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**Design, Synthesis and Evaluation of Potential Dual Drugs Targeting the
Haemoglobin Degradation Pathway in the Malaria Parasite *Plasmodium
falciparum***

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**University of Cape Town
December 2006**

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Haemoglobin Degradation Pathway in the Malaria Parasite *Plasmodium
falciparum***

**A thesis submitted to the
University of Cape Town
In partial fulfillment of the requirements of the
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By

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Abstract

For many years malaria has been a major cause of human suffering. Despite significant advances in understanding the disease and the parasite, malaria still remains one of the leading causes of morbidity and mortality, particularly in the tropics. Approximately 500 million people are afflicted and almost 3 million people die from the disease annually. Of the four causative species, *Plasmodium falciparum* is the most lethal. Recent trends indicate rapid emergence of drug-resistant and more virulent strains of the parasite to further intensify the problem. The choice of therapies currently available for the treatment of malaria is highly limited, and several of these may eventually be lost or compromised due to drug resistance. New antimalarial drugs with proven clinical efficacy against current drug-resistant cases of malaria including *Plasmodium falciparum* infections is critical to combat the disease and cope with the problem of further development of resistance.

Novel antimalarial agents were designed and synthesized based on the dual/double drug strategy *via* three key methods, i.e. peptide coupling, Biginelli multi-component reaction and click chemistries. In this strategy two antimalarial pharmacophores are hybridized *via* an appropriate linker in one single molecule with the aim of targeting two mechanisms of action. Compounds with promising dual antimalarial activities were identified against the chloroquine-resistant strains (W2 and K1) and β -haematin formation.

(i) A series of novel peptidyl-based compounds with promising inhibitory activities against various plasmodia strains and enzymes (FP-2 and FP-3) were identified. From the series several 4-aminoquinoline containing bisquinolines have revealed potency against the chloroquine-resistant (W2) strain of *Plasmodium falciparum*. The most potent peptidyl-based 4-aminoquinoline derivatives, (4.46, 4.47, 4.50, and 4.48) have IC_{50} values less than 2.48 μ M against chloroquine-resistant (W2) strain with the peptidyl-based derivative (4.48) [IC_{50} = 0.2313 μ M]] exhibiting the best activity among the compounds. Evidently, the data of these compounds have suggested a significant enhanced activity governed by the presence of the 4-aminoquinoline derived substrate. The consistent moderate activities revealed by the peptidyl-based derivatives (4.49),

(4.50) and (4.53) against both enzymes (FP-2 and FP-3) and W2 strain compared to the other compounds in the series, suggests that these compounds may exert dual antimalarial activities. The peptidyl-based bisquinoline derivative (4.46) with an IC_{50} of 0.003 μ M, displayed the greatest antiplasmodial activity in the chloroquine-resistant (K1) strain while the peptidyl-based bisquinoline derivative (4.48) with an IC_{50} of 0.035 μ M was the most active compound in the chloroquine-sensitive (3D7) strain. Screening against β -haematin formation revealed that all the compounds tested were several folds more active than chloroquine, with the most efficacious compound (4.46), being 24 times more active than chloroquine. There is fairly a good correlation between the antimalarial activity against chloroquine-resistant (W2) strain and inhibition of β -haematin formation. The observed correlation may be a result of similar pKa values and thus comparable vacuolar accumulation of the compounds investigated in this study without regard to the chloroquine resistance mechanism. Slight deviations in the correlation between the activity and β -haematin inhibition may be a result of the small differences in pKa values. No significant antiprotozoal activities were shown against *T.b. brucei* and *T. b. rhodesiense*. The most active within the series were compounds (4.50) [IC_{50} = 6.17 μ M)] and (4.45) [IC_{50} = 2.00 μ M)] against *T.b. brucei* and *T. b. rhodesiense* respectively.

(ii) A class of compounds was designed in such a way as to hybridize the privileged dihydropyrimidinone (DHPM) scaffold and 4-aminoquinolines containing variable chain lengths. The Biginelli three multicomponent reaction (MCR) was used as the basic method to synthesize the DHPM scaffold. The 4-aminoquinoline-containing DHPMs and their salt derivatives were synthesized and evaluated for their abilities to inhibit parasite growth *in vitro* and also their abilities to inhibit β -haematin formation and *T.b. brucei* cultures. Moderate to high inhibitory activities of the DHPM derivatives were displayed against chloroquine-sensitive, 3D7 and chloroquine-resistant, K1 strains. Within the series, the DHPM derivatives (4.112) [IC_{50} = 0.007 μ M)] and (4.107) [IC_{50} = 0.004 μ M)] exhibit the best activity in the respective 3D7 and K1 strains. All the DHPM derivatives were more active than chloroquine against β -haematin formation; especially compound (4.112) which were 5 times more active than chloroquine. No significant *in vitro* antiprotozoal activity of the DHPMs were revealed against *T.b. brucei*, with the most active compound (4.72) within the series having an IC_{50} value of 0.6 μ M.

(iii) Novel isatin-derived triazoles were designed and synthesized using “click” chemistry. The “click” approach provides a means for the quick assembly of a diversity of pharmacophores, enabling rapid SAR profiling of synthesized compounds. From the analog libraries evaluated, the thiosemicarbazone derivative (4.159) exhibited the best antiplasmodial activity with IC_{50} values of 0.0614, 0.395 and 0.022 μM against the respective *P. falciparum* strains, D10, W2 and 3D7. The isatin-derived triazoles (4.145b) and (4.145g) were the most active compounds against the K1 strain with comparable IC_{50} values of 0.02 μM . The thiosemicarbazone derivative (4.159) [$IC_{50} = 2.97 \mu M$] was also trypanocidal towards *T. b. rhodesiense*. With the exception of the thiosemicarbazone (4.159), semicarbazones (4.158) and (4.160), isatin-derived triazoles (4.156) and (4.157) and acetylene precursor (4.142), all the isatin-derived triazoles displayed more superior inhibitory activity than chloroquine against β -haematin formation.

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List of Abbreviations

Amu	Atomic mass units
µg	Microgram(s)
µM	Micromolar
Ar	Aromatic
Boc ₂ O	Ditertiarybutyldicarbonate (boc-anhydride)
br s	Broad singlet (in ¹ H NMR)
bt	Broad triplet (in ¹ H NMR)
CD ₃ OD	Deuteromethanol
CDCl ₃	Deuteriochloroform
CHCl ₃	Chloroform
CH ₃ I	Methyl iodide
CH ₃ CN	Acetonitrile
CuSO ₄ ·5H ₂ O	Copper(II) sulphate pentahydrate
Cys	Cysteine
d	Doublet (in ¹ H NMR)
D ₂ O	Deuterowater
DCM	Dichloromethane
dd	Double doublet or double of doublet (in ¹ H NMR)
ddd/td	Doublet of doublet of doublet or triplet of doublets (in ¹ H NMR)
DIEA	Diisopropyl ethylamine
DMF	Dimethylformamide
DMSO- <i>d</i> ₆	Deuterodimethylsulfoxide
DMSO	Dimethylsulfoxide
ED ₅₀	50% Effective Dose
EDC·HCl	<i>N</i> - (3-Dimethylaminopropyl)- <i>N</i> '-ethylcarbodiimide hydrochloride
EI	Electron Impact
eq	Equivalent(s)
Et ₃ N	Triethylamine
EtOAc	Ethyl acetate
EtOH	Ethanol

FAB	Fast Atomic Bombardment
FV	Food Vacuole
^1H NMR	Proton Nuclear Magnetic Resonance
H	Hour(s)
Hex	Hexane
His	Histidine
HOBt	1-Hydroxybenzotriazole
HRMS	High Resolution Mass Spectrometry
IC ₅₀	50% Inhibitory Concentration
K ₂ CO ₃	Potassium carbonate
LRMS	Low Resolution Mass Spectrometry
m	Multiplet (in ^1H NMR)
MCR	Multicomponent Reactions
m. p.	Melting Point
m/z	Mass to charge ratio
MeOH	Methanol
MgSO ₄	Magnesium sulfate
min	Minute(s)
mM	Millimolar
mL	Millilitre
NaN ₃	Sodium Azide
NaH	Sodium Hydride
Na ₂ SO ₄	Sodium Sulfate
nM	Nanomolar
NMP	N-methyl -2-pyrrolidine
NMR	Nuclear magnetic resonance
ppm	Parts per million
PFV	Parasitic food vacuole
Ph	Phenyl
Ph ₃ P	Triphenyl phosphine
PLC	Preparative layer chromatography
q	Quartet (in ^1H NMR)
R _f	Retention factor

s	Singlet (in ^1H NMR)
t	Triplet (in ^1H NMR)
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMSCl	Tetramethylsilyl chloride

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CHAPTER 1

INTRODUCTION

1.1 History of Malaria

Malaria was evident very early in the history (6000-5500 B.C) of humankind and has persistently inflicted disease among the human population.¹ The name originates from the "*mala aria*", which means bad air; a suitable name since it was thought to arise from exhalation of swamps. In 1880,¹ the first true sighting of the malaria parasite was made in Algeria by a French Army physician, Charles-Louis-Alphonse Laveran (Nobel Prize in Medicine 1907), who discovered the malaria parasite in human blood. In later years (1897) ¹, the English physician Dr. Ronald Ross (Nobel Prize in Medicine 1902) and Italian scientist Giovanni Batista Grassi (1898)¹ demonstrated that the malaria parasite was transmitted into the human bloodstream through the bite of an infected female mosquito.²

Malaria is caused by parasitic protozoa of the genus *Plasmodium*, and alternates between human and mosquito hosts. Four species of *Plasmodium* are responsible for human malaria, of which three (*P. vivax*, *P. ovale*, *P. malariae*) may cause serious illness, but are rarely fatal. The fourth, *P. falciparum*, is the most highly pathogenic and causes much more severe and progressive illness in non-immune persons. The natural vectors of the malaria parasites are female *Anopheles* mosquitoes. Approximately 60 of the 400 species transmit malaria under natural conditions.³

1.1.1 The Global Burden of Malaria

According to the World Health Organization (WHO)⁴, between 300 and 500 million clinical cases of malaria occur every year. Recent empirical estimates have suggested the total could run as high as 660 million.⁵ Malaria is estimated to kill more than 1

million people annually and possibly as many as 3 million, with most of the deaths occurring among children under age six living in sub-Saharan Africa. About 90% of malaria-related deaths occur in Africa and are caused by *P. falciparum*. If one survives multiple infections in childhood, such exposure leads to a natural immunity that limits the severity of the disease. It is estimated that over 40% of the world's population lives in malaria endemic areas, which are centered throughout the tropics and subtropical regions (Fig. 1).⁵ The global distribution as well as the endemicity are largely dependent on the type of mosquito, the parasite species and the climate.⁶

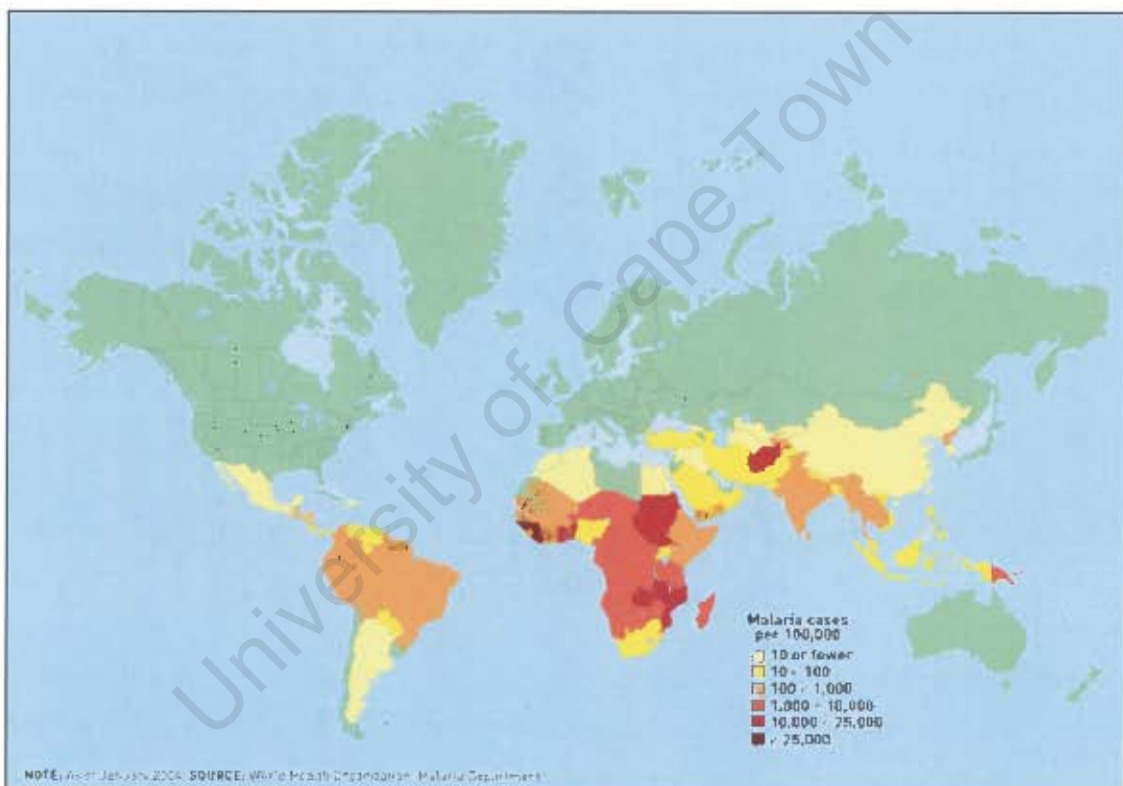


Figure 1: Global distribution of malaria transmission risk

The disease takes an economic toll as well because of reduced productivity, which is responsible for an estimated average loss of 1.3% of economic growth annually in countries with intense transmission.⁷ Malaria control is increasingly recognized as playing a key role in poverty reduction in high burden countries. Several organized

efforts to control the transmission of the disease have been made throughout history.⁸ Two major approaches have been employed, viz., killing the parasite and killing the parasite vector. In addition, various attempts to develop antimalarial vaccines have also been employed.⁹ The first large multilateral initiative was the WHO Malaria Eradication Program (1955-1969), which aimed at the total eradication of malaria mainly by vector control with dichlorodiphenyltrichloroethane (DDT, an insecticide) residual spraying.⁸ This effort failed but achieved regional eradication in Southern Europe and some countries in North Africa and the Middle East. Subsequent important initiatives were the creation of the Special Program for Training and Research in Tropical Diseases (TDR) in 1975, the Multilateral Initiative on Malaria (MIM) in 1997, the Roll Back Malaria (RBM) launched in 1998 by WHO, and the Global Fund, initiated in 2002.⁸ Despite all the efforts to reduce the adverse effect of the disease, the number of malaria cases is constantly rising. Factors contributing to the increase in malaria include: (i) resistance of parasites to commonly used antimalarial drugs; (ii) breakdown of control programmes; (iii) complex emergencies; (iv) collapse of local primary health services; and (v) resistance of mosquito vectors to insecticides.¹⁰⁻¹³

1.2 The Malaria Parasite's Life Cycle

The life cycle of the malaria parasite is complex and consists of several distinct phases. Two basic cycles, an asexual cycle in man and a sexual cycle in the female *Anopheles* mosquito, constitute the total life cycle. In man the asexual cycle can be further divided into a liver stage or pre-erythrocytic stage and an erythrocytic stage. A summary of the malaria parasite life cycle is depicted in Fig. 1.2 (a).^{14, 15, 16}

A malaria infection in the mammalian host starts with the bite by a malaria-infected *Anopheles* mosquito. Parasites, the so called sporozoites are injected together with mosquito saliva into the bloodstream of a human. These sporozoites, circulate for a short time in the blood stream, and then invade the liver cells, where they develop into exoerythrocytic schizonts during the next 5 to 15, days.

The hepatic (exoerythrocytic) schizonts contain 10 000 to 30 000 merozoites which are released into the blood stream and invade red blood cells. This is the beginning of the

blood (erythrocytic) stage. The entire invasion process takes about 30 seconds. Once the merozoites invade erythrocytes, they begin to undergo their asexual reproduction which leads to the formation of erythrocyte schizonts. The erythrocyte containing the mature schizont then ruptures and releases the merozoites which in turn invade other red blood cells. Over the next 48 hours, internalized parasites differentiate (ring stage), metabolize hemoglobin (trophozoite stage) and replicate (schizont stage) to produce expanded populations of invasive merozoites that are released upon rupture of the cell.

In the course of events, some merozoites which have invaded the erythrocytes could differentiate themselves into microgametocytes (male) and macrogametocytes (female). These gametocytes can be transmitted to an *Anopheles* mosquito during a blood meal, and then complete their life cycle. Mature gametocytes taken to the midgut of the *Anopheles* mosquito escape from the erythrocyte to form gametes, Fig. 1.2 (a).

The microgametes move quickly to fertilize a microgamete and form a zygote. Within 18 to 24 hours, the zygote elongates into a slowly moving ookinete. The ookinete crosses the peritrophic membrane and the epithelial cell of the midgut, and then transform into an oocyst beneath the basement membrane of the midgut epithelium. Between 7 and 15 days post infection, depending on the plasmodium species and ambient temperature, a single oocyst forms more than 10 000 sporozoites. The motile sporozoites migrate into the salivary glands and accumulate in the acinar cells of the salivary glands. When an infected mosquito bites a susceptible vertebrate host, the plasmodium life cycle begins, Fig. 1.2 (c).

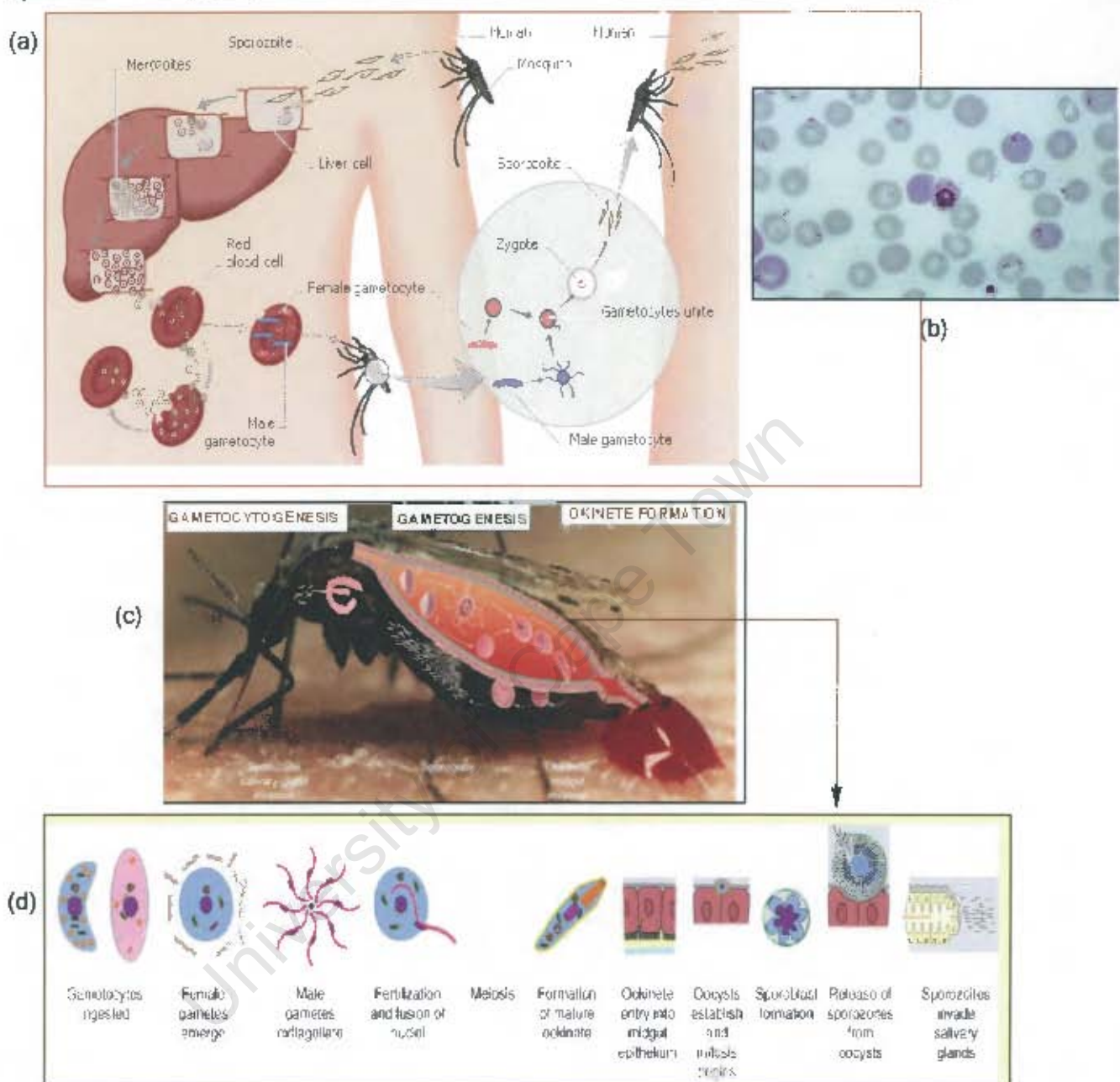


Figure 1.2: (a) Life cycle of the malaria parasite.¹⁴ (b) A photomicrograph of a blood smear shows erythrocytes containing developing *Plasmodium falciparum* parasites, magnified 1,000 times.¹⁶ (c) The metamorphoses of malaria within the mosquito vector.¹⁵ (d) Malaria parasite development in the mosquito, from the ingestion of parasite by the mosquito to the end of sporogony, when the sporozoites reach the salivary glands.¹⁵

1.3 Classification of Antimalarial Drugs

1.3.1 Clinically Established Drugs

Clinically established antimalarial drugs can be classified in terms of (i) their activity at different stages of the parasite life cycle or (ii) according to their chemical structures and mechanism of action.^{17, 18}

1.3.1.1 Classification based on the Stages of the Parasite Life cycle

(i) *Causal prophylaxis*

A causal prophylactic is an agent that has a lethal effect on the parasites in the pre-erythrocytic stages and thereby prevents the development of symptoms. Examples of causal prophylactic agents include primaquine, pyrimethamine, proguanil, dapsone and doxycycline, (see section 1.3.2). The term true casual prophylaxis refers to the killing of sporozoites before they infect the hepatocytes. No such drug is available today, although true casual prophylaxis may be achieved in the future with vaccines.⁹

(ii) *Suppressive treatment*

Suppressive treatment refers to the inhibition of the erythrocytic stage keeping the individual free from symptoms by early treatment. Drugs used for this purpose include chloroquine and mefloquine, (see section 1.3.2). This class, together with the casual prophylactic agents, belongs to the drugs used for the chemoprophylaxis when traveling to malaria endemic areas.

(iii) *Clinical cure*

Agents in this category are also called blood schizonticides as they interrupt erythrocytic schizogony and terminate the clinical attack. This is the largest group including the quinoline methanols (e.g. quinine and mefloquine), the 4-aminoquinolines (e.g. chloroquine), the phenanthrenes (e.g. halofantrine), the antifolates (e.g. pyrimethamine, proguanil, dapsone and sulfadoxine), the artemisinin group (e.g. dihydroartemisinin, artesunate and artemether) and some antibiotics (e.g. tetracycline and doxycycline), (see section 1.3.2).

(iv) *Radical cure*

Radical cure refers to the eradication of not only the erythrocytic parasites but also those in the pre-erythrocytic stage such as hypnozoites. Only primaquine has this action, (see section 1.3.2).

(v) *Prevention of transmission*

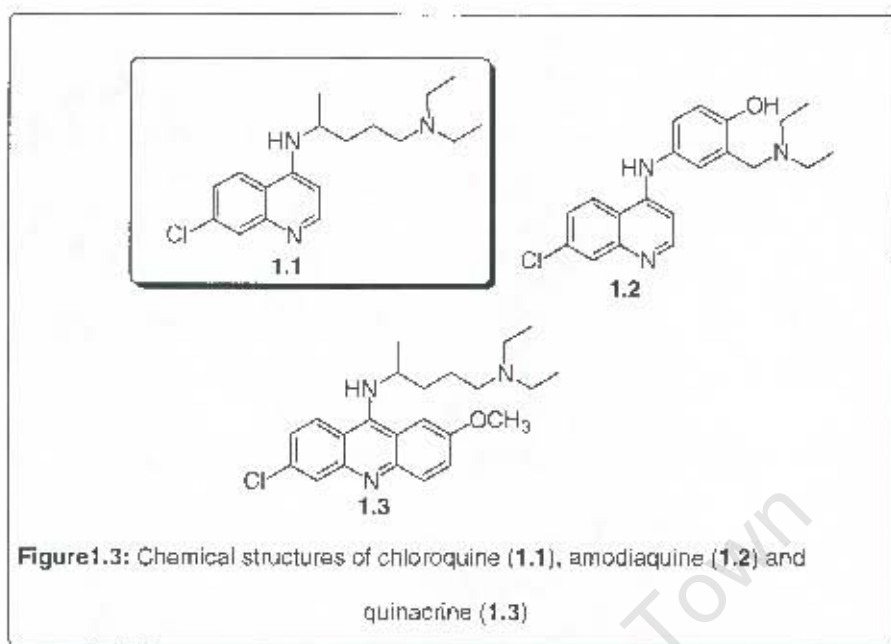
These agents prevent transmission *via* the mosquito by destroying the gametocytes (e.g. primaquine, proguanil, the artemisinins and pyrimethamine), (see section 1.3.2).

1.3.2 Classification based on Chemical Structure and Mode of Action

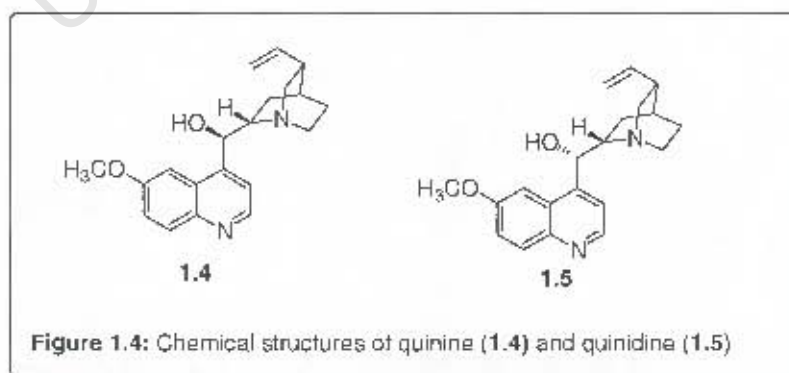
Antimalarial drugs can also be classified according to their chemical structure and mode of action that they exert.

1.3.2.1 Quinoline-containing Antimalarial Drugs

Quinoline antimalarials consist of all those antimalarial drugs containing the quinoline heteroaromatic nucleus.^{19, 20} They can be divided into three groups: (i) 4-aminoquinolines and closely related acridines including chloroquine (1.1), amodiaquine (1.2) and quinacrine (1.3). (ii) Quinoline methanols that include quinine (1.4), quinidine (1.5) mefloquine (1.6) and the closely related halofantrine (1.7). (iii) 8-Aminoquinoline that include pamaquine (1.8) and primaquine (1.9). The principal mode of action of some of the quinoline-containing drugs is to bind to haeme and to inhibit haeme polymerization.



Quinacrine (1.3) was one of the first synthetic antimalarials which were introduced in 1933. Chloroquine (CQ) (1.1, Fig. 1.3) was introduced in 1944-1945 and soon became the mainstay of therapy and prevention, since this drug was cheap, non-toxic, and active against all strains of the malaria parasite.²¹ Until a decade ago, chloroquine was the first-line treatment in most parts of the world. Today, the extensive spread of parasite resistance has severely limited its use. Amodiaquine (1.2, Fig. 1.3) is chemically related to CQ, but is more effective than CQ for clearing parasitemia in cases of uncomplicated malaria; even against some chloroquine-resistant strains.^{22, 23} However, drug resistance and potential hepatic toxicity limits its use.



Quinine (1.4, Fig. 1.4), and its isomers including quinidine (1.5) and related compounds are found in the bark of the cinchona tree.¹⁹ For almost three centuries, quinine, either in its purified form or as a component of the cinchona bark, was the only widely available medication for clinical use.

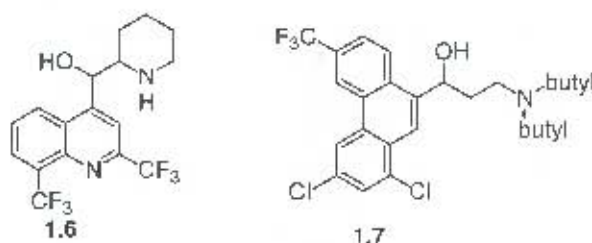


Figure 1.5: Chemical structures of mefloquine (1.6) and halofantrine (1.7)

The search for new antimalarials continued and eventually led to the synthesis of mefloquine (1.6, Fig. 1.5) and halofantrine (1.7). Mefloquine is structurally related to quinine (1.4) and its long half-life (14-21 days) has contributed to the rapid development of resistance. Halofantrine is effective against chloroquine-resistant malaria.²¹ Despite this, cardio toxicity has limited its use as a therapeutic agent.²¹

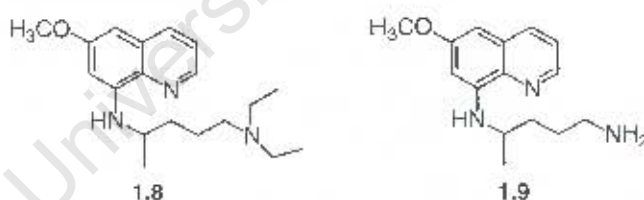
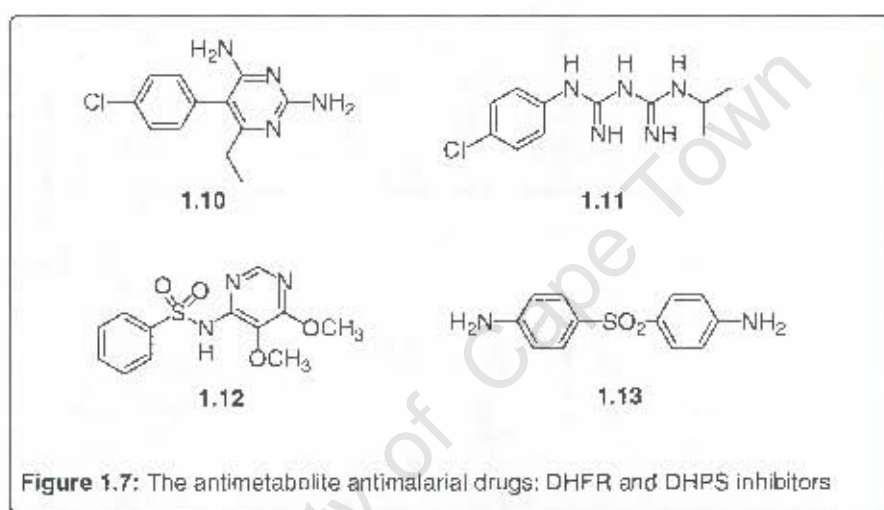


Figure 1.6: Chemical structures of pamaquine (1.8) and primaquine (1.9)

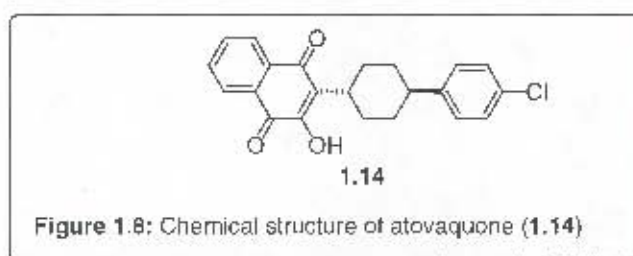
Pamaquine (1.8, Fig. 1.6) was shown to be capable of radical cure, and was first used in 1926. It was later found to be too toxic, and primaquine (1.9) was synthesized as a less toxic analog.

1.3.2.2 Folate Antagonists

These compounds (Fig. 1.7) inhibit the synthesis of parasitic pyrimidines, and thus of parasitic DNA.²¹ There are two groups of antifolates: (i) the dihydrofolate reductase (DHFR) inhibitors like pyrimethamine (1.10) and proguanil (1.11) and (ii) the dihydropteroate synthase (DHPS) that are sulfones and sulphonamides like sulfadoxine (1.12) and dapsone (1.13), respectively. Due to a marked synergistic effect, a drug of the first group is usually used in combination with a drug of the second.



Unfortunately resistance is widespread in Asia, India, and now in Africa.^{24, 25} Pyrimethamine-sulfadoxine (SP) is the most widely used combination. It is both cheap and practical (only one dose is needed because of the slow elimination from the body), and currently efficient in many parts of Africa. However, it is poorly active against highly chloroquine-resistant strains. The mechanism of resistance has been shown to be due to mutations in the genes of DHFR²⁶ and DHPS.²⁷



Development of the combination of proguanil and atovaquone (1.14) (Malarone®), provides another useful way of treatment.^{26, 27} Atovaquone is hydroxynaphthoquinone which acts by inhibiting parasite mitochondrial electron-transport. The mechanism of synergy of proguanil with atovaquone is complex and is still under investigation.^{30, 31} This combination is well tolerated and more effective than CQ alone, CQ-SP,³² proguanil³⁰ or mefloquine³³. Unfortunately, atovaquone is expensive and not easily affordable in most African countries.

1.3.2.3 Tetracyclines (Antibiotics)

In this group are found the antibiotics, which have a long history in the treatment of malaria. Interest in the use of antibiotics to treat malaria re-emerged with the appearance of chloroquine-resistance and multidrug resistant strains of *P. falciparum*. The tetracyclines (doxycycline or tetracycline) inhibit protein synthesis in the mitochondria and/or apicoplast.³⁴

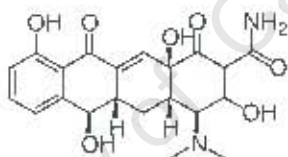
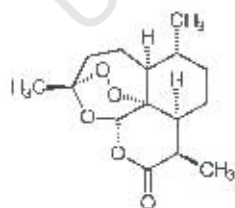
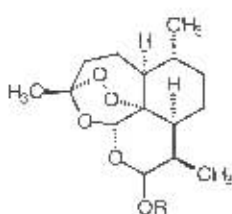


Figure 1.9: Chemical structure of tetracycline (1.15)

1.3.2.4 Artemisinin derivatives



1.16



1.17, R = H, Dihydroartemisinin

1.18, R = Me, Artemether

1.19, R = Et, Arteether

1.20, R = $\text{OCO}(\text{CH}_2)_2\text{COONa}$
Sodium Artesunate

Figure 1.10: Artemisinin (1.16) and its semisynthetic derivatives (1.17-1.20)

Artemisinin, a sesquiterpene lactone has been employed historically in China as a treatment for malaria.³⁵ The semisynthetic derivatives also have been increasingly employed during the past 20 years.³⁶ Four compounds have been used, the parent one, artemisinin (1.16), extracted from *Artemisia annua*³⁷ and three derivatives that are actually more active than artemisinin itself.^{38, 39} One of them is a water-soluble hemisuccinate: artesunate (1.20), two others are oil-soluble ethers: artemether (1.18) and arteether (1.19). All of them are readily metabolized to the biologically active metabolite, dihydroartemisinin (DHA) (1.17). The semisynthetic derivative artemether (1.18) and arteether (1.19) are effective against both chloroquine sensitive and chloroquine resistant *P. falciparum* strains and are clinically used for cerebral malaria. Artemisinins contain an endoperoxide bridge that is essential for antimalarial activity and that appears to undergo an iron-catalyzed decomposition into free radicals.⁴⁰

1.3.2.4.1 Mechanism of Action of Artemisinin-Like Compounds

Even though the mechanism of action is not completely understood, several theories have been proposed for the mechanism of the antimalarial action of artemisinin (QHS)-like compounds.^{41, 42, 43} Among the different proposed mechanisms, some researchers suggested that the endoperoxide group play a key role in the antimalarial activity of these types of compounds. This prevailing hypothesis revealed that reductive activation of the O-O bond by Fe^{III}-haeme (Fig. 1.11) generate the haeme derivatives that could be alkylated by a C-centered radical derived from QHS-like compounds, (Scheme 1.1).⁴⁴⁻⁴⁶ Lending support to this mechanistic findings, was work done by Taylor *et al.*⁴⁷ who have shown that some novel endoperoxide antimalarials interact with oxidized haeme in an analogous fashion to chloroquine while other were shown to interact with reduced haeme. Recently an experiment was conducted on the mechanistic studies of endoperoxides. In this study, parasite growth and chemosensitivity were performed under different oxygen tension: anaerobiosis, microaerophilia (1% O₂), high oxygen content (20%) or in the presence of carbon monoxide. The results indicated that endoperoxides were not activated by haeme iron and free radicals are not needed for its toxicity.⁴⁸ To add more confusion to the "real" mechanism was an observation of specific binding between artemisinin and the *P. falciparum* homologue of the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), a protein which is not located in the

digestion vacuole of the parasite.⁴⁸ Furthermore,⁴⁹ another alternative mechanism proposed for artemisinin is the inhibition of inducible nitric oxide synthase and nuclear factor, NK-kB .⁵⁰ These results suggest that in addition to the direct antimalarial activity of artemisinin, this drug could also exert a therapeutic effect on neurological complications in malaria.

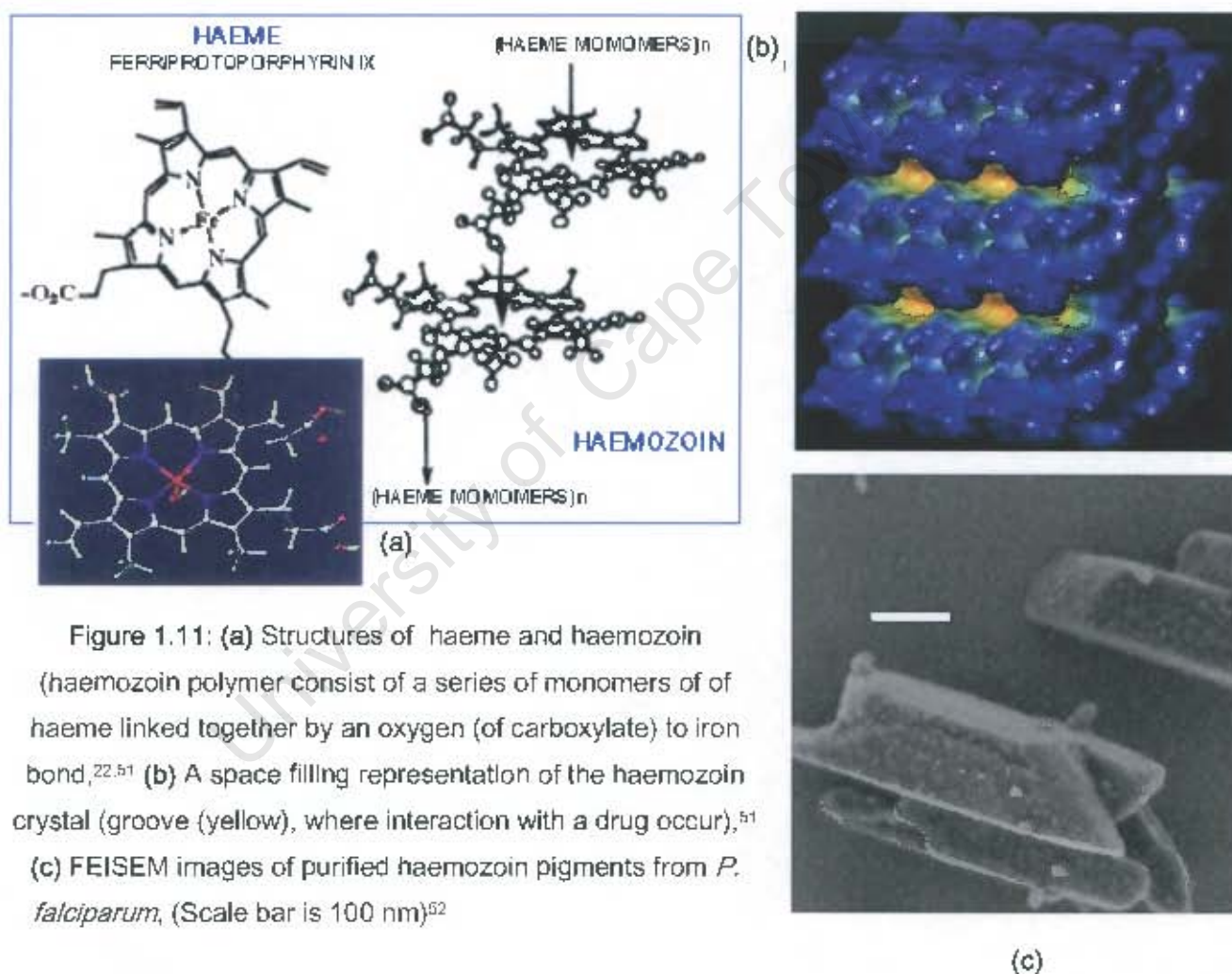
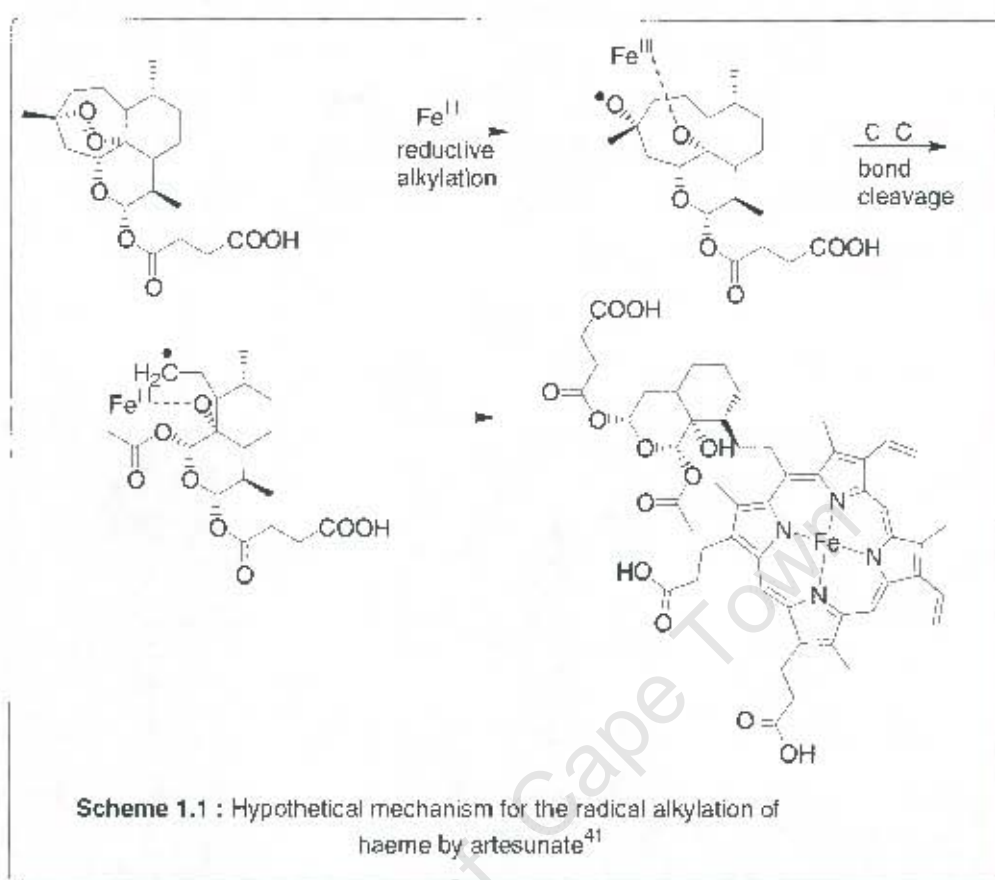


Figure 1.11: (a) Structures of haeme and haemozoin (haemozoin polymer consist of a series of monomers of of haeme linked together by an oxygen (of carboxylate) to iron bond,^{22,51} (b) A space filling representation of the haemozoin crystal (groove (yellow), where interaction with a drug occur),⁵¹ (c) FESEM images of purified haemozoin pigments from *P. falciparum*, (Scale bar is 100 nm)⁵²



1.4 Drug Resistance

The emergence of drug resistance poses a major obstacle in the control of malaria. *Plasmodium falciparum* has demonstrated the capacity to develop resistance to most of the antimalarial drugs that are presently available. Resistance has emerged towards all classes of antimalarial drugs except for the artemisinins.³⁷ Resistance to chloroquine, one of the most effective and used drugs was observed in Southeast Asia and South America at the end of the 1950's and in Africa in the late 1970's. Chloroquine resistance has since then spread across Sub-Saharan Africa but is still used for the treatment of *Plasmodium falciparum*. The effectiveness of chloroquine was enhanced by demonstrating that it has a synergic effect with the tumor necrosis factor (TNF), a cytokine responsible for some cerebral damages and which is produced by the immune system during the malaria crisis.⁵³

The molecular mechanisms behind resistance depend on the chemical class of the drug and its mechanism of action.³⁷ Generally, resistance arises from mutations in the genes encoding the parasite drug target or influx/efflux pumps that affect the concentration of the drug at that target.

1.4.1 Mechanism of Action of Quinoline-containing Antimalarials

The quinoline-containing drug class includes some of the most significant and common drugs which are the chloroquine-type 4-aminoquinolines, 8-aminoquinolines, and aryl-amino quinine type alcohols. The mechanism of action of chloroquine is only partially understood. Various mechanisms have been proposed for the action for chloroquine and related compounds, either extra- or intravascular.²² These include inhibition of protein synthesis⁵¹, inhibition of the food vacuole lipase⁵⁵ and aspartic proteinase⁵⁶, and inhibition of DNA and RNA synthesis.⁵⁷ The most commonly accepted hypothesis is that quinoline-containing drugs act primarily on haeme disposal, a process whereby intra-erythrocytic-stage malaria parasites detoxify haeme in the food vacuole, (Fig. 1.12).⁵² It is believed that in this process chloroquine is selectively taken up inside the parasite food vacuole where the parasite actively digests haemoglobin. The weak base properties of chloroquine ($pK_{a1}=8.1$; $pK_{a2}=10.2$) would explain its selective accumulation in the acidic food vacuole. At neutral pH, chloroquine is uncharged and can diffuse freely across the membrane against a pH gradient using energy and once inside the acidic vacuole, the protonated drug becomes trapped.⁵⁵ Upon proteolysis of haemoglobin, toxic haeme is released that is polymerized into an inert, nontoxic crystal called malarial pigment or haemozoin. Chloroquine would thus interfere with polymerization by binding to haeme and thus forming a haeme-chloroquine complex, thereby causing an accumulation of haeme, which is toxic to the parasite.

It was shown⁵⁸ that only a fraction of haeme is polymerized and that unpolymerized haeme in the food vacuole is degraded by glutathione in the parasite or host-cell cytosol.⁵⁹ Chloroquine would act primarily by competitively inhibiting glutathione-dependent haeme degradation outside the food vacuole.^{60, 61} Thus, chloroquine treatment would result in increased accumulation of haeme into membranes, which in turn, would alter $[Na^+]/[K^+]$ concentration in the cytoplasm. This hypothesis is supported

by the fact that chloroquine concentrations needed to kill parasites are inversely related to glutathione levels.⁶²

Recent data by Gao-De,⁶³ suggests that the nucleus may be the key site of chloroquine antimalarial action and resistant development while the lysosome in which chloroquine action has been well studied might be the secondary important site.

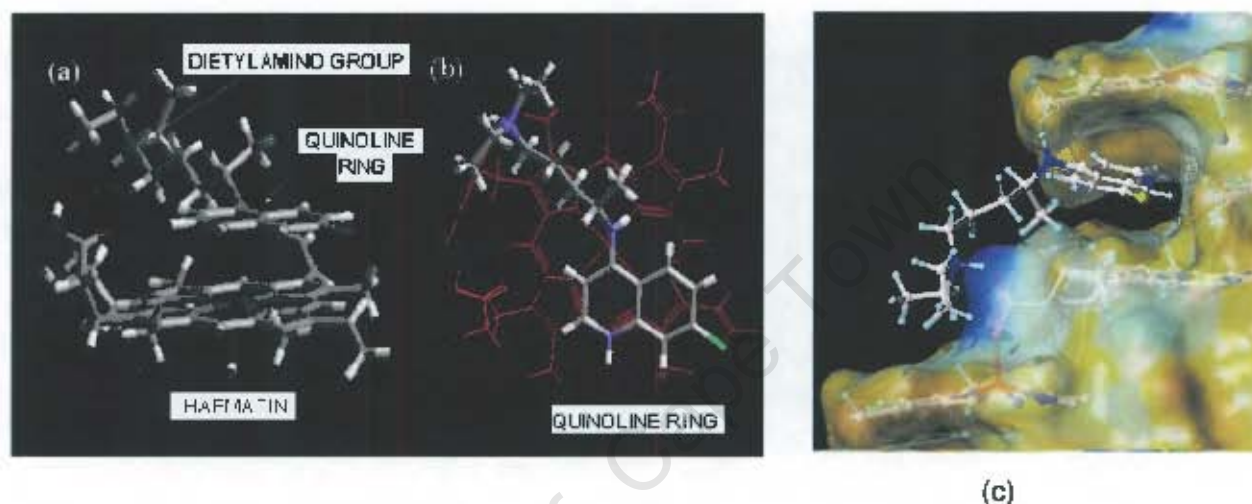


Figure 1.12: (a) Lowest energy complex of haematin (Fe(III)FPIX) (in vacuo) with CQ showing the π -stacking interaction between the porphyrin ring of haematin and the quinoline ring of CQ. The figure on the left is the side on view (a) with the top down view on the right (b) For panel b, all atoms of haematin are displayed in red for clarity,⁶⁴ (c) The mode of binding or interaction between haemozoin and CQ is illustrated with CQ sitting on the growing face of haemozoin.⁵²

1.4.2 Mechanism of Chloroquine Resistance

Resistant parasites accumulate markedly less CQ than their sensitive counterparts enabling them to survive at much higher concentrations of the drug.⁶⁵ Several studies have suggested that this could be due to an elevated vacuolar pH⁶⁶, leading to the reduced accumulation of CQ or an enhanced drug efflux from the infected cells.⁶⁷ CQ resistance can be reversed *in vitro*^{68, 69} and in animal models⁷⁰ by co-administration of compounds such as verapamil, desipramil and chlorpromazine. These agents can also be used to modulate drug resistance in human tumour cell lines, leading to the suggestion that the CQ-resistance phenotype may have a parallel with tumour multidrug resistance (MDR).⁷¹ MDR is thought to be due to the overexpression of an ATP-dependent drug effluxer known as P-glycoprotein.

P. falciparum was found to express a homologue of the human MDR protein, referred to as *P. falciparum* P-glycoprotein homolog-1 (PfPgh-1).⁷¹ Pgh-1 appears not to be the major effector of CQ resistance,⁷² however, particular alleles of Pfmdr1 modulate the level of resistance to CQ and the protein is involved in resistance to a related quinoline, mefloquine.⁷³

Recently, a protein referred to as the *P. falciparum* chloroquine resistance transporter (PfCRT) was identified as the major player in the phenomenon of CQ resistance. PfCRT is an integral protein with 10 predicted transmembrane domains and is located in the digestive vacuole membrane, i.e. in a suitable location to exert an effect on CQ accumulation. The precise role of this protein has not been delineated, however, it has been suggested that it may function as a transporter that removes CQ from its site of action.⁷⁴

PfCRT is predicted to be a member of the drug/metabolite transporter superfamily and may also be involved in the export of amino acids and/or peptides from the digestive vacuole. The mutant PfCRT allele expressed by CQ resistant parasites has fewer basic residues on the surface predicted to face the digestive vacuole lumen. This may allow entry of the positively charged species, such as CQ, thus facilitating exit of the drug down an electrochemical gradient.⁷⁴

Another recent hypothesis suggests that an increase in glutathione levels is met with increased resistance to CQ.⁷⁵ It was found that an increase in glutathione was accompanied by a corresponding increase in the activity of glutathione-S-transferase, an enzyme that catalyzes the synthesis of glutathione.⁷⁵

Other hypotheses suggested, include reduced Na⁺/H⁺ exchanger (NHE)⁷⁶, increase vascular pH⁷⁷ and reduced access to hemozoin.⁷⁸

1.5 Approaches to Antimalarial Chemotherapy

At present, multiple approaches to the identification of novel antimalarials are being pursued. Anti-infective drugs rely for their efficacy and specificity on their ability to interfere with aspects of metabolism that differ significantly from the human host. Many examples of different approaches and additional detail on some promising ones are discussed below.

1.5.1 Optimization of therapy with existing agents⁷⁹

Combination therapy, including newer agents (e.g. artemisinin derivatives, atovaquone) and new combinations of older agents (e.g. amodiaquine/sulfadoxine/pyrimethamine, chlorproguanil/dapsone), are under study as first-line therapies for Africa and other areas with widespread drug resistance. The use of combination antimalarial therapy offers two important potential advantages. First, the combination should improve antimalarial efficacy, providing additive or, ideally, synergistic antiparasitic activity. Second, the use of combination therapy should slow the progression of parasite resistance to the new agents.

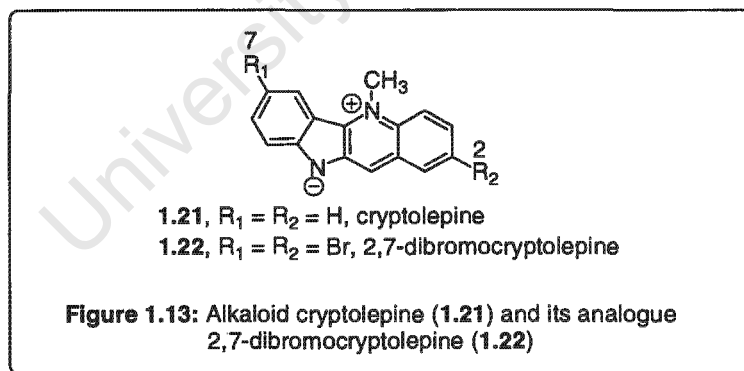
1.5.2 Development of analogs of existing agents

In this approach, existing antimalarials are improved by chemical modifications and do not require the knowledge of the mechanism of action or the biological target of the parent compound. For example, tafenoquine (an 8-aminoquinoline analog of primaquine) and new endoperoxides related to artemisinin.

1.5.3 Natural products⁸⁰

Plant-derived compounds offer a third approach to chemotherapy. Natural products are the source of two most important drugs currently available to treat severe *falciparum* malaria, i.e. quinine and derivatives of artemisinin. In the case of artemisinin, relatively simple chemical modifications of the natural parent molecule have led to a series of highly potent antimalarials.

Other potential natural product leads include the alkaloid cryptolepine (1.21, Fig. 1.13) a constituent of *Cryptolepis sanguinolenta*, which is a West African shrub and its analogue 2,7-dibromocryptolepine (1.22, Fig. 1.13). Although cryptolepine has potent *in vitro* antiplasmodial activity, it failed to cure malaria in mice when given orally and was toxic when given by the intraperitoneal (i.p.) route. On the other hand, its analogue 2,7-dibromocryptolepine is approximately nine-fold more potent than cryptolepine against chloroquine-resistant *Plasmodium falciparum* (strain K1) and when tested in mice infected with *Plasmodium berghei*, parasitaemia was suppressed by 90% with no apparent toxicity to mice.⁸⁰ Recent studies also suggest that the antimalarial mode of action of this and other cryptolepine analogues involves other mechanisms in addition to the inhibition of haemozoin formation.⁸¹



1.5.4 Compounds active against other diseases

A fourth approach to antimalarial chemotherapy is to identify agents that are developed or marketed as treatments for other diseases. The advantage of these compounds is

that whatever their mechanism, they have already been developed for a human indication and thus inexpensive to develop as antimalarials. Folate antagonists, tetracyclines and other antibiotics were developed for their antibacterial properties and were later found to be active against the malaria parasite.⁸²

1.5.5 Compounds active against new targets

In this approach it is important to identify novel drug targets and subsequently design chemical entities that are active on these targets. Progress towards the characterization of the biology of the malaria parasites has been stimulated by the development of technology to disrupt plasmodial genes and the sequencing and annotation of the *P. falciparum* genome. The readily accessible genome sequence posed the viability to assist in the drug discovery of novel targets for antimalarial therapy. Several target locations, target molecules and compounds active on these targets have already been identified. Of particular interest is the lysosomal food vacuole, the site of action for a number of existing antimalarials and also offers opportunities for therapies directed against new targets. The principal metabolic pathways in the food vacuole include haeme polymerization, hemoglobin degradation and free radical generation.

1.5.6 Drug Resistance reversers

Combining effective antimalarial agents with compounds that reverse parasite resistance offers another approach to chemotherapy. This was demonstrated in chloroquine (CQ)⁸³, in which its activity could be recovered by the use of a CQ resistance reverser, a compound which lowers the IC₅₀ value of CQ in CQ-resistant parasites to a value comparable to that of CQ-sensitive parasites. The substance is not itself active against the parasite at the concentration at which it restores activity and it does not alter activity against CQ-sensitive parasites. In a recent report, Bray *et al*,⁸⁴ have shown that primaquine (PQ) is a CQ-resistant reverser in *P. falciparum* *in vitro*. This study also involves the investigation of several analogues of PQ, for example tafenoquine (TQ), a 8-aminoquinoline, 8-amino-2-methylquinoline, a derivative of 8-amino-2-methylquinoline containing the aminoalkyl side chain of PQ and a carboxyprimaquine (CPQ), which is a

major metabolite of PQ. Many other drugs have been shown to reverse the resistance of *P. falciparum* to chloroquine *in vitro*, most notably the antihypertensive verapamil and the antidepressant desipramine as mentioned before. Compounds that appear to inhibit drug efflux can also be employed as an alternative strategy to modulate drug resistance or chemosensitize resistant parasites to conventional drugs.

1.6 Other Parasitic Protozoan Diseases

There is evidence that suggest that compounds found to be active antimalarials can also act against other protozoan parasites, for example *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania*.⁸⁴ Although the main focus of this thesis is on malaria, it is worth discussing these protozoan parasites and their associated parasitic diseases.

