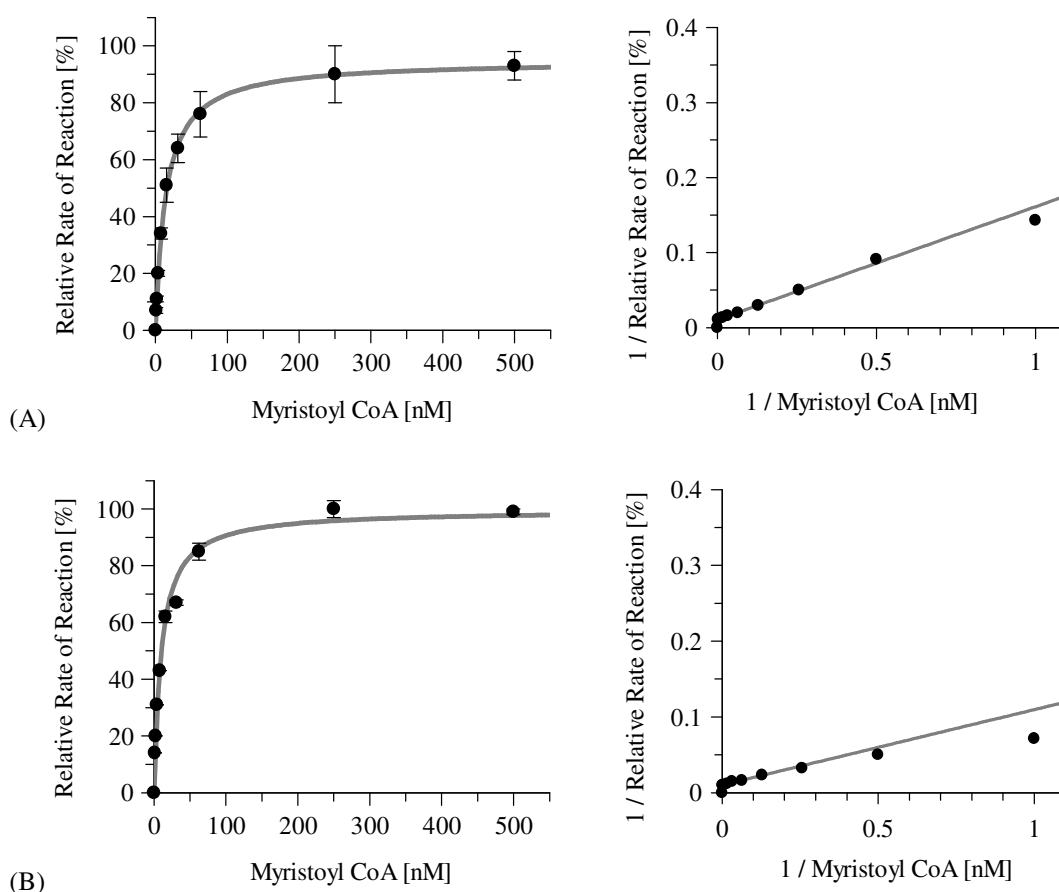


Figure V-7: Determination of K_m for myristoyl CoA

The rate of reaction was measured by varying the concentration of myristoyl CoA; peptide concentration was kept constant. K_m was determined for PfNMT (A) and CaNMT (B) from the fitted data by GraFit from the Michaelis Menten equation. The double reciprocal (Lineweaver-Burk) plot is pictured on the right.

V.3.2 Optimisation Procedures for SPA

V.3.2.1 The SPA Buffer

The highest count of signal was achieved when the SPA buffer was made up fresh every day prior to *in vitro* experiments (Figure V-8A). It was furthermore found that the signal increased by using 1.25 mM instead of 2.5 mM DTT (Figure V-8B). As an alternative to DTT, TCEP, a milder reducing agent for disulfides, was also tried; however, it did not improve enzyme activity (Figure V-8B). 2.5 mM TCEP was even worse than the control without a reducing agent. Overall, the best results were obtained using 1.25 mM DTT.

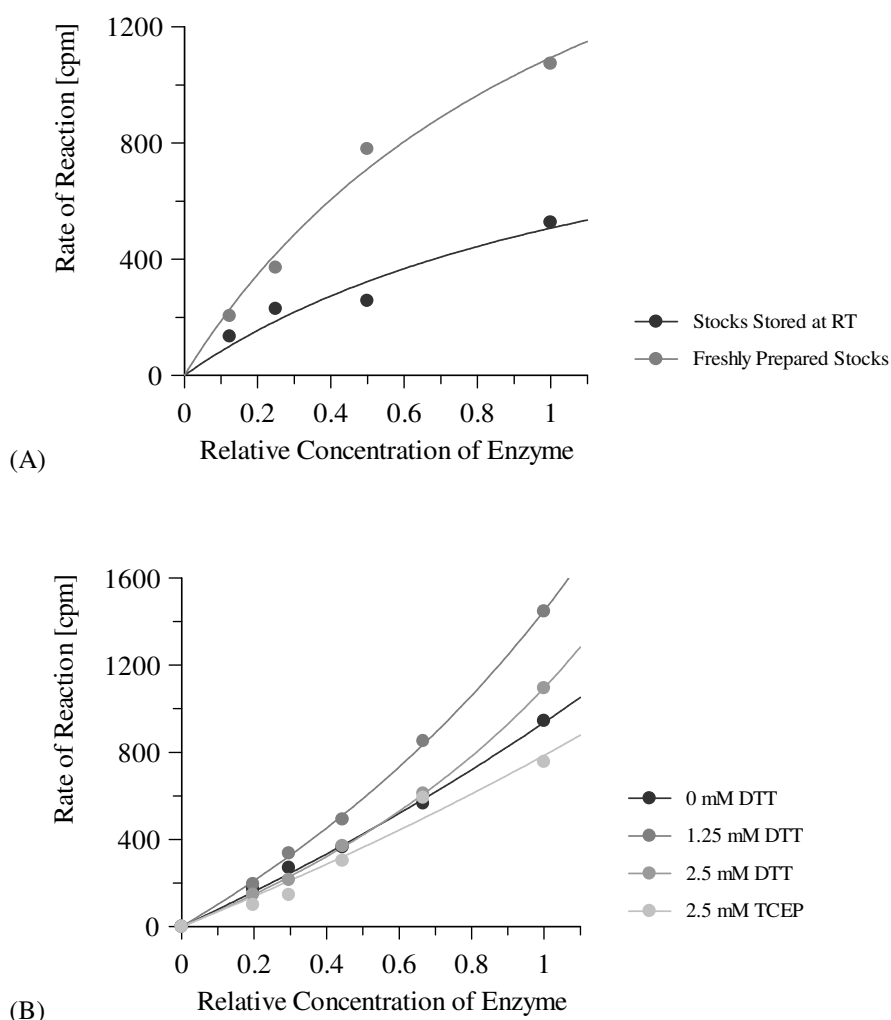


Figure V-8: In the search for optimum concentration of buffer components

Buffer conditions (*CaNMT*, A) were examined as well as different reducing agents (*PfNMT*, B).

V.3.2.2 Substrate Optimisation

The assay aimed for a high signal count that was at least ten times the level of background noise so that inhibition could be easily detected. Signal intensity can also be varied with enzyme concentration in such way that larger amounts of enzyme generally lead to an increase in signal (Figure V-9). However, upon increase of the enzyme concentration substrate depletion may occur represented by the tailing off of the straight line; the amount of 135 ng PfNMT was too high for the SPA. For lower concentrations (45 and 90 ng) however, the graph could be approximated to a straight line throughout the reaction time of 30 min, whereby the amount of enzyme is rate limiting. Thus the gradient can be estimated as the initial rate of reaction.

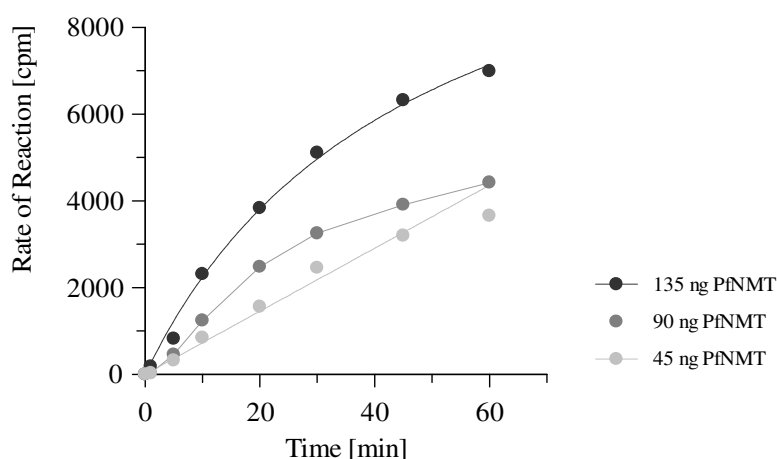


Figure V-9: Signal dependence on enzyme concentration

The rate of reaction was examined with three different amounts of PfNMT; 45 ng (light grey-coloured), 90 ng (medium grey-coloured) and 135 ng (dark grey-coloured). The reaction was stopped at various time points.

Aliquots of enzyme were constantly tested for activity in a dilution series (Figure V-10A) and over time (Figure V-10B). It was observed that *Plasmodium falciparum* NMT was more stable on ice than *Candida albicans* or human NMT. Again, plotted values need to resemble a straight line in the dilution series and be linear up to 30 min for the time series to confirm stable enzyme activity.

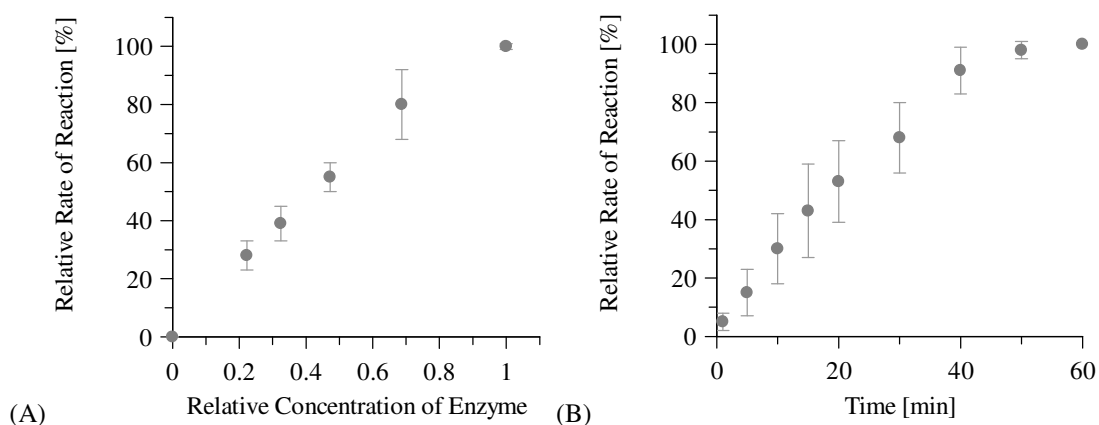


Figure V-10: Time and dilution series

These graphs represent control reactions performed prior to any inhibition studies. Aliquots of PfNMT were analysed by dilution (A) and time (B).

The K_m -value for the peptide substrate GLYVSRLFNRLFQKK(biotin)-NH₂ is $1.2 \pm 0.4 \mu\text{M}$ (Bowyer 2006). To determine the optimum amount of peptide for the assay, the peptide concentration was varied from 500–62.5 nM using 2-fold dilution at four different myristoyl CoA concentration points (62.5 nM, 125 nM, 250 nM, 500 nM). Figure V-11A displays the results, whereby the signal strength of peptide concentration at 62.5 nM and 125 nM were similar at all four myristoyl CoA concentrations. However on increasing the peptide substrate to 250 nM, at least 125 nM myristoyl CoA had to be present for optimum *N*-myristoylation. Best results were achieved with 500 nM peptide and 250–500 nM myristoyl CoA.

However an increase in peptide also meant the addition of more beads to the reaction mixture to sufficiently bind all biotinylated peptides. Results in Figure V-11B show a linear trend, the higher the peptide concentration, the more beads were required to capture everything for evaluation. At a peptide concentration of 500 nM, at least 0.475 mg beads were needed.

As this is a cost-related issue, it was decided to work with 250 nM peptide substrate (well below the K_m of $1.2 \mu\text{M}$ in PfNMT), 0.1 mg streptavidin-coated scintillation beads and 125 nM myristoyl CoA at 8 Ci mmol^{-1} (well above the K_m of 14.3 nM in PfNMT).

V IN VITRO EVALUATION OF INHIBITORS

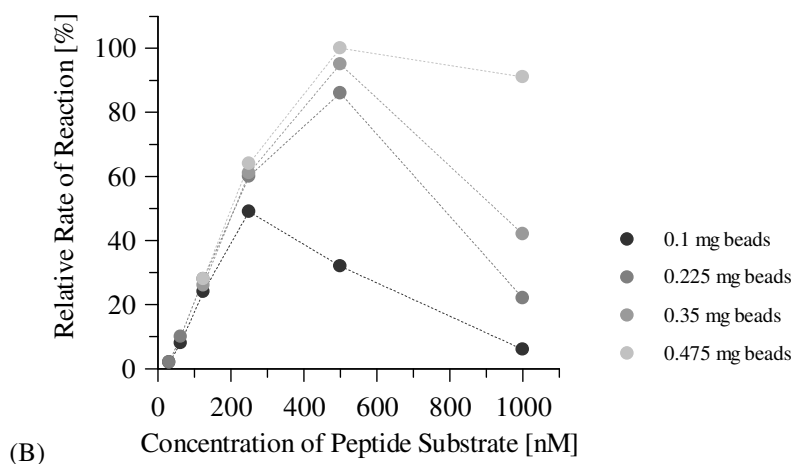
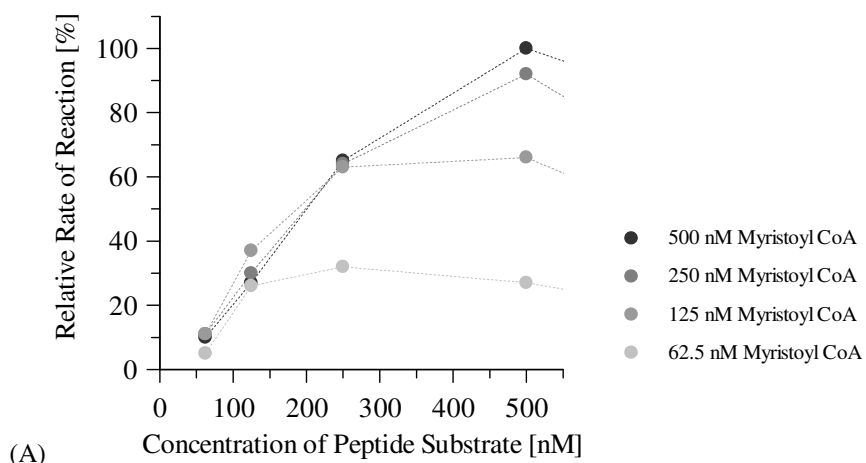


Figure V-11: Substrate optimisation for the SPA

The optimum amount of myristoyl CoA was determined (A) as well as the required amount of scintillation beads for specific peptide concentrations at 250 nM myristoyl CoA (B).

V.3.2.3 The Inhibitors

DMSO is a vital component of the assay, being needed to aid solubility of inhibitors in the buffer and prevent precipitation. It had to be borne in mind that future *in vivo* experiments could not be carried out at high DMSO concentrations as it interferes with the viability of cells. The original 1% DMSO final concentration (Fig. 4.18, (Bowyer 2006)) was increased to 4% to maximise compound solubility.

To further improve solubility of inhibitors, the buffer was warmed up to 50 °C in a heating block before the addition of inhibitors to the Standard SPA Buffer. Sonication was tried but improved dissolution little for moderately soluble compounds (Oldenburg, Pooler et al. 2005). Vigorous vortexing in between heating stages of up to 2 h at 50 °C was employed, a method that appeared to facilitate dissolution.

One possible reasons for precipitation of compounds is too much water present in DMSO stocks (Oldenburg, Pooler et al. 2005). Compounds were therefore prepared in anhydrous DMSO ($\geq 99.9\%$, Sigma-Aldrich) which greatly improved solubility. However DMSO absorbs water readily in freeze–thaw cycles and new batches of inhibitors would have needed to be prepared constantly. Inhibitors were tested in the SPA straight after preparation of the DMSO stocks and freeze–thaw cycles were kept to a minimum.

Nevertheless, precipitation still occurred for many compounds when used at a final concentration of 1 mM. Stepwise addition of Standard SPA Buffer to inhibitor stocks dissolved in 100% DMSO was unsuccessful; compounds were found to precipitate typically at 80% DMSO concentration.

V.3.3 Potency and Selectivity Studies of Inhibitors

V.3.3.1 Interplate Control Reactions

A positive control experiment using non-hydrolysable myristoyl CoA (Figure V-5A) was included whenever the *in vitro* SPA was performed; this was to observe steady enzyme activity and ensure optimal and consistent conditions for the assays. Figure V-12 displays inhibition curves of non-hydrolysable myristoyl CoA against NMT of various organisms, from which IC_{50} -values were deduced. The values are summarised in Table V-2. Non-hydrolysable myristoyl CoA is a potent inhibitor against NMT with activity in the low nanomolar region from 2–17 nM. The inhibition values from all species were within the same range, which is consistent with the highly conserved myristoyl CoA binding site in the enzyme.

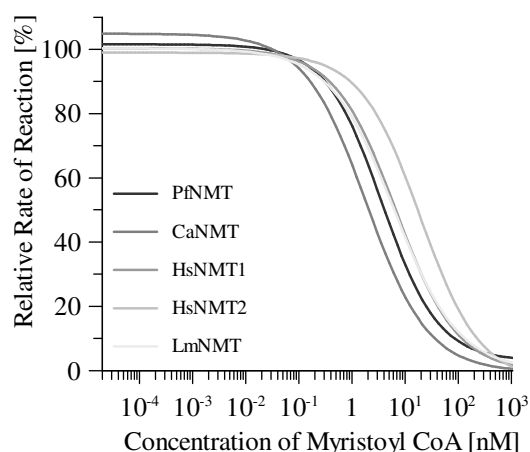


Figure V-12: IC_{50} determination of non-hydrolysable myristoyl CoA against a range of NMTs

The activity of non-hydrolysable myristoyl CoA (A) was examined against NMT of various organisms; PfNMT, CaNMT, HsNMT1, HsNMT2 and LmNMT. Error bars have been omitted for clarity.

Table V-2: Inhibition data of non-hydrolysable myristoyl CoA against NMT

Non-hydrolysable myristoyl CoA was tested against N-myristoyl transferase as an indicator of stable enzyme activity. The values were determined with GraFit.

N-Myristoyl Transferase	PfNMT	HsNMT1	HsNMT2	CaNMT	LmNMT
IC_{50} -Value [nM]	3.7 ± 0.7	6.7 ± 1.5	17.3 ± 1.5	1.9 ± 0.4	6.2 ± 2.3

V.3.3.2 The Lead Compound

Lead structure **35** for inhibition studies of this project was based on Pfizer compound UK-370,509 (Figure V-13A, B). Out of several compounds originally produced by Pfizer as fungal NMT inhibitors and tested for activity against PfNMT, UK-370,509 was one of the most active with an IC_{50} -value of $30 \pm 3 \mu M$ (Bowyer 2006).

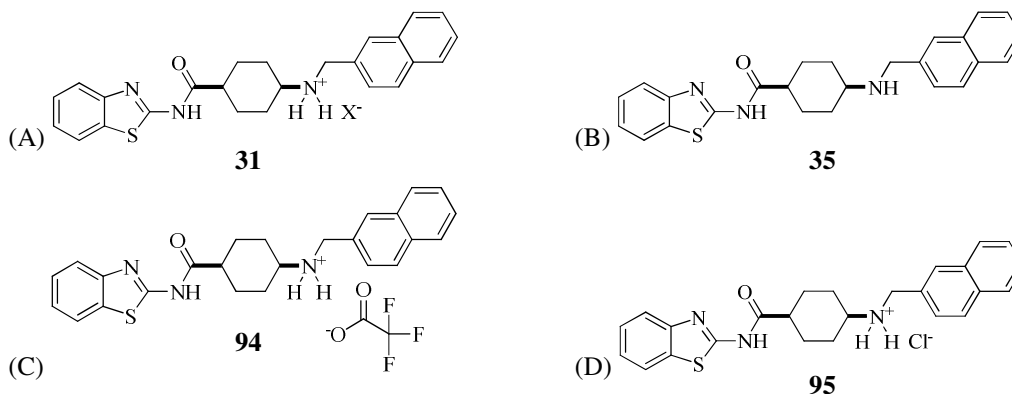


Figure V-13: The lead compound

The lead compound (**35**, B) was based on Pfizer compound UK-370,509 (**31**, A), which was provided as an unknown salt composition (Pfizer, unpublished results). Since inhibitor **31** is of unknown salt composition, various salts were tested to investigate differences in activity; namely the TFA salt (**94**, C) and the HCl salt (**95**, D).

The inhibitor was re-synthesised (IV.3.1, p. 88) and tested in the SPA against PfNMT for comparison with data gathered previously from UK-370,509. Expected inhibition should occur in the low micromolar range. Initial inhibition studies resulted in an IC_{50} of $75.0 \pm 8.2 \mu M$, a value in the same range to SPA data of UK-370,509 with $30 \mu M$ (Figure V-14A).

However, the inhibition data could not be replicated (Figure V-14B). An apparent IC_{50} of $490 \mu M$ was determined from a separate set of assays performed in triplicate. Reasons for non-reproducible results may lie in solubility issues with the compound at higher concentrations ($>100 \mu M$). By diluting a highly concentrated stock of 1 mM in DMSO 25-fold with Standard SPA Buffer, precipitation was noticed for both UK-370,509 and **35**. Furthermore, different salt combinations (Figure V-13C, D) were examined to investigate solubility issues further; a TFA salt (**94**), an HCl salt (**95**) and a free amine (**35**) were tested and activity was compared (Figure V-15).

Some improvement was noted for the HCl salt, but all showed limited and variable solubility at the higher concentrations needed to obtain reasonable inhibition.

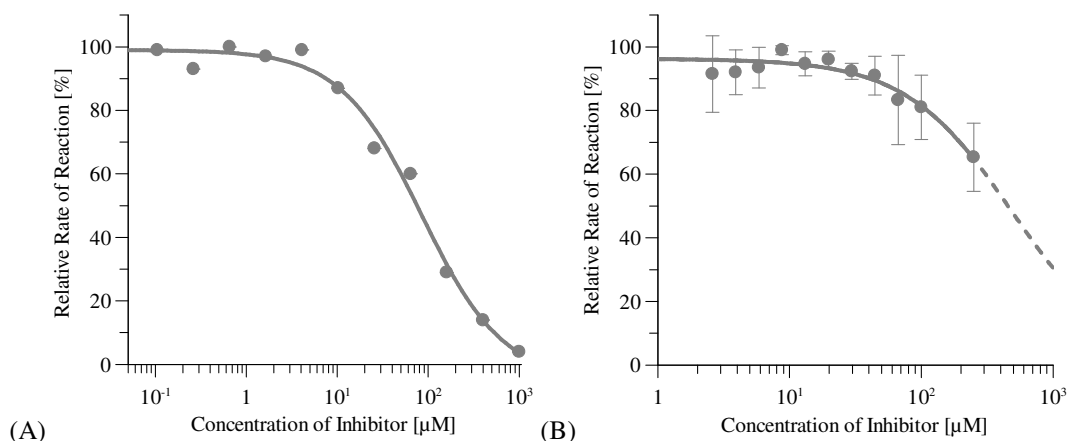


Figure V-14: Inhibition studies of lead compound 35

Inhibition of PfNMT was validated for the lead compound 35. However comparable data was only produced in one attempt ($IC_{50} = 75.0 \pm 8.2 \mu M$, A). In more extensive experiments it was found that inhibition results were not repeatable, probably due to solubility issues at higher concentrations ($IC_{50} = 490 \pm 105 \mu M$, B).

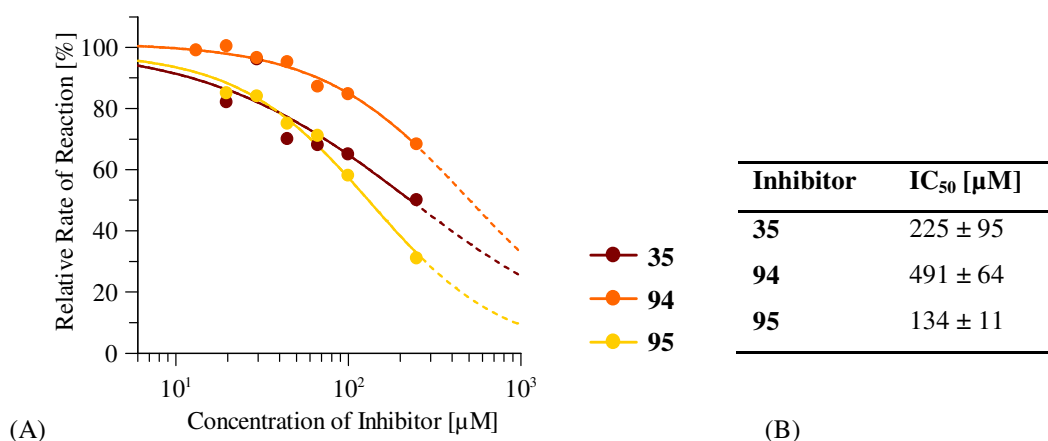


Figure V-15: Inhibition studies of different salt combinations of the lead compound

To study the effects of different salts on solubility and activity the free amine (35), the TFA salt (94) and the HCl salt (95) of the lead compound were studied. Data points represent a mean of triplicate determinations. The standard deviation for each individual point was <15% of the value; error bars have been omitted for clarity. Figure A shows the inhibition data and Figure B states the inhibitory data for each of the three compounds tested.

V.3.3.3 Activity against *Plasmodium falciparum* NMTV.3.3.3.1 In Vitro Analysis of 1st Generation Library – Variations of Unit A

The 1st generation library of potential antimalarial drugs was based on **35** with the original *cis*-1,4-cyclohexane attached to the benzothiazole scaffold *via* an amide bond and variations of building block A; for synthesis see Chapter IV. The collection investigated several aspects to systematically explore the peptidic binding domain such as size, aromaticity, electron withdrawing and donating properties, as well as H-donor and acceptor groups. Variations of building block A were furthermore subject to availability. The IC₅₀ data is documented in Table V-3 and inhibition curves displayed in Figure V-17.

To probe the dimensions of the peptidic binding pocket, the size of the aromatic ring (**49**, **94**, **68** in Table V-3) as well the spacing of the aromatic ring from the amine (**49**, **70** in Table V-3) were investigated first. For variations in size the following groups were chosen: methylbenzene (**49**), methylnaphthalene (**94**) and methylantracene (**68**). A tendency towards larger aromatic ring systems to increase potency in PfNMT was noted (Figure V-17A). Compound **68** was more active against PfNMT than **94** and **49**. GraFit was used to estimate IC₅₀-values of some compounds as they were largely insoluble at a top concentration of 1 mM in the buffer system. It was estimated that **68** had an IC₅₀ of 177 µM, whereas the smaller-sized aromatic substituents, such as naphthyl and benzyl, both showed weaker inhibition characteristics with 0.5 mM and 1 mM respectively. The small increase of activity could be driven by an increase of logP. Solubility issues prohibited thorough examination.

Ethylbenzene substituent (**70**) as opposed to methylbenzene (**49**) was selected to investigate possible CH–π interactions further into the cavity. No change in activity was observed with an IC₅₀ of 1.66 mM for compound **70** compared to 1 mM for **49** (Figure V-17B). The spacer unit was perhaps too small to have significant impact on more optimal binding.

Next, aliphatic vs. aromatic character was compared with building blocks methylcyclohexane (**48**) and methylbenzene (**49**) respectively (Table V-3). There was no enhancement in activity against PfNMT observed with an IC₅₀ of 1 mM for both compounds **48** and **49** (Figure V-17C).

The introduction of heteroatoms gives aromatic ring systems opportunities for polar interactions such as hydrogen bonding, 5-methylbenzofuran (**54**) was assessed (Table V-3). Compound **54** was far more potent against PfNMT than the lead compound **94** (Figure V-17D) with an IC_{50} of $19.9 \pm 2.7 \mu M$ (Figure V-16).

The degree of aromaticity of **49** was explored through groups that may either increase or decrease the electron density of the ring system (**49**, **61**, **63**, **67**, **59**, **58**, **64** in Table V-3). The effects of *p*-fluoromethylbenzene (**61**) and pentafluoromethylbenzene (**63**) were investigated. Fluoride for example is highly electronegative and decreases the overall electron density of the aromatic ring as it pulls them towards itself, also called negative inductive effect. The positive mesomeric effect, by which the aromatic ring is activated, is overruled by the electronegativity of the element because fluoride has such a high electronegative value. Other groups that exhibit a $-I/+M$ effect and additional H-donor properties in case of the first substituent were 2,3-dihydroxymethylbenzene (**67**), *p*-methoxybenzene (**59**) and *p*-methylsulfonylbenzene (**58**). Compound **64**, *m*-nitromethylbenzene, deactivates the aromatic ring system due to the $-I/-M$ character. Compounds **61** and **63** were not active at the highest concentration of 1 mM (Figure V-17E, i). All other inhibitors (**67**, **59**, **58** and **64**) displayed some activity at 1 mM (Figure V-17E, ii). However, they were not sufficiently potent against PfNMT to determine an IC_{50} -value, apart from **59** with a determined IC_{50} of $702 \pm 49 \mu M$. No distinct conclusion could be drawn about electron-withdrawing or electron-donating substituents that favour interactions with surrounding amino acids in the target binding site.

Overall, only compound **54** was active against PfNMT and inhibited *N*-myristoylation in the low micromolar region with an IC_{50} of $19.9 \pm 2.7 \mu M$ (Figure V-16). Ideally, the working concentration of a good inhibitor should be in the nanomolar range for optimum target binding. For purpose of this discussion, in this thesis definition of inhibitors is as follows; strong inhibitors display inhibition at a concentration $<100 \mu M$, moderate inhibitors show activity $100\text{--}500 \mu M$ and weak inhibitors have an IC_{50} in the range of $500\text{--}1\ 000 \mu M$.

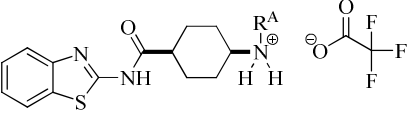
Analyses of results so far suggested that building block A should possess an aromatic core in combination with a heteroatom such as a benzofuran group. Favourable interactions of the aromatic ring system could potentially be with other

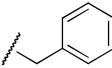
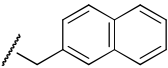
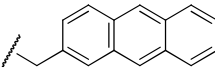
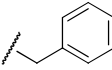
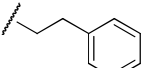
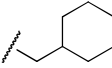
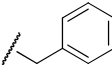
aromatic groups of nearby amino acids in the peptidic binding pocket for CH- π interactions. An ionic interaction of the heteroatom with surrounding amino acids is also likely. The next library focused on analogues of **54**.

Table V-3: Evaluation of activity against PfNMT of the 1st generation library – Variations of building block A

This table summarises results of the SPA for compounds of the 1st generation library against PfNMT. Several properties were evaluated such as ring size and aromaticity. The inhibitors in this table are ordered in groups to compare these characteristics. The SPA was carried out in triplicate, whereby the average, normalised to the uninhibited reaction, was quoted with standard deviation error. Many samples were insoluble at >100 μ M, thus the IC₅₀ was estimated with GraFit to observe trends; extrapolated curves are marked with *.

The active compound against PfNMT is highlighted in grey.



Compound Number	R ^A	IC ₅₀ Determination [μ M]	Trend
49		1 005 $\pm$ 124	Size of aromatic ring system
94		481 $\pm$ 62 *	
68		177 $\pm$ 3 *	
49		1 005 $\pm$ 124	Size of spacer
70		1 655 $\pm$ 375	
48		1 043 $\pm$ 123	Aromatic character
49		1 005 $\pm$ 124	

Compound Number	R ^A	IC ₅₀ Determination [μ M]	Trend
94		481 $\pm$ 62 [*]	Introduction of heteroatoms ↓
54		19.9 $\pm$ 2.7	
49		1 005 $\pm$ 124	Variation of degree of aromaticity ↓
61		>1 000	
63		>1 000	
67		993 $\pm$ 135	
59		687 $\pm$ 32	
58		350 $\pm$ 359 [*]	
64		>1 000	

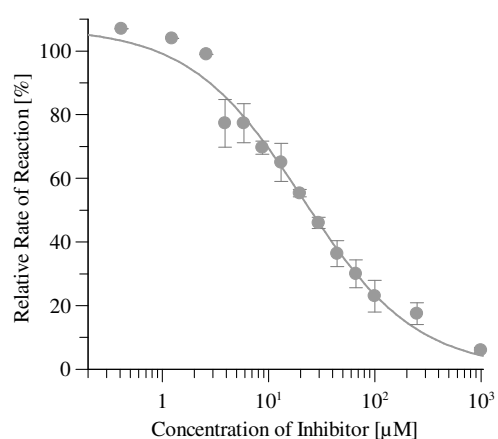
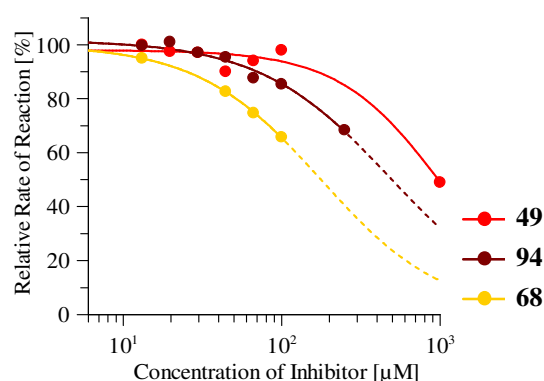


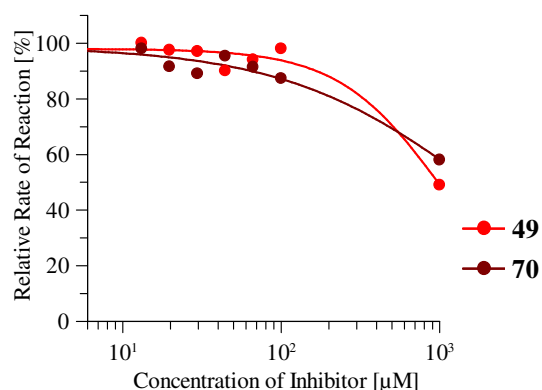
Figure V-16: IC₅₀ determination of compound 54

This figure shows the inhibition curve of **54** against PfNMT generated with GraFit. The IC₅₀ was determined as 19.9 $\pm$ 2.7 μ M.

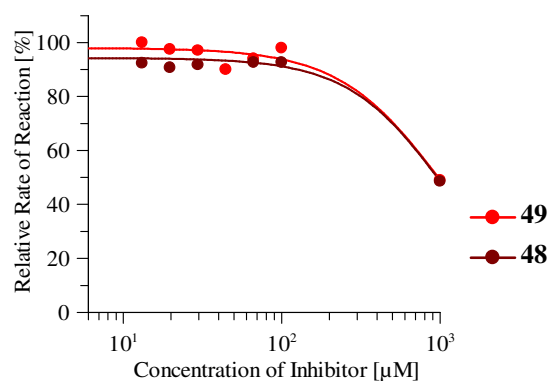
(A) Size of aromatic ring system



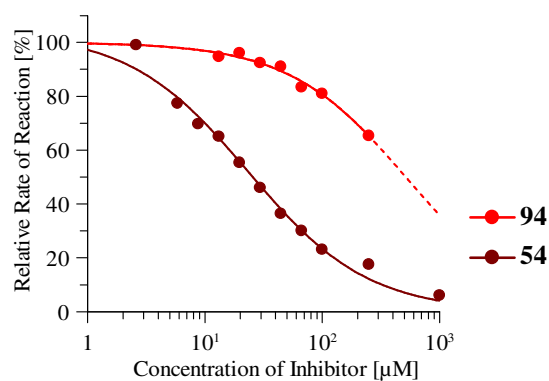
(B) Size of spacer



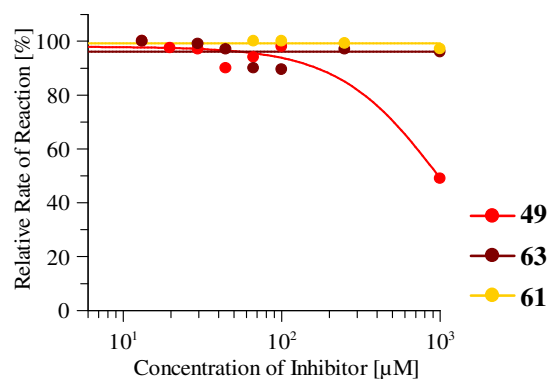
(C) Aromatic character



(D) Introduction of heteroatoms



(E) i) Variation of degree of aromaticity



(E) ii)

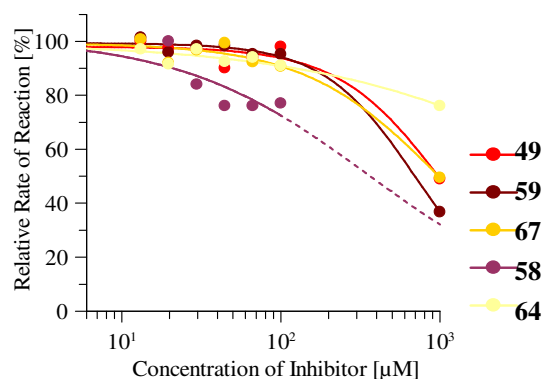


Figure V-17: Inhibition curves of compounds tested against PfNMT

Various compounds were tested for potency against PfNMT and the inhibition curves are shown. The size of the aromatic ring (A), the spacing out of the aromatic unit (B), aromatic character (C), influence of heteroatoms (D) and differing electron density of the aromatic ring system (E) were investigated. Data points represent the mean of triplicate samples. The standard deviation on each individual point was <15% of the value; error bars have been omitted for clarity.

V.3.3.3.2 *In Vitro Analysis of 2nd Generation Library – New Variations of Building Block A*

The next step included investigations of close analogues to compound **54** as this was the only compound from the 1st generation library to show enhanced activity against PfNMT. The results are shown in Table V-4 and inhibition curves are displayed in Figure V-18.

Heteroatoms play a role in binding as results suggest (V.3.3.3.1, p. 143). Replacing the oxygen atom (**54**) with a heteroatom from the same group, sulfur (**72**), may affect binding. Due to the larger size of the soft sulfur atom, it may be a more favourable electron acceptor than oxygen. The activity against PfNMT of the inhibitor with 5-methylbenzothiophene as building block A was equal to 5-methylbenzofuran. The calculated IC₅₀ of **54** was $19.9 \pm 2.7 \mu\text{M}$ (Figure V-19A) compared to $24.5 \pm 4.9 \mu\text{M}$ for **72** (Figure V-19B). Subtle differences in the slope of the inhibition curves were noted. The Hill slope found for **72** was much shallower than for **54** (Figure V-18A). This may be due to different kinetics of the inhibitors or solubility issues.

Compound **73**, the nitrogen analogue, showed moderate signs of inhibition with an IC₅₀ of $116 \pm 11 \mu\text{M}$ but no overall improvement of activity compared to **54** (Figure V-18A, Figure V-19C). It can be assumed that the additional proton donor moiety in that position was of no use for effective interactions with surrounding AA of the target binding site.

Next, size and character of the aromatic system including differing heteroatoms were examined a bit closer with **54** and **72** as well as **51** and **65**. A loss of activity was noted for both 5-methylbenzofuran and 5-methylbenzothiophene lead compound analogues with IC₅₀ > 1 mM when the system was reduced to methylfuran or methylthiophene as unit A. An increase in size (**69**) did not improve inhibition of PfNMT either (Figure V-18B); neither did the loss in aromaticity (**71**) compared to inhibitor **54** (Figure V-18B), IC₅₀ > 1 mM was observed for both.

The insertion of yet another heteroatom (**56**), however, led to moderate activity with an IC₅₀-value of $379 \pm 42 \mu\text{M}$ though **56** is not as active as **54** (Figure V-18B, Figure V-19D).

By excluding the heteroatom from the ring system to a methoxy-group, –OCH₃ acted as an electron donating group (–I/+M effect). Inhibition was not improved.

Compound **59** weakly inhibited PfNMT with an IC_{50} of $687 \pm 32 \mu M$ (Figure V-18B).

In general, the tested analogues of compound **54**, the 2nd generation library, did not reveal more potent *N*-myristoyl transferase inhibitor candidates. Nevertheless, four compounds were discovered that display activity against PfNMT in the low micromolar region with 5-methylbenzofuran, 5-methylbenzothiophene, 5-methylindole and methylbenzodioxole as building block A; the inhibition curves of each are displayed in Figure V-19. Of those inhibitors, two compounds are very active, **54** and **72**, with an IC_{50} of $19.9 \pm 2.7 \mu M$ and $24.5 \pm 4.9 \mu M$ respectively. The results suggest that building block A should contain a heteroatom such as oxygen or sulfur for ideal ionic interactions. Additionally the position of the heteroatom is also important as increase or decrease in size of the aromatic core resulted in loss of activity.

Table V-4: Evaluation of activity against PfNMT of the 2nd generation library – Variations of building block A

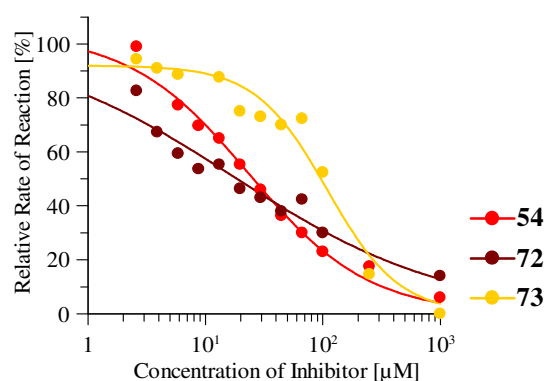
This table summarises results of the SPA for inhibitors of the 2nd generation library against PfNMT. The compounds were based on **54**, whereby analogues were tested differing in heteroatoms, aromaticity and ring size for example. The inhibitors in this table are ordered in groups to compare these characteristics. The SPA was carried out in triplicate, whereby the average, normalised to the uninhibited reaction, was quoted with standard deviation error.

Active compounds against PfNMT are highlighted in grey.

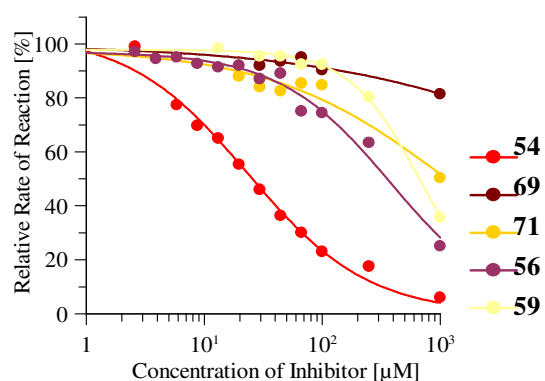
Compound Number	R ^A	IC ₅₀ Determination [μM]	Trends
54		19.9 ± 2.7	Different heteroatoms
72		24.5 ± 4.9	
73		116 ± 11	
54		19.9 ± 2.7	Various changes
69		>1 000	
71		1 206 ± 395	
56		379 ± 42	
59		687 ± 32	
54		19.9 ± 2.7	Different heteroatom & size of ring
51		1 090 ± 114	

Compound Number	R ^A	IC ₅₀ Determination [μM]	Trends
72		24.5 ± 4.9	Different heteroatom & size of ring
65		1 488 ± 206	

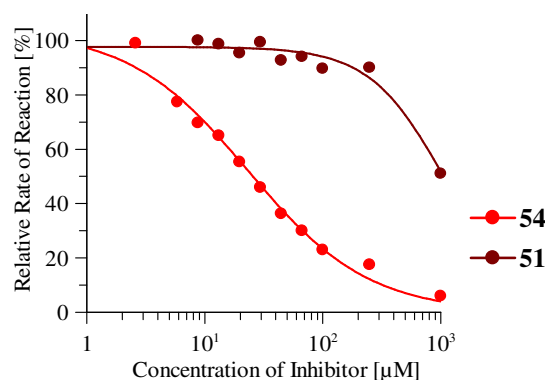
(A) Different heteroatoms



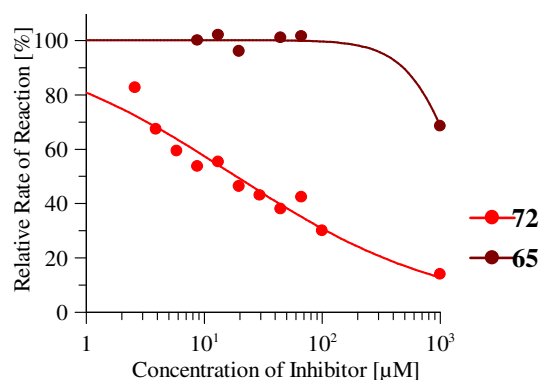
(B) Various changes



(C) i) Changes in size with different heteroatom

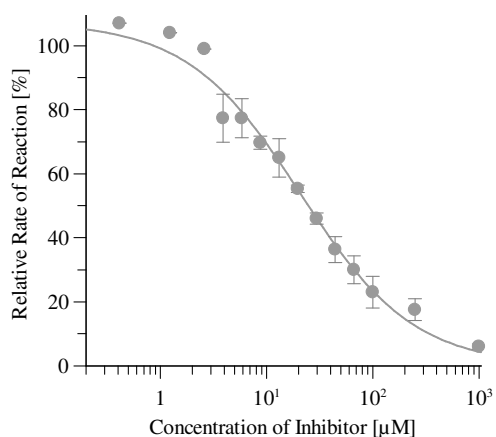


ii) Changes in size with different heteroatom

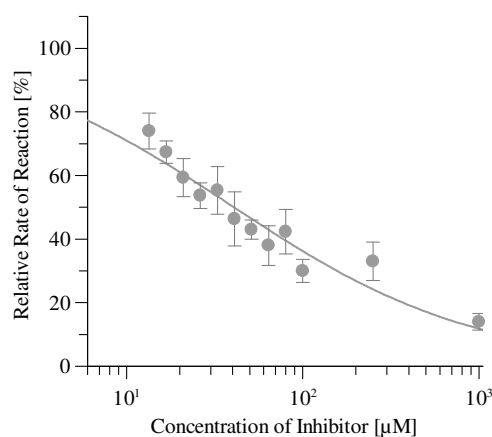

Figure V-18: Inhibition curves of compounds tested against PfNMT

Analogues of **54** were tested for potency against PfNMT and the inhibition curves are shown. Different heteroatoms (A) and size of aromatic ring system (C) among other various factors (B) were explored. Data points represent the mean of triplicate samples. The standard deviation on each individual point was <15% of the value; error bars have been omitted for clarity.

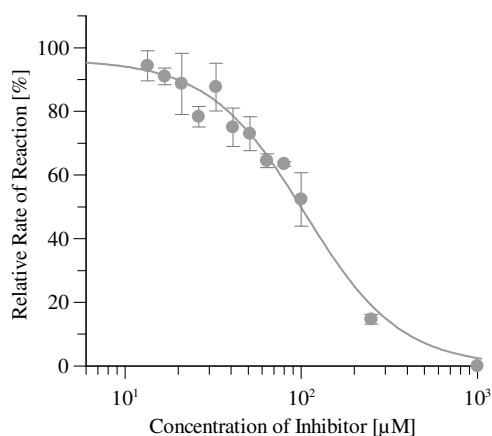
(A) IC_{50} of **54** against PfNMT = $19.9 \pm 2.7 \mu M$



(B) IC_{50} of **72** against PfNMT = $24.5 \pm 4.9 \mu M$



(C) IC_{50} of **73** against PfNMT = $116 \pm 11 \mu M$



(D) IC_{50} of **56** against PfNMT = $379 \pm 42 \mu M$

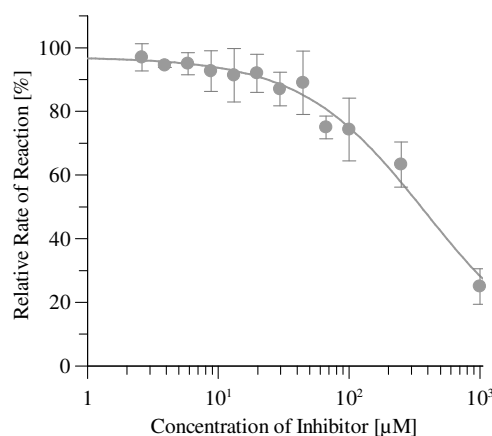


Figure V-19: IC_{50} determination of **54, **72**, **73** and **56****

This figure shows the inhibition curve of **54**, **72**, **73** and **56** against PfNMT generated with GraFit.

V.3.3.3.3 Examination of Remaining Building Blocks – Linkage of Units A and B

Building blocks A and B were connected to each other *via* an amine bond. The secondary amine was created during the reaction of a primary amine moiety with an aldehyde after subsequent reduction, namely reductive amination (Figure IV-25). The secondary amine moiety was examined closely as it was thought to interact with the C-terminal carboxylate of *N*-myristoyl transferase (Hasegawa, Morikami et al. 2003).

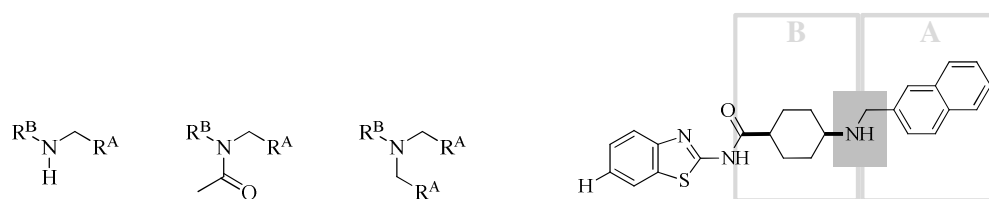


Figure V-20: Modifications of the secondary amine moiety

The secondary amine can be either acetylated or alkylated (from left to right). The modified compounds were tested for activity against PfNMT to investigate the importance of the secondary amine moiety.

Consequently, the 2° amine moiety was modified and investigated for three compounds, **94**, **49** and **54** (Figure V-20). The most straightforward alteration was acetylation with acetic anhydride which yielded **83**, **84** and **85** respectively (IV.3.4, p. 116). The acetyl group was chosen as it is a fairly small and may not sterically hinder interactions between the nitrogen atom and surrounding AA. However, a complete loss in activity was recorded for **85** (Figure V-21).

The other modification of the secondary amine moiety tested was a tertiary amine with two R^A groups attached, the by-product of the reductive amination, **97**, **53** and **55** were tested but no activity against PfNMT was observed (Figure V-21). The large-sized aromatic unit may possibly interfere with the correct orientation in the target site for optimal binding.

Another reason for loss in activity in both cases of acetylation and alkylation is the removal of the 2° amine functionality. The findings suggest that the 2° amine is needed. It may function as a proton donor and interacts most likely with the C-terminal carboxylate (Figure II-17).

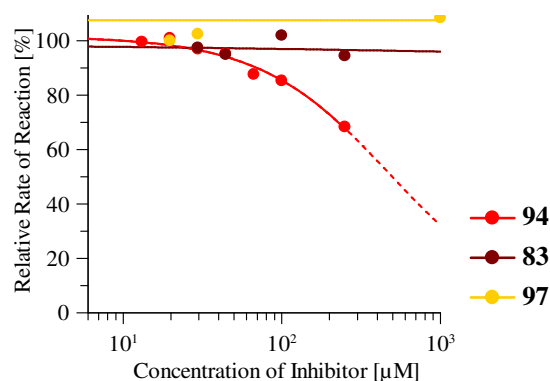
Table V-5: Evaluation of activity against PfNMT – The R^A-R^B-Linkage

This table summarises results of the SPA for assorted inhibitors against PfNMT to evaluate the influence of the 2° amine moiety. The compounds were based on **94**, **49** and **54**. The inhibitors in this table are ordered in groups to compare these characteristics. The SPA was carried out in triplicate, whereby the average, normalised to the uninhibited reaction, was quoted with standard deviation error. Many samples were insoluble at >100 µM, thus the IC₅₀ was estimated with GraFit to observe trends; extrapolated curves are marked with *.

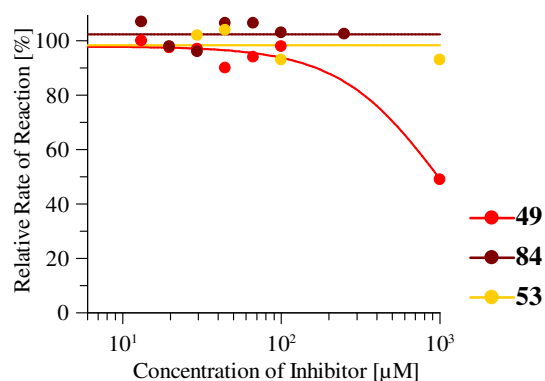
The active compound against PfNMT is highlighted grey.

Compound Number	R'	R ^A	IC ₅₀ Determination [µM]	Trends
94			481 ± 62*	Size of amine residue ↓
83			>1 000	
97			>1 000	
49			1 005 ± 124	Size of amine residue ↓
84			>1 000	
53			>1 000	
54			19.9 ± 2.7	Size of amine residue ↓
85			>1 000	
55			>1 000	

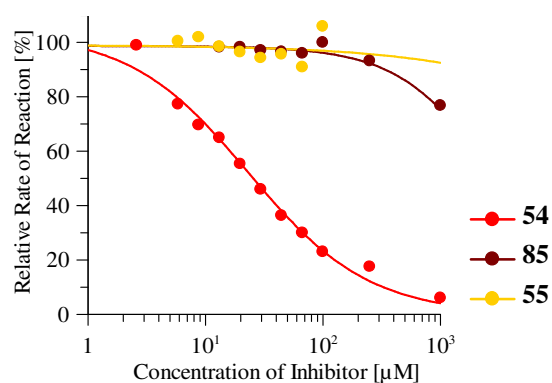
(A) Naphthyl derivatives



(B) Benzyl derivatives



(C) Benzofuran derivatives

**Figure V-21: Inhibition curves of compounds tested against PfNMT**

Analogues of **94**, **49** and **54** with a modified 2° amine moiety were tested for potency against PfNMT, the inhibition curves are shown. The acetylated version (**83**, **84**, **85**) was investigated as well as the dialkylated species (**97**, **53**, **55**). Data points represent the mean of triplicate samples. The standard deviation on each individual point was <15% of the value; error bars have been omitted for clarity.

V.3.3.3.4 Examination of Remaining Building Blocks – Variations of Unit B

The starting point for building block B was the *cis*-1,4-aminocyclohexyl unit (Figure V-22A), as it had proven to be the most effective group out of the “Pfizer series”, which included alkyl chains, aliphatic ring or heterocyclic systems (Pfizer, unpublished research). In addition to further explore the cavity of the peptidic binding domain another building block was chosen that had a different orientation of substituents; the *cis*-1,3-aminocyclohexyl unit (Figure V-22B). The *trans*-isomer of either was not readily available. To investigate the need for building block A in general, Figure V-22C, D and E were also examined. To further explore the binding site a different size of the aliphatic ring system, as well as the introduction of heteroatoms and linker molecules, were utilised (Figure V-22F, G and H).

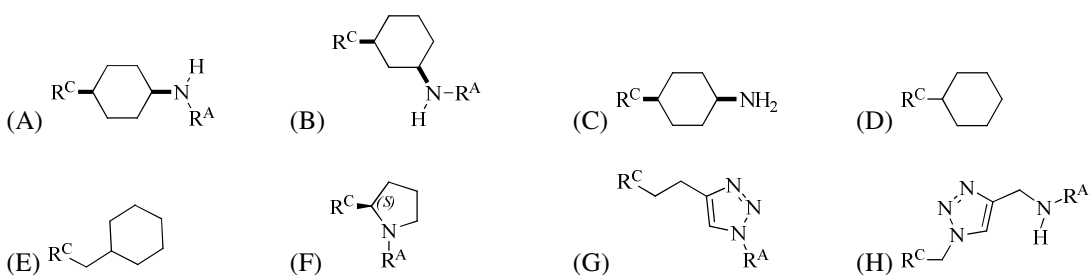


Figure V-22: A wide selection of building block B

Various building blocks have been used and tested such as the *cis*-1,4-aminocyclohexyl (A), *cis*-1,3-aminocyclohexyl (B), 1,4-aminocyclohexyl (C), cyclohexyl (D), methylcyclohexyl (E), proline (F) and triazole (G and H) units.

They were tested *in vitro* against PfNMT in combination with three different building blocks A (methylbenzene, methylnaphthalene, 5-methylbenzofuran). Several conclusions may be gathered from the experiments. First of all, the results showed compounds that lack unit A (**39**, **40**, **38**, **37**, **44**) inhibited PfNMT weakly. Compound **37** displayed the highest activity out of the five compounds with an IC_{50} -value of 743 μ M.

The level of inhibition could not be improved much with triazoles or proline as unit B. This may be due to the lack of the secondary amine functionality. To investigate this, compound **89** was synthesised and tested. Activity was restored with 1196 μ M although not as active as **94** with 481 μ M. Presumably, **96** would be even

more active against PfNMT because 5-methylbenzofuran is a better choice as building block A compared to methyl-benzene and methylnaphthalene. Nevertheless, compounds with 5-methyl-benzofuran did not guarantee activity. The substituents of building block B need to have optimal orientation in the binding pocket. This was not the case for either proline (Figure V-22F) or triazole (Figure V-22G).

From the range of building blocks B, *cis*-1,4- and *cis*-1,3-aminocyclohexyl units were the most appropriate for the particular section of the molecule as all of them were active; 1005 μM for **49** and 473 μM for **76** with methylbenzene, 481 μM for **94** and 359 μM for **74** with methylnaphthalene as well as $19.9 \pm 2.7 \mu\text{M}$ for **54** and $35.9 \pm 1.9 \mu\text{M}$ for **78** with 5-methylbenzofuran as unit A.

These correlations were investigated thoroughly with more building blocks A. The results of activity against PfNMT are listed in Table V-6 and the inhibition curves are shown in Figure V-23. Results indicate that there is no considerable difference in inhibition between the *cis*-1,4- and *cis*-1,3-aminocyclohexyl unit. Inhibition data was very similar, especially for the most potent inhibitor from the library so far. Compound **54** and **72** from the the *cis*-1,4-aminocyclohexyl series inhibited PfNMT at $19.9 \pm 2.7 \mu\text{M}$ and $25.5 \pm 4.9 \mu\text{M}$ respectively. The *cis*-1,3-amino-cyclohexyl analogue **78** and **81** displayed equal activity against PfNMT with IC_{50} -values of $35.9 \pm 1.9 \mu\text{M}$ and $44.8 \pm 3.1 \mu\text{M}$.

Table V-6: Evaluation of activity against PfNMT

This table summarises results of the SPA for inhibitors against PfNMT comparing the activity of *cis*-1,3-cyclohexyl vs. *cis*-1,4-cyclohexyl building block B. The inhibitors in this table are ordered in groups to compare these characteristics. The SPA was carried out in triplicate, whereby the average, normalised to the uninhibited reaction, was quoted with standard deviation error. Many samples were insoluble at >100 μ M, thus the IC_{50} was estimated with GraFit to observe trends; extrapolated curves are marked with *. All compounds were tested as the TFA salt.

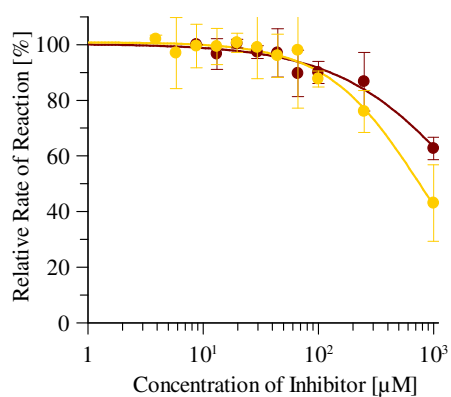
Active compounds against PfNMT are highlighted grey.

Compound Number	R ^B	R ^A	IC ₅₀ Determination [μ M]
38			1946 $\pm$ 346
43			743 $\pm$ 54
37			2317 $\pm$ 348
41			>1 000
49			1005 $\pm$ 124
76			473 $\pm$ 46
94			481 $\pm$ 62*
74			359 $\pm$ 57

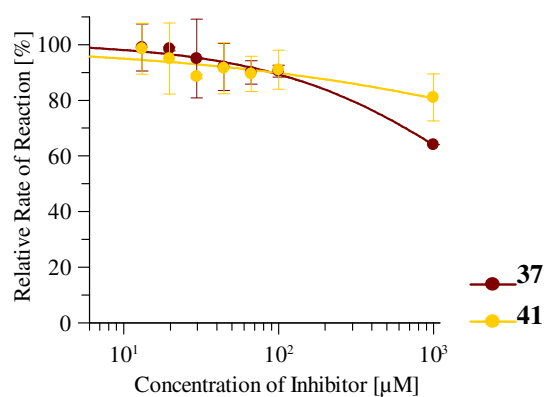
V IN VITRO EVALUATION OF INHIBITORS

Compound Number	R ^B	R ^A	IC ₅₀ Determination [μM]
54			19.9 ± 2.7
78			35.9 ± 1.9
72			24.5 ± 4.9
81			44.8 ± 3.1
73			116 ± 11
80			275 ± 48
56			379 ± 42
82			620 ± 159

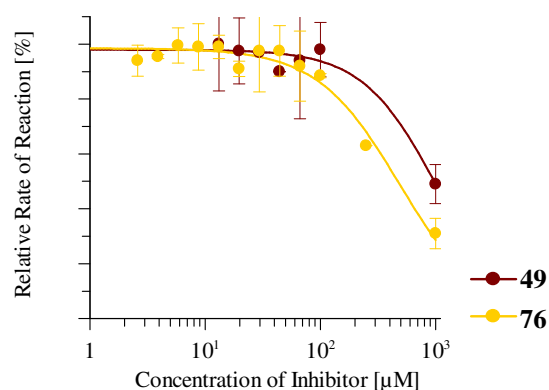
(A) No unit A



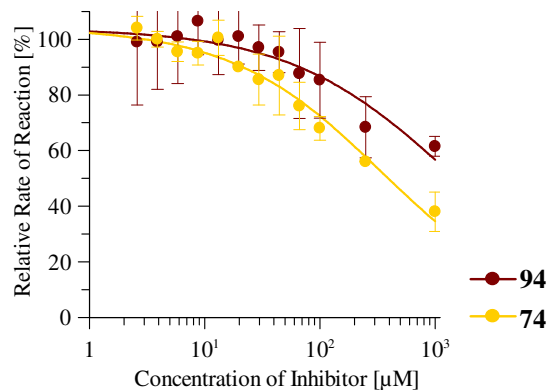
(B) *tert*-Butyl derivatives



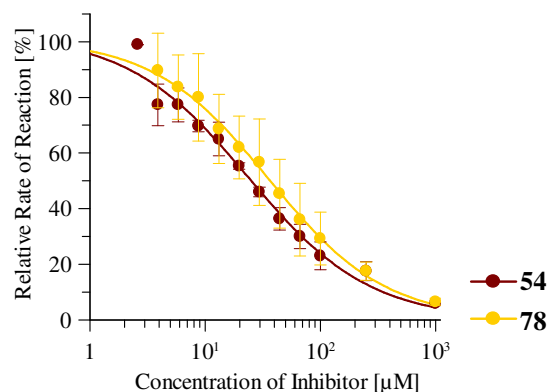
(C) Benzyl derivatives



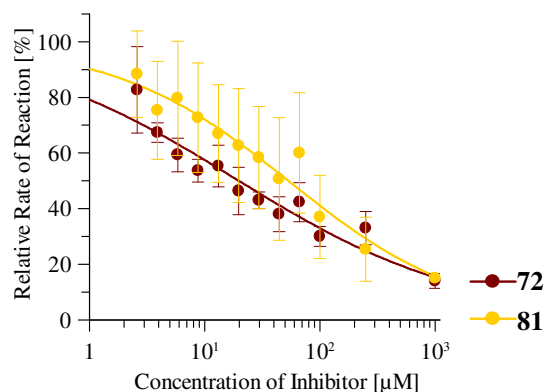
(D) Naphthyl derivatives



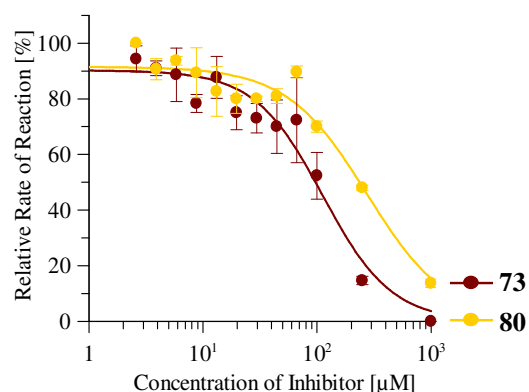
(E) Benzofuran derivatives



(F) Benzothiophene derivatives



(G) Indole derivatives



(H) Benzodioxole derivatives

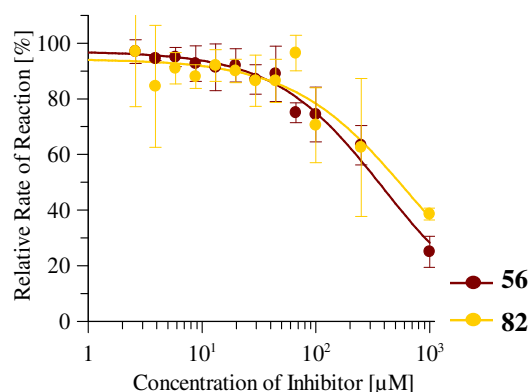


Figure V-23: Comparison of IC₅₀ data between *cis*-1,3- and *cis*-1,4-cyclohexyl building block B

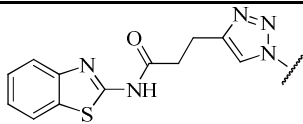
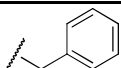
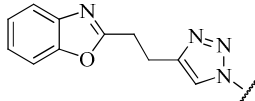
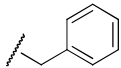
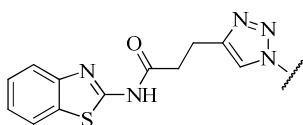
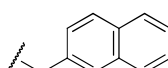
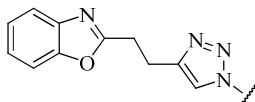
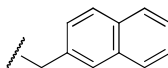
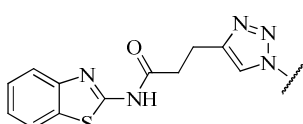
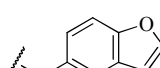
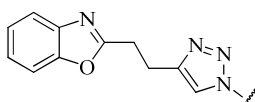
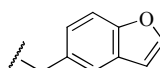
This figure compares inhibition curves between the *cis*-1,4- and *cis*-1,3-cyclohexyl building block B against PfNMT generated with GraFit. The following compounds were tested: 38 and 43 (A), 37 and 41 (B), 49 and 76 (C), 94 and 74 (D), 54 and 78 (E), 72 and 81 (F), 73 and 80 (G), as well as 56 and 82 (H).

V.3.3.3.5 Examination of Remaining Building Blocks – Variations of Unit C

Not much is known about the benzothiazole scaffold, but research has shown vital interactions of the aromatic ring system as well as ionic interactions with the heteroatoms (Hasegawa, Morikami et al. 2003). The benzoxazole scaffold, a close analogue, was synthesised and tested for activity against PfNMT; however, there was no inhibition recorded (Table V-7). This may be due to the missing secondary amine moiety in building block B that is likely to be necessary for interactions with the C-terminus, from which part of the peptidic binding domain is formed. Hence no conclusion could be drawn about the improvement of building block C.