1.6.1 African Trypanosomiasis

Most of the African trypanosomes are transmitted to humans by the tsetse fly vector (*Glossina ps.*) and infects the central nervous system (CNS). The species that cause human African Trypanosomiasis, also known as 'sleeping sickness' are represented by two main subspecies of *trypanosoma*: *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. It has been estimated that 300 000 – 500 000 people are infected in Africa, mostly in the sub-Sahara regions. African Trypanosomes also infect wild and domestic animals (*Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei brucei*) responsible for 'nagana' in cattle which can be transmitted to humans from these animals. The only drugs to treat African Trypanosomiasis are melarsoprol, pentamidine (1.23, Fig. 1.14) and eflornithine. Melarsoprol is restricted in use due to toxicity and adverse side affects including chest pain, abdominal pain and phlebitis.

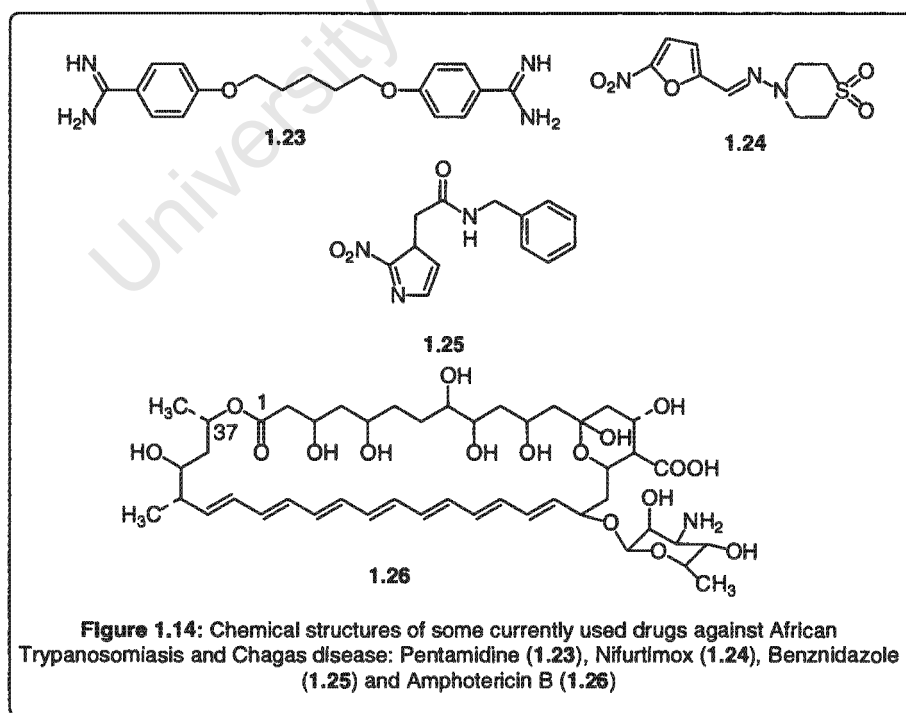
1.6.2 American Trypanosomiasis (Chagas disease)

Chagas disease is caused by the parasite of the species *Trypanosoma cruzi* (*T. cruzi*) and is found throughout most of Central and South America where conditions favour the

growth of reduviid insects, called kissing bugs. While biting the human host, the infected bugs deposit faeces on the skin surface. The protozoan parasite, *T. cruzi*, from the faeces of these bugs enters the human body through the bite wound or penetrates the mucous membranes of the eyes, nose or mouth. Chagas disease manifests itself in the acute (mild symptoms of fever and swelling of glands) and chronic forms. The chronic stage is the most fatal as it affects the heart muscle, oesophagus and lower intestine causing severe damage. Nifurtimox (1.24) and benznidazole (1.25) are effective against the acute stages of the disease and are inadequate to treat the chronic infections.

1.6.3 Leishmaniasis

Trypanosomatid flagellated protozoa of the genus *Leishmania* are the etiological agents of human leishmaniasis. *Leishmania* species are present world-wide in tropical and several temperate climate zones and infect about 12 million people annually.⁸⁵ The lifecycle of these protozoa involve a blood sucking sand fly as vector that transmits the parasite to vertebrate hosts. The disease commonly manifests either in a cutaneous (skin) form or in a visceral (internal organ) form. Presently, the principal medication for the disease is amphotericin B, (1.26, Fig 1.14).



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CHAPTER 2

PARASITIC CYSTEINE PROTEASES AND THEIR INHIBITORS

2.1 Introduction

Proteases, also referred to as peptidase or proteinases, are enzymes that catalyze the hydrolysis of amide bonds linking amino acids in peptides and proteins. Approximately 2% of the genes in all kinds of organisms encode proteases.¹ These enzymes are involved in a number of essential processes in both humans and pathogens, e. g. in replication or growth, cell differentiation, signaling and host invasion, making them attractive as drug targets.²

Proteases can be categorized based on their substrate specificities or mechanism of catalysis. Enzymes cleaving within a polypeptide chain are named endopeptidases, and those cleaving at the ends of polypeptides are named exopeptidases. Aminopeptidases cleave at the N-terminus and carboxypeptidases cleave at the C-terminus. Proteases have been divided into groups on the basis of the catalytic mechanism used during the hydrolytic process. The main catalytic types are serine, threonine, aspartic, metallo and cysteine proteases. Structural homology further distinguishes each subclass into families and similar families into clans.²

Interest in cysteine proteases as targets, derives from the recognition that they are critical to the life cycle or pathogenicity of many parasites. This functional diversity is in turn derived from their unique nucleophilicity, adaptability to different substrates, and stability in different biological environments.

Cysteine proteases exist in a range of organisms including, plants, viruses, parasitic protozoa, mammals, fungi and bacteria. Of particular interest to the work described in this dissertation are the cysteine proteases (falcipains) found in the malaria parasite *Plasmodium falciparum*.

2.1.1 Parasitic Cysteine Protease Nomenclature

Parasitic cysteine proteases contain two major amino acid residues, Cys 25 and His159 that are actively involved in the hydrolysis of peptide bonds. The first cysteine protease was purified and characterized in 1879, from the *Carica papaya*, the papaya fruit and was thus named papain.³ Numerous proteases that have sequences in common with papain have been called 'papain-like'. Parasitic cysteine proteases are divided into two main groups referred to as clans, CA and CD.⁴ The clans are sub-divided into families based on sequence identities and similarities. Clan CA super family contains the majority of cysteine proteases in which the catalytic residues, (Cys 25 and His 159) are highly conserved among all the members. Clan CA, papain-family cysteine proteases are well characterized for many eukaryotic organisms⁵ and these are amongst the best characterized cysteine proteases of *Plasmodium*. In *P. falciparum*, analysis of the genome sequence suggests that clan CA cysteine proteases include as many as four falcipains, three dipeptidyl peptidases, nine proteins related to the serine-rich antigen (SERA) and a calpain homolog.⁵ Clan CD proteases include caspases in higher organisms, and sequence analyses suggest that members of the C13 and C14 families are present in plasmodia.

2.1.1.1 Protease Binding and Classification of Binding Pockets

Prior to hydrolysis of peptide bonds, a protease must bind the protein or peptide substrate in its active site. The binding efficiency is a function of both the respective chemical environments that the protease subsites create, and the chemical nature of the peptide that interacts directly with the active site groove. Important factors affecting interactions include; size, polarity, charge, hydrophobicity and accessibility. Although a single peptide bond is cleaved during catalysis, a number of amino acids on either side of the site of cleavage are critical to fix the register and specificity of an enzyme. Sequences that directly flank the active site cysteine and histidine are also highly conserved to maintain catalytic register.³

Schechter and Berger² have established a nomenclature to designate substrate residues and the corresponding enzyme subsites based on their position relative to the scissile peptide bond as shown in Fig. 2.1, (a), (b). Amino acid residues from the peptide are denoted by 'P' and the subsites that the peptide interact with is given the letter 'S'. The active site cysteine sulfhydryl nucleophile is represented as -SH, and the corresponding peptide bond that is attacked prior to hydrolysis (scissile bond) is labeled. The carboxyl side of the peptide and corresponding subsites are conventionally referred to as the *primed side* and termed P'₁, P'₂, P'₃, P'_n and S'₁, S'₂, S'₃, S'_n, respectively. The amino side of the peptide and corresponding subsites are assigned the *non-prime side* and are designated P₁, P₂, P₃, P_n and S₁, S₂, S₃, S_n, respectively. Cysteine proteases require an essential cysteine residue in the active site for hydrolysis. The thiol-group is enhanced as a nucleophile due to the close proximity of an active site histidine which acts as a proton donor/general base. The sulfhydryl or '-SH' group of the cysteine side chain and the imidazole of histidine give rise to a thiolate-imidazolium charge relay diad, as depicted in Fig. 2.1 (c).³

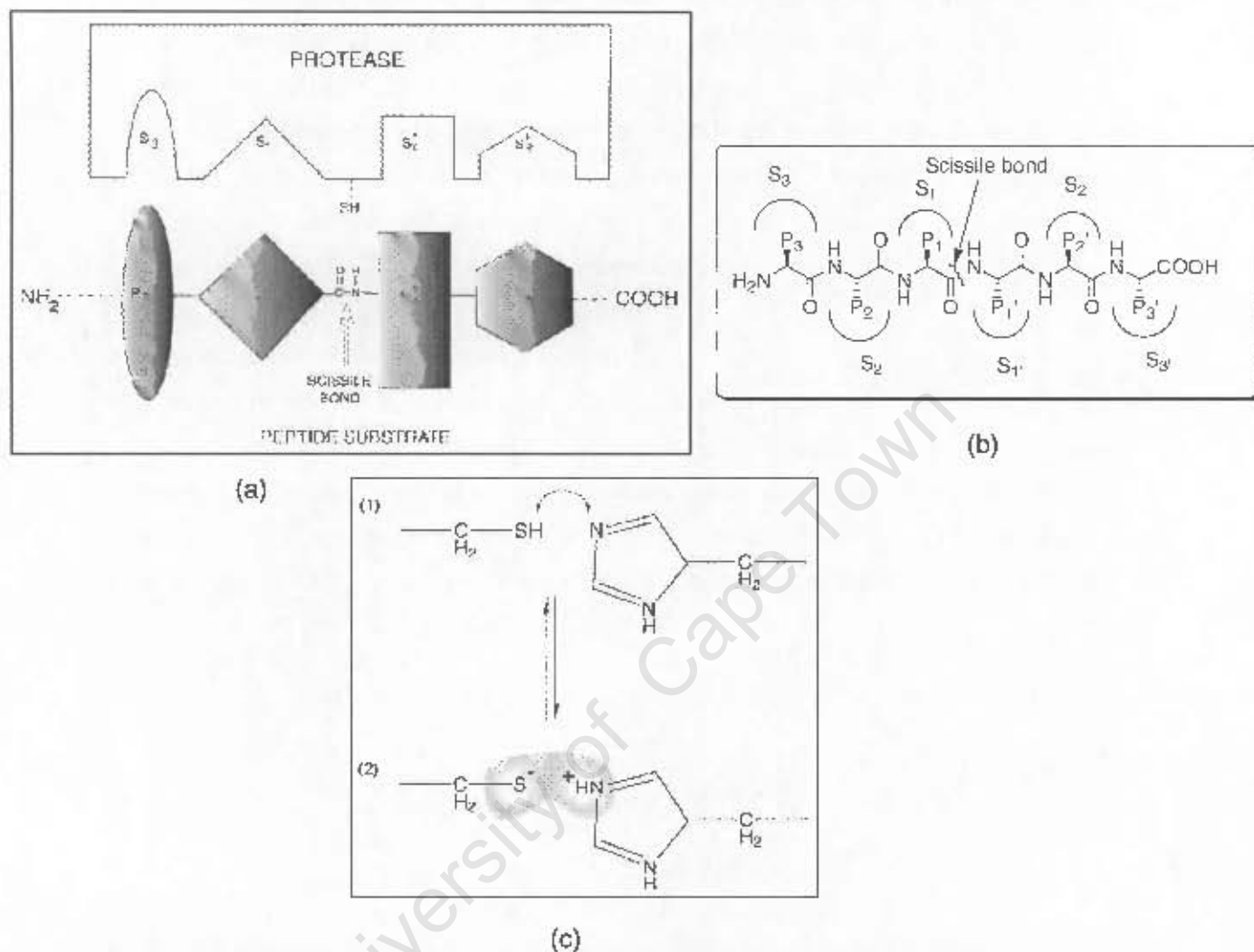
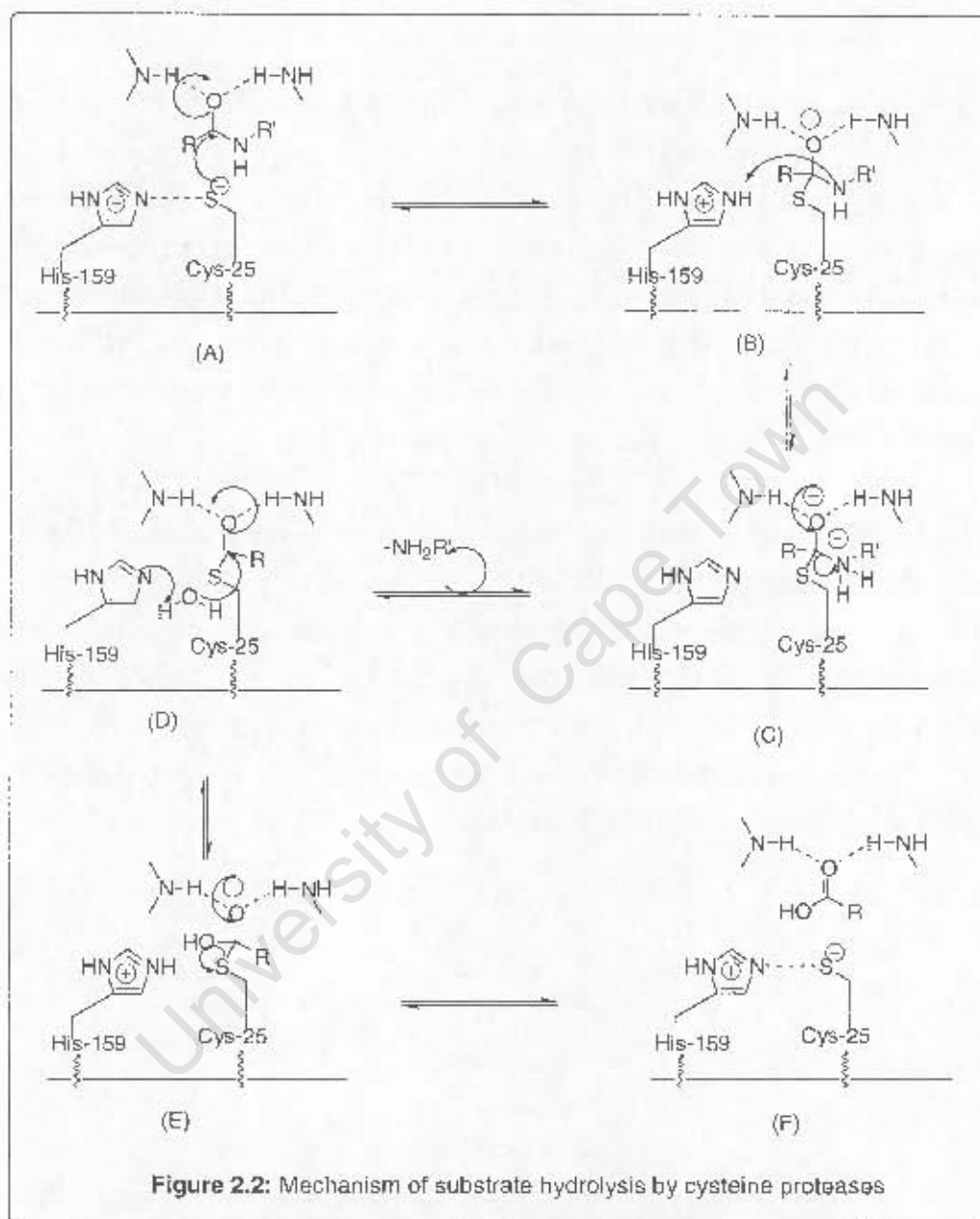


Figure 2.1: (a) Diagrammatic representation of peptide substrate interaction with the active site pockets of the cysteine protease, (b) Schechter and Berger's nomenclature⁶ for substrate residues and corresponding binding sites, (c) Schematic presentation of the close spatial proximity in the active site showing: (1) the sulfhydryl of cysteine to the imidazole group of histidine, and the equilibrium with (2) the thiolate-imidazolium charge relay diad. The shaded area represents a delocalised electron dense cloud.

2.1.1.2 Mechanism of Proteolysis

A clear understanding of the mechanism of the enzyme can assist in the rational design and development of novel potent inhibitors. The proposed mechanism of substrate hydrolysis by papain-like cysteine proteases is depicted in Fig. 2.2, in which an active site cysteine thiol is involved during hydrolysis. His-159 in close proximity polarizes the enzyme cysteine thiol group allowing deprotonation to occur even at near neutral pH to produce a highly nucleophilic thiolate/imidazolium ion pair.⁷⁻⁹ (Fig. 2.2A). This highly nucleophilic thiolate cysteine attacks the carbonyl carbon of the scissile bond of the bound substrate and forms a tetrahedral intermediate (Fig. 2.2B).

Stabilization of this transition state oxy-anion occurs by H bonding to the N-H backbone of Cys-25 and to the NH₂ group of the Gln-19. The tetrahedral intermediate transforms into an acyl enzyme (enzyme-substrate thiol ester) with the simultaneous release of the C-terminal portion of the substrate (acylation) (Fig. 2. 2C). This step is followed by the hydrolysis of the acyl enzyme with water, forming a second intermediate (Fig. 2.2D). Finally the second tetrahedral intermediate collapses (Fig. 2.2E) into free enzyme and the N-terminal portion of the substrate (deacylation) (Fig. 2.2F).



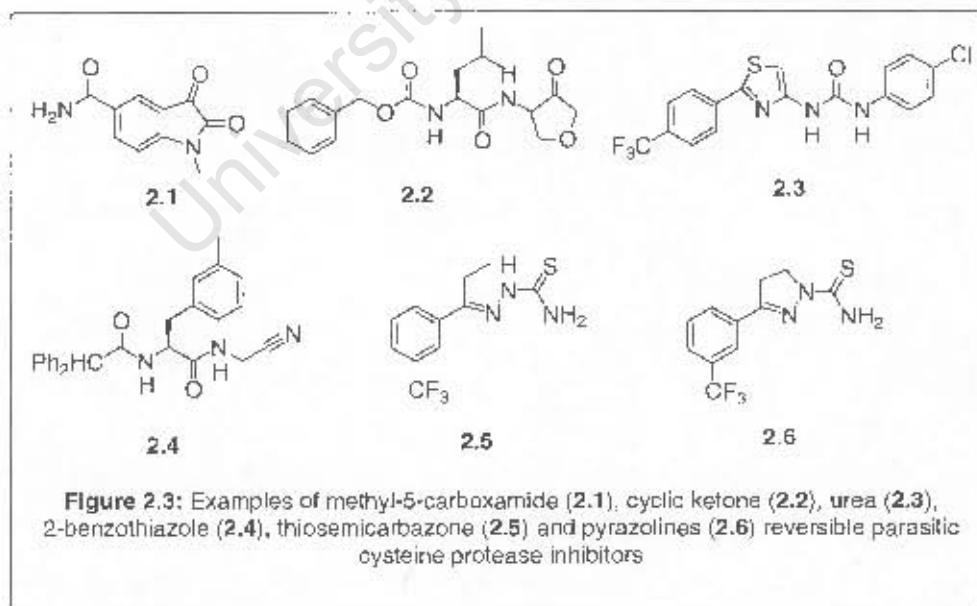
2.2 Inhibition Strategies of Cysteine Proteases (CP's)

Recently, the development in CP inhibitors has significantly increased, driven by the advances in knowledge, computer-aided design and the use combinatorial syntheses.

Most of the protease inhibitors that have been developed comprise a peptide segment for recognition by the enzyme, corresponding to the sequence of a good substrate. It contains two or more amino acids to achieve good affinity, at least for endopeptidases. The peptide segment bound to an electrophilic group which can react with the protease active site cysteine residue. Inhibitors can be directed towards the active site or elsewhere on the enzyme (allosteric inhibitors).¹⁰ A variety of CP inhibitors have been developed which may be broadly categorized into three mechanistically distinct groups which include reversible, irreversible and slow-turnover inhibitors.^{11, 12}

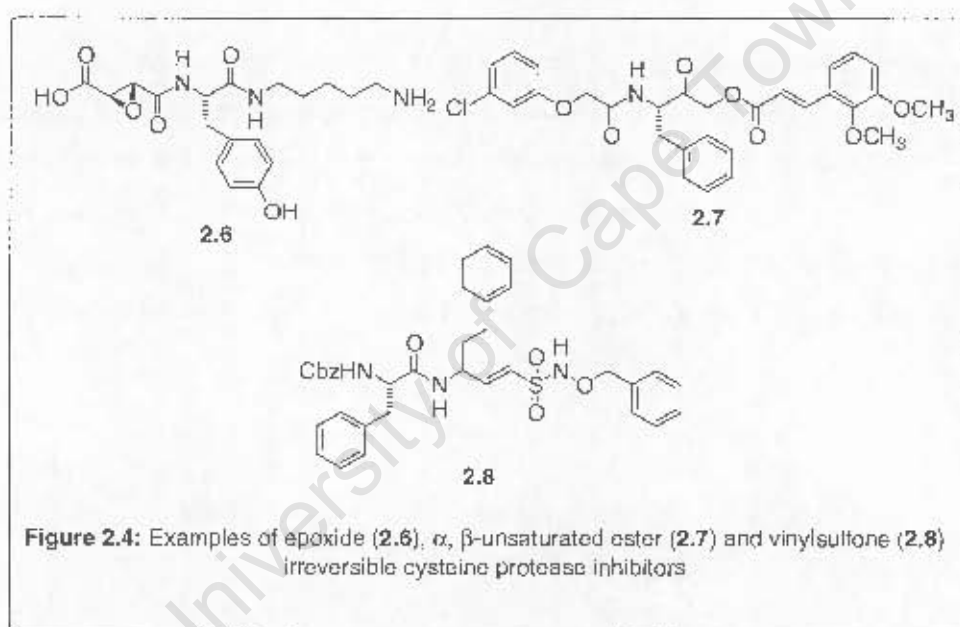
2.2.1 Reversible Inhibitors

The first inhibitor class relies on the formation of non-covalent or covalent¹¹, yet reversible, interaction between the active site of the cysteine protease and the inhibitor. They form weak bonds and do not permanently disable the enzyme. Potent reversible inhibitors have been designed based on their highly electrophilic warheads.^{13, 14} Methylisatin-5-carboxamide (2.1)¹⁵, cyclic ketones (2.2)¹⁶, urea (2.3)¹⁷, 2-benzothiazole (2.4),¹⁸ thiosemicarbazones (2.5) and pyrazolines (2.6)¹⁹ serve as examples of reversible inhibitors.



2.2.2 Irreversible Inhibitors

The second class of inhibitors binds to the enzyme covalently¹¹. This type of inhibitor binds to the active site of the enzyme with a high affinity causing the enzyme-inhibitor to dissociate very slowly, whilst inactivating its catalytic activities. These types of inhibitor stay in the active site for a longer time and disable the enzyme permanently. Examples include epoxide (2.6)²⁰, α , β -unsaturated ester and vinyl sulfone Michael acceptors (2.7)²¹ and (2.8) respectively.



2.2.3 Slow Turnover Inhibitors

The third class is a series of inhibitors that form a stable thioacyl-enzyme complex with the active site cysteine that is slow to hydrolyze. Aza-substituted peptides, such as peptidyl carbamate ester (2.9)²² and diacyl hydrazine derivative (2.10)²³ are the principle examples of this class of inhibitors, (Fig. 2.5).

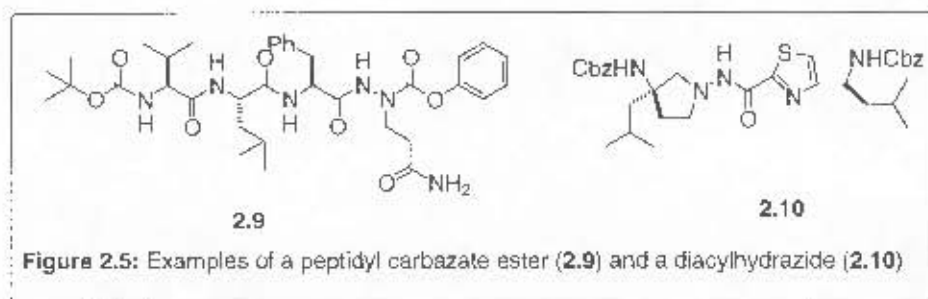


Figure 2.5: Examples of a peptidyl carbazate ester (2.9) and a diacylhydrazide (2.10)

2.3 The Role of Falcipains in Haemoglobin Degradation

2.3.1 Utilization of Host Amino Acids by the Parasite

Given that the malaria parasite has only a limited capacity to synthesize amino acids *de novo* or scavenge them exogenously, the parasite must catabolise them from the host. As haemoglobin is processed, its haeme component is converted into haemozoin pigment, and globin is hydrolyzed to its constituent amino acids. Haemoglobin degradation is necessary to provide amino acids for protein synthesis, to maintain osmotic stability of malaria parasites and to provide space for the growing intraerythrocytic parasite. Haemoglobin constitutes more than 90% of the total protein in a red cell. At 20% parasitaemia, during the trophozoite stage, more than 60% of the host haemoglobin in a red cell is digested by the parasite.²⁴

The degradation of haemoglobin occurs within a specialized acidic lysosomal organelle called the digestive food vacuole (pH 4.5 – 5.2).²⁵ The food vacuole is one of the essential organelles of the parasite, hosting important biochemical processes on which parasite survival is dependent. These important processes include acidification, haemoglobin degradation, peptide transport, haeme polymerization and quinoline action. Haemoglobin is taken up by simple endocytosis of the host cell during the ring stage of the parasite, with the help of specialized organelles known as cytostomes during the trophozoite stage. The endocytotic vesicles empty their contents into the acidic food vacuole of the parasite.

2.3.2 The Haemoglobin Degradation Pathway

2.3.2.1 Classification of Proteases Participating in the Haemoglobin Degradation Pathway

Haemoglobin degradation appears to be the result of a cooperative process involving proteases of multiple catalytic classes comprising food vacuole aspartic (plasmepsins),²⁶ cysteine (falcipains)^{27, 28} and metalloprotease (falcilysin)²⁹ and at least one cytosolic metalloaminopeptidase.³⁰ Biochemical studies and the availability of the full *P. falciparum* genome sequence have identified additional members of these families. Four plasmepsins³¹ were proposed to be involved in the onset of haemoglobin hydrolysis which include, plasmepsins (PM's) I, II, IV and histo-aspartic protease (HAP).³² HAP and PM IV are homologous to PM I and PM II.³³ HAP is 60% identical to PM I and II, but has several substitutions, including replacement of a catalytic aspartate with a histidine and changes in a conserved flap region that lies over the binding cleft.³⁴ Three papain-family cysteine proteases, falcipain1, -2 and -3 were also identified. Expanded analysis of the completed *P. falciparum* genome revealed a fourth member of the falcipains family,³⁵ which is 96% identical at the nucleotide level and 93.4% identical at the amino acid level to falcipain-2. The original gene (PF11-0165) is referred to as falcipain 2A, and upstream homologue (PF11-0161) is referred to as falcipain 2B. Recently another gene homologous to falcipain-2, falcipain-2' has been reported.³⁶ It was shown that falcipain-2 and falcipain-2' (differing by only 3 amino acids) is much more similar in sequence compared to falcipain-2 and falcipain-3.

2.3.2.2 Functions of proteases participating in the Haemoglobin Degradation Pathway

Haemoglobin degradation is initiated by the action of two homologous aspartic proteases, plasmepsins I and II which make an initial cleavage between residue α 33 Phe and α 34 Leu amino acids. This cleavage destabilizes the globin molecule, making it susceptible to further enzymatic action.³⁷ PM I prefers phenylalanine in the P1 position, while PM II prefers hydrophobic residues, particularly leucine in the P1' position.

Inhibition studies have shown that blocking PM I is lethal to the parasite and that PM II cannot compensate for the loss of PM I activity. Evidence suggests that PM IV and HAP act later in the haemoglobin pathway. PM IV and HAP prefers to cleave denatured globin rather than native haemoglobin at a site known to be the essential primary cleavage site in the haemoglobin degradation pathway, (Fig. 2.6). However, more recent results have demonstrated that haemoglobin-degrading plasmepsins are not promising targets, since minimal effect on parasite growth was observed when plasmepsin was treated with a potent inhibitor, pepstain A.³⁸

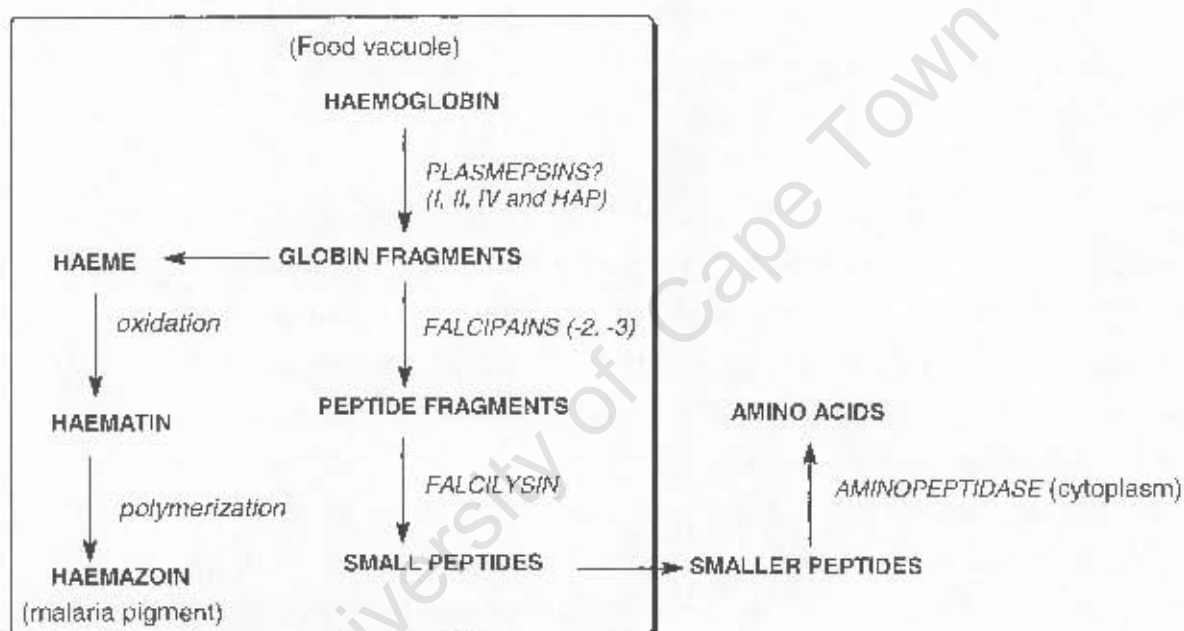


Figure 2.6: The haemoglobin degradation pathway

The globin fragments produced by the action of the plasmepsins are substrates for the cysteine proteases, falcipains -2 and -3. Falcipain-2 and -3 are the two major cysteine proteases active during haemoglobin degradation and are expressed sequentially by erythrocytic parasites. Both proteases require a reducing environment and acidic pH for optimal activity, and both prefer peptide substrate with leucine at the P (2) position.²⁴ These enzymes are similar in sequence (68% identity) and in substrate specificity, but differ markedly in activity, such that falcipains -2 has a much greater activity against a

range of peptide substrates.³⁹ Falcipain-2 cleaves denatured haemoglobin and falcipain-3 undergoes efficient processing to an active form only at acidic pH, thus it is more active and stable at acidic pH and has much lower specificity activity against peptide substrates, but has a greater activity against native haemoglobin.⁴⁰ Both falcipain-2 and falcipain-3 contain amino-terminal extensions that mediate folding of the mature proteases independent of the prodomain^{41, 42} and an insertion near the carboxy terminus that mediates interaction with haemoglobin.⁴⁰ Recently discovered falcipain-2' has similar biochemical properties to those of falcipains-2 (99% homology), including pH optima (pH 5.5-7.0), reducing requirements; substrate preference and it also readily hydrolyzes haemoglobin. Evaluation with cysteine protease inhibitors showed similar inhibition of falcipain-2 and falcipain-2', suggesting that falcipain-2' is also an active haemoglobinase,³⁸(Fig. 2.6).

The resulting globin peptides serve as substrates for the zinc metalloprotease, falcilysin^{43, 44} (FLN) also found in the digestive vacuole.^{45, 46} Falcilysin appears to function downstream of the family of aspartic proteases, the plasmepsins and cysteine proteases, falcipains-2 and -3 in the haemoglobin proteolytic pathway, (Fig. 2.6). FLN prefers to degrade small globin peptides up to 20 amino acids in length at a pH optimum of 5.2²⁹ consistent with the acidic environment of the food vacuole.^{47, 31} Falcilysin cleaves sites along the α - and β - chains of haemoglobin and are polar in character, with charged residues located in the P1 and/or P4' positions.⁴⁸ In contrast, the plasmepsins I, II, falcipains-2 and -3 prefer hydrophobic residues around the scissile bond. It was also demonstrated that the enzyme is robustly active at neutral pH, but with a substantially different substrate specificity. Strongly supporting this hypothesis is imaging studies which suggest that falcilysin may have an extended role beyond globin catabolism and may function as two different proteases in two locations.⁴³

2.4 Additional Functions of Cysteine proteases Determined from Inhibitor Studies

The timing of effects of cysteine protease inhibitors provides clues regarding protease functions. In a detailed characterization of effects of leupeptin and E-64 against cultured *P. falciparum* parasites, both inhibitors were maximally active against mature

trophozoites and schizonts,⁴⁹ suggesting a critical role for cysteine proteases in trophozoites hemoglobin, as discussed above, but also suggesting that additional cysteine protease functions may be important and also targets for inhibitor effects. Some additional functions of cysteine proteases are discussed below.

2.4.1 Erythrocyte Rupture

Cysteine protease inhibitors have been shown to inhibit the rupture of erythrocytes that accompanies the completion of the erythrocytic cycle. Cysteine protease activity therefore appears to be required for the release of merozoites, which rapidly invade other erythrocytes to reinitiate the asexual cycle. Previous studies showed the accumulation of mature schizonts in cultures treated with leupeptin.⁵⁰ A further analysis of this study suggested that in mature schizont-stage parasites, E-64 blocked the lysis of the parasitophorous vacuole membrane, which surrounds the intraerythrocytic parasite.⁵¹ This result suggested that cysteine protease activity is required for the hydrolysis of parasitophorous vacuole membrane-associated proteins to mediate merozoite release. This idea is strongly supported by data emanating from a recent study that suggest that a cysteine protease, viz., falcipain-2, may play a role in merozoite release (egression).⁵²

2.4.2 Erythrocytic Invasion

Most reports have not identified an effect of cysteine protease inhibitors on the invasion of erythrocytes by merozoites.⁵³ This process has generally been shown to be inhibited by serine protease inhibitors such as chymostatin.⁵³ It was proposed by Greenbaum *et al.*⁵⁴ that falcipain-1 is involved in host cell invasion, but further investigation has demonstrated that this may not be the case.⁵⁵ It still remains unclear whether cysteine proteases are required for erythrocyte invasion.

2.4.3 Non-erythrocytic Parasite Stages

During gametogenesis, hydrolysis of the gametocyte surface protein Pfs230 generates two polypeptides which remain associated with the newly formed gamete.⁵⁶ The cleavage of Pfs230 is blocked by the membrane-permanent cysteine protease inhibitor E-64, suggesting that a cysteine protease is responsible for this cleavage, but relevant enzymes have not been identified.⁵⁷ Recently, the inhibition of falcipain-1 was shown to lead to markedly decreased oocyst production, proposing a specific role for this protease in sexual-stage parasites.⁵⁸

2.5 Potential for Cysteine protease Inhibitors as Antimalarial Drugs

Cysteine protease inhibitors arrest the erythrocytic life cycle of *P. falciparum*.^{59,60} Examination of inhibitor-treated parasites revealed abnormally swollen food vacuoles filled with undigested haemoglobin, indicating that the block in parasite development was due to the inhibition of haemoglobin hydrolysis.⁶⁰ Numerous peptide-based cysteine protease inhibitors, including fluoromethyl ketones,^{61(a), (b), (c)} vinyl sulfones,^{59, 62,} and aldehydes⁶³ inhibited falcipain activities and blocked the development of cultured parasites at nanomolar concentrations. Some non-peptide falcipain inhibitors showed more modest antiparasitic activity.⁶⁴ In nearly all cases with potent inhibitors, the inhibition of parasite development was accompanied by a specific block in haemoglobin hydrolysis, marked by the appearance of swollen, dark-staining food vacuoles. More recent analysis has shown that antiparasitic effects correlate with potent inhibition of falcipain-2 and falcipain-3. In an *in vivo* study utilizing a murine malaria model, a fluoromethyl ketone cysteine protease inhibitor cured 80% of *Plasmodium vinckei-infected mice* at high and frequent dosages.^{61(b)} Similarly, an orally administered vinyl sulfone cysteine protease inhibitor cured 40% of *Plasmodium vinckei-infected mice*, treated twice-a-day for four days.^{62(a)} Inhibitors of cysteine and aspartic proteases showed synergistic antimalarial effects both *in vitro*^{65(a), (b)} and *in vivo*,^{65(b)} suggesting the possibility of combined protease inhibitor antimalarial therapy.

Concerning the potential for resistance to antimalarial cysteine protease inhibitors, a

study described the selection of parasites resistant to a vinyl sulfone falcipain inhibitor, but the selection was slow, and the mechanism was complex.⁶⁵ This result might indicate that resistance to antimalarial cysteine protease inhibitors will be slow to develop, and that the best means of avoiding resistance will likely be the use of combination antimalarial therapy.

From previous studies, it is clear that potent falcipain inhibitors may serve as promising therapeutics and thus form the basis for future drug discovery efforts. Ultimately, a better understanding of the biochemical properties and biological roles of malaria proteases will foster the development of protease inhibitors that specifically inhibit parasite enzymes and thus are the most suitable candidates for chemotherapy.

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CHAPTER 3

DUAL DRUGS

3.1 The 'Dual Drug Approach'

In the 'Dual Drug Approach' active molecules with independent modes of action are joined together, *via* a covalent linker, (Fig. 3.1).

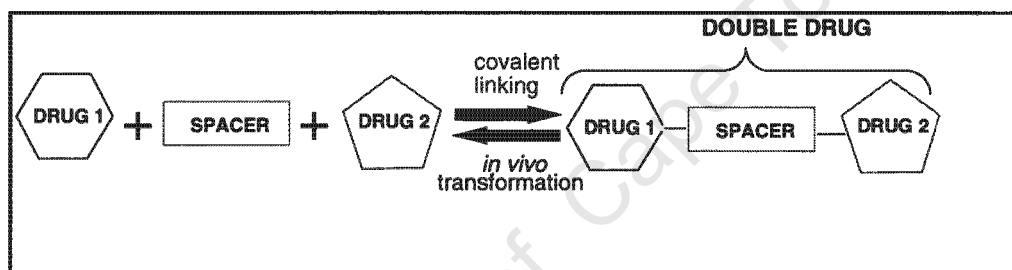


Figure 3.1: Diagrammatic representation of The Dual (Double Drug) Concept

This approach is exemplified by cysteine protease and haemozoin formation inhibitors, HIV protease inhibitors, primaquine-containing plasmepsin inhibitors, phosphatidylcholine/haeme inhibitors and quinoline-containing zinc metallo-aminopeptidase inhibitors. In these examples discussed below researchers employed this double drug approach to enhance the activity of the former drug by linking it to a second drug with known activity.

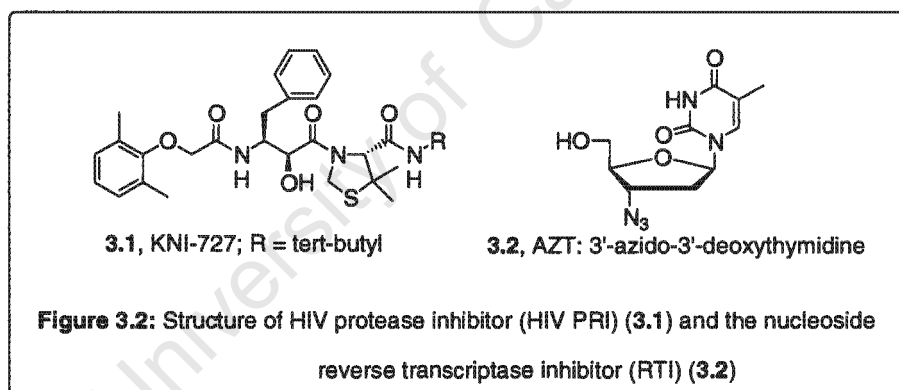
3.2 Examples of the 'Double Drug Approach'

3.2.1 HIV Protease Inhibitors

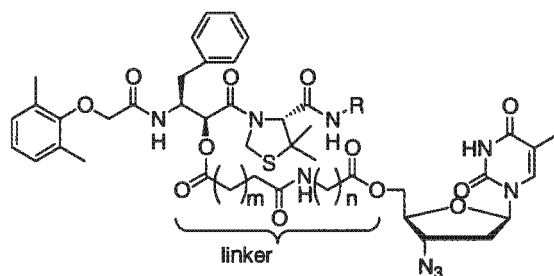
The 'double-drug' approach has been successfully used to improve poor membrane permeation of some HIV protease inhibitors.⁵ HIV protease (HIV PR) and reverse transcriptase (RT) are major targets for the prevention of HIV proliferation, and inhibitors of these enzymes are widely employed for the chemotherapy of AIDS.⁶⁻⁸ Billelo *et al.*⁹

have reported that the antiviral efficacy of HIV PR inhibitors depends not only on the enzyme inhibitor activity, but also on their intracellular concentrations closely related to the membrane permeability.¹⁰ To improve antiviral activity Matsumoto and coworkers suggested a 'double-drug' strategy that combined an HIV PR inhibitor and a nucleoside RT inhibitor within a single molecule.¹¹⁻¹³ In their work they applied the 'double-drug' concept to the dipeptide HIV PR inhibitor KNI-727 (3.1),¹⁴⁻¹⁶ which is devoid of a carbonyl group.

Although Inhibitor (3.1) exhibited excellent HIV PR inhibitory activity it showed poor anti-HIV activity. It was speculated that this might be due to its poor water solubility and high lipophilic nature which would restrict penetration across the cell membrane. To enhance the anti-HIV activity and improve the physicochemical characteristics of inhibitor (3.1), i.e. the KNI-727 (3.1) a strategy was developed to link it to AZT (3.2), (Fig. 3.2).

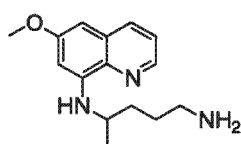


Both HIV PR inhibitor (3.1) and nucleoside RT inhibitor AZT (3.2) contain hydroxyl groups essential for enzyme inhibitory activity,¹³ and therefore both of their hydroxyl groups were connected through a cleavable linker (i.e. enzymatically labile ester groups, presumably subject to intracellular hydrolysis by target cell acylases), (3.3, Fig. 3.3).

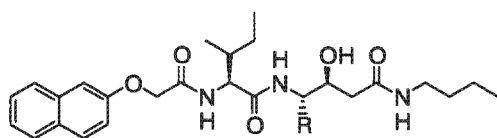
3.3, $m = 1$, $n = 1$, $R = \text{tert-butyl}$ **Figure 3.3:** Structure of double-drugs containing a spontaneously cleavable linker

3.2.2 Antiplasmodial Primaquine-Statine 'Double Drugs'

Plasmeprin II (PLM II) is one of the aspartic proteases involved in the degradation of haemoglobin during the intraerythrocytic cycle of *Plasmodium falciparum*.¹⁷ In an effort aimed at improving the inhibitory activity of PLM II inhibitors, including those with a statine-based core,¹⁸ Romeo *et al.*¹⁹ reported the synthesis of PLM II and *Plasmodium falciparum in vitro* inhibitors based on the 'double drug' approach, in which a statine-based inhibitor of PLM II was linked to the well known antimalarial, Primaquine (PQ). Several statine-based inhibitors for example, PS777621(3.4) and PS189863 (3.5), (Fig. 3.4) have been developed, which showed inhibition of PLM II at low micromolar concentrations.²⁰ The toxicity of PQ initially limited its use, and was later reevaluated as a safe and efficient agent.²¹

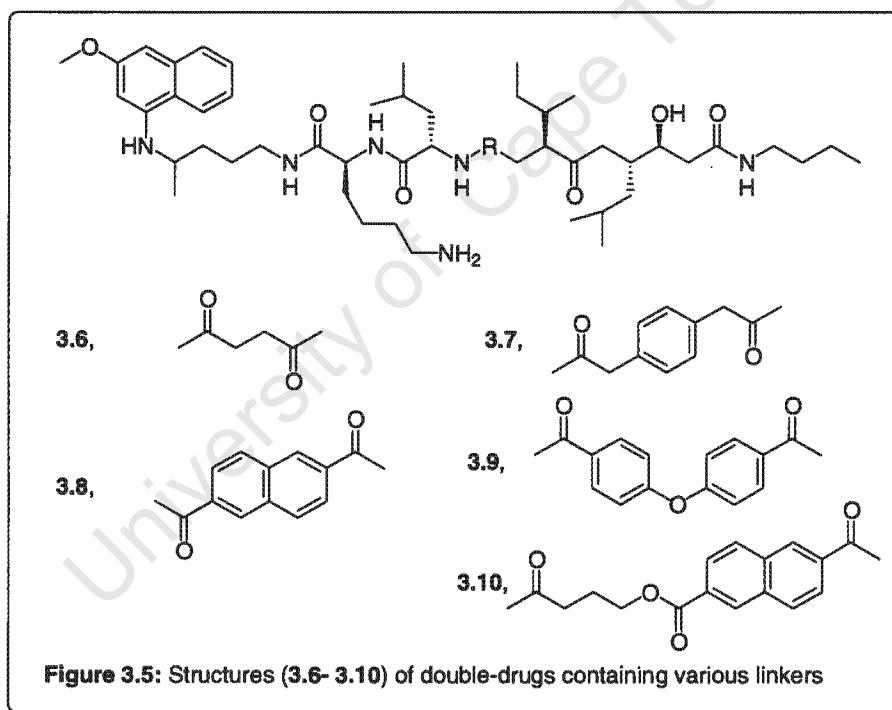


Primaquine (PQ)

3.4, PS 777621; $R = \text{i-Butyl}$; $K_i = 180\text{nM}$ (Plasmeprin II)3.5, PS 189863; $R = \text{Benzyl}$; $K_i = 110\text{nM}$ (Plasmeprin II)**Figure 3.4:** Chemical structures of Primaquine and Plasmeprin inhibitors (3.4 and 3.5)

It was shown that the toxicity of PQ is diminished and the activity increased by the conversion of PQ to peptide prodrugs.²² It was reasoned that the linkage of the peptide derivative HLeuLysPQ to the Ile residue of PS777621 (3.4) would provide a potential 'double-drug'.

Compounds (3.6-3.10, Fig. 3.5) were thus synthesized that incorporate linkers derived from diketones that should mimic the P₃ residue (P₃ refer to the amino side of the peptide that binds to the corresponding S₃ subsite) of PS777621 (3.4). The IC₅₀ values for PLM II inhibition ranged from 0.59 to 400 nM and the best IC₅₀ value for inhibition of *Plasmodium falciparum* growth *in vitro* was 0.4 μ M, for compound (3.9), which represents a remarkable improvement compared to other statine-based PLM II inhibitors.



3.2.3 Phosphatidyl-Choline/Haeme Dual Inhibitors

The efficiency of the 'double drug' concept was further demonstrated by studies done by Salom-Roig *et al.*²³ who designed and synthesized three generations of compounds exemplified by, bisquaternary ammonium salt, (3.11), bis-amino-imine, (3.12) and bis-thiazolium salt, (3.13), (Fig. 3.6).

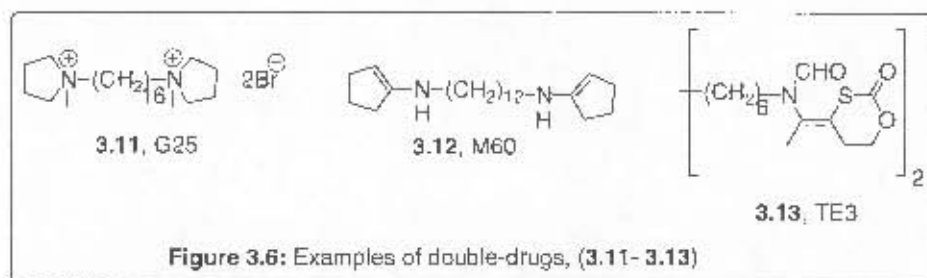
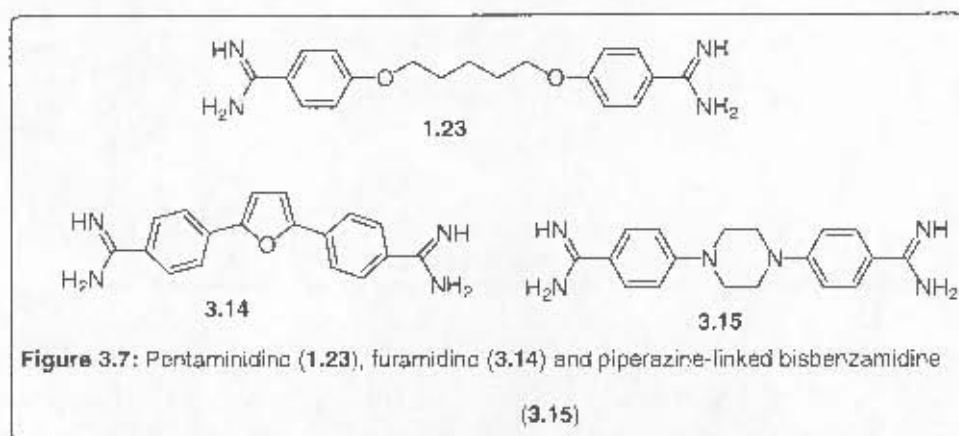


Figure 3.6: Examples of double-drugs, (3.11- 3.13)

These dual compounds were found to target two mechanisms: (i) they interact with the plasmodial phospholipid (PL) metabolism and also with (ii) malarial pigment. The first target was selected based on the synthesis of substantial quantities of membrane through the phospholipid (PL) mechanism^{24, 25} by the malarial parasite during the asexual development within the erythrocytes. The dual drugs (3.11-3.13) target the *de novo* phosphatidylcholine biogenesis necessary for membrane synthesis in the erythrocytic stage of the parasite, which is crucial for development and growth.²⁶ The second target can also be found in the erythrocytic stage of the parasite, where the free haeme produced from haemoglobin digestion is rapidly oxidized into ferriprotoporphyrin IX (FPIX), which is toxic for the parasite. As stated earlier, detoxification of haeme occurs in the acidic food vacuole of the parasite *via* formation of a polymer called haemozoin. Bis-quaternary ammonium compounds have shown that in addition to their selective inhibition of *de novo* phosphatidylcholine biosynthesis, their potent antimalarial activity could be attributed to their compartmentalization into the parasite's food vacuole, where they would bind to ferriprotoporphyrin IX blocking the formation of malaria pigment (haemozoin).²⁷

3.2.3.1 Proposed Mechanism for Three Generation drugs

Pentamidine (1.23) and pentamidine-like compounds (3.14) and (3.15) have been demonstrated to exhibit potent antimalarial activities, (Fig. 3.7).^{28, 29, 30}



These types of compounds possess a common framework with the three generation drugs. They are constituted by two heads, each with two different pharmacophore groups, (Fig 3.8): an ammonium group and a π -electron rich site (benzamidine or a thiazolium cycle). Both of active heads are separated by a linker.

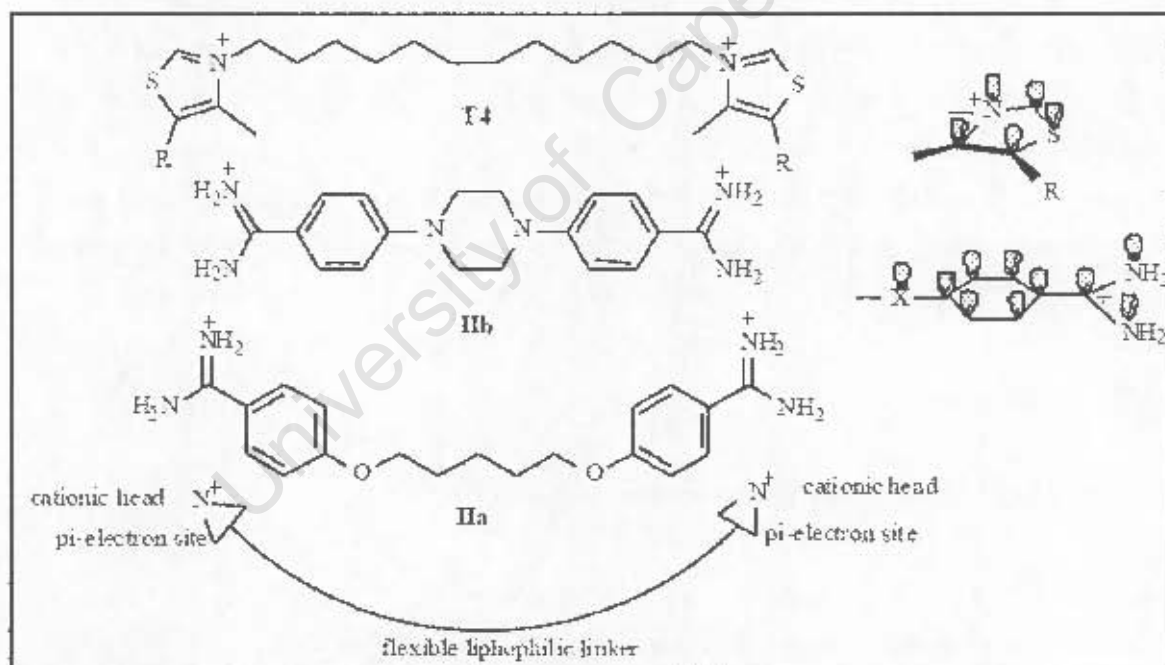


Figure 3.8: Common framework between pentaminidino-like compounds and our drugs

Previous studies have shown that modifications to the linker by introducing an aromatic ring and heteroatoms, resulted in a decrease in antimalarial activity.^{31, 32} The three generation compounds^{33, 34} like pentaminidino biscations³⁵ bind to ferriprotoporphyrin IX

(FPIX). Ferriprotoporphyrin IX possesses two propionate side chains. Linker flexibility in bis cationic drugs could allow the formation of a kind of sandwich-type structure 1:1 (a) or 2:1 (b) haeme-drug³⁶ which would block FPIX unities avoiding their polymerization, (Fig. 3.9). Thus, the mechanism of the interaction of antimalarial bis cationic agents with FPIX could involve an ionic interaction between the positive charged ammonium heads of the drug and the haeme carboxylates of the target. A second interaction could also be envisaged: formation of a π - π complex between drugs and FPIX could increase the drug-target interaction, analogous to the classical antimalarial quinolines, quinine and chloroquine, which are believed to act *via* complexation to haeme by π - π stacking of the coplanar aromatic molecules.³⁷ A similar interaction model has been proposed to explain the haeme binding affinity of xanthenes, which also exhibit antimalarial activity.³⁸ The three generation bis cationic compounds inhibit *de novo* phosphatidylcholine biosynthesis and bind to ferriprotoporphyrin IX.²³ The biological activity observed is the addition of both mechanisms of action. In the case of the first generation, where the π -electron rich site is absent, just the ionic interaction could be responsible of the ferriprotoporphyrin IX binding. Following this assumption, they would be active mainly by the choline transport mechanism. Second and third generation compounds would strongly interact with both targets since they are capable of giving ionic and π - π interactions.

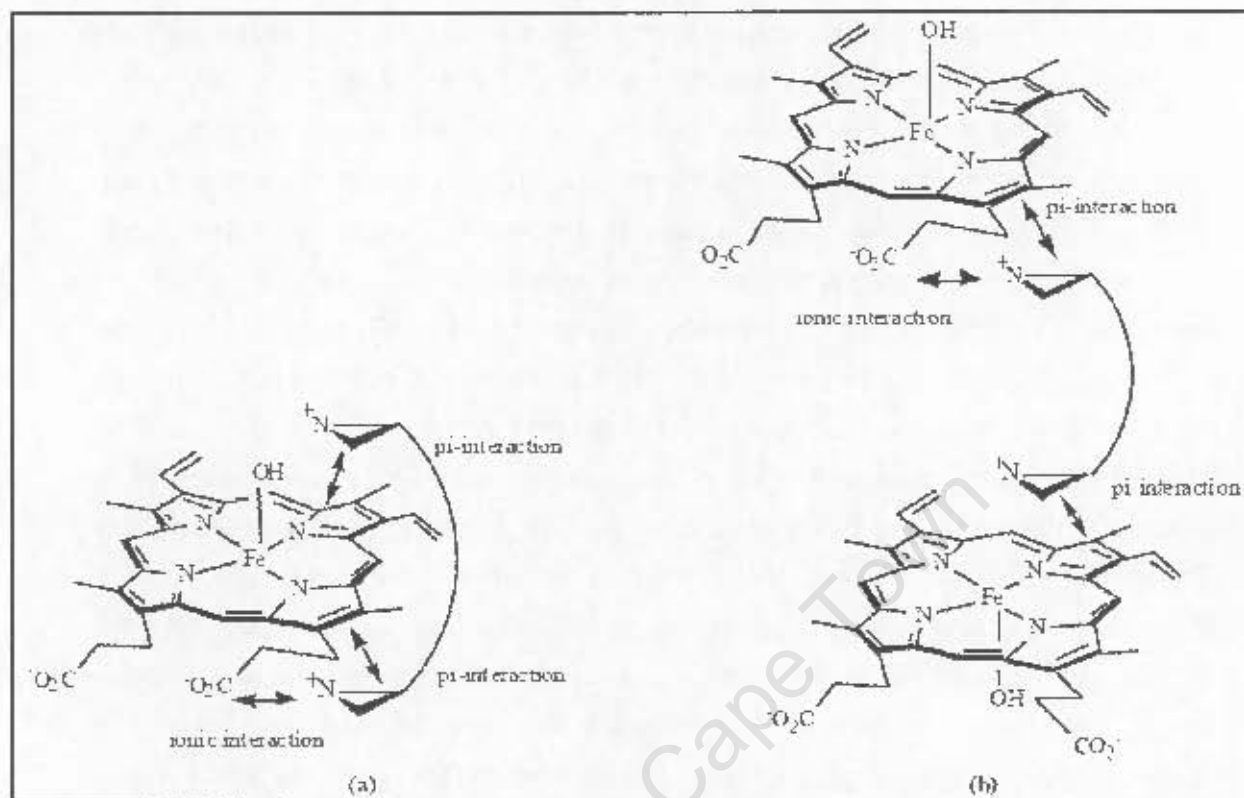


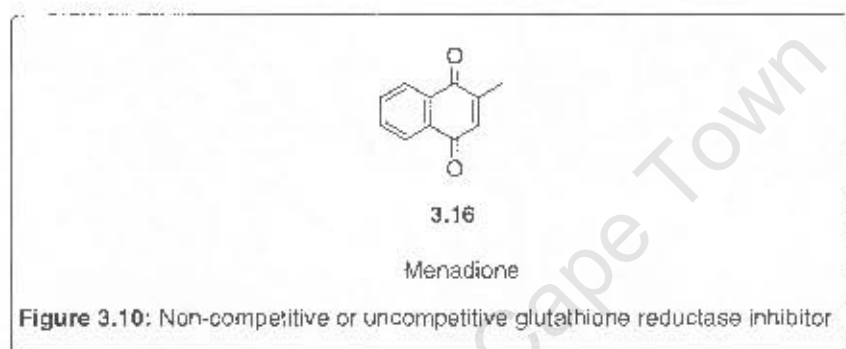
Figure 3.9: Hypothetical interaction mechanism between drugs and FPIX. Two sandwich structures with a 1:1 (a) or 2:1 (b) haeme-drug stoichiometry are possible

3.2.4 Antioxidant enzymes/haem dual inhibitors

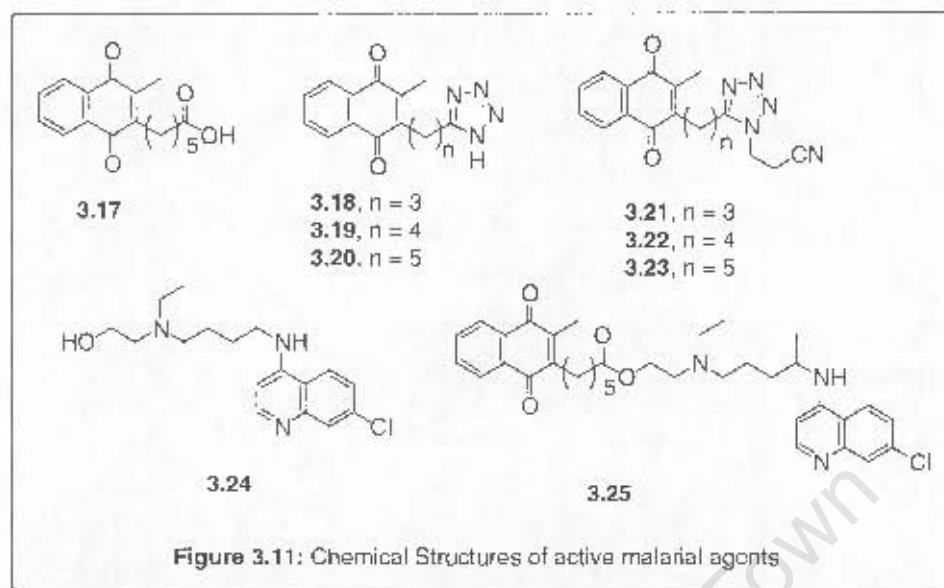
Further investigation commenced by Biot and coworkers,³⁰ focused on the synthesis of double-headed prodrugs by linking bioreversibly a glutathione reductase (GR) inhibitor to a 4-aminoquinoline moiety with the expectation of a synergistic or additive effect. These types of dual drugs were designed to be directed against two essential pathways, namely glutathione regeneration and haeme detoxification. Glutathione (GSH) is a thiol responsible for detoxification of reactive oxygen species in *P. falciparum* and is also involved in the degradation of toxic ferriprotoporphyrin IX released during host haemoglobin digestion. It was shown that the glutathione-dependent haeme modification is inhibited by chloroquine (CQ) and related 4-aminoquinoline inhibitors, suggesting that GSH is involved in the development of chloroquine resistance. It was also shown that the elevation in GSH levels leads to an increase in resistance to CQ while GSH

depletion in resistant strains restores sensitivity to CQ, thus a decrease in GSH intracellular level may lead to a reversal of CQ resistance. High intracellular concentration of GSH is regulated by the efficient reduction of glutathione disulfide (GSSG) by glutathione reductase (GR).

Up to now the most potent reversible GR inhibitors known to display antimalarial activity *in vivo* are the non- and uncompetitive inhibitors. An example of these types of inhibitors is the 1,4-naphthoquinone menadione (**3.16**, Fig. 3.10).



A series of drugs were designed and synthesized based on the menadione core and then tested *in vitro* against *P. falciparum* FcBIR strains using CQ as the control. Amongst those that exhibited significant activity were the single drugs, cyanoethyl-protected tetrazoles (**3.21-3.23**). In this case the carboxylic acid functional group in compound (**3.17**), which is important for GR recognition was bio-isoterically replaced with a tetrazole moiety, depicted in compounds (**3.18-3.20**), to improve the bioavailability but unfortunately led to poor antimalarial activity. However, the introduction of a cyanoethyl moiety enhanced the activity. One of the most active compounds in the double drug series was compound (**3.25**), which was prepared by linking the GR inhibitor **M₅** (**3.17**) to a potent 4-aminoquinoline antimalarial drug hydroxychloroquine (**3.24**), (Fig. 3.8).⁴⁹



Aims and Objectives

The overall aim of this study is:

- To design and synthesize new antimalarial agents targeting the falcipains and/or haemozoin formation.

The specific aims of this study were:

- To synthesize antimalarials based on the 'Dual Drug Approach', in which quinoline inhibitors of haemozoin formation are combined with cysteine protease inhibiting moieties in the same molecule.
- To improve the solubility of selected compounds by salt formation and by inclusion into selected cyclodextrins.
- To evaluate the biological activities of the synthetic compounds in order to identify potential new antimalarials.
- To explore structure-activity relationships (SARs) for antimalarial activities within the different compound libraries.

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CHAPTER 4

DESIGN AND SYNTHESIS OF DUAL ACTING ANTIMALARIAL AGENTS TARGETING CYSTEINE PROTEASES AND HAEMOZOIN FORMATION

4.1 Introduction

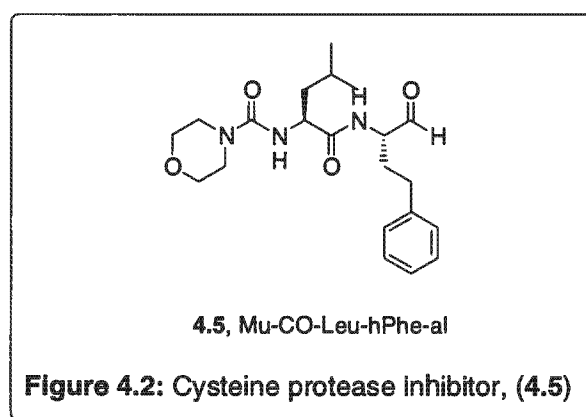
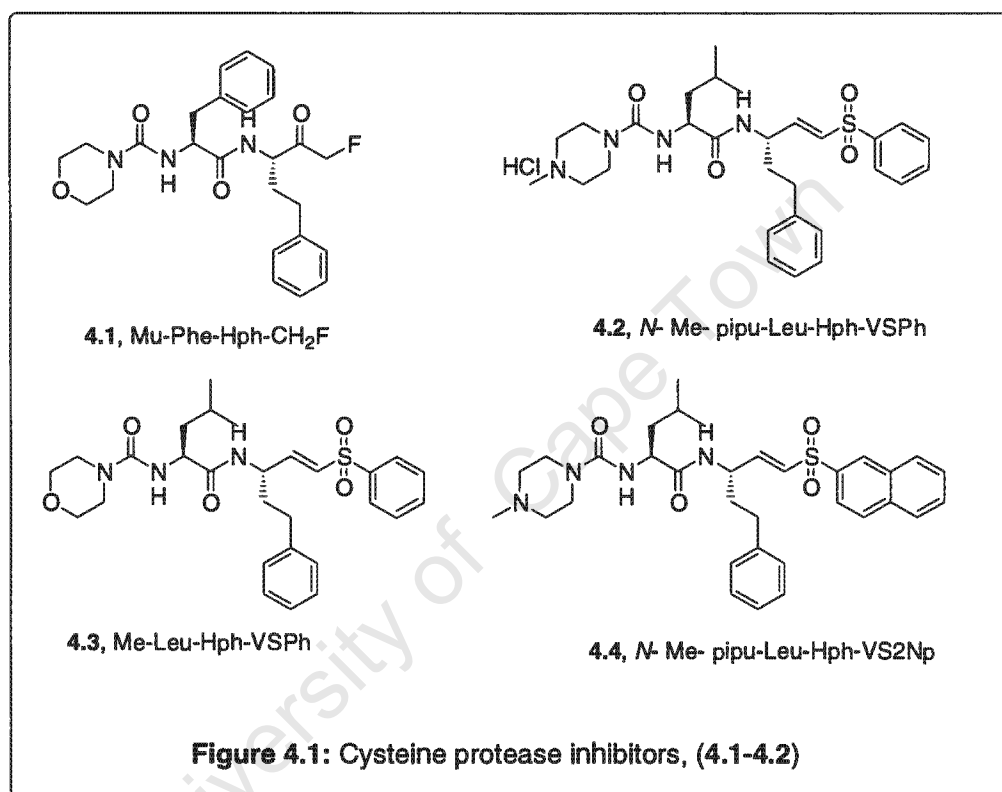
The emergence of resistance to current antimalarial drugs, in particular chloroquine and related quinoline-based agents, has accentuated the need to identify novel, potential antimalarial drugs. Hence, the primary focus of this chapter is to discuss the attempts made to design and synthesize potential new inhibitors that target the falcipain cysteine proteases, FP-2 and FP-3 and haemozoin formation, with the hope of decreasing and/or slowing down drug resistance. The envisaged inhibitors were designed based on the 'dual/double drug approach' which target two mechanisms of action and which contain pharmacophores that are derived from established antimalarial agents. As earlier indicated in Chapter 3, (page 48) such a strategy has been developed by Avery for the design of malarial cysteine protease inhibitors based on mefloquine and chloroquine.^{1, 2}

4.2 Peptidyl-Based Cysteine Inhibitors

4.2.1 Rationale and Background of Drug Design

To simplify SAR studies, we limited our research to the investigation of two scaffolds, namely the 4-aminoquinoline and morpholine peptide-derived moieties. Earlier studies have shown that the antimalarial cysteine protease inhibitors Mu-Phe-Hph-CH₂F (4.1)³ cured mice when administered parentally and Mu-Leu-HphVSPH (4.2)⁴ blocked hemoglobin degradation and development in cultured *P. falciparum* parasites at low nanomolar concentrations. However, the *in vivo* effectiveness of these compounds (4.1) and (4.2) were improved, by the synthesis of

their analogues *N*-Me-pipu-Leu-HphVSPh (4.3) and *N*-Me-pipu-Leu-HphVS-2Np (4.4). One of these compounds, *N*-Me-pipu-Leu-HphVS-2Np (4.4), cured about 40% of mice when administered orally twice-a-day for four days.⁵ In an expanded study, α -ketoamide cysteine protease inhibitor, morpholino-CO-Leu-HPhe-al (4.5), exhibited excellent activity against falcipain-2 and against cultured parasites.⁶



Based on established antimalarial cysteine protease inhibitors, (4.1–4.4, Fig 4.1) we reasoned that linking the two antimalarial pharmacophores could result in enhanced antimalarial activities through an additive or synergistic effect, (Fig. 4.3).

4.2.1.1 Designing of the Peptide-Based 4-Aminoquinoline Amine Scaffold

Designing of the Peptide-Based 4-Aminoquinoline Amine Scaffold was based on the following rationale:

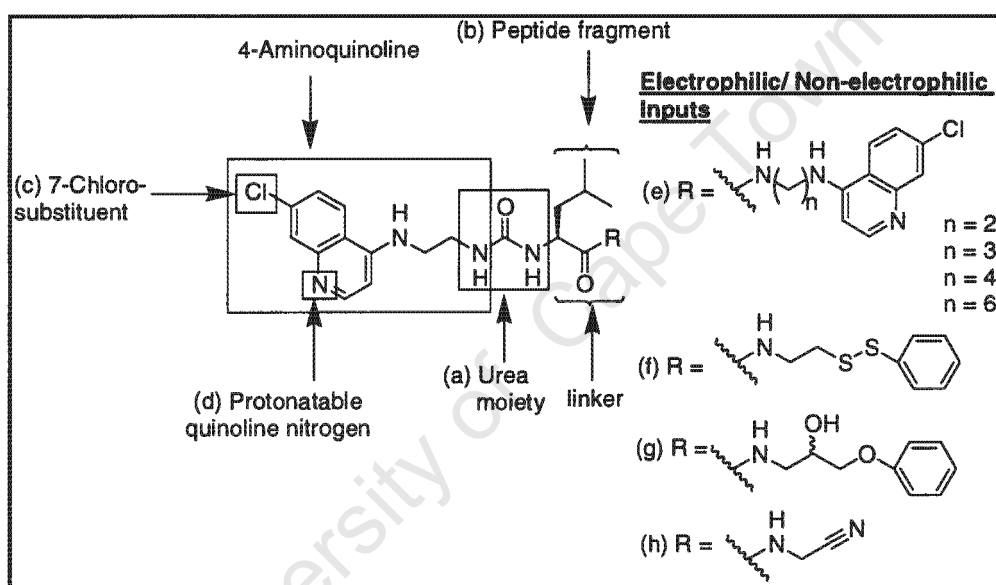


Figure 4.3: Important chemical features and inputs for peptidyl-derived 4-aminoquinolines

The following is a discussion of the various chemical features highlighted in Figure 4.3:

- (a) The presence of the urea moiety potentially offers more resistance towards enzymatic hydrolysis than a peptide bond in the gastro intestinal tract, and therefore enhances bioavailability.⁷ The urea moiety also provides an increase in water solubility *via* hydrogen bonding.
- (b) A peptidyl fragment, which forms part of the linker/spacer, is common in all the designed inhibitors. As mentioned previously, the effectiveness of peptidyl cysteine protease inhibitors such as (4.1–4.5) against cultured *P. falciparum* and in murine malaria models

has been demonstrated.^{5, 6} These studies have shown that compounds with a Leu residue at the P2 position were consistently more effective than those with a Phe at P2, having a strong preference in falcipain-2, falcipain-3 and vinkepain-2 (cysteine protease in rodent malaria parasites)^{8, 9} In this regard, falcipain-2 differ from the host cysteine proteases cathepsin L and B and many other papain family cysteine proteases.¹⁰

- (c) The 7-chloro substituent at position 7 in the 4-aminoquinoline moiety has been shown to be important for inhibition of haemozoin formation.¹¹ Further research by Kashula *et al.*¹² have concluded that strongly electron-donating groups at the C-7 position inhibit β -haematin formation the most, whilst moderately electron-withdrawing groups such as Cl, Br and I displayed better antiplasmodial activities.
- (d) The protonatable nitrogen atom in the quinoline ring aids in the accumulation of the compound in the acidic food vacuole *via* pH trapping. In addition, the protonation of the nitrogen atom can also increase the aqueous solubility of the compound.

4.2.1.2 Design of Peptidyl-Based Morpholines

The peptidyl-based morpholine scaffold was designed based on similar considerations as discussed above, (Fig. 4.3) with the only difference being the incorporation of morpholine. In addition, previous studies already mentioned in section 4.2.1 (page 62, 63) have shown that morpholine containing peptidyl cysteine protease inhibitors such as (4.1), (4.3), (Fig. 4.2) and (4.5, Fig. 4.3) exhibit potent antimalarial activities.^{5, 6}

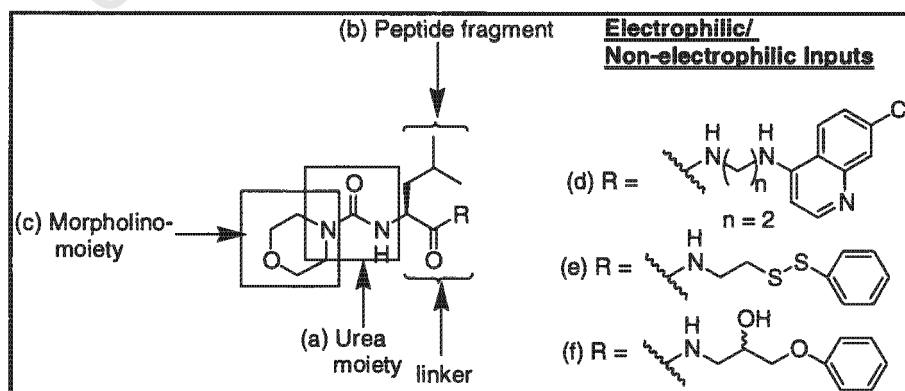


Figure 4.4: Important chemical features and inputs for peptidyl-based morpholine scaffolds

4.2.2 Incorporation of Various Inputs

Designing of the inputs represented in Fig. 4.3 and 4.4 was based on the following rationale and background:

4.2.2.1 Incorporation of 4-Aminoquinoline Amine Input

Interest in the 4-aminoquinoline containing inhibitors is derived from the well established quinoline antimalarials, (e. g. quinine, chloroquine and mefloquine) which have shown to exhibit potent antimalarial activities. The 7-chloroquinoline functionality has been shown to be important for optimal efficacy both *in vitro* and *in vivo*.¹³ Fig. 4.5 depicts the general numbering of the positions around the quinoline ring.

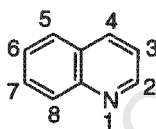
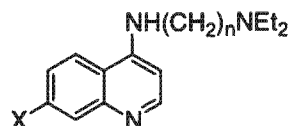


Figure 4.5: General numbering of the quinoline ring

Modification to the quinoline ring has been extensively studied. Independent studies carried out by De *et al.*¹⁴ and Ridley *et al.*¹⁵ have shown that chloroquine analogs having shortened side chains retain their haeme polymerization inhibitory activity against chloroquine resistant strains of *P. falciparum*. Subsequent research done by De *et al.*¹⁶ have suggested that aminoquinolines with short ($n = 2 - 4$), (4.6 and 4.7, Fig. 4.6) and long ($n = 10 - 12$), (4.8 and 4.9, Fig. 4.6) carbon chains were active against chloroquine-sensitive (Haiti 135) and resistant (Indochina I) *P. falciparum* strains compared to those with intermediate ($n = 5 - 8$) chain lengths.

General Structure:



4.6, X = Br, n = 2

4.7, X = I, n = 3

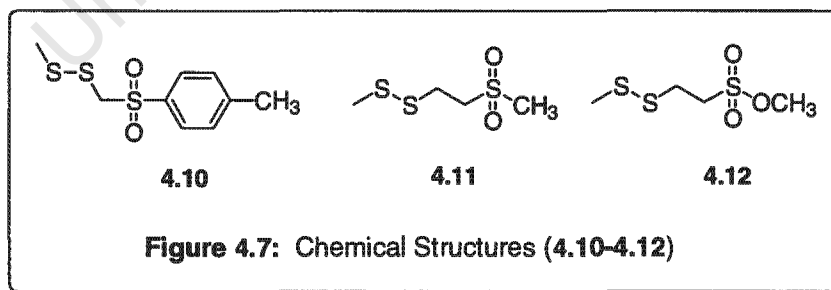
4.8, X = Br, n = 12

4.9, X = I, n = 10

Figure 4.6: Aminoquinolines with short (4.6 and 4.7) and long (4.8 and 4.9) diaminoalkane side chains

4.2.2.2 Incorporation of Disulfide Input

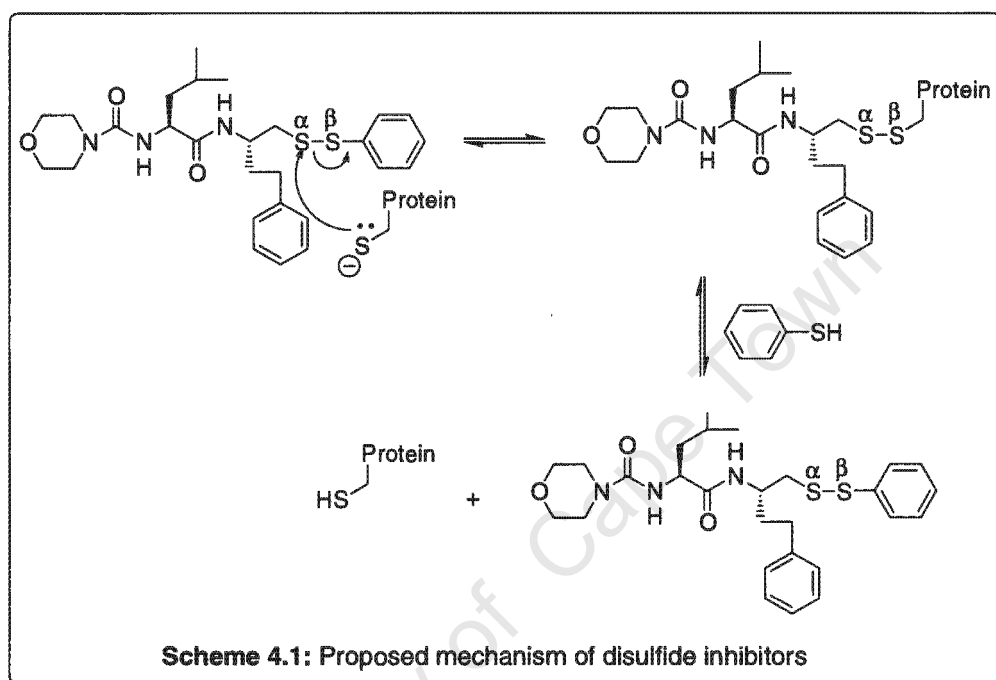
Some naturally occurring disulfides such as ajoene and dysoxysulfone, found in garlic,¹⁷ onions and mahogany trees^{18, 19} have shown promise as antifungal,²⁰ anticancer,²¹ and antithrombotic²² agents. Previous work has suggested that α -sulfone disulfides are particularly active against rapidly replicating cells a condition that is present during the second half of the intra-erythrocytic cycle of malaria. *P. falciparum* (human malaria) and, separately, C32 (human skin cancer) cultures were exposed to 11 sulfur compounds to determine their respective IC_{50} concentrations. Some compounds displayed minimum to no antimalarial/anticancer activity while others were highly active with IC_{50} values in the low micromolar range. Three disulfides (4.10, 4.11 and 4.12, Fig.4.7) were found to show promising activities against human malaria and human skin cancer.²²



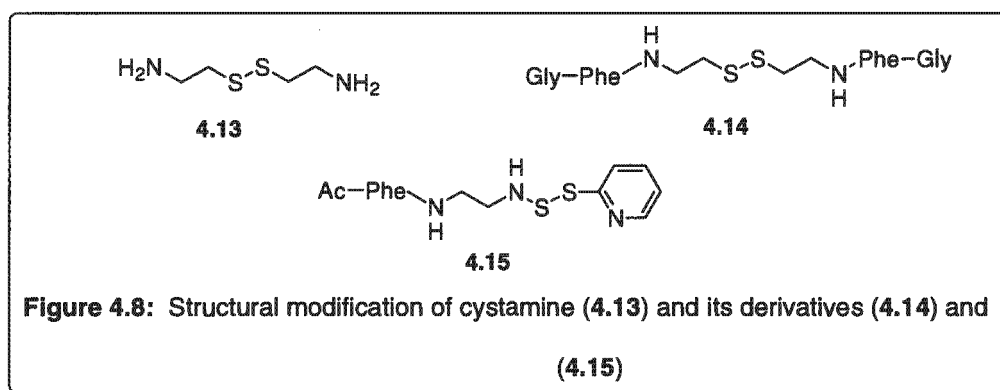
Mixed disulfides like (4.10-4.12) could be considered to be reduced to free thiols by the high intracellular concentrations of glutathione (upon entering the parasite). This is worth mentioning since it could be that in whole cell experiments the disulfides facilitates drug delivery and is then reduced to in the cell in which cases it would be the free thiol that exerts observed biological

activity.

The disulfide moiety acts as an electrophilic warhead which can be readily attacked by the thiolate anion of cysteine proteases. A proposed mechanism for the interaction of the enzyme and substrate is depicted in Scheme 4.1.

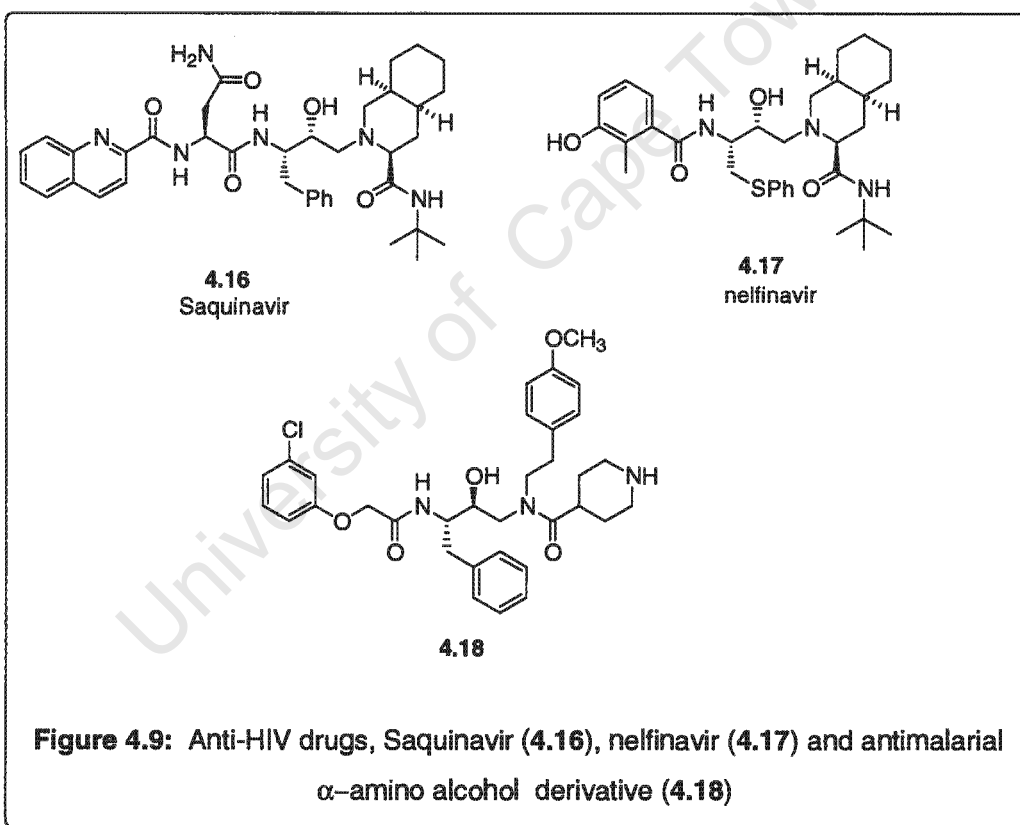


Cystamine (4.13) can also inactivate papain by blocking the cysteine residue at the active site.²³ Modification of cystamine resulted in improved inhibition activity. Inhibition was thus found to be stronger if cystamine derivatives were used with hydrophobic peptide residues, for example (4.14) which corresponds to the P2 specificity of the protease. However these symmetrical disulfides react very slowly.²⁴ But when the one leaving group was replaced by the smaller 2-pyridyl residue for example, (4.15), much more effective inhibitors were obtained,²⁴ (Fig. 4.8).



4.2.2.3 Incorporation of β -Amino Alcohol Input

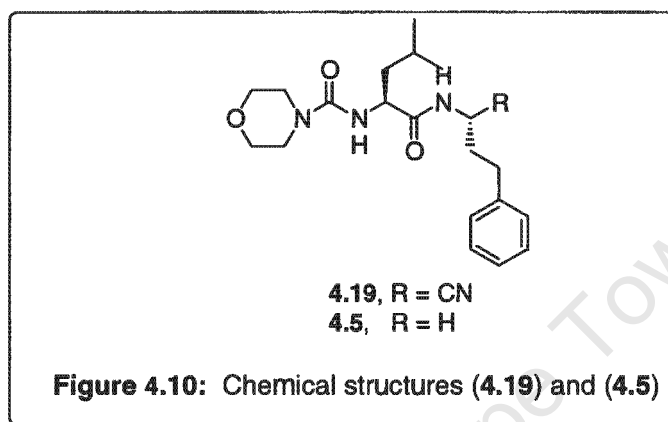
The β -amino alcohol pharmacophore stems from rich chemistry background. It functions as a significant building block in various drugs, such as the HIV-protease inhibitor drugs,²⁵ which include saquinavir (4.16) and nelfinavir (4.17)²⁶ (Fig. 4.9), angiotensin converting enzymes (ACE)²⁷ and well-established old antimalarial drugs including quinine, quinidine and mefloquine. Studies by Haque and coworkers²⁸ have shown that the β -amino alcohol derivative (4.18) inhibited the activities of malarial aspartic proteases plasmepsin I and plasmepsin II in *in vitro* assays. They have also shown that this β -amino alcohol derivative was active against parasite growth in cultured parasite-infected human erythrocytes.²⁸



4.2.2.4 Incorporation of Nitrile Input

Nitriles are known to be inhibitors of cysteine protease and were first reported by Hanzlik²⁹ as inhibitors of the plant protease papain. Evident from NMR analysis, these compounds were shown to form covalent bonds with the active site cysteine. To explore the importance of the

nitrile functionality for activity against Cathepsin S enzyme, Ward *et al.*³⁰ designed and synthesized some reversible dipeptide nitrile inhibitors. Compound (4.19, Fig. 4.10) was found to be a potent and reversible inhibitor of Cathepsin S ($IC_{50} = 42$ nM). Replacement of the nitrile with a hydrogen atom, (4.5, Fig.4.10) resulted in a dramatic loss in potency ($IC_{50} > 28000$ nM), lending support to the proposal that the nitrile functions as an electrophilic warhead.

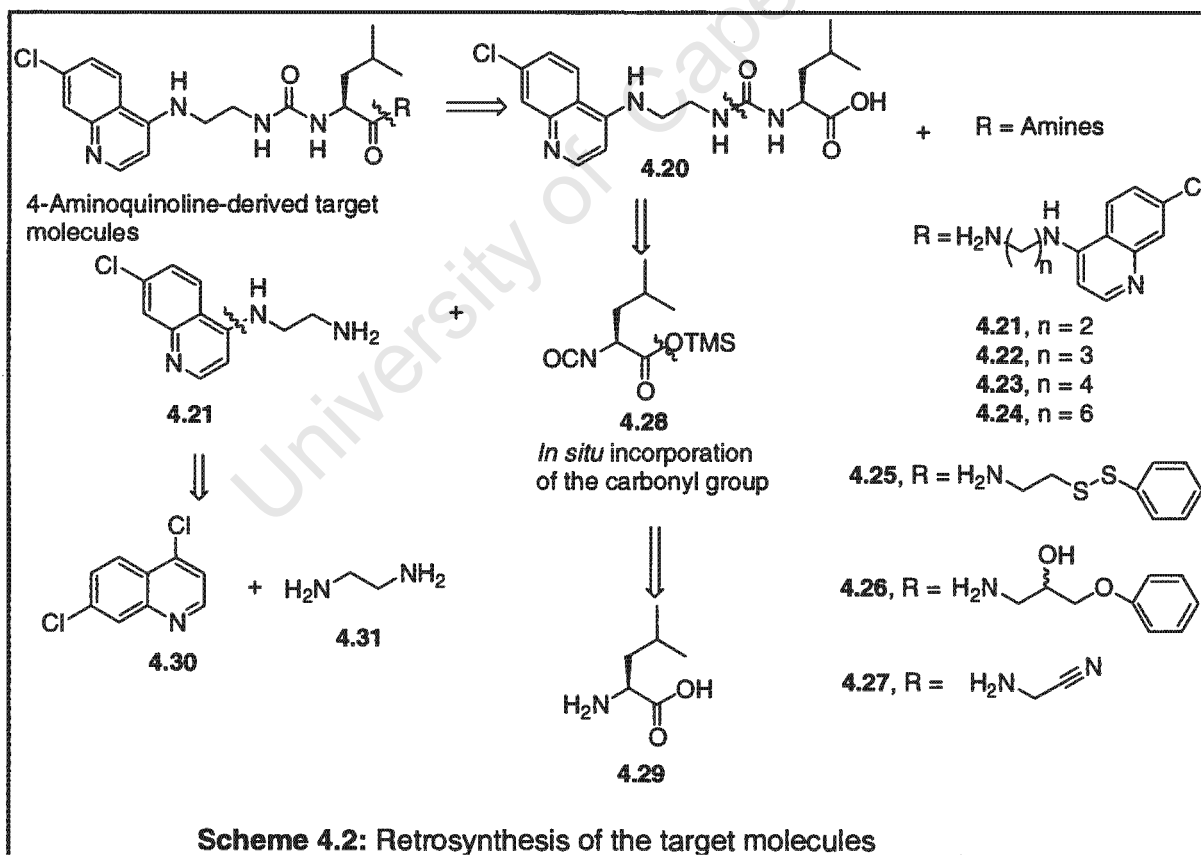


The effectiveness of the presence of a nitrile was also demonstrated in the activity of glutathione reductase inhibitors (see section 3.2.4, page 56 and 57).

4.3 Chemical Synthesis and Characterization

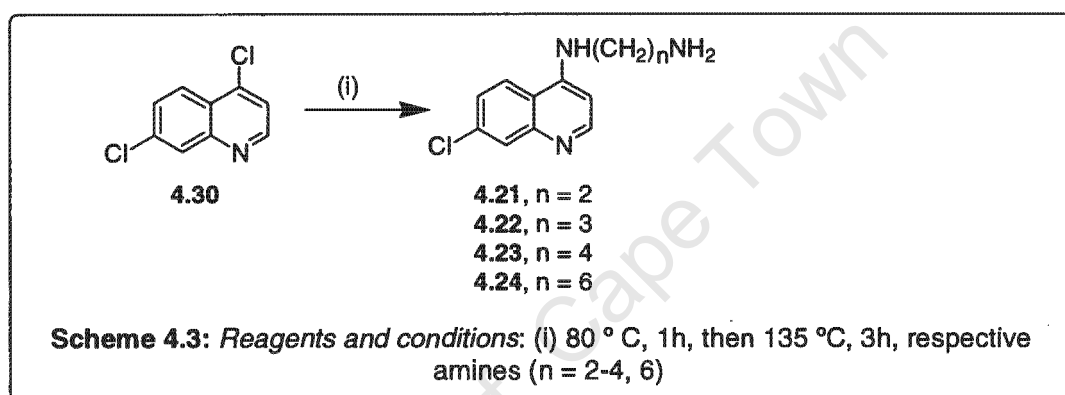
4.3.1 Retro-synthesis of the Peptidyl-based 4- Aminoquinoline Amine Scaffold

Retrosynthetically, we envisaged target compounds as arising from the commercially available starting materials, (4.29, 4.30 and 4.31), depicted in Scheme 4.2. Peptide coupling of the carboxylic acid derivative, (4.20) with the respective amines (4.21–4.27) gives rise to the desired 4-aminoquinoline-derived target molecules. Further analysis of the 4-aminoquinoline derived carboxylic acid (4.20) leads to the isocyanate (4.28) and the diamine (4.21). The isocyanate carbonyl group in (4.28) was incorporated *in situ* without isolation and is derived from the commercially available *L*-leucine (4.29). Diamine (4.21) is obtainable from a nucleophilic substitution reaction of 4, 7-dichloroquinoline (4.30) and the ethylene diamine (4.31), which are both commercially available.



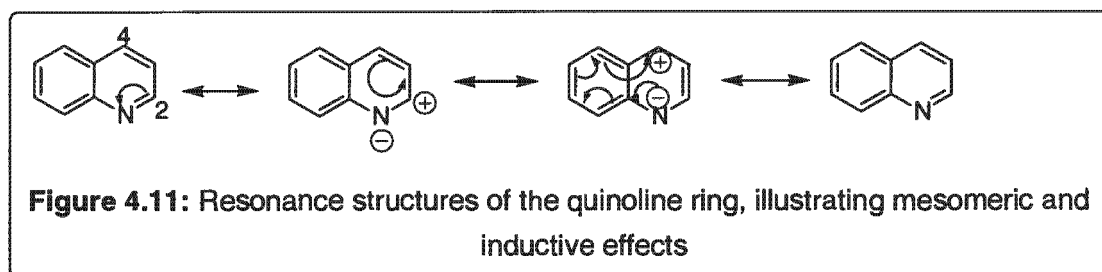
4.3.2 Synthesis of 4-Aminoquinoline Diamines

The synthesis of 1-(4-amino-7-chloroquinoline)-diamines (4.21–4.24, Scheme 4.3) was achieved by a method reported by De *et al.*³¹ In this procedure a large excess (> 5.0 mol equivalence) of the respective amines were used to suppress the formation of terminally disubstituted amines. The reactions were performed in the neat, thus using the amines as reactant and solvent. Heating under reflux at high temperature also ensures complete conversion of the starting material over a short period of time in the absence of an additional base.



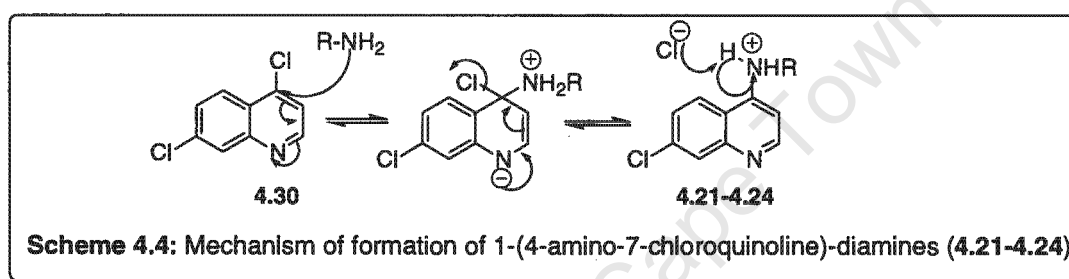
4.3.2.1 Reactivity of Quinoline Ring

The quinoline ring is a fused benzo[b]pyridine ring and thus demonstrates similar properties to that of both benzene and pyridine from which it is derived. The quinoline ring offers access for electrophilic reactions to occur on the benzene ring and for nucleophilic reactions to occur on the pyridine ring. The nitrogen atom in the heterocyclic ring creates a decrease in the electron density *via* the mesomeric and inductive effects at the C-2 and C-4 position. The reactivity of these positions is further increased by the presence of electron withdrawing groups, for example halogens at position 7, (Fig. 4.11).



4.3.2.1.1 Mechanistic Comments

The mechanism of formation of 1-(4-amino-7-chloroquinoline)-diamines (4.21-4.24), is illustrated in Scheme 4.4. This involves nucleophilic attack of the alkyldiamine nitrogen to the 4-position of the 4, 7-dichloroquinoline, followed by elimination of the chloride anion. As mentioned above, the C-2 and C-4 position of the quinoline is more susceptible to nucleophilic attack, which is further enhanced by the presence of an electronegative group at those positions. The influence of the nitrogen atom *via* resonance stabilization is a major contributor for observed regioselectivity.



In this reaction the use of an additional base was omitted. The role of the base is to 'mop up' the acid formed as a byproduct. It was found that using a base directly in the reaction mixture resulted in low yielding products. Hence the base was introduced during the work-up procedure, which involves washing with 1 M NaOH, followed by extraction with EtOAc. Table 3.1 shows the isolated yields and melting points obtained for 1-(4-amino-7-chloroquinoline)-diamines (4.21-4.24).

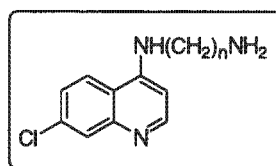
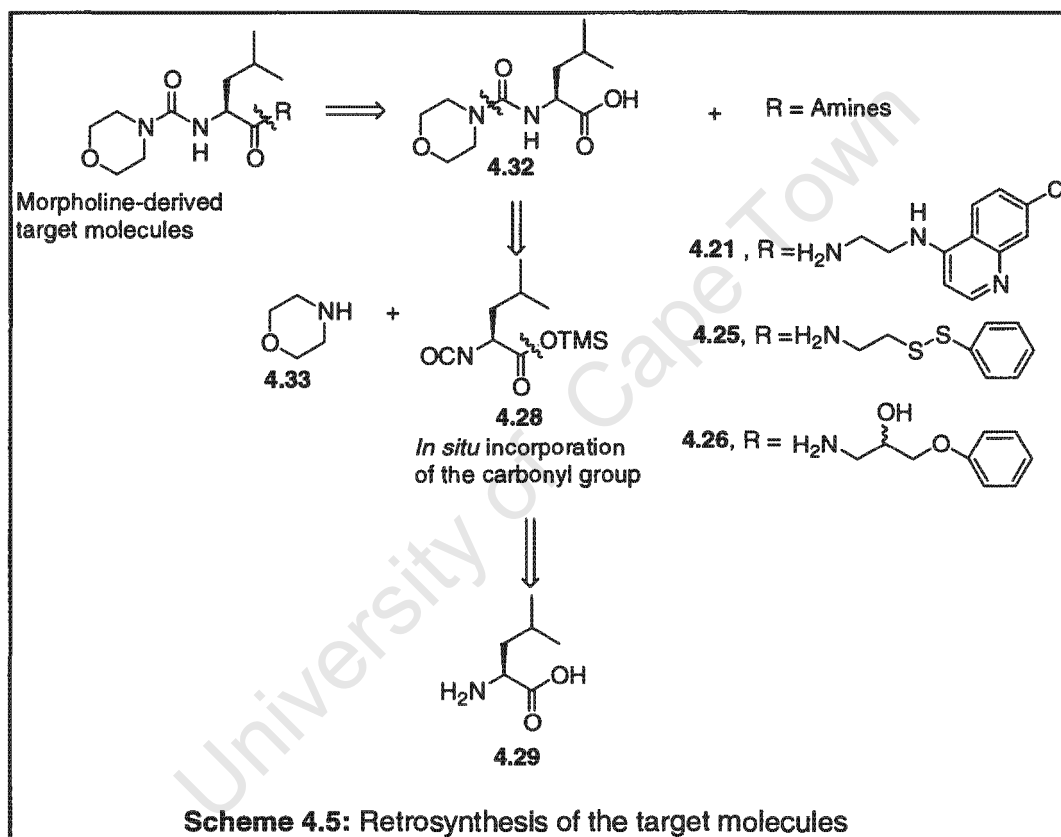


Table 4.1: Isolated yields and melting points of the diamines (4.21-4.24)

Compound	n	Yield (%)	M.p. (lit m.p.) ³²
4.21	2	94	94-97 °C
4.22	3	83	55-57 °C
4.23	4	95	75-80 °C
4.24	6	93	137-138 °C

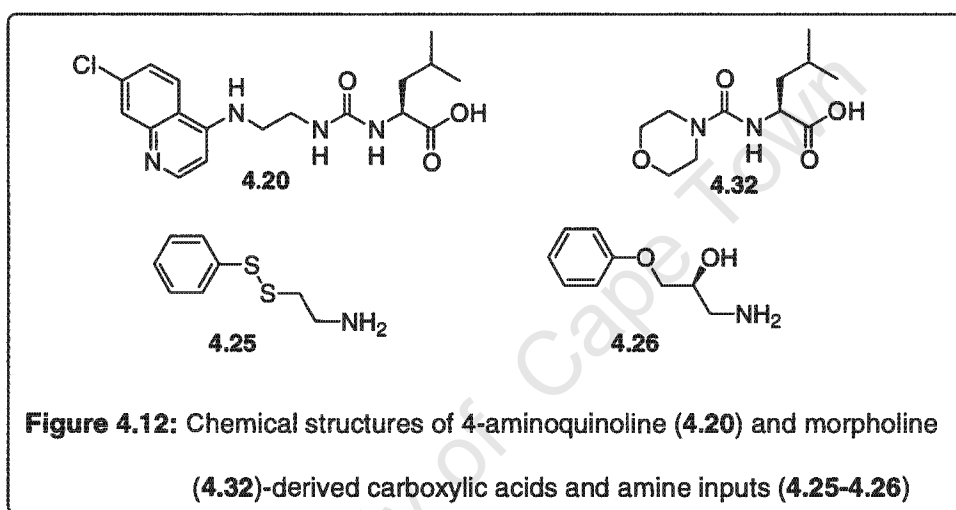
4.3.3 Retrosynthesis of the Morpholine-Based Target Molecules

The retrosynthesis of the morpholine-based target molecules is presented in Scheme 4.5. The target molecules were obtained *via* peptide coupling of the carboxylic acid (4.32) with the respective amines (4.21, 4.25–4.26). The carboxylic acid derivative (4.32) was formed from a “one-pot” reaction of the morpholine (4.33) and *in situ*-generated intermediate, isocyanate (4.28).



4.3.4 Synthesis of 4-Aminoquinoline (4.20) and Morpholine (4.32)-Derived Carboxylic acids and Amine Inputs (4.25-4.26)

The synthesis of 4-aminoquinoline (4.20, Scheme 4.2) and morpholine-derived carboxylic acid (4.32, Scheme 4.5) was achieved by following the same methodology. The synthesis of the 4-aminoquinoline amines (4.21-4.24) has already been discussed section (section 4.3.2, page 72). The nitrile amine (4.27, Scheme 4.2) was commercially available.

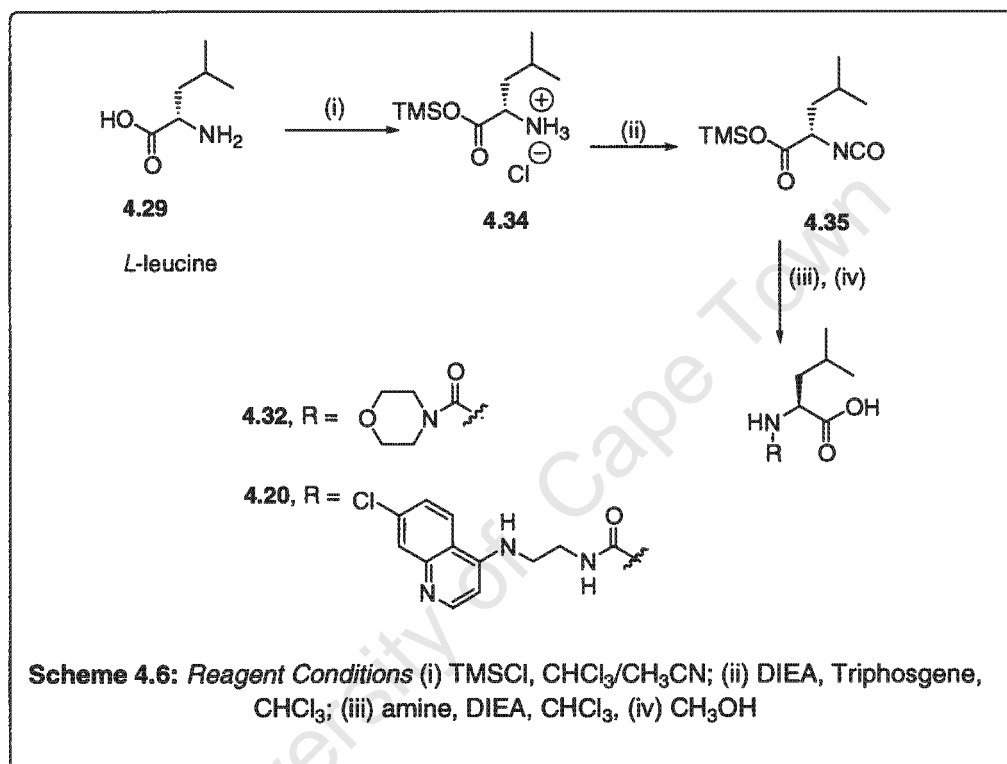


4.3.4.1 Synthesis of 4-Aminoquinoline (4.20) and Morpholine (4.32)-Derived Carboxylic acids via *N*-(Carbonylamino)amino acids

N-(Carbonylamino)amino acids are starting materials or structural components for a variety of pharmacologically active compounds, including, HIV-protease inhibitors,³² renin inhibitors,³³ selective antagonists for the endothelin receptors,³⁴ and cysteine protease inhibitors.³⁵

N-(Carbonylamino)amino acids are often prepared in step-wise fashion transforming α -amino acid esters to imidazole carbonates using 1,1-carbonyldiimidazole,³⁶ followed by reaction with amines, and then esterification. In this reaction *N*-(carbonylamino)amino acids were directly prepared from α -amino acids³⁷ as a one-pot procedure *via* the isocyanate using triphosgene.³⁸ To achieve this, the carboxylic acid functionality would have to be blocked momentarily so as not to interfere with isocyanate formation. The ideal *O*-protecting group, in the context of this

desired one-pot procedure, is one that can be installed selectively in the presence of an amine, and is sufficiently labile to be removed during work-up. Trimethylsilyl chloride (TMSCl) was chosen as suitable protecting reagent, since it was previously demonstrated to selectively *O*-silylate amino acids.³⁹ The “one-pot” synthesis of *N*-(Carbonylamino)amino acids directly from α -amino acids by employing TMS as a “transient” protecting group is outlined in Scheme 4.6.



Treatment of leucine (**4.29**) with 1.0-1.1 mol equivalence of Me_3SiCl (TMSCl) in 1:5 acetonitrile/ CHCl_3 with stirring for 12 h at room temperature, gave a solution of the selectively *O*-protected amino acid hydrogen chloride (**4.34**).⁴⁰ At this stage a heterogeneous mixture was obtained due to salt formation. This solution in the presence of base, DIEA was added to a stirred solution of triphosgene⁴¹ in chloroform. Due to the highly reactive nature of triphosgene, it was added very slowly to the reaction mixture at low temperature (0°C). The resulting solution was treated with 1.0-1.2 mol equivalence of the amine (**4.33**, Scheme 4.5) or (**4.21**, Scheme 4.2) in the presence of additional base, followed by mild deprotection using MeOH, gave the *N*-(carbonylamino)amino acids (**4.32**) and (**4.20**) respectively. A moderate yield for both *N*-(carbonylamino)amino acids, (**4.32**) and (**4.20**) was obtained.

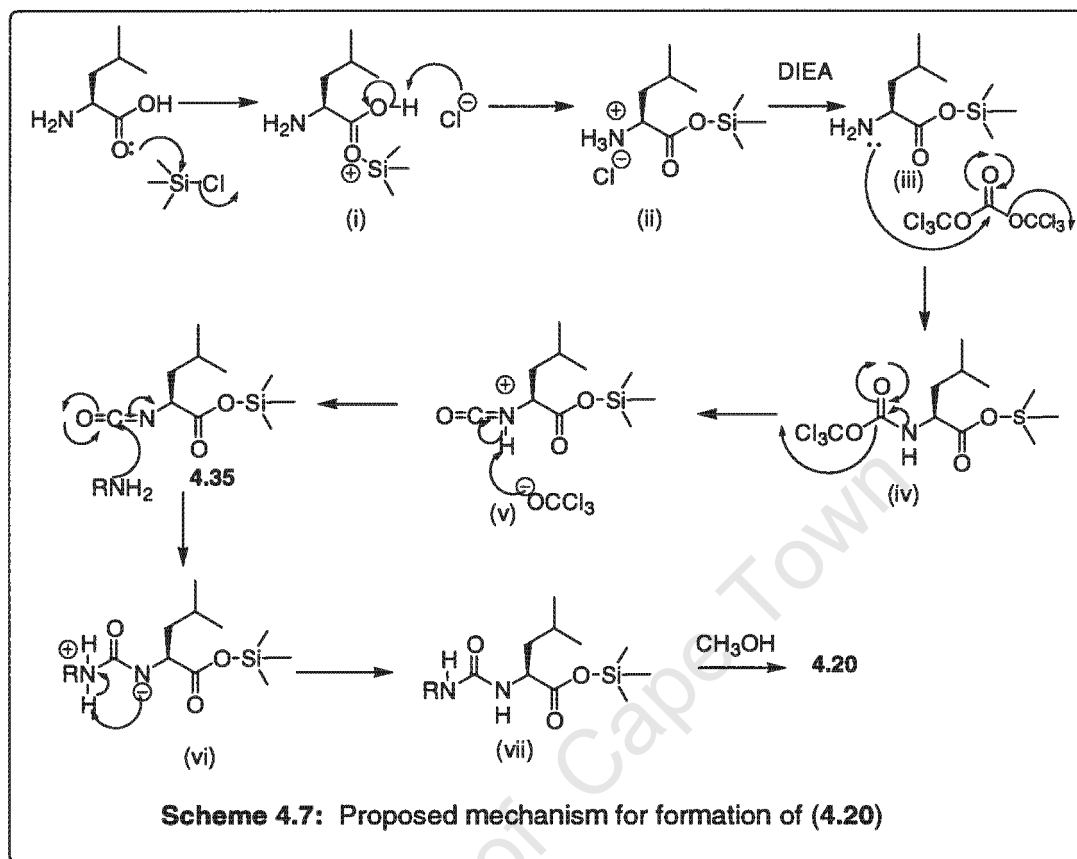
Table 4.2 Isolated yields of compounds (4.20) and (4.32)

Compound	Yield (%)
4.20	50
4.32	67

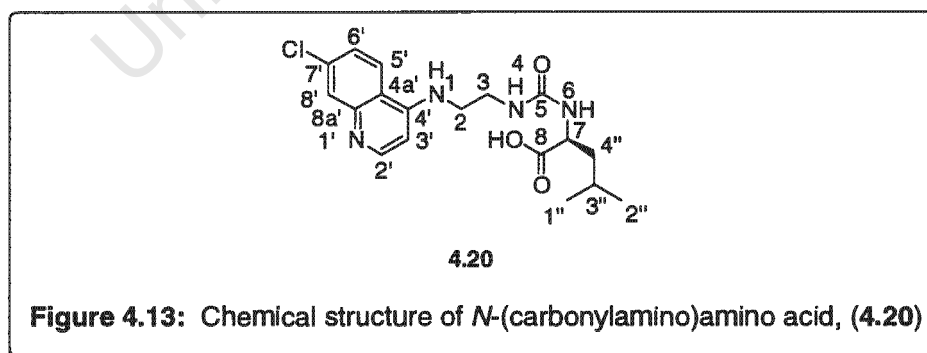
The acid functionality of the products conveniently provides the opportunity during acid/base extractive work up to remove any unreacted amine an amino acid with an acid wash of the organic extracts, and any neutral by-products with an organic wash of the basic aqueous phase. Thus the isolated products are obtained as pure products, as confirmed by TLC analysis, indicating a single product spot in 10% MeOH in DCM as the mobile phase.

4.3.4.1.1 Mechanistic Comments

The mechanism is initiated *via* a nucleophilic attack of the oxygen lone pair at the silicon center of the trimethylsilylchloride with subsequent removal of the chlorine atom to produce the intermediate product (i). The presence of the chloride ion activates the formation of intermediate product (ii) to form the hydrochloride salt which is neutralized by addition of the base, diethylisopropylamine (DIEA) to produce the free amine (iii). Incorporation of the carbonyl group proceeds *via* a nucleophilic attack of the nitrogen lone pair at the electrophilic carbonyl of triphosgene with contaminant release of the trichlorocarbonate ion to produce the intermediate product (iv). Donation of the nitrogen lone pair in the intermediate product (iv) promotes the formation of isocyanate (4.35), the key intermediate product. Attack by the primary amine then produces the intermediate product (vi), which undergoes hydrogen transfer, followed by deprotection of the OTMS group to afford the target product (4.20).



Characteristic IR bands for compound (4.20) were observed as broad peaks at approximately 3500 cm^{-1} and 3261 cm^{-1} due to OH and NH stretching respectively. Another characteristic peak was observed for the carboxylic and amide carbonyl, at approximately 1722 cm^{-1} and 1645 cm^{-1} respectively.



The ^1H NMR spectrum obtained was in agreement with the proposed structure, (Fig. 4.13). The ^1H NMR spectrum of the compound (4.13) revealed the following characteristic signals; a one-proton multiplet at δ 4.20 ppm assigned to H-7, a four-proton multiplet at δ 3.53 ppm assigned

to CH_2 -2 and CH_2 -4, a three-proton multiplet at δ 1.58 ppm assigned to CH_2 -3'' and CH_4 '', three-proton doublet at δ 0.93 ppm assigned to CH_3 -1'' and another three-proton doublet at δ 0.91 ppm assigned to CH_3 -2''. All five quinoline protons were observed in the downfield region, (Fig.4.14). The ^{13}C NMR spectrum confirmed the two carbonyl groups, assigned to the two carbonyl peaks at δ 174.0 and at δ 154.0 respectively. Low resolution electron ionization mass spectroscopy further revealed the presence of the parent molecular ion (M^+ 378.).