Table V-7: Evaluation of activity against PfNMT

This table summarises results of the SPA for assorted inhibitors against PfNMT to evaluate building block C. The compounds were based on **94**, **49** and **54**. The inhibitors in this table are ordered in groups to compare these characteristics. The SPA was carried out in triplicate as a single point assay at 100 μ M.

$R^{CB}R^A$			
Compound Number	R^{CB}	R^A	IC ₅₀ Estimation [μ M]
87			>1 000
91			>1 000
88			>1 000
92			>1 000
90			>1 000
93			>1 000

V.3.3.4 Activity against *Homo sapiens* NMT

Four compounds were found to be very active against PfNMT: **54**, **78**, **72** and **81**. They inhibit *in vitro* N-myristoylation in the low micromolar region. Drug molecules should not only be potent against the enzyme of the target parasite but also display selectivity towards the human analogue. Potential antimalarials are ideally very specific towards the target. To evaluate selectivity of the synthesised compounds **54**, **78**, **72** and **81** were tested against HsNMT1 and HsNMT2. The results are listed in Table V-8 and inhibition curves are displayed in Figure V-24.

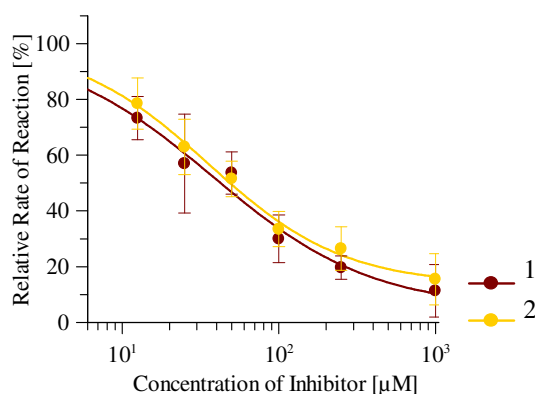
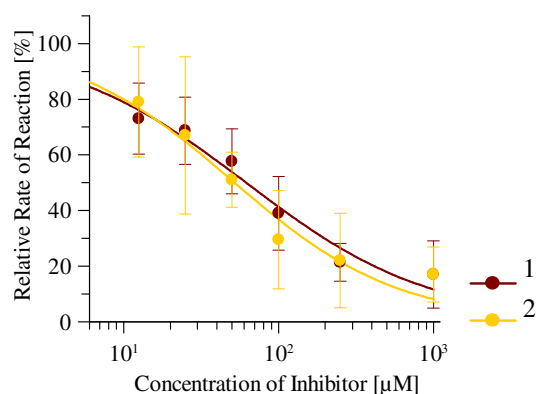
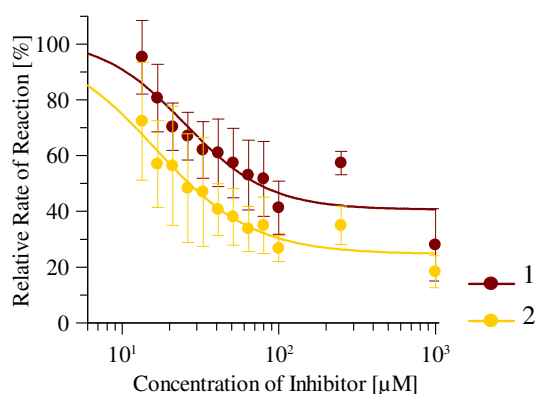
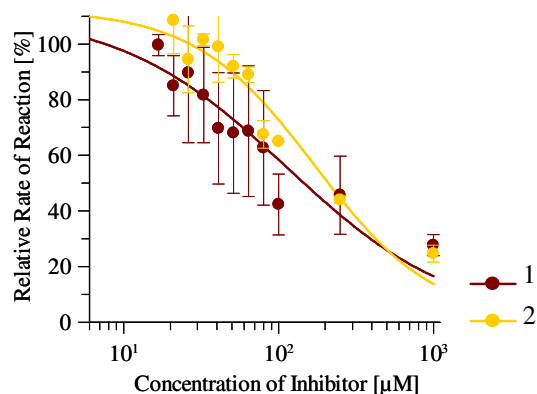
All four compounds are shown to be very active against both isomers of human NMT. The IC₅₀-value of **54** was calculated as $37.8 \pm 11.8 \mu\text{M}$ for HsNMT1 and $35.6 \pm 4.7 \mu\text{M}$ for HsNMT2 (Figure V-24A). Also equally active against both forms of human NMT was the sulfur analogue **72** with an IC₅₀ of $25.2 \pm 6.8 \mu\text{M}$ for HsNMT1 and $16.3 \pm 2.3 \mu\text{M}$ for HsNMT2 (Figure V-24B). The IC₅₀ of **78** was determined as $48.8 \pm 16.4 \mu\text{M}$ for HsNMT1 and $35.3 \pm 4.3 \mu\text{M}$ for HsNMT2 (Figure V-24C). However, the sulfur analogue of **78**, **81**, did not display such high activity against HsNMT2 with an IC₅₀ of $170 \pm 33 \mu\text{M}$ and against HsNMT1 $50.2 \pm 11.1 \mu\text{M}$ (Figure V-24D).

Table V-8: Evaluation of activity against human NMT

This table summarises the results of the SPA where the most potent inhibitors found against PfNMT were tested against human NMT, both HsNMT1 and HsNMT2. The inhibitors in this table are ordered in groups for easier comparison. The SPA was carried out in triplicate, whereby the average, normalised to the uninhibited reaction, was quoted with standard deviation error. All compounds were tested as the TFA salt.

Compound Number	R ^B	R ^A	IC ₅₀ Determination [μM]	
			HsNMT1	HsNMT2
54			37.8 ± 11.7	35.6 ± 4.7
78			48.8 ± 16.4	35.3 ± 4.3

Compound Number	R^B	R^A	IC ₅₀ Determination [μ M]	
			HsNMT1	HsNMT2
72			25.2 $\pm$ 6.8	16.3 $\pm$ 2.3
81			50.2 $\pm$ 11.1	170 $\pm$ 33

(A) IC₅₀ data of compound 54

(B) IC₅₀ data of compound 78

(C) IC₅₀ data of compound 72

(D) IC₅₀ data of compound 81

Figure V-24: Inhibition curves of selected inhibitors against human NMTs

Inhibition curves of four compounds against PfNMT are displayed: 54 (A), 78 (B), 72 (C) and 81 (D). (Legend: brown = HsNMT1 (1), yellow = HsNMT2 (2))

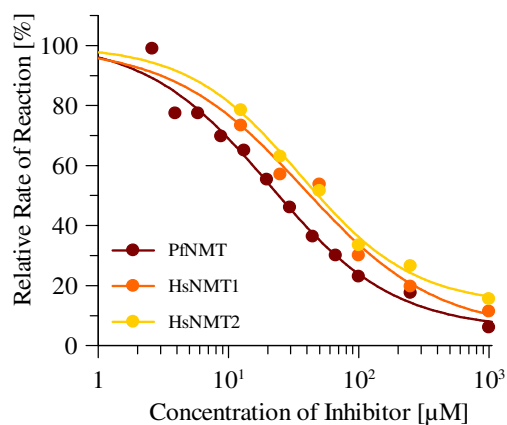
Overall, all four compounds were very effective against human NMT with activity in the low micromolar region. They did not display high selectivity towards PfNMT in comparison with the human homologue (Table V-9, Figure V-25).

Table V-9: Comparison of potency and selectivity against malarial and human NMT

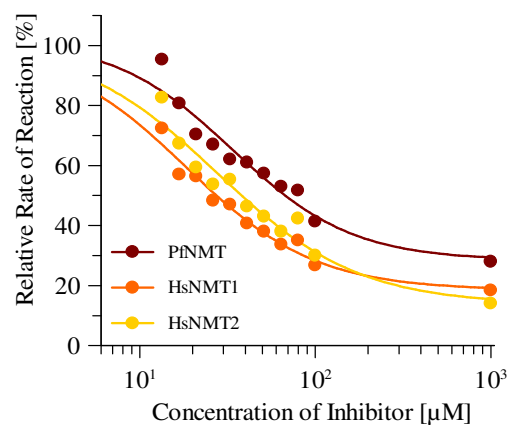
The rate of inhibition of the four most potent inhibitors against PfNMT was compared against human NMT for selectivity, *cis*-N-(benzo[d]thiazol-2-yl)-4-(benzofuran-5-ylmethylamino)cyclohexanecarboxamide (**54**), *cis*-N-(benzo[d]thiazol-2-yl)-4-(benzothiophene-5-ylmethylamino)cyclohexanecarboxamide (**78**), *cis*-N-(benzo[d]thiazol-2-yl)-3-(benzofuran-5-ylmethylamino)cyclohexanecarboxamide (**72**) and *cis*-N-(benzo[d]thiazol-2-yl)-3-(benzo-thiophene-5-ylmethylamino)cyclohexanecarboxamide (**81**).

Compound Number	R ^B	R ^A	IC ₅₀ -Value (PfNMT) [μM]	IC ₅₀ -Value (HsNMT1) [μM]	IC ₅₀ -Value (HsNMT2) [μM]
54			19.9 ± 2.7	37.8 ± 11.7	35.6 ± 4.7
72			24.5 ± 4.9	25.2 ± 6.8	16.3 ± 2.3
78			35.9 ± 1.9	48.8 ± 16.4	35.3 ± 4.3
81			44.8 ± 3.1	50.2 ± 11.1	170 ± 33

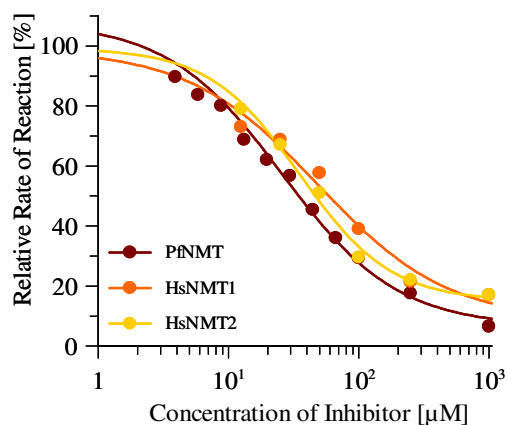
(A) Activity of **54**



(B) Activity of **78**



(C) Activity of **72**



(D) Activity of **81**

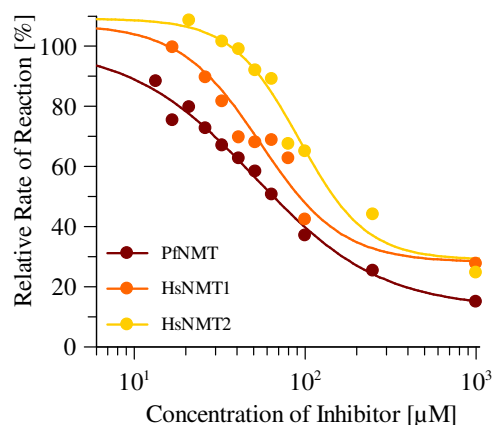


Figure V-25: Comparison of potency and selectivity against malarial and human NMT

Inhibition curves of four compounds are displayed against PfNMT, HsNMT1 and HsNMT2: **54** (A), **78** (B), **72** (C) and **81** (D). Data points represent the mean of triplicate samples. The standard deviation error on each individual point was <15% of the value; error bars have been omitted for clarity.

V.3.3.5 Activity against NMT of other organisms

Inhibitors were also tested against *Candida albicans* NMT (CaNMT), *Leishmania major* NMT (LmNMT) and *Trypanosoma brucei* NMT (TbNMT). Inhibitory studies against the latter organism were conducted by Dr S Elroy (Drug Discovery Centre, University of Dundee, UK). The data is listed in Table V-10.

Only **54** and **78**, the most active inhibitors against PfNMT, were tested against *Leishmania major* NMT, an *N*-myristoyl transferase of another species. Both compounds inhibited the enzyme moderately with an IC₅₀ of 270 µM and 180 µM respectively.

Compounds **54** and **78** displayed strong inhibition against fungal NMT; an IC₅₀-value of below 4 µM was recorded as the most potent. In fact, numerous compounds were active against CaNMT with a selection listed in Table V-10.

However no inhibitors showed any signs of inhibition against TbNMT (Table V-10) at a top concentration of 50 µM. It is likely that the peptidic binding pocket from TbNMT differs largely from the malarial enzyme peptidic binding domain as phylogenetic analysis had indicated (Figure II-10).

Table V-10: Evaluation of activity against CaNMT, LmNMT and TbNMT

This table summarises results of the SPA for assorted inhibitors against CaNMT, LmNMT and TbNMT. The inhibitors in this table are ordered in groups to compare these characteristics. The SPA was carried out in triplicate unless otherwise stated, whereby the average, normalised to the uninhibited reaction, was quoted with standard deviation error. All compounds were tested as the TFA salt.

Compound Number	R ^B	R ^A	IC ₅₀ Determination [µM]		
			CaNMT*	LmNMT**	TbNMT***
49			134 ± 23	n.t.	>50
94			62.0 ± 15.2	n.t.	>50

Compound Number	R ^B	R ^A	IC ₅₀ Determination [μM]		
			CaNMT [*]	LmNMT ^{**}	TbNMT ^{***}
48			211 ± 54	n.t.	n.t.
54			10.8 ± 2.2	268 ± 106	>50
67			443 ± 119	n.t.	n.t.
56			203 ± 34	n.t.	>50
59			211 ± 8	n.t.	n.t.
51			495 ± 53	n.t.	n.t.
43			190 ± 28	n.t.	>50
74			14.0 ± 2.4	n.t.	>50
76			140 ± 19	n.t.	>50
78			3.8 ± 0.6	180 ± 96	>50

^{*}SPA against CaNMT, multiple point analysis for IC₅₀ determination, in triplicate; ^{**}SPA against LmNMT, single point analysis for IC₅₀ determination; ^{***}SPA carried out by Dr S Elroy at University of Dundee; n.t. = not tested

V.4 Conclusion and Discussion

Optimal concentrations of all substrates have been investigated; 125 nM [³H]-myristoyl CoA at a specific activity of 8 Ci mmol⁻¹, 250 nM PfARF1 substrate (GLYVSRLFNRLFQKK(biotin)-NH₂) and 0.1 mg streptavidin-coated scintillation beads were employed in the SPA to monitor *N*-myristoylation. Additionally DMSO was added at a final concentration of 1% to increase solubility of inhibitors. To further maximise solubility of compounds tested, they were incubated in the buffer at 50 °C and subjected to regular heat–vortex cycles over 1 h. Enzymes were thawed on ice shortly before the assay was conducted. The most favourable concentration for NMTs of various enzymes was established with enzyme dilution and time series. It was found that 73 ng (1.40 μmol) PfNMT, 3 ng CaMNT (0.06 μmol), 6 ng (0.11 μmol) HsNMT1, 83 ng (1.46 μmol) HsNMT2 and 300 ng (6.12 μmol) LmNMT gave satisfactory results in the SPA.

The recombinant PfARF1 substrate was used as a peptide substrate in previous studies for CaNMT and human NMT (Bowyer, Gunaratne et al. 2007). Hence it was used as a peptide substrate against all enzymes in this project. However, for more accurate inhibition results different peptide substrates should be synthesised for human NMT and LmNMT. Synthetic peptide substrates for the latter may be based on HASPA, HASPB or ARL (Panethymitaki, Bowyer et al. 2006). Adequate peptide substrates for human NMT may be based on the N-terminus of p60^{Scr} (McIlhinney, Patel et al. 1994; Giang and Cravatt 1998). Results of interactions with the peptidic binding pocket of NMT of various organisms may be more precise if more specific substrates were used.

Non-hydrolysable myristoyl CoA is an inhibitor that targets the myristoyl CoA binding pocket to inhibit *N*-myristoylation. As this site is highly conserved throughout species, non-hydrolysable myristoyl CoA is not a drug candidate for malaria (Figure II-10). Selectivity, especially between PfNMT and the human analogue is an essential criterion in drug development. The compound was assayed as a positive control to confirm high activity of different batches of enzyme as each assay was carried out with a new aliquot of enzyme to avoid degradation due to frequent freeze–thaw cycles. The activity of the enzyme over time was investigated to assure comparable enzyme activity levels. The inhibition values were all within

the same range and highlight the vast level of conservation of the myristoyl CoA site in NMT (Figure V-12).

About 50 inhibitors were synthesised and tested for activity against PfNMT. The 1st generation library incorporated a variety of building blocks A with diverse characteristics to investigate the binding pockets more thoroughly.

Research indicated that the size of the aromatic group is important, suggesting that larger-sized aromatic groups increase interactions with AA in the target binding site: anthracyl > naphthyl > benzyl, benzofuran > furan, benzothiophene > thiophene.

No conclusions can be drawn about the level of aromaticity as building block A compared to an aliphatic unit. Aromaticity was explored by utilising building block A with differing substituents, such as fluoro-substituted groups to decrease aromaticity and methoxy-groups to enhance electron density of the ring.

Activity study showed that the insertion of heteroatoms greatly increased activity, although the number of heteroatoms was not as important as the position of the heteroatom for vital polar interactions: benzofuran > benzodioxole > naphthyl. The most active inhibitors against PfNMT had IC₅₀-values in the low micromolar range: **54** ($19.9 \pm 2.7 \mu\text{M}$), **78** ($35.9 \pm 1.9 \mu\text{M}$), **72** ($24.5 \pm 4.9 \mu\text{M}$) and **81** ($44.8 \pm 3.1 \mu\text{M}$). The inhibition curves for **72** and **81** were quite shallow. More data points should be collected as 13 μM , the last data point, of **72** still inhibited PfNMT by ~30%. The shape of the curve would change, leading to a more precise IC₅₀-value. The Hill slope factor for the graph was 0.5, too inaccurate for comparison.

In addition, it was also found that the secondary amine functionality was vital for strong inhibition of PfNMT as modifications of **54** lead to a complete loss in activity. It is thought to interact with the C-terminus of the enzyme which partially forms the peptide binding site.

The four most potent inhibitors (**54**, **78**, **72** and **81**) from the small-sized library were tested for selectivity against both human NMT enzymes, HsNMT1 and HsNMT2. All compounds tested displayed activity within the same range as with regards to PfNMT, hence **54**, **78**, **72** and **81** did not exhibit selectivity towards the malarial *N*-myristoyl transferase.

VI *IN VIVO* EVALUATION OF INHIBITORS

VI.1 Introduction

The antimalarial drug target development aims at the erythrocytic cycle. The target enzyme, *N*-myristoyl transferase, will be inhibited by optimised potent inhibitors possibly leading to parasitic death. Previous work with small molecule NMT inhibitors incubated in *Plasmodium falciparum* infected erythrocytes resulted in a significant reduction of parasitemia (Bowyer 2006; Bowyer, Gunaratne et al. 2007).

The complex life cycle of the parasite was briefly discussed in Chapter II.1.3 (p. 22). After rupture of hepatocytes, merozoites reach the bloodstream and invade erythrocytes, a complex multi-step process referred to as erythrocytic cycle, where the asexual reproduction occurs. For *Plasmodium falciparum* this is typically 48 h (Figure VI-1). After invasion, a parasitophorous vacuole is formed during the ring stage or so-called early-stage trophozoites (Figure VI-2A). The rapid maturation of trophozoites also leads to changes in erythrocytes. As haemoglobin is consumed the crystalline by-product haemozoin accumulates, also called malaria pigment (Pagola, Stephens et al. 2000; Wiesner, Ortmann et al. 2003; Baton and Ranford-Cartwright 2005). After multiplication, the trophozoites form schizonts (Figure VI-2B) and mature to merozoites over time. The cell prepares for re-invasion of new erythrocytes by replicating and dividing into typically 16–32 merozoites (Bozdech, Llinas et al. 2003). After cell rupture, the merozoites quickly and selectively invade uninfected erythrocytes (Cowman and Crabb 2006). Induced by stress factors, a small proportion undergoes sexual development; they differentiate into female and

male gametocytes (Figure VI-2C) and are taken up by feeding mosquitoes (Talman, Domarle et al. 2004).

This section describes *in vivo* assays, namely FACS, with *Plasmodium falciparum* infected erythrocytes to analyse the effect of synthesised potential antimalarials on parasitemia.

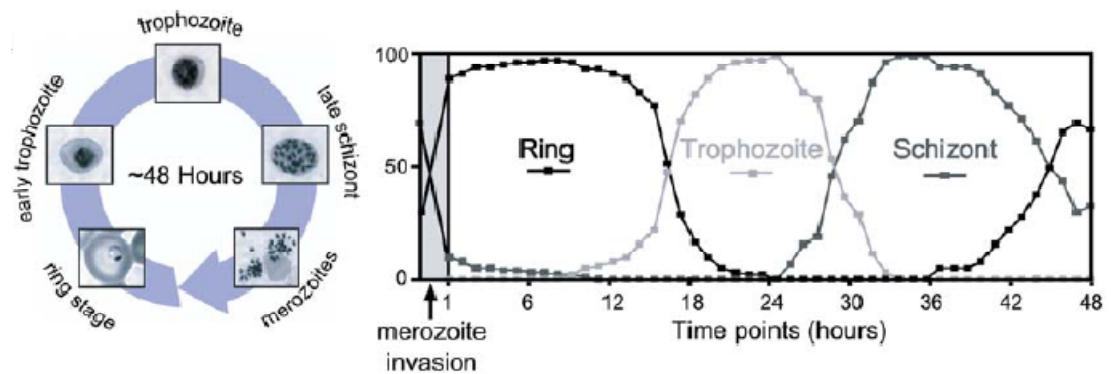


Figure VI-1: Life stages of *Plasmodium falciparum* during the erythrocytic cycle I

The diagram shows the major morphological stages; ring-, trophozoite- and schizont-stage over time as well as Giesma stains of each stage (Bozdech, Llinas et al. 2003).

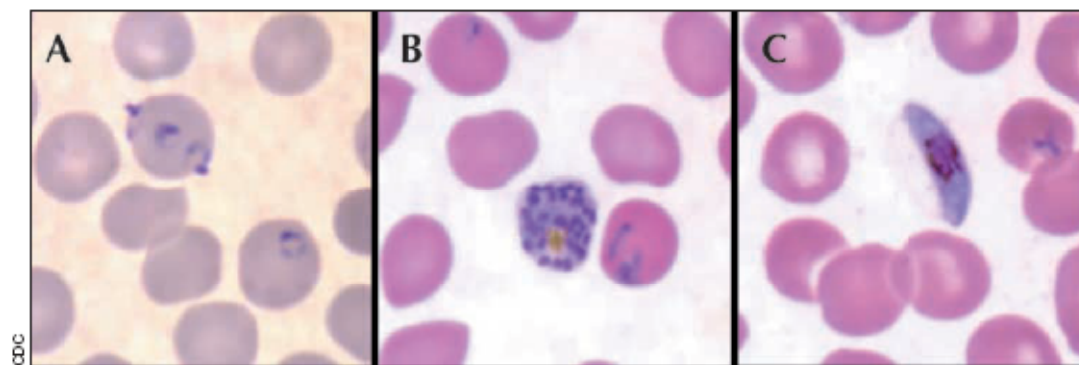


Figure VI-2: Life stages of *Plasmodium falciparum* during the erythrocytic cycle II

A close-up of ring forms (A), mature schizonts (B) and a gametocyte (C) (Suh, Kain et al. 2004).

VI.2 Material & Methods

In vivo studies were entirely carried out by Dr D Moss at The National Institute of Medical Research (NIMR), London, UK.

VI.2.1 Chemicals and Reagents

Dry DMSO for inhibitor stocks was purchased from Sigma Aldrich. 20 mM stocks of inhibitors in 100% DMSO were provided to Dr D Moss at NIMR.

Hydroethidine (HE) fluorescent stain was obtained from Polyscience. Stocks of 10 mg mL⁻¹ in DMSO were stored at -70 °C. 2% Haematocrit are 2% red blood cells in media, i.e. 2 mL erythrocytes in 100 mL media. 70% Percoll was purchased from Amersham Biosciences (formerly known as GE Healthcare). Albumax was purchased from GIBCO and Giesma from BDH.

VI.2.2 Methods and Equipment

VI.2.2.1 Theory

Fluorescent-activated cell sorting (FACS), the *in vivo* assay of choice for the analysis of parasitemia, was based on a published procedure (Bergmann-Leitner, Duncan et al. 2006). FACS is a flow cytometric method to analyse cell populations based on light scattering properties and fluorescent characteristics of each cell. The machine can rapidly separate cells based on size and on colour of the fluorophore.

FACS is used to study growth inhibition of intraerythrocytic protozoan parasites that have been HE-stained. HE is taken up by living cells and metabolically converted to ethidium by NADPH oxidase. Ethidium intercalates into DNA, hence the red fluorescence distinguishes infected erythrocytes with viable parasites from uninfected or infected erythrocytes containing dead parasites (Wyatt, Goff et al. 1991; van der Heyde, Elloso et al. 1995; Bergmann-Leitner, Duncan et al. 2006).

VI.2.2.2 Equipment

The FACS assay was performed with BD Bioscience FACSCalibur system and FACS-flow (BD Bioscience, 342003). FACS reaction tubes were used BD Falcon 5 mL Polystyrene from BD Bioscience.

VI.2.2.3 Method

Plasmodium falciparum parasites were routinely cultured in 2% haematocrit at 37 °C in RPMI 1640 containing albumax and glutamine. To synchronise parasites, heterogeneous populations containing late stages were first centrifuged onto a 70% Percoll cushion to separate late trophozoite/schizont from rings. Late trophozoite/schizonts were then harvested from the Percoll cushion, pelleted and washed twice in media to remove residual Percoll. These parasites were re-cultured for 3–5 h with fresh red blood cells allowing invasion to occur, assessed using Giemsa-stained smears. Following invasion the culture was pelleted and incubated in a final concentration of 90% sorbitol, at 37 °C for 10 min to remove remaining schizonts. Post sorbitol treatment, the culture was pelleted and washed twice in fresh media prior to re-suspension in media to 2% haematocrit for culturing.

Synchronised cultures were grown for approximately 40 h or until entering the next late trophozoite stage. To accurately determine the cultures parasitemia HE labelling and FACS counts were made. 50 µL culture was added to 500 µL freshly diluted HE (1:200 dilution of 10 mg mL⁻¹ DMSO stock in PBS) and incubated for 20 min at 37 °C. Samples were then diluted with 1 mL PBS to enable appropriate counts (5 000 counts s⁻¹). Staining was stopped by storing samples on ice. For negative controls non-infected red blood cells were HE-stained.

Parasitemia was calculated using the FACSCalibur flow cytometer. In brief, counts were initial screened using forward and side scatter parameters and gated for erythrocytes. Of the gated population HE-staining was determined using FL2 detectors at 585 and 542 nm. When expressed as a histogram of HE intensity vs. number of erythrocytes, a distinct peak representing late trophozoite/schizont infected erythrocytes is evident and can be used to calculate parasitemia of cultures. This FACS calculated parasitemia was used to appropriately dilute 1% parasitemia in 2% haematocrit per one cycle assay.

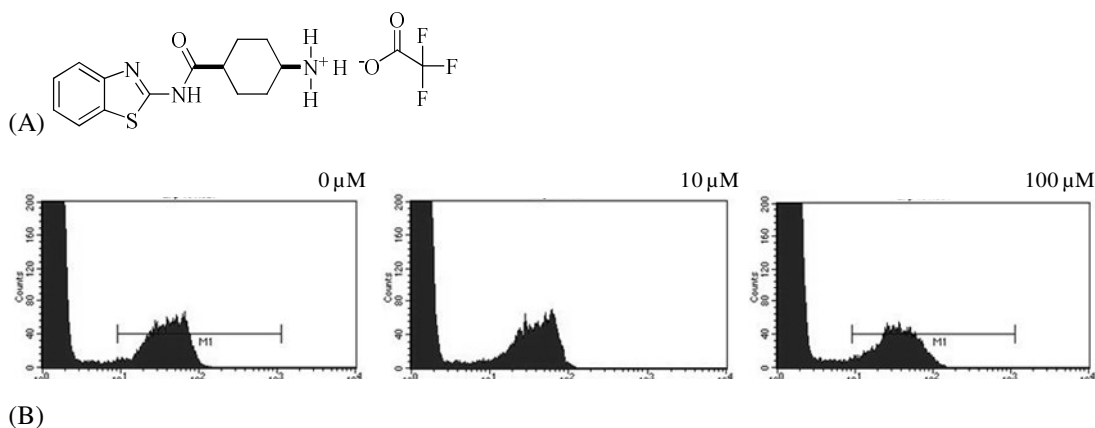
The compound inhibition assays were carried out in a 96-well plate format, in a final volume of 100 μL for each culture (90 μL of stock culture plus 10 μL of 10 $\times$ inhibitor stock). All assays were carried out in triplicate. For each compound a serial dilution was made from 20 mM stocks in 100% DMSO. The maximum final working concentration was 10 μM , a 200-fold dilution, to ensure the in culture DMSO concentration did not exceed 0.5% DMSO. Serial dilutions of inhibitors were made with the 10 $\times$ stocks of DMSO media (5%) to make certain the DMSO concentration remained constant. To each well 10 μL of this 10 $\times$ stock was added and mixed thoroughly. Following set-up plates were incubated in gassed humidity chambers at 37 $^{\circ}\text{C}$ for one invasion cycle typically 44–48 h, to the next trophozoite stage, prior to FACS analysis as previously described.

VI.3 *Results*

VI.3.1 Preliminary Tests

VI.3.1.1 The Negative Control

Compound **38** (Figure VI-3A) displayed no significant inhibitory effect against PfNMT at a top concentration of 1 mM in the *in vitro* SPA ($\text{IC}_{50} = 1\,946 \pm 436\, \mu\text{M}$), hence it was used as an additional negative control for the *in vivo* FACS assay. As expected, no signs of inhibition were observed (Figure VI-3C). Figure VI-3B shows histograms of HE intensity vs. number of erythrocytes at varying inhibitor concentrations (0 μM , 10 μM , 100 μM from left to right) with a distinct peak characterising infected erythrocytes. The parasitemia in cultures was deduced from the histogram to create a bar chart shown in from which the inhibitory effects of the tested compound were easily deduced.



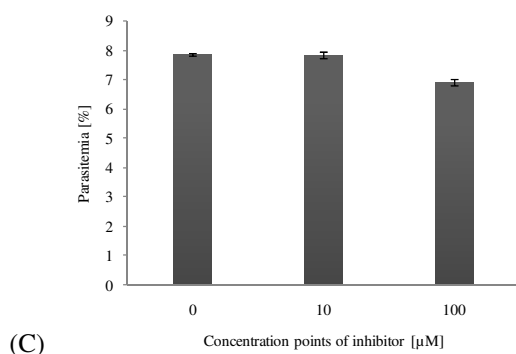


Figure VI-3: The negative control

Cis-4-amino-N-(benzo[d]thiazol-2-yl)cyclohexanecarboxamide · TFA, **38**, (A) showed no signs of inhibition during the *in vivo* studies. The histograms in (B) represent FL2-H vs. number of erythrocytes at varying inhibitor concentrations, 0 μM, 10 μM, 100 μM from left to right. From it a bar chart (C) was prepared to make analysis of parasitemia more straightforward.

VI.3.1.2 The Salt Effect

VI.3.1.2.1 The Effects of TFA in the FACS Assay

The first issue to be addressed was the effect of TFA on cell growth and parasitemia, as all compounds for inhibition assays were TFA salts. As illustrated in Figure VI-4B, TFA had no considerable effect on parasitemia, did not inhibit cell growth and no sign of toxicity at a top concentration of 1 mM was recorded. Hence TFA salts could be used in the FACS *in vivo* assay to determine the effects of potential inhibitor on parasite growth in infected erythrocytes.

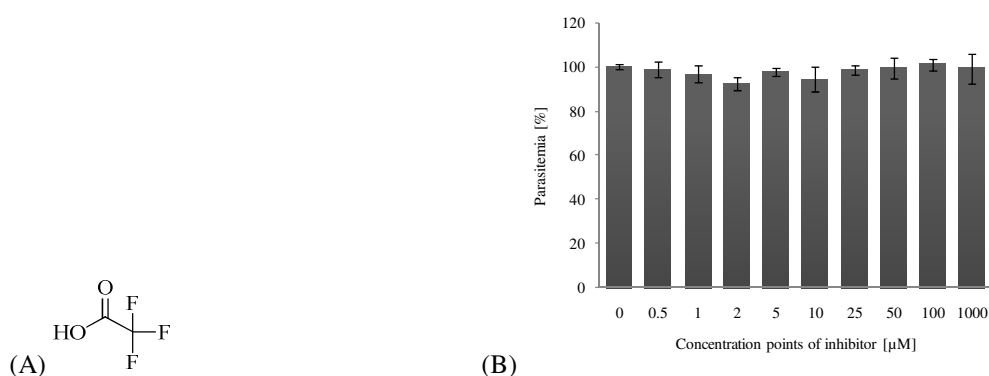


Figure VI-4: The effects of TFA in the FACS assay

TFA (A) displays no inhibitory/toxic effects on *P. falciparum* infected erythrocytes as seen in the histogram (B). FACS assay data was obtained from two separate experiments carried out in triplicate.

VI.3.1.2.2 The Effects of Different Salt Compositions in the FACS assay

Due to unknown salt composition of the “Pfizer series”, tests were carried out with the lead compound in various salt compositions to investigate possible harmful effects of the anion with regards to the *in vivo* assay. The free amine (**35**), the HCl (**95**) and the TFA (**94**) salts were studied (Figure VI-5 from left to right). *In vitro* results showed no considerable difference in activity between the three inhibitors with an IC_{50} for **94** of 491 ± 64 (V.3.3.2, p. 141). Likewise **35**, **94** and **95** showed similar inhibition results *in vivo* (Figure VI-6). At a concentration of 20 μ M nearly full inhibition was noted. The IC_{50} for **94** was calculated as 2.73 ± 0.39 μ M (Figure VI-7).

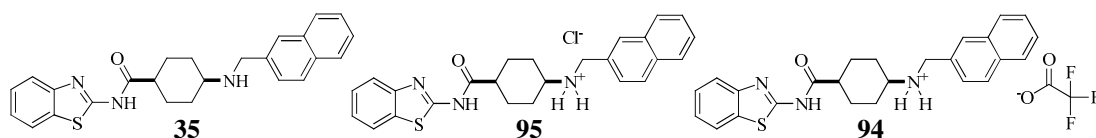


Figure VI-5: Diverse salt combinations of *cis*-N-(benzo[d]thiazol-2-yl)-4-(naphthalen-2-ylmethylamino)cyclohexanecarboxamide (35**, **95**, **94**)**

The lead compound was tested in different salt compositions: the free amine (**35**, left), HCl salt (**95**, middle) and TFA salt (**94**, right).

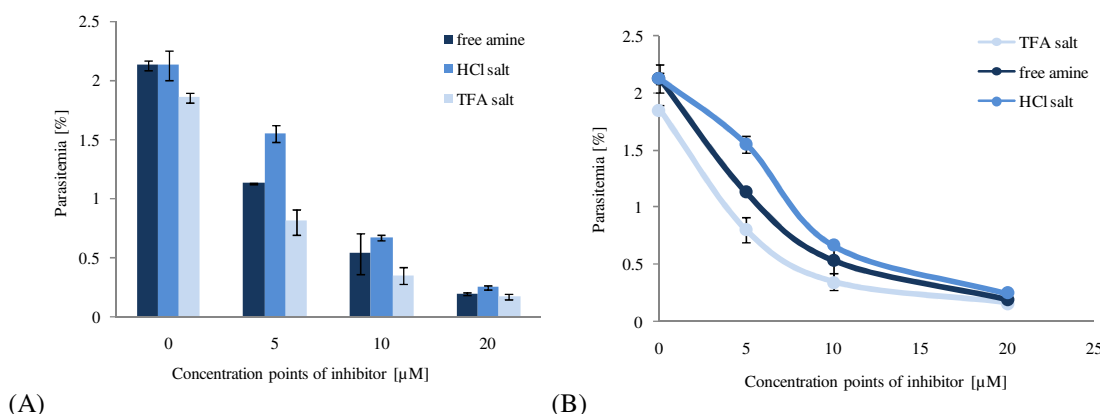


Figure VI-6: FACS data of compounds (35**, **95**, **94**)**

The lead compound was tested in various salt compositions, such as the free amine (**35**, dark blue-coloured, left), HCl salt (**95**, medium blue-coloured, middle) and TFA salt (**94**, light blue-coloured, right). As can be seen in the bar graph (A) as well as the line graph (B) the inhibitory action of all salts are comparable.

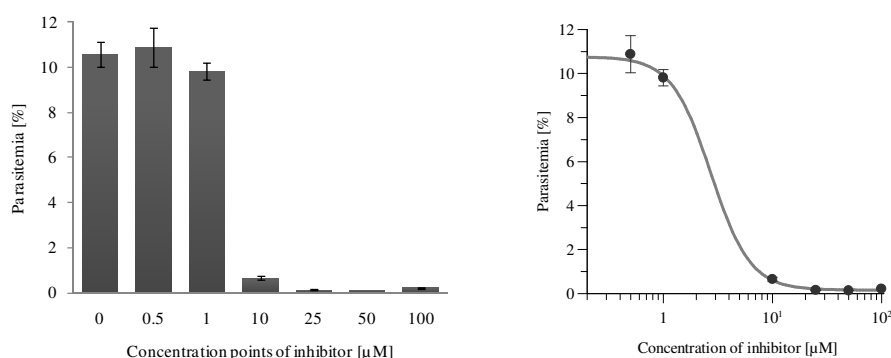


Figure VI-7: More detailed FACS data of compound 94

On the left hand side is a histogram of the inhibition data of compound **94** in *Plasmodium falciparum* infected erythrocytes. The data was plotted in GraFit (Leatherbarrow 2007) to create an inhibition curve, illustrated on the right, from which the IC_{50} -value could be determined.

VI.3.1.3 Positive Controls

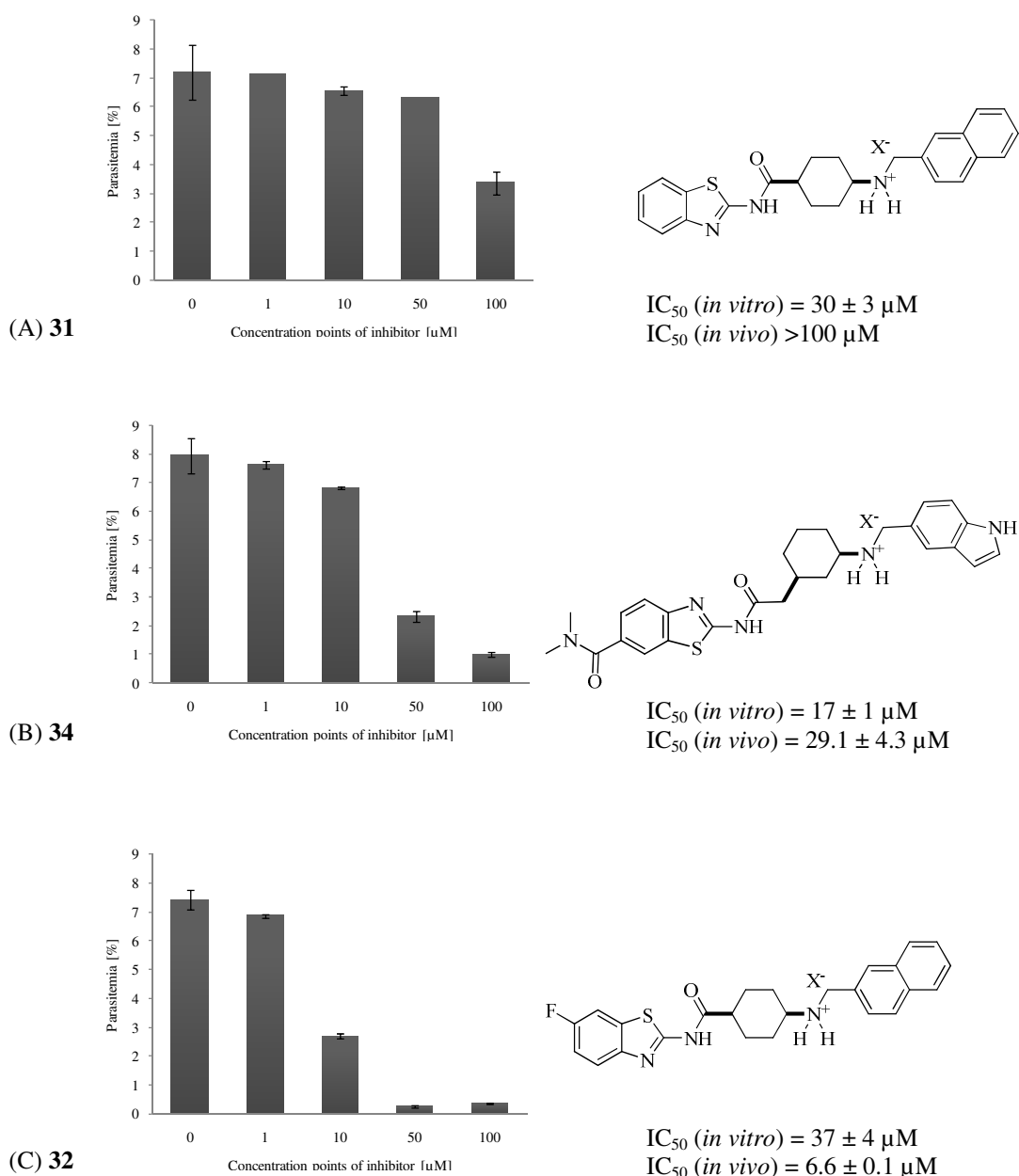
Preceding the newly established FACS assay, another *in vivo* assay was carried out where the parasite was visualised by Giesma-staining and the life stages analysed with light microscopy (Bowyer 2006). This time-consuming procedure was to be replaced by the FACS assay. For comparative studies for *in vivo* results from compounds previously tested (Bowyer 2006; Bowyer, Gunaratne et al. 2007), **31–33** from the “Pfizer series” were assayed with the latest conditions of the FACS assay. The percentage of parasitemia was plotted against the concentration of inhibitor the infected erythrocytes were incubated in (Figure VI-8). Overall, results were consistent with previous observations.

Compounds **34–33** show resemblance between *in vitro* and *in vivo* data as listed in Figure VI-8. The calculated IC_{50} -values for **34** are in the same order of magnitude. Full inhibition was noticed at 50 μ M and 100 μ M, with slight reduction in parasitemia at a concentration point of 10 μ M. Full parasitemia was noted at 1 μ M (Figure VI-8B). The estimated IC_{50} -value of 29 μ M correlates with the IC_{50} *in vitro* value of 17 μ M. The results for compound **32** and **33** are about 10-fold off. Both showed complete inhibition at 50 μ M and 100 μ M. They still inhibited parasite growth at 10 μ M; however **32** did not completely stop parasite growth. Full parasitemia was observed at 1 μ M (Figure VI-8C and D). The IC_{50} data of **32** and **33** are 68 μ M (*in vitro*)/7 μ M (*in vivo*) and 37 μ M (*in vitro*)/3 μ M (*in vivo*) respectively.

VI *IN VIVO* EVALUATION OF INHIBITORS

Even though the results are not in the same order of magnitude, they are by no means significantly dissimilar.

However, one compound does not follow the correlation of *in vitro* vs. *in vivo* data. Inhibitor **31**, with *in vitro* IC₅₀ of 30 ± 3 μM, did not show any signs of inhibition at 50 μM and only a slight reduction on parasitemia occurred at 100 μM (Figure VI-8A). Yet in previous *in vivo* assays significant reduction in parasitemia was noticed at concentration points 10 μM and 100 μM (Bowyer, Gunaratne et al. 2007).



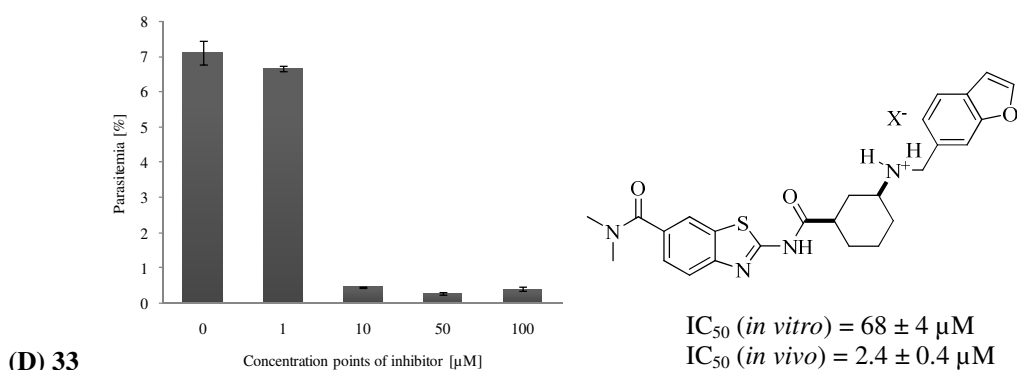


Figure VI-8: FACS data of compounds 31-33

On the left hand side is a histogram of the inhibition data of compound **31** (A), **34** (B), **32** (C) and **33** (D) in *Plasmodium falciparum* infected erythrocytes. The IC_{50} -values are compared from *in vitro* SPA data (Bowyer, Gunaratne et al. 2007) and *in vivo* FACS data.

The dissimilar behaviour of compound **31** is also noted in the raw data of the histograms. Figure VI-9 compares the effects of parasitemia with **31** and **33**. For inhibitor **31**, there was a general peak shift noted to the left, indicating the presence of more ring-stage parasites as opposed to late trophozoite/schizont stages (Figure VI-9A). Figure VI-9B clearly illustrates inhibitory effects against the parasite. The shift may represent a delay or halt in the growth cycle. Smears from **31** were taken and analysed under a microscope. Analysis revealed a reduction in parasitic growth, whereby the number of ring-stage parasites increases yet the number of trophozoite-stage parasites decreases (Table VI-1). Compound **31** may have a different kinetic mechanism on *P. falciparum* compared to the other tested inhibitors.

In general, the FACS assay was used to gather inhibition data at various concentrations points. Plots of the level of reduction in parasitemia relative to the inhibitor concentration enabled the estimation of IC_{50} -values *in vivo*. Similarities between *in vitro* and *in vivo* results were found.

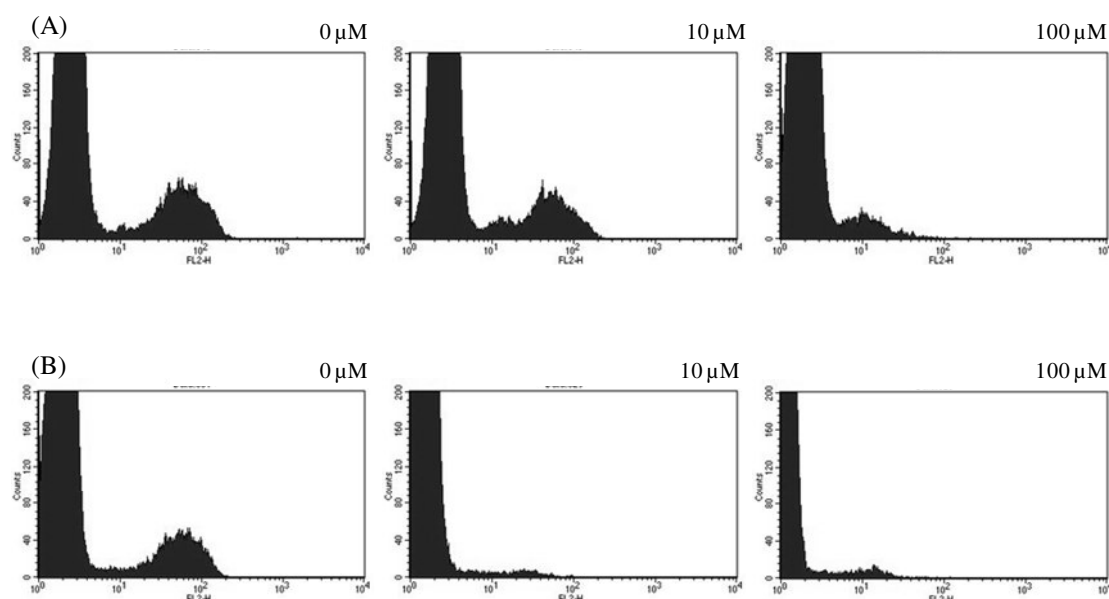


Figure VI-9: FACS histograms of 31 and 33

The FACS histograms were used to compare levels of parasitemia between two compounds, **31** (A) and **33** (B), at different concentrations. As can be seen in (A) the distinct peak of parasite infected erythrocytes shifted to the left. With increasing inhibitor concentration the level of parasitemia was also reduced. Nevertheless the compound exhibits different kinetic properties compared to (B) where parasitemia drops immediately without a shift

Table VI-1: Giesma-stained analysis of inhibitory action of 31

The table analyses *P. falciparum* infected erythrocytes, whereby the life stage was recorded.

Concentration of Inhibitor [μM]	No. of Ring-Stage Parasites	No. of Trophozoite- Stage Parasites	No. of Schizont- Stage Parasites
0	0.9	14	1.3
10	2.5	10	n.d.
50	12	4	n.d.

n.d. not detected

VI.3.2 *In vivo* studies of potential antimalarials

Due to anticipated results from all negative controls such as TFA, non active inhibitors and a range of salt compositions as well as comparable data to published results of potent inhibitors (Bowyer, Tate et al. 2008), it was concluded that the FACS assay gave repeatable and valid information about inhibition of *P. falciparum*

infected erythrocytes. The following sections describe *in vivo* inhibition experiments carried out with synthesised potential antimalarials.

VI.3.2.1 Analysis of the Best Antimalarial Candidates

Four compounds (**54**, **78**, **72**, **81**) have been discovered to inhibit PfNMT in the low micromolar region in *in vitro* studies using the SPA. They were then analysed *in vivo* employing the FACS method. Results showed consistency with previously gathered data. No cell toxicity was observed at the highest concentrations used. Figure VI-10A displays the activity of compound **54** against *P. falciparum* in the FACS assay. A noticeable drop in parasitemia was observed for concentration points at 100 μ M, 50 μ M and 25 μ M with near complete inhibition of parasitic growth. At 10 μ M the level of parasitemia was reduced one third. This correlated to the *in vitro* results where the IC₅₀ for **54** was $19.9 \pm 2.7 \mu\text{M}$. The estimated IC₅₀ *in vivo* was calculated to be $11.9 \pm 0.7 \mu\text{M}$ (Figure VI-10A). They are in the same range and confirm that **54** inhibits *P. falciparum* growth.

The analogue of **54**, compound **78**, with a *cis*-1,3-cyclohexyl group instead of *cis*-1,4-cyclohexyl unit showed in the *in vitro* tests more activity against PfNMT; however **78** was far more active, about 10-fold, in the *in vivo* experiments. Parasitemia was reduced considerably at 25 μ M, 10 μ M and 2 μ M. An IC₅₀-value of $1.74 \pm 0.79 \mu\text{M}$ was estimated (Figure VI-10B).

The oxygen atom in the benzofuran-based inhibitors was substituted with a sulfur atom to make benzothiophene analogues **72** and **81**. The inhibitors demonstrated activity against PfNMT in the *in vitro* tests which are in the same range as the inhibition results of the oxygen analogues. The FACS assay with *Plasmodium falciparum* infected erythrocytes incubated with **72** and **81** also had an effect on parasitemia; complete reduction in parasitemia was recorded at 25 μ M and 10 μ M and low levels for 2 μ M. The IC₅₀-values were calculated as $1.43 \pm 1.99 \mu\text{M}$ (Figure VI-10C) and $1.99 \pm 0.11 \mu\text{M}$ (Figure VI-10D) respectively. Compound **81** displayed the most potent effects on *Plasmodium falciparum* infected erythrocytes out of the series of compounds tested.

VI *IN VIVO* EVALUATION OF INHIBITORS

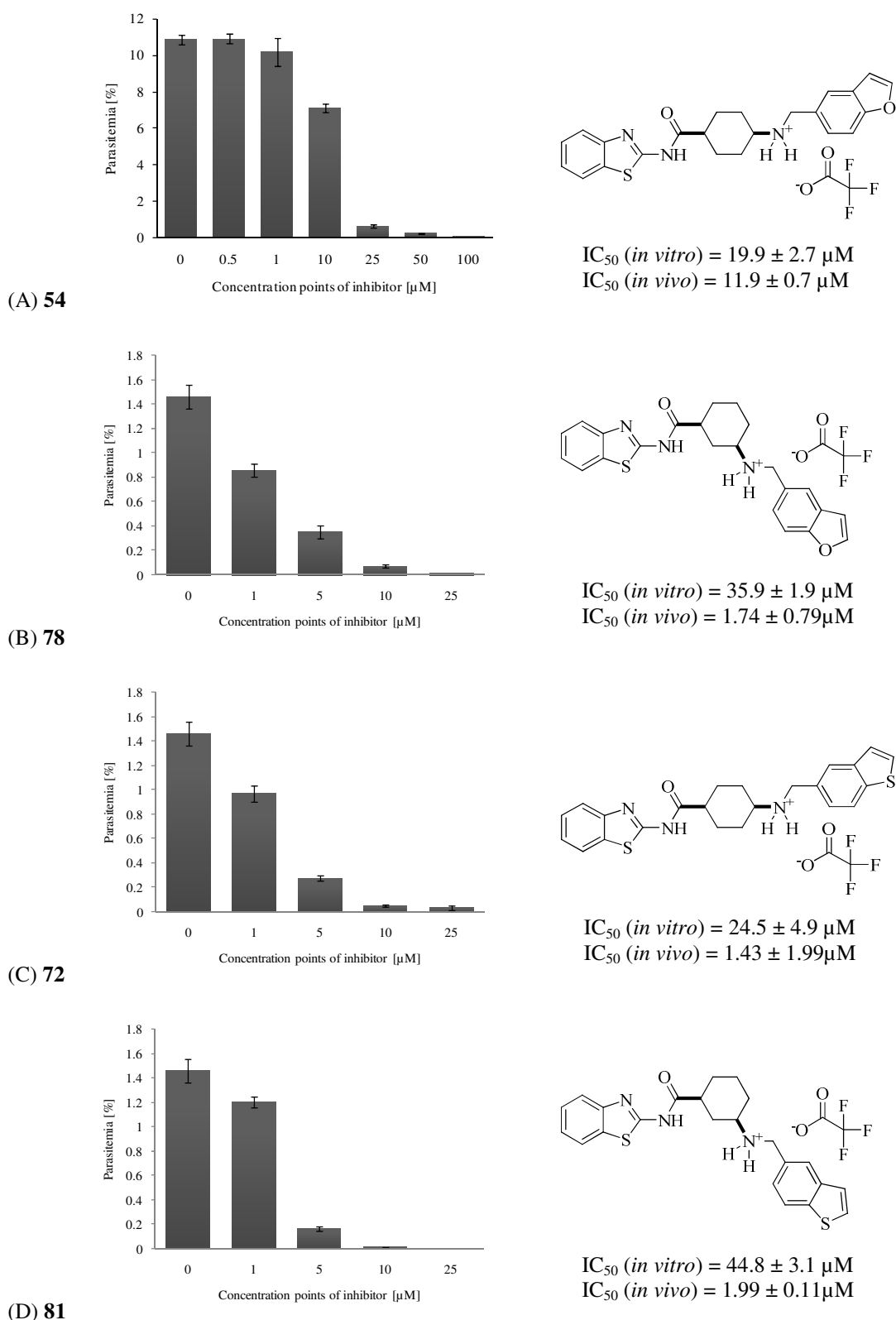


Figure VI-10: FACS data of compounds 54, 78, 72 and 81

On the left hand side is a histogram of the inhibition data of compound **54** (A), **78** (B), **72** (C) and **81** (D) in *Plasmodium falciparum* infected erythrocytes. The IC_{50} -values are compared from *in vitro* SPA data and *in vivo* FACS data.

VI.3.2.2 *In Vivo* Studies of Other Antimalarials

Other compounds (**56**, **71**, **49**) were also tested in the *in vivo* FACS assay (see Figure VI-11). They were not as potent in the *in vitro* tests as the previous four candidates (**54**, **78**, **72**, **81**). Nevertheless *in vivo* experiments were conducted with the intention of gaining information about inhibition patterns. Weakly inhibiting compounds with *in vitro* IC₅₀-values in the upper micromolar scale should behave similar in *in vivo* experiments. However, IC₅₀ results determined from *in vitro* SPA and *in vivo* FACS assays do not correlate with each other. Compound **56** inhibited parasite growth 30-fold more *in vivo* than *in vitro*; a larger difference was observed for **71** and **49**.

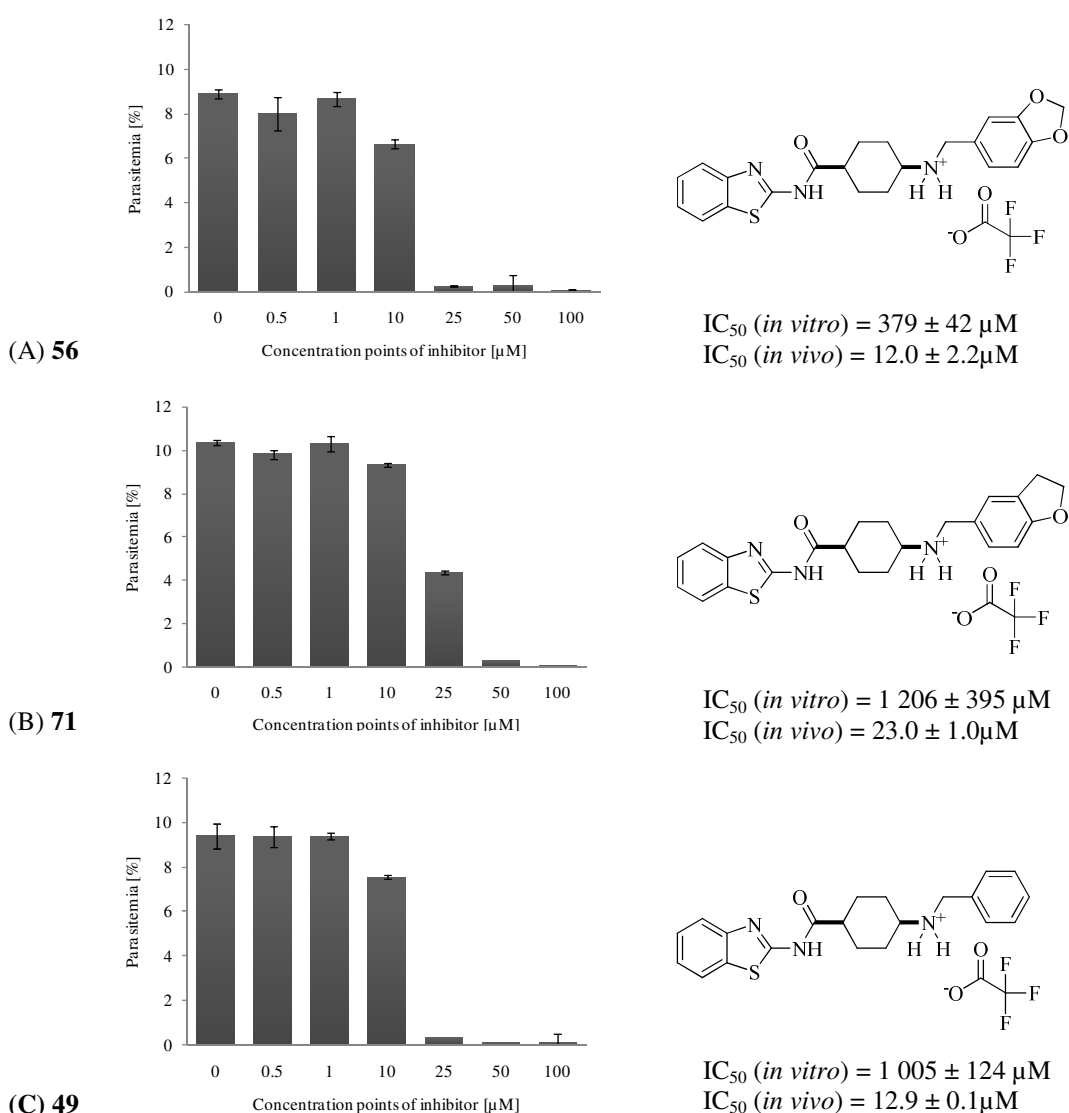


Figure VI-11: FACS data of compounds 56, 71, 49

On the left hand side is a histogram of the inhibition data of compound **56** (A), **71** (B) and **49** (C) in *Plasmodium falciparum* infected erythrocytes. The IC₅₀-values are compared from *in vitro* SPA data and *in vivo* FACS data.

VI.4 Conclusion and Discussion

The FACS methodology, an alternative to assay the effect of potential inhibitors in *P. falciparum* infected erythrocytes, was set up by our collaborators Dr A A Holder and Dr D Moss at NIMR. In previous years a different method was employed, the parasite was visualised by Giesma-staining and analysed with light microscopy. Parasite-infected erythrocytes were counted as well as the life stage of the parasite was noted (Bowyer 2006). This procedure is very time consuming. FACS is more efficient as the machine sorts each cell based on their size and fluorescent properties.

For comparative studies previously compounds tested by Dr P W Bowyer (Bowyer 2006; Bowyer, Gunaratne et al. 2007) were analysed with the FACS assay. And indeed, results are consistent and thus the developed FACS assay was used to determine the effects of potential antimalarials on parasitemia. Assays were carried out by Dr D Moss at NIMR.

A number of negative controls were also investigated to further establish conditions of the FACS assay. *In vivo* effects of one inhibitor as a free amine, HCl salt and TFA salt were evaluated because different salt compositions may have influenced inhibition. There was no significant change noted; all three compounds had similar inhibition patterns. Hence TFA salts of inhibitors were used in the *in vivo* studies of *P. falciparum* infected erythrocytes. An investigation also ruled out that TFA negatively influenced the assay.

In vitro SPA resulted in four compounds (**54**, **78**, **72**, **81**) with IC_{50} -values in the low micromolar region. Excitingly, potency in the *in vivo* FACS assay showed consistency with data from the *in vitro* tests; inhibition in the low micromolar region was observed. Additionally no cell toxicity was observed by a final concentration of maximum 100 μ M inhibitor in 0.5% DMSO. However, the assumption the sole reason of parasitic death was due to inhibition of NMT cannot be made; an off-target effect could also be likely and needs to be investigated further in future projects.

The most active inhibitor *in vitro*, IC_{50} (**54**) = $19.9 \pm 2.7 \mu$ M, retained antimalarial potency *in vivo* against *P. falciparum* infected erythrocytes. The estimated IC_{50} was calculated as $11.9 \pm 0.7 \mu$ M; with complete inhibition of parasite growth at 25 μ M, ~30% inhibition at 10 μ M and little inhibition at 1 μ M. However, the inhibitor which was most effective in the FACS assay was compound **81** (IC_{50} = $1.99 \pm 0.11 \mu$ M).

VII STRUCTURE-ACTIVITY RELATIONSHIPS – A FINAL SUMMARY

VII.1 SAR Analysis

As discussed previously (Figure IV-1) the lead compound from the “Pfizer series”, UK-370,509, was segmented into three parts R^A – R^C and systematically optimised. Table VII-1 shows a summary of *in vitro* inhibition results against PfNMT. The information was divided up into three sections; the size of the aromatic group R^A was investigated first, followed by studies of aromaticity and the influence of heteroatoms on inhibition.

Initially, crystal structures have shown that through the addition of a building block R^A a conformational change of the AA in the peptidic binding pocket was induced to extend the cavity (Figure II-19, Pfizer, unpublished results). Thus the first group in Table VII-1 examined this information by comparing inhibition behaviour with R^A substituted as methylbenzene (**49**), methylnaphthalene (**94**) and methylantracene (**68**) compared to no unit R^A (**38**). Inhibition studies suggested a trend, whereby a larger size of the aromatic group favoured binding in the target site resulting in lower IC_{50} -values; the inhibition was doubled with each additional C_6H_6 -group. However, the scale of improvement of inhibition was minor. The small increase in activity may have been caused by an increase of logP. A noted side-effect however, was the increase in insolubility of the compounds with a larger hydrophobic group. Ethylbenzene (**70**) was also tested to probe further into the binding pocket but it was not considerably more active than **49**. Either an additional

CH₂-group was not sufficiently long enough for favourable interactions of the benzyl ring with possible AA for π - π stacking interactions or the size of the aromatic system is vital.

The level of aromaticity was investigated next with various building blocks R^A such as aliphatic groups or alterations of aromatic character by substitution of the benzyl ring with electron-withdrawing and electron-donating groups. A tendency could not be recorded as all compounds inhibited PfNMT poorly; IC₅₀-values were >1 mM.

However, the insertion of heteroatoms yielded more active inhibitors; oxygen (**54**), sulfur (**72**) and nitrogen (**73**) derivatives of **94** exhibited inhibition in the low micromolar region. Nevertheless, **73** was a 5-fold weaker inhibitor. The Group VI atoms must be necessary for vital polar interactions such as hydrogen bonding with surrounding AA of the target binding site. Hence the position of the heteroatom is crucial as studies showed weakened inhibition with methylfuran (**51**) and methylthiophene (**65**) as unit R^A with 100-fold less activity than their larger analogues **54** and **72**.

Yet the inclusion of an oxygen atom was no guarantee for inhibition. Compounds with a second oxygen atom were also tested and although they displayed inhibition below 1 mM, they were not as active as **54**.

The size of the aromatic system seemed also important as 5-methylbenzyl-dihydrofuran (**71**), a less aromatic system than 5-methylbenzylfuran (**54**), was not very active against PfNMT during the *in vitro* studies as well as the sulfur substitute. But increasing the aromatic system with 5-methyldibenzofuran (**69**) resulted in a complete loss in activity also. Hence 5-methylbenzofuran as group R^A is a valid choice. The positions of the oxygen atom as well as the π -clouds lead to enhanced binding compared to the lead compound, although there is still room for improvement. Studies could go into the investigation of 3-methylbenzofuran whereby the oxygen atom is in a different position in the ring which may lead to different interactions.

Compounds with a *cis*-1,4-cyclohexyl group as building block R^B that showed some activity against PfNMT (IC₅₀ <1 mM) were subsequently synthesised with a *cis*-1,3-orientation of the substituents to further explore the binding cavity. *In vitro* inhibition results are listed in the third column of Table VII-1.

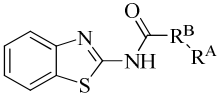
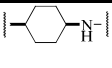
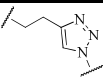
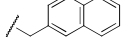
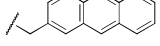
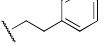
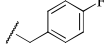
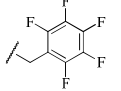
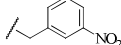
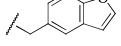
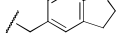
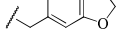
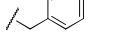
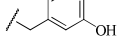
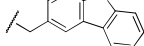
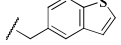
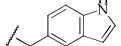
Results showed that the orientation of substituents on the cyclohexyl ring (*cis*-1, 4 vs. *cis*-,1,3) had no major impact on inhibition of PfNMT. This probably indicates that the orientation of the substituents is similar, irrespective of the cyclohexyl linker used.

Another very important aspect is the secondary amine which joins units R^A and R^B. Building blocks R^B proline (Column IV, Table VII-1) and triazole derivatives (Column V, Table VII-1) lack this moiety which resulted in a complete loss in activity. Further studies examined the linkage more closely; compound **94**, **49** and **54** were acetylated and were found to be inactive against PfNMT.

Altogether several factors are very important for binding in the peptidic pocket of *Plasmodium falciparum* NMT. The π -electron clouds of the benzothiazole unit, R^C, as well as the heteroatoms probably interact with surrounding AA of the binding site. Building block R^B possibly serves as a linker to space out the interactive sections of the molecule, whereby a rigid unit was preferred over a flexible chain (Pfizer, unpublished research). The ideal orientation of the substituents has not been fully examined and needs to be explored further, but the *cis*-1,4-cyclohexyl group was the most successful. The linkage from R^B to R^A, in this case a 2° amine, is needed most likely for interactions with the C-terminus of the enzyme that partially forms the peptidic binding site (Hasegawa, Morikami et al. 2003). Unit R^A appears to be the key factor for inhibition against PfNMT. 5-Methylbenzofuran and the sulfur analogue attached to *cis*-4-amino-*N*-benzo[*d*]thiazol-2-yl)cyclohexylcarboxamide resulted in inhibitors with IC₅₀-values in the low micromolar region.