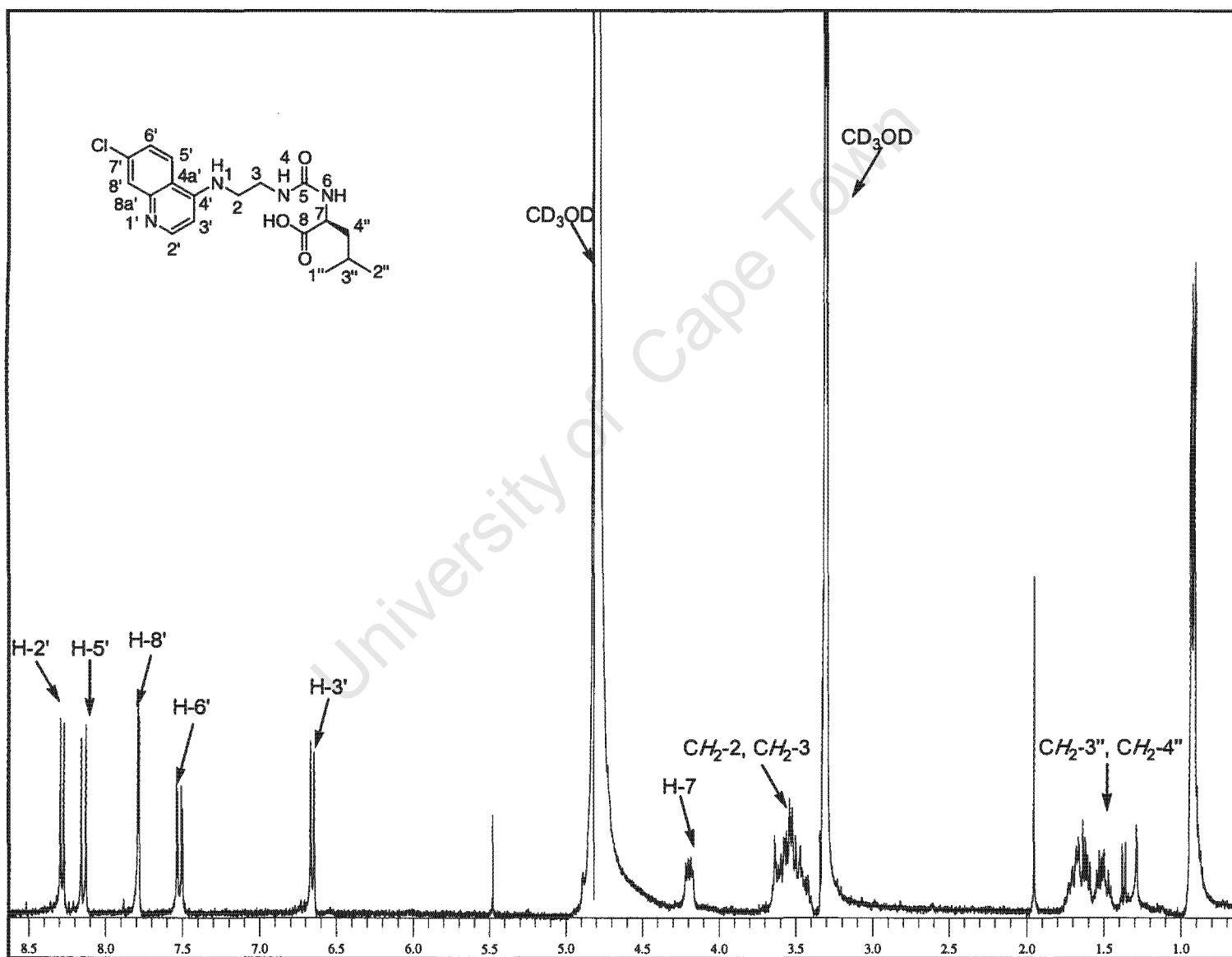
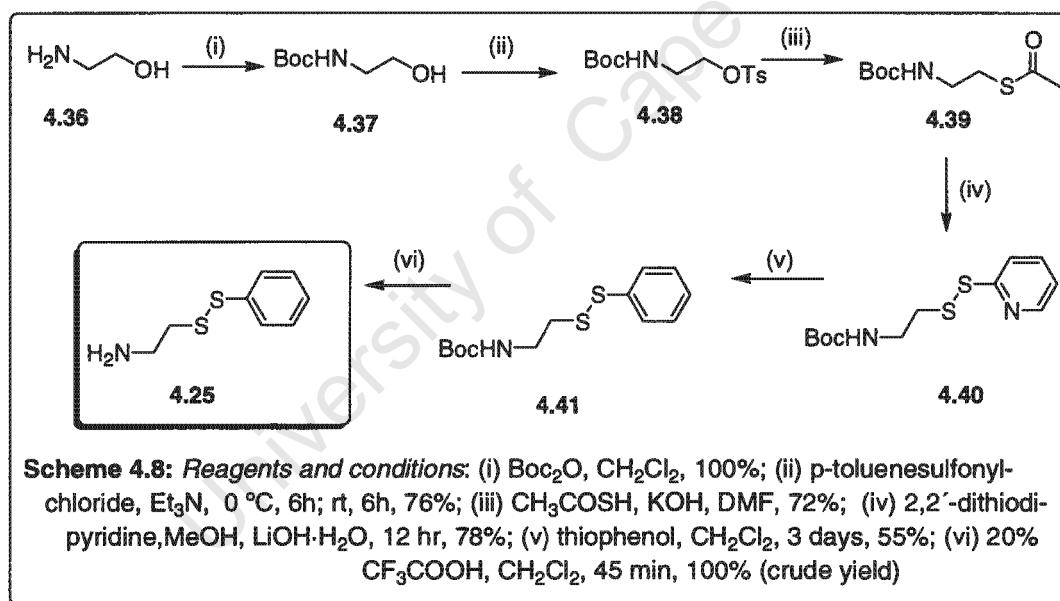


Figure 4.14: ^1H NMR spectrum of compound (4.20) in CD_3OD

4.3.5 Synthesis of Amines (4.25 and 4.26)

4.3.5.1 Synthesis of Disulfide amine (4.25)

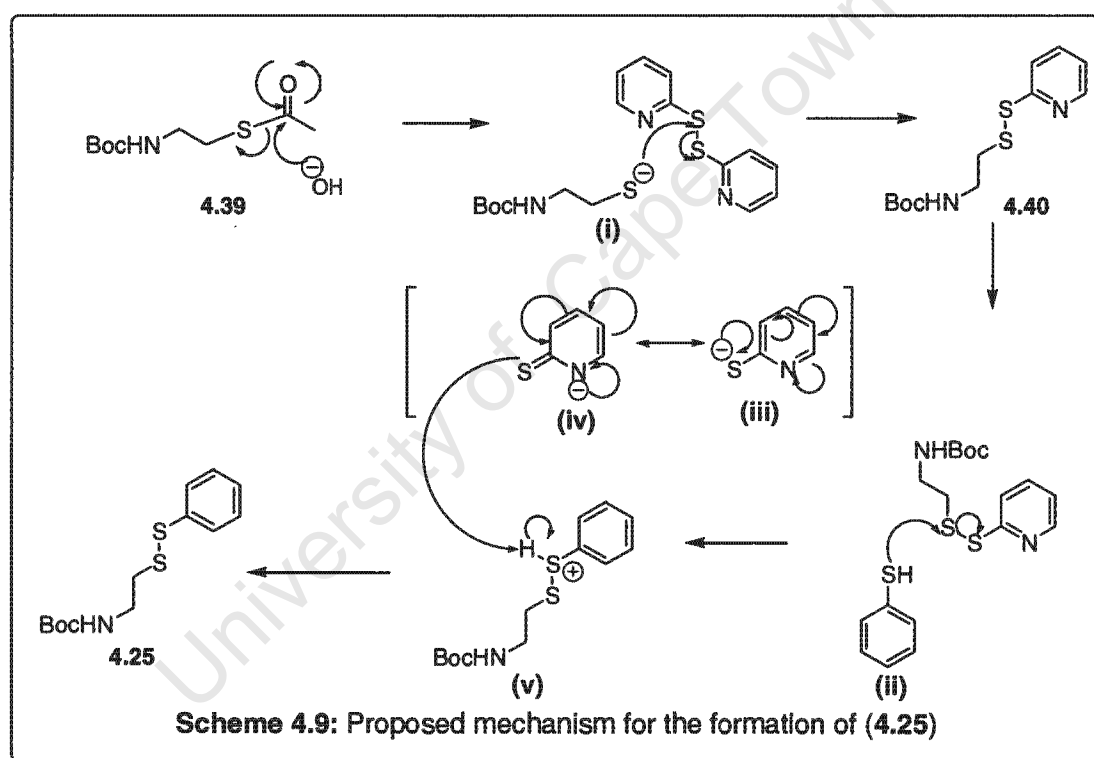
The reaction steps in this synthesis were straightforward and the intermediate products were obtained in moderate to high yields. Ethanolamine (4.36) was boc-protected to afford the alcohol (4.37), which was converted into a better leaving group by treatment with *p*-toluenesulfonyl chloride in the presence of triethylamine to afford the protected tosylate (4.38). The first sulfur was introduced by a reaction of tosylate (4.38) with thiolacetic acid to produce the thiolacetate (4.39). Deacetylation of the intermediate thiolacetate (4.39) and disulfide formation with thiolacetate (4.39) and subsequent disulfide formation with 2,2'-dithiodipyridine led to the formation of the labile disulfide (4.40).⁴² Treatment of (4.40) with thiophenol followed by deprotection led to the desired disulfide amine (4.25, Scheme 4.8).⁴³

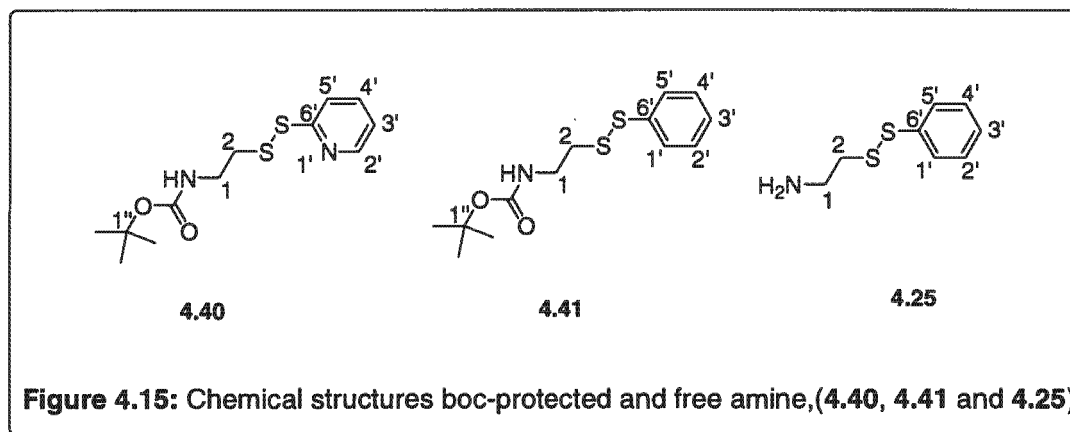


The disulfide amine (4.25) was used directly in the subsequent reaction step without isolation or characterization. TLC analysis indicated complete consumption of the starting boc-protected amine (4.40) with a R_f value higher than that of the product (4.25) in 50% EtOAc in hexane as the mobile phase.

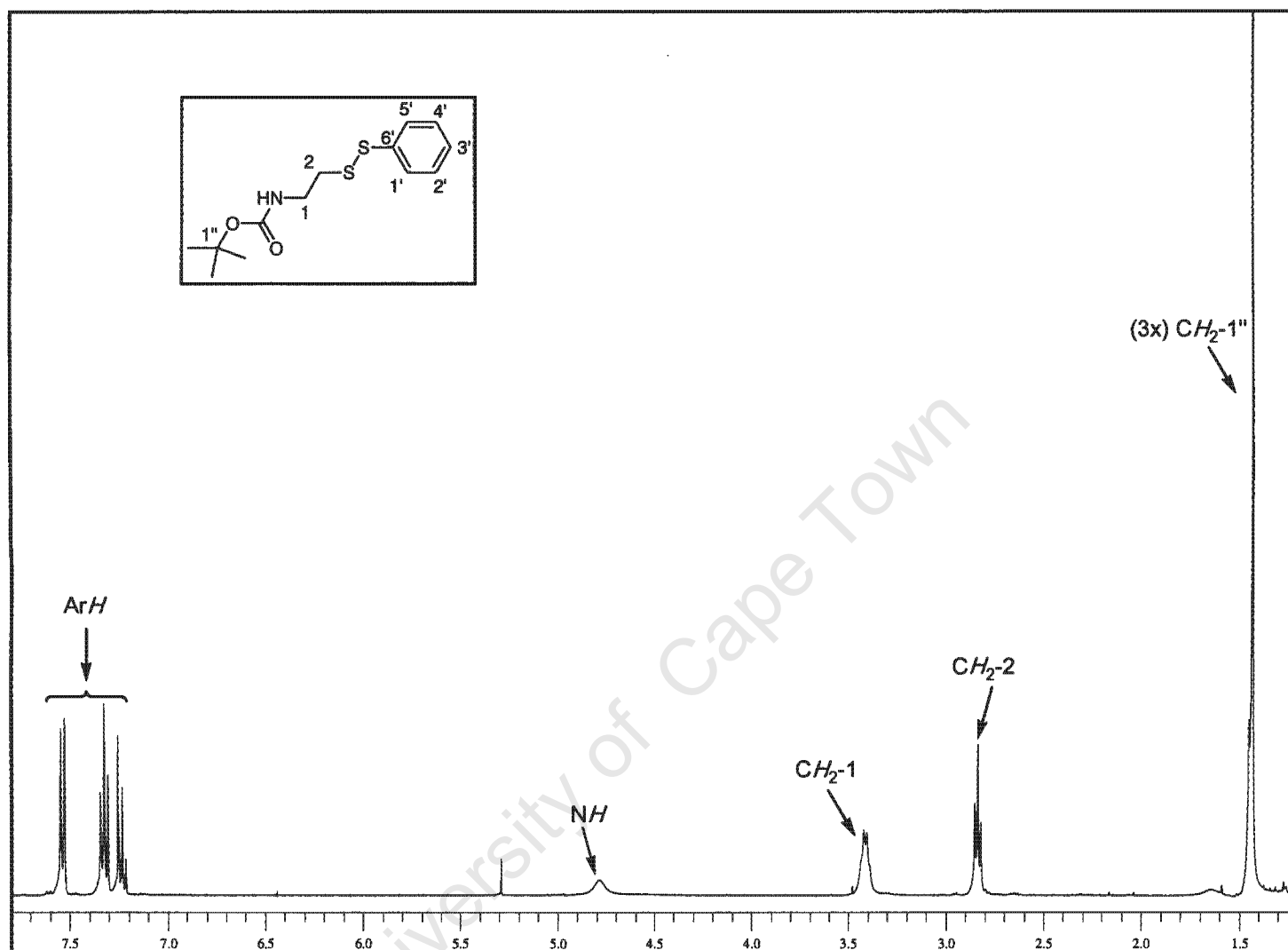
4.3.5.1.1 Mechanistic Details

The key steps in reaction sequence depicted in Scheme 4.9, are the introduction of the disulfide moiety and subsequent substitution by the aromatic thiol group. The proposed mechanism is illustrated in Scheme 4.9. In this mechanism the lithium hydroxide hydrolyses the thiolacetate (4.39) and the resulting thiolate anion (i) then attacks the pyridyldisulfide reagent to form the disulfide (4.40). Nucleophilic attack of the thiophenol sulfur (ii) produces the intermediate product (v) which is deprotonated by the resonance stabilized intermediate (iv) to give the desired product (4.25).





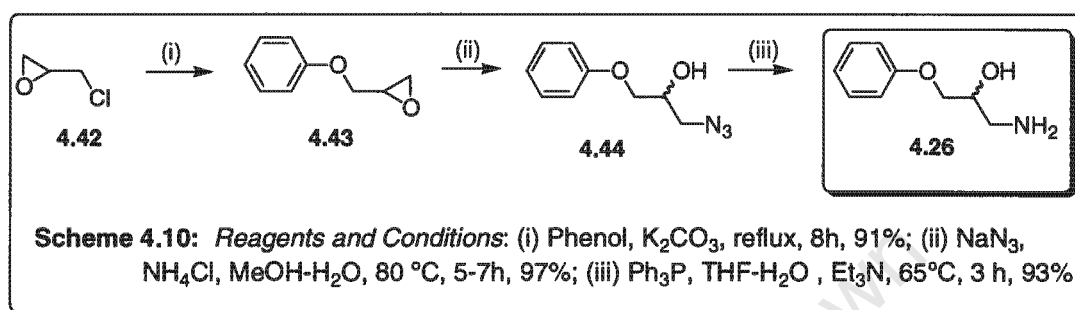
Noticeable differences in the ^1H NMR spectra of compounds (4.40) and (4.41) were observed, (Fig. 4.15). The broad singlet assigned to NH in (4.40) formally at δ 5.78 was shifted upfield to δ 4.78 in (4.25). A similar effect was observed for the other characteristic signals in (4.40). This observation may be due to the strong deshielding effect of the nitrogen pyridine causing the proton signals in (4.40) to appear more downfield. However, in the absence of the nitrogen pyridine, the proton signals in (4.25) appeared more upfield. In the ^1H NMR spectrum of the boc-protected amine (4.41), (Fig. 4.16) the following characteristic signals were observed; a one-proton broad singlet at δ 4.78 assigned to NH , a two-proton quartet at δ 3.14 (J 5.6) assigned to CH_2 -1, a two-proton triplet at δ 2.83 (J 5.6) assigned to CH_2 -2 and a sharp singlet integrating for nine protons at δ 1.43 assigned the three CH_3 -1'''. The ^{13}C NMR spectrum revealed the characteristic $\text{C}=\text{O}$ signal to appear at δ 159.3 ppm and the three methyl carbons as a single peak at δ 28.2 ppm. The proposed structure was further confirmed by low resolution electron ionization spectroscopy which showed the mass of the parent ion (M^+ 285).

Figure 4.16: ^1H NMR spectrum of compound (4.41) in CDCl_3

4.3.5.2 Synthesis of β -Amino alcohol Amine (4.26)

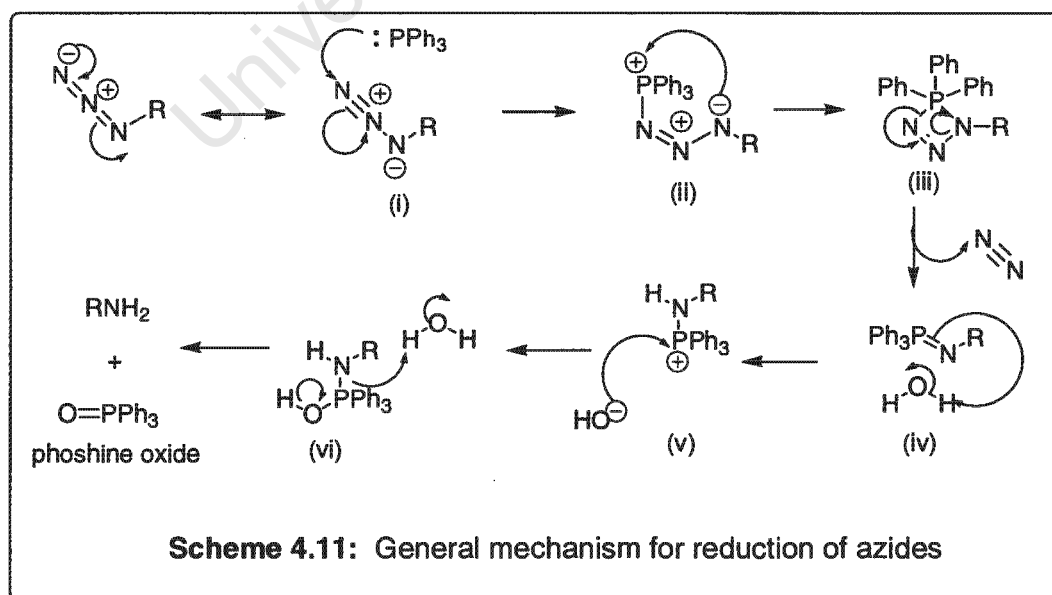
Treatment of phenol with 1.1 equivalence of epichlorohydrin (4.42) in the neat followed by reflux at high temperature produced the product (4.43) in an excellent yield of 91%. It was found that this reaction can proceed in the absence of a base at a high temperature. Azide formation was achieved by treatment of (4.43) with NaN_3 in the presence of NH_4Cl to afford the product (4.44) in almost quantitative yield. The reaction was carried out in a mixture of water and organic solvent ($\text{MeOH-H}_2\text{O}$) with NH_4Cl as buffer to provide a proton for the Intermediate (4.44). Many

procedures were found in the literature for azide formation; however this method proved to be more suitable for this type of substrate.⁴⁴ Reduction of the azide (**4.44**) was accomplished in a simple one-step reaction, in which the azide (**4.44**) in THF:H₂O (20:0.1 ml) was heated in the presence of Ph₃P at 65 °C to obtain (**4.26**) in high yield,⁴⁵ (Scheme 4.10).



4.3.5.2.1 Mechanistic Comments

The resonance stabilized azide (i) is attacked by the lone pair of electrons on the phosphine atom to produce the intermediate product (ii) which affords the intermediate product (iii) *via* cyclization. Collapsing of the cyclized imine intermediate (iii) followed by contaminant lost of a molecule of nitrogen produces intermediate (iv). Further steps involve a molecule of water, which is 'dismembered' and shared between the reagents to yield the desired amine and phosphine oxide, (Scheme 4.11).



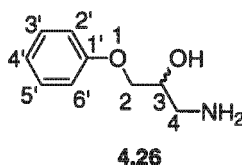
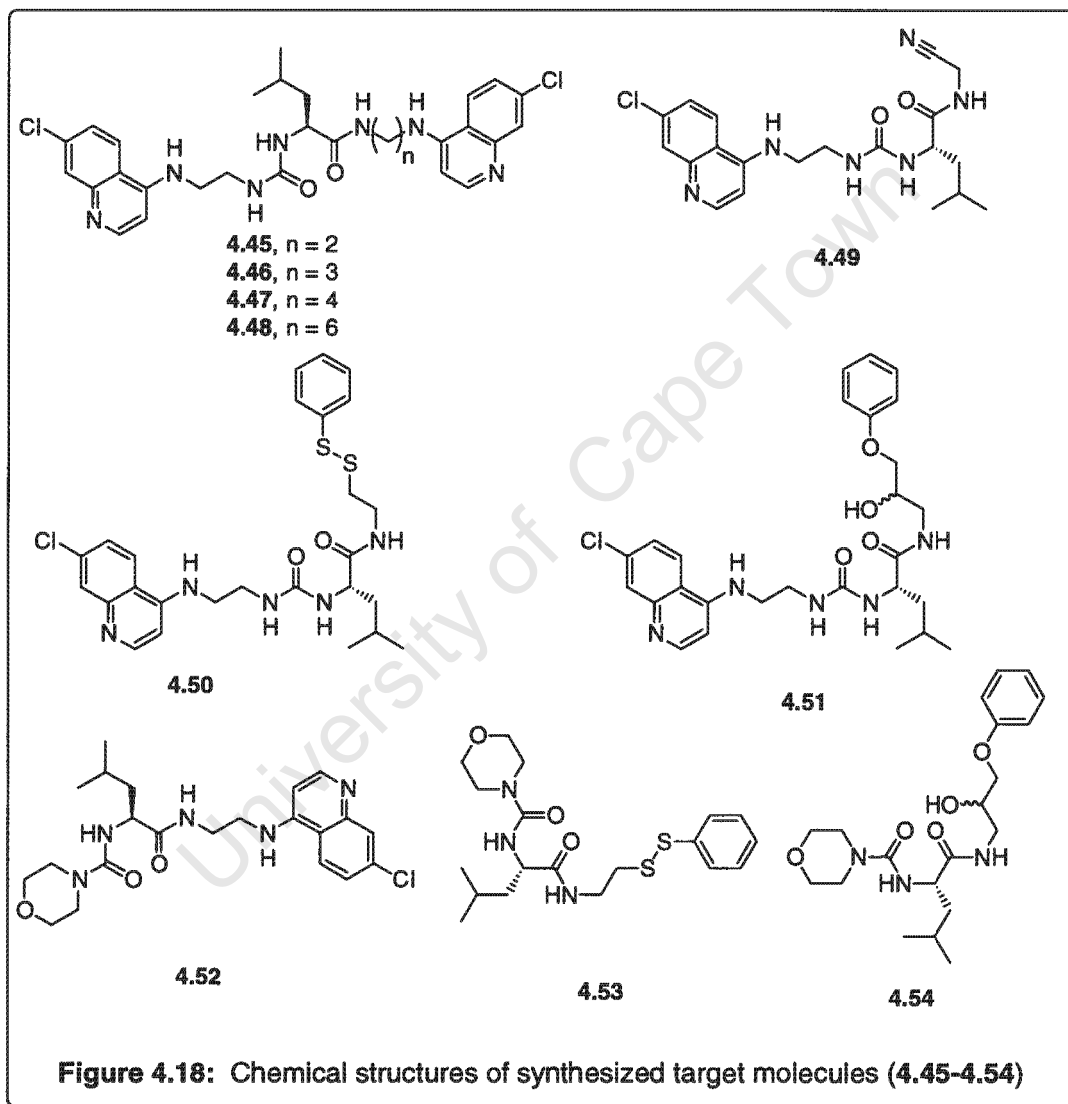


Figure 4.17: Chemical structure of α -amino alcohol, (4.26)

The ^1H NMR spectrum of (4.26) shows a characteristic signal which as a one-proton multiplet at approximately δ 4.00 and is assigned to H-3. The two pairs of diastereotopic protons CH_2 -2 and CH_2 -4 on either side of the stereogenic center would appear as doublets of doublets; however it was found that they appear as multiplets integrating for two hydrogens at δ 4.00 and 2.98 assigned to CH_2 -4 and CH_2 -2 respectively. The appearance of multiplets instead of doublets of doublets could be due to poor resolution. ^{13}C NMR also confirms the formation of the proposed structure, indicating characteristic signals to appear at δ 70.5, 70.0 and 44.0 assigned to C-4, C-3 and C-2 respectively. Further proof was provided by the low resolution mass spectrum which confirms the molecular ion as the base peak at 417 amu.

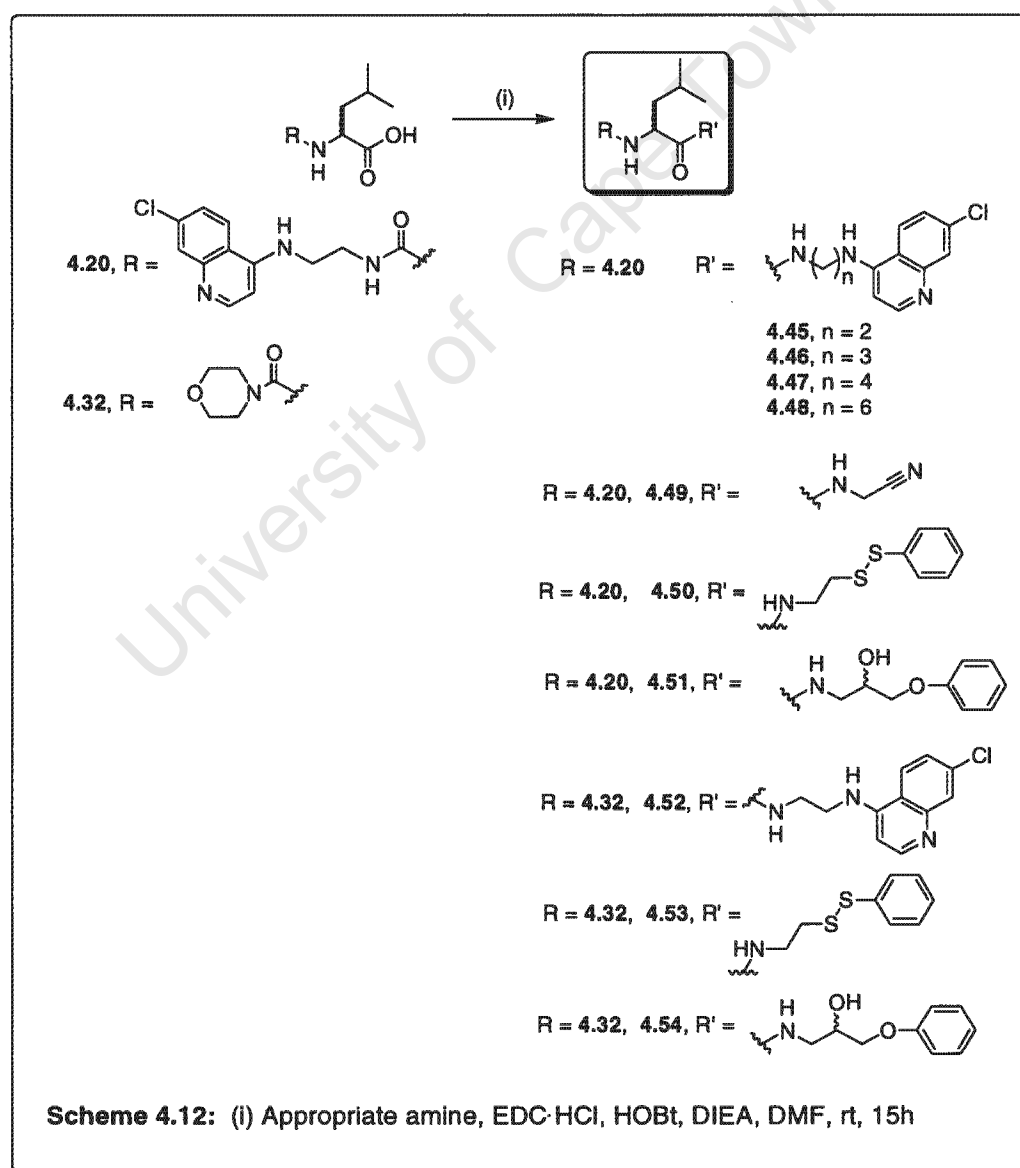
4.3.6 Synthesis of Target Molecules (4.45–4.54)

All of the compounds presented in Fig. 4.18 followed the same method of synthesis which involved a one-step peptide coupling reaction. The general synthesis of these compounds is depicted in Scheme 4.12. This reaction procedure was straightforward to perform with no complications.



4.3.6.1 General Synthesis of 4-Aminoquinoline-and-Morpholine-Derived target molecules (4.45-4.54)

Due to the insoluble nature of the starting materials and in some cases the reactants, DMF was used as the most suitable solvent, due to its high polarity. DMF was removed upon work up, by washing the organic extracts several times with water while the products were dissolved in EtOAc. Respective treatment of the carboxylic acids (4.20) and (4.32) with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloric salt (EDC-HCl) in the presence of DIEA as a base and hydroxybenzotriazole (HOBt) gave the desired target molecules (4.45-4.54).⁴⁶ Scheme 4.12 depicts the general synthesis of these target molecules (4.45-4.54).



The yields and melting points obtained for target molecules (4.45–4.54) are tabulated in Table 4.3. The yields obtained vary from low to moderate to high. Noticeably high yields for the β -amino alcohols (4.51) and (4.52) were obtained. Also, the melting points were generally high, with the lowest ones around 100 °C for the 4-aminoquinoline containing amines (4.50) and (4.52), where $n = 2$.

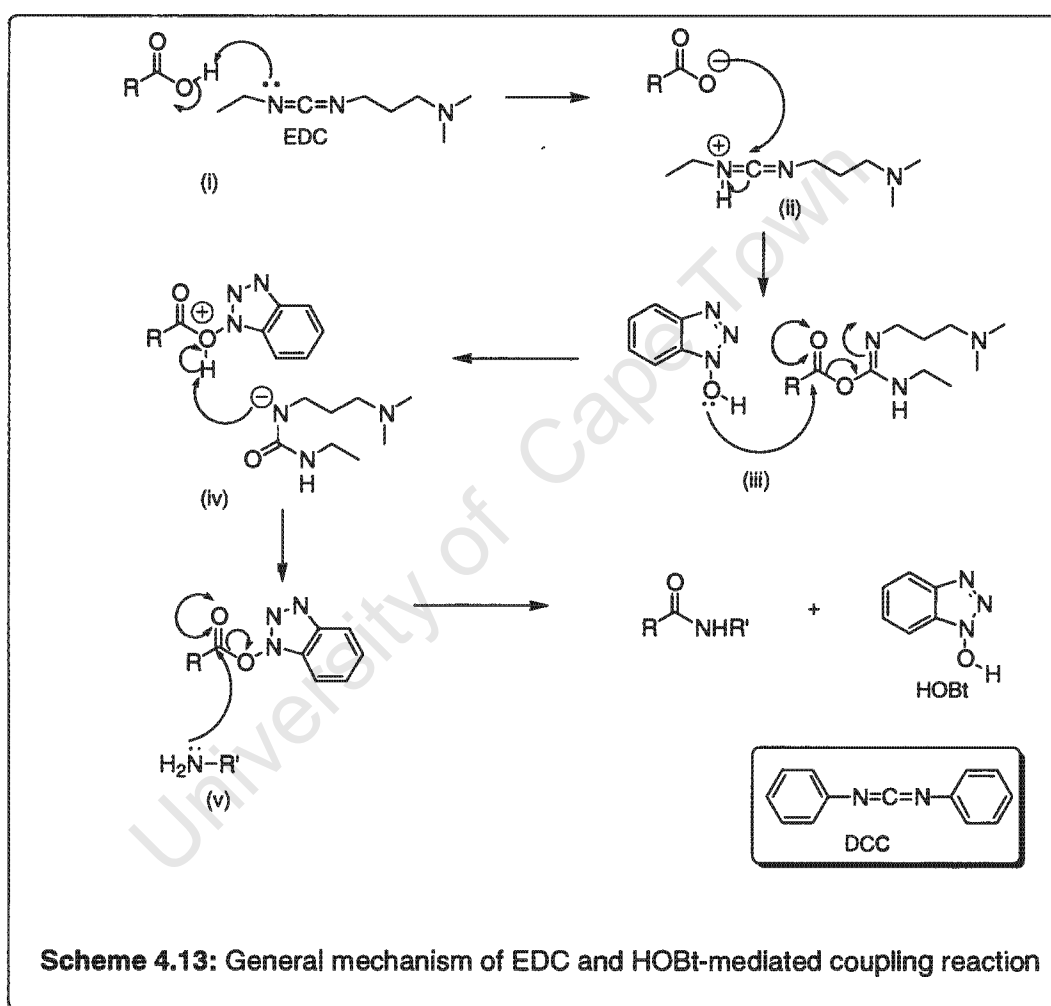
Table 4.3: Recrystallized yields and melting points of target molecules (4.45–4.54)

Compound	Yield (%)	Melting point (M.p.) (°C)
4.45	45	207-209
4.46	41	150-151
4.47	22	146-148
4.48	34	209-211
4.49	36	206-209
4.50	35	99-102
4.51	89	139-142
4.52	54	100-103
4.53	44	121-125
4.54	71	oil

4.3.6.1.1 Mechanistic Comments

Scheme 4.13 depicts the general mechanism of peptide coupling. In step (i), the nitrogen of the carbodiimide removes the proton of the acid, giving rise to the intermediate salt (ii), which is in turn attacked by the carboxylate anion to form intermediate product (iii). The lone pair of electrons of the oxygen of HOBt attacks the electrophilic carbonyl carbon producing the urea as a byproduct and then affording the intermediate product (iv). Proton transfer to the urea then renders the intermediate product (v) representing the activated carboxylate, which is attacked by a nitrogen atom of the primary/ secondary amine to form the intermediate product (iv), which undergoes proton transfer to produce the desired amide and the recycled HOBt. A number of points are worth mentioning about this reaction; (1) EDC·HCl proved to be a vastly superior coupling reagent to dicyclohexyldiimide (DCC) and was thus the reagent of choice. Utilizing EDC·HCl as a coupling reagent produces the water soluble urea as a by product, which can be

removed upon work up. However, the urea byproduct obtained from using DCC as a coupling reagent is not water soluble and requires column chromatography for purification purposes (Scheme 3.13). (2) HOBt which intercepts the activated ester (intermediate iii) is used to prevent racemization by preserving the chiral integrity of the cystyl moiety of the molecule and it also enhances the rate of reaction. Recycled HOBt (Scheme 4.13) is water soluble and can be easily removed by water washings during the work up procedure.



All the ^1H NMR spectra obtained were in agreement with the proposed structures. Fig. 4.19 depicts a ^1H NMR spectrum of the bisquinoline target molecule, (4.48). The ^1H NMR spectra of the bisquinoline series showed the same general trends of peaks. Some of the signals assigned to the quinoline protons of the two quinoline rings overlap, and appear at the same chemical shift value. The ^1H NMR spectrum of (4.48) gave the following characteristic signals; five D_2O

exchangeable *NH* peaks, which appear at δ 7.88, 7.45, 7.28, 6.28 and 6.88 respectively. Ambiguous assignments were made for four of these *NH*s which appear as broad singlets. A clear assignment was made for *NH*-6, which appears as a doublet ($J = 8.5$) due to the single neighboring influence of the methine proton at H-7. The structure was further confirmed by ^{13}C NMR, which revealed the two carbonyl signals at δ 173.8 and δ 158.2. IR spectroscopy also showed the presence of the carbonyl peaks at 1648 and 1583 cm^{-1} .

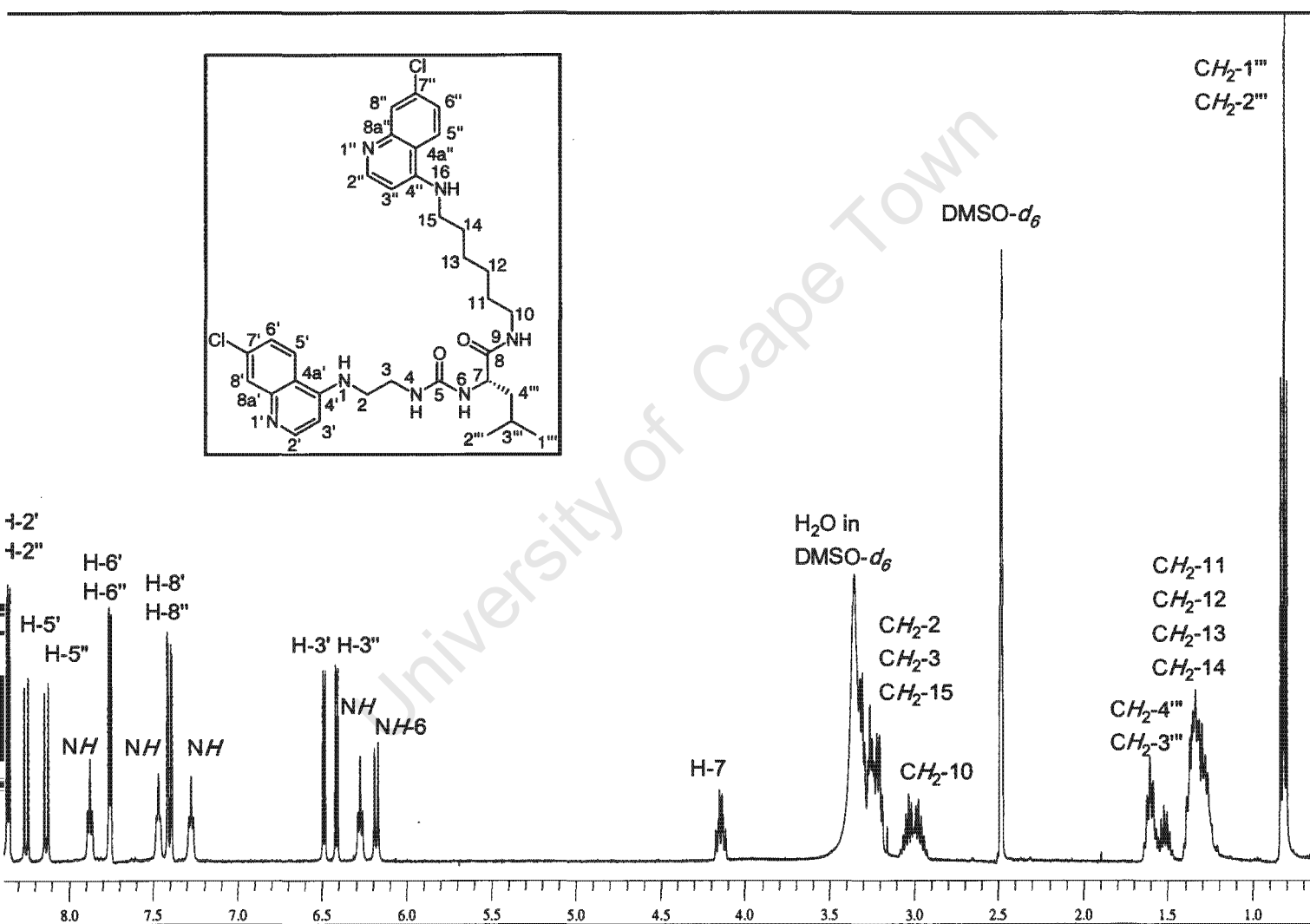
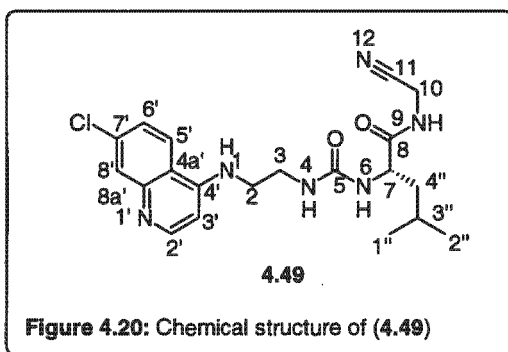
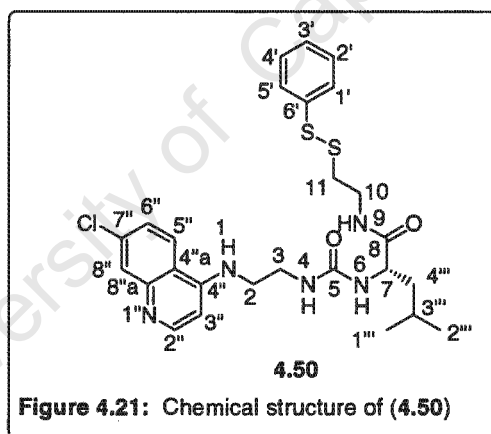


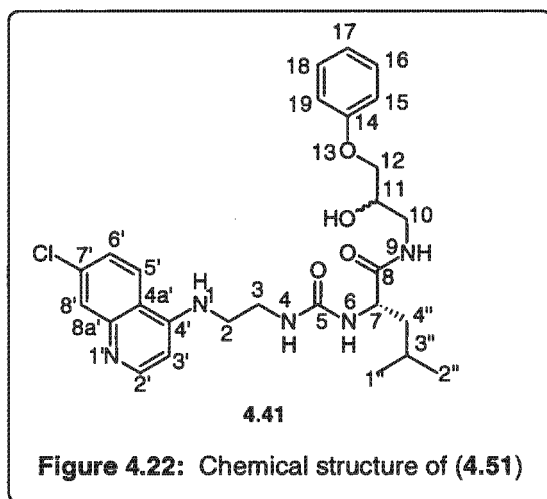
Figure 4.19: ^1H NMR spectrum of target molecule (4.48) in $\text{DMSO}-d_6$



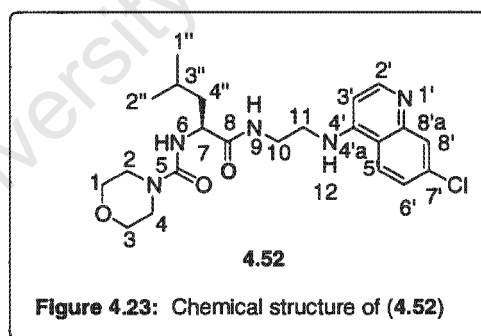
The ^1H NMR spectrum of (4.49) revealed the following characteristic signals; a one-proton multiplet at δ 4.25 assigned to H-7, a two-proton singlet at δ 4.10 assigned to CH_2 -10 and the ethylene protons, CH_2 -2 and CH_2 -3 at δ 3.52-3.44 as a four-proton multiplet. The ^{13}C NMR spectrum revealed the two carbonyl signals at δ 176.5 and δ 161.1.



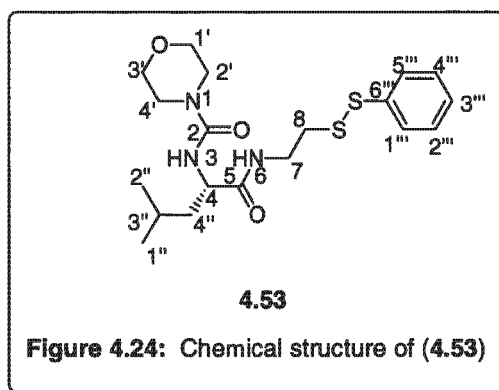
The ^1H NMR spectrum of (4.50) showed the following characteristic signals; five-aromatic-protons at δ 7.42-7.19 as a multiplet, a one-proton multiplet at δ 4.21 assigned to H-7, a six-proton multiplet at δ 3.54-3.35, assigned to CH_2 -2, CH_2 -3 and CH_2 -10 and a two-proton triplet ($J = 6.8$) at δ 2.77 assigned to CH_2 -11. The ^{13}C NMR spectrum indicated the carbonyl carbons at δ 175.0 and 160.0. The presence of the proposed structure (4.50) was further supported by mass spectroscopy analysis which clearly indicated the molecular and base peak ion at m/z 546.



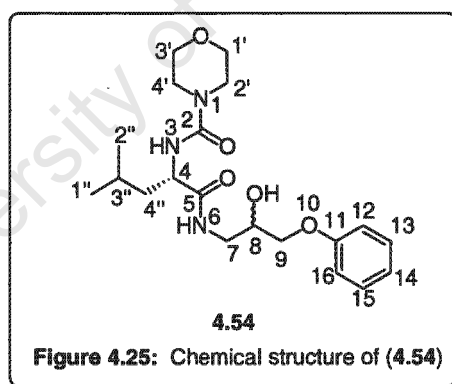
The ^1H NMR spectrum of (4.51) indicated the following characteristic signals; a one-proton multiplet at δ 4.25 assigned to H-7, a one-proton multiplet at δ 3.92 assigned to H-11, the one set of methylene protons, CH_2 -12, appear as a two-proton multiplet at δ 3.92, while the other set of methylene protons, CH_2 -10, and the ethylene protons, CH_2 -2 and CH_2 -3, appear as a six-proton multiplet at δ 3.53-3.43. The ^{13}C NMR spectrum revealed the carbonyl carbons at δ 176.6 and 160.3.



The ^1H NMR spectrum of (4.52) revealed the following characteristic signals; a one-proton broad triplet ($J = 5.2$) at δ 8.04 assigned to NH -12, a one-proton broad triplet ($J = 5.2$), at δ 7.28 assigned to NH -9, a one-proton doublet ($J = 7.6$) at δ 6.43 assigned to NH -6, one-proton multiplet at δ 4.14-4.08 assigned to H-7, a four-proton triplet ($J = 5.6$) at δ 3.47 assigned to NCH_2 -2, and NCH_2 -4, and an eight-proton multiplet at δ 3.33-3.88 assigned to OCH_2 -1, OCH_2 -3, CH_2 -10 and CH_2 -11. The ^{13}C NMR spectrum showed the carbonyl carbons at δ 174.8 and 158.0.



The ^1H NMR spectrum of (4.53) showed the following characteristic signals; a five-aromatic-proton multiplet at δ 7.45-7.15, a one-proton broad triplet ($J = 5.1$) at δ 6.59 assigned to NH -6, a one-proton broad doublet ($J = 7.8$) appears at δ 4.94 assigned to NH -3, a one-proton multiplet at δ 4.26-4.19 assigned to H-4, a four-proton triplet ($J = 5.1$) at δ 3.47 assigned to NCCH_2 -2' and NCCH_2 -4', a two-proton multiplet at δ 3.52-3.40, assigned to CH_2 -7, a four-proton triplet ($J = 5.1$) at δ 3.30-3.26 assigned to OCH_2 -1' and OCH_2 -3' and a two-proton multiplet at δ 2.81-2.75 assigned to CH_2 -8. The ^{13}C NMR spectrum showed the carbonyl carbons at δ 174.0 and 164.2.

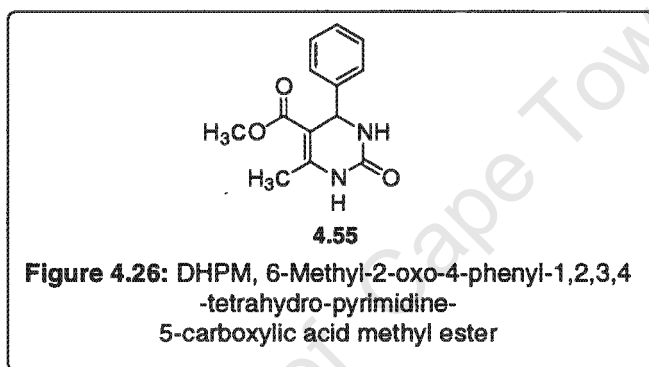


The ^1H NMR spectrum of (4.54) showed the following characteristic signals; a two-proton multiplet at δ 7.29-7.25 assigned to H-12 and H-16, a three-proton multiplet at δ 6.97-6.88 assigned to H-13, H-14 and H-15, a one proton multiplet at δ 4.31 assigned to H-4, a one-proton multiplet at δ 4.12 assigned to H-8, a two-proton doublet ($J = 5.7$) assigned to CH_2 -9, a four proton triplet ($J = 4.8$) at δ 3.64 assigned to NCCH_2 -2', and NCCH_2 -4', a six-proton multiplet at δ 3.38-3.28 assigned to OCH_2 -1', OCH_2 -3' and CH_2 -7 and a two-proton broad singlet at δ 2.19 assigned to NH and O-H . The ^{13}C NMR spectrum revealed carbonyl carbons at δ 174.8 and 158.2.

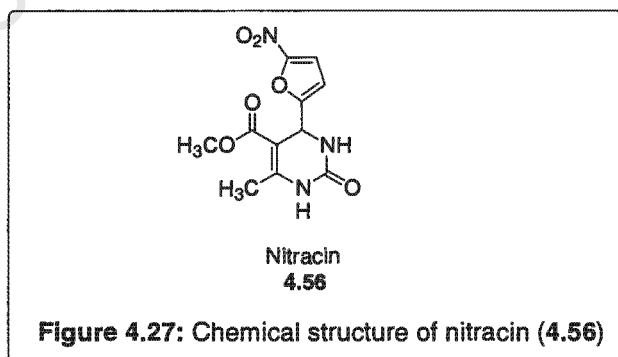
4.4 4-Aminoquinoline containing compounds *via* the Biginelli Multi-Component (MCR) Reaction

This section describes the synthesis and characterization of novel 4-aminoquinoline dihydropyrimidinone-based compounds *via* the Biginelli multi-component reaction (MCR). The envisaged heterocyclic privileged scaffold presents multiple positions onto which pharmacophores with known antimalarial activity can be appended.

4.4.1 Biological Significance of Dihydropyrimidinones (DHPMs) and their derivatives

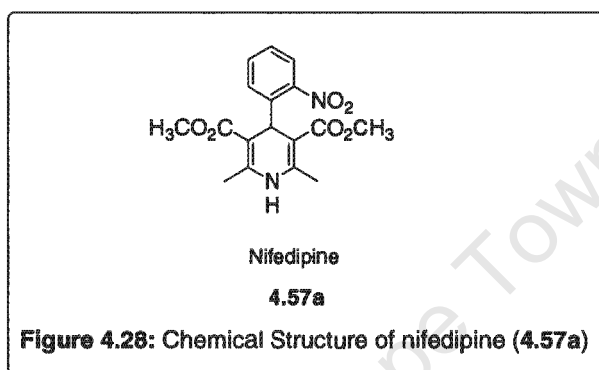


As early as the 1940's DHPMs of type (4.55, Fig. 4.26) were shown to possess antiviral activity. Eventually, the nitrofuryl-substituted analog nitracin (4.56) was developed, which displayed good activity against the viruses of the trachom group, in addition to showing modest antibacterial activity, (Fig. 4.27).⁴⁷

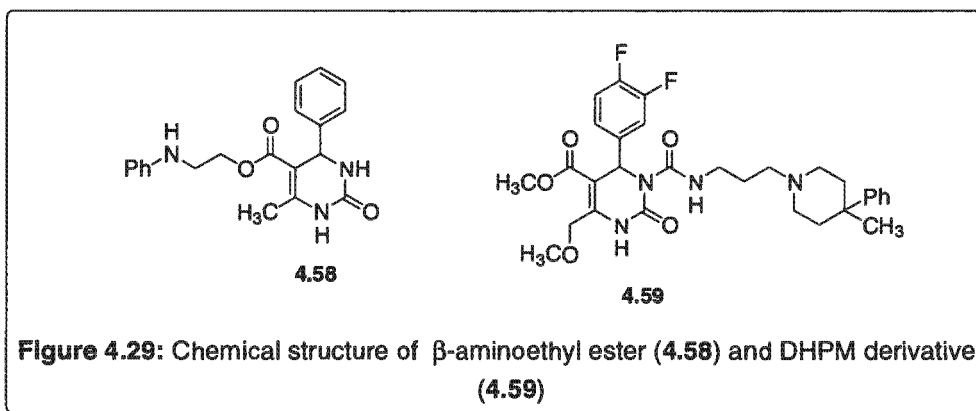


Since the 1980's interest in dihydropyrimidones has increased significantly due to the remarkable pharmacological properties associated with this heterocyclic scaffold.⁴⁸ It was

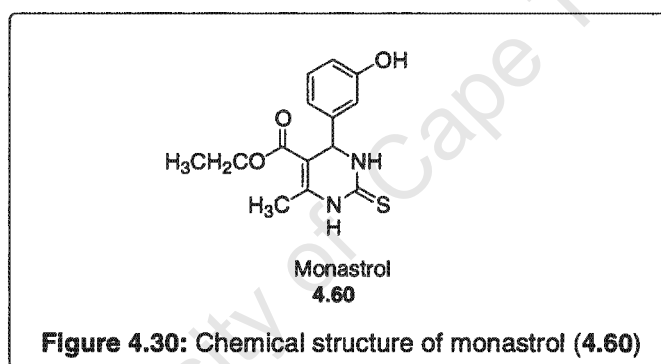
established that interest stems from the apparent structural similarity of DHPMs to the well-known dihydropyridine (DHP) calcium channel modulators.⁴⁸ It was also shown that DHPMs exhibit similar pharmacological properties to DHP calcium modulators of the nifedipine type (Fig. 4.28). The DHP, nifedipine (4.57) are the most studied class of organic calcium channel modulators. Since 1975 it has been used for the treatment of cardiovascular diseases, such as hypertension, cardiac arrhythmias or angina.⁴⁹



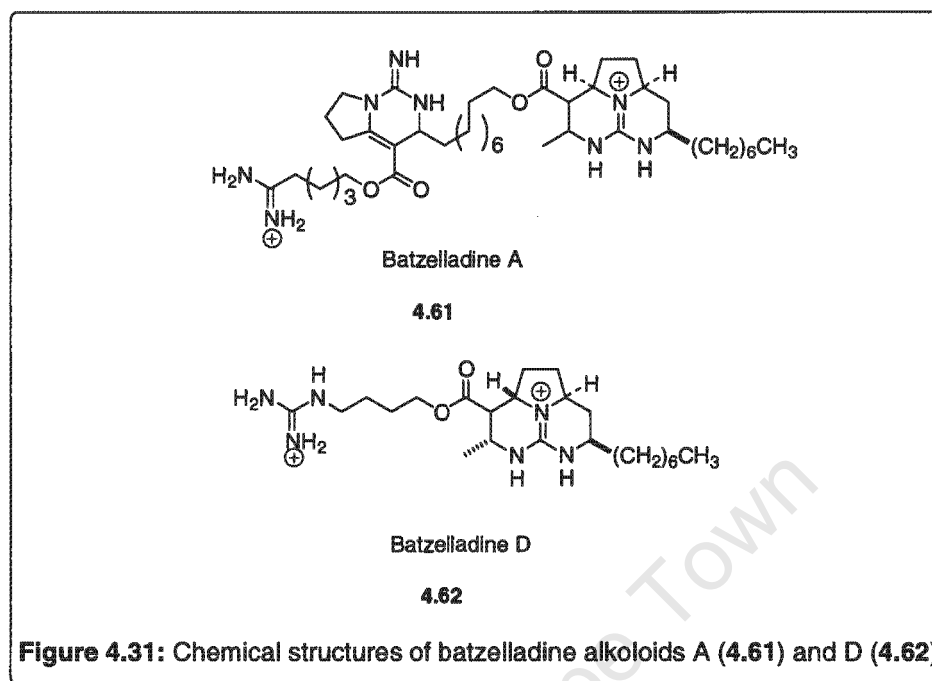
In 1978, the cardiovascular activity of DHPMs was first recognized by Khanina *et al*⁵⁰, who reported the moderate hypertensive activity and coronary dilatory properties of β -aminoethyl esters of type (4.58). *In vivo* studies of the DHPM derivative (4.59) have suggested that DHPMs of this type have significant potential to relieve the symptoms of benign prostatic hyperplasia (BHP) without eliciting effects on the cardiovascular system. BHP is the progressive enlargement of the prostate, resulting in a number of obstructive and irritative symptoms,^{51, 52} Figure 4.29.



A common strategy for cancer therapy is the development of drugs that interrupt the cell cycle during the mitosis stage. Compounds that perturb microtubule shortening (depolymerization) or lengthening (polymerization) cause arrest of the cell cycle in mitosis due to perturbation off the normal microtubule dynamics necessary for chromosome movement. A variety of such drugs that bind to tubulin and inhibit spindle assembly are currently used in cancer therapy (e.g. paclitaxel, docetaxel).⁵³ The structurally rather simple DHPM monastrol (4.60, Fig. 4.30), was identified as a novel cell-permeable molecule, that blocks normal bipolar mitotic spindle assembly in mammalian cells and therefore, cause cell cycle arrest.⁵⁴ Monastrol blocks mitosis by specifically inhibiting the motor activity of the mitotic kinesin Eg5, a motor protein required for spindle bipolarity. Monastrol is the only cell-permeable molecule currently known to specifically inhibit mitotic kinesin Eg5 and can therefore be considered as a lead for the development of new anticancer drugs.