Table VII-1: Summary of inhibition studies *in vitro*

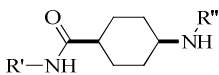
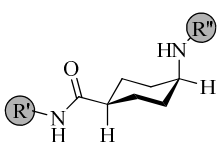
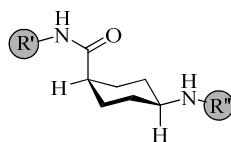
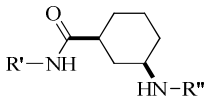
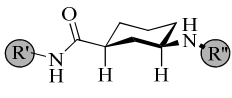
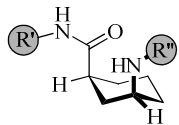
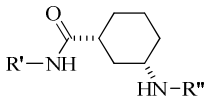
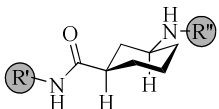
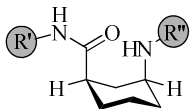
This table sums up the compounds that were tested in the SPA against PfNMT. R^A was split up into three groups of certain criteria; first the size of the aromatic group, second the level of aromaticity and third the inclusion of heteroatoms. The table is colour coded as follows: $IC_{50} < 100 \mu M$ (red-coloured); $IC_{50} < 500 \mu M$ and $> 100 \mu M$ (orange-coloured); $IC_{50} < 1 mM$ and $> 500 \mu M$ (gold-coloured); $IC_{50} > 1 mM$ (pale yellow-coloured); white/blank – not tested.

					
R^A	R^B				
	38	43 743 ± 54	44		
	49 1005 ± 124	76 473 ± 46	46	87	
	35 481 ± 62	74 359 ± 57	45	88	
	68 177 ± 3				
	70				
	61				
	63				
	64				
	48				
	51				
	54 19.9 ± 2.7	78 35.9 ± 1.9	47	90	
	71				
	56 379 ± 42	82 620 ± 159			
	59 687 ± 32				
	67 993 ± 135				
	69				
	65				
	72 24.5 ± 4.9	81 44.8 ± 3.1			
	73 116 ± 11	80 275 ± 4.8			

The four top inhibitors against PfNMT (**54**, **72**, **78** and **81**) were investigated more closely. The main difference between **54/78** and **72/81** is the orientation of the substituents, which are either arranged in a 1,4 or 1,3-position of the cyclohexyl ring. The *cis*-1,4-compound is a single stereoisomer due to a planar symmetry. The substituents are orientated one axial and one equatorial. Both possible chair conformations are illustrated in Table VII-2A. The “*cis*-1,3” series has two enantiomeric carbon atoms. The cyclohexyl unit R^B was purchased as a racemic mixture. Substituents are either both orientated in an axial or equatorial position (Table VII-2B, C). The di-equatorial arrangement (#1) is more likely than the di-axial one (#2) due to steric hinderance. Orientation of the substituents are similar for compounds of the “*cis*-1,4” and “*cis*-1,3” series with di-equatorial arrangement. And indeed, similar inhibition patterns *in vitro* were noted.

Table VII-2: Comparison of *cis*-1,4- and *cis* -1,3-cyclohexyl building block

The conformation of two building blocks B, *cis*-1,4-cyclohexane (A) and *cis*-1,3-cyclohexane (B and C), are compared and visualised in the chair conformation. The 1,4 compound is a single stereoisomer, but the 1,3 version is a mixture of two enantiomers (B and C). For each compound there are two possible chair conformations of the cyclohexyl ring: in the 1,4 series it is possible that either or both conformations are present; for the 1,3 compounds it is likely that the di-equatorial conformations (#1) will predominate over the crowded di-axial ones (#2).

Chemical Structure	Chair Conformation #1	Chair Conformation #2
(A) 		
(B) 		
(C) 		

Inhibitors with an $IC_{50} < 1$ mM were tested in the *in vivo* FACS assay with *Plasmodium falciparum* infected erythrocytes. But precise correlations between *in vitro* SPA and *in vivo* data were difficult to propose. This is due to the fact that *in vitro* evaluations are carried out on specific targets alone. *In vivo* experiments are conducted in a cell environment where numerous factors are in competition with the target. Similarities were noted between compounds that inhibited PfNMT *in vitro* in the low micromolar region; they also exhibited high reductions in parasitemia at the calculated *in vitro* IC_{50} . Non-inhibitors, that were ineffective against PfNMT *in vitro*, displayed no activity *in vivo* as well. But data was not consistent with regards to weak inhibitors *in vitro* as they were more active *in vivo*, as expected.

Reduction in levels of parasitemia may be the cause of different interactions. First it could be caused by inhibition of *Plasmodium falciparum* *N*-myristoyl transferase in which case the inhibitor competes with the peptide substrate in the NMT binding site, thus inhibiting *N*-myristoylation. Inhibition results may differ though due to several possibilities such as solubility, transport and metabolism of the compound. For cases where the activity *in vivo* is higher than *in vitro*, effects of solubility may be responsible. The environment in the parasitic cell may increase solubility of the inhibitor compared to the buffer system in the *in vitro* assay. A higher concentration of compound would then lead to increased inhibition in *N*-myristoylation. Alternatively, not the compound but by-products of metabolised inhibitor may influence the function of NMT strongly. The inhibitor could also accumulate in the cell and reach toxic levels. However, no negative impact was noted on the integrity of the erythrocytes.

If the compound does not reduce parasitemia solely *via* the mechanism of *N*-myristoyl transferase, the target site needs to be identified. Studies would need to be carried out in future projects to investigate the mode of inhibition in a cell. It is important to investigate the specificity of the drug molecule for NMT, a different target in the parasite or the display of a non-specific inhibitory effect.

VII.2 Conclusions

This project had the goal of examining the peptidic binding pocket of *N*-myristoyl transferase of the malarial organism *Plasmodium falciparum* in more depth; a small-sized library was synthesised to explore the target site systematically, to develop potent inhibitors that in future could be formulated into antimalarial drugs. Co-crystal structures of PfNMT and inhibitors have not yet been published that could lead to valid information about preferential binding partners. Nevertheless successful co-crystal structures of NMT of other organisms such as *Candida albicans* have been solved. Phylogenetic analysis revealed similarities between the peptidic binding pocket of CaNMT and PfNMT; hence, a piggy-back approach was carried out whereby established potent inhibitors for CaNMT were applied to the malarial system. In this thesis the strategic development of the lead compound to more potent equivalents against PfNMT yet retaining selectivity over human NMT was described.

Inhibitors were based on a benzothiazole scaffold; the “Pfizer series” compounds, which have been shown to interact with AA from the peptidic binding pocket of NMT, have been methodically altered to create a small-sized library with approximately 50 inhibitors. Potency against PfNMT and selectivity over human NMT were determined in an established scintillation proximity assay that monitors the transfer of [³H]-labelled myristoyl CoA to the N-terminus of a polypeptide substrate. The plot of inhibitor concentration with respect to remaining enzyme activity gave inhibition curves from which IC₅₀-values were derived. Enzymes required for the assay were overexpressed in *Escherichia coli* and purified yielding 1.0 mg L⁻¹ *P. falciparum* NMT, 0.6 mg L⁻¹ *Homo sapiens* NMT1 and 1.5 mg L⁻¹ *Homo sapiens* NMT2. *In vitro* SPA revealed four compounds that inhibited PfNMT in the low micromolar region, **54** (IC₅₀ = 19.9 ± 2.7 µM), **78** (IC₅₀ = 35.9 ± 1.9 µM), **72** (IC₅₀ = 24.5 ± 4.9 µM) and **81** (IC₅₀ = 44.8 ± 3.1 µM). They were taken forward to be tested *in vivo* against *Plasmodium falciparum* infected erythrocytes. They showed a significant reduction in parasitemia at concentration levels of 25 µM, 10 µM and 5 µM inhibitor. Unfortunately the inhibitors were not exceptionally selective over human NMT with IC₅₀-values *in vitro* in similar micromolar regions.

VII.3 Outlook

Lead compound optimisation showed limited success; the improved compounds inhibit PfNMT in the low micromolar region with an IC_{50} of 19.9 μ M reported for the best inhibitor. It is essential for potential drug candidates to inhibit the target site at least in the nanomolar region to be considered for further drug validation processes.

The aspiration will not only be an increase in activity against PfNMT but also create a larger selectivity against human NMT. Unfortunately, the best four hits with activity against PfNMT in the low micromolar region also showed activity against human NMT in the same range. This needs to be addressed in upcoming drug development projects. Potentially, modifications at the C⁶-position of the benzothiazole-scaffold could decrease activity against the human enzyme and further investigations into building block A can yield higher potency against malarial NMT. A different scaffold as R^C should also be examined. Various amines could be employed to probe possible AA interactions in the peptide binding pocket; in particular interactions with Ser should be investigated (Pfizer, unpublished work).

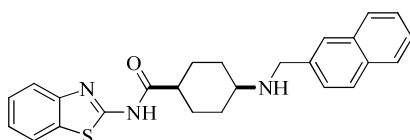
A large problem was the solubility of compounds. Sections should be included to increase solubility in aqueous assay buffer such as ether, ketones or alcohol moieties.

Also in future projects, libraries can be produced by solid phase assisted synthesis instead of combinatorial solution phase chemistry (II.3.3., pg. 44, (Yamazaki, Kaneko et al. 2005)).

A different series, based on benzofuran scaffolds, is analysed in the Leatherbarrow Research Group as previously mentioned. There has been no solubility issue noted so far. Zhiyong Yu has reported an inhibitor with an IC_{50} -value of 15 μ M; a good starting point for optimising the inhibitors further (Yu 2009).

VIII APPENDIX

VIII.1 Compound Synthesis and Characterisation



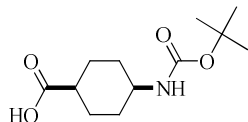
cis-N-(Benzo[d]thiazol-2-yl)-4-((naphthalen-2-yl)methylamino)cyclohexane-carboxamide (35). Compound **38** (102 mg, 0.262 mmol) was dissolved in DCE (1.4 mL), with a final concentration of 0.2 M. NEt₃ (76 μ L, 0.545 mmol), 2-naphthaldehyde (40 mg, 0.256 mmol), NaBH(OAc)₃ (91 mg, 0.429 mmol) were added to the reaction mixture. Product precipitation was observed over time. After 24 h, the precipitate was collected by filtration. The crude product was purified by chromatography on silica gel (0–70% EtOAc in Hex) to afford **35** (0.73 g, 67%) as a white crystalline solid.

TFA salt (**94**). Compound **35** was dissolved in TFA and stirred for 10 min. All volatiles were removed *in vacuo*.

HCl salt (**95**). Compound **35** was dissolved in DCM. HCl_(conc) was added and stirred for 10 min. All volatiles were removed *in vacuo*.

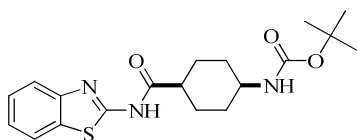
35. >98% Purity (t_r = 10.8 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.03 (EtOAc–Hexane, 2:1); mp 196–198 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2927 (w, br), 1674 (m, sh), 1544 (m, sh), 1183 (s, br), 1133 (s, br), 721 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.85 (1H, m, PhH), 7.77–7.83 (4H, m, PhH), 7.73 (1H, m, PhH), 7.42–7.48 (4H, m, PhH), 7.32 (1H, t, J 11.4, PhH), 3.90 (2H, s, NHCH₂), 2.78 (1H, m, NHCOCH), 2.48 (1H, m, CHNH), 2.04 (2H, m, CH₂), 1.73 (2H, m, CH₂), 1.59 (2H, m, CH₂), 1.39 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 174.7 (C), 159.8 (C), 147.7 (C), 137.8 (C), 131.8 (C), 128.1 (PhH), 127.7 (PhH), 126.7 (PhH), 126.5 (PhH), 126.3 (PhH), 125.6 (PhH), 124.0 (PhH), 121.7 (PhH), 120.4 (PhH), 51.5

(CH), 51.2 (NHCH₂), 43.5 (CH), 29.1 (2C, CH₂), 24.1 (2C, CH₂); *m/z* (FAB) 416 (40%, [M + H]⁺), 289 (15%, [M – naphthyl]), 149 (30%, [M – ABT]⁺), 141 (75%, [M – methyl naphthyl]), 136 (75%, [benzothiazole]⁺); HRMS found 416.1798 (C₂₅H₂₆N₃OS, [M+H]⁺, requires 416.1797).



***cis*-4-*N*-*tert*-Butylcarbamatecyclohexylcarboxylic acid (36).** *rac-cis*-4-Aminocyclohexanecarboxylic acid (2.06 g, 14 mmol) was dissolved in NEt₃ (11.8 mL, 85 mmol) and MeOH (14 mL), with a final concentration of 1.0 M. A solution of di-*tert*-butyl dicarbonate (6.95 g, 32 mmol) in MeOH was added drop wise to the cream-coloured suspension. The reaction mixture was refluxed at 60 °C. Gas formation was noticed. After 18 h reaction time, the reaction mixture was filtered and concentrated *in vacuo*. The oily, yellow-coloured substance was dissolved in 2% NaHCO₃ (50 mL) and washed with hexane (3 × 50 mL). The aqueous phase was acidified with cold 1 M HCl_(aq) to a pH of 2, whereupon precipitation was noticed. The product was extracted with EtOAc. The organic phase was dried over MgSO₄ and filtered. The solid was dried *in vacuo* to yield **36** (2.37 g, 70%) as a white amorphous solid.

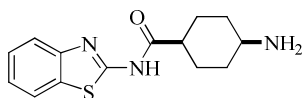
>98% Purity; *R_f* = 0.62 (EtOAc–Hex, 2:1); mp 169–172 °C; *v*_{max}/cm^{–1} (solid) 3295 (w, br), 2928 (m, br), 2857 (w, br), 1694 (vs, sh), 1635 (m, sh), 1407 (m, sh), 1367 (m, sh), 1162 (s, sh), 962 (m, sh), 752 (s, sh); *δ*_H/ppm (500 MHz, DMSO-*d*₆) 6.15 (NH-Boc), 3.32 (1H, m, CHCOOH), 2.35 (1H, m, CHNH), 1.82–1.88 (2H, m, CH₂), 1.40–1.55 (6H, m, CH₂), 1.36 (9H, s, C(CH₃)₃), *δ*_C/ppm (125 MHz, DMSO-*d*₆) 175.8 (C), 154.7 (C), 77.1 (C(CH₃)₃), 47.8 (CH), 39.7 (CH), 29.7 (2C, CH₂), 28.7 (3C, CH₂), 25.1 (2C, CH₂); *m/z* (FAB) 244 (10%, [M + H]⁺), 188 (40%, [M + *tert*-butyl]⁺), 126 (50%), 57 (100%, [*tert*-butyl]⁺); HRMS found 406.1591 (C₂₃H₂₄N₃O₂S, [M + H]⁺, requires 406.1589).



cis-tert-Butyl-4-(benzo[d]thiazol-2-ylcarbonyl)cyclohexylcarbamate (37).

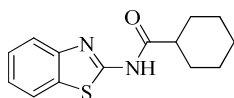
Compound **36** (2.20 g, 9 mmol) was dissolved in DMF–DCM (1/1, V/V, 18 mL), with a final concentration of 0.5 M, at 0 °C. 2-Aminobenzothiazole (1.51 g, 10 mmol), PyBOP (5.16 g, 10 mmol), DIPEA (3.15 mL, 18 mmol) were added. After 10 min the clear, yellow-coloured solution was allowed to warm to RT. After 19 h, the pink-coloured suspension was filtered. The precipitate was washed with H₂O (3 × 20 mL), brine (1 × 10 mL), dried over MgSO₄ and filtered. The solid was dried *in vacuo* to yield **37** (3.07 g, 90%) as a white amorphous solid.

>98% Purity (t_r = 13.4 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.35 (EtOAc–Hexane, 2:1); mp 239–240 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 3049 (*m*, br), 2932 (*m*, br), 2863 (*w*, sh), 1672 (*s*, sh), 1550 (*s*, sh), 1441 (*m*, sh), 1399 (*m*, sh), 1362 (*m*, sh), 1258 (*m*, sh), 1167 (*s*, sh), 1071 (*m*, sh), 962 (*m*, sh), 753 (*s*, sh); $\delta_{\text{H}}/\text{ppm}$ (500 MHz, DMSO-*d*₆) 12.10 (1H, *s*, NHCO), 7.85 (1H, *d*, *J* 7.5, *PhH*), 7.62 (1H, *d*, *J* 7.5, *PhH*), 7.32 (1H, *t*, *J* 7.5, *PhH*), 7.18 (1H, *t*, *J* 7.0, *PhH*), 6.72 (1H, *s*, NH-Boc), 2.78 (1H, *m*, NHCOCH), 2.40 (1H, *m*, CHNH-Boc), 1.78 (2H, *m*, CH₂), 1.50–1.56 (4H, *m*, CH₂), 1.42 (2H, *m*, CH₂), 1.28 (9H, *s*, C(CH₃)₃); $\delta_{\text{C}}/\text{ppm}$ (125 MHz, DMSO-*d*₆) 174.9 (*C*), 158.2 (*C*), 155.1 (*C*), 148.6 (*C*), 130.5 (*C*), 126.1 (*PhH*), 123.5 (*PhH*), 121.7 (*PhH*), 120.4 (*PhH*), 77.5 (C(CH₃)₃), 45.9 (*CH*), 41.7 (*CH*), 28.4 (3C, C(CH₃)₃), 28.9 (CH₂), 24.0 (CH₂); m/z (FAB) 376 (85%, [M + H]⁺), 151 (90%, [ABT + H]⁺), 57 (85%, [*tert*-butyl + H]⁺); HRMS found 376.1711 (C₁₉H₂₆N₃O₃S, [M + H]⁺, requires 376.1695).



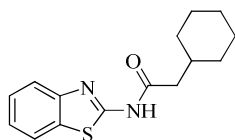
cis-4-Amino-N-(benzo[d]thiazol-2-yl)cyclohexylcarboxamide (38). Compound **37** (0.10 g, 0.3 mmol) was dissolved in TFA–H₂O–TIPS (90/5/5, V/V/V, 0.50 mL, 6.5 mmol TFA) and DCM (0.20 mL), with a final concentration of 0.4 M for **37**. The reaction mixture was stirred for 30 min at RT until gas formation stopped. Cold diethyl ether was added until precipitation was observed (~10 mL). The precipitate was collected by centrifugation and washed with cold diethyl ether (3 × 10 mL). The solid was dried *in vacuo* to afford **38** (0.06 g, 79%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 5.3 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.02 (EtOAc–Hexane, 2:1); mp 197–199 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2926 (*w*, br), 1676 (*m*, sh), 1563 (*m*, sh), 1134 (*s*, sh), 720 (*s*, sh); $\delta_{\text{H}}/\text{ppm}$ (500 MHz, DMSO-*d*₆) 12.40 (1H, s, NHCO), 8.03 (2H, br s, NH₂), 7.94 (1H, d, *J* 7.5, PhH), 7.72 (1H, d, *J* 9.0, PhH), 7.41 (1H, t, *J* 7.5, PhH), 7.28 (1H, t, *J* 7.5, PhH), 3.19 (1H, m, CHNH₂), 2.76 (1H, m, NHCOCH), 1.98 (2H, m, CH₂), 1.73 (4H, m, CH₂), 1.67 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (125 MHz, DMSO-*d*₆) 174.5 (C), 158.1 (C), 148.6 (C), 131.5 (C), 126.2 (PhH), 123.6 (PhH), 121.7 (PhH), 120.5 (PhH), 47.6 (CH), 25.0 (2C, CH₂), 24.0 (2C, CH₂); m/z (ESI) 391 (20%, [M + TFA]⁺), 276 (20%, [M + H]⁺), 151 (100%, [ABT + H]⁺); HRMS found 276.1178 (C₁₄H₁₈N₃OS, [M + H]⁺, requires 276.1171).



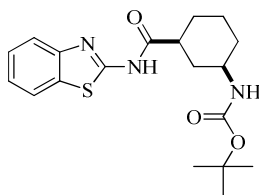
***N*-(Benzo[*d*]thiazol-2-yl)cyclohexylcarboxamide (39).** 2-Aminobenzothiazole (1.49 g, 10 mmol) and 4-pyrrolidinopyridine (1.77 g, 12 mmol) were each dissolved in DMF (20 mL), with a final concentration of 0.5 M for 2-aminobenzothiazole, and added together, upon which a white precipitate formed. Cyclohexanecarbonyl chloride (1.470 mL, 12 mmol) was added drop wise. After 12 h the reaction mixture was diluted with DCM (80 mL), washed with 1 M HCl_(aq) (3 × 100 mL), H₂O (3 × 100 mL) and brine (1 × 100 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was purified by chromatography on silica gel (0–98% DCM in MeOH) to afford yellow oil. Re-crystallisation in MeOH resulted in **39** (2.04g, 78%) as a cream-coloured crystalline solid.

>98% Purity (*t_r* = 13.6 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); *R_f* = 0.82 (EtOAc–Hexane, 2:1); mp 137.6–138.4 °C; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 3179 (*w*, *br*), 3056 (*w*, *br*), 2928 (*m*, *sh*), 1693 (*s*, *sh*), 1542 (*s*, *sh*), 1443 (*s*, *sh*), 1262 (*s*, *sh*), 1162 (*s*, *sh*), 1126 (*s*, *sh*), 760 (*s*, *sh*); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 11.63 (1H, *s*, NHCO), 7.86 (1H, *d*, *J* 8.0, *PhH*), 7.79 (1H, *d*, *J* 8.0, *PhH*), 7.46 (1H, *t*, *J* 7.5, *PhH*), 7.34 (1H, *t*, *J* 7.5, *PhH*), 2.32 (1H, *tt*, *J* 12.0, *J* 3.5, NHCOCH), 1.80 (2H, *m*, CH₂), 1.67 (2H, *m*, CH₂), 1.47–1.55 (3H, *m*, CH₂), 1.13 (1H, *m*, CH₂), 0.96 (2H, *m*, CH₂), $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 175.2 (*C*), 160.2 (*C*), 147.4 (*C*), 132.0 (*C*), 126.4 (*PhH*), 124.1 (*PhH*), 121.7 (*PhH*), 120.4 (*PhH*), 45.0 (NHCOCH), 29.2 (2C, CH₂), 25.1 (2C, CH₂); *m/z* HRMS found 262.1067 (C₁₄H₁₇N₂OS, [M + H]⁺, requires 262.1062).



***N*-(Benzo[*d*]thiazol-2-yl)-2-cyclohexylacetamide (40).** Cyclohexylacetic acid (0.65 g, 5 mmol) was dissolved in DMF–DCM (1/1, V/V, 18 mL), with a final concentration of 0.25 M. DIPEA (1.55 mL, 9 mmol), HOBt (0.70 g, 6 mmol), and HBTU (2.57 g, 7 mmol) were added sequentially. The clear, yellow-coloured solution was stirred for 30 min at RT. 2-Aminobenzothiazole (0.62 g, 4 mmol) was added to the reaction mixture. After 20 h the solution was diluted with DCM (100 mL), washed with H₂O (3 × 100 mL), and dried *in vacuo*. The crude product was purified by chromatography on silica gel (0–95% DCM in MeOH) to afford **40** (0.024 g, 2%) as a white crystalline solid.

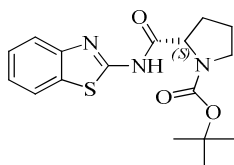
>98% Purity (t_r = 14.9 min, 2–98% MeOH–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.65 (EtOAc–Hexane, 2:1); mp 174–179 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 3184 (w, br), 3056 (w, br), 2926 (*m*, br), 2850 (*m*, sh), 1657 (*m*, sh), 1543 (*s*, br), 1456 (*w*, br), 1116 (*m*, br), 721 (*s*, sh); $\delta_{\text{H}}/\text{ppm}$ (300 MHz, CDCl₃) 7.83 (1H, d, *J* 8.1, PhH), 7.75 (1H, d, *J* 8.1, PhH), 7.74 (1H, t, *J* 7.2, PhH), 7.32 (1H, t, *J* 7.2, PhH), 2.33 (1H, m, CH₂), 1.69–1.83 (2H, m, CH₂), 1.01–1.37 (6H, m, CH₂), 0.86 (3H, m, CH₂), $\delta_{\text{C}}/\text{ppm}$ (75 MHz, CDCl₃) 172.1 (C), 146.2 (C), 130.4 (PhH), 126.1 (PhH), 120.1 (PhH), 114.6 (PhH), 64.3 (CH₂), 42.9 (CH), 33.1 (CH₂), 29.7 (2, CH₂), 26.0 (CH₂); *m/z* (FAB) 275 (30%, [M + H]⁺), 151 (25%, [ABT + H]⁺); HRMS found 275.1229 (C₁₅H₁₉N₂OS, [M + H]⁺, requires 275.1218).



cis-tert-Butyl-3-(benzo[d]thiazol-2-ylcarbamoyl)cyclohexylcarbamate (41).

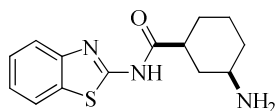
3-(*rac-cis-N-tert-Butylcarbamate*)cyclohexylcarboxylic acid (0.49 g, 2.0 mmol) was dissolved in DMF (14 mL), with a final concentration of 0.5 M, at 0 °C. 2-Aminobenzothiazole (0.34 g, 2.0 mmol), PyBOP (1.19 g, 2.0 mmol), DIPEA (0.696 mL, 4.0 mmol) were added. After 10 min the clear, yellow-coloured solution was allowed to warm to RT. After 19 h, the clear, brown-coloured reaction mixture was diluted with DCM (100 mL), washed 0.1 M HCl_(aq) (3 × 100 mL), H₂O (3 × 100 mL), brine (1 × 100 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was dissolved in a minimal amount of DCM (1 mL) and added to cold diethyl ether until precipitation was observed (~10 mL). The precipitate was collected by centrifugation and washed with cold diethyl ether (1 × 10 mL). The solid was dried *in vacuo* to yield **41** (0.50 g, 66%) as a white flocculent solid.

>98% Purity (*t_r* = 13.5 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); *R_f* = 0.51 (EtOAc–Hexane, 2:1); mp 190–194 °C; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 3274 (*m*, br), 3141 (*vw*, br), 2935 (*m*, br), 2866 (*w*, br), 1675 (*s*, sh), 1557 (*s*, sh), 1441 (*s*, sh), 1364 (*s*, sh), 1315 (*s*, sh), 1266 (*s*, sh), 1167 (*s*, sh), 757 (*vs*, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, DMSO-*d*₆) 12.31 (1H, *s*, NHCO), 7.95 (1H, *d*, *J* 7.6, *PhH*), 7.72 (1H, *d*, *J* 8.0, *PhH*), 7.42 (1H, *t*, *J* 8.8, *PhH*), 7.29 (1H, *t*, *J* 7.6, *PhH*), 6.84 (1H, *d*, *J* 8.0, NH-*tert*-Bu), 3.28 (1H, *m*, CHNH-Boc), 2.60 (1H, *m*, CH), 1.94 (1H, *m*, CH₂), 1.77 (3H, *m*, CH₂), 1.37 (9H, *s*, C(CH₃)₃), 1.29 (3H, *m*, CH₂), 1.10 (1H, *m*, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, DMSO-*d*₆) 174.7 (*C*), 158.3 (*C*), 155.0 (*C*), 131.5 (*PhH*), 126.2 (*PhH*), 123.6 (*PhH*), 121.8 (*PhH*), 120.6 (*PhH*), 77.7 (C(CH₃)₃), 55.0 (CH), 48.6 (CH₂), 43.0 (CH), 35.2 (CH₂), 28.4 (3C, C(CH₃)₃), 27.9 (CH₂), 24.0 (CH₂); *m/z* (FAB) 376 (85%, [M + H]⁺), 151 (90%, [ABT + H]⁺), 57 (85%, [^{*t*}Bu + H]⁺); HRMS found 376.1711 (C₁₉H₂₆N₃O₃S, [M + H]⁺, requires 376.1695).

**(S)-tert-Butyl 2-(benzo[d]thiazol-2-ylcarbamoyl)pyrrolidine-1-carboxylate (42).**

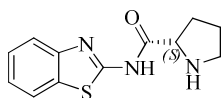
Boc-L-Pro-OH (1.01 g, 4.7 mmol) was dissolved in DMF (10 mL), with a final concentration of 0.5 M, at 0 °C. 2-Aminobenzothiazole (0.79 g, 5.2 mmol), PyBOP (2.75 g, 5.3 mmol), DIPEA (1.628 mL, 9.4 mmol) were added sequentially. After 10 min the clear, yellow-coloured solution was allowed to warm up to RT. After 4 h, the brown-coloured reaction mixture was diluted with DCM (100 mL), washed 0.1 M HCl_(aq) (3 × 100 mL), H₂O (3 × 100 mL), brine (1 × 100 mL), and dried over Na₂SO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified with flash column chromatography (DCM–MeOH, 0–40%) to yield **42** (1.39 g, 86%) as a white amorphous solid.

>98% Purity (*t_r* = 12.4 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); *R_f* = 0.42 (EtOAc–Hexane, 2:1); mp 199–201 °C; *v*_{max}/cm^{−1} (solid) 3188 (w, br), 3058 (w, br), 2967 (m, br), 2880 (w, br), 1720 (m, sh), 1652 (m, sh), 1555 (m, sh), 1424 (m, sh), 1264 (s, sh), 1164 (m, sh), 755 (s, sh); δ_H/ppm (400 MHz, CDCl₃) 11.39 (1H, s, NHCO), 7.78 (2H, m, PhH), 7.35 (1H, m, PhH), 7.24 (1H, m, PhH), 4.52 (1H, app d, *J* 4.8, CH), 3.48 (2H, m, CH₂N), 1.82–2.29 (4H, m, CH₂), 1.39–1.45 (9H, m, C(CH₃)₃); δ_H/ppm (400 MHz, CDCl₃, 50 °C) 7.77 (2H, d, *J* 8.0, PhH), 7.40 (1H, t, *J* 8.0, PhH), 7.27 (1H, t, *J* 8.0, PhH), 4.50 (1H, s, CH), 3.48 (2H, m, CH₂), 2.38 (1H, m, CH₂), 2.12 (1H, m, CH₂), 1.93 (2H, m, CH₂), 1.39 (9H, s, C(CH₃)₃); δ_C/ppm (100 MHz, CDCl₃) 172.3 (C), 158.5 (C), 156.2 (C), 148.5 (C), 132.1 (C), 126.2 (PhH), 124.0 (PhH), 121.4 (PhH), 120.9 (PhH), 81.2 (C(CH₃)₃), 60.9 (CH), 46.4 (NHCH₂), 28.4 (3C, C(CH₃)₃), 26.5 (CH₂), 24.6 (CH₂); *m/z* (FAB) 348 (100%, [M + H]⁺), 292 (15%, [M – *tert*-butyl]⁺), 248 (50%, [M – Boc]⁺), 151 (95%, [ABT + H]⁺), 57 (40%, [*tert*-butyl]⁺); HRMS found 348.1396 (C₁₇H₂₂N₃O₃S, [M + H]⁺, requires 348.1382).



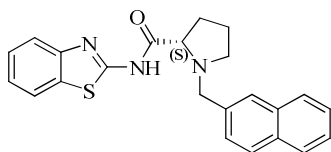
cis-3-Amino-N-(benzo[d]thiazol-2-yl)cyclohexylcarboxamide (43). Compound **41** (0.41 g, 1.0 mmol) was dissolved in TFA–H₂O–TIPS (90/5/5, V/V/V, 1.67 mL, 22 mmol TFA), with a final concentration of 0.6 M for **41**. The reaction mixture was stirred for 30 min at RT until gas formation stopped. Cold diethyl ether was added until precipitation was observed (~17 mL). The precipitate was collected by centrifugation and washed with cold diethyl ether (3 × 10 mL). The solid was dried *in vacuo* to afford **43** (0.42 g, 99%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 6.0 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.02 (EtOAc–Hexane, 2:1); mp 190–194 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2962 (w, br), 2874 (w, br), 1685 (w, sh), 1175 (m, br), 1123 (s, br), 758 (s, sh), 720 (s, sh); δ_H/ppm (400 MHz, DMSO- d_6) 12.44 (1H, s, NHCO), 8.01 (1H, br s, NH₂), 7.95 (1H, d, J 8.0, PhH), 7.43 (1H, d, J 8.0, PhH), 7.43 (1H, t, J 8.2, PhH), 7.29 (1H, t, J 8.0, PhH), 3.09 (1H, m, CHNH₂), 2.67 (1H, m, NHCOCH), 2.09 (1H, m, CH₂), 1.93 (2H, m, CH₂), 1.84 (1H, m, CH₂), 1.53 (1H, q, J 12.4, CH₂), 1.20–1.41 (3H, m, CH₂); δ_C/ppm (100 MHz, DMSO- d_6) 173.8 (C), 158.0 (C), 148.9 (C), 131.6 (C), 126.3 (PhH), 123.7 (PhH), 121.8 (PhH), 120.6 (PhH), 48.7 (CH), 42.0 (CH), 32.2 (CH₂), 29.7 (CH₂), 28.0 (CH₂), 23.2 (CH₂); m/z (FAB) 391 (10%, [M + TFA]⁺), 276 (100%, [M + H]⁺), 151 (90%, [ABT + H]⁺); HRMS found 276.1174 (C₁₄H₁₈N₃OS, [M + H]⁺, requires 276.1171).



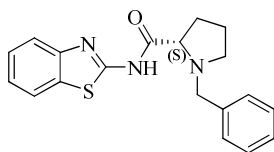
(S)-N-(Benzo[d]thiazol-2-yl)pyrrolidine-2-carboxamide (44). Compound **42** (0.83 g, 2.4 mmol) was dissolved in TFA–H₂O–TIPS (90/5/5, V/V/V, 4.0 mL, 47 mmol TFA) and DCM (2.4 mL), with a final concentration of 0.4 M for **42**. The clear, slightly yellow-coloured reaction mixture was stirred for 30 min at RT until gas formation stopped. Cold diethyl ether was added until precipitation was observed (~60 mL). The precipitate was collected by centrifugation and washed with cold diethyl ether (3 × 10 mL). The solid was dried *in vacuo* to afford **44** (0.73 g, 84%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 4.9 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.02 (EtOAc–Hexane, 2:1); mp 144–145 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2997 (*w*, br), 1663 (*s*, sh), 1180 (*s*, sh), 1130 (*s*, sh), 721 (*m*, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, DMSO-*d*₆) 9.45 (1H, br s, *NH*), 8.00 (1H, d, *J* 7.6, *PhH*), 7.78 (1H, d, *J* 8.0, *PhH*), 7.47 (1H, t, *J* 7.6, *PhH*), 7.35 (1H, t, *J* 7.0, *PhH*), 4.52 (1H, app dd, *J* 7.6, *CH*), 3.30 (2H, m, *NHCH*₂), 2.42 (1H, m, *CH*₂*CH*), 2.04 (1H, m, *CH*₂*CH*), 1.95 (2H, m, *CH*₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, DMSO-*d*₆) 158.6 (*C*), 131.5 (*C*), 126.6 (*PhH*), 124.2 (*PhH*), 122.0 (*PhH*), 59.7 (*CH*), 46.1 (*NHCH*₂), 29.4 (*CH*₂), 23.7 (*CH*₂); *m/z* (FAB) 248 (100%, [*M* + *H*]⁺), 151 (25%, [*ABT* + *H*]⁺), 70 (50%, [*pyrrolidine*]⁺); HRMS found 248.0869 (C₁₂H₁₄N₃OS, [*M* + *H*]⁺, requires 248.0858).



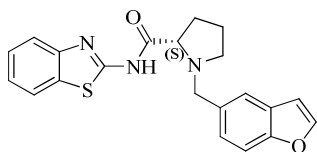
(S)-N-(Benzo[d]thiazol-2-yl)-1-((naphthalen-2-yl)methyl)pyrrolidine-2-carboxamide (45). Compound **42** (30.5 mg, 0.084 mmol) was dissolved in NEt₃ (35 μ L, 0.251 mmol) and DCM (420 μ L), with a final concentration of 0.2 M, at 0 °C. Bromomethylnaphthalene (21.3 mg, 0.101 mmol) was added after 5 min. Aqueous work-up procedure followed after overnight reaction at 4 °C; the reaction mixture was diluted with DCM (20 mL), washed 0.1 M HCl_(aq) (3 $\times$ 20 mL), H₂O (3 $\times$ 20 mL), brine (1 $\times$ 20 mL), and dried over Na₂SO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **45** (9 mg, 28%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 10.0 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.53 (EtOAc–Hexane, 2:1); mp 74–76 °; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2962 (w, br), 1670 (s, sh), 1177 (s, br), 1126 (s, sh), 750 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 10.74 (1H, m, NHCO), 7.81 (3H, m, PhH), 7.75 (3H, m, PhH), 7.52 (1H, m, PhH), 7.38–7.47 (3H, m, PhH), 7.30 (1H, m, PhH), 4.04 (1H, d, J 12.4, NCH₂), 3.87 (1H, d, J 12.8, NCH₂), 3.50 (1H, app dd, J 4.4, CH), 3.17 (1H, m, NC_{cyclopentyl}H₂), 2.59 (1H, m, NC_{cyclopentyl}H₂), 2.28–2.38 (1H, m, CH₂), 2.01–2.08 (1H, m, CH₂), 1.76–1.90 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 173.8 (C), 157.4 (C), 148.8 (C), 135.0 (C), 133.5 (C), 133.0 (C), 132.5 (C), 128.7 (PhH), 128.1 (PhH), 128.0 (PhH), 127.9 (PhH), 127.2 (PhH), 126.1 (PhH), 124.0 (PhH), 121.6 (PhH), 121.2 (PhH), 66.8 (CH), 54.4 (NCH₂), 29.9 (CH₂), 24.6 (CH₂); m/z (FAB) 338 (55%, [M + H]⁺), 210 (55%, [M – furan-methylnaphthalene]⁺), 141 (100%, [methylnaphthalene]⁺), 83 (25%, [methylfuran]⁺), 69 (45%, [furan]⁺); HRMS found 388.1414 (C₂₃H₂₂N₃OS, [M + H]⁺, requires 388.1414).

**(S)-N-(Benzo[d]thiazol-2-yl)-1-benzylpyrrolidine-2-carboxamide (46).**

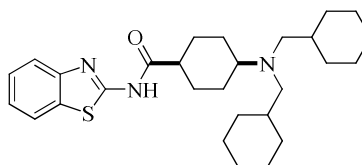
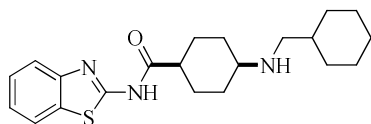
Compound **42** (49.6 mg, 0.137 mmol) was dissolved in NEt₃ (58 μ L, 0.416 mmol) and DCM (1.4 mL), with a final concentration of 0.1 M. Benzylbromide (20 μ L, 0.168 mmol) was added after 10 min. Aqueous work-up procedure followed after overnight reaction; the reaction mixture was diluted with DCM (20 mL), washed 0.1 M HCl_(aq) (3 $\times$ 20 mL), H₂O (3 $\times$ 20 mL), brine (1 $\times$ 20 mL), and dried over Na₂SO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **46** (13 mg, 14%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 16.0 min, 2–98% MeOH–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.58 (EtOAc–Hexane, 2:1); $\nu_{\max}/\text{cm}^{-1}$ (solid) 2976 (w, br), 1664 (*m*, sh), 1180 (*s*, br), 1130 (*s*, br), 763 (*m*, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.74 (1H, t, *J* 9.4, PhH), 7.46 (3H, m, PhH), 7.31 (5H, m, PhH), 4.32 (1H, d, *J* 12.8, NCH₂), 4.04 (1H, d, *J* 12.8, NCH₂), 3.69 (1H, m, CH), 2.99 (1H, m, CH₂), 2.39 (1H, m, CH₂), 2.25 (1H, m, CH₂), 2.03 (2H, m, NC_{cyclopentyl}H₂), 2.03 (1H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 170.3 (*C*), 162.5 (*C*), 156.3 (*C*), 138.7 (*C*), 129.5 (*PhH*), 127.7 (*PhH*), 125.1 (*PhH*), 122.3 (*PhH*), 117.5 (*PhH*), 58.1 (NCH₂), 54.6 (CH), 46.1 (NC_{cyclopentyl}H₂), 29.5 (CH₂), 23.1 (CH₂); m/z (ESI) 338 (100%, [M + H]⁺), 360 (100%, [M + Na]⁺); HRMS found 338.1315 (C₁₉H₂₀N₃OS, [M + H]⁺, requires 338.1327).



(S)-N-(Benzo[d]thiazol-2-yl)-1-((benzofuran-5-yl)methyl)pyrrolidine-2-carboxamide (47). Compound **42** (33.1 mg, 0.092 mmol) was dissolved in acetic acid (27.6 μ L, 0.460 mmol), molecular sieves (~10 mg) and DMF–DCM (0.46 mL), with a final concentration of 0.2 M. 1-Benzofuran-5-carbaldehyde (40.3 mg, 0.248 mmol) was added after 5 min. After 1 h at RT, NaBH(OAc)₃ (118 mg, 0.557 mmol) was added to the reaction mixture. Aqueous work-up procedure followed after overnight reaction; the reaction mixture was diluted with DCM (20 mL), washed 0.1 M HCl_(aq) (3 $\times$ 20 mL), H₂O (3 $\times$ 20 mL), brine (1 $\times$ 20 mL), and dried over Na₂SO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **47** (35.0 mg, 77%) as a white flocculent solid (TFA salt).

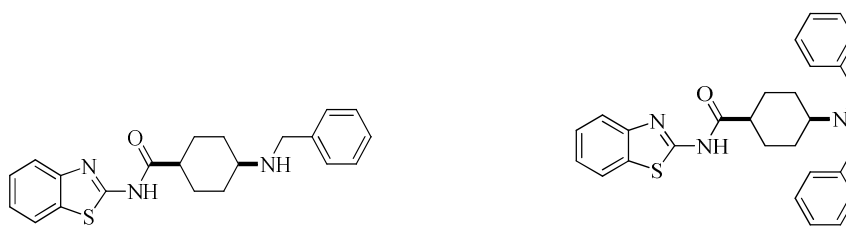
>90% Purity (t_r = 8.8 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); δ_H /ppm (400 MHz, MeOH-d₄) 7.80 (2H, m, PhH), 7.70 (2H, m, PhH), 7.65 (1H, m, PhH), 7.57 (1H, m, PhH), 7.52 (1H, d, J 8.2, PhH), 7.41–7.46 (3H, m, PhH), 7.30 (2H, m, PhH), 4.67 (2H, s, NCH₂), 4.57 (2H, s, NCH₂), 4.50 (1H, app dd, J 7.6, CH), 3.70 (1H, m, NC_{cyclopentyl}H₂), 3.47 (1H, m, NC_{cyclopentyl}H₂), 2.68 (1H, m, CH₂), 2.25 (1H, m, CH₂), 2.11 (1H, m, CH₂), 2.11 (1H, m, CH₂); δ_C /ppm (100 MHz, MeOH-d₄) 169.1 (C), 157.1 (C), 148.0 (PhH), 146.9 (PhH), 137.5 (C), 132.8 (C), 129.8 (PhH), 129.0 (PhH), 128.1 (PhH), 127.6 (PhH), 125.9 (PhH), 123.0 (PhH), 121.0 (PhH), 113.1 (PhH), 107.8 (PhH), 68.4 (CH), 65.6 (NCH₂), 60.0 (NCH₂), 56.1 (CH₂), 30.4 (CH₂), 23.9 (CH₂); m/z (ESI) 378 (100%, [M + H]⁺); HRMS found 378.1264 (C₂₁H₂₀N₃O₂S, [M + H]⁺, requires 378.1276).



cis-N-(Benzo[d]thiazol-2-yl)-4-(cyclohexylmethylamino)cyclohexanecarboxamide (48) and cis-N-(benzo[d]thiazol-2-yl)-4-(cyclohexylmethylamino)-cyclohexanecarboxamide (50). Compound **38** (33.8 mg, 0.087 mmol) was dissolved in NEt_3 (24.3 μL , 0.174 mmol), molecular sieves (~ 10 mg) and DMF (0.435 mL), with a final concentration of 0.2 M. Cyclohexanecarbaldehyde (15.7 μL , 0.130 mmol) was added after 15 min. After 1 h $\text{NaBH}(\text{OAc})_3$ (36.1 mg, 0.170 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO_3 (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3×10 mL), H_2O (3×10 mL), brine (1×10 mL), and dried over MgSO_4 , filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm) and lyophilised to yield **48** (11.7 mg, 29%) and by-product **50** (<1 mg) as white flocculent solids (TFA salts).

48. >98% Purity ($t_r = 10.3$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.01$ (EtOAc–Hexane, 2:1); mp 186–188 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2930 (w, br), 1669 (s, sh), 1178 (s, br), 1132 (s, br), 720 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 7.82 (1H, d, J 8.0, PhH), 7.69 (1H, d, J 8.0, PhH), 7.51 (1H, t, J 7.6, PhH), 7.41 (1H, t, J 7.6, PhH), 3.11 (1H, m, CHNH), 2.85 (3H, m, NHCH_2 , NHCOCH), 2.28 (1H, m, CH_2), 2.05 (1H, m, CH_2), 1.87 (5H, m, CH_2), 1.70 (5H, m, CH_2), 1.10–1.25 (4H, m, CH_2), 0.98 (2H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 173.4 (C), 126.9 (PhH), 125.3 (PhH), 122.3 (PhH), 118.1 (PhH), 66.5 (CHNH), 50.8 (NHCH_2), 39.6 (NHCOCH), 34.9 (CH_2), 30.3 (3C, CH_2), 25.4 (3H, CH_2); m/z (ESI) 372 (100%, $[\text{M} + \text{H}]^+$), 325 (20%); HRMS found 372.2122 ($\text{C}_{21}\text{H}_{30}\text{N}_3\text{OS}$, $[\text{M} + \text{H}]^+$, requires 372.2110).

50. >98% Purity ($t_r = 14.2$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.52$ (EtOAc–Hexane, 2:1); mp 79–82 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2933 (w, br), 1671 (s, sh), 1544 (s, sh), 1178 (s, br), 1134 (s, br), 719 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 7.84 (1H, d, J 7.6, PhH), 7.81 (1H, d, J 8.0, PhH), 7.53 (1H, t, J 7.2, PhH), 7.41 (1H, t, J 7.6, PhH), 3.53 (1H, m, CHNH), 2.92 (3H, m, NCH_2 , NHCOCH), 2.73 (2H, m, NCH_2), 2.29 (2H, m, CH_2), 2.12 (2H, m, CH_2), 1.93 (4H, m, CH_2), 1.83 (6H, m, CH_2), 1.74 (4H, m, CH_2), 1.67 (2H, m, CH_2), 1.29 (4H, m, CH_2), 1.17 (2H, m, CH_2), 1.02 (4H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 127.2 (PhH), 124.9 (PhH), 121.0 (PhH), 119.5 (PhH), 62.6 (CHN), 58.8 (2C, NCH_2), 37.7 (NHCOCH), 31.7 (CH_2), 26.4 (CH_2), 25.4 (3C, CH_2), 22.5 (CH_2); m/z (ESI) 468 (100%, $[\text{M} + \text{H}]^+$); HRMS found 468.3036 ($\text{C}_{28}\text{H}_{42}\text{N}_3\text{OS}$, $[\text{M} + \text{H}]^+$, requires 468.3049).

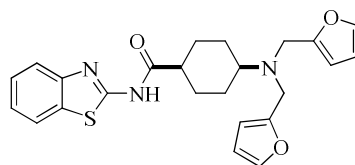
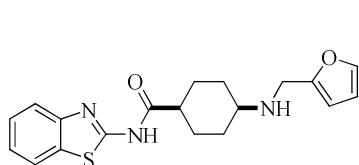


***cis*-N-(Benzo[d]thiazol-2-yl)-4-(benzylamino)cyclohexanecarboxamide (49) and *cis*-N-(benzo[d]thiazol-2-yl)-4-(dibenzylamino)cyclohexanecarboxamide (53).**

Compound **38** (55.2 mg, 0.142 mmol) was dissolved in NEt₃ (43.5 μ L, 0.312 mmol) and DMF (0.71 mL), with a final concentration with 0.2 M. Benzaldehyde (28.8 μ L, 0.284 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (119.8 mg, 0.565 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **49** (16 mg, 23%) and by-product **53** (5 mg, 8%) as white flocculent solids (TFA salts).

49. >98% Purity (t_r = 8.9 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.04 (EtOAc–Hexane, 2:1); mp 191–192 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 3545 (w, br), 2954 (w, br), 1657 (s, sh), 1179 (s, br), 1130 (m, sh), 754 (m, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 9.63 (1H, s, N⁺H₂ TFA[−]), 7.82 (1H, d, J 7.5, Ph_{ABTH}), 7.73 (1H, d, J 8.0, Ph_{ABTH}), 7.52 (1H, t, J 8.0, Ph_{ABTH}), 7.39–7.44 (3H, m, PhH), 7.32 (3H, m, PhH), 4.01 (2H, s, NHCH₂), 3.02 (1H, m, CHNH), 2.82 (1H, m, NHCOCH), 2.17–2.23 (2H, m, CH₂), 1.84 (4H, m, CH₂), 1.68 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 173.4 (C), 130.1 (PhH), 128.9 (PhH), 127.4 (PhH), 125.8 (PhH), 122.1 (PhH), 117.9 (PhH), 54.8 (CHNH), 48.1 (NHCH₂), 40.3 (NHCOCH), 25.0 (2C, CH₂), 24.0 (2C, CH₂); m/z (ESI) 366 (100%, [M + H]⁺); HRMS found 366.1649 (C₂₁H₂₄N₃OS, [M + H]⁺, requires 366.1640).

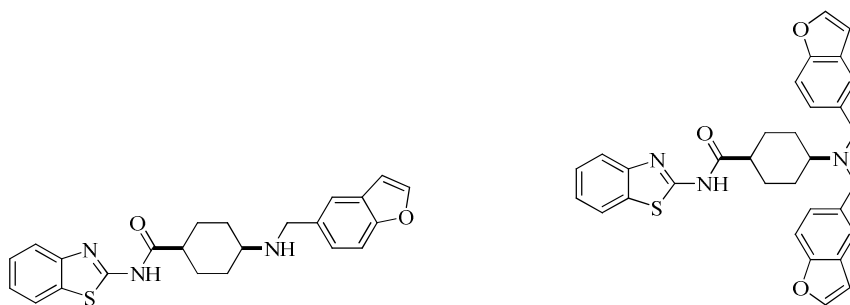
53. >98% Purity (t_r = 11.7 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.67 (EtOAc–Hexane, 2:1); mp 168–170 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 1669 (m, sh), 1178 (s, br), 1133 (s, br), 701 (m, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.82 (1H, d, J 7.2, Ph_{ABTH}), 7.73 (1H, d, J 8.0, Ph_{ABTH}), 7.50–7.55 (5H, m, PhH), 7.49 (1H, t, J 8.0, Ph_{ABTH}), 7.39 (1H, t, J 8.0, Ph_{ABTH}), 7.38 (5H, m, PhH), 4.12 (4H, s, NCH₂), 3.26 (1H, m, CHN), 2.86 (1H, m, NHCOCH), 2.26 (2H, m, CH₂), 2.12 (2H, m, CH₂), 1.99 (2H, m, CH₂), 1.58 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 174.0 (C), 130.7 (3C, PhH), 129.0 (3C, PhH), 126.9 (PhH), 121.9 (PhH), 118.5 (PhH), 61.1 (CHN), 54.2 (2C, NCH₂), 38.7 (NHCOCH), 26.2 (2C, CH₂), 22.0 (2C, CH₂); m/z (ESI) 456 (100%, [M + H]⁺); HRMS found 456.2008 (C₂₈H₃₀N₃OS, [M + H]⁺, requires 456.2110).



cis-N-(Benzo[d]thiazol-2-yl)-4-((furan-2-yl)methylamino)cyclohexanecarboxamide (51) and cis-N-(benzo[d]thiazol-2-yl)-4-(bis(furan-2-yl)methylamino)-cyclohexanecarboxamide (52). Compound **38** (42.2 mg, 0.108 mmol) was dissolved in NEt_3 (33.1 μL , 0.237 mmol) and DMF (0.54 mL), with a concentration of 0.2 M. Furan-2-carbaldehyde (17.9 μL , 0.216 mmol) was added after 15 min. After 1 h $\text{NaBH}(\text{OAc})_3$ (81.9 mg, 0.386 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO_3 (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M $\text{HCl}_{(\text{aq})}$ (3×10 mL), H_2O (3×10 mL), brine (1×10 mL), and dried over MgSO_4 , filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm) and lyophilised to yield **51** (1.3 mg, 3%) and **52** (36.5 mg, 78%) as white flocculent solids (TFA salts).

51. >98% Purity ($t_r = 7.6$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.02$ (EtOAc–Hexane, 2:1); mp 64–66 °C; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 1668 (s, sh), 1544 (s, sh), 1179 (m, br), 1133 (m, br), 722 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 7.77 (1H, d, J 8.0, $\text{Ph}_{\text{ABT}}H$), 7.69 (1H, d, J 8.0, $\text{Ph}_{\text{ABT}}H$), 7.49 (1H, t, J 7.6, $\text{Ph}_{\text{ABT}}H$), 7.38 (1H, t, J 8.0, $\text{Ph}_{\text{ABT}}H$), 7.43 (1H, m, $\text{Ph}H$), 6.57 (1H, m, $\text{Ph}H$), 6.36 (1H, m, $\text{Ph}H$), 4.20 (2H, s, NHCH_2), 3.12 (1H, m, CHNH), 2.84 (1H, m, NHCOCH), 2.25 (2H, m, CH_2), 1.94 (4H, m, CH_2), 1.77 (2H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 144.5 ($\text{Ph}H$), 127.3 ($\text{Ph}_{\text{ABT}}H$), 125.3 ($\text{Ph}_{\text{ABT}}H$), 121.9 ($\text{Ph}_{\text{ABT}}H$), 118.1 ($\text{Ph}_{\text{ABT}}H$), 112.7 ($\text{Ph}H$), 111.3 ($\text{Ph}H$), 54.0 (CHNH), 40.4 (NHCH_2), 40.8 (NHCOCH), 25.2 (2C, CH_2), 24.2 (2C, CH_2); m/z (ESI) 356 (100%, $[\text{M} + \text{H}]^+$), 206 (30%), 151 (20%, $[\text{ABT} + \text{H}]^+$); HRMS found 356.1429 ($\text{C}_{19}\text{H}_{22}\text{N}_3\text{O}_2\text{S}$, $[\text{M} + \text{H}]^+$, requires 356.1433).

52. >98% Purity ($t_r = 10.0$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.53$ (EtOAc–Hexane, 2:1); mp 70–72 °C; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 1669 (m, br), 1546 (m, br), 1182 (s, br), 1134 (s, br), 720 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 7.86 (1H, d, J 7.6, $\text{Ph}_{\text{ABT}}H$), 7.79 (1H, d, J 8.0, $\text{Ph}_{\text{ABT}}H$), 7.53 (1H, t, J 7.6, $\text{Ph}_{\text{ABT}}H$), 7.47 (2H, m, $\text{Ph}H$), 7.42 (1H, t, J 7.6, $\text{Ph}_{\text{ABT}}H$), 6.67 (2H, m, $\text{Ph}H$), 6.42 (2H, m, $\text{Ph}H$), 4.31 (4H, s, NCH_2), 3.15 (1H, m, CHN), 2.86 (1H, m, NHCOCH), 2.28 (2H, m, CH_2), 1.98 (2H, m, CH_2), 1.82 (2H, m, CH_2), 1.65 (2H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 143.7 (2C, $\text{Ph}H$), 126.7 ($\text{Ph}H$), 124.9 ($\text{Ph}H$), 121.3 ($\text{Ph}H$), 118.7 ($\text{Ph}H$), 114.4 ($\text{Ph}H$), 111.3 (2C, $\text{Ph}H$), 60.3 (CHN), 45.4 (2C, NCH_2), 38.8 (NHCOCH), 26.6 (2C, CH_2), 22.3 (2C, CH_2); m/z (ESI) 436 (100%, $[\text{M} + \text{H}]^+$), 151 (25%, $[\text{ABT} + \text{H}]^+$); HRMS found 436.1701 ($\text{C}_{24}\text{H}_{26}\text{N}_3\text{O}_3\text{S}$, $[\text{M} + \text{H}]^+$, requires 436.1695).

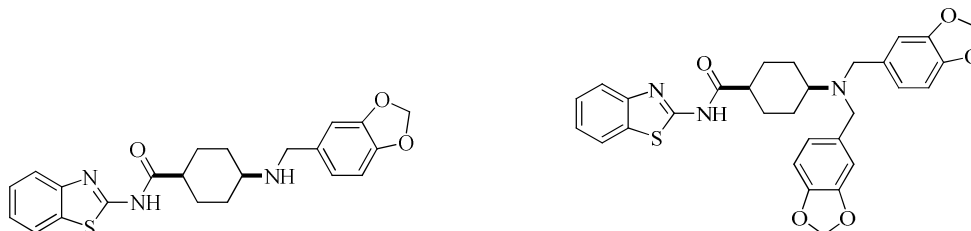


cis-N-(Benzo[d]thiazol-2-yl)-4-((benzofuran-5-yl)methylamino)cyclohexanecarboxamide (54) and cis-N-(benzo[d]thiazol-2-yl)-4-(bis(benzofuran-5-yl)methylamino)cyclohexanecarboxamide (69). Compound **38** (55.4 mg, 0.142 mmol) was dissolved in NEt₃ (43 μ L, 0.309 mmol) and DMF (0.71 mL), with a concentration of 0.2 M. 5-Benzofurancarbaldehyde (40.8 mg, 0.279 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (122.2 mg, 0.577 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **54** (23 mg, 31%) and by-product **69** (31 mg, 41%) as white flocculent solids (TFA salts).

54. >98% Purity (t_r = 9.8 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.04 (EtOAc–Hexane, 2:1); mp 184–186 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 3405 (w, br), 2960 (w, br), 1674 (s, sh), 1533 (m, sh), 1448 (m, sh), 1179 (s, br), 1127 (s, br), 721 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 9.65 (1H, s, NH), 7.83 (1H, d, J 8.0, PhH), 7.69 (1H, d, J 8.0, PhH), 7.64 (1H, m, PhH), 7.58 (1H, m, PhH), 7.48 (1H, t, J 8.4, PhH), 7.39 (2H, m, PhH), 7.37 (1H, m, PhH), 6.71 (1H, m, OCH), 4.10 (1H, s, NHCH₂), 3.64 (1H, s, NHCH₂), 2.96 (1H, m, CHNH), 2.72 (1H, m, NHCOCH), 2.14 (2H, m, CH₂), 1.80 (2H, m, CH₂), 1.70 (2H, m, CH₂), 1.53 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 145.8 (PhH), 126.7 (PhH), 126.5 (PhH), 124.8 (PhH), 123.2 (PhH), 122.7 (PhH), 121.2 (PhH), 118.9 (PhH), 106.8 (OCH), 53.4 (CHNH), 48.8 (NHCH₂), 39.6 (NHCOCH), 25.1 (2C, CH₂), 24.4 (2C, CH₂); m/z (ESI) 406 (100%, [M + H]⁺), 256 (20%); HRMS found 406.1587 (C₂₃H₂₄N₃O₂S, [M + H]⁺, requires 406.1589).

69. >98% Purity (t_r = 18.0 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.53 (EtOAc–Hexane, 2:1); mp 221–226 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2947 (w, br), 1665 (m, sh), 1180 (s, br), 1130 (s, br), 762 (m, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 8.05 (2H, m, PhH), 7.97 (1H, d, J 7.6, Ph_{ABT}H), 7.73 (3H, m, PhH), 7.64 (2H, m, PhH), 7.44 (1H, t, J 8.0, Ph_{ABT}H), 7.39 (2H, m, PhH), 7.31 (1H, t, J 8.0, Ph_{ABT}H), 6.98 (2H, m, PhH), 4.56 (2H, s, NCH₂), 4.35 (2H, s, NCH₂), 3.21 (1H, m, CHNH), 2.86 (1H, m, NHCOCH), 2.21 (2H, m, CH₂), 2.05 (4H, m, CH₂), 1.55 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 174.3 (C), 158.0 (C), 154.6 (C), 147.5 (PhH), 127.6 (PhH), 126.8 (PhH), 124.9 (PhH), 123.7 (PhH), 122.1 (PhH), 120.8 (PhH), 112.1 (PhH), 107.2 (2C, PhH), 61.9 (CHN), 54.3 (2C, NCH₂), 36.8 (NHCOCH),

26.6 (2C, CH₂), 23.0 (2C, CH₂); *m/z* (ESI) 536 (100%, [M + H]⁺), 338 (30%), 206 (20%); HRMS found 536.2016 (C₃₂H₂₉N₃O₃S, [M + H]⁺, requires 536.2008).

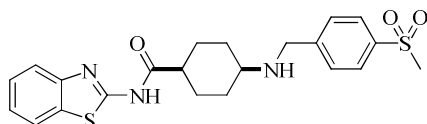


cis-N-(Benzo[d]thiazol-2-yl)-4-((benzo[d][1,3]dioxol-5-yl)methylamino)cyclohexanecarboxamide (56) and cis-N-(benzo[d]thiazol-2-yl)-4-(bis(benzo[d][1,3]dioxol-5-yl)methylamino)cyclohexanecarboxamide (57). Compound **43** (40.7 mg, 0.105 mmol) was dissolved in NEt₃ (32.2 μ L, 0.230 mmol) and DMF (0.525 mL), with a final concentration of 0.2 M. Piperonal (32.1 mg, 0.214 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (84.1 mg, 0.397 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **56** (13.9 mg, 25%) and the by-product **57** (3.9 mg, 7%) as white flocculent solids (TFA salts).

56. >98% Purity (*t_r* = 9.1 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); *R_f* = 0.25 (EtOAc–Hexane, 2:1); mp 71–72 °C; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2962 (w, br), 1671 (s, br), 1448 (s, sh), 1181 (s, br), 1135 (s, br), 721 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.83 (1H, d, *J* 7.2, Ph_{ABTH}), 7.66 (1H, d, *J* 8.0, Ph_{ABTH}), 7.48 (1H, t, *J* 7.2, Ph_{ABTH}), 7.34–7.43 (2H, m, PhH), 6.94 (1H, m, PhH), 6.87 (1H, d, *J* 8.4, PhH), 6.73 (1H, d, *J* 8.0, PhH), 5.99 (1H, s OCH₂O), 6.08 (1H, s OCH₂O), 4.04 (2H, s, NHCH₂), 3.02 (1H, m, CHNH), 2.77 (1H, m, NHCOCH), 2.17 (2H, m, CH₂), 1.88 (2H, m, CH₂), 1.75 (2H, m, CH₂), 1.63 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 126.9 (Ph_{ABTH}), 124.8 (PhH), 123.9 (PhH), 121.9 (Ph_{ABTH}), 118.7 (Ph_{ABTH}), 110.1 (PhH), 108.5 (PhH), 102.2 (OCH₂O), 54.2 (CHNH), 48.3 (NHCH₂), 40.0 (NHCOCH), 25.3 (2C, CH₂), 24.7 (2C, CH₂); *m/z* (ESI) 410 (100%, [M + H]⁺), 280 (20%); HRMS found 410.1541 (C₂₂H₂₄N₃O₃S, [M + H]⁺, requires 410.1538).

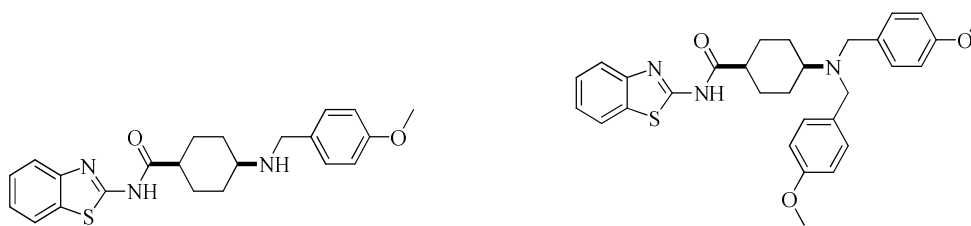
57. >98% Purity (*t_r* = 11.8 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); mp 114–121 °C; $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.82 (1H, d, *J* 7.2, Ph_{ABTH}), 7.73 (1H, d, *J* 8.0, Ph_{ABTH}), 7.49 (1H, t, *J* 8.0, Ph_{ABTH}), 7.39 (1H, t, *J* 8.0, Ph_{ABTH}), 7.05 (2H, m, PhH), 6.93 (2H, m, PhH), 6.78 (2H, m, PhH), 5.97 (4H, s, OCH₂O), 4.08 (4H, s, NCH₂), 3.24 (1H, m, CHN), 2.85 (1H, m, NHCOCH), 2.26 (2H, m, CH₂), 2.09 (2H, m, CH₂), 1.98 (2H, m, CH₂), 1.60 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz,

CDCl₃) 174.3 (C), 148.8 (C), 126.8 (*PhH*), 124.9 (*PhH*), 124.7 (*PhH*), 121.5 (*PhH*), 120.0 (*PhH*), 110.7 (2C, *PhH*), 108.5 (2C, *PhH*), 101.4 (2C, OCH₂O), 60.3 (CHN), 54.0 (2C, NCH₂), 37.9 (NHCOCH), 25.9 (2C, CH₂), 21.9 (2C, CH₂); *m/z* (ESI) 544 (100%, [M + H]⁺); HRMS found 544.1896 (C₃₀H₃₀N₃O₅S, [M + H]⁺, requires 544.1906).



***cis*-N-(Benzo[d]thiazol-2-yl)-4-(4-methylsulfonyl)benzylamino)cyclohexane-carboxamide (58).** Compound **38** (35.0 mg, 0.090 mmol) was dissolved in NEt₃ (25.1 μL, 0.180 mmol) and DMF (0.45 mL), with a concentration of 0.2 M. 4-Methylsulfonylbenzaldehyde (28.5 mg, 0.155 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (37.7 mg, 0.178 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3 × 10 mL), H₂O (3 × 10 mL), brine (1 × 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **58** (2.7 mg, 5%) as a white flocculent solid (TFA salt).