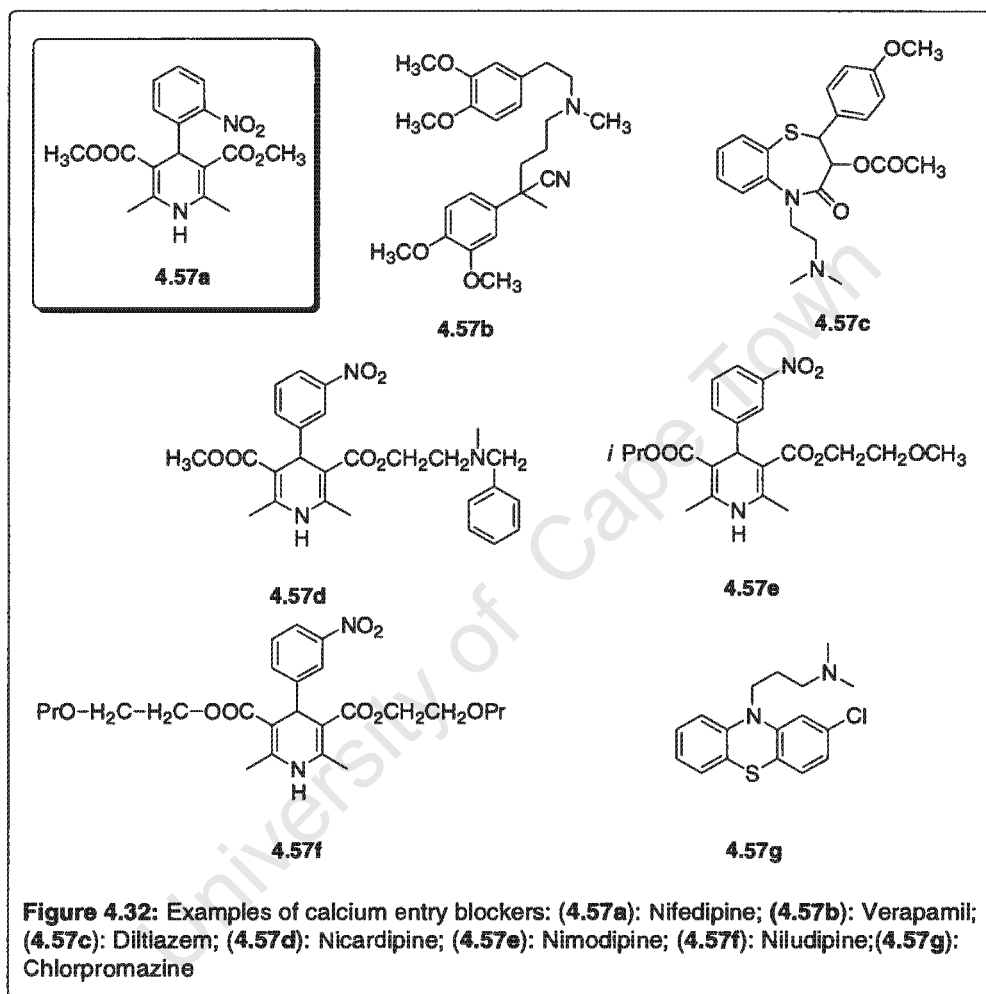
More recently, several natural marine alkaloids containing the dihydropyrimidine-5-carboxylate core and possessing interesting biological activities have been isolated, among them the batzelladine alkaloids A (4.61) and D (4.62), Fig. 4.31, which were found to be potent HIV gp-120-CD4 inhibitors.⁵⁵



4.4.2 Reversing Agents in the Treatment of Drug-Resistant Malaria

Most strategies in malaria control focus on the various approaches to overcome the dilemma of drug resistance. A rational approach is the search of drugs named reversing agents or chemosensitizers which in combination with the existing antimalarials, could reverse drug resistance.⁵⁶ Drug resistances in malaria have been identified as the “ability of a parasite strain to survive and/or to multiply despite the administration or absorption of a drug given in a dose equal to or higher than those usually recommended but within the limits of tolerance of the subject.”⁵⁷ This phenomenon is not specific to malaria but may be observed in cancer and other parasitic diseases such as bilharziasis, amoebiasis, trypanosomiasis and leishmaniasis. In cancer chemotherapy, the results of various studies have led to suggest that a decrease in drug accumulation within the tumor cells is the mechanism of anticancer drug resistance and cross resistance.⁵⁸ In the mechanism of chloroquine (CQ) resistance, there are also strong evidences through several investigations that resistant malaria parasites accumulate less CQ in their food vacuole than sensitive parasites so that CQ concentration does not reach the required dose level to kill the parasite resistant strains, (see section 1.42, page 17).⁵⁹⁻⁶² Researchers suggested that the expulsion mechanism of CQ may proceed *via* P-glycoprotein mediated pump efflux (see section 1.42, page 17) or through vacuole proton leak which is unfavorable to the

diffusion of CQ into the parasite vesicle.^{63, 64} Interest in the synthesis of reversing agents was initiated the observation that verapamil (**4.57b**, Fig. 4.32) and various Ca^{2+} antagonists (**4.57a**, **c-g** Fig. 4.32) used in the treatment of cardiovascular diseases such as hypertension, arrhythmia and angina pectoris reverse drug resistance in cancer.

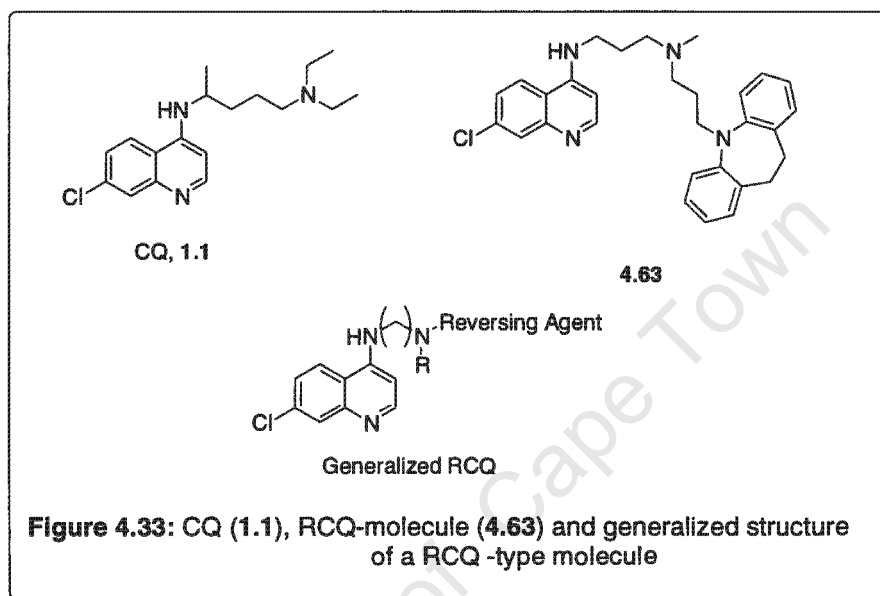


It has been established that these calcium antagonists enhance drug accumulation within the tumor cell and consequently restore drug sensitivity to resistant cell by reducing the inhibitory concentration to that of the susceptible cells.⁶⁵ Similar patterns of cross-patterns of cross-resistance have been identified between drug resistance in cancer and malaria, which suggest that Ca^{2+} channel blockers might also restore CQ sensitivity to resistant *Plasmodium* strains. A study by Martin *et al.*⁶⁶ showed that the combination of CQ and verapamil at subinhibitory concentrations markedly enhances *in vitro* the CQ effectiveness against cloned CQ-resistant strains of *P. falciparum* W2 (from Indochina and IEC-306 from Brazil) but had no action on CQ-

sensitive strain D6 (from West Africa). However, verapamil alone had a weak intrinsic antiplasmodial effect with an IC_{50} of 6-8 $\mu\text{g/ml}$. On the other hand verapamil (4.57b, Fig. 4.32) and other Ca^{2+} channel blockers diltiazem and nifedipine (4.57c and 4.57d, Fig. 4.32) have been shown to be effective in the reversing CQ resistance *in vivo* in mice using a resistant line of *Plasmodium chabaudi*.⁶⁷ However, this observation was in contrast to the results obtained *in vitro* with CQ-sensitive strains of *P. falciparum* in which verapamil and several Ca^{2+} channel blockers, when combined with CQ, fail to increase CQ susceptibility in CQ-sensitive strains of *P. falciparum*.⁶⁸ Two main hypothesis have been proposed for the mechanism of CQ resistance: the first one was based on the calcium ions in which a model was used to demonstrate the cycling of calcium ions to control the pH gradient over which CQ may diffuse⁶⁹ and the second hypothesis postulated the existence of P-glycoprotein CQ carrier in the resistant parasite (alluded earlier) similar to that proposed in drug resistant tumor cells.⁷⁰ Kyle *et al.*⁷¹ evaluated interactions between various Ca^{2+} modulators and quinoline-containing antimalarials against the resistant strain W2 (from Indochina) and the sensitive line D6 (Sierra Leone). It was found that verapamil and two analogs, diltiazem, chlorpromazine, (4.57c and 4.57g, Fig 4.32) and one analog act synergistically *in vitro* with CQ, desethyl-CQ, quinine and quinidine against the resistant strain. Interestingly, it was found that chlorpromazine (4.57g, Fig 4.32) at concentrations much lower than those clinically achievable increases markedly the susceptibility of clone W2 to CQ, desethyl-CQ, quinine and quinidine. In contrast, no effect was observed in the sensitive strain.

4.4.2.1 Chloroquine-Derived Reversal Agents (RA) or 'Reversed Chloroquine' (RCQ) Molecules in the Treatment of Drug-Resistant *P. falciparum*

More recently the effectiveness of reversal agents in drug-resistant malaria was demonstrated by linking a CQ-like moiety to a reversal agent (RA) entity which may overcome CQ-resistance, (Fig. 4.33).⁷²



Such a design would deliver imipramine, (RA-like portion) in a 1:1 ratio with the quinoline moiety, (CQ-like-portion) thereby lowering the dose required when the two molecules are given separately since both low digestive vacuole (DV) pH and binding of CQ to haeme and/or haemozoin in the DV would favor both components in the DV.⁷³ In addition to promoting drug accumulation in the DV, it was speculated that the RA component would interfere with export from the DV of the CQ moiety by the mutated CQ-resistant PfCRT (a DV membrane protein, alluded in section 1.4.2, page 17).^{62, 66, 74-77} Together, these effects could allow a curative dose against CQ-resistance strains to be much lower than that of its components given separately, with resultant reduced cost and toxicity. The RCQ molecule, (4.63) demonstrated *in vitro* inhibitory activities against both CQ-resistant and CQ-resistant strains at low-nM concentrations. A preliminary study in mice showed oral efficacy against *P. chabaudi* and the absence of apparent toxicity making this approach feasible for further investigation.

4.4.3 Multi-Component reactions (MCRs)

MCR strategies offer significant advantages over conventional linear-type syntheses since it provide easy and rapid access to large libraries of organic compounds with diverse substitution patterns.⁷⁸

4.4.3.1 Defining MCRs

MCRs are convergent reactions, in which three or more starting materials react to form a product in which all or most of the atoms contribute to the newly formed product.⁷⁹ The starting materials do not react all at the same time but in a series of ordered steps. In an MCR, a product is assembled according to a cascade of elementary chemical reactions. Thus, there is a network of reaction equilibria, which all finally flow into an irreversible step yielding the product. The challenge is to conduct an MCR in such a way that the network of pre-equilibrated reactions channel into the main product and do not yield side products. MCRs can be basically grouped into three separate classes, considering the reversibility of the reactions leading to intermediary products P^1 , P^2 ,.... and the final product P^N ,⁷⁹ Table 4.4.

Table 4.4: The basic types of MCRs

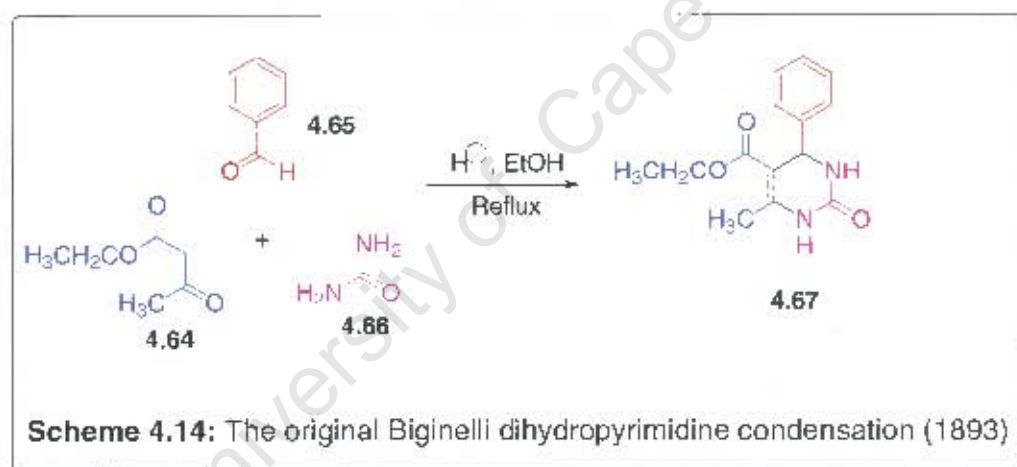
MCR Types	General reaction scheme
Type I	$A + B \rightleftharpoons P^1 + C \rightleftharpoons P^2 + D \dots \rightleftharpoons P^N$
Type II	$A + B \rightleftharpoons P^1 + C \rightleftharpoons P^2 + D \rightleftharpoons \dots \xrightarrow{\text{O}} P^N$
Type III	$A + B \longrightarrow P^1 + C \longrightarrow P^2 + D \longrightarrow \dots \xrightarrow{\text{O}} P^N$

In type I MCRs the starting materials, intermediates and products are in constant equilibrium. In this case yields between 0-100% is obtainable as different states of equilibrium can exist causing most products to occur as mixtures with the starting materials and/or intermediates. Type II MCRs are advantageous since the total equilibrium is shifted in the direction of the products by the irreversible step. Type III MCRs reactions are sequences of irreversible elementary reactions that rarely occurs in preparative synthetic chemistry. These reactions include biochemical reactions in the living world, many of whose irreversibility occur from either thermodynamic circumstances or due to a combination of endothermal and exothermal reactions.

The products of multicomponent reactions are dependent on the reaction conditions: viz., solvent, temperature, catalyst, concentration, the kind of starting materials and functional groups. Such considerations are of particular importance in relation to the design and discovery of novel MCRs.⁸⁰

4.4.4 Biginelli Multicomponent Synthesis of DHPMs

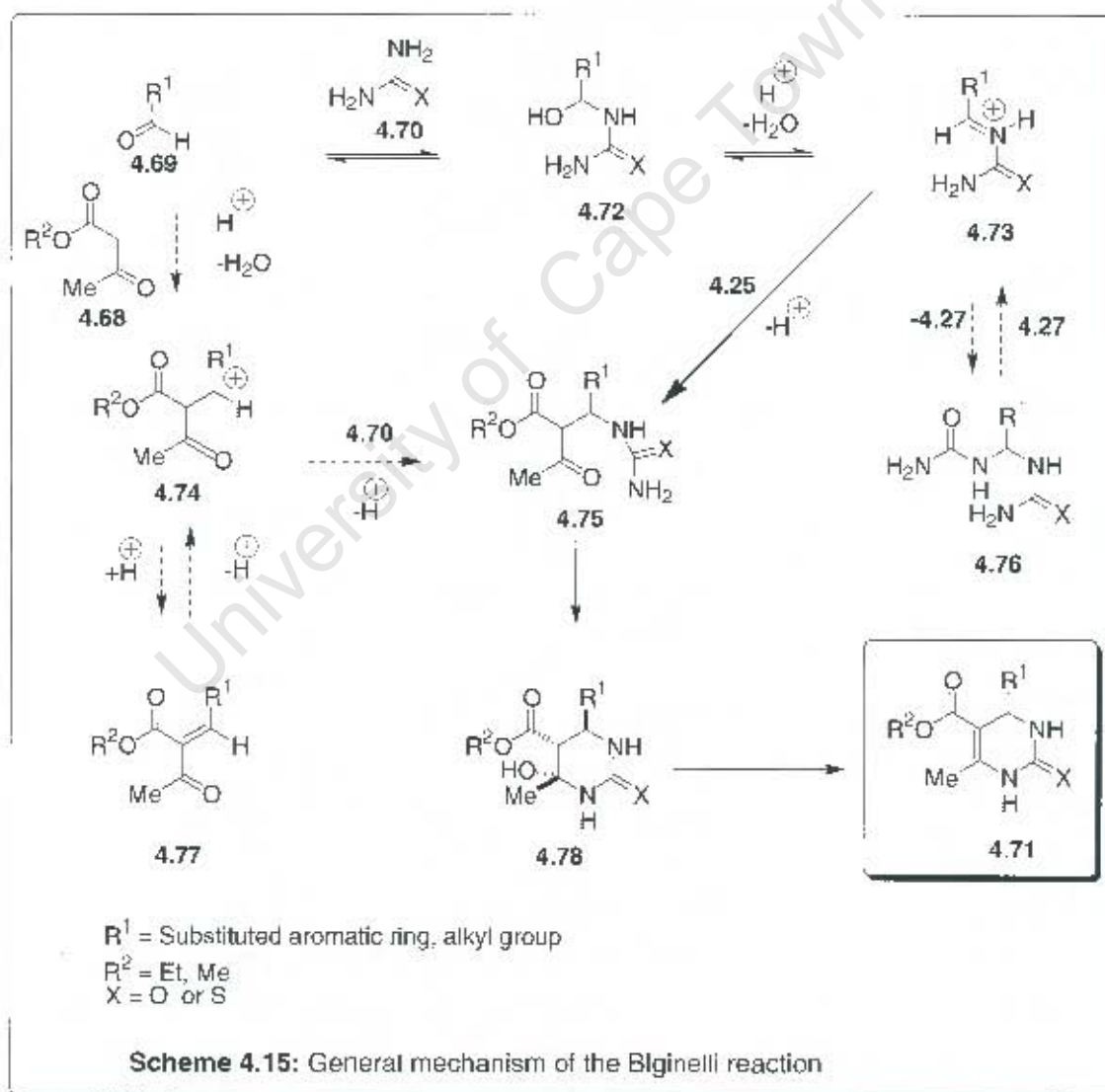
In 1893, Pietro Biginelli reported on the acid catalyzed cyclocondensation reaction between ethyl acetoacetate (4.64), benzaldehyde (4.65), and urea (4.66).⁸¹ The reaction was carried out by simply heating a mixture of the three components dissolved in ethanol with catalytic amounts of HCl under reflux. The product of this novel, one-pot, three-component synthesis that precipitated on cooling of the reaction mixture was identified correctly by Biginelli as 3,4-dihydropyrimidin-2 (1*H*)-one (4.67) (Scheme 4.6).⁸²



4.4.4.1 Development of Biginelli Mechanism

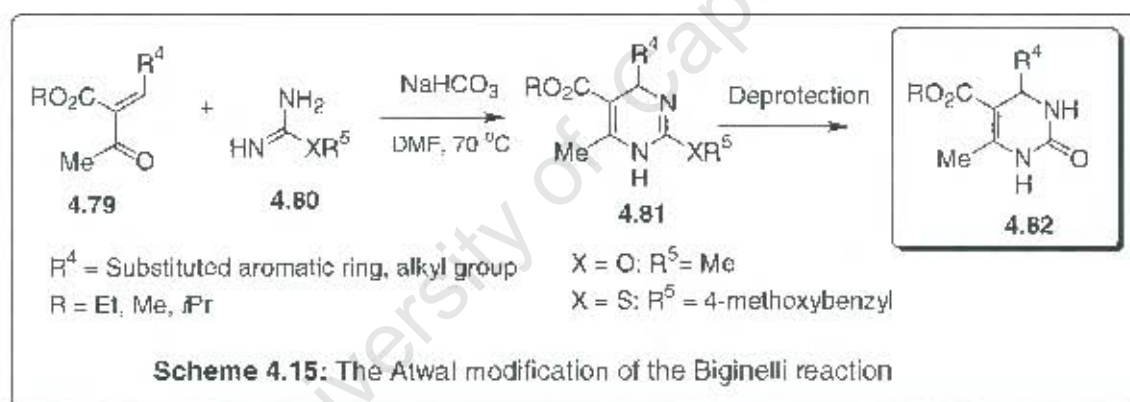
The mechanism of the Biginelli reaction has been under investigation for many years. Early work done by Folkers and Johnson suggested that bisureide (4.76), the primary bimolecular condensation product of benzaldehyde (4.69) and urea (4.70) is the first intermediate in this reaction.⁸³ In 1973 Sweet and Fissekis proposed a different pathway and suggested that carbonium ion (4.74), produced by acid-catalyzed aldol reaction of benzaldehyde (4.69) with ethyl acetoacetate (4.68) is formed in the first and rate-limiting step of the Biginelli condensation (4.69 → 4.74 → 4.75).⁸⁴ The mechanism was reinvestigated in 1997 using ¹H/¹³CNMR spectroscopy trapping experiments. It has since been established that the key step in this

sequence involves the acid-catalyzed formation of an *N*-acyliminium ion intermediate of type (4.73) from the aldehyde (4.69) and urea (4.70) precursors, Scheme 4.15.⁸⁵ Interception of the iminium ion (4.73) by ethyl acetoacetate (4.68), presumably through its enol tautomer, produces an open-chain ureide (4.75) which subsequently cyclizes to the hexahydropyrimidine (4.78). Acid-catalyzed elimination of water from (4.78) ultimately leads to the final DHPM product (4.71). The mechanism can therefore be classified as an α -amidoalkylation, or more specifically as an α -ureidoalkylation. The alternative "carbenium ion mechanism" (4.69 $\rightarrow$ 4.74 $\rightarrow$ 4.75)³⁴ does not constitute a major pathway; however small amounts of enone (4.77) are sometimes observed as a byproduct.⁸⁵

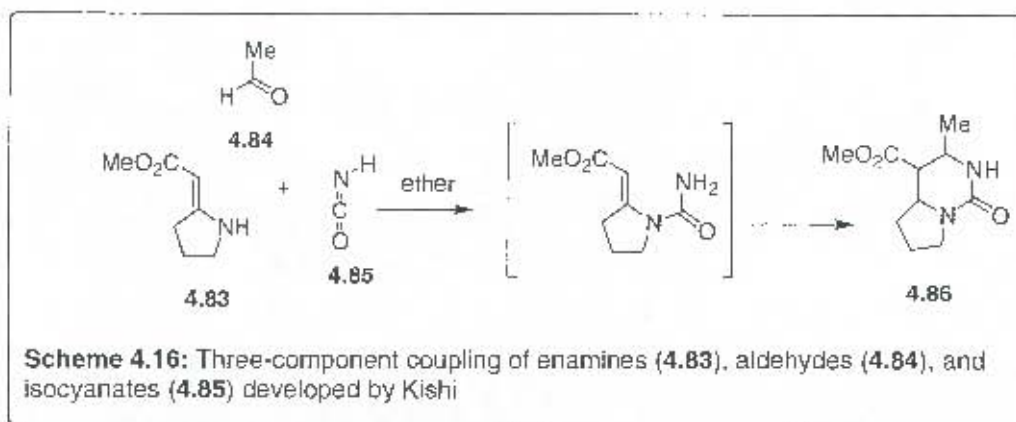


4.4.5 Alternative Synthetic Strategies

Apart from employing the traditional Biginelli three-component protocol, a few alternative synthetic methods also lead to DHPMs. However, these protocols lack the experimental and conceptual simplicity of the Biginelli method. An alternative method was reported in 1987 by K. Atwal and coworkers called the Atwal modification of the Biginelli reaction.⁸⁶⁻⁸⁸ Here, an enone of type (4.79) is first condensed with a suitable protected urea or thiourea derivative (4.80) under mildly basic conditions. Deprotection of the resulting 1, 4-dihydropyrimidine (4.81) leads to the desired DHPMs (4.82). Although this method requires prior synthesis of enone (4.79), its reliability and broad applicability makes it an attractive alternative to the traditional one-step Biginelli condensation. In addition, 1, 4-dihydropyrimidines (4.81) can be alkylated or acylated regiospecifically at N-3 by various electrophiles, thereby making pharmacologically important DHPM analogues readily accessible, (Scheme 4.15).⁸⁶

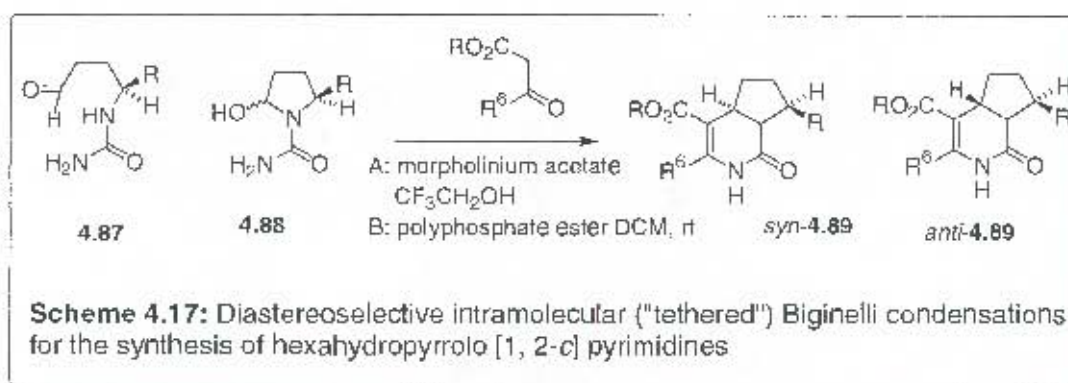


A conceptually different approach to DHPM analogues was developed by Kishi and co-workers. The trimolecular room temperature condensation of an enamine (4.83), acetaldehyde (4.84), and isocyanic acid (4.85) provides the bicyclic DHPM derivative (4.86), Scheme 4.16). With some modification, this strategy was initially employed towards a stereospecific^{89, 90} and later an enantioselective⁹¹ synthesis of the natural product saxitoxin. Recent investigations by Elliot and coworkers have shown that substituted isocyanates can also be employed in this method.⁹²⁻⁹⁷

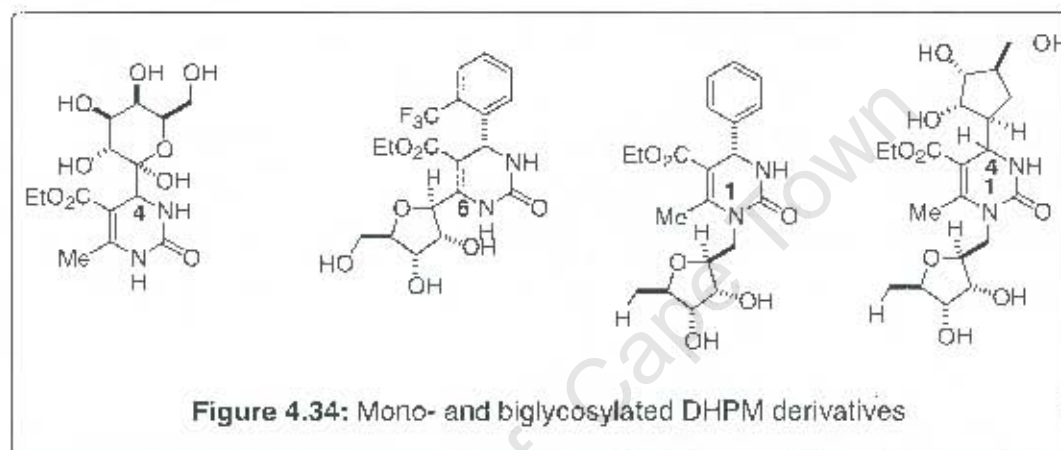


4.4.6 Related MCRs

There are a number of related processes in which similar building blocks are employed but the structure of the final product differs from a Biginelli DHPM. Special variants of the Biginelli reaction are the intramolecular or so-called tethered Biginelli condensations developed by Overman and coworkers, in which the aldehyde and urea components are linked together in one building block (Scheme 4.17).⁹⁸⁻¹⁰⁸ The "tethered Biginelli strategy" has been used in the synthesis of various polycyclic guanidinium marine natural products such as the batzelladine alkaloids, which all have the hexahydropyrrolo[1,2-*c*]pyrimidine fragment in common and display a range of interesting biological activities as mentioned earlier, (Fig. 4.31). For example, condensation of the chiral hemiaminal precursor (4.88) obtained from the pre-synthesized aldehyde derivative (4.87) with a suitable β -ketoester leads to the desired hexahydropyrrolo [1, 2-*c*] pyrimidine scaffold (4.89).⁹⁸ Importantly, depending on the reaction conditions (A or B), both the *syn* and *anti* stereoisomers of (4.89) can be obtained in high selectivities.



Dondoni and coworkers have prepared a number of dihydropyrimidone conjugates where the sugar residues were installed at the N1, C4, or C6 position in the monoglycosylated derivatives and at both the C4 and C6 positions in the biglycosylated products (Fig 435).^{108, 110} The mono- and biglycosylated products were obtained as mixtures of diastereomers with good to excellent selectivities due to asymmetric induction by the sugar residue in the formation of the C4 stereocenter of the DHPM ring.



4.4.7 Asymmetric Biginelli Synthesis

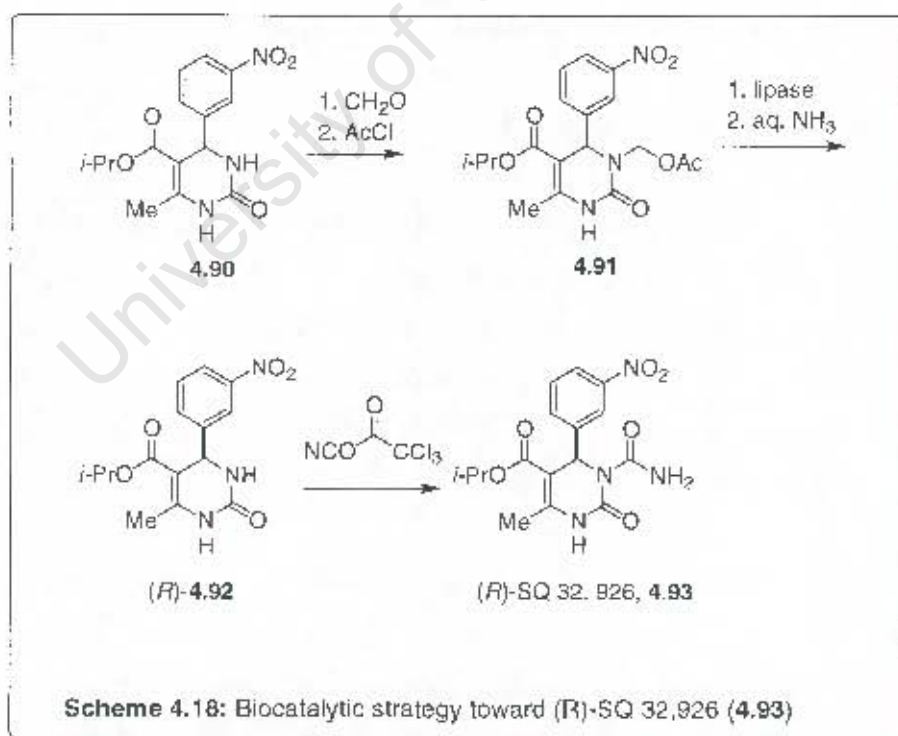
DHPMs of the Biginelli type are inherently asymmetric molecules and the influence of the absolute configuration at the stereogenic center at C4 on biological activity is well documented.¹¹¹ In the calcium channel blocker SQ 32926 (4.93), for example it is exclusively the (*R*)-enantiomer that carries the therapeutically desired antihypertensive effect.¹¹² In some related DHPM analogues, the individual enantiomers have demonstrated opposing antagonist/agonist pharmacological activity.¹¹³

Resolution strategies have been chosen as the method to obtain enantiomerically pure DHPMs. Optically pure DHPMs were obtained by resolution of corresponding racemic 5-carboxylic acids *via* fractional crystallization of the corresponding diastereomeric α -methylbenzylammonium salts.¹¹⁴ The absolute configurations of those acids were proven by single-crystal X-ray analysis of suitable salts. Analytical separation of DHPM derivatives can be readily achieved by enantioselective HPLC using a variety of different chiral stationary phases

(CSPs),¹¹⁵ including “designer-made” CSPs that are based on the principle of “reciprocal” recognition of chirality using the immobilized DHPM derivatives.¹¹⁶ Alternatively, chiral separation can be performed by capillary electrophoresis (CE) with for example, quaternary ammonium- β -cyclodextrin as chiral buffer addition.¹¹⁷

As an alternative to the chemical resolution methods, a biocatalytic strategy towards the preparation of enantiopure (*R*) and (*S*)-SQ 32,926 (**4.93**) was developed. The key step in the synthesis is the enzymatic resolution of an N3-acetoxymethyl-activated dihydropyrimidinone precursor by *Thermomyces lanuginosus* lipase.¹¹⁸ The readily available racemic DHPM (**4.90**) was hydroxymethylated at N3 with formaldehyde, followed by standard acetylation with acetyl chloride. The resulting N3-acetoxymethyl-activated DHPM (**4.91**) was then cleaved enantioselectively by *Thermomyces lanuginosus* with excellent selectivity (*E*>200).

Degradation of unreacted (*R*)-(**4.91**) with aqueous ammonia produced (*R*)-(**4.92**) which was converted into the desired target structure (*R*)-SQ 32,926 in one step by N3-carbamoylation with trichloroacetyl isocyanate,¹¹⁹ (Scheme 4.18).



4.4.8 Privileged Substructures in Drug Discovery

Earlier work done by various researchers led to the conclusion that a common pharmacophore existed throughout diverse drug classes, and that specificity resulted from secondary binding groups attached to the basic pharmacophore. The original concept of privileged substructures was put forth by Evans¹²⁰ and more recently reviewed by Patchett.¹²¹ Evans defined the term privileged structure as a "single molecular framework able to provide ligands for diverse receptors". Further elaboration of the privileged substructure was postulated to lead to selectivity toward a specific target receptor. Currently the term has been used to describe ligands that bind multiple, unrelated proteinaceous enzymes and receptors with high affinity.¹²²

An investigation of the structural diversity has suggested that most of the privileged substructures known thus far are natural products or derivatives of natural products. It was suggested that the binding abilities of these substructures to multiple targets are a result of evolutionary selection over time. It was also proposed that binding may result from biosynthetic processes used in the preparation. Biosynthesis of natural products involves intermediates that bind to proteins to catalyze their assembly. In this process preferential selection for physiochemical features, favoring protein binding would occur.

Non-natural organic scaffolds, for example, biphenyls, arylpiperidines, benzopyrans, 1, 4-dihydropyridines and dihydropyrimidines have the ability to selectively bind diverse receptors just like natural products.¹²⁰

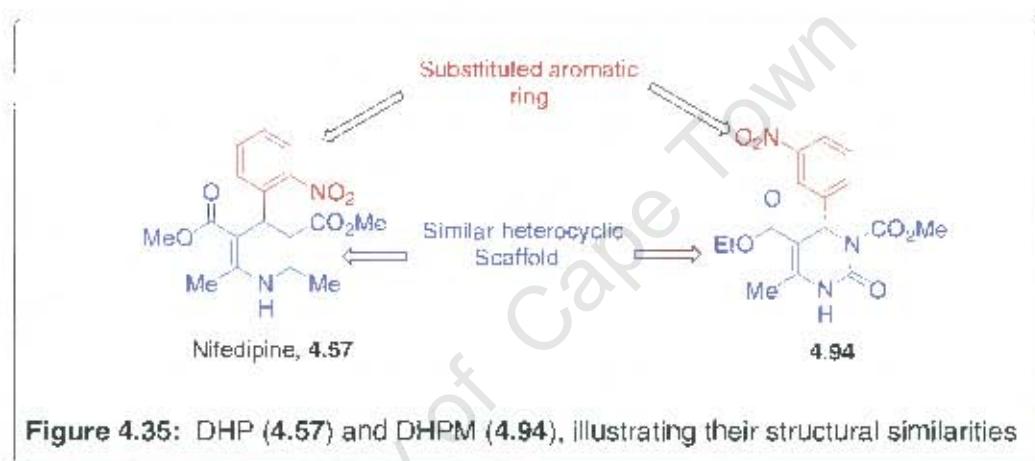
4.4.8.1 DHPMs as Privileged Substructures in Drug Discovery

DHPMs are very attractive compounds in drug discovery because: (i) they possess a privileged scaffold since several natural marine products with interesting biological activities exemplified by anti-HIV alkaloid batzelladine contain the DHPM core; (ii) the related dihydropyridine (DHP) scaffold is present in known CQ resistance reversal agents; (iii) the simplicity of the three component MCRs facilitates rapid exploration of structure-activity relationship (SAR).

4.4.9 Rationale for Drug Design

4.4.9.1 Based on Calcium Channel Modulator Reversing Agents

It has been demonstrated that RAs in combination with existing antimalarials could reverse drug resistance. In terms of cancer, it was established that nifedipine-type calcium channel modulators, are able to serve as RAs by enhancing drug accumulation within tumor cells and hence restore drug sensitivity to resistant cells. It was suggested that nifedipine-type calcium channel modulators in combination with CQ could act in a similar manner to restore CQ sensitivity to resistant *Plasmodium* strains in malaria (see section 4.4.2).⁵⁶



Based on these findings we decided to turn our focus on the synthesis of a similar type of compound, the DHPMs which displayed comparable biological activities. Biological interests in DHPMs stemmed from the most studied class of organic calcium channel modulators, the 4-aryl-1, 4-dihydropyridines (DHPs). DHPMs closely mimic the DHP scaffold, (Fig. 4.35) and later it was found that they are potent calcium blockers and also exhibit similar levels of cardiovascular activity. Figure 4.36 presents examples of DHPs and DHPMs analogs with potent calcium modulatory activity.

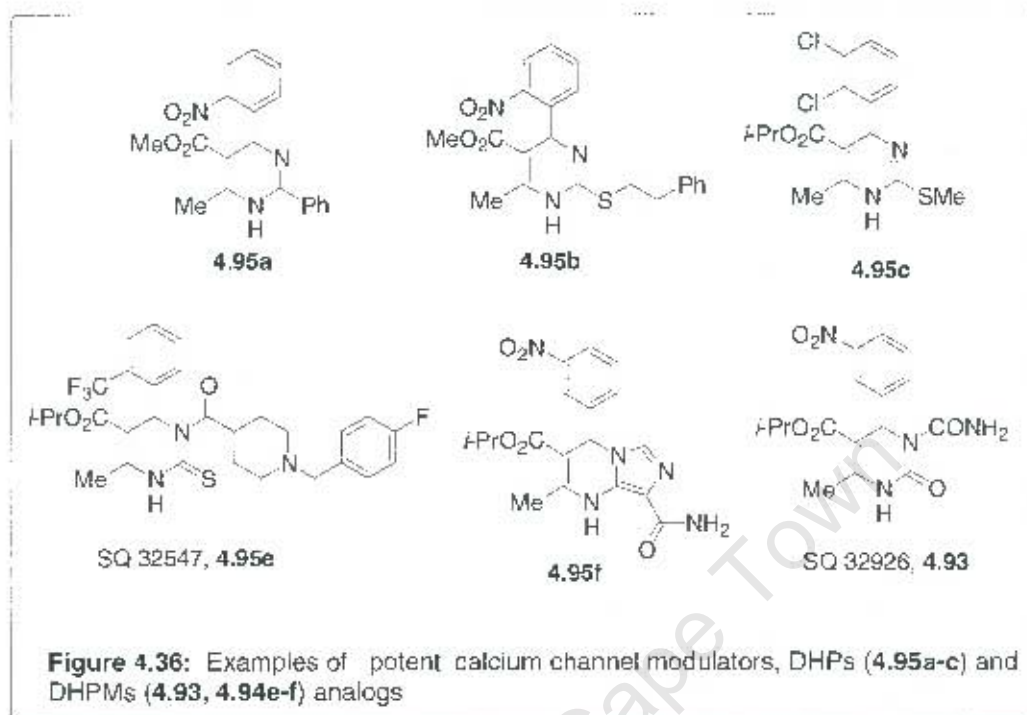
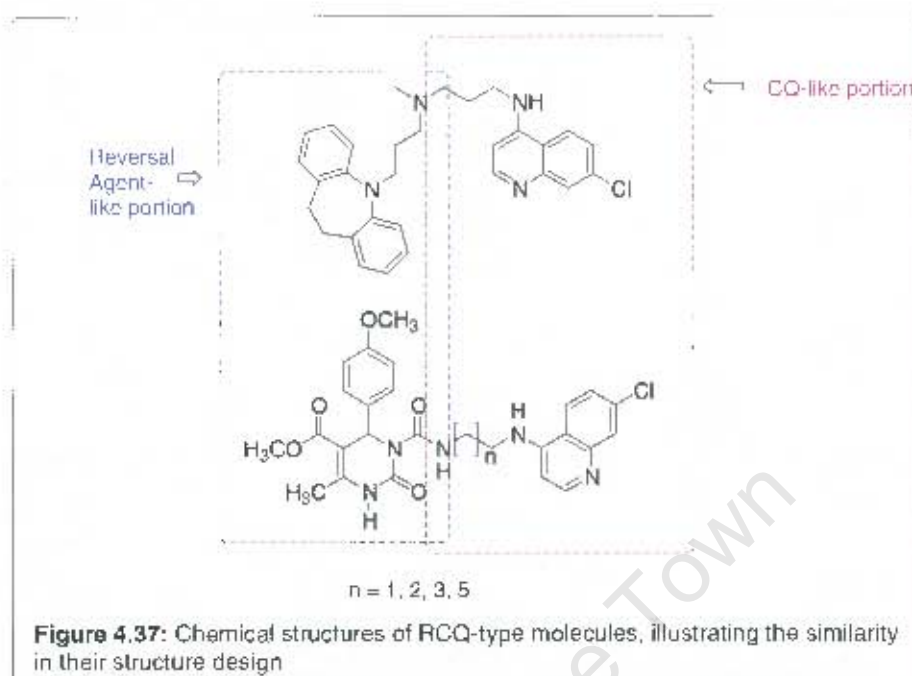


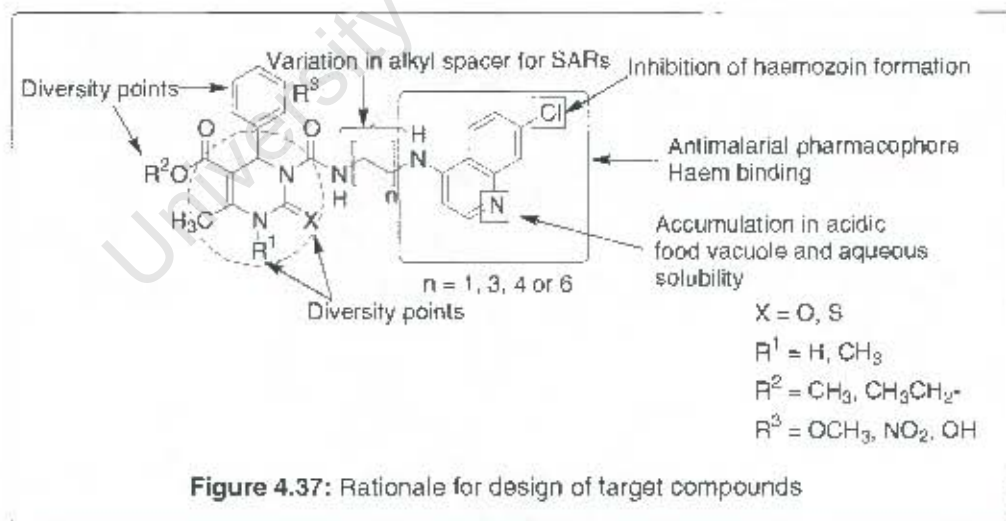
Figure 4.36: Examples of potent calcium channel modulators, DHPs (4.95a-c) and DHPMs (4.93, 4.94e-f) analogs

4.4.9.2 Based on 'Reversed Chloroquines' (RCQs)

Furthermore we reasoned that the same effect can be achieved by linking the DHPM analog, which will serve as the RA to a CQ analog in one molecule instead of separately. Recently the effectiveness of this approach was demonstrated by Burgess and coworkers.⁷² Their work entail the design and synthesis of 'reversed chloroquines' (RCQs) in which a CQ-like-moiety and RA were linked to yield a highly effective drug against malaria (see section 4.4.2.1).



4.4.9.3 Based on the Important Chemical Features



The design of this class of compounds was based on the following rationale:

- (i) The basic quinoline nitrogen may assist in the accumulation of the compounds in the acidic vacuole *via* pH trapping and may also enhance the aqueous solubility of the

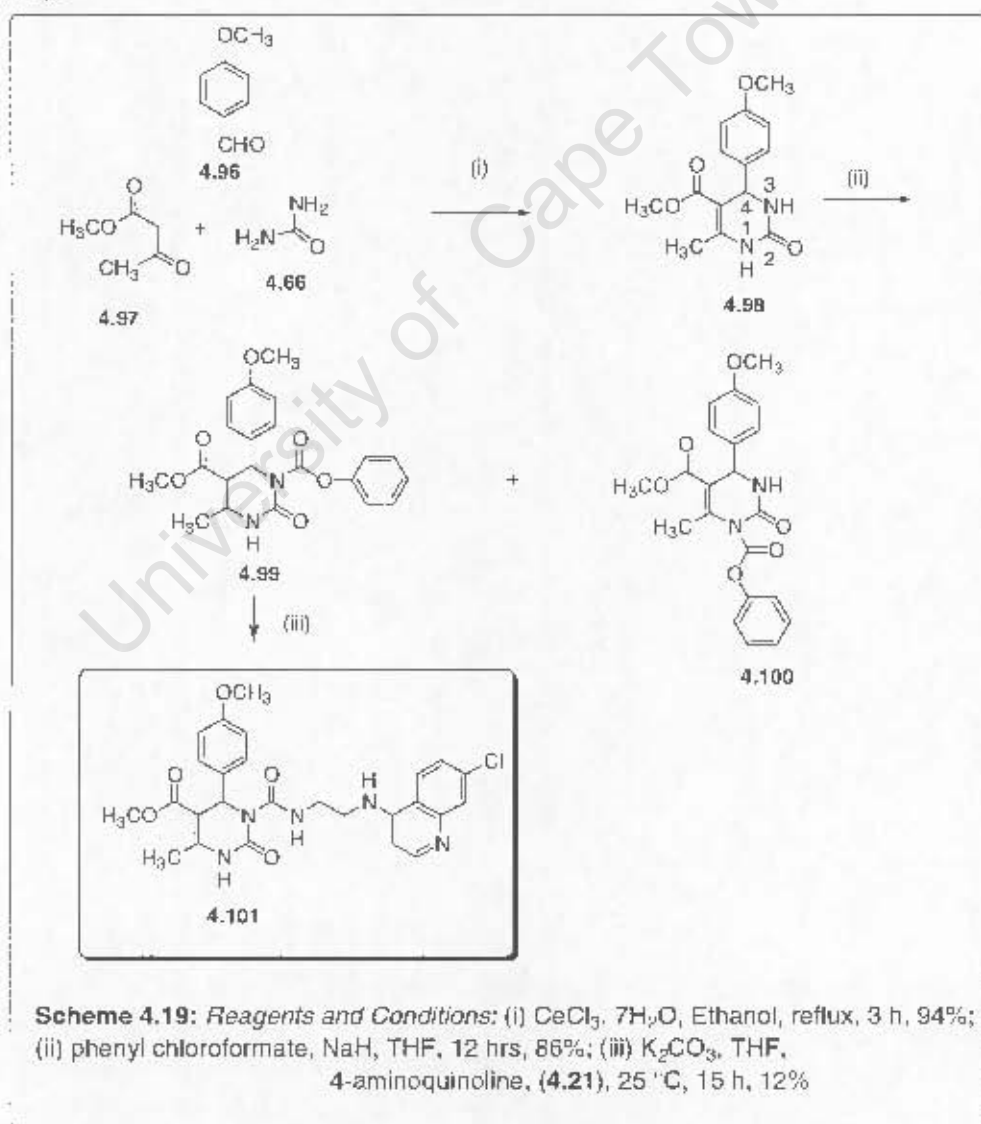
compounds *via* salt formation.

- (ii) Presence of the 4-amino-7-dichloroquinoline antimalarial pharmacophore which is important for haem binding and inhibition of haemozoin formation.
- (iii) Variation in the alkyl chain length may help to circumvent chloroquine resistance and lipophilicity.
- (iv) The DHPM heterocyclic scaffold provides a number of diversity points which creates the opportunity for SARs which may ultimately assist in the search for novel drugs.

University of Cape Town

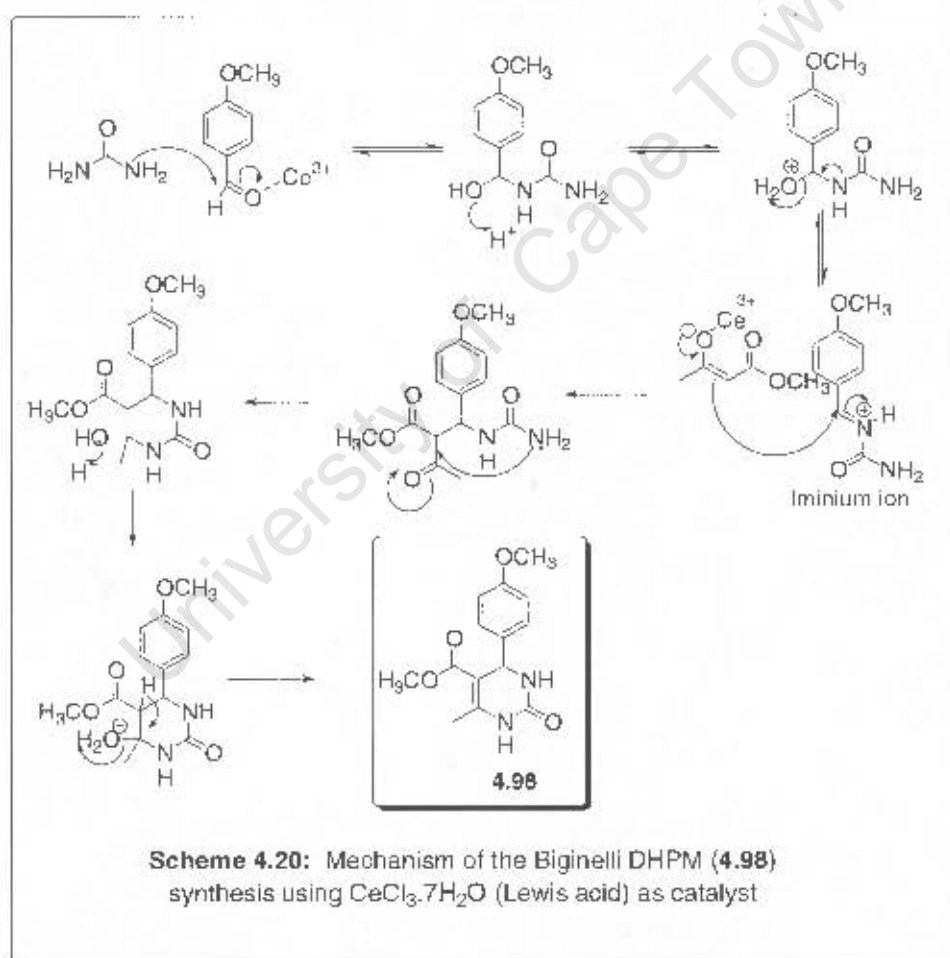
4.4.10 Synthesis of DHPM (4.101)

The first step involve a one-pot reaction between the aldehyde (4.96), β -ketoester (4.97) and urea (4.67) in the presence of a catalytic amount of $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ to afford the Biginelli dihydropyrimidinone (DHPM) (4.98).¹²³ To introduce a carbonyl group at N3, DHPM (4.98) was reacted with phenyl chloroformate in the presence of NaH as base to produce the carbamates (4.99) and (4.100) respectively.¹²⁴ The yield in this reaction was optimized by increasing the molar equivalence of both reactants, NaH and acid chloride and by rising the temperature. In the final reaction step the carbamate (4.99) was reacted with the 4-aminoquinoline, (4.21, Scheme 4.2, page 70) to produce the desired target compound (4.101)¹²⁴ in a low yield of 12%, (Scheme 4.19).



4.4.10.1 Mechanistic Details

The mechanism of the DHPM (**4.98**) synthesis is illustrated in Scheme 4.20. The first step involves coordination of the CeCl_3 Lewis acid to the aldehyde carbonyl, thereby enhancing the electrophilicity of the carbonyl carbon and facilitating imine formation with the urea nitrogen. The second step proceeds in a similar fashion to the first, with nucleophilic attack of the Lewis acid-coordinated ketone resulting in the formation of a second imine-like functionality. DHPM (**4.98**) is obtained in a further three intermediate steps involving intramolecular cyclisation of the nitrogen lone-pair and ester carbonyl, followed by protonation of the hydroxyl group at C-6, and finally elimination of the proton α to the ester carbonyl and water at C-6.¹²³



The intermediate products (**4.98**- **4.100**) and the target product (**4.101**) were characterized by IR, NMR, mass spectrometry and elemental analysis. The ^1H NMR analysis of intermediate products (**4.99**) revealed the following signals; one D_2O -exchangeable nitrogen proton as a

singlet at δ 9.09 assigned to $NH-1$ and one D_2O -exchangeable nitrogen protons to appear as a singlet δ 7.60 ppm assigned to $NH-3$. The observation of a stronger deshielding effect of the $NH-1$ proton is due to electronic influence of the conjugated enamine system, Fig. 4.38.

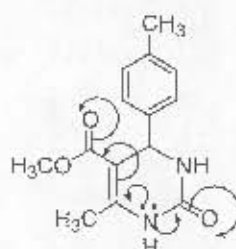
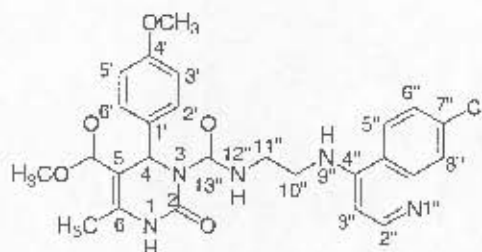


Figure 4.38: Electronic influence of neighboring groups

The ^1H NMR spectra depicted in Fig. 4.40 (a) and (b) of intermediate products (4.99) and (4.100) revealed the following signals for the D_2O -exchangeable nitrogen protons; a one proton singlet at δ 7.80 assigned to $NH-3$ and a one proton singlet at δ 8.60 assigned to $NH-1$ respectively. The ^1H NMR spectrum of intermediate products (4.100) revealed the $NH-3$ signal formally at δ 7.60 shifted slightly downfield to δ 7.80 and the disappearance of the signal due to $NH-1$. The ^1H NMR spectrum of intermediate products (4.99) revealed the $NH-1$ signal formally at δ 9.09 shifted upfield to δ 8.60 and the disappearance of the signal due to $NH-3$. The ^1H NMR spectrum of DHPM (4.101) indicated the following characteristic signals; one broad triplet (J 5.4) at δ 9.26 assigned to $NH-12''$, one broad triplet (J 5.4) at δ 5.90 assigned to $NH-9''$ and a singlet at δ 6.49 assigned to $NH-1$, which appeared formally at δ 8.60 in the intermediate product (4.99), (Fig. 4.39).



4.101

Figure 4.39: Chemical structure of compound (4.101)

The one proton doublet (J = 3.2) assigned to H-4 in DHPM (4.98) that formally appears at δ 5.10 is shifted downfield to δ 6.41 and δ 6.40 respectively in the intermediate products (4.99) and (4.101), where it appears as singlets. The high resolution mass spectrum also confirmed the

molecular formula of the target molecule (4.101) ($C_{26}H_{26}ClN_5O_5$, Found: $[M+1]^+$, 523.16212; Requires: 523.16225).

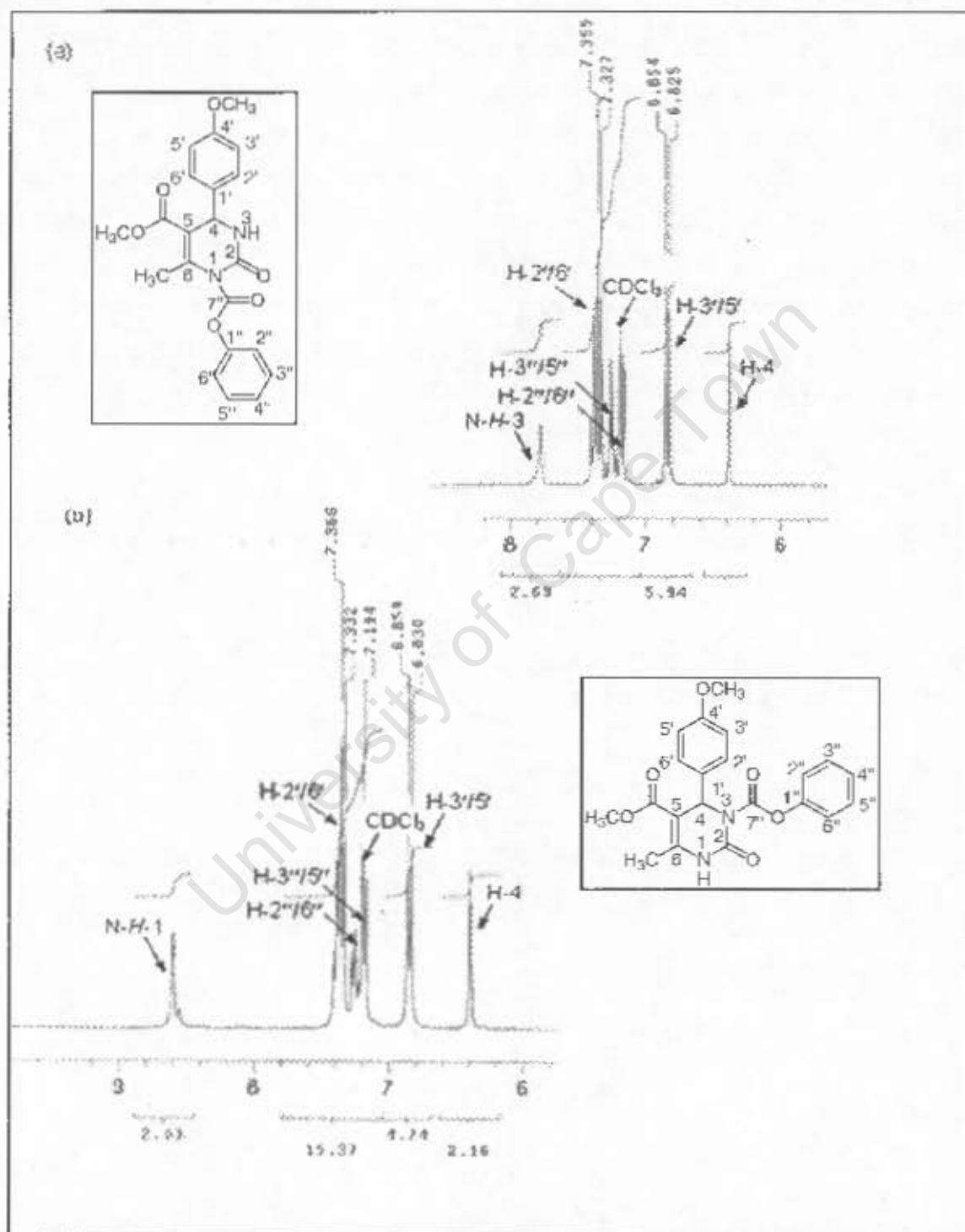


Figure 4.40: Expanded regions in the ^1H NMR spectra of two regioisomers 4.100 (a) and 4.99 (b) in $CDCl_3$

4.4.11 nSynthesis of 4-Aminoquinoline DHPM Derivatives (4.105-4.108)

In order to optimize the yield of target compound (4.101), we decided to methylate N1 to avoid the formation of regioisomers. There was a significant improvement in the yield, as expected and the series was continued with N1 methylated. Monomethylated (4.102) and the byproduct, dimethylated (4.103), (13:1) DHPMs, were obtained by methylation of DHPM (4.98) in the presence of 1.0 equivalence methyl iodide.¹²⁵ The monomethylated DHPM (4.102) was converted into monomethylated carbamate (4.104) which was subsequently converted into the respective 4-aminoquinoline DHPM derivatives (4.105-4.108). The reaction was performed under argon or nitrogen atmosphere in the presence of a weak base and the respective, (4.21-4.24, Scheme 4.2, page 71).¹²⁷ Due to the poor solubility of the diamine starting materials, DMF was chosen as the most suitable solvent, (Scheme 4.21).