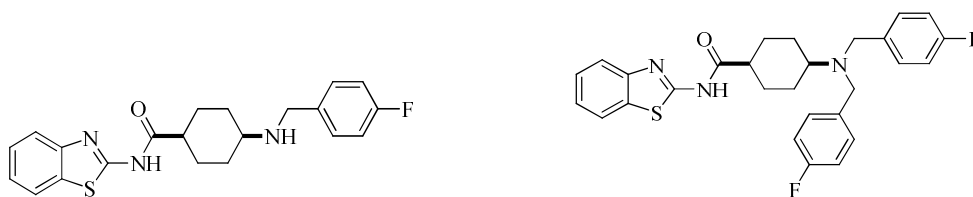
>98% Purity (*t_r* = 7.8 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); *R_f* = 0.41 (EtOAc–Hexane, 2:1); mp 135–136 °C; *v*_{max}/cm^{−1} (solid) 2933 (w, br), 1656 (*m*, sh), 1542 (*m*, sh), 1295 (*m*, br), 1147 (*s*, br), 762 (*s*, sh); δ_H/ppm (400 MHz, MeOH-*d*₄) 8.18 (1H, *m*, *PhH*), 8.08 (1H, *d*, *J* 8.4, *PhH*), 7.95 (1H, *m*, *PhH*), 7.87 (1H, *d*, *J* 7.6, *PhH*), 7.77 (3H, *m*, *PhH*), 7.60 (1H, *m*, *PhH*), 7.45 (1H, *t*, *J* 8.4, *PhH*), 7.32 (1H, *t*, *J* 8.4, *PhH*), 4.40 (2H, *s*, NHCH₂), 3.15 (1H, *m*, CHNH), 2.94 (1H, *m*, NHCOCH), 2.26 (2H, *m*, CH₂), 2.12 (2H, *m*, CH₂), 2.02 (2H, *m*, CH₂), 1.84 (2H, *m*, CH₂); δ_C/ppm (100 MHz, MeOH-*d*₄) 135.2 (*PhH*), 130.7 (*PhH*), 127.9 (*PhH*), 125.0 (*PhH*), 120.2 (*PhH*), 116.0 (*PhH*), 121.0 (*PhH*), 47.2 (NHCH₂), 44.0 (CHNH), 39.2 (NHCOCH), 25.0 (4C, CH₂); *m/z* (ESI) 466 (100%, [M + Na]), 444 (50%, [M + H]⁺), 276 (50%), 151 (25%, [ABT + H]⁺); HRMS found 444.1425 (C₂₂H₂₆N₃O₃S₂, [M + H]⁺, requires 444.1416).



cis-N-(Benzo[d]thiazol-2-yl)-4-(4-methoxybenzylamino)cyclohexanecarboxamide (59) and cis-N-(benzo[d]thiazol-2-yl)-4-(bis(4-methoxybenzyl)amino)cyclohexanecarboxamide (60). Compound **38** (39.0 mg, 0.100 mmol) was dissolved in NEt₃ (31.0 μ L, 0.222 mmol) and DMF (0.50 mL), with a concentration of 0.2 M. *p*-Anisaldehyde (24.3 μ L, 0.200 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (37.5 mg, 0.177 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **59** (3.8 mg, 7%) and **60** (11.6 mg, 23%) as white flocculent solids (TFA salts).

59. >98% Purity (t_r = 9.2 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.02 (EtOAc–Hexane, 2:1); mp 86–88 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2961 (*w*, br), 2848 (*w*, br), 1669 (*s*, sh), 1540 (*m*, sh), 1178 (*s*, br), 1131 (*s*, br), 721 (*s*, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.84 (1H, m, *PhH*), 7.69 (1H, d, *J* 8.0, *PhH*), 7.48 (1H, t, *J* 8.0, *PhH*), 7.39 (1H, d, *J* 7.6, *PhH*), 7.34 (2H, m, *PhH*), 7.01 (1H, d, *J* 8.4, *PhH*), 6.84 (1H, d, *J* 8.4, *PhH*), 4.02 (2H, s, NHCH₂), 2.99 (1H, m, CHNH), 2.77 (1H, m, NHCOCH), 3.90 (1H, s, OCH₃), 3.73 (2H, s, OCH₃), 2.17 (2H, m, CH₂), 1.86 (2H, m, CH₂), 1.75 (2H, m, CH₂), 1.60 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 131.3 (*PhH*), 124.7 (*PhH*), 122.0 (*PhH*), 118.8 (*PhH*), 114.5 (*PhH*), 55.5 (OCH₃), 54.1 (CHNH), 47.6 (NHCH₂), 40.1 (NHCOCH), 24.6 (2C, CH₂), 24.6 (2C, CH₂); m/z (ESI) 396 (100%, [M + H]⁺), 246 (30%); HRMS found 396.1751 (C₂₂H₂₆N₃O₂S, [M + H]⁺, requires 396.1746).

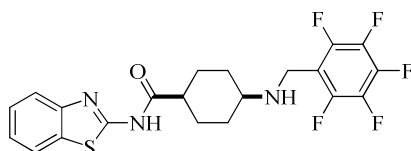
60. >98% Purity (t_r = 12.1 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.53 (EtOAc–Hexane, 2:1); mp 92–96 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2968 (*w*, br), 1669 (*m*, sh), 1517 (*m*, sh), 1257 (*s*, sh), 1179 (*s*, br), 1130 (*s*, br), 719 (*s*, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.83 (1H, d, *J* 8.0, *Ph_{ABT}H*), 7.72 (1H, d, *J* 8.0, *Ph_{ABT}H*), 7.49 (1H, t, *J* 7.6, *Ph_{ABT}H*), 7.39 (1H, t, *J* 8.0, *Ph_{ABT}H*), 7.43 (4H, d, *J* 8.8, *PhH*), 6.99 (4H, d, *J* 8.8, *PhH*), 4.01 (4H, s, NCH₂), 3.76 (6H, s, OCH₃), 3.22 (1H, m, CHN), 2.83 (1H, m, NHCOCH), 2.24 (2H, m, CH₂), 2.09 (2H, m, CH₂), 1.97 (2H, m, CH₂), 1.55 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 173.7 (C), 160.8 (C), 132.5 (4C, *PhH*), 126.8 (*PhH*), 125.2 (*PhH*), 121.5 (*PhH*), 118.4 (*PhH*), 114.4 (4C, *PhH*), 55.1 (2C, OCH₃), 59.8 (CHNH), 53.7 (2C, NCH₂), 38.2 (NHCOCH), 26.2 (2C, CH₂), 22.2 (2C, CH₂); m/z (ESI) 516 (100%, [M + H]⁺); HRMS found 516.2321 (C₃₀H₃₄N₃O₃S, [M + H]⁺, requires 516.2321).



cis-N-(Benzo[d]thiazol-2-yl)-4-(4-fluorobenzylamino)cyclohexanecarboxamide (61) and cis-N-(benzo[d]thiazol-2-yl)-4-(bis(4-fluorobenzyl)amino)cyclohexanecarboxamide (62). Compound **38** (32.0 mg, 0.082 mmol) was dissolved in NEt₃ (23.0 μ L, 0.165 mmol) and DMF (0.41 mL), with a concentration of 0.2 M. *p*-Fluorobenzaldehyde (17.6 μ L, 0.164 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (70.5 mg, 0.333 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **61** (8.5 mg, 21%) and by-product **62** (6.7 mg, 17%) as white flocculent solids (TFA salts).

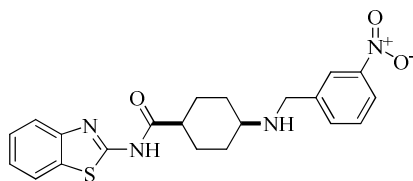
61. >98% Purity (t_r = 9.2 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.02 (EtOAc–Hexane, 2:1); mp 180–182 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2971 (w, br), 1669 (s, sh), 1545 (m, sh), 1181 (s, br), 1132 (s, br), 721 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, MeOH-*d*₄) 7.86 (1H, d, J 8.0, Ph_{ABTH}), 7.75 (1H, d, J 8.0, Ph_{ABTH}), 7.55 (1H, m, PhH), 7.44 (1H, t, J 7.2, Ph_{ABTH}), 7.32 (1H, t, J 8.0, Ph_{ABTH}), 7.22 (2H, m, PhH), 4.25 (2H, s, NHCH₂), 3.26 (1H, m, CHNH), 2.92 (1H, m, NHCOCH), 2.25 (2H, m, CH₂), 2.10 (2H, m, CH₂), 1.99 (2H, m, CH₂), 1.84 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, MeOH-*d*₄) 174.3 (C), 164.7 (C), 162.2 (C), 158.5 (C), 148.6 (C), 132.0 (PhH), 125.8 (PhH), 123.5 (PhH), 120.9 (PhH), 120.3 (PhH), 115.8 (PhH), 115.5 (PhH), 55.7 (CHNH), 48.0 (NHCH₂), 38.3 (NHCOCH), 25.0 (2C, CH₂); m/z (ESI) 384 (100%, [M + H]⁺), 276 (20%), 234 (20%), 151 (20%, [M + ABT]⁺); HRMS found 384.1554 (C₂₁H₂₃N₃OSF, [M + H]⁺, requires 384.1546).

62. >98% Purity (t_r = 12.0 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.64 (EtOAc–Hexane, 2:1); mp 102–106 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2976 (w, br), 1669 (s, sh), 1542 (m, sh), 1515 (m, sh), 1181 (s, br), 1131 (s, br), 759 (s, sh), 720 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.84 (1H, d, J 8.0, Ph_{ABTH}), 7.79 (1H, d, J 8.0, Ph_{ABTH}), 7.52 (1H, t, J 7.6, Ph_{ABTH}), 7.39–7.46 (5H, m, PhH), 7.05 (4H, m, PhH), 4.03 (4H, s, NCH₂), 3.08 (1H, m, CHN), 2.82 (1H, m, NHCOCH), 2.27 (2H, m, CH₂), 2.02 (2H, m, CH₂), 1.94 (2H, m, CH₂), 1.73 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 132.1 (PhH), 126.9 (PhH), 125.0 (PhH), 121.7 (PhH), 119.1 (PhH), 116.1 (PhH), 53.4 (2C, NCH₂), 38.4 (NHCOCH), 26.9 (2C, CH₂), 23.0 (CH₂); m/z (ESI) 492 (100%, [M + H]⁺); HRMS found 492.1921 (C₂₈H₂₈N₃OSF₂, [M + H]⁺, requires 492.1921).



***cis*-N-(Benzo[d]thiazol-2-yl)-4-(perfluorobenzylamino)cyclohexanecarboxamide (63).** Compound **38** (40.1 mg, 0.103 mmol) was dissolved in NEt₃ (31.6 μ L, 0.227 mmol) and DMF (0.515 mL), with a concentration of 0.2 M. 2,3,4,5,6-Pentafluorobenzaldehyde (25.4 mg, 0.206 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (92.8 mg, 0.438 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **63** (6.2 mg, 11%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 9.9 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.42 (EtOAc–Hexane, 2:1); mp 183–185 $^{\circ}$ C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 3266 (w, br), 2868 (w, br), 1681 (s, sh), 1521 (s, sh), 1128 (s, br), 954 (m, br), 755 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, MeOH- d_4) 7.87 (1H, d, J 7.6, Ph_{ABT}H), 7.76 (1H, d, J 8.0, Ph_{ABT}H), 7.45 (1H, t, J 8.4, Ph_{ABT}H), 7.32 (1H, t, J 8.4, Ph_{ABT}H), 4.44 (2H, s, NHCH₂), 3.41 (1H, m, CHNH), 2.94 (1H, m, NHCOCH), 2.27 (2H, m, CH₂), 2.13 (2H, m, CH₂), 2.02 (2H, m, CH₂), 1.87 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, MeOH- d_4) 174.1 (C), 131.4 (C), 125.7 (Ph_{ABT}H), 123.6 (Ph_{ABT}H), 120.9 (Ph_{ABT}H), 120.3 (Ph_{ABT}H), 48.5 (CHNH), 37.9 (NHCOCH), 35.1 (NHCH₂), 24.9 (4C, CH₂); m/z (ESI) 456 (100%, [M + H]⁺), 222 (20%), 151 (20%, [M + ABT]⁺); HRMS found 456.1177 (C₂₁H₁₉N₃OSF₅, [M + H]⁺, requires 456.1169).



***cis*-N-(Benzo[d]thiazol-2-yl)-4-(3-nitrobenzylamino)cyclohexanecarboxamide**

(64). Compound **38** (39.8 mg, 0.102 mmol) was dissolved in NEt₃ (31.0 μL, 0.222 mmol) and DMF (0.51 mL), with a concentration of 0.2 M. *o*-Nitrobenzaldehyde (30.2 mg, 0.200 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (83.0 mg, 0.392 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 × 10 mL), H₂O (3 × 10 mL), brine (1 × 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **64** (25.8 mg, 48%) as a white flocculent solid (TFA salt).

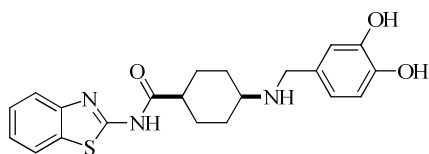
>98% Purity (t_r = 9.1 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.02 (EtOAc–Hexane, 2:1); mp 87–89 °C; ν_{max}/cm^{−1} (solid) 3266 (*w*, *br*), 2868 (*w*, *br*), 1681 (*s*, *sh*), 1512 (*s*, *sh*), 1128 (*s*, *br*), 954 (*m*, *br*), 755 (*s*, *sh*); δ_H/ppm (400 MHz, CDCl₃) 8.17 (1H, *d*, *J* 9.6, *PhH*), 7.87 (1H, *d*, *J* 7.2, *Ph_{ABT}H*), 7.81 (1H, *d*, *J* 7.6, *PhH*), 7.76 (1H, *d*, *J* 8.4, *Ph_{ABT}H*), 7.71 (1H, *t*, *J* 6.4, *PhH*), 7.63 (1H, *t*, *J* 7.2, *PhH*), 7.54 (1H, *t*, *J* 7.2, *Ph_{ABT}H*), 7.44 (1H, *t*, *J* 8.0, *Ph_{ABT}H*), 4.30 (2H, *s*, *NHCH₂*), 3.50 (1H, *m*, *CHNH*), 2.88 (1H, *m*, *NHCOCH*), 2.27 (2H, *m*, *CH₂*), 2.09 (4H, *m*, *CH₂*), 1.87 (2H, *m*, *CH₂*); δ_C/ppm (100 MHz, CDCl₃) 174.2 (*C*), 163.2 (*C*), 148.6 (*C*), 135.2 (*PhH*), 134.7 (*PhH*), 131.4 (*PhH*), 128.4 (*PhH*), 126.0 (*PhH*), 125.6 (*PhH*), 122.3 (*PhH*), 117.1 (*PhH*), 57.2 (*CHNH*), 47.2 (*NHCH₂*), 40.0 (*NHCOCH*), 25.0 (2*C*, *CH₂*), 23.5 (2*C*, *CH₂*); *m/z* (ESI) 411 (100%, [M + H]⁺), 261 (25%); HRMS found 411.1504 (C₂₁H₂₃N₃O₃S₂, [M + H]⁺, requires 411.1491).



cis-N-(Benzo[d]thiazol-2-yl)-4-((thiophene-2-yl)methylamino)cyclohexanecarboxamide (65) and cis-N-(benzo[d]thiazol-2-yl)-4-(bis(thiophene-2-yl)methylamino)cyclohexanecarboxamide (66). Compound **38** (42.3 mg, 0.109 mmol) was dissolved in NEt_3 (33.4 μL , 0.240 mmol) and DMF (0.545 mL), with a concentration of 0.2 M. Thiophene-2-carbaldehyde (20.4 μL , 0.218 mmol) was added after 15 min. After 1 h $\text{NaBH}(\text{OAc})_3$ (92.5 mg, 0.436 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO_3 (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3×10 mL), H_2O (3×10 mL), brine (1×10 mL), and dried over MgSO_4 , filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm) and lyophilised to yield **65** (5.4 mg, 10%) and **66** (6.4 mg, 13%) as white flocculent solids (TFA salts).

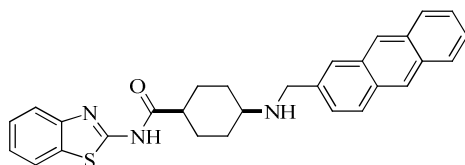
65. >98% Purity ($t_r = 8.3$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.01$ (EtOAc-Hexane , 2:1); mp 178–181 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2952 (w, br), 1666 (m, br), 1540 (m, sh), 1443 (m, sh), 1267 (s, br), 1134 (s, br), 718 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, MeOD-d_4) 7.87 (1H, d, J 7.6, $\text{Ph}_{\text{ABT}}H$), 7.65 (1H, d, J 8.0, $\text{Ph}_{\text{ABT}}H$), 7.59 (1H, m, $\text{Ph}H$), 7.47 (1H, t, J 7.2, $\text{Ph}_{\text{ABT}}H$), 7.32 (2H, m, $\text{Ph}H$), 7.14 (1H, m, $\text{Ph}H$), 4.51 (2H, s, NHCH_2), 3.28 (1H, m, CHNH), 2.91 (1H, m, NHCOCH), 2.24 (2H, m, CH_2), 2.01 (2H, m, CH_2), 1.99 (2H, m, CH_2), 1.84 (2H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, MeOD-d_4) 130.2 ($\text{Ph}H$), 128.1 ($\text{Ph}H$), 127.4 ($\text{Ph}H$), 125.9 ($\text{Ph}_{\text{ABT}}H$), 123.5 ($\text{Ph}H$), 120.8 ($\text{Ph}_{\text{ABT}}H$), 120.4 ($\text{Ph}_{\text{ABT}}H$), 47.7 (CHNH), 41.9 (NHCH_2), 38.5 (NHCOCH), 25.1 (4C, CH_2); m/z (ESI) 372 (100%, $[\text{M} + \text{H}]^+$), 222 (40%), 151 (30%, $[\text{ABT} + \text{H}]^+$); HRMS found 372.1206 ($\text{C}_{19}\text{H}_{22}\text{N}_3\text{OS}_2$, $[\text{M} + \text{H}]^+$, requires 372.1204).

66. >98% Purity ($t_r = 11.0$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.70$ (EtOAc-Hexane , 2:1); mp 82–87 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2966 (w, br), 1668 (s, sh), 1543 (s, sh), 1182 (s, br), 1135 (s, br), 720 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 7.87 (1H, d, J 7.6, $\text{Ph}_{\text{ABT}}H$), 7.83 (1H, d, J 8.0, $\text{Ph}_{\text{ABT}}H$), 7.56 (1H, t, J 8.0, $\text{Ph}_{\text{ABT}}H$), 7.44 (1H, t, J 8.0, $\text{Ph}_{\text{ABT}}H$), 7.33 (2H, m, $\text{Ph}H$), 7.18 (2H, m, $\text{Ph}H$), 7.02 (2H, m, $\text{Ph}H$), 4.26 (2H, s, NHCH_2), 3.67 (2H, s, NHCH_2), 3.06 (1H, m, CHNH), 2.86 (1H, m, NHCOCH), 2.30 (2H, m, CH_2), 1.96 (2H, m, CH_2), 1.68 (2H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 174.1 (C), 130.3 ($\text{Ph}H$), 127.4 ($\text{Ph}H$), 125.1 ($\text{Ph}H$), 121.9 ($\text{Ph}H$), 118.4 ($\text{Ph}H$), 58.9 (CHNH), 47.1 (2C, NHCH_2), 38.7 (NHCOCH), 26.0 (2C, CH_2), 23.7 (CH_2); m/z (ESI) 468 (100%, $[\text{M} + \text{H}]^+$); HRMS found 468.1228 ($\text{C}_{24}\text{H}_{26}\text{N}_3\text{OS}_3$, $[\text{M} + \text{H}]^+$, requires 468.1238).



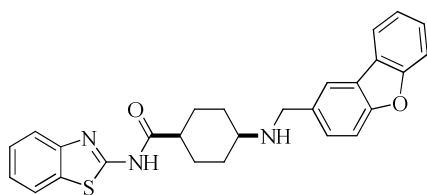
***cis*-N-(Benzo[d]thiazol-2-yl)-4-(3,4-dihydroxybenzylamino)cyclohexane-carboxamide (67).** Compound **38** (35.8 mg, 0.103 mmol) was dissolved in NEt₃ (25.6 μ L, 0.184 mmol) and DMF (0.46 mL), with a concentration of 0.2 M. 3,4-Dihydroxybenzaldehyde (19.0 mg, 0.138 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (37.5 mg, 0.177 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **67** (1.0 mg, 2%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 7.0 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.01 (EtOAc–Hexane, 2:1); mp 135–137 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 1672 (w, sh), 1573 (w, sh), 1183 (m, br), 1132 (m, br), 721 (w, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, MeOH-d₄) 7.86 (1H, d, J 8.4, Ph_{ABTH}), 7.74 (1H, d, J 8.4, Ph_{ABTH}), 7.44 (1H, d, J 8.4, Ph_{ABTH}), 7.31 (1H, t, J 7.2, Ph_{ABTH}), 6.91 (1H, m, PhH), 6.81 (2H, m, PhH), 4.01 (2H, s, NHCH₂), 3.22 (1H, m, CHNH), 2.90 (1H, m, NHCOCH), 2.22 (2H, m, CH₂), 2.05 (2H, m, CH₂), 1.95 (2H, m, CH₂), 1.83 (2H, m, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, MeOH-d₄) 127.2 (Ph_{ABTH}), 124.7 (Ph_{ABTH}), 122.1 (Ph_{ABTH}), 122.7 (PhH), 121.6 (Ph_{ABTH}), 117.4 (PhH), 116.3 (PhH), 56.2 (CHNH), 49.2 (NHCH₂), 39.4 (NHCOCH), 26.2 (4C, CH₂); m/z (ESI) 396 (100%, [M + H]⁺), 276 (80%); HRMS found 396.1525 (C₂₁H₂₄N₃O₃S, [M + H]⁺, requires 396.1538).



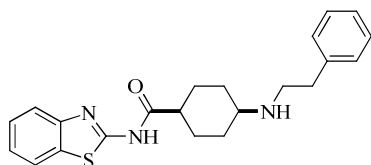
***cis*-N-(Benzo[d]thiazol-2-yl)-4-((anthracen-3-yl)methylamino)cyclohexane-carboxamide (68).** Compound **38** (35.1 mg, 0.090 mmol) was dissolved in NEt₃ (25.1 μ L, 0.180 mmol), molecular sieves (~10 mg) and DMF (0.45 mL), with a final concentration of 0.2 M. 9-Anthraldehyde (25.5 mg, 0.124 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (35.5 mg, 0.168 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **68** (1.7 mg, 3%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 12.3 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.71 (EtOAc–Hexane, 2:1); $\nu_{\max}$ /cm^{–1} (solid) 2917 (w, br), 2359 (w, br), 1677 (s, sh), 1286 (s, sh), 1171 (m, sh), 938 (m, sh), 695 (s, sh); m/z (ESI) 466 (100%, [M + H]⁺), 316 (30%), 276 (25%), 191 (30%); HRMS found 466.1947 (C₂₉H₂₈N₃OS, [M + H]⁺, requires 466.1953).



cis-N-(Benzo[d]thiazol-2-yl)-4-((dibenzo[b,d]furan-2-yl)methylamino)cyclohexanecarboxamide (69). Compound **38** (35.1 mg, 0.090 mmol) was dissolved in NEt₃ (25.1 μ L, 0.180 mmol), molecular sieves (~10 mg) and DMF (0.450 mL), with a final concentration of 0.2 M. Dibenzo[b,d]furan-2-carbaldehyde (26.3 mg, 0.134 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (44.6 mg, 0.210 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **69** (9.2 mg, 17%) as a white flocculent solid (TFA salt).

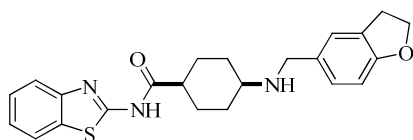
>98% Purity (t_r = 11.7 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.57 (EtOAc–Hexane, 2:1); mp 110–112 $^{\circ}$ C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 1671 (*s*, sh), 1540 (*m*, sh), 1450 (*m*, sh), 1267 (*s*, br), 1196 (*s*, br), 1130 (*s*, br), 720 (*s*, sh); δ_H/ppm (400 MHz, MeOH-*d*₄) 8.22 (1H, *m*, PhH), 8.08 (1H, *d*, *J* 8.4, PhH), 8.04 (1H, *m*, PhH), 7.87 (1H, *d*, *J* 8.4, PhH), 7.70 (1H, *m*, PhH), 7.64 (2H, *m*, PhH), 7.58 (2H, *m*, PhH), 7.45 (2H, *m*, PhH), 7.45 (1H, *t*, *J* 8.0, PhH), 7.37 (1H, *t*, *J* 7.6, PhH), 7.32 (1H, *t*, *J* 8.0, PhH), 4.78 (1H, *s*, NHCH₂), 4.44 (1H, *s*, NHCH₂), 3.34 (1H, *m*, CHNH), 2.94 (1H, *m*, NHCOCH), 2.26 (2H, *m*, CH₂), 2.14 (2H, *m*, CH₂), 2.04 (2H, *m*, CH₂), 1.84 (2H, *m*, CH₂); δ_C/ppm (100 MHz, MeOH-*d*₄) 174.6 (*C*), 156.7 (*C*), 148.6 (*C*), 128.8 (*PhH*), 127.8 (*PhH*), 125.8 (*PhH*), 122.2 (*PhH*), 120.9 (*PhH*), 120.5 (*PhH*), 120.3 (*PhH*), 111.8 (*PhH*), 111.4 (*PhH*), 111.2 (*PhH*), 110.8 (*PhH*), 55.9 (CHNH), 47.8 (NHCH₂), 38.0 (NHCOCH), 24.9 (4C, CH₂); m/z (ESI) 456 (100%, [M + H]⁺), 306 (40%); HRMS found 456.1758 (C₂₇H₂₆N₃O₂S, [M + H]⁺, requires 456.1746).



cis-N-(Benzo[d]thiazol-2-yl)-4-(phenethylamino)cyclohexanecarboxamide (70).

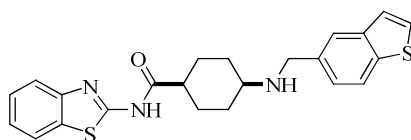
Compound **38** (34.8 mg, 0.059 mmol) was dissolved in NEt₃ (24.8 μ L, 0.178 mmol), molecular sieves (~10 mg) and DMF (0.45 mL), with a final concentration of 0.2 M. Phenylacetaldehyde (14.9 μ L, 0.133 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (54.6 mg, 0.258 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **70** (1.3 mg, 3%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 9.8 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.01 (EtOAc–Hexane, 2:1); mp 135–138 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2948 (w, br), 1541 (s, sh), 1187 (s, br), 1136 (s, br), 756 (m, sh); $\delta_{\text{H}}/\text{ppm}$ (500 MHz, CDCl₃) 9.69 (NH), 7.85 (1H, m, PhH), 7.71 (1H, m, PhH), 7.61 (1H, m, PhH), 7.50 (1H, m, PhH), 7.35 (1H, t, J 11.5, PhH), 7.18–7.24 (4H, m, PhH), 4.20 (2H, s, CH₂), 3.26 (1H, m, CHNH), 2.84 (1H, m, NHCOCH), 2.42 (2H, m, CH₂), 1.80 (2H, m, CH₂), 1.70 (2H, m, CH₂), 1.53 (2H, m, CH₂), 1.27 (2H, s, CH₂); $\delta_{\text{C}}/\text{ppm}$ (125 MHz, CDCl₃) 136.4 (C), 128.1 (PhH), 128.0 (PhH), 127.0 (PhH), 124.7 (PhH), 122.2 (PhH), 121.9 (PhH), 117.8 (PhH), 117.4 (PhH); m/z (ESI) 380 (100%, [M + H]⁺), 230 (20%); HRMS found 380.1801 (C₂₂H₂₆N₃OS, [M + H]⁺, requires 380.1797).



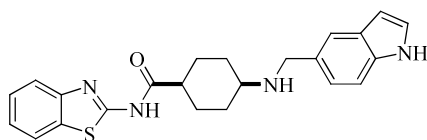
cis-N-(Benzo[d]thiazol-2-yl)-4-((2,3-dihydrobenzofuran-5-yl)methylamino)-cyclohexanecarboxamide (71). Compound **38** (35.0 mg, 0.090 mmol) was dissolved in NEt_3 (25.1 μL , 0.180 mmol), molecular sieves (~ 10 mg) and DMF (0.450 mL), with a final concentration of 0.2 M. 2,3-Dihydrobenzofuran-5-carbaldehyde (17 μL , 0.135 mmol) was added after 15 min. After 1 h $\text{NaBH}(\text{OAc})_3$ (43.7 mg, 0.206 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO_3 (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M $\text{HCl}_{(\text{aq})}$ (3×10 mL), H_2O (3×10 mL), brine (1×10 mL), and dried over MgSO_4 , filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm) and lyophilised to yield **71** (12.4 mg, 26%) as a white flocculent solid (TFA salt).

>98% Purity ($t_r = 9.2$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.01$ (EtOAc-Hexane , 2:1); mp 88–100 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2966 (w, br), 1671 (s, sh), 1543 (m, sh), 1179 (s, br), 1131 (s, br), 721 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 9.53 (1H, s, NH), 7.81 (1H, d, J 8.0, PhH), 7.75 (1H, m, PhH), 7.68 (1H, m, PhH), 7.48 (1H, t, J 8.0, PhH), 7.38 (1H, t, J 7.6, PhH), 7.12 (1H, d, J 7.6, PhH), 6.88 (1H, d, J 8.0, PhH), 6.68 (1H, d, J 8.0, PhH), 4.69 (1H, t, J 8.8, CH_2), 4.51 (1H, t, J 8.8, CH_2), 3.99 (2H, s, NHCH_2), 3.28 (1H, t, J 8.8, CH_2), 3.14 (1H, t, J 8.8, CH_2), 3.04 (1H, m, CHNH), 2.79 (1H, m, NHCOCH), 2.19 (2H, m, CH_2), 1.85 (4H, m, CH_2), 1.66 (2H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 173.5 (C), 164.4 (C), 160.9 (C), 130.2 (PhH), 127.0 (PhH), 124.9 (PhH), 121.9 (PhH), 118.6 (PhH), 113.4 (PhH), 109.3 (PhH), 71.4 (OCH_2), 53.5 (CHNH), 47.6 (NHCH_2), 40.5 (NHCOCH), 28.8 (CH_2), 25.3 (2C, CH_2), 24.2 (2C, CH_2); m/z (ESI) 408 (100%, $[\text{M} + \text{H}]^+$), 258 (50%); HRMS found 408.1739 ($\text{C}_{23}\text{H}_{26}\text{N}_3\text{O}_2\text{S}$, $[\text{M} + \text{H}]^+$, requires 408.1746).



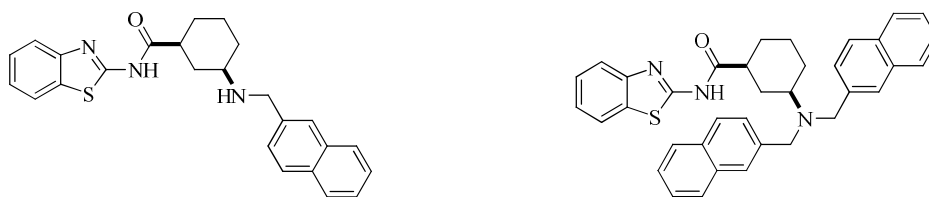
***cis*-N-(Benzo[d]thiazol-2-yl)-4-(benzo[b]thiophen-5-ylmethylamino)cyclohexanecarboxamide (72).** Compound **38** (36.3 mg, 0.093 mmol) was dissolved in NEt₃ (25.9 μ L, 0.186 mmol), molecular sieves (~10 mg) and DMF (0.450 mL), with a concentration of 0.2 M. 1-Benzothiophene-5-carbaldehyde (22.2 mg, 0.137 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (44.9 mg, 0.212 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **72** (9.5 mg, 19%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 10.4 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.01 (EtOAc–Hexane, 2:1); mp 163–166 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 1671 (s, sh), 1540 (m, sh), 1450 (m, sh), 1196 (s, br), 1130 (s, br), 720 (s, sh); δ_H/ppm (400 MHz, CDCl₃) 9.64 (1H, s, NH), 7.89 (1H, m, PhH), 7.81 (1H, m, PhH), 7.75 (2H, m, PhH), 7.57 (1H, t, J 8.4, PhH), 7.39–7.44 (3H, m, PhH), 7.28 (1H, m, PhH), 4.05 (2H, s, NHCH₂), 3.00 (1H, m, CHNH), 2.79 (1H, m, NHCOCH), 2.18 (2H, m, CH₂), 1.96 (2H, m, CH₂), 1.84 (2H, m, CH₂), 1.67 (2H, m, CH₂); δ_C/ppm (100 MHz, CDCl₃) 174.0 (C), 163.1 (C), 162.3 (C), 140.8 (C), 139.6 (C), 128.0 (PhH), 127.7 (PhH), 125.7 (PhH), 125.5 (PhH), 123.4 (PhH), 123.1 (PhH), 121.5 (PhH), 117.8 (PhH), 53.7 (CHNH), 48.0 (NHCH₂), 40.3 (NHCOCH), 24.5 (2C, CH₂), 23.9 (2C, CH₂); m/z (ESI) 422 (100%, [M + H]⁺), 272 (60%), 151 (25%, [ABT + H]⁺); HRMS found 422.1351 (C₂₃H₂₄N₃OS₂, [M + H]⁺, requires 422.1361).



***cis*-N-(Benzo[d]thiazol-2-yl)-4-((1*H*-indole-5-yl)methylamino)cyclohexane-carboxamide (73).** Compound **38** (31.3 mg, 0.080 mmol) was dissolved in NEt₃ (22.4 μ L, 0.160 mmol), molecular sieves (~10 mg) and DMF (0.450 mL), with a final concentration of 0.2 M. Indole-5-carbaldehyde (10.4 mg, 0.172 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (33.7 mg, 0.159 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **73** (21.3 mg, 51%) as a white flocculent solid (TFA salt).

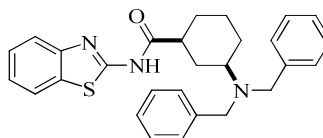
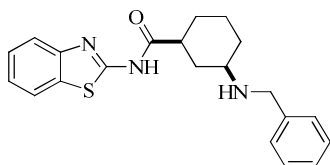
>98% Purity (t_r = 16.3 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.53 (EtOAc–Hexane, 2:1); mp 123–128 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 1674 (s, sh), 1199 (s, br), 1135 (s, br), 724 (s, sh); δ_H/ppm (400 MHz, CDCl₃) 9.23 (1H, s, NH), 7.81 (1H, m, PhH), 7.61 (1H, m, PhH), 7.50 (2H, m, PhH), 7.34 (2H, m, PhH), 7.23 (2H, m, PhH), 6.74 (1H, m, PhH), 4.12 (2H, s, NHCH₂), 3.01 (1H, m, CHNH), 2.82 (1H, m, NHCOCH), 2.09 (2H, m, CH₂), 1.82 (2H, m, CH₂), 1.75 (2H, m, CH₂), 1.60 (2H, m, CH₂); δ_C/ppm (100 MHz, CDCl₃) 192.3 (C), 126.4 (PhH), 125.9 (PhH), 124.4 (PhH), 121.9 (PhH), 112.2 (PhH), 104.8 (PhH), 48.8 (NHCH₂), 34.0 (CH₂), 24.0 (CH₂); m/z (ESI) 405 (100%, [M + H]⁺), 151 (25%, [ABT + H]⁺); HRMS found 405.1754 (C₂₃H₂₅N₄OS, [M + H]⁺, requires 405.1745).



***cis*-N-(Benzo[d]thiazol-2-yl)-3-((naphthalen-2-yl)methylamino)cyclohexanecarboxamide (74) and *cis*-N-(benzo[d]thiazol-2-yl)-3-(bis(naphthalen-2-yl)methylamino) cyclohexanecarboxamide (75).** Compound **43** (49.7 mg, 0.128 mmol) was dissolved in NEt_3 (39 μL , 0.280 mmol) and DMF (0.64 mL), with a final concentration of 0.2 M. 2-Naphthaldehyde (40.1 mg, 0.257 mmol) was added after 15 min. After 1 h $\text{NaBH}(\text{OAc})_3$ (92.0 mg, 0.434 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO_3 (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M $\text{HCl}_{(\text{aq})}$ (3×10 mL), H_2O (3×10 mL), brine (1×10 mL), and dried over MgSO_4 , filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN– H_2O (+0.1% TFA) in 30 min, $\lambda = 220$ nm) and lyophilised to yield **74** (7 mg, 10%) and by-product **75** (10 mg, 14%) as white flocculent solids (TFA salts).

74. >98% Purity ($t_r = 10.9$ min, 2–98% ACN– H_2O (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.02$ (EtOAc–Hexane, 2:1); mp 138–139 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2958 (w, br), 1674 (m, sh), 1547 (m, sh), 1179 (m, br), 1131 (m, br), 721 (m, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 9.72 (1H, s, NH), 7.73–7.81 (5H, m, PhH), 7.42–7.50 (3H, m, PhH), 7.36–7.42 (2H, m, PhH), 4.11 (1H, d, J 13.2, NHCH_2), 4.03 (1H, d, J 13.2, NHCH_2), 2.94 (1H, m, NHCOCH), 2.46 (1H, m, CHNH), 1.85 (3H, m, CH_2), 1.56 (1H, m, CH_2), 1.30 (1H, m, CH_2), 1.13 (2H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 130.0 (PhH), 128.2 (PhH), 127.8 (PhH), 127.1 (PhH), 126.5 (PhH), 125.2 (PhH), 48.1 (NHCH_2), 43.3 (NHCOCH), 30.2 (CH_2), 27.3 (CH_2), 22.3 (CH_2); m/z (ESI) 438 (30%, $[\text{M} + \text{Na}]^+$), 416 (60%, $[\text{M} + \text{H}]^+$), 155 (100%); HRMS found 416.1811 ($\text{C}_{25}\text{H}_{26}\text{N}_3\text{OS}$, $[\text{M} + \text{H}]^+$, requires 416.1797).

75. >98% Purity ($t_r = 14.5$ min, 2–98% ACN– H_2O (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.78$ (EtOAc–Hexane, 2:1); mp 137–139 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2957 (w, br), 1668 (m, sh), 1545 (m, sh), 1179 (s, br), 1128 (m, br), 719 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 7.95 (2H, s, PhH), 7.78–7.84 (7H, m, PhH), 7.76 (1H, d, J 12.0, PhH), 7.59 (2H, d, J 8.0, PhH), 7.46–7.52 (4H, m, PhH), 7.42 (1H, t, J 8.0, PhH), 7.35 (1H, t, J 8.0, PhH), 4.37 (2H, d, J 12.0, NCH_2), 4.26 (2H, d, J 12.0, NCH_2), 3.32 (1H, m, CHNH), 2.84 (1H, m, CH_2), 2.54 (1H, m, NHCOCH), 2.31 (1H, m, CHCH_2), 1.94–2.03 (3H, m, CH_2), 1.42–1.62 (2H, m, CH_2), 1.24 (1H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 173.3 (C), 133.5 (PhH), 133.2 (PhH), 130.6 (PhH), 129.2 (PhH), 128.2 (PhH), 127.6 (PhH), 127.4 (PhH), 127.1 (PhH), 126.8 (PhH), 125.5 (PhH), 121.8 (PhH), 119.0 (PhH), 60.2 (CHN), 54.5 (2C, NCH_2), 43.7 (NHCOCH), 29.2 (CH_2), 27.9 (CH_2), 26.1 (CH_2), 23.9 (CH_2); m/z (ESI) 556 (60%, $[\text{M} + \text{H}]^+$); HRMS found 556.2399 ($\text{C}_{36}\text{H}_{34}\text{N}_3\text{OS}$, $[\text{M} + \text{H}]^+$, requires 556.2423).



cis-N-(Benzo[d]thiazol-2-yl)-3-(benzylamino)cyclohexanecarboxamide (76) and cis-N-(benzo[d]thiazol-2-yl)-3-(dibenzylamino)cyclohexanecarboxamide (77). Compound **43** (48.4 mg, 0.142 mmol) was dissolved in NEt_3 (38 μL , 0.273 mmol) and DMF (0.62 mL), with a final concentration with 0.2 M. Benzaldehyde (25 μL , 0.246 mmol) was added after 15 min. After 1 h $\text{NaBH}(\text{OAc})_3$ (105.3 mg, 0.45 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO_3 (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3×10 mL), H_2O (3×10 mL), brine (1×10 mL), and dried over MgSO_4 , filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm) and lyophilised to yield **76** (27 mg, 45%) and by-product **77** (18 mg, 32%) as white flocculent solids (TFA salts).

76. >98% Purity ($t_r = 9.1$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.09$ (EtOAc–Hexane, 2:1); mp 130–132 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2956 (w, br), 1670 (s, sh), 1547 (s, sh), 1179 (s, br), 1130 (s, sh), 721 (m, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 9.72 (1H, br s, NH), 7.78 (1H, d, J 8.0, PhH), 7.61 (1H, d, J 8.0, PhH), 7.39 (1H, d, J 7.8, PhH), 7.31 (3H, m, PhH), 7.28 (3H, m, PhH), 3.98 (2H, s, NHCH_2), 3.02 (1H, m, CHNH), 2.54 (1H, m, NHCOCH), 2.40 (1H, m, CH_2), 2.02 (1H, m, CH_2), 1.83 (3H, m, CH_2), 1.42 (2H, m, CH_2), 1.23 (1H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 130.5 (PhH), 130.1 (PhH), 129.6 (PhH), 129.3 (PhH), 126.8 (PhH), 124.5 (PhH), 121.9 (PhH), 55.5 (CHNH), 48.2 (NHCH_2), 43.2 (NHCOCH), 30.9 (CH_2), 28.0 (CH_2), 23.3 (CH_2); m/z (ESI) 366 (60%, $[\text{M} + \text{H}]^+$); HRMS found 366.1631 ($\text{C}_{21}\text{H}_{24}\text{N}_3\text{OS}$, $[\text{M} + \text{H}]^+$, requires 366.1640).

77. >98% Purity ($t_r = 12.0$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.82$ (EtOAc–Hexane, 2:1); mp 118–120 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2962 (w, br), 1667 (s, sh), 1546 (m, sh), 1164 (s, br), 1128 (s, br), 750 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 7.85 (1H, d, J 7.2, PhH), 7.77 (1H, d, J 8.4, PhH), 7.50–7.55 (5H, m, PhH), 7.43 (1H, t, J 7.2, PhH), 7.38 (6H, m, PhH), 4.21 (4H, s, NCH_2), 3.26 (1H, m, CHN), 2.86 (1H, NHCOCH), 2.26 (1H, m, CH_2), 2.12 (1H, m, CH_2), 2.00 (2H, m, CH_2), 1.58 (2H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 173.9 (C), 131.2 (PhH), 130.8 (4C, PhH), 129.1 (4C, PhH), 126.9 (PhH), 122.2 (PhH), 118.5 (PhH), 60.7 (CHN), 53.9 (2C, NCH_2), 38.1 (NHCOCH), 26.2 (2C, CH_2), 22.3 (2C, CH_2); m/z (FAB) 456 (100%, $[\text{M} + \text{H}]^+$); HRMS found 456.2068 ($\text{C}_{28}\text{H}_{30}\text{N}_3\text{OS}$, $[\text{M} + \text{H}]^+$, requires 456.2110).

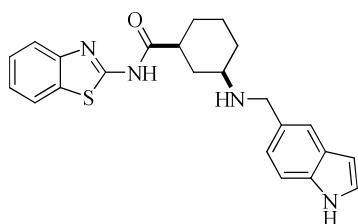


cis-N-(Benzo[d]thiazol-2-yl)-3-((benzofuran-5-yl)methylamino)cyclohexanecarboxamide (78) and cis-N-(benzo[d]thiazol-2-yl)-3-(bis(benzofuran-5-ylmethyl)amino)cyclohexanecarboxamide (79). Compound **43** (56.3 mg, 0.145 mmol) was dissolved in NEt_3 (44 μL , 0.316 mmol) and DMF (0.723 mL), with a concentration of 0.2 M. 5-Benzofurancarbaldehyde (40.9 mg, 0.280 mmol) was added after 15 min. After 1 h $\text{NaBH}(\text{OAc})_3$ (120.3 mg, 0.568 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO_3 (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M $\text{HCl}_{(\text{aq})}$ (3×10 mL), H_2O (3×10 mL), brine (1×10 mL), and dried over MgSO_4 , filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm) and lyophilised to yield **78** (2.5 mg, 3%) and by-product **79** (28 mg, 36%) as white flocculent solids (TFA salts).

78. >98% Purity ($t_r = 10.0$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.09$ (EtOAc–Hexane, 2:1); mp 134–135 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 2955 (w, br), 1670 (s, sh), 1547 (m, sh), 1200 (s, br), 1129 (s, br), 721 (m, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 9.62 (1H, br s, NH), 7.82 (1H, d, J 8.0, PhH), 7.74 (1H, d, J 8.0, PhH), 7.61 (2H, m, PhH), 7.53 (1H, t, J 8.0, PhH), 7.43 (2H, m, PhH), 7.28 (1H, m, PhH), 6.73 (1H, m, OCHCH), 4.12 (1H, d, J 12.8, NHCH_2), 4.03 (1H, d, J 12.8, NHCH_2), 2.98 (1H, m, CHNH), 2.53 (1H, m, NHCOCH), 2.53 (1H, m, CH_2), 1.98 (1H, m, CH_2), 1.88–1.73 (3H, m, CH_2), 1.40 (1H, m, CH_2), 1.21 (2H, m, CH_2); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, DMSO-d_6) 8.87 (1H, br s, NH), 8.08 (1H, m, PhH), 7.98 (1H, d, J 7.6, PhH), 7.80 (1H, m, PhH), 7.74 (1H, d, J 8.0, PhH), 7.69 (1H, d, J 8.4, PhH), 7.45 (2H, m, PhH), 7.31 (1H, t, PhH), 7.05 (1H, m, OCHCH), 4.32 (2H, m, NHCH_2), 3.18 (1H, m, CHNH), 2.67 (1H, m, NHCOCH), 2.37 (1H, m, CH_2), 2.17 (1H, m, CH_2), 1.92 (2H, m, CH_2), 1.62 (1H, m, CH_2), 1.35 (3H, m, CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl_3) 128.4 (PhH), 126.5 (PhH), 125.0 (PhH), 123.6 (PhH), 122.5 (PhH), 117.7 (PhH), 112.1 (PhH), 106.9 (PhH), 55.0 (CHNH), 48.4 (NHCH_2), 43.3 (NHCOCH), 31.2 (CH_2), 28.3 (CH_2), 27.6 (CH_2), 23.3 (CH_2); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, DMSO-d_6) 136.0 (PhH), 131.3 (PhH), 127.6 (PhH), 126.6 (PhH), 123.1 (PhH), 121.7 (PhH), 120.6 (PhH), 111.5 (PhH), 106.7 (PhH), 55.1 (CHNH), 47.0 (NHCH_2), 41.9 (NHCOCH), 30.3 (CH_2), 28.1 (CH_2), 23.1 (CH_2); m/z (ESI) 406 (100%, $[\text{M} + \text{H}]^+$); HRMS found 406.1591 ($\text{C}_{23}\text{H}_{24}\text{N}_3\text{O}_2\text{S}$, $[\text{M} + \text{H}]^+$, requires 406.1589).

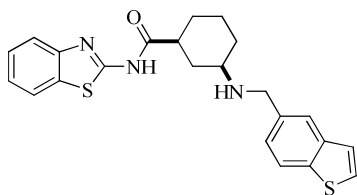
79. >98% Purity ($t_r = 13.0$ min, 2–98% $\text{ACN-H}_2\text{O}$ (+0.1% TFA) in 30 min, $\lambda = 220$ nm); $R_f = 0.78$ (EtOAc–Hexane, 2:1); mp 113–115 $^\circ\text{C}$; $\nu_{\text{max}}/\text{cm}^{-1}$ (solid) 3468 (w, br), 3018 (w, br), 2971 (m, sh), 1741 (s, sh), 1366 (s, sh), 1217 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl_3) 7.81 (4H, m, PhH), 7.66 (2H, m, PhH), 7.50 (3H, m, PhH), 7.41 (3H, m, PhH), 6.77 (2H, m, PhH), 4.35 (2H, m, NCH_2), 4.24 (2H, m, NCH_2), 3.35 (1H, m, CHN), 2.57 (1H, NHCOCH), 2.86 (1H, m, CH_2), 2.32 (1H, m, CH_2), 2.00

(3H, m, CH₂), 1.34–1.64 (2H, m, CH₂), 1.22 (1H, m, CH₂); δ_C /ppm (100 MHz, CDCl₃) 146.1 (2C, PhH), 126.7 (PhH), 124.2 (PhH), 121.7 (PhH), 118.7 (PhH), 112.0 (2C, PhH), 106.7 (2C, PhH), 59.9 (CHN), 54.3 (2C, NCH₂), 43.3 (NHCOCH), 29.2 (CH₂), 27.8 (CH₂), 25.6 (CH₂), 23.9 (CH₂); m/z (ESI) 536 (100%, [M + H]⁺), 155 (15%); HRMS found 536.2008 (C₃₂H₂₉N₃O₃S, [M + H]⁺, requires 536.2008).



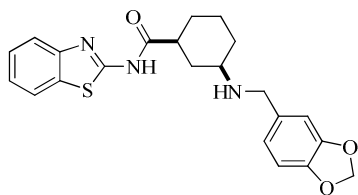
***cis*-N-(Benzo[d]thiazol-2-yl)-3-((1*H*-indole-5-yl)methylamino)cyclohexane-carboxamide (80).** Compound **43** (35.0 mg, 0.090 mmol) was dissolved in NEt₃ (25.1 μ L, 0.180 mmol), molecular sieves (~10 mg) and DMF (0.450 mL), with a final concentration of 0.2 M. Indole-5-carbaldehyde (20.6 mg, 0.142 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (37.0 mg, 0.175 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **80** (7.0 mg, 15%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 9.6 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.58 (EtOAc–Hexane, 2:1); mp 116–121 °C; $\nu_{\max}$ /cm^{−1} (solid) 2953 (w, br), 2863 (w, br), 1673 (s, sh), 1201 (s, br), 1182 (s, br), 702 (*m*, sh); δ_H /ppm (400 MHz, MeOH-*d*₄) 7.86 (1H, d, *J* 8.0, PhH), 7.74 (1H, d, *J* 8.0, PhH), 7.71 (1H, m, PhH), 7.48 (1H, t, *J* 8.4, PhH), 7.44 (1H, m, PhH), 7.32 (2H, m, PhH), 7.22 (1H, m, PhH), 4.33 (2H, s, NHCH₂), 3.26 (1H, m, NHCOCH), 2.70 (1H, m, CHNH), 2.41 (1H, m, CH₂), 2.28 (1H, m, CH₂), 2.05 (2H, m, CH₂), 1.78 (1H, m, CH₂), 1.51 (3H, m, CH₂); δ_C /ppm (100 MHz, MeOH-*d*₄) 127.4 (PhH), 125.2 (PhH), 123.7 (PhH), 123.3 (PhH), 122.5 (PhH), 122.0 (PhH), 113.2 (PhH), 102.9 (PhH), 57.0 (CHNH), 50.8 (2H, s, NHCH₂), 44.1 (NHCOCH), 32.2 (CH₂), 29.9 (CH₂), 24.8 (CH₂); m/z (ESI) 405 (100%, [M + H]⁺); HRMS found 405.1747 (C₂₃H₂₅N₄OS, [M + H]⁺, requires 405.1745).



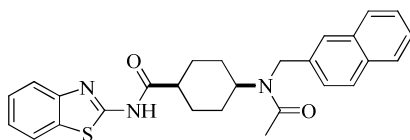
***cis*-N-(Benzo[*d*]thiazol-2-yl)-3-(benzo[*b*]thiophen-5-ylmethylamino)cyclohexanecarboxamide (**81**).** Compound **43** (34.3 mg, 0.088 mmol) was dissolved in NEt₃ (24.5 μ L, 0.176 mmol), molecular sieves (~10 mg) and DMF (0.440 mL), with a concentration of 0.2 M. 1-Benzothiophene-5-carbaldehyde (22.4 mg, 0.138 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (35.0 mg, 0.165 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl_(aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **81** (5.9 mg, 13%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 10.6 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.01 (EtOAc–Hexane, 2:1); mp 124–126 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2965 (*w*, *br*), 1670 (*s*, *sh*), 1547 (*m*, *sh*), 1184 (*s*, *br*), 1133 (*s*, *br*), 722 (*m*, *sh*); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 8.04 (1H, *d*, *J* 8.4, *PhH*), 8.01 (1H, *m*, *PhH*), 7.87 (1H, *d*, *J* 8.0, *PhH*), 7.71 (1H, *t*, *J* 5.6, *PhH*), 7.49–7.42 (3H, *m*, *PhH*), 7.32 (1H, *t*, *J* 7.0, *PhH*), 4.41 (2H, *s*, NHCH₂), 3.36 (1H, *m*, CHNH), 2.72 (1H, *m*, NHCOCH), 2.45 (1H, *m*, CH₂), 2.30 (1H, *m*, CH₂), 2.06 (2H, *m*, CH₂), 1.78 (1H, *m*, CH₂), 1.53 (3H, *m*, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 173.53 (*C*), 140.8 (*C*), 140.2 (*C*), 128.0 (*PhH*), 126.3 (*PhH*), 126.6 (*PhH*), 126.0 (*PhH*), 124.1 (*PhH*), 123.3 (*PhH*), 122.4 (*PhH*), 117.8 (*PhH*), 55.2 (CHNH), 48.1 (NHCH₂), 43.2 (NHCOCH), 30.2 (CH₂), 28.2 (CH₂), 27.7 (CH₂), 23.3 (CH₂); m/z (ESI) 422 (100%, [M + H]⁺); HRMS found 422.1367 (C₂₃H₂₄N₃OS₂, [M + H]⁺, requires 422.1361).



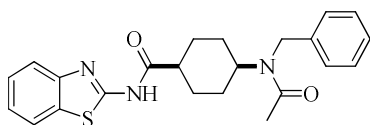
***cis*-N-(Benzo[*d*]thiazol-2-yl)-3-(benzo[*d*][1,3]dioxol-5-yl)methylamino)cyclohexanecarboxamide (82).** Compound **43** (30.7 mg, 0.079 mmol) was dissolved in NEt₃ (22.0 μ L, 0.158 mmol), molecular sieves (~10 mg) and DMF (0.395 mL), with a final concentration of 0.2 M. Piperonal (18.0 mg, 0.120 mmol) was added after 15 min. After 1 h NaBH(OAc)₃ (30.0 mg, 0.142 mmol) was added to the reaction mixture. After 24 h, the reaction was quenched with NaHCO₃ (sat) (1 mL), diluted with DCM (10 mL), washed with 0.1 M HCl (aq) (3 $\times$ 10 mL), H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), and dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **82** (11.2 mg, 28%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 9.4 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.62 (EtOAc–Hexane, 2:1); mp 89–96 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 2970 (*w*, *br*), 2870 (*w*, *br*), 1670 (*s*, *sh*), 1549 (*m*, *sh*), 1448 (*s*, *sh*), 1256 (*m*, *sh*), 1180 (*s*, *br*), 1128 (*s*, *br*), 1038 (*m*, *sh*), 721 (*m*, *sh*); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, CDCl₃) 7.82 (1H, *d*, *J* 8.0, *PhH*), 7.75 (1H, *d*, *J* 8.0, *PhH*), 7.54 (1H, *m*, *PhH*), 7.43 (1H, *m*, *PhH*), 6.98 (1H, *m*, *PhH*), 6.94 (1H, *m*, *PhH*), 6.79 (1H, *m*, *PhH*), 6.08 (1H, *s*, OCH₂O), 5.93 (1H, *s*, OCH₂O), 4.04 (2H, *s*, NHCH₂), 3.07 (1H, *m*, NHCOCH), 2.62 (1H, *m*, CHNH), 2.15–1.90 (5H, *m*, CH₂), 1.58 (2H, *m*, CH₂), 1.42 (1H, *m*, CH₂); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, CDCl₃) 190.5 (*C*), 173.3 (*C*), 148.7 (*C*), 126.9 (*PhH*), 124.3 (*PhH*), 121.9 (*PhH*), 119.2 (*PhH*), 110.1 (*PhH*), 108.8 (*PhH*), 101.5 (OCH₂O), 55.0 (NHCOCH), 48.2 (NHCH₂), 43.0 (CHNH), 30.8 (CH₂), 28.2 (CH₂), 23.4 (CH₂); m/z (ESI) 410 (100%, [M + H]⁺); HRMS found 410.1533 (C₂₂H₂₄N₃O₃S, [M + H]⁺, requires 410.1538).



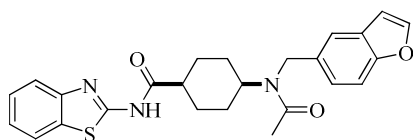
cis-N-(Benzo[d]thiazol-2-yl)-4-((N-(naphthalene-3-yl)methyl)acetamido)cyclohexanecarboxamide (83). Compound **35** (49.1 mg, 0.118 mmol) was dissolved in Ac₂O (0.500 mL, 5.29 mmol), with a final concentration of 0.2 M. After 1 h, all volatiles were removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **83** (33 mg, 61%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 14.7 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.04 (EtOAc–Hexane, 2:1); mp 216–218 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 3538 (w, br), 3373 (w, br), 3183 (w, br), 3061 (w, br), 2921 (m, sh), 1600 (m, sh), 1548 (m, sh), 1440 (m, sh), 1267 (s, sh), 758 (s, sh); m/z (ESI) 480 (100%, [M + Na]), 458 (60%, [M + H]⁺), 276 (30%); HRMS found 458.1917 (C₂₇H₂₇N₃O₂S, [M + H]⁺, requires 458.1902).



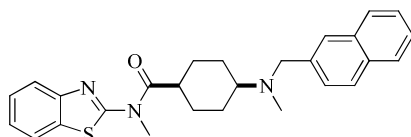
cis-N-(Benzo[d]thiazol-2-yl)-4-(N-benzylacetamido)cyclohexanecarboxamide (84). Compound **49** (6.2 mg, 0.013 mmol) was dissolved in Ac₂O (0.250 mL, 2.64 mmol), with a final concentration of 0.04 M. After 1 h, all volatiles were removed *in vacuo*. The crude product was purified by preparative HPLC (30–70% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **84** (3.5 mg, 66%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 13.0 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.01 (EtOAc–Hexane, 2:1); mp 135–137 °C; m/z (ESI) 430 (80%, [M + Na]), 408 (100%, [M + H]⁺); HRMS found 408.1751 (C₂₃H₂₆N₃O₂S, [M + H]⁺, requires 408.1756).



cis-N-(Benzo[d]thiazol-2-yl)-4-((N-(benzofuran-5-yl)methyl)acetamido)cyclohexanecarboxamide (85). Compound **54** (5.0 mg, 0.010 mmol) was dissolved in NEt₃ (13.2 μ L, 0.100 mmol) and Ac₂O (3.0 mL, 32 mmol) in a final concentration of 3 mM for **54**. After 1 h, all volatiles were removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **85** (0.6 mg, 14%) as a white flocculent solid (TFA salt).

>98% Purity (t_r = 13.3 min, 2–98% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm); R_f = 0.09 (EtOAc–Hexane, 2:1); mp 216–218 °C; $\nu_{\max}/\text{cm}^{-1}$ (solid) 3538 (w, br), 3373 (w, br), 3183 (w, br), 3061 (w, br), 2921 (m, sh), 1600 (m, sh), 1548 (m, sh), 1440 (m, sh), 1267 (s, sh), 758 (s, sh); $\delta_{\text{H}}/\text{ppm}$ (400 MHz, MeOH-*d*₄) 7.88 (2H, m, PhH), 7.83 (1H, m, PhH), 7.76 (1H, d, *J* 8.0, Ph_{ABTH}), 7.66 (1H, d, *J* 8.4, Ph_{ABTH}), 7.45 (1H, m, PhH), 7.33 (1H, t, *J* 8.0, Ph_{ABTH}), 6.95 (1H, m, OCH), 4.62 (1H, d, *J* 13.2, NCH₂), 4.39 (1H, d, *J* 13.6, NCH₂), 2.96 (1H, m, NHCOCH), 3.48 (1H, m, CHN), 2.34 (2H, m, CH₂), 2.22 (2H, m, CH₂), 1.84 (2H, m, CH₂), 1.34 (3H, t, *J* 7.5, COCH₃); $\delta_{\text{C}}/\text{ppm}$ (100 MHz, MeOH-*d*₄) 174.6 (C), 146.2 (PhH), 126.8 (PhH), 125.6 (PhH), 124.1 (PhH), 123.5 (PhH), 121.1 (PhH), 120.3 (PhH), 111.8 (PhH), 106.4 (PhH), 61.2 (CHN), 53.1 (NCH₂), 36.9 (NHCOCH), 26.1 (2C, CH₂), 21.9 (CH₂); *m/z* (ESI) 480 (100%, [M + Na]), 458 (60%, [M + H]⁺), 276 (30%); HRMS found 448.1712 (C₂₅H₂₆N₃O₃S, [M + H]⁺, requires 448.1695).



cis-N-(Benzo[d]thiazol-2-yl)-4-((N-(naphthalene-3-yl)methyl)amino)-N-methylcyclohexanecarboxamide (86). Compound **35** (7.9 mg, 0.015 mmol) was dissolved in DMF (0.150 mL), with a final concentration of 0.1 M. K₂CO₃ (6.2 mg, 0.045 mmol) and MeI (1.87 μ L, 0.030 mmol) were added, the round bottom flask wrapped in aluminium foil and left to stir at RT for 18 h. The reaction mixture was diluted with DCM (10 mL), washed with H₂O (3 $\times$ 10 mL), brine (1 $\times$ 10 mL), dried over MgSO₄, filtered and all volatiles removed *in vacuo*. The crude product was purified by preparative HPLC (30–50% ACN–H₂O (+0.1% TFA) in 30 min, λ = 220 nm) and lyophilised to yield **86** (1.0 mg, 15%) as a white flocculent solid (TFA salt).

R_f = 0.09 (EtOAc–Hexane, 2:1); *m/z* (ESI) 444 (100%, [M + H]⁺), 276 (30%); HRMS found 444.2126 (C₂₇H₃₀N₃OS, [M + H]⁺, requires 444.2110).

VIII.2 Miscellaneous Information

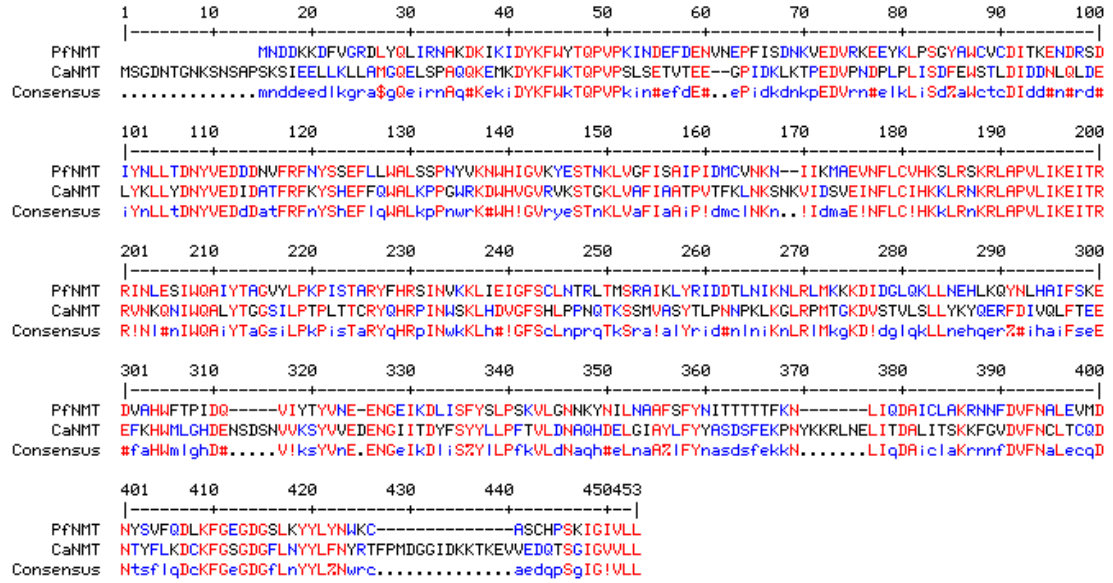
Table VIII-1: Bacterial strains

The table references *E. coli* strains used during enzyme expressions. The legend defines the abbreviations. (Source: Novagen, *E. coli* genotypes www.openwetware.org 14.07.2008)

<i>E. coli</i> Strain	Genotype
BL21(DE3) (Studier and Moffatt 1986)	F ⁻ <i>ompT hsdS_B(r_B-m_B-) gal dcm</i> (DE3)
DH5α TM (Grant, Jessee et al. 1990)	F ⁻ Φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ , m _K ⁺) <i>phoA supE44 λ- thi-1 gyrA96 relA1</i>

Legend: F⁻ = does not carry the F plasmid which is a self-transmissible, low-copy plasmid used for generation of single-stranded DNA when infected with M13 phage, often carries *lacI* and *lacZ*ΔM15 genotypes; *lacI* = encodes the *lac* repressor that controls expression from promoters that carry *lac* operator, IPTG binds *lac* repressor and derepresses promoter; *lacZ* = β-D-galactosidase gene, *lacZ*ΔM15 = required for β-galactosidase complementation, carried on lambdoid prophage φ80; *ompT* = mutation in outer membrane protein protease VII, reduces proteolysis of expressed proteins; *hsdS* = both restriction and methylation of certain sequences is deleted from strain; r_{B/K}⁻-m_{B/K}⁻ = B/K defines the strain lineage, r₊/– indicates that it has/has not got the restriction system, m₊/– indicates that it has/has not got the modification (methylation) system; *dcm* = cytosine methylation at second C of CCWGG sites abolished; DE3 = λ prophage carrying the T7 RNA polymerase gene and *lacI*, used to induce expression in T7 driven expression systems; *argF* = ornithine carbamoyl transferase mutation blocks ability to use arginine; *recA* = mutation in a gene responsible for general recombination of DNA; *endA* = mutation in non-specific endonuclease endonuclease I, eliminates non-specific endonuclease activity, resulting in improved plasmid preps; *hsd* = mutations in the system of methylation and restriction that allow *E. coli* to recognize DNA as foreign, *hsd* genotype allows efficient transformation of DNA generated from PCR reactions; *supE* = tRNA glutamine suppressor of amber; *thi-1* = requires thiamine for growth on minimal media; *gyrA96* = DNA gyrase mutant produces resistance to nalidixic acid; *relA* = regulates the coupling between transcription and translation.

(E)

**Figure VIII-1: Sequence alignments of NMT proteins**

(A) *HsNMT1*, *HsNMT2*; (B) *ScNMT*, *HsNMT1*, *HsNMT2*; (C) *PfNMT*, *HsNMT1*, *HsNMT2*; (D) *ScNMT*, *CaNMT*, *PfNMT*; (E) *PfNMT*, *CaNMT*.

FASTA sequences are: *PfNMT* (AAAF18461), *CaNMT* (P30418), *HsNMT1* (P30419), *HsNMT2* (O60551), *ScNMT* (P14743). They have been aligned with the program MultAlign (Version 5.4.1. (Corpet 1988)). The colour code is as follows: Red = exact match, blue = AA of similar properties, black = no match, with a consensus level of 90% high and 50% low. The consensus symbols are as follows: ! = any of IV, \$ = any of LM, % = any of FY and # = any of NDQEBZ.

Table VIII-2: PfNMT construct

PfNMT was sequenced by Dr R S Gunaratne (Gunaratne, Sajid et al. 2000; Bowyer 2006).

PfNMT Construct					
10	20	30	40	50	60
MGGSHHHHHH	GMASMTGGQQ	MGRDLYDDDD	KDRWGSNDDK	KDFVGRDLYQ	LIRNAKD KIK
70	80	90	100	110	120
IDYKFWYTQP	VPKINDEFDE	NVNPEFISDN	KVEDVRKEEY	KLPSGYAWCV	CDITKENDRS
130	140	150	160	170	180
DIYNLLTDNY	VEDDDNVFRF	NYSSEFLLWA	LSSPNYVKNW	HIGVKYESTN	KLVGFISAIP
190	200	210	220	230	240
IDMCVNKNII	KMAEVNFCV	HKSLRSKRLA	PVLIKEITRR	INLESIWQAI	YTAGVYLPKP
250	260	270	280	290	300
ISTARYFHRS	INVKKLIEIG	FSCLNTRLTM	SRAIKLYRID	DTLNKLNRL	MKKKDIDGLQ
310	320	330	340	350	360
KLLNEHLKQY	NLHAIFSKED	VAHWFTPIDQ	VIITYVNEEN	GEIKDLISFY	SLPSKVLGNN
370	380	390	400	410	420
KYNILNAAFS	FYNITTTTTF	KNLIQDAICL	AKRNNFDVFN	ALEVMDNYSV	FQDLKFGECD
430	440				
GSLKYYLYNW	KCASCHPSKI	GIVLL			

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unpublished work, Pfizer PLC.

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