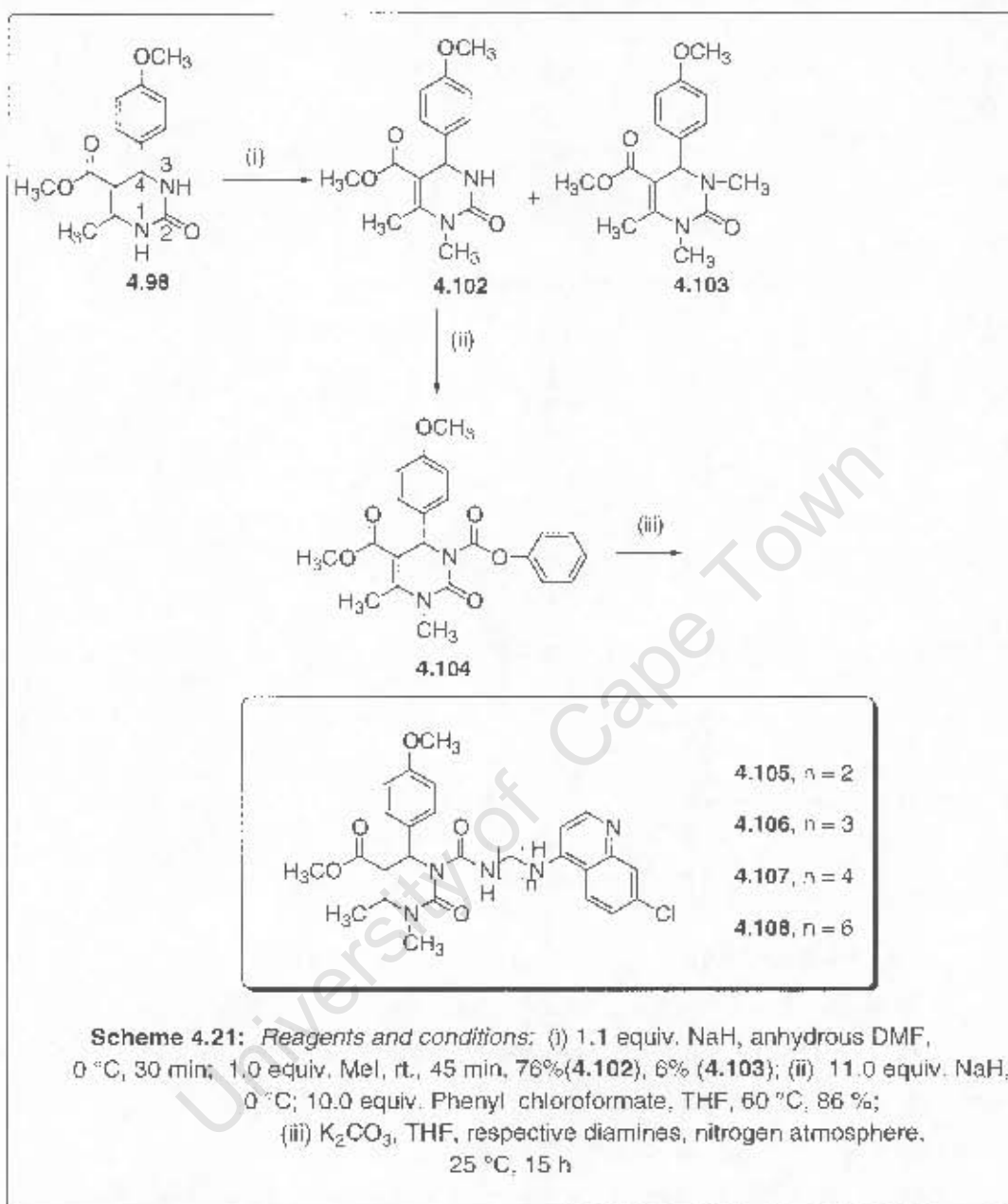
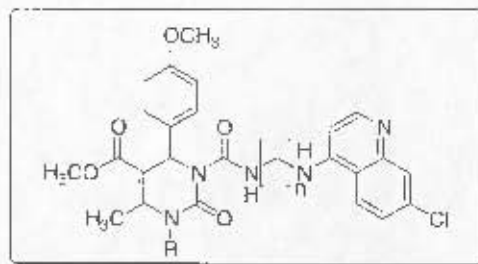


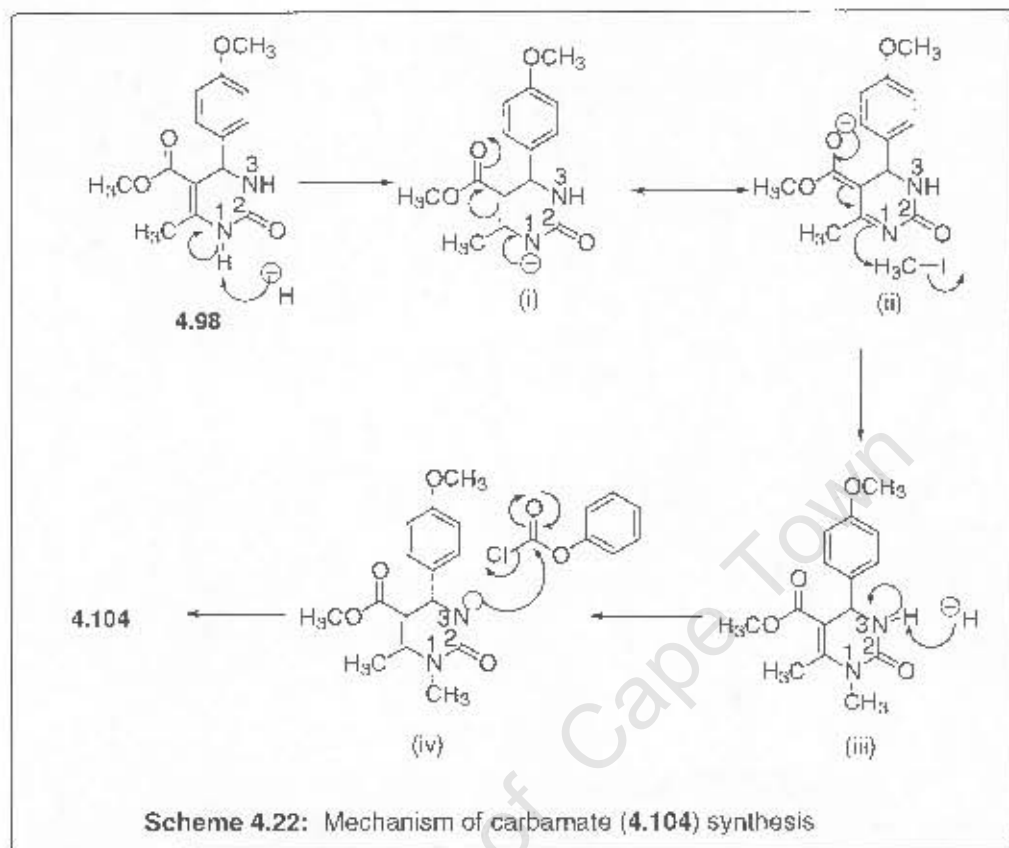
Table 4.5 shows the isolated yields and melting points of target compounds (4.105-4.108). Significant increase in the yields of target molecules (4.105-4.108) was observed compared to target molecule (4.101), attributed to the presence of a methyl group at N1 as mentioned above. Protection of N1, by methylation, eliminates formation of byproducts compelling the reaction to occur only at N3.

**Table 4.5:** Yields and melting points of DHPM derivatives (4.101 and 4.105- 4.108)

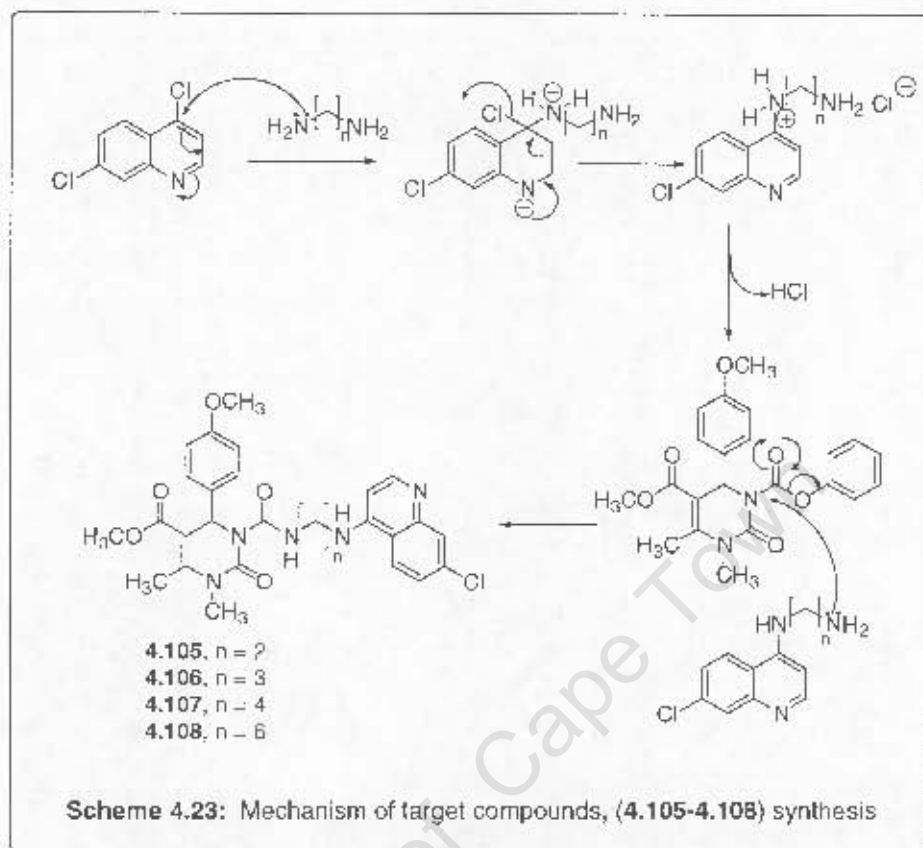
Compound	R	n	Yield	Melting Point
4.101	H	2	12%	72-74 °C
4.105	CH ₃	2	97%	174-176 °C
4.106	CH ₃	3	78%	120-123 °C
4.107	CH ₃	4	70%	50-53 °C
4.108	CH ₃	6	70%	50-52 °C

4.4.11.1 Mechanistic Details

The mechanism of steps (i) and (ii) is depicted in Scheme 4.22. The N-H at N1 of (4.98) is regioselectively deprotonated using 1.1 equivalents of NaH to generate the more resonance stabilized anion. Deprotonation of N3 DHPM (4.98) leads to a less stabilized anion compared to N1 deprotonation. Reaction of the more resonance stabilized anion with methyl iodide in a typical S_N2 reaction delivers intermediate (iii). N3 deprotonation of this intermediate, followed by nucleophilic attack of the resulting anion at the electrophilic carbonyl of phenyl chloroformate resulted in the target compound *via* intermediate (iv). The by-product, (4.103) of the *N*-methylation reaction, resulted from both nitrogen atoms being deprotonated, with subsequent methylation at N1 and N3 in the presence of methyl iodide.



The mechanism of target compounds (4.105-4.108) is depicted in Scheme 4.23. This mechanism is initiated by a nucleophilic attack of the lone pair of electrons on the nitrogen of the diamines onto position 4 of quinoline, followed by elimination of the chloride anion which in turn reacts with methylated carbamate (4.104) in a simple one-step process to produce the series of target compounds, (4.105-4.108).



The regioselectivity of monomethylated (4.102) and the by-product, dimethylated (4.103) DHPMs were confirmed by X-ray crystallography. (see Crystallography Section). Further support for these structures was evident from IR, NMR, and mass spectral data as well as elemental analysis.

Infrared analysis for monomethylated DHPM (4.102) showed four characteristic functional groups, secondary N-H (3300 cm^{-1}), aromatic C-H (3052 cm^{-1}), ester C=O (1696 cm^{-1}) and amide CONH (1628 cm^{-1}). Key signals in the ^1H NMR spectrum of this compound were assigned to the D_2O exchangeable N-H3 which appeared as a singlet at δ 5.60, the methyl protons at N1, which appear as a singlet at 3.21 and the methyl protons at C6, which also appear as a singlet at δ 2.50. The ^1H NMR spectrum of (4.102) revealed the singlet signal assigned to N-H3 formally at δ 7.60 shifted upfield to δ 5.60 and also the disappearance of the singlet signal assigned to N-H1, (Fig. 4.41).

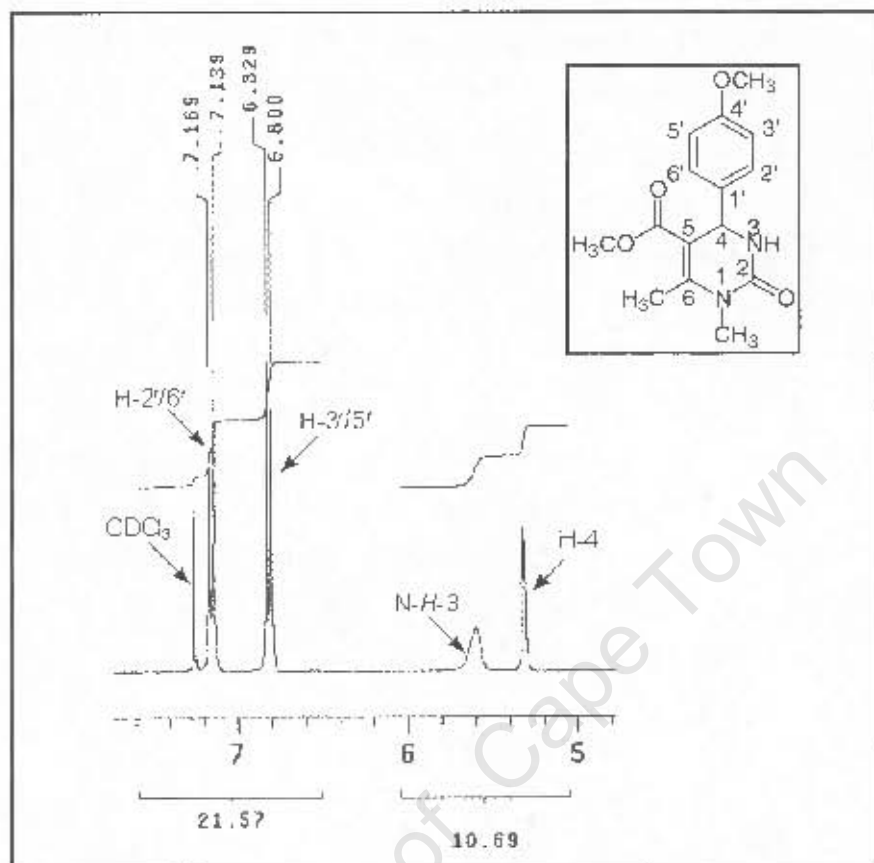


Figure 4.41: An expanded region in the ^1H NMR of monomethylated DHPM (4.102) in CDCl_3

IR analysis for dimethylated DHPM (4.103) showed three characteristic functional groups, aromatic C-H (3052 cm^{-1}), ester C=O (1778 cm^{-1}) and amide CONH (1638 cm^{-1}). Analysis of ^1H NMR spectrum of this compound revealed three sets of methyl protons which appeared as singlets at δ 3.26 (N-CH₃-1), δ 2.90 (N-CH₃-3) and δ 2.73 (CH₃-6). The absence of the NH peak in the ^1H NMR spectrum provided further proof of the dimethylated DHPM (4.105). Mass spectrometry for both compounds (4.102 and 4.103) confirmed the molecular formulae of the proposed structures, with molecular ions observed at m/z 290.0 and m/z 304.0 respectively.

The target molecules (4.105-4.108) were fully characterized by IR, NMR, and mass spectral data as well as elemental analysis data. Infrared spectra indicated characteristic peaks of functional groups, secondary N-H (3200 cm^{-1}), aromatic C-H (3052 cm^{-1}), C-H (2987), ester

C=O (1693 cm^{-1}) and amide CONH (1638 cm^{-1}). The ^1H NMR spectral data of one target compound (**4.106**), Fig 4.42, revealed the following key signals; the D_2O exchangeable N-H protons: a 1-proton triplet at δ 9.08 (J 5.6) assigned to N-H13'' and a 1-proton triplet at δ 6.11 assigned to N-H9'', the quinoline protons: a 1-proton doublet at δ 8.48 (J 5.6) assigned to H-2'', 1-proton doublet at δ 7.95 (J 2.4) assigned to H-8'', a 1-proton doublet at δ 7.87 (J 9.2) assigned to H-5'', a 1-proton doublet of doublets at 7.52 (J 2.4 and 9.2) assigned to H-6'' and 1-proton doublet at δ 6.39 (J 5.6) assigned to H-3'', the aromatic protons: a 2-protons doublet at δ 7.14 ppm (J 8.4) assigned to Ar-H2' and Ar-H6' and a 2-protons doublet at δ 6.82 (J 8.4) assigned to Ar-H3' and Ar-H5' and a heterocyclic proton: 1-proton singlet at δ 6.75 assigned to H-4. High resolution mass spectrometry was helpful in confirming the molecular formula of the proposed structure of (**4.106**) with the molecular ion being observed at m/z 553.20919 ($\text{C}_{28}\text{H}_{30}\text{ClN}_5\text{O}_5$). The characterization data obtained for the target compounds (**4.105**, **4.107** and **4.108**) are similar to (**4.106**) with no significant changes in chemical shift values for common protons observed in ^1H NMR, (Fig. 4.42).

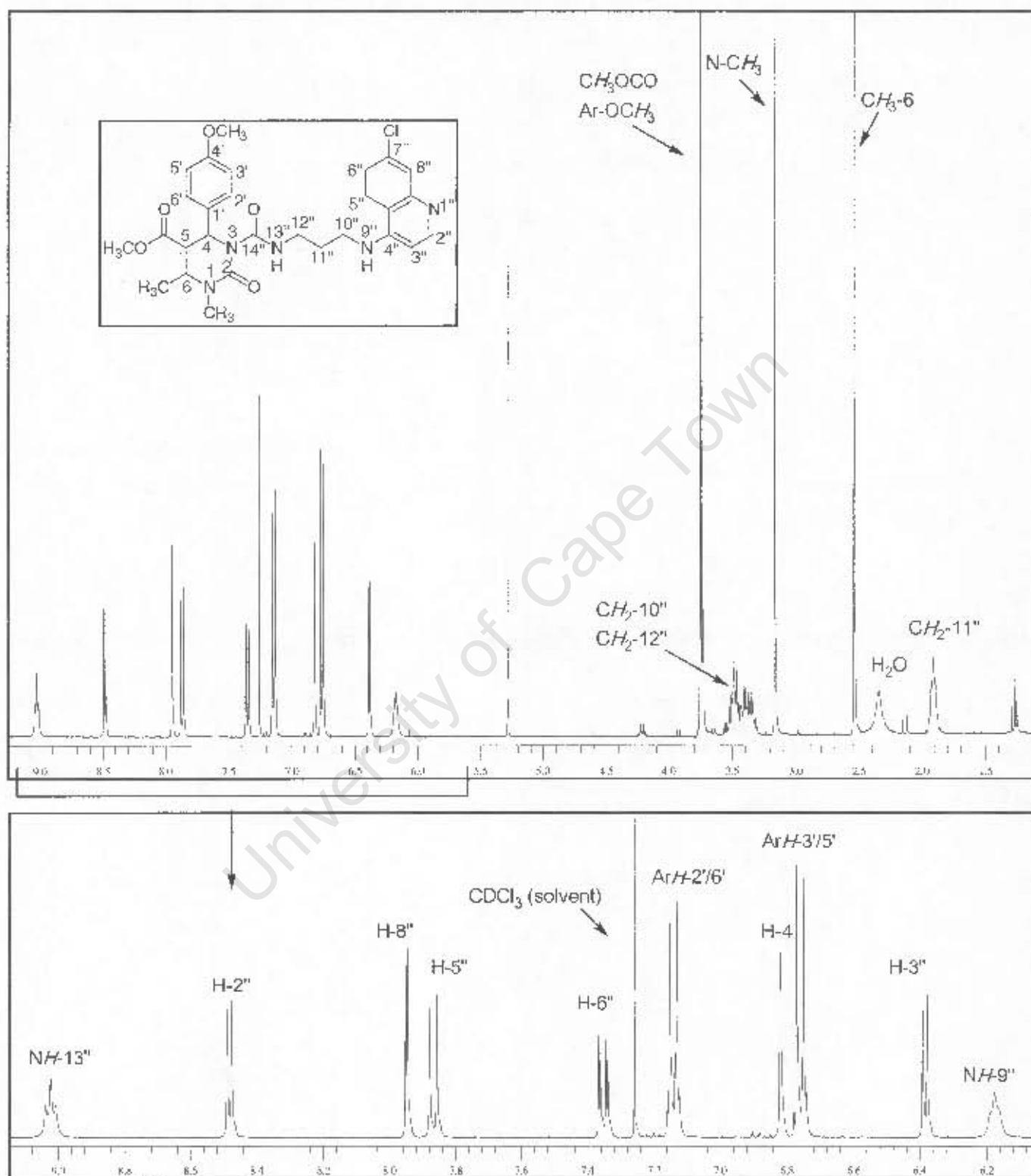


Figure 4.42: An expanded region in the ^1H NMR spectrum of DHPM derivative, 4.106 in CDCl_3

The ^{13}C NMR data for all the DHPM target molecules (4.105-4.108) were also consistent with the proposed structures. The only difference occurs in the number of aliphatic carbons within each target molecule. As the chain length increased, an additional carbon peak in the alkyl region was evidenced in respective ^{13}C NMR spectra. A representative ^{13}C NMR obtained for DHPM derivative (4.107) is shown in Fig. 4.43.

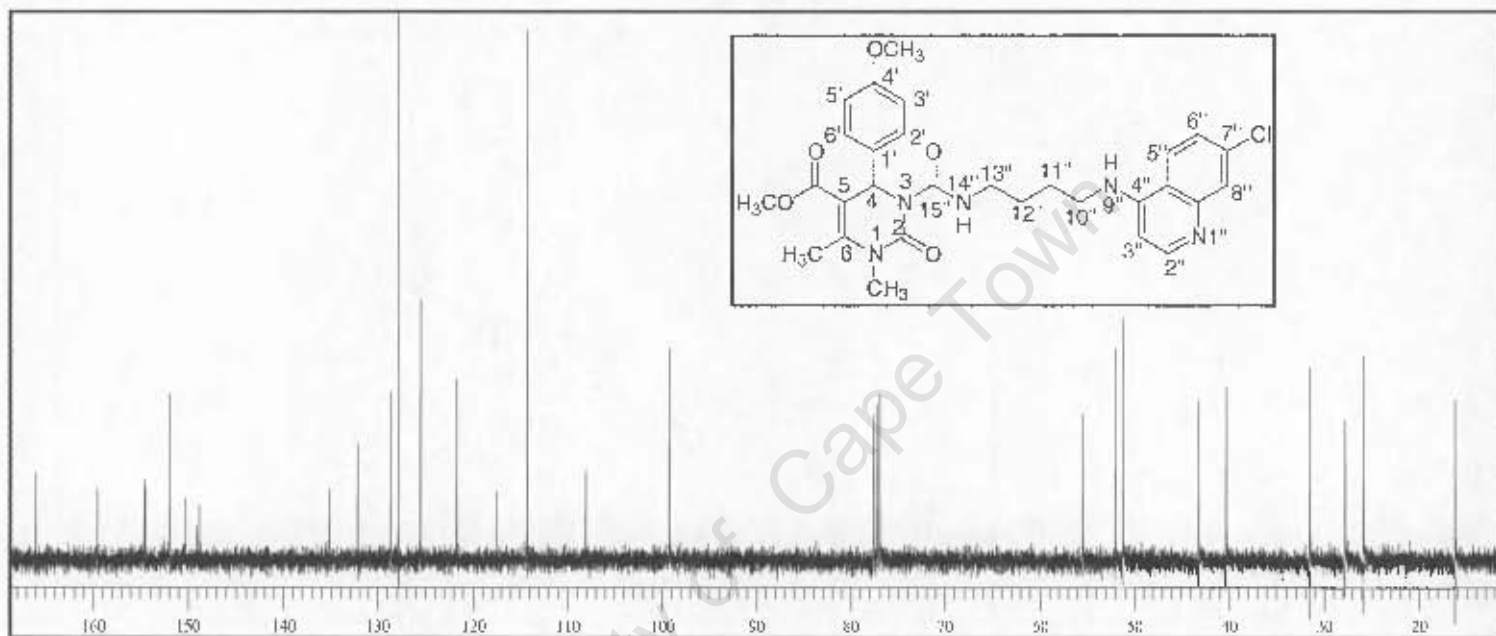


Figure 4.43: ^{13}C NMR of 4-aminoquinoline DHPM derivative, 4.107 in CDCl_3

4.4.12 Attempts at improving drug solubility¹²⁶

One of the drawbacks of the target compounds, (4.105-4.108) is that they are insoluble in aqueous media, which would potentially limit their bioavailability and thus reduce efficacy. This is a common problem affecting many drugs, because it affects their ability to enter the bloodstream and reach the desired target system. Three methods can be employed to improve the solubility of a drug. These include the formation of salt derivatives of the compound,^{127, 128} inclusion into cyclodextrins,¹²⁹ and isolation of crystalline polymorphs of higher solubility.¹³⁰

4.4.12.1 Salt formation

Salt formation is a relatively simple procedure involving the reaction of the compound of interest with an acid or base. The acid creates a charged centre on the molecule, converting it into an ion, which then improves its water solubility. Different salt forms have different solubility profiles and dissolution rates which affect the bioavailability and absorption of the drug.¹²⁷ At high concentrations, some cations (e.g. Li⁺) and anions (e.g. tartrate) can be toxic, and therefore appropriate acids and bases should be chosen to ensure that the salt that is formed is 'pharmaceutically acceptable'.¹²⁸ Different salt forms of a drug can have different stabilities. Salts of mineral acids such as hydrochlorides and sulphates are highly polar and as such have high water solubility. These exposed polar groups create a hydrophilic surface on the crystal, and can cause hygroscopicity. This may reduce the stability of the drug if it is susceptible to hydrolysis. In this project, citric acid salts were prepared, because of their known low toxicity and the ready availability of citric acid.

4.4.12.2 Cyclodextrins (CDs)

Cyclodextrin (CD) inclusion is a powerful method for increasing the solubility of drugs because it is compatible with a wide range of compounds, without altering their structure. CDs are cyclic oligosaccharides consisting of 6, 7 or 8 1, 4-linked α -D-glucose units corresponding to α -, β - and γ -CD. The CD structure (Fig. 4.44) is cone-shaped, with a hydrophobic interior, allowing complexation of poorly soluble drugs, and a hydrophilic exterior that enables solubilisation.^{129 (a)} As a result of the conformation of the glycosidic linkage of glucose rings, all the secondary hydroxyl groups are positioned along one edge of the cone, and all the primary hydroxyl groups

along the other.^{129 (a)} It is these hydroxyl groups that impart hydrophilic character to the exterior of the cone. The inner surface of the cone is lined with hydrogens and the glycosidic oxygen bridges, which result in its hydrophobic character. The non-bonding electron pairs of the oxygen bridges point towards the centre of the structure, creating high electron density, and giving the centre of the CD some Lewis-base character. The structure is held in position by hydrogen bonding between the C₂ and C₃-OH groups on adjacent glucose units. These bonds form a belt around the structure that holds it rigidly in place. The alignment of the OH groups in β -CD produces the strongest H-bonding, which is probably why it is the least soluble of the three CDs. The belt is incomplete in the α -CD molecule, due to the distorted position of one of the glucopyranose units, resulting in four out of the six possible H-bonds forming. γ -CD has the most flexible structure of the three. It is also the most soluble.^{129 (u)}

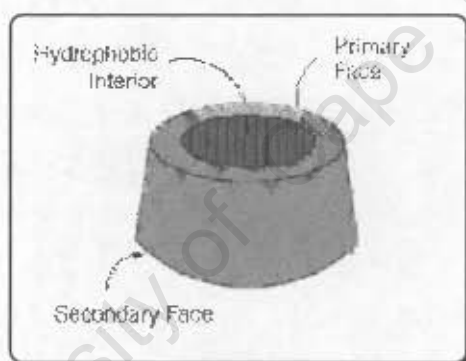


Figure 4.44: Schematic depicting β -CD structure¹²⁹⁽ⁿ⁾

The choice of which CD to use for complexation of a particular drug should take into account differences in toxicity and bioavailability of the various CDs. Due to the small size of its cavity, α -CD is only able to complex small molecules, and generally has limited usage in drug solubilisation, although it is used in the stabilisation of prostaglandins. β -CD is the most commonly used form for drug inclusion complexes, and has been used for a number of oral and topical products, although it cannot be used for parenteral delivery due to its renal toxicity.^{129 (d)} As mentioned above, it has the lowest water solubility of the three, and so di- and trimethyl derivatives (DIMEB and TRIMEB respectively) are often used to improve the solubility of the β -CD host. Although γ -CD is the most soluble, its enlarged size often prevents it from forming easily characterised inclusion complexes, as a result of fractional host: guest ratios. The

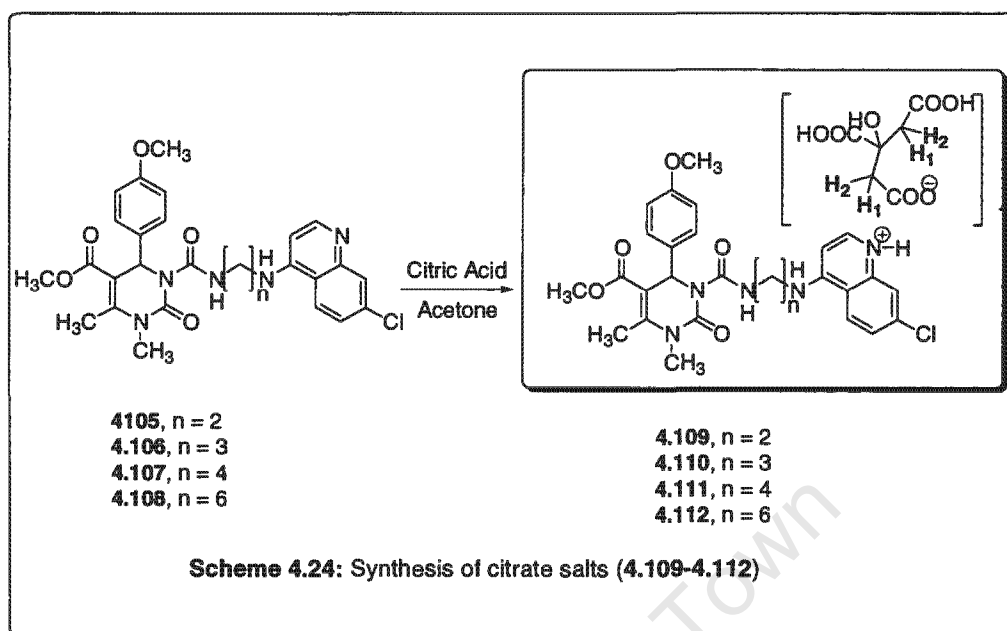
tendency for guest disorder in complexes of this host also makes accurate crystal structure determination difficult.

4.4.12.3 Polymorphism

Polymorphism is a phenomenon that can greatly affect the solid state characteristics of a compound. Only one form of a compound is stable at a given temperature and pressure, the others being metastable; however, interconversion between forms may be slow or unapparent due to kinetic barriers. As a result, several polymorphic forms of a compound may be crystallised under the same laboratory conditions, each having potentially a different solubility, melting point, density and chemical reactivity (among others).¹³⁰ In the pharmaceutical industry, this has a great effect on the efficacy of a particular drug, since the synthesis of an undesired polymorph with a different solubility, may result in a change in its bioavailability. This could result in a pharmaceutical dosage being either ineffective or toxic.¹³⁰ Determination of whether or not a specific compound is prone to polymorphism should always be investigated. Alternative forms of a drug may prove to have greater solubility and bioavailability, which could enhance its pharmacological properties.

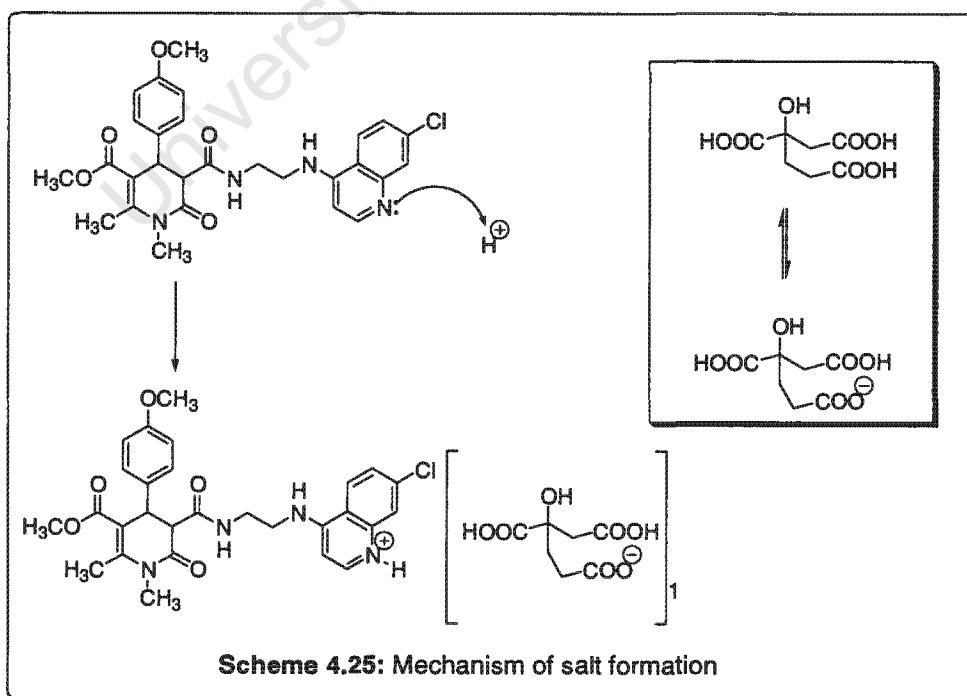
4.4.13 Synthesis of Citrate Salts¹²⁶

In order to improve the aqueous solubility, and therefore bioavailability, of target compounds, (4.105–4.108), citrate salts of these compounds were prepared for biological evaluation. Surprisingly, the salt formation process was more challenging than initially expected. The method employed involved dropwise addition of an acetone solution of citric acid to a stirring acetone solution of respective compounds (4.105–4.108).¹³¹ The salts formed precipitated from solution as a sticky oil, which made filtration and purification difficult, and resulted in lower than expected yields (e.g. 52 % for 4.112 citrate). The filtration step was consequently omitted from subsequent salt formation protocols (for citrate salts 4.7), and the mother liquor decanted off instead of filtered. Residual starting material remained in solution when discarded, and therefore did not affect the purity of the final salts.



4.4.13.1 Mechanistic Details

The salt forming process relies on the basicity of the quinoline nitrogen, and since there is only one such nitrogen in compounds (**4.105- 4.108**), the salts form a 1:1 molar ratio of citric acid:free base. In this one step mechanism, the basic quinoline nitrogen is protonated in the presence of the citric acid, resulting in the formation of the salt, (Scheme 4.25).



The salts (4.109–4.112) were characterised by ^1H and ^{13}C NMR spectroscopy, and by C, H and N elemental analysis. NMR analysis of the salts was performed in CDCl_3 . The success of the salt forming process was confirmed in each respective ^1H NMR spectrum by the presence of two doublets, arising from geminal coupling of each pair of heterotopic protons (Table 4.6). Each doublet integrated for two protons, which indicated that the salts were indeed obtained in a 1:1 molar ratio of citric acid:free base.

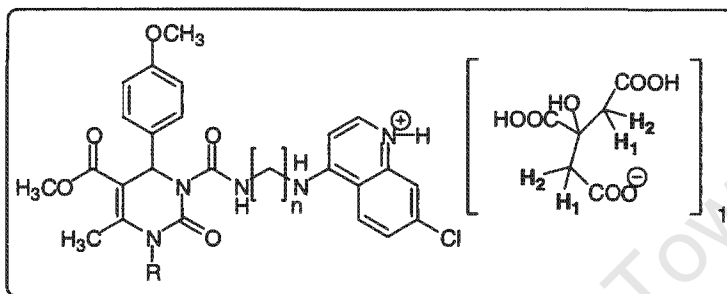
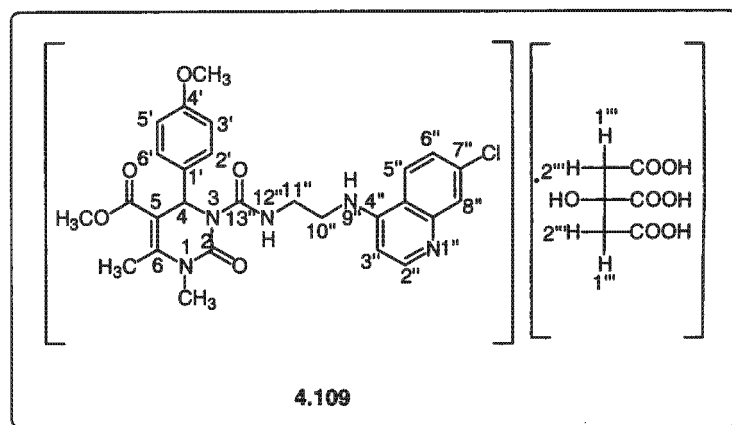


Table 4.6: Chemical shifts and J -values of characteristic citrate salt doublets (H_1 and H_2)

Citrate salt	n	Chemical Shift of Doublets (ppm) and Corresponding J -values (Hz)	
		H_1	H_2
4.109	2	2.90, J 15.3	3.01, J 15.6
4.110	3	2.87, J 12.4	3.00, J 14.8
4.111	4	2.92, J 15.2	3.03, J 14.4
4.112	6	2.94, J 15.3	3.03, J 15.6

Due to the poor solubility of the salts, (4.109–4.112) in CDCl_3 the resolution of many signals was reduced, resulting in inaccurate descriptors (e. g. broad s instead of a d). In this regard, it was decided to change the NMR solvent to $\text{DMSO}-d_6$ to obtain a clearer spectrum, but almost similar resolutions of the signals were obtained.

^1H NMR analysis indicates downfield shifting of the chloroquine proton signals for each of the salts. Examples of these shifts are presented in Table 4.7. The chemical shift of the signals of other hydrogens in the molecules remains relatively unchanged, as illustrated by the negligible change in chemical shift of the $\text{H}-2'/\text{H}-6'$ and $\text{H}-3'/\text{H}-5'$ protons. This phenomenon confirms salt formation *via* protonation of the quinoline nitrogen.



Proton	H-2'	H-3'	H-5'	H-6'	H-8'	H-3'/H-5'	H-2'/H-6'
4.105	8.51	6.32	7.72	7.30	7.94	6.61	7.10
4.109 citrate salt	8.75	7.73	7.87	7.18	8.22	6.66	7.06

4.4.14 Crystallographic Analysis¹²⁶

Crystals of compounds (4.102) and (4.103) (see Scheme 4.21, page 146) and compound (4.105), were grown for structure determination. Their crystal data are summarized in Table 4.8. Each of the three compounds crystallizes in a centrosymmetric space group, therefore containing one or more enantiomeric pairs of molecules in the unit cell.

The Ortep diagram of (4.102) (Fig. 4.45) indicates that the resonance-stabilized nitrogen, N1, of the DHPM ring was regioselectively methylated. The byproduct of the reaction, (4.103), was shown by crystal structure analysis to be the dimethylated DHPM, as illustrated in Fig. 4.47 below.

Note: Crystal structure numbering schemes used in this section of the report are different from those in the synthetic sections. Fig. 4.45 indicates the two numbering schemes used for compound (4.102). These differences are due to the requirement that each atom in the crystal structure diagram be labeled, whereas synthetic numbering schemes are based on ¹H NMR assignments, Fig. 4.45 (ii) and therefore omit certain non-protonated atoms (e.g. oxygen). In this crystallographic analysis section of the report, the crystal structure numbering scheme will be used, Fig.4.45 (i).

Table 4.8: Crystal data of compounds (4.102), (4.103) and (4.105)

Compound	4.102	4.103	4.105
Molecular formula	C ₁₅ H ₁₈ N ₂ O ₄	C ₁₆ H ₂₀ N ₂ O ₄	C ₂₇ H ₂₈ N ₅ O ₅ Cl
Formula weight (g.mol ⁻¹)	290.31	304.34	537.99
Crystal system	Monoclinic	Monoclinic	Triclinic
Space group	P2 ₁ /c	P2 ₁ /c	P(1)
a (Å)	11.7710 (2)	16.6529 (3)	11.1775 (3)
b (Å)	5.5290 (1)	10.9426 (4)	13.6470 (4)
c (Å)	22.9500 (5)	8.2819 (7)	16.3680 (6)
α (°)	90	90	82.645 (1)
β (°)	104.342 (1)	91.395 (1)	86.423 (1)
γ (°)	90	90	88.415 (2)
V (Å) ³	1447.08 (5)	1508.7 (1)	2470.9 (1)
Z	4	4	4
D _c (g.mol ⁻³)	1.333	1.340	1.446
λ (Mo-Kα) (Å)	0.71073	0.71073	0.71073
F (000)	616	648	1128
Crystal size (mm)	0.12 x 0.15 x 0.20	0.15 x 0.22 x 0.25	0.10 x 0.12 x 0.25
Range scanned θ (°)	3.7 - 26.0	3.1 - 25.3	1.3 - 26.8
Range of indices	h: ±14 k: ±6 l: ±28	h: ±20 k: -12; 13 l: ±9	h: -14; 13 k: -16; 17 l: ±20
No. reflections collected	5320	10088	61222
No. unique reflections	2834	2734	10424
No. parameters	194	204	693
S	1.01	1.03	0.92
R ₁	0.0437	0.0434	0.0507
wR ₂	0.1197	0.1174	0.1259
Δρ excursions /e.Å ⁻³	-0.26, 0.19	-0.25, 0.39	-0.32, 0.39

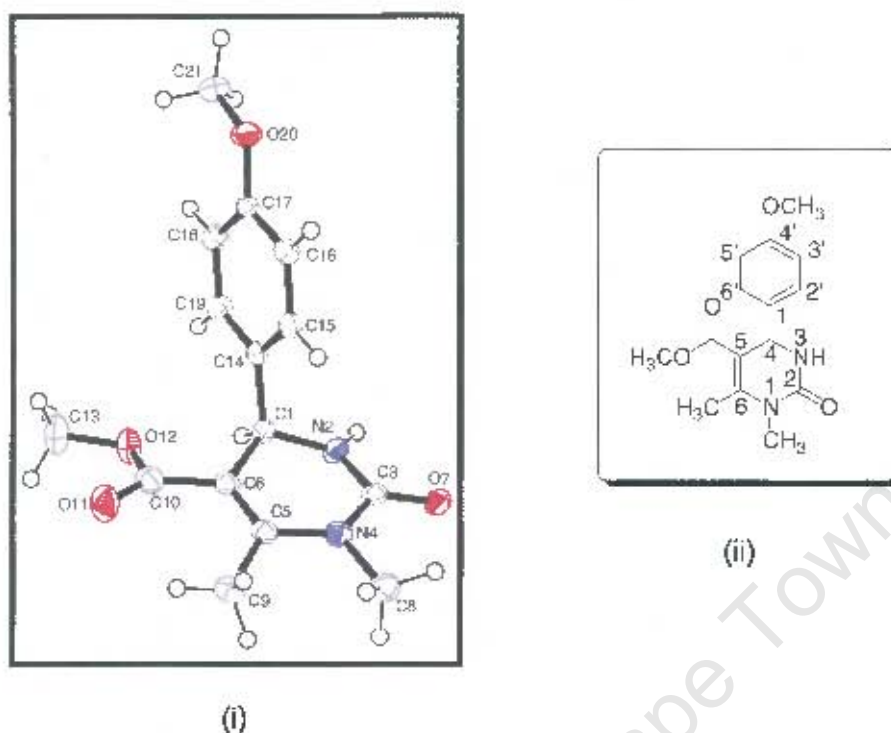


Figure 4.45: Ortep diagram of (4.102) with thermal ellipsoids at the 50% probability level and crystallographic numbering scheme.

Structure determination indicated that (4.102) crystallizes in a monoclinic unit cell in space group $P2_1/c$, with four molecules per cell. The orientation of the molecule is such that the methoxy phenyl ring is approximately orthogonal to the DHPM ring, as indicated by the C3-N2-C1-C14 torsion angle of 109.3° (Note: All torsion angles quoted with an average e.s.d of approximately 0.3). The plane of the methoxy phenyl ring is twisted off-centre from the middle of the DHPM ring, at an angle of 55.4° (torsion angle: N2-C1-C14-C15). The DHPM ring itself is slightly puckered, with the C1 carbon elevated above the plane of the other atoms (torsion angles: C1-N2-C3-N4 = 9.2° ; N4-C5-C6-C1 = -1.0°).

Hydrogen bonding in the crystal structure of (4.102) is illustrated in Figure 4.46, which identifies the presence of intermolecular H-bonds (N-H $\cdots$ O) between the carbonyl oxygen, O7, of the DHPM urea group and the NH hydrogen atom of N2, on an inversion-related molecule. The intramolecular H-bonds between O11 and the C9 methyl hydrogen atom, indicated in Fig. 4.46, can be considered too weak to be significant. The presence of the intermolecular H-bonds is

likely to have been a stabilizing factor contributing to the crystallization of (4.102). H-bond distances are indicated in Table 4.9.

Table 4.9: Hydrogen bond distances and angle in (4.102)

Atoms	D...A (Å)	D-H (Å)	H...A (Å)	D-H...A (°)
N2-H2...O7	2.8349(2)	0.88	1.96	172.00

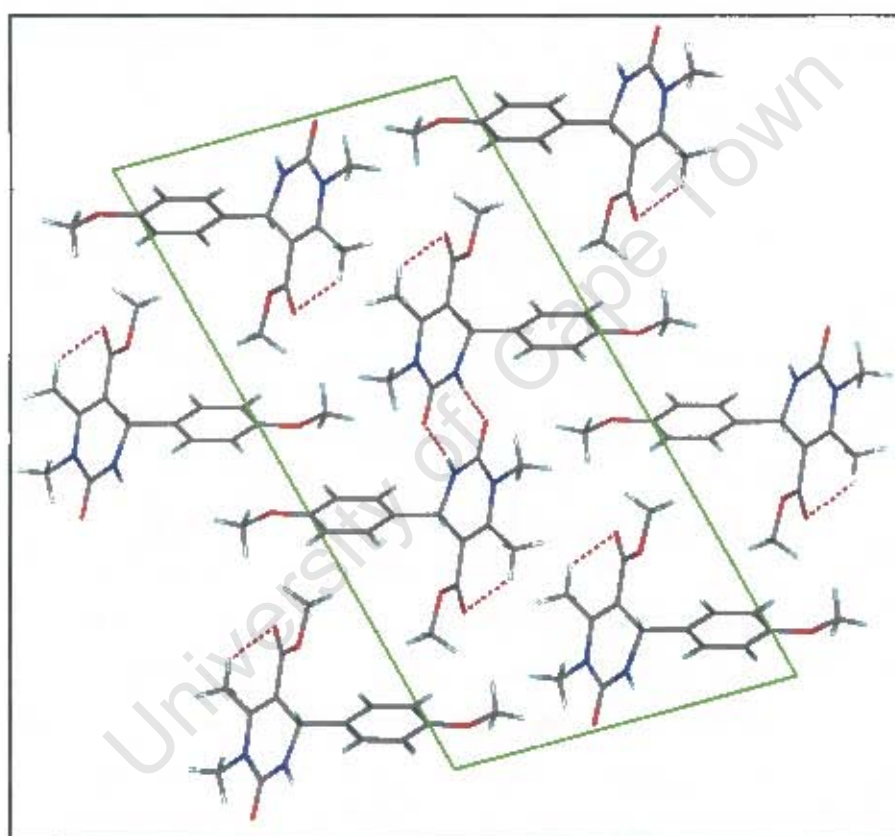


Figure 4.46: [010] Projection of the crystal structure of (4.102) illustrating the hydrogen bonding scheme

Crystal structure analysis of (4.103) confirmed the expected dimethylated DHPM structure, as illustrated in Fig. 4.17 below. The compound was found to crystallize in the same system as that of (4.102), namely $P2_1/c$, and to have a similar molecular conformation.

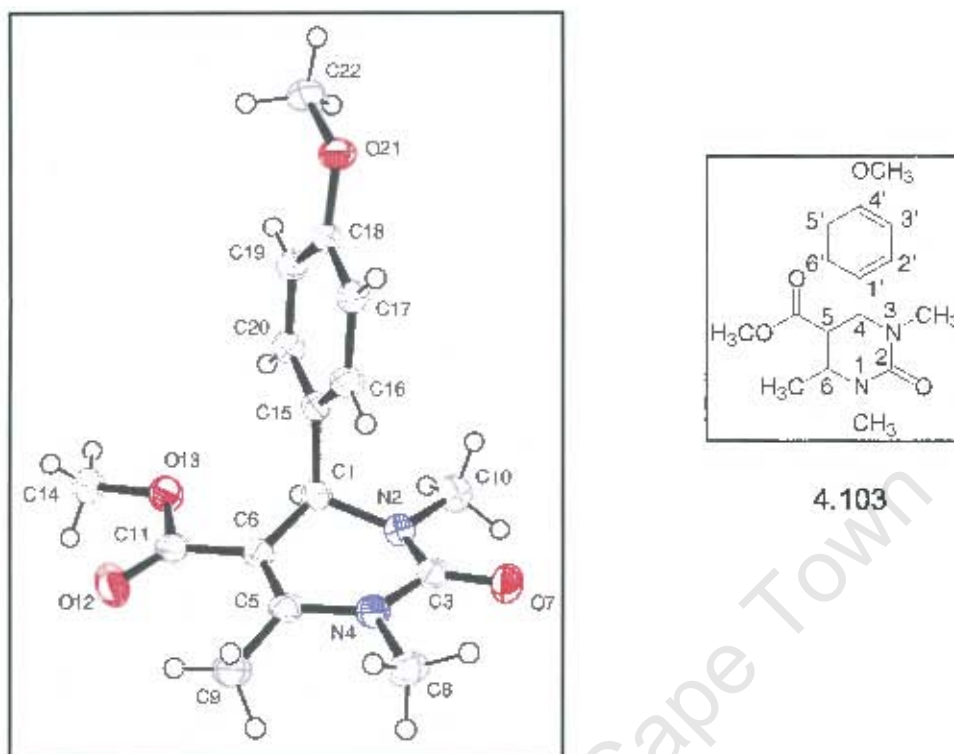


Figure 4.47: Ortep diagram of (4.103) with thermal ellipsoids at the 50% probability level

Due to methylation of the N2 nitrogen, the intermolecular H-bonding of (4.102) is absent in the (4.103) structure. Of the four target molecules comprising the homologous series, only crystals of (4.105) and (4.106) were successfully grown. The X-ray analysis of (4.105) (see Fig. 4.48) was used to provide a representative structure for the series.

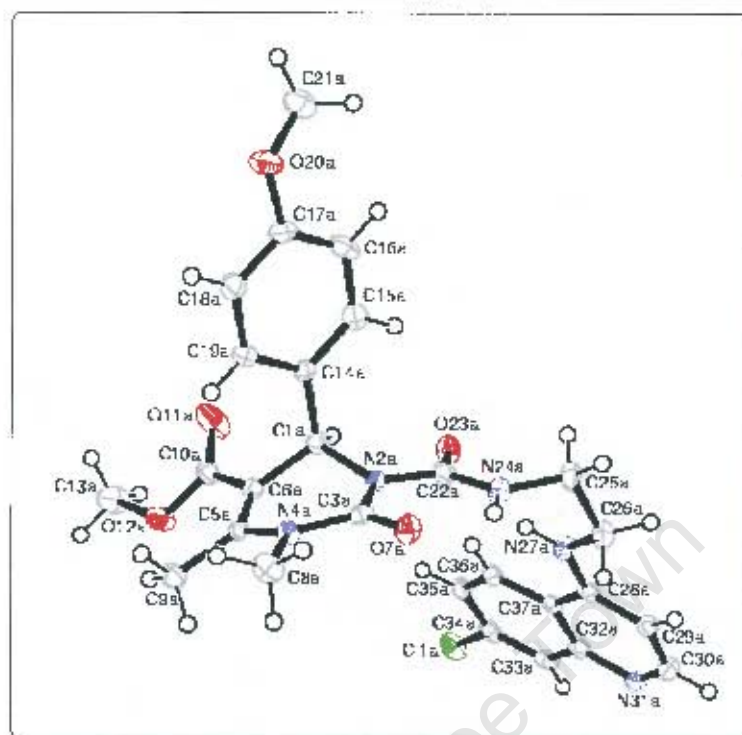


Figure 4.48: Ortep diagram of (4.105) with thermal ellipsoids at the 50% probability level

The structure of (4.105) differs from (4.102) and (4.103) in the conformation of the DHPM scaffold at the methyl ester substituent (C6a). In (4.105) carbonyl group points in the opposite direction. Also, the methoxy phenyl group is twisted with respect to the centre of the DHPM at a new angle of 114.9° (torsion angle N2a-C1a-C14a-C15a).

The structure of (4.105) has the attached chloroquine group positioned below (torsion angle C22a-N24a-C25a-C26 = 90.5°) and behind (N24a-C25a-C26a-N27a = -66°) the DHPM moiety, as viewed in Fig. 4.48. This conformation results from intramolecular hydrogen bonding between the hydrogen atom of N27a and the alkyl urea carbonyl oxygen, O23a, which forms a seven-membered ring. A second hydrogen bond is present between the DHPM carbonyl oxygen, O7a, and the alkyl urea nitrogen, N24a, forming a five-membered ring. Since all H-bond donors are involved in bonding, there are no intermolecular H-bonds. This is illustrated in Fig. 4.49.

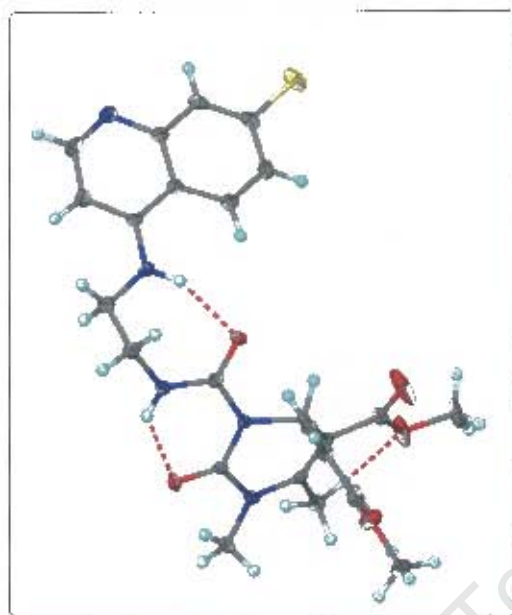


Figure 4.49: Hydrogen bonding in (4.105), viewed parallel to the plane of the methoxy phenyl DHPM substituent. The resulting 5- and 7-membered rings are clearly visible. Bonding between the ester oxygen, O11a, and C9a methyl hydrogen is negligible and can be ignored.

The H-bond lengths and angles present in the crystal structure of (4.105) are given in Table 4.10.

Table 4.10: Hydrogen bond distances and angles in the crystal structure of (4.105)

Atoms	D...A (Å)	D-H (Å)	H...A (Å)	D-H...A (°)
N27a-H27a...O23a	3.060 (3)	0.88	2.30	144
N24a-H24a...O7a	2.617 (3)	0.88	1.94	132

The stabilizing effect of these intramolecular H-bonds is likely to have contributed to the crystallization of (4.105). It is possible that crystals of (4.105-4.108) were not obtained because the increased size of the rings that might form by the N27a-H27a...O23a H-bond, as *n* increases above 3, could cause it to lose stability and prevent the compound from crystallizing. Crystallization of (4.105) and (4.106) produced only one crystal type per compound. As a result,

polymorphism was not detected for either compound during this project. Due to the lack of crystallinity of (4.105-4.108), polymorphism in these compounds could not be evaluated.

4.4.14.1 Cyclodextrin Inclusion

Compound (4.108) and its citrate salt were selected for attempted cyclodextrin inclusion based on its biological importance. Kneading and co-precipitation methods were employed using β - and γ -CD in both 1:1 and 2:1 molar ratios (CD: compound), and all attempts were unsuccessful for both (4.108) and its citrate salt. It was then postulated that the strong non-polar character of the compounds was inhibiting inclusion, and so the less polar methylated CDs, DIMEB and TRIMEB, were employed using the protocol outlined in chapter 6 of this report. These attempts also proved unsuccessful.

Successful inclusion of a guest often requires special design of a procedure 'tailor-made' to the particular guest. This involves pH, temperature and solvent optimization, which was outside the scope of this project. Future work aimed at successful inclusion of compounds (4.105-4.108) could be centered on optimizing such a process.

4.5 Pharmacophore Hybridization

The following section describes a fast and efficient method for generating novel compounds based on the "click" chemistry strategy. The rationale and synthesis of the envisaged series of compounds are described and presented by schematic illustrations.

4.5.1 "Click" Chemistry Strategy

"Click" chemistry as defined by K. B. Sharpless is "a set of powerful, virtually 100% reliable, selective reactions for the rapid synthesis of new compounds *via* heteroatom links (C-X-C)."¹³² This approach involves the efficient production of molecules by joining small repeating units together as nature does. For lead optimization, it enables rapid structure-activity relationship (SAR) profiling, through generation of analog libraries. It works in conjunction with structure-based design and combinatorial chemistry techniques, and through the choice of appropriate building blocks, can provide derivatives or mimics of 'traditional' pharmacophores, drugs and natural products.¹³³⁻¹³⁵ "Click" chemistry-based products are wide-ranging, due to the use of highly selective reactions of broad scope, allowing a much greater diversity of the resultant target compounds.

4.5.1.1 Defining a "Click" Chemistry Reaction

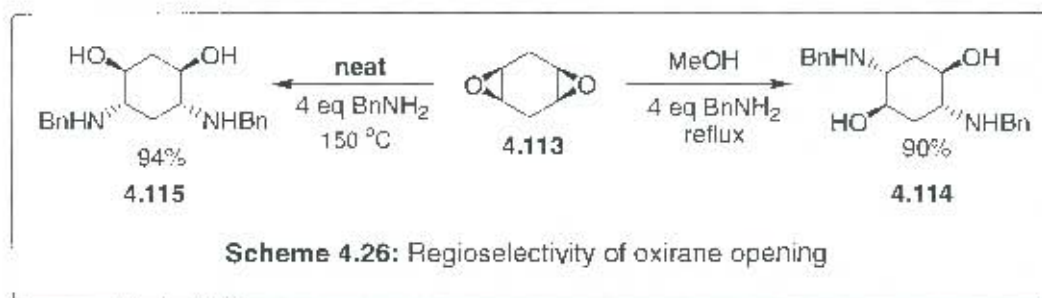
A set of stringent criteria have been defined to view a process in the context of "click" chemistry. A "click" chemistry reaction must be "modular, wide in scope, high yielding; only generate easily removal byproducts and be stereospecific (but not necessarily enantioselective)." The required process characteristics include simple reaction conditions (ideally the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or an aqueous solvent, and convenient product isolation. "Click" reactions achieve their required characteristics by having a high thermodynamic driving force, usually greater than 20 kcal/mol⁻¹. Such processes proceed rapidly to completion and also tend to be highly selective for a single product.^{136, 137}

4.5.1.2 "Click" Chemistry Reaction Types

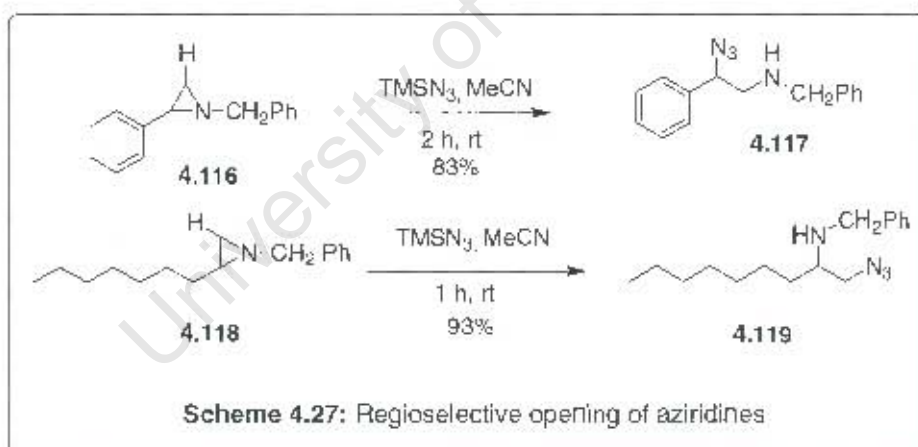
Three principal types of "click" reactions have been identified, and include nucleophilic ring opening,^{133, 138} "protecting group" reactions¹³³ and cycloaddition reactions.¹³⁹ The two most useful "click" reactions in drug discovery are the nucleophilic opening of strained ring systems and 1,3-dipolar cycloadditions. These are the most extensively studied "click" reactions to date. Preparation of potential drug candidates by these reactions utilizes building blocks such as acetylenes (for 1,3-dipolar cycloadditions) and olefins (for nucleophilic ring opening). These starting materials are readily available in nature¹⁴⁰ or can be accessed through "steam cracking" of alkanes in the petrochemical industry¹⁴¹ and can be functionalized by oxidative or addition reaction.

4.5.1.2.1 Nucleophilic Ring Opening

The ring opening of three membered heterocycles is generally facilitated by the release of strain energy¹⁴² and therefore these compounds have been termed spring-loaded electrophiles.¹³³ Great diversity can be achieved from the nucleophilic opening of epoxides, aziridines, cyclic sulfates as well as episulfonium and aziridinium ions. Of these heterocycles, epoxides (eg. 4.113) and aziridines (eg. 4.116 and 4.118, Scheme 4.27) are the most common substrates for "click" reactions and their regioselective ring opening is highly useful for the formation of diverse compounds. In a number of cases the regioselectivity can be controlled by the choice of solvent. For example the regioselective oxirane opening to generate constitutional isomers can be regulated by the reaction conditions as demonstrated by the nucleophilic opening of diepoxide (4.113) with benzylamine (Scheme 4.26). In the presence of methanol, the regioisomer, (1,4-diol) (4.114) is obtained in 90% yield whereas in the absence of solvent, the amino alcohol, (1,3-diol) (4.115) is obtained in 94%. Generation of the first hydroxyl group in (4.113) allows intramolecular activation of the remaining epoxide. The effect is less prevalent when the reaction is carried out in protic solvents which allows for nucleophilic attack from a more stable chair conformation, leading to the regioisomer (4.114).¹³³



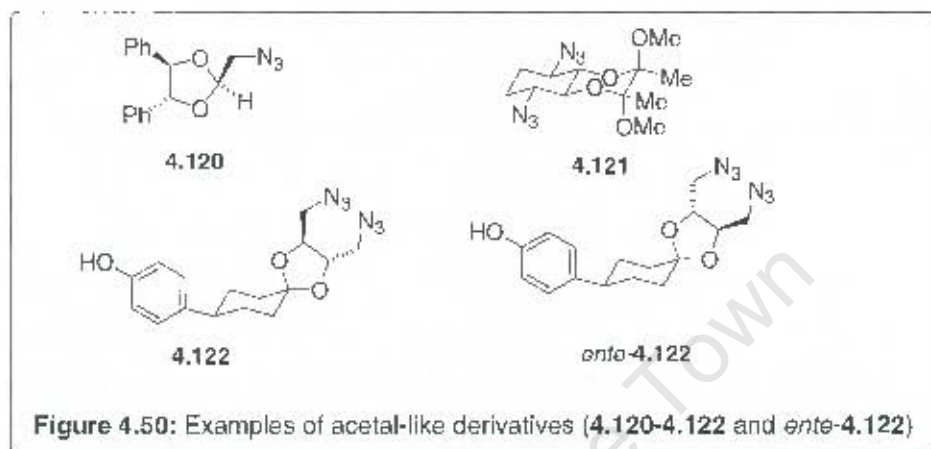
Unlike the reactions of epoxides, regioselective opening of aziridines is primarily substrate controlled as shown by the opening of unsymmetrical aziridines (**4.116** and **4.118**) using TMS azides (Scheme 4.27).¹⁴³ Silicon coordination to the aziridine nitrogen weakens the bond to the more substituted carbon in the aziridine ring system. The developing positive charge in (**4.116**) is better stabilized at the benzylic position in (**4.116**). This favors benzylic position attack in (**4.116**) since the corresponding position in (**4.118**) is secondary in nature. In addition greater product diversity can be achieved through variation of the nitrogen substituent. Nucleophilic ring opening reaction of oxiranes and aziridines can be high yielding, stereospecific, and regioselective, thus fulfilling the requirements for “click” chemistry.



4.5.1.2.2 “Protecting Group” Reactions

Acetals, ketals and some of their aza-analogs have been found to form part of this type of click reactions. They are generally stable at physiological pH,^{144(a)} have already appeared as components in orally available drugs,^{144(b)} contribute several hydrogen bond acceptor sites and interesting dipole effects. These types of compounds also provide constrained scaffolds with

well-defined projections and spatial orientations of their substituents. They represent one of the rare “click” chemistry modules based on *reversible* carbonyl chemistry. Examples of acetal-like derivatives (**4.120-4.122** and *ente-4.122*) were prepared from the appropriate diols or hydroxysulfonamides, (Fig. 4.50).



4.5.1.2.3 Cycloaddition Reactions

A cycloaddition is a pericyclic chemical reaction, in which two π bonds are lost and two σ bonds are gained,¹⁴⁵ the resulting reaction is a cyclization reaction. A reaction in which two or more unsaturated molecules (or parts of the same molecule) combine with the formation of a cyclic adduct in which there is a net reduction of the bond multiplicity. The following two systems of notations have been used for the more detailed specification of cycloadditions, of which the second, more recent system [described under (2)] is preferred:

(1) A ($i + j + \dots$) cycloaddition is a reaction in which two or more molecules (or parts of the same molecule), respectively, provide units of $i, j, \dots$ linearly connected atoms: these units become joined at their respective termini by new σ -bonds so as to form a cycle containing ($i + j + \dots$) atoms. In this notation, the for example Diels–Alder reaction is a (4+2) cycloaddition. (N.B.: parentheses (...) are used in the description based on numbers of atoms.)

(2) The symbolism [$i + j + \dots$] for a cycloaddition identifies the numbers $i, j, \dots$ of electrons in the interacting units that participate in the transformation of reactants to products. In this notation

the reaction (a) of the preceding paragraph would be described as [2+4] cycloadditions. The symbol a or s (a = antarafacial, s = suprafacial) is often added (usually as a subscript after the number to designate the stereochemistry of addition to each fragment. A subscript specifying the orbitals, viz., s, p (sigma, pi) with their usual significance) or n (for an orbital associated with a single atom only), may be added as a subscript before the number. Thus the normal Diels-Alder reaction is a $[4_s + 2_s]$ or $[p4_s + p2_s]$ cycloaddition. Cycloadditions may be *pericyclic reactions* or (non-concerted) *stepwise reactions*. The term 'dipolar cycloaddition' is used for cycloadditions of 1,3-dipolar compounds.

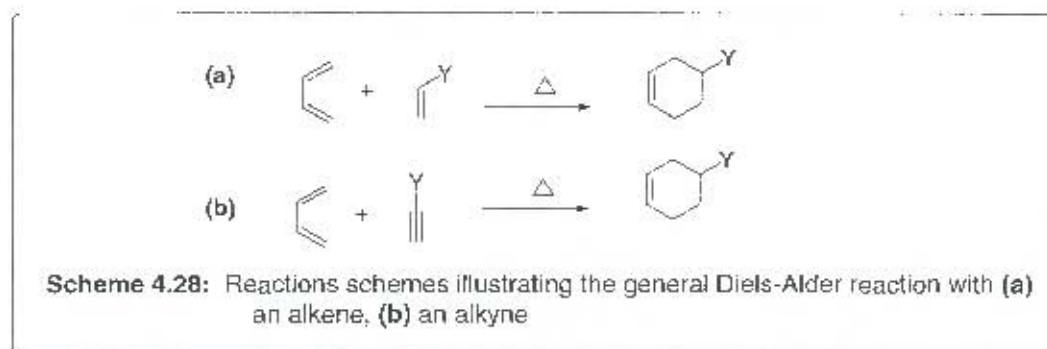
4.5.1.2.3.1 Types of cycloaddition

Two major cycloaddition reactions are:

- (i) Diels-Alder reaction
- (ii) 1,3-dipolar cycloaddition

(i) Diels-Alder reaction

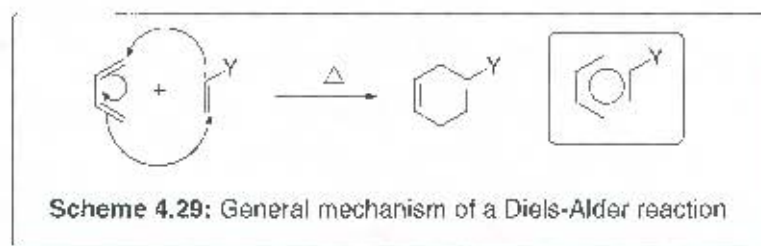
The [4+2]-cycloaddition of a conjugated diene and a dienophile (an alkene or alkyne), an electrocyclic reaction that involves the 4 π -electrons of the diene and 2 π -electrons of the dienophile. The driving force of the reaction is the formation of new σ -bonds, which are energetically more stable than the π -bonds.¹⁴⁶ In the case of an alkynyl dienophile, the initial adduct can still react as a dienophile if not too sterically hindered. In addition, either the diene or the dienophile can be substituted with cumulated double bonds, such as substituted allenes, (Scheme 4.28).



With its broad scope and simplicity of operation, the Diels-Alder is the most powerful synthetic method for unsaturated six-membered rings. A variant is the hetero-Diels-Alder, in which either

the diene or the dienophile contains a heteroatom, most often nitrogen or oxygen. This alternative constitutes a powerful synthesis of six-membered ring heterocycles.

Mechanism:



Overlap of the molecular orbitals (MOs) is required:

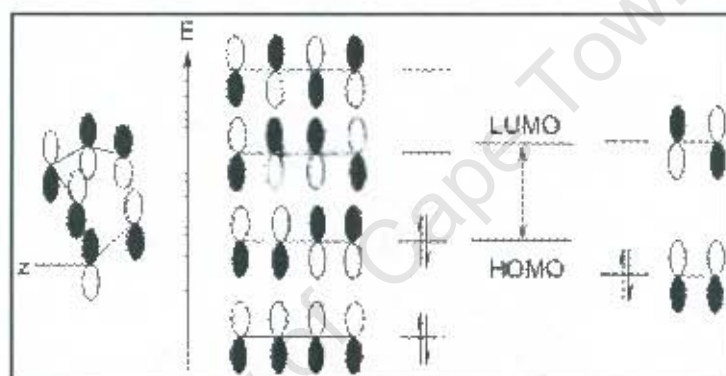


Figure 4.51: Schematic illustration of MOs overlap

Overlap between the highest occupied MO of the diene (HOMO) and the lowest unoccupied MO of the dienophile (LUMO) is thermally allowed in the Diels Alder reaction, provided the orbitals are of similar energy. The reaction is facilitated by electron-withdrawing groups on the dienophile, since this will lower the energy of the LUMO. Good dienophiles often bear one or two of the following substituents: CHO, COR, COOR, CN, C=C, Ph, or halogen. The diene component should be as electron-rich as possible, (Fig. 4.51).

There are "inverse demand" Diels Alder reactions that involve the overlap of the HOMO of the dienophile with the unoccupied MO of the diene. This alternative scenario for the reaction is favored by electron-donating groups on the dienophile and an electron-poor diene, (Fig. 4.52).

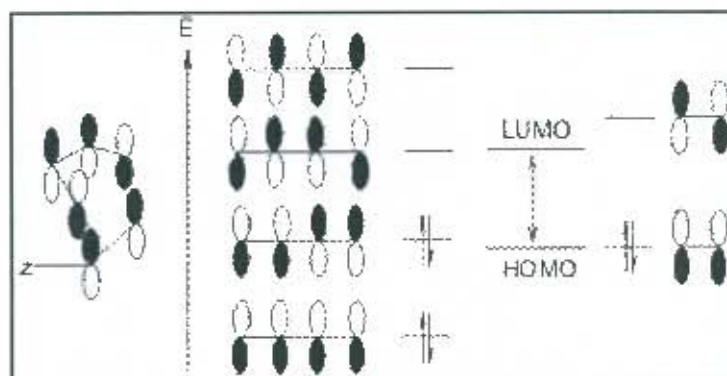
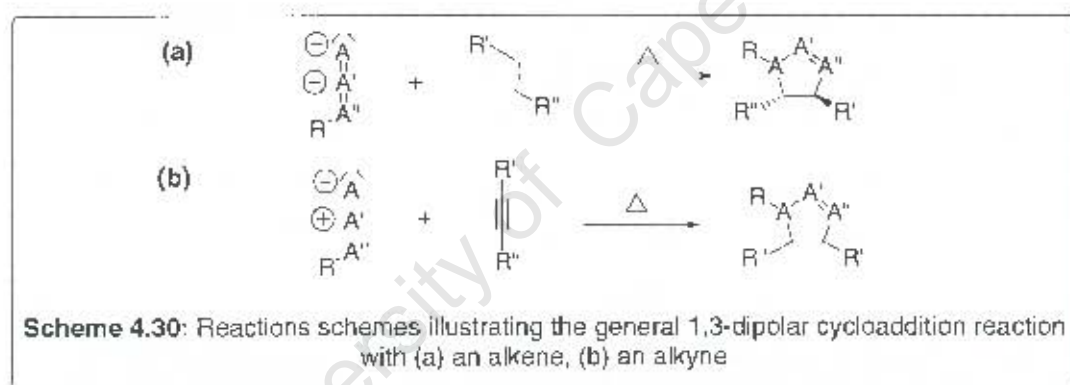


Figure 4.52: Schematic illustration the overlap of the HOMO of the dienophile with the unoccupied MO of the diene ("reverse demand")

(ii) 1,3-Dipolar Cycloaddition



The 1,3-dipolar cycloaddition, also known as the Huisgen cycloaddition or Huisgen reaction,¹⁴⁷ is an organic chemical reaction belonging to the larger class of cycloadditions.¹⁴⁸ It is the reaction between a 1,3-dipole and a dipolarophile, most of which are substituted alkenes, to form a five-membered ring, Scheme 4.30. Rolf Huisgen first saw the prospects of varying the 1,3-dipole and its high value for synthesis of 5-membered heterocycles. Examples of dipolarophiles are alkenes and alkynes and molecules that possess related heteroatom functional groups (such as carbonyls and nitriles). 1,3-Dipolar compounds contain one or more heteroatoms and can be described as having at least one mesomeric structure that represents a charged dipole, (Fig 4.53).

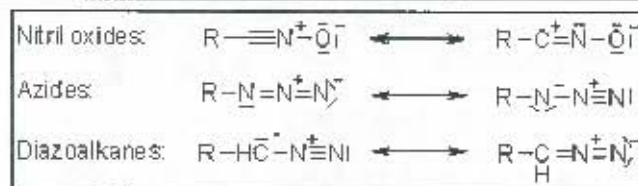
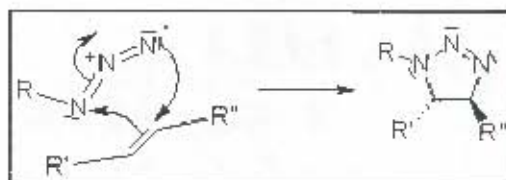


Figure 4.53: Examples of dipolarophiles that possess related heteroatom functional groups

Mechanism:



Scheme 4.31: General mechanism of a 1,3-dipolar reaction

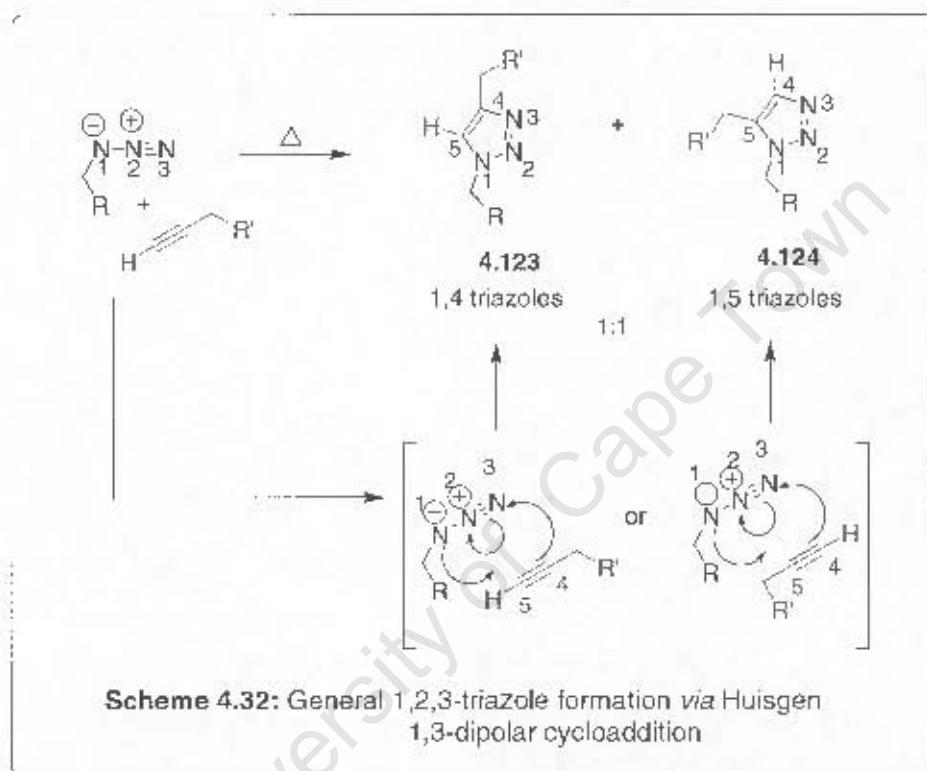
The 2 π -electrons of the dipolarophile and the 4 electrons of the dipolar compound participate in a concerted, pericyclic shift. The addition is stereoconservative (suprafacial), and the reaction is therefore a $[2_s+4_s]$ cycloaddition similar to the Diels-Alder Reaction. (Attention: many authors still use "[2+3] cycloaddition", which counts the number of involved atoms but does not follow IUPAC recommendations. IUPAC recommends the use of "(2+3)" for the number of involved atoms instead.)

A condition for such a reaction to take place is a certain similarity of the interacting HOMO and LUMO orbitals, depending on the relative orbital energies of both the dipolarophile and the dipole. Electron-withdrawing groups on the dipolarophile normally favor an interaction of the LUMO of the dipolarophile with the HOMO of the dipole that leads to the formation of the new bonds, whereas electron donating groups on the dipolarophile normally favor the inverse of this interaction. Diazomethane as an electron-rich dipolar compound therefore rapidly reacts with electron-poor alkenes, such as acrylates. Relative reactivity patterns may be found in the literature.^{149, 150}

4.5.1.2.4 Huisgen [3+2] cycloaddition in "Click" Chemistry

The Huisgen [3+2] cycloaddition between a terminal alkyne and an azide has rapidly become the most popular "click" reaction to date. This reaction has been termed the "cream of the crop"

of "click" reactions¹³³ and has found application in various facets of drug discovery.¹⁵¹ The formation of triazoles *via* the cycloaddition of an azide and acetylene was first reported by Dimroth in the early 1900's but the generality, scope, and mechanism of these cycloadditions was not fully realized until the 1960's.¹³⁹ The reaction generates a mixture of 1,4- and 1,5-disubstituted triazoles (4.123 and 4.124, Scheme 4.32).



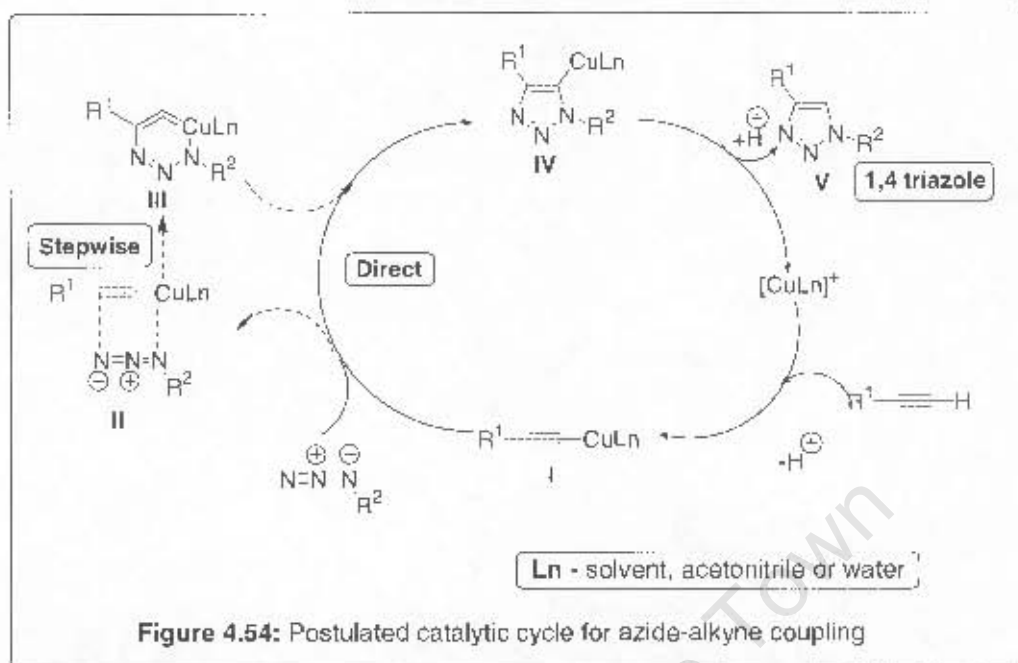
4.5.1.2.4.1 Variant of Huisgen [3+2] Cycloaddition- The use of Copper(I) Catalyst

A variant of the Huisgen 1,3-dipolar cycloaddition is the copper(I) catalyzed variant, which reacts azides and terminal alkynes to afford 1,4-triazoles as the sole product. While the copper(I) catalyzed variant gives rise to a triazole from a terminal alkyne and an azide, it is not a 1,3-dipolar cycloaddition and thus should not be termed a Huisgen cycloaddition. This reaction is better termed the copper(I) catalyzed azide and terminal alkyne cycloaddition (CuAAC), but is certainly a "click" reaction.¹⁵² Various attempts to control the regioselectivity have been reported without much success until the discovery of the copper(I)-catalyzed reaction in 2002, which exclusively yields the 1,4-disubstituted 1,2,3-triazoles (4.123).^{153(a), 153(b)} Several copper(I) salts

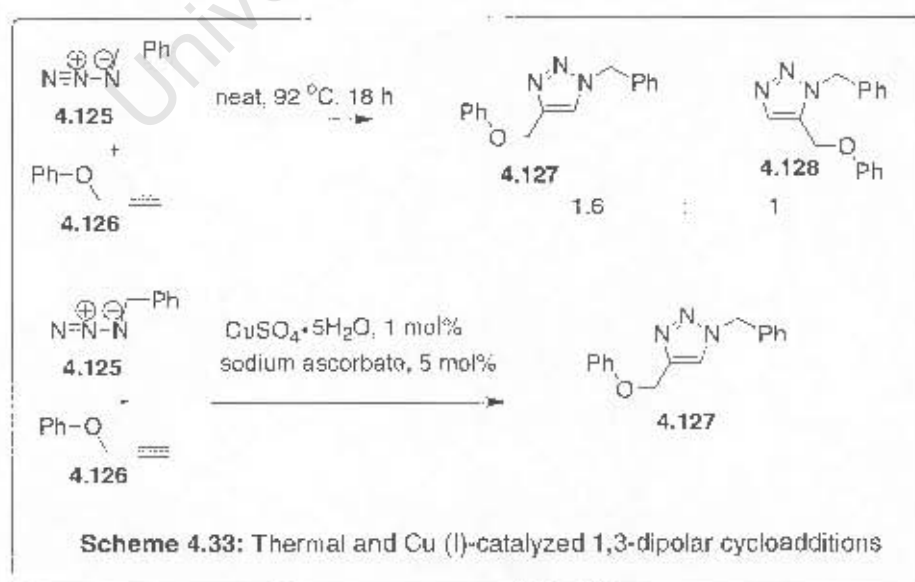
such as CuI, CuOTf·C₆H₆, and [Cu(NCCH₃)₄][PF₆] can be employed but the reactions generally must be carried out with acetonitrile as co-solvent, require a nitrogen base, and sometimes generate undesired diacetylene, bis-triazole and 5-hydroxytriazoles as byproducts.^{155(a)} The *in situ* reduction of copper(II) salts such as CuSO₄·5H₂O with sodium ascorbate in aqueous alcoholic solvents allows the formation of 1,4-triazoles at room temperature in high yields with less than 2 mol % catalyst loading. Primary, secondary, and tertiary substituted azides as well as aromatic azides can be utilized. Numerous terminal acetylene components participate in the transformation and the reaction is compatible with various functional groups such as esters, acids, alkenes, alcohols, and amines. The copper-catalyzed reaction was later expanded by Yamamoto and coworkers using a bimetallic catalyst so that 1,4,5-substituted triazoles could be obtained from internal alkynes.¹⁵⁴

4.5.1.2.4.1.1 Proposed Mechanism

The proposed catalytic cycle for azide-alkyne coupling is depicted in Fig. 4.54.^{153(a), 155, 156} The copper-catalyzed reaction is proposed to proceed in a stepwise manner starting with the coordination of the alkyne to the Cu(I) generating the Cu(I) acetylide species, intermediate (I), displacing one of the acetonitrile or water ligands. In the following step, the azide replaces another one of the ligands and binds to the copper atom *via* the nitrogen proximal to the carbon, forming intermediate (II). The distal nitrogen of the azide in intermediate (II) attacks the C-2 carbon of the acetylide, yielding the unusual six-membered Cu(III) metallocycle, intermediate (III). Proteolysis of intermediate (IV) releases the triazole product, thereby completing the catalytic cycle. Density functional theory calculations have shown a preference for the stepwise addition (I→II→III→IV) over the concerted cycloaddition (I→IV) by approximately 12 to 15 kcal mol⁻¹, leading to the six-membered metallocycle (III), (Fig. 4.54).



Scheme 4.33 demonstrates the effectiveness of the copper catalyst in a regioselective “click” reaction by comparing the thermal reaction between benzyl azide (4.125) and phenyl propargyl ether (4.126) with the copper-catalyzed reaction of the same substrate. The thermal reaction leads to the formation of two disubstituted triazole isomers (4.127) and (4.128) while the copper (I)-catalyzed reaction selectively produces the 1,4-isomer (4.127) in 91% yield after 8 hours, (Scheme 4.33).^{153(a)}

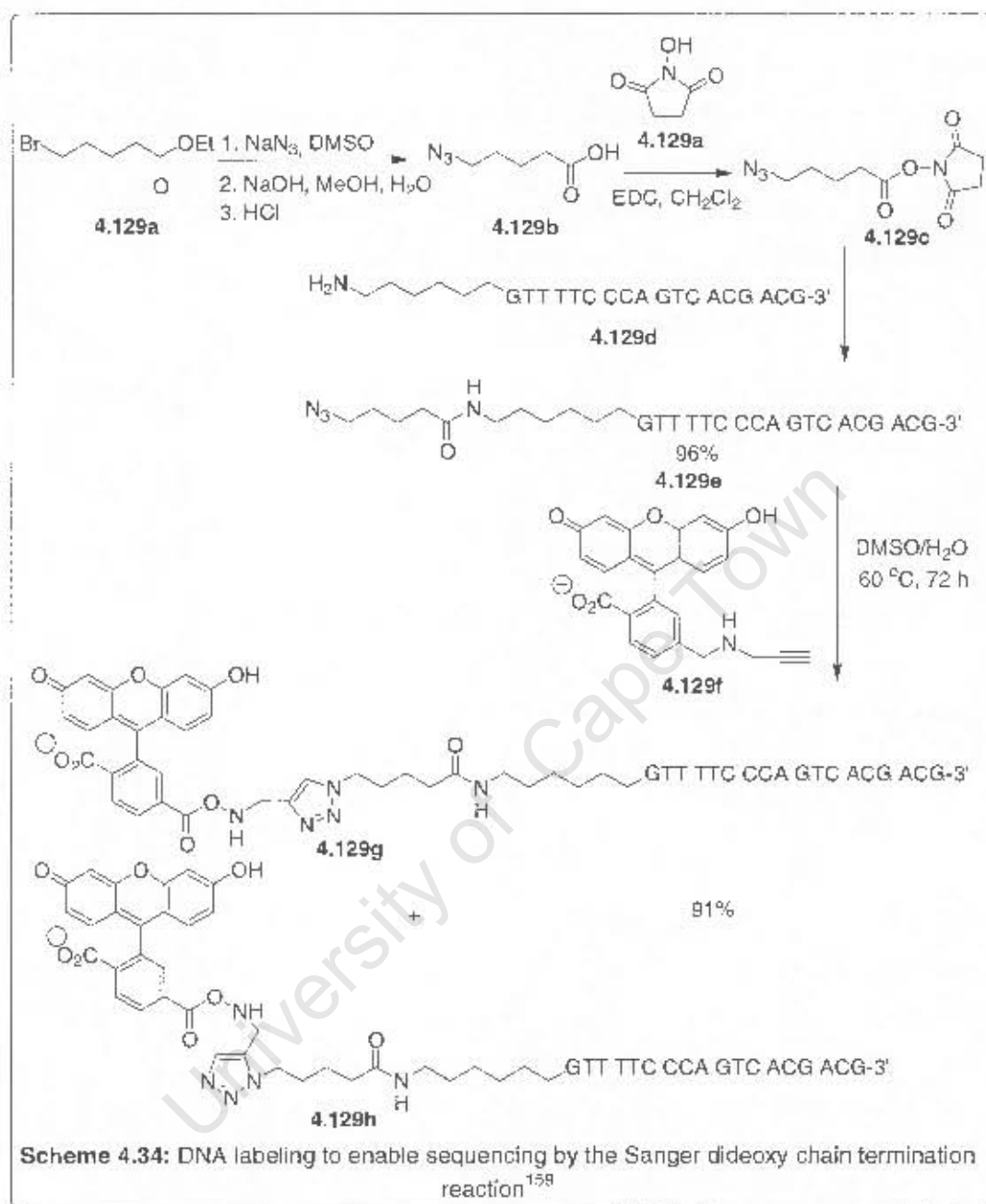


4.5.2 Application of Triazoles and Biological Importance

The development of the copper(I)-catalyzed cycloaddition reaction between azides and terminal alkynes has led to many interesting applications of "click" reactions including in a wide range of industrial applications such as agrochemicals, corrosion inhibitors, dyes, optical brighteners as well as biologically active agents.^{153(b)} Their applications in many areas of biology have also demonstrated their versatility. Areas of particular interest include, (i) DNA sequence, (ii) biological inhibitors and (iii) *in situ* 'click' approach.^{157, 158}

4.5.2.1 DNA sequence

Recently the use of "click" chemistry as in the 1,3-dipolar cycloaddition reaction was explored by Ju *et al.*¹⁵⁷ who synthesized fluorescent single stranded DNA (ssDNA) and applied the same as a primer in the Sanger dideoxy chain termination reaction¹⁵⁹ to produce DNA sequencing fragments (4.129g and 4.129h, Scheme 4.34).

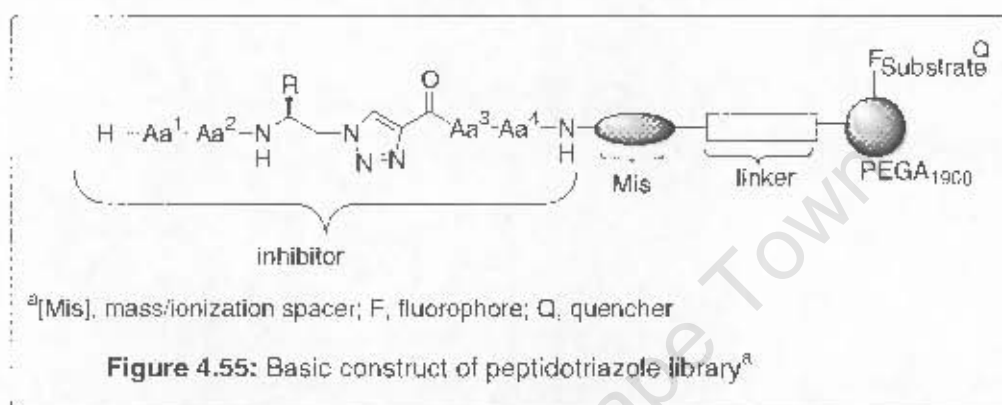


4.5.2.2 Biological Inhibitors

4.5.2.2.1 Leishmania Cysteine Protease Inhibitors

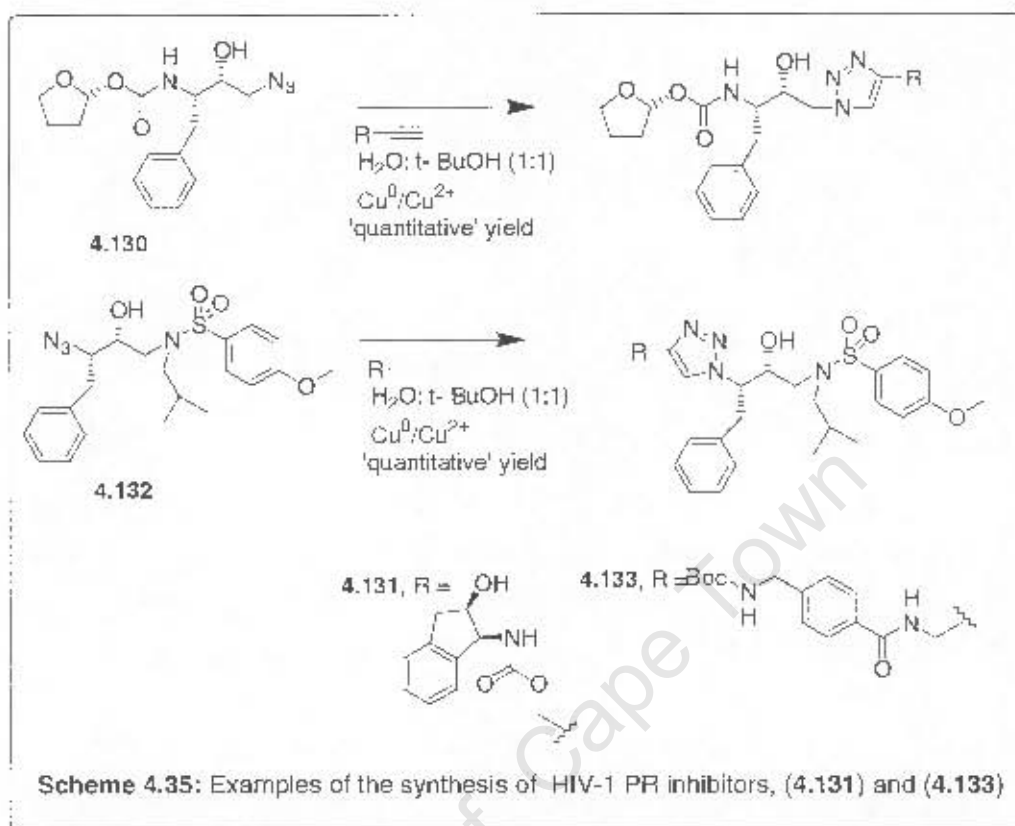
A tandem array of 19 genes express a cysteine protease, (cathepsin-like cysteine protease, termed CPb) which has been identified as an excellent target for drugs against leishmaniasis, a

disease caused by the protozoan parasite *Leishmania*.^{60, 61} A library of potential peptidotriazoles cysteine protease inhibitors was prepared by solid-phase peptide synthesis combined with a regiospecific copper(I)-catalyzed 1, 3-dipolar cycloaddition between a resin-bound alkyne and a protected amino azide, (Fig. 4.55). The library was screened against a recombinant cysteine protease, designated *Leishmania Mexicana* CPB2.8ΔCTE, affording novel inhibitors with K_i values in the low nanomolar to high micromolar range.



4.5.2.2.2 HIV-1 Protease (HIV-1 PR) Inhibitors

HIV-1 PR is responsible for the final stages of the virus maturation and, thus, its inhibitors are useful drugs for the treatment of AIDS.⁶² The emergence of drug-resistant mutant HIV-1 PRs increases the demand for new inhibitors.¹⁶³ Brik *et al.*¹⁶⁴ prepared two focused libraries of 50 compounds each, each based on hydroxyethylamine peptide isosteres, Scheme 4.35. The library was generated from appropriate azide-bearing scaffolds which were fused *via* the copper(I) catalyzed process with acetylenes. Screening against wild type HIV-1 PR and three mutants (G48V, V82F, and V82A), revealed compounds (4.131) and (4.133) derived from the scaffold (4.132) to be more active than compounds derived from scaffold (4.130) in the low nanomolar range.



4.5.2.2.3 *In Situ* synthesis in biomolecular active sites *via* 'Click' reactions

Target-guided synthesis (TGS) is the use of small molecule building blocks that can be assembled by specifically targeted enzymes to synthesize their own inhibitors. Only building blocks that interact with the active site of the protein will be in close proximity to react with one another and form potent inhibitors.¹⁶⁵ Installation of azides and alkynes within organic building blocks has allowed the use of the Huisgen [3+2] cycloaddition to discover an inhibitor of acetylcholinesterase (AChE),¹⁶⁶ an enzyme that hydrolyzes acetylcholine, a neurotransmitter, that plays a vital role in the central nervous system.¹⁶⁷ Out of all possible azide-alkyne cycloaddition products, only the 1,5-triazole (4.136) was formed from the combination of (4.134) and (4.135) in the presence of AChE, (Fig. 4.56).¹⁶⁶ This inhibitor (4.136), is currently the most potent inhibitor of AChE and has led to the discovery of three new inhibitors of AChE.¹⁶⁶ Also subsequent co-crystallization studies of (4.136) with AChE demonstrated how it bound to the enzyme in a previously unknown intermediate enzyme conformation. Thus the enzyme template inhibitor selection methodology enables the discovery of a potent inhibitor that would

not have been expected/predicted as a promising inhibitor using standard structure-based drug design.

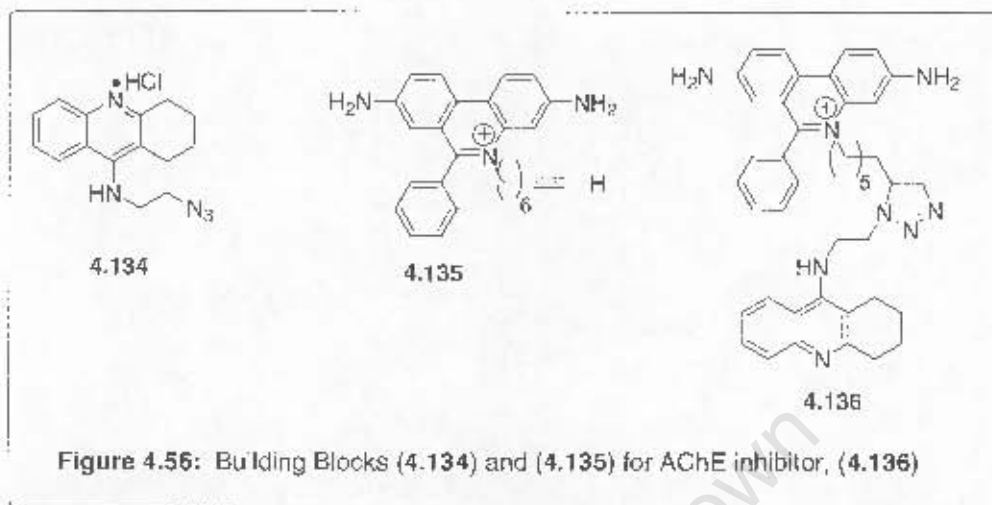


Figure 4.56: Building Blocks (4.134) and (4.135) for AChE inhibitor, (4.136)

4.5.3 Rationale for Drug Design

The “click” approach seems to be an ideal strategy for rapid access to SAR compounds due its simplicity, diversity and efficiency. The primary focus of this design is based on the independent synthesis of antimalarial and/or cysteine protease inhibitor-based “click” building blocks which are incorporated into a single molecule to produce bi-therapeutic/dual compounds which may exhibit enhanced inhibitory activity, (Fig. 4.57).

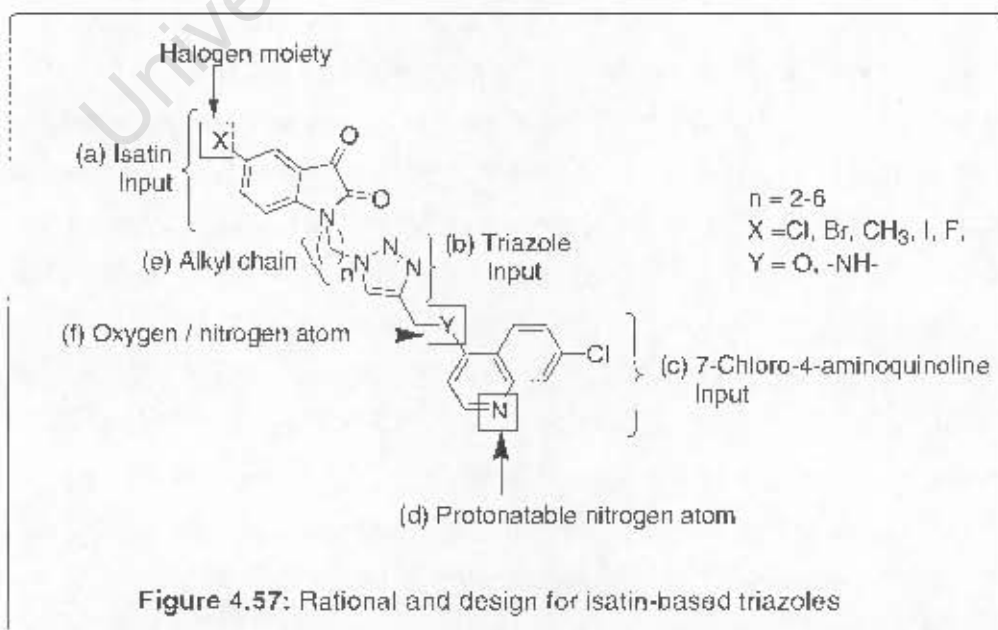


Figure 4.57: Rational and design for isatin-based triazoles

The rationale and background of the various inputs are summarized as follows:

(a) The isatin input provides a hydrophobic aromatic ring template potential for binding to hydrophobic sites of the target. In addition isatin is a privileged natural product and through variation of the substituents on the scaffold enable greater product diversity. Isatins have particularly shown to be effective enzyme inhibitors of cysteine and serine proteases.¹⁶⁸ A library of thiosemicarbazones isatin derivatives synthesized in our laboratories have also shown to display inhibitory activities against the parasitic cysteine proteases cruzain, falcipain-2 and rhodesain.¹⁶⁹ More recently, work done also in our laboratories produced a series of 4-aminoquinoline isatin derivatives which showed anti-plasmodial activities with IC_{50} values in the low micromolar range against a chloroquine-sensitive (D10) and two resistant (K1 and W2) strains of *P. falciparum*, respectively.¹⁷⁰

(b) As previously indicated (section 4.5.2, page 152), the triazole moiety has demonstrated a diversity of properties, especially in drug discovery. The triazole pharmacophore also provides an increase in water solubility properties of a compound.¹⁷¹

(c) The 7-chloro-4-aminoquinoline input has been shown to be important for inhibition of haemozoin formation which is crucial for parasite survival.¹⁷²

(d) The protonatable nitrogen atom in the quinoline ring assists in the accumulation of the compound in the acidic food vacuole via pH trapping. In addition, the protonation of the nitrogen atom can also increase the aqueous solubility of the compound.

(e) Variation of the alkyl chain (spacer) between the isatin and triazole moieties could lead to more active compounds that are able to circumvent parasite resistance to the aminoquinoline drugs.¹⁷³

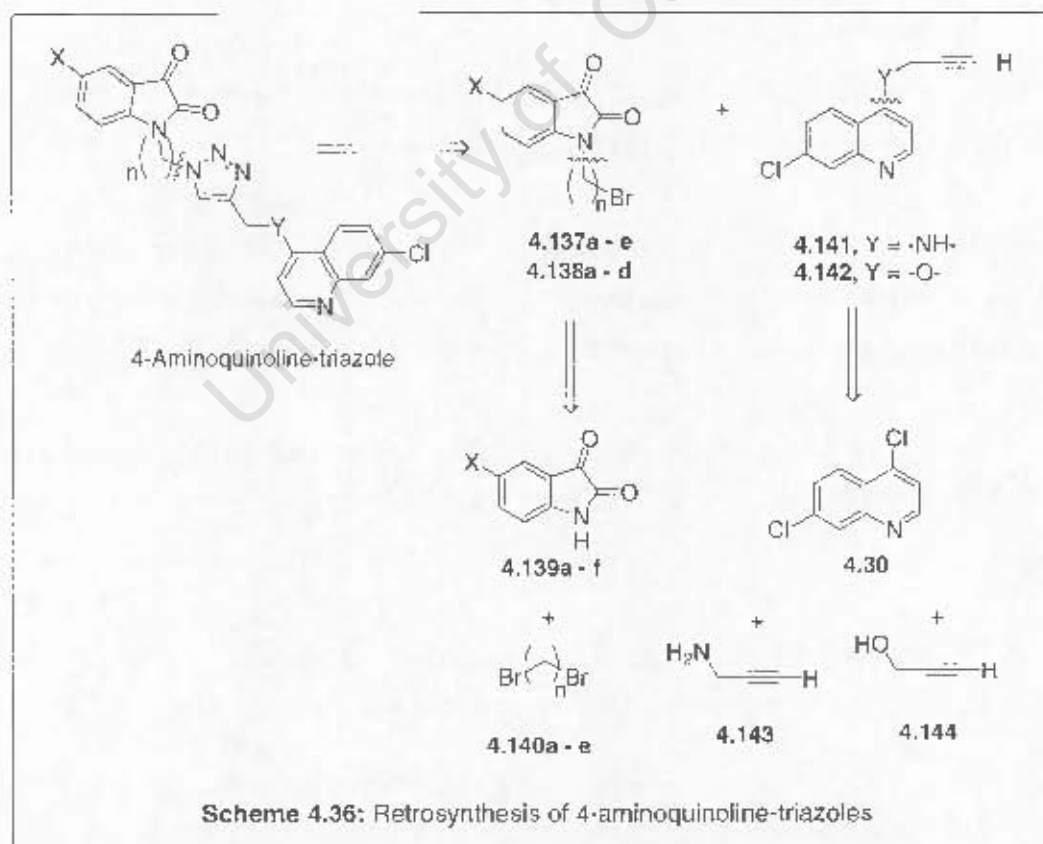
(f) Variation of the oxygen and nitrogen atoms would allow comparison of the antimalarial properties of the respective compounds since the two atoms have different effects on the basicity of the quinoline nitrogen.

4.5.4 Synthesis and Characterization

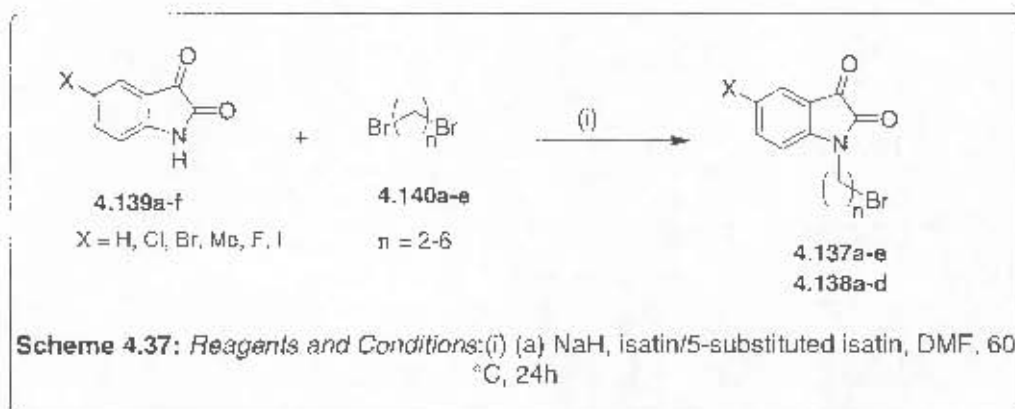
Three related methods were employed for the synthesis of the target triazoles which were mainly governed by the type of inputs used to assemble the envisaged compounds.

4.5.4.1 Retro-synthesis of 4-Aminoquinoline-Containing Triazoles

Scheme 4.36 presents the retrosynthesis of the envisaged target 4-aminoquinoline-containing triazoles. 1,3-Dipolar cycloaddition of respective acetylenes, (4.141), (4.142) and isatin halides (4.137a-e), (4.138a-d) *via in situ* generated azides leads to the desired triazoles. Retrosynthetic analysis of the relevant intermediate acetylenes (4.141) and (4.142), results in 4,7-dichloroquinoline (4.30), propargyl amine (4.143) and propargyl alcohol (4.144). The isatin halides (4.137a-e) and (4.138a-d) are obtainable from isatins (4.139a-f) and dibromoalkanes (4.140a-e).



4.5.4.2 Synthesis of Isatin-Derived Alkyl Halides (4.137a-e) and (4.138a-d)



In this straightforward synthesis (Scheme 4.37) commercially available isatins ((4.139a-f)) were treated with 1, 3-dibromopropane in the presence of NaH as the base in DMF as the solvent to generate the desired isatin-derived alkyl halide inputs (4.137a-e), (Fig. 4.58)¹⁷⁰ respectively in moderate (recrystallized) yields, (Table 4.11).

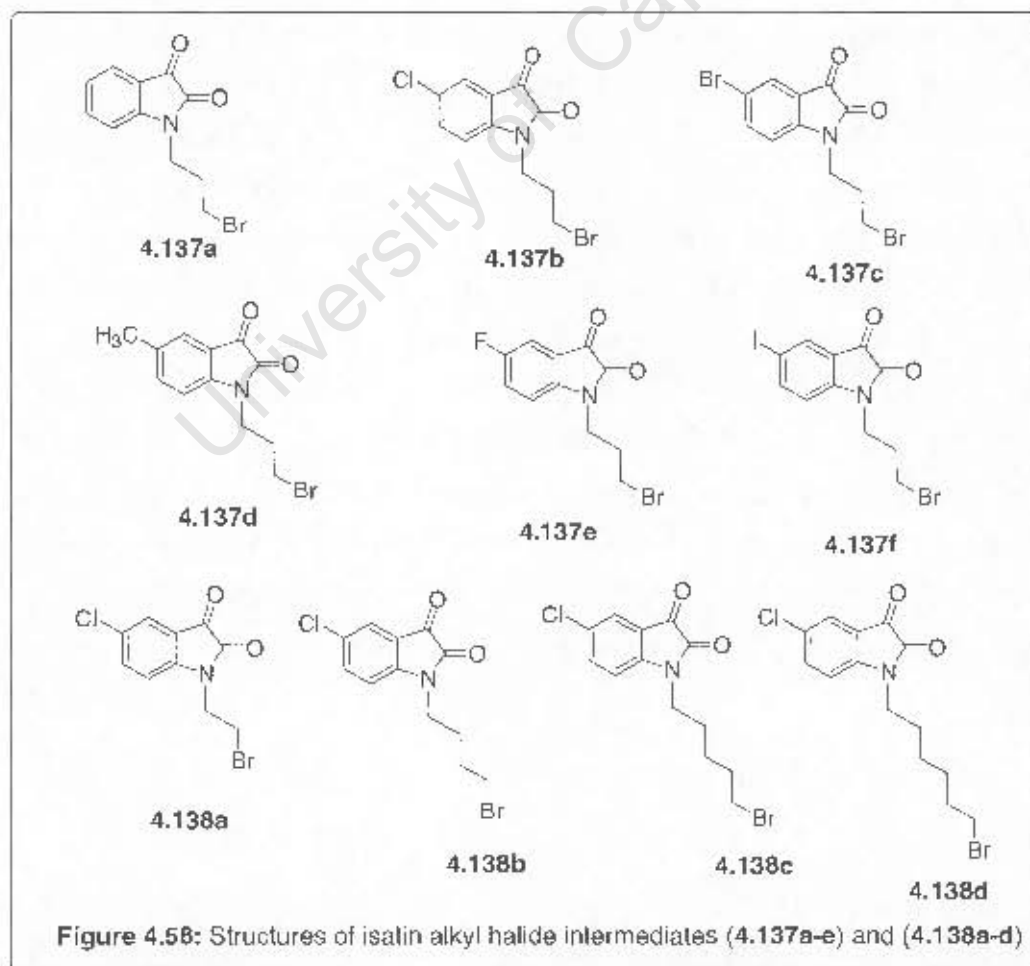
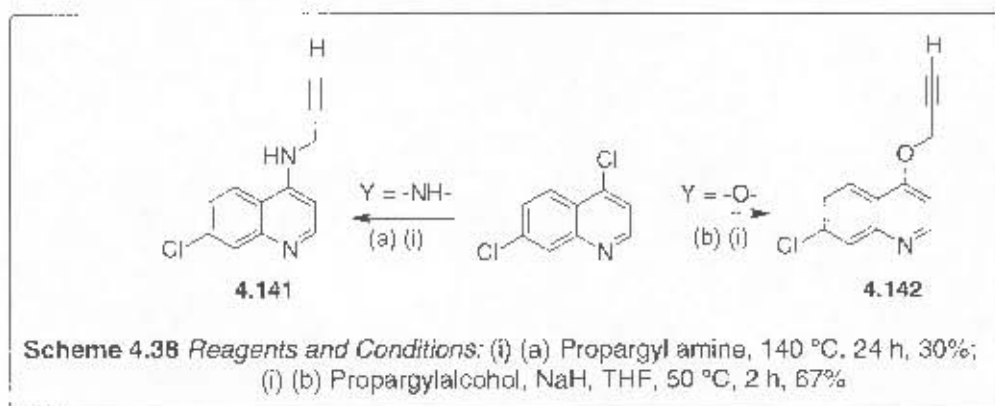


Table 4.11: Recrystallized yields obtained for compounds (4.137a- e) and (4.138a- d)

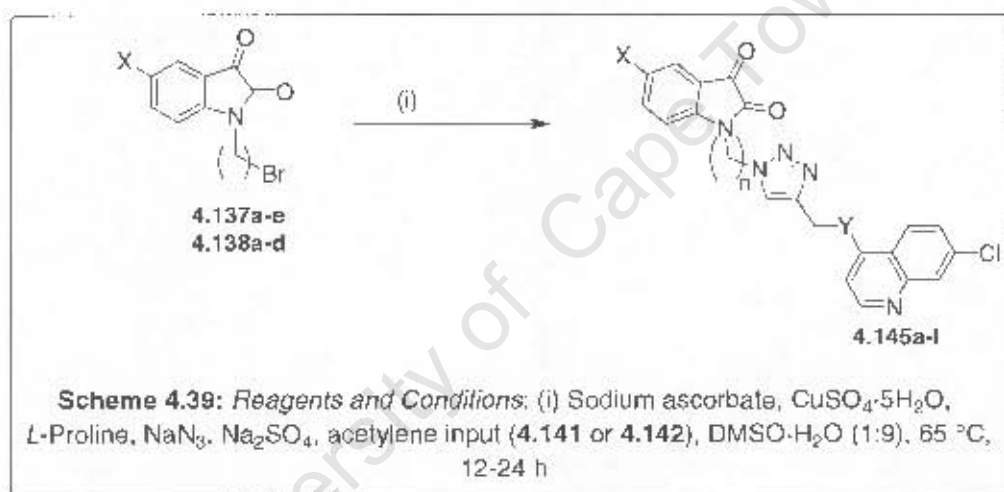
Compound	Yield (%)
4.137a	68
4.137b	69
4.137c	55
4.137d	52
4.137e	47
4.138a	42
4.138b	80
4.138c	62
4.138d	66
4.138e	75

4.5.4.3 Synthesis of Acetylenes (4.141) and (4.142)

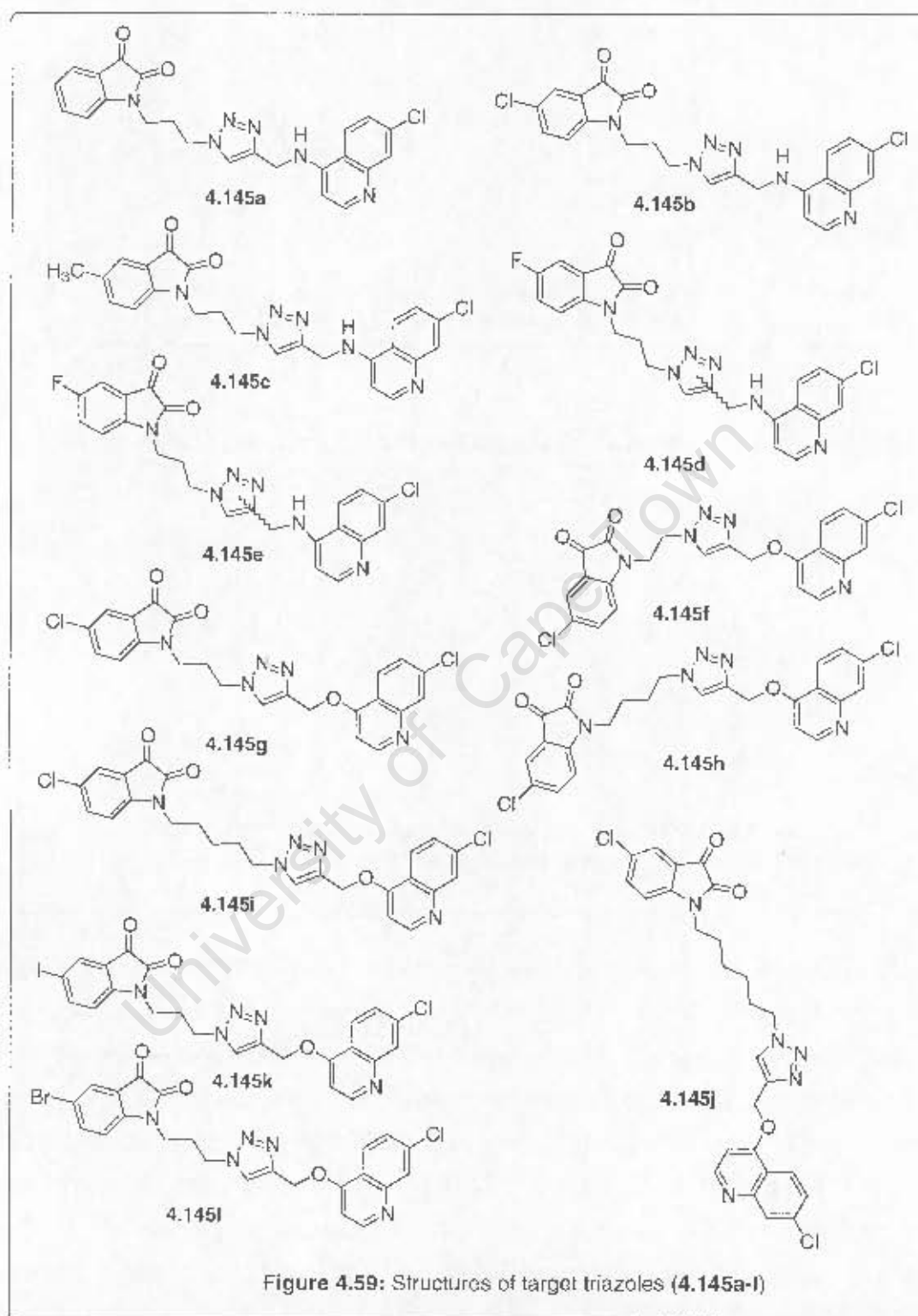
The method described by De *et al.*¹⁷³ was the most suitable one for this synthesis, Scheme 4.38, (a), since a procedure reported by Biot *et al.*¹⁷⁴ which involved the use of *N*-methylpyrrolidinone (NMP) as the solvent in the presence of Et₃N and K₂CO₃ as the base, failed to produce the desired product. In this approach 4,7-dichloroquinoline was treated with propargyl amine (4.143) under reflux at 140 °C for 24 h to yield the intermediate acetylene (4.141) in low yield. The reaction was performed in the neat in the absence of base, which was introduced in the work up to remove the acid, formed as a byproduct. Product formation is dependent on refluxing at high temperatures for the required time period. On the other hand acetylene (4.142) was prepared by adapting literature methodology reported by Kaval *et al.*,¹⁷⁵ Scheme 4.38 (b). This procedure involves treatment of 4,7-dichloroquinoline with propargyl alcohol (4.144) in the presence of sodium hydride using THF as the solvent. Slight heating of the resulting reaction mixture at 50 °C for 2 h afforded the desired product (4.142). Exceeding the reaction time (>5 h) gave rise to the formation of unwanted byproducts.



4.5.4.4 One-Pot Synthesis of Triazoles (4.145a-l) from *In Situ* Generated Azides



Although organic azides are generally safe compounds, those with low molecular weight can be unstable and, therefore, difficult to handle.¹⁷⁶ Thus, methodology that avoids isolation of organic azide intermediates is desirable. An one-pot, two-step procedure was employed for trapping generated azides by alkynes to obtain the corresponding triazole products.¹⁷⁷ This method entails the preparation of *in situ* generated azides from the corresponding halides (by nucleophilic substitution) *via* a Cu(I)-catalyzed proline-promoted reaction, (Scheme 4.39).¹⁷⁸ Even though this method¹⁷⁹ has been reported to be suitable for producing 1,4 regioisomers exclusively, a mixture of 1,4 and 1,5 regioisomers, (4.145d) and (4.145e) were obtained in one case, (Fig. 4.59).



The desired triazoles were obtained in low to moderate (recrystallized) yields, Table 4.12. In all cases the pure compounds were isolated by column chromatography. The melting points were

generally ranged from low to high with the two regioisomers, (4.145d, 221-222 °C) and (4.145e, 118-120 °C) differing significantly.

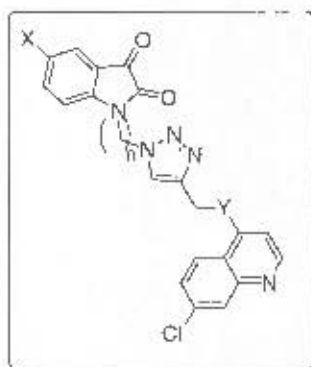


Table 4.12: Yields obtained after recrystallization for compounds (4.145a-l)

Compound	n	X	Y	Yield (%)	Melting point (°C)
4.145a	3	H	NH	40	229-233
4.145b	3	Cl	NH	45	106-109
4.145c	3	CH ₃	NH	38	215-217
4.145d	3	F	NH	36	221-222
4.145e	3	F	NH	15	118-120
4.145f	2	Cl	NH	20	98-100
4.145g	3	Cl	O	36	106-109
4.145h	4	Cl	O	44	176-177
4.145i	5	Cl	O	45	57-59
4.145j	6	Cl	O	54	136-138
4.145k	3	I	O	31	81-84
4.145l	3	Br	O	31	86-88

Infrared spectroscopy was employed to confirm the presence of key functional groups in the proposed structures. The IR spectra indicated the characteristic stretching bands at; 1725 cm⁻¹ and 1617 cm⁻¹ assigned to the two isatin carbonyl groups. The ¹H NMR spectrum (Fig. 4.60) of compound (4.145j) revealed the following characteristic signals; a one-proton singlet at δ 8.34 assigned to H-10, a two-proton singlet at δ 5.44 assigned to CH₂-12, a two-proton triplet (*J* = 7.0) at δ 4.36 assigned to CH₂-6 and a two-proton triplet (*J* = 7.0) at δ 3.59 assigned to CH₂-1.

The ^{13}C NMR spectrum also showed the characteristic isatin carbonyl groups at δ 182.8 and δ 161.1. Further evidence for the proposed structure was given by the low resolution mass spectrum which indicated the molecular ion at m/z 524 (M').

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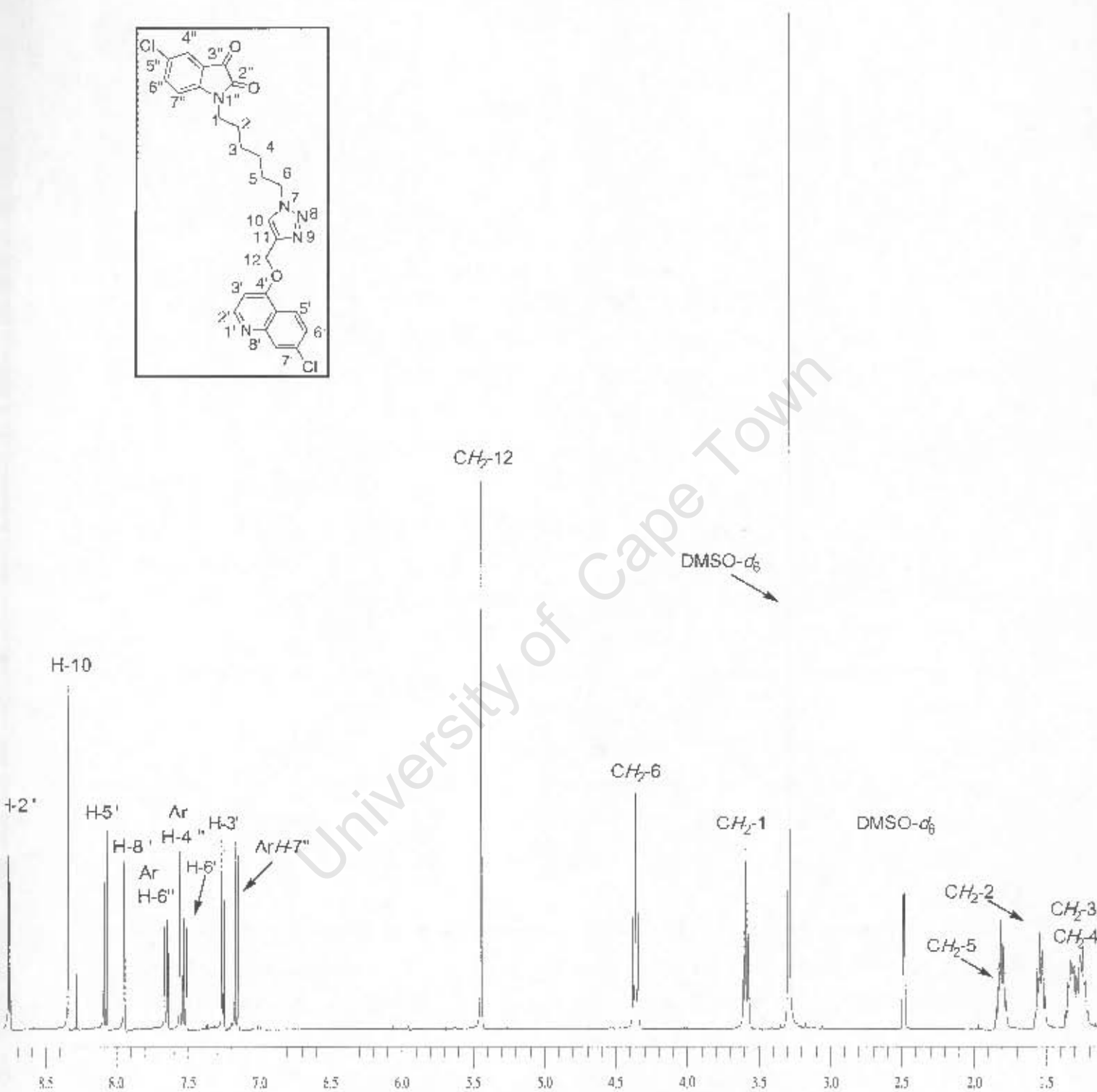
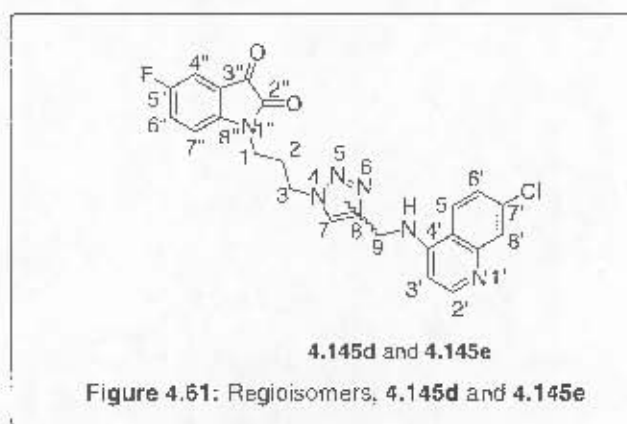


Figure 4.60: ^1H NMR of (4.145j) in $\text{DMSO}-d_6$



Attempts to grow crystals of the two regioisomers, (4.145d) and (4.145e), (Fig. 4.61) were unsuccessful. The ^1H NMR spectra of the two regioisomers, (4.145d) and (4.145e) indicated comparable chemical shift values for the respective signals except in the aromatic region where minor differences were observed, (Fig. 4.62). One of the most noticeable differences was the D_2O -exchangeable nitrogen proton in (4.145d) that formally appeared at δ 7.86 as a triplet ($J = 5.6$) was shifted more downfield to δ 7.94 in (4.145e) where it appears as a broad triplet ($J = 5.6$). Although the aromatic protons, ArH-4'' and ArH-7'' appear at the chemical shift values, (δ 7.48-7.43) their respective multiplicity pattern was different, (Fig. 4.62). The chemical shift values for the H-7 protons only differ by one digit, δ 4.56 in (4.145d) and δ 4.55 in (4.145e) where they appear as singlets. Table 4.13 demonstrates that since the characteristic signals in related structures (4.145a-c) appear at nearly the same chemical shift values as the characteristic signals in regioisomers, (4.145d) and (4.145e), a general trend was difficult to establish in order to confirm the regioselectivity of this particular reaction. Also, the ^{13}C NMR revealed minor differences in the chemical shift values for the characteristic C-7, C-8 and C-9 carbons. Various attempts to grow crystals is still under investigation.

The regioisomers, (4.145d) and (4.145e) were obtained after repeating the reaction for several times in the presence of the Cu(I) catalyst under click conditions. The reproducibility of the results proves that the fluoro-containing substrate may proceed *via* the cycloaddition of an azide and acetylene as proposed by Huisgen *et al.*¹³⁹ to yield the regioisomers rather than the non-concerted (stepwise) mechanism, suggested by Rostovtsev *et al.*^{153(a)} to yield the single 1,4-regioisomer. Since this effect was solely observed in the fluoro-containing substrate, it is assumed, that the nature of the fluoro-substituent influences the mechanistic pathway in such a way as to favor the pericyclic mechanism.

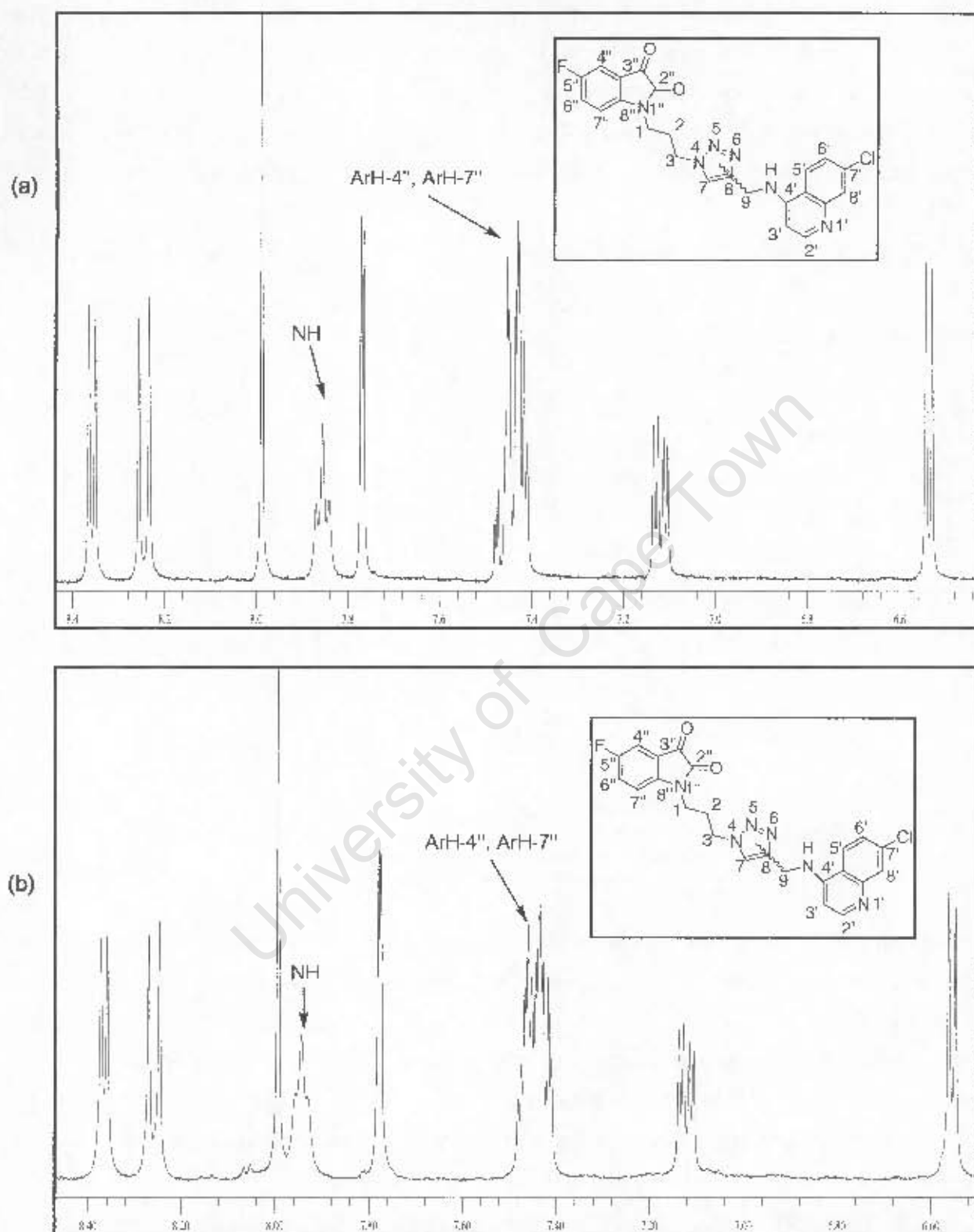
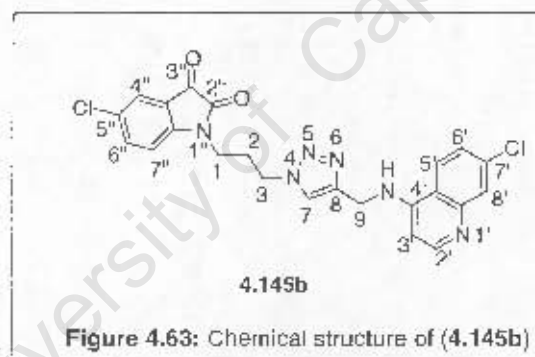


Figure 4.62: Expanded aromatic regions in ¹H NMR of (a) 4.145d and (b) 4.145e in DMSO-*d*₆

Another possibility worth highlighting regarding the structures of triazoles (4.145d) and (4.145e) is that both these compounds might be 1,4-triazoles but cis/trans isomers about the amide bond. The aryl-fluoro-substituent may influence the rotation barrier about the tertiary amide. The triazole peaks (assigned 3, 7 and 9) in the $^{13}\text{C}/^1\text{H}$ NMRs appear at the same chemical shift values for both (4.145d) and (4.145e) triazoles, suggesting that they may be cis/trans isomers.

Table 4.13: Chemical shift values of characteristic signals of structurally related compounds (4.145a-e)

Compound	N-H	H-7	CH ₂ -9
4.145a	7.78	7.87	4.58
4.145b	7.89	7.98	4.56
4.145c	7.71	7.96	4.56
4.145d	7.86	7.99	4.56
4.145e	7.94	7.99	4.55



A noticeable observation in both the ^1H NMRs and ^{13}C NMRs of the target triazoles were the duplication of individual peaks in the spectra, for example (4.145b, Fig. 4.63). This observation is ascribed to the slow interconversion between the *cis* and *trans* isomers about the tertiary amide bond which gave rise to two rotamers (major and minor). This phenomenon of conformation rotation, also demonstrated by Musonda *et al.*¹⁶⁰ was further proven by the different chemical shift values (observed in some peaks) and same coupling constants obtained for the duplicating peaks. However, this effect was prevalent in deuterated CH_3OH NMR solvent, run at 25 °C while running variable temperature (VT) NMR at 25, 30, 90 and 60 °C in deuterated DMSO, indicated coalescence of the duplicating peaks at these temperatures, (Fig.4.64 and Fig. 4.65). Another interesting observation was the upfield shifting (δ 7.91-7.60) of

the nitrogen proton with increasing temperatures, 25, 30, 90 and 60 °C, (Fig. 4.65). This observation may be attributed to the intramolecular hydrogen bonding that exists between the nitrogen triazole and nitrogen proton, (Fig. 4.66) which result in the formation of a stable 5-membered ring. Hence, at low temperatures the nitrogen proton appears more downfield due to the strong deshielding effect contributed by the two adjacent nitrogen atoms. As the temperature increases, the hydrogen bond breaks and this effect becomes less due to the influence of only one hydrogen atom, causing the proton to appear more upfield.

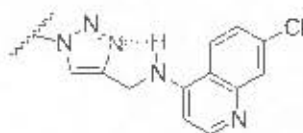


Figure 4.66: Diagram illustrating the intramolecular hydrogen bonding between the nitrogen triazole and nitrogen proton

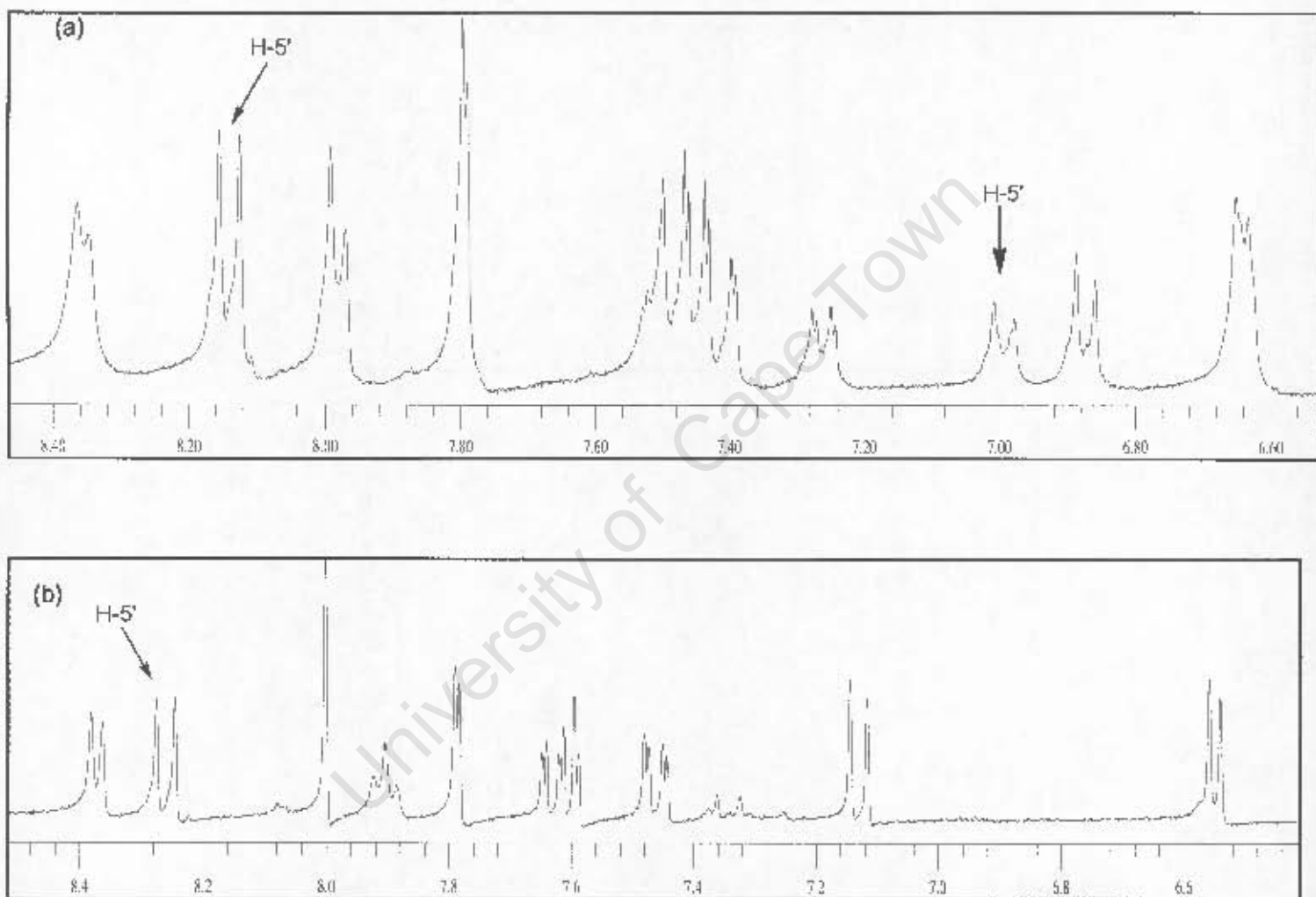
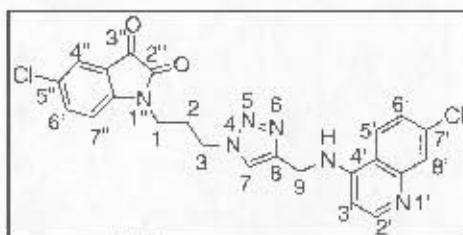


Figure 4.64: ^1H NMR of (4.145b) in (a) CD_3OD , (b) and $\text{DMSO}-d_6$ both at 25°C

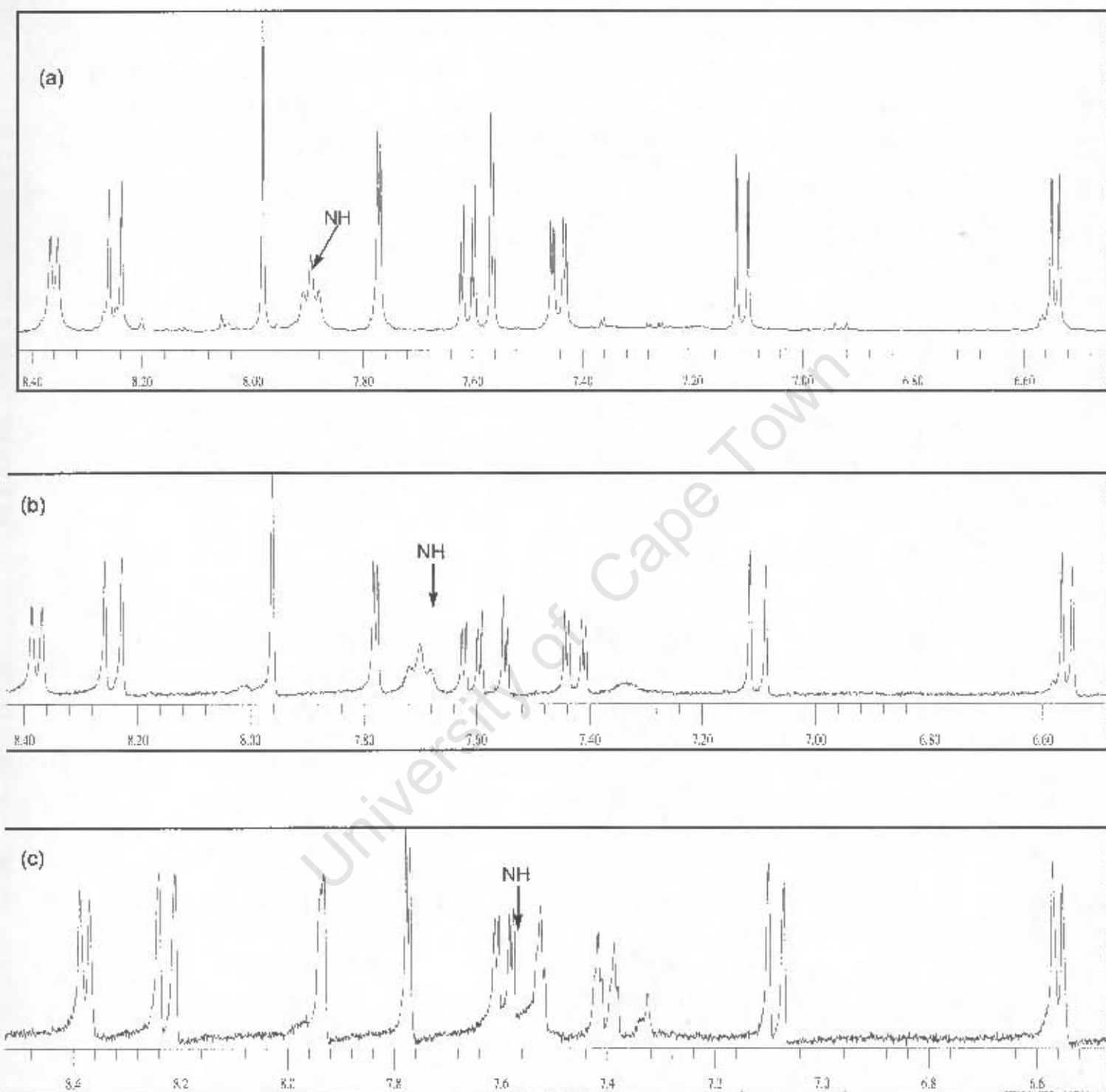
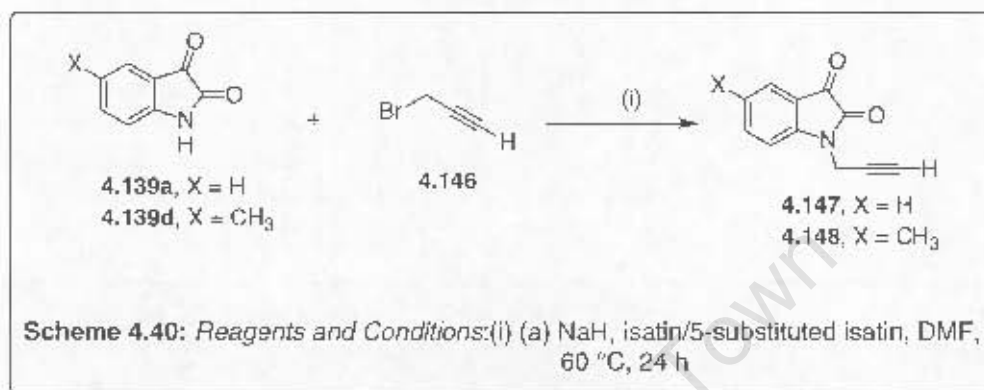


Figure 4.65: ^1H NMR of (4.145b) in $\text{DMSO}-d_6$ (a) at 30 °C, (b) at 60 °C and (c) at 60 °C

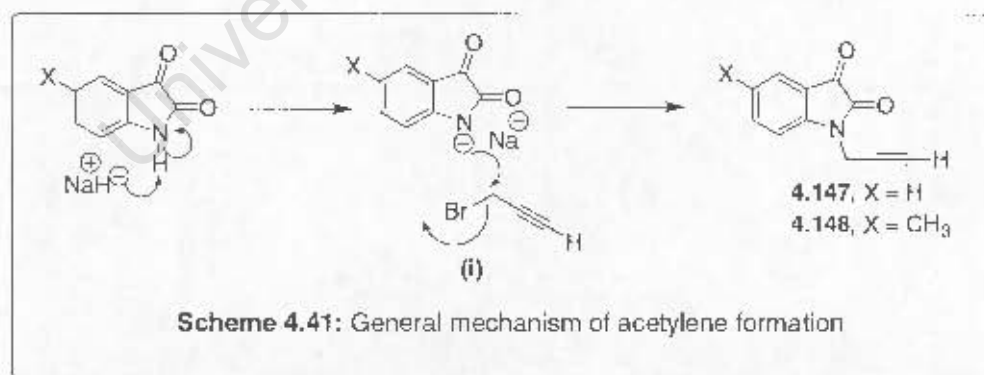
4.5.4.5 Synthesis of Acetylene Inputs (4.147) and (4.148)

Under similar experimental conditions described for the isatin-derived alkyl halides (4.137a- e), the acetylenes, (4.147) and (4.148) were synthesized from isatins (4.139a) and (4.139d) and propargyl bromide (4.146) in moderate yields of 48% and 47% respectively, (Scheme 4.40).¹⁷⁰

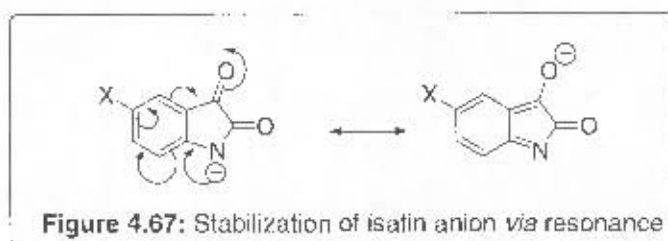


4.5.4.5.1 Mechanism for the Alkylation

The amide group of the starting substituted isatin is deprotonated by the base (sodium hydride) to form the intermediate (i), which then attacks the propargyl bromide carbon to form the desired acetylenic compounds (4.147) and (4.148) via a S_N2 nucleophilic substitution reaction, (Scheme 4.41).

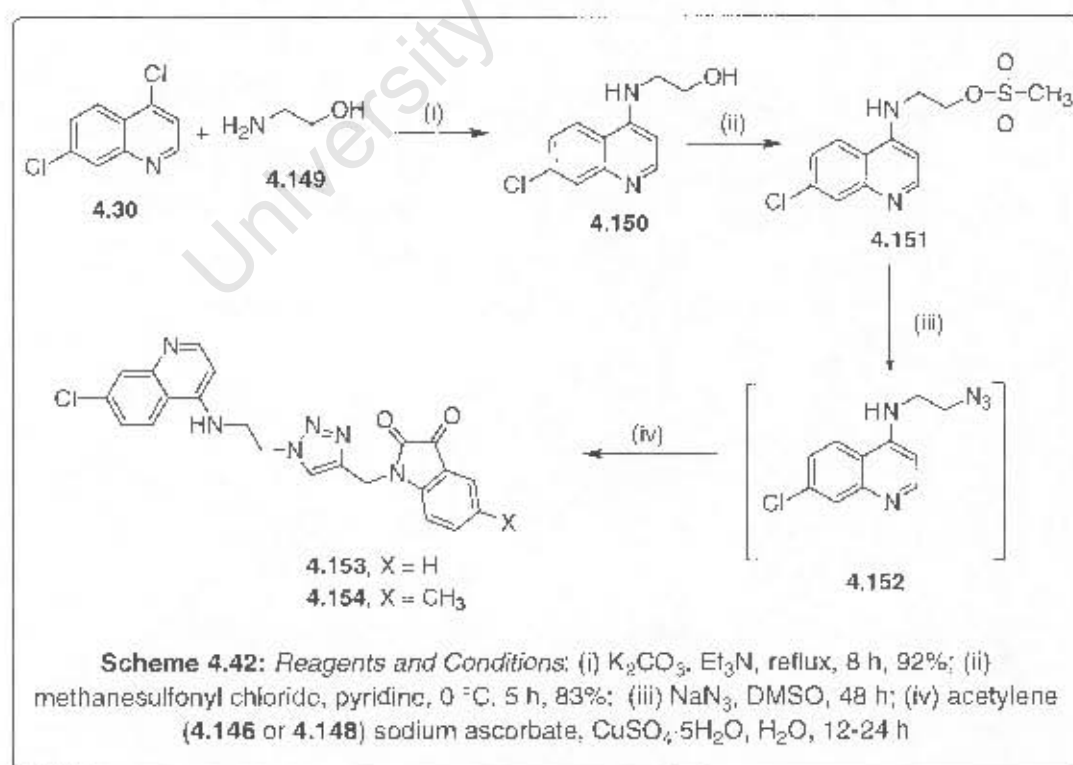


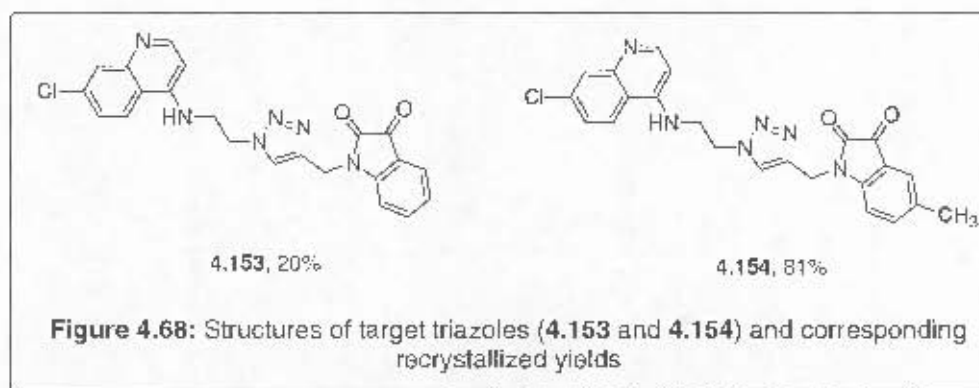
The amide proton in isatins is particularly acidic because the resulting anion is stabilized resonance as depicted in Fig. 4.67.



4.5.4.6 One-Pot Synthesis of Triazoles (4.153) and (4.154)

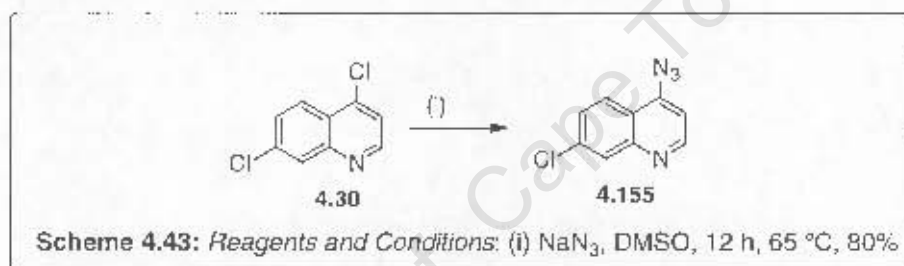
The mesylate intermediate (4.151) was prepared according to a method depicted in Scheme 4.42.^{170, 181} Treatment of 4,7-dichloroquinoline (4.30) with excess 2-aminoethanol (4.149) afforded (4.150) in a good yield of 92%. This was followed by *o*-mesylation in pyridine at 0 °C to provide the mesylate (4.151) in a yield of 82%. The isolated mesylate (4.151) was converted to the triazoles (4.153) and (4.154) by an one-pot synthesis in which it was first converted to the corresponding *in situ* generated azide, using a slight excess (1.1 equiv) of NaN₃ in anhydrous DMSO at 65 °C for 48 h. After the azidation step was completed, water, alkyne, sodium ascorbate, and CuSO₄ solution were added and stirring was continued for 12-24 h to produce the desired triazoles (4.153) and (4.154).¹⁸² Compound (4.153) was obtained in a good yield of 81% while compound (4.154) was obtained in very low yield of 20%, (Fig. 4.68).





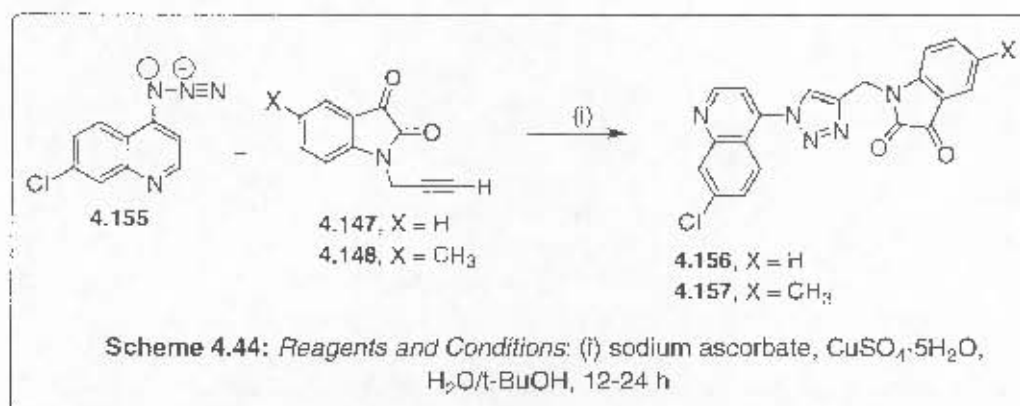
4.5.4.7 Synthesis of Azide Input (**4.155**)

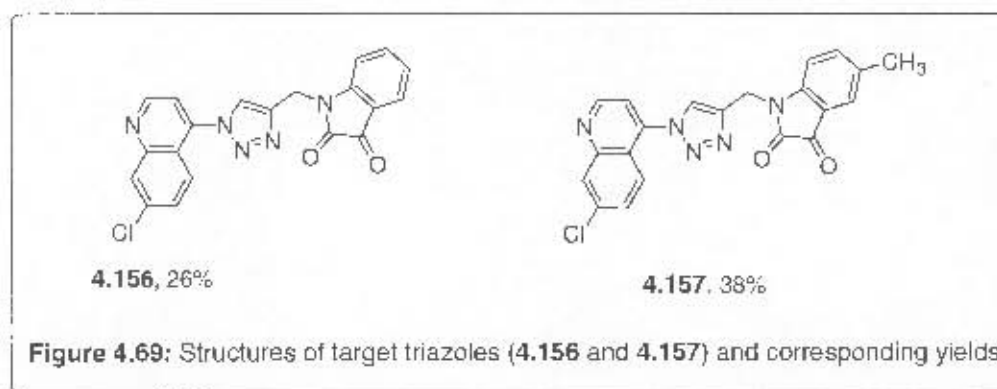
Azide (**4.155**) was prepared by reacting 4,7-dichloroquinoline with NaN_3 in DMSO at 65 °C for 12 h, (Scheme 4.43) to obtain the product in a good yield of 80%.



4.5.4.8 Synthesis of Triazoles (**4.156**) and (**4.157**)

In this procedure the desired azide (**4.155**) was prepared separately (Scheme 4.43) before subjecting it to "click" chemistry conditions. The reaction between the azide (**4.155**) and respective acetylenes (**4.147**) and (**4.148**) in the presence of 5 mol % of sodium ascorbate and 1 mol % of copper (II) sulfate in a 2:1 mixture of water and *tert*-butyl alcohol furnished the triazole products (**4.156**) and (**4.157**), (Scheme 4.44) in low yields of 26% and 38%, (Fig.4.69).¹⁵²

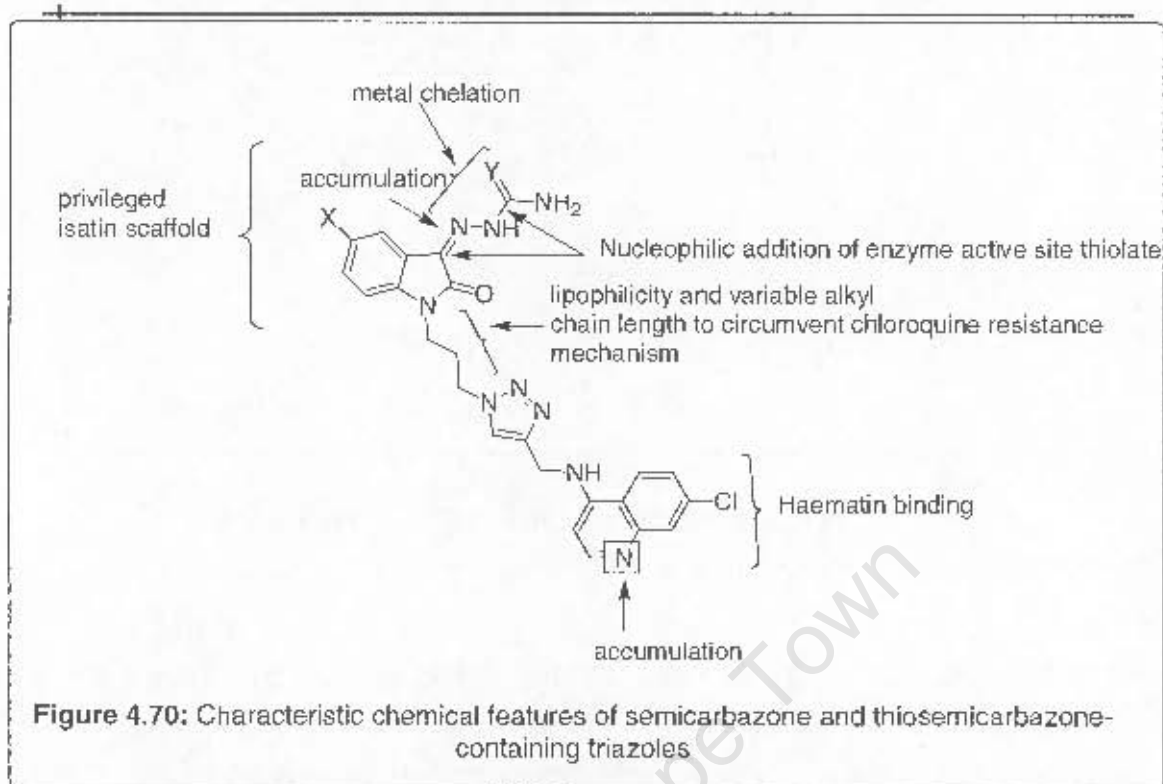




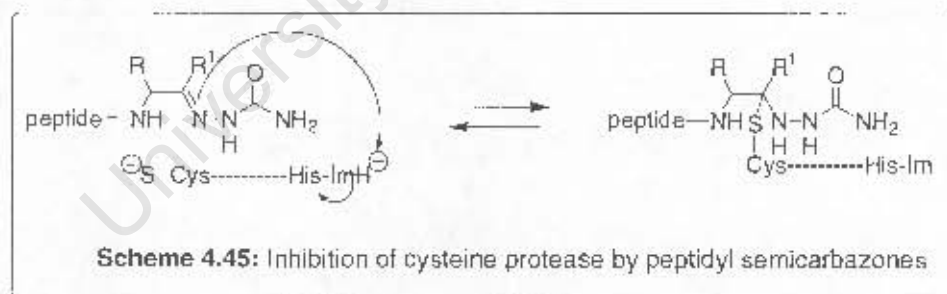
4.5.4.9 Semicarbazone and Thiosemicarbazone-Containing Triazoles

4.5.4.9.1 Background and Rationale

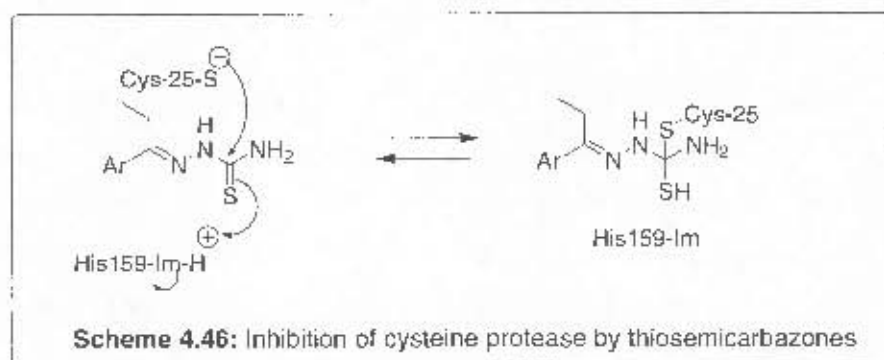
In an attempt to expand the synthetic design of the SAR library, a semicarbazone or thiosemicarbazone moiety was appended to the triazole-isatin scaffold and assessed for inhibitory activities. Evaluation of thiosemicarbazones over the years has demonstrated this class of small molecules to be potent antivirals,^{183, 184} anticancer therapeutics and parasitic cysteine protease inhibitors.¹⁸⁵⁻¹⁹⁰ More recently semicarbazones were found to be potent inhibitors of rhodesain and cruzain.¹⁹⁹ Their marked inhibitory activities may be attributed to the presence of two potential sites of attack by nucleophilic thiolate anions of parasitic proteases. Fig. 4.70 depicts the characteristic chemical features from which the rationales for the design is based and are discussed below.



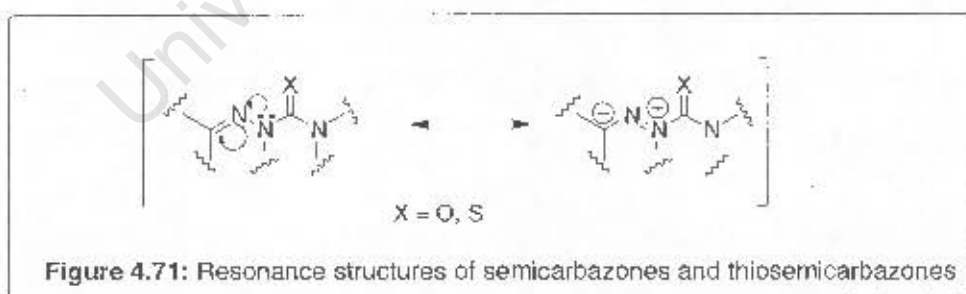
- (i) It has been shown that inhibition of cysteine proteases by peptidyl semicarbazone results from the formation of a tetrahedral adduct by attack of the active site thiolate on the imine carbon of the semicarbazone, (Scheme 4.45).¹⁰¹



- (ii) Non-peptidic thiosemicarbazones have also been shown to be effective antitrypanosomal cysteine protease inhibitors and the thiocarbonyl group of thiosemicarbazones has been suggested to be the site of attack by the thiolate anion resulting in the formation of the tetrahedral adduct, (Scheme 4.46). This mechanism is supported by experimental and computer modeling results.¹⁸⁸

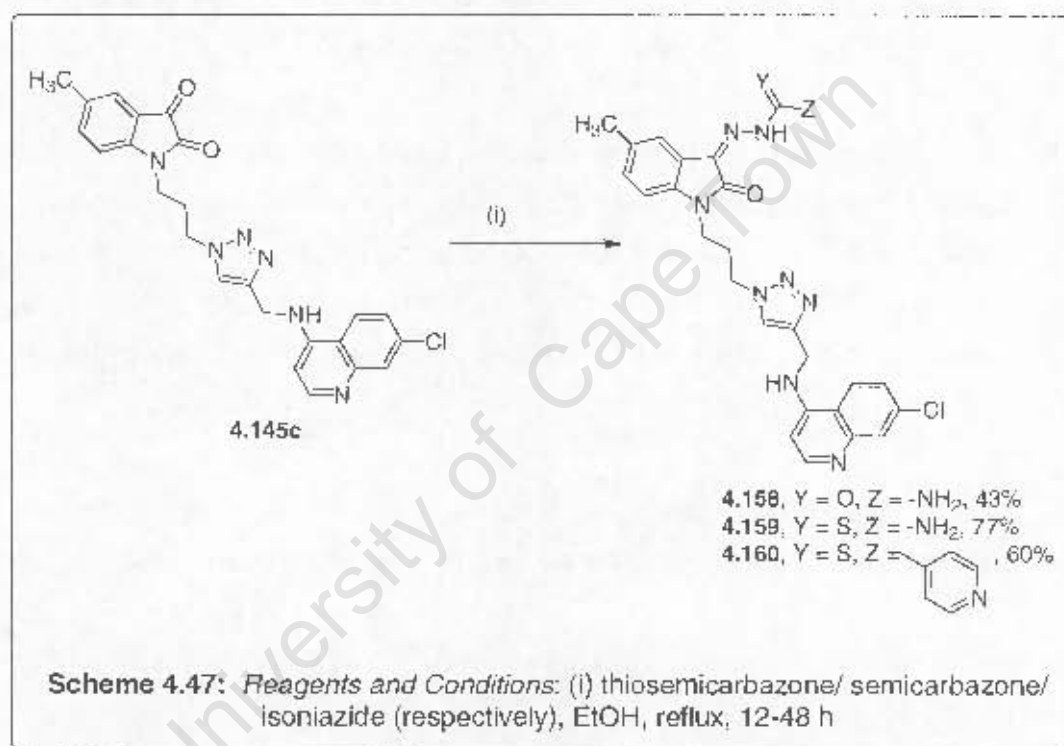


- (iii) Irrespective of the site of nucleophilic attack (i.e. imine C=N or thiocarbonyl C=S), both mechanisms described above in (i) and (ii) are reversible only in the host.
- (iv) The thiosemicarbazones are more stable and less susceptible to hydrolysis than corresponding imines suggesting that thiosemicarbazones would be more bioavailable. The greater stability of thiosemicarbazones stems from delocalization, and therefore, stabilization of the imine (C=N) double bond by the electronegative nitrogen, making the imine (C=N) bond more resistant to nucleophilic attack, (Fig. 4.71).
- (v) Thiosemicarbazones are metal chelators (*via* the sulfur and hydrazinic nitrogen atoms) and as such which may act as metal-interactive inhibitors of cysteine proteases.¹⁹²



4.5.4.10 Synthesis of Semicarbazone and Thiosemicarbazone-Containing Triazoles

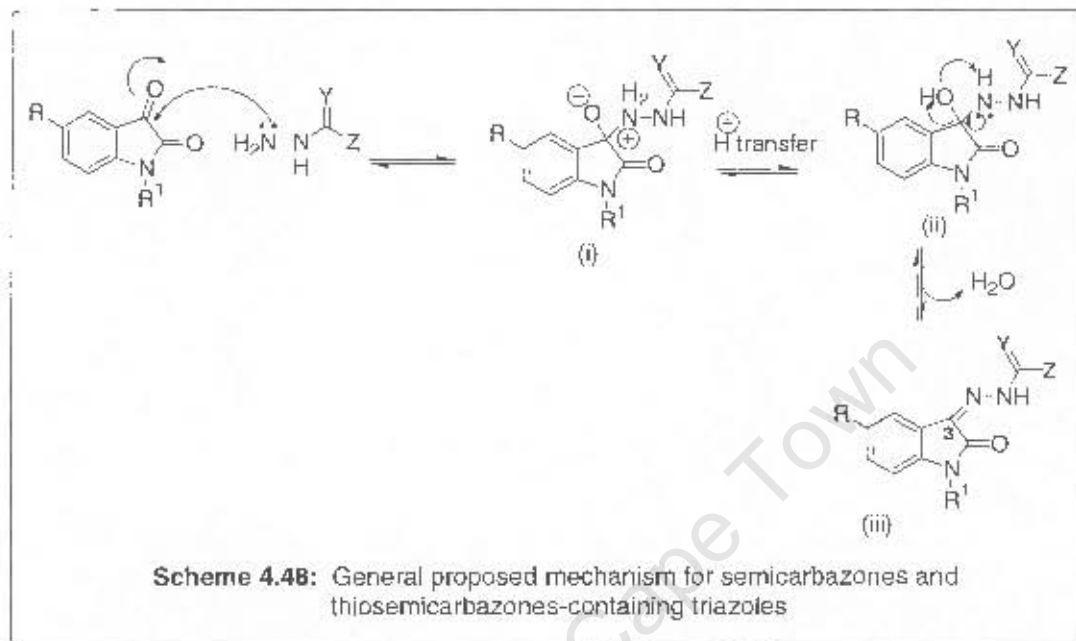
Semicarbazone and thiosemicarbazone-containing triazoles (**4.158-4.160**) were synthesized by condensation of equimolar quantities of a selected triazole isatin (**4.145c**) with the respective thiosemicarbazone, semicarbazone and isoniazid in refluxing ethanol for 12-48 h.¹⁸⁰ The crystalline products formed upon cooling were filtered, washed with ethanol and recrystallized from acetonitrile to give the desired products (**4.158-4.160**). The overall yield ranged from low to good (43-77%), (Scheme 4.47).



4.5.4.10.1 Mechanism Details

A general proposed mechanism for the formation of compounds (**4.158-4.160**) is presented in Scheme 4.48. Nucleophilic addition of the more reactive terminal amino group of the semicarbazone at the isatin carbonyl ketone results in the formation of the unstable intermediate (i), which undergoes proton transfer to give rise to the neutral molecule, intermediate (ii). Elimination of water enhanced by the delocalization of the nitrogen lone pair of electrons affords the imine, intermediate (iii), (Scheme 4.50). Nucleophilic attack occurs preferentially at C-3 of

the carbonyl ketone of the isatin since this carbonyl is more electrophilic than the amide carbonyl group.



The proposed structures of the compounds were confirmed by spectroscopy means. For example the IR spectra have stretching bands at 3655 (N-H), 3410 (N-H), 1637 (C=O), 1517 (C=N) and 1132 (C=S). The ^1H NMR spectrum presented in Fig. 4.72 shows the following characteristic signals; a one-proton singlet at δ 12.35 assigned to N-H/12, a one-proton singlet at δ 8.98 assigned to N-H/14 and a one-proton singlet at δ 8.64 assigned to NH/14. All the other peaks correspond to the precursor, (4.145c). Further evidence for the proposed structure was revealed by the ^{13}C NMR spectrum which has peaks at δ 179.4 (C=S) and at δ 160.8 (C=O). Additional support was provided by the mass spectrum, which gave a molecular ion peak at m/z 534.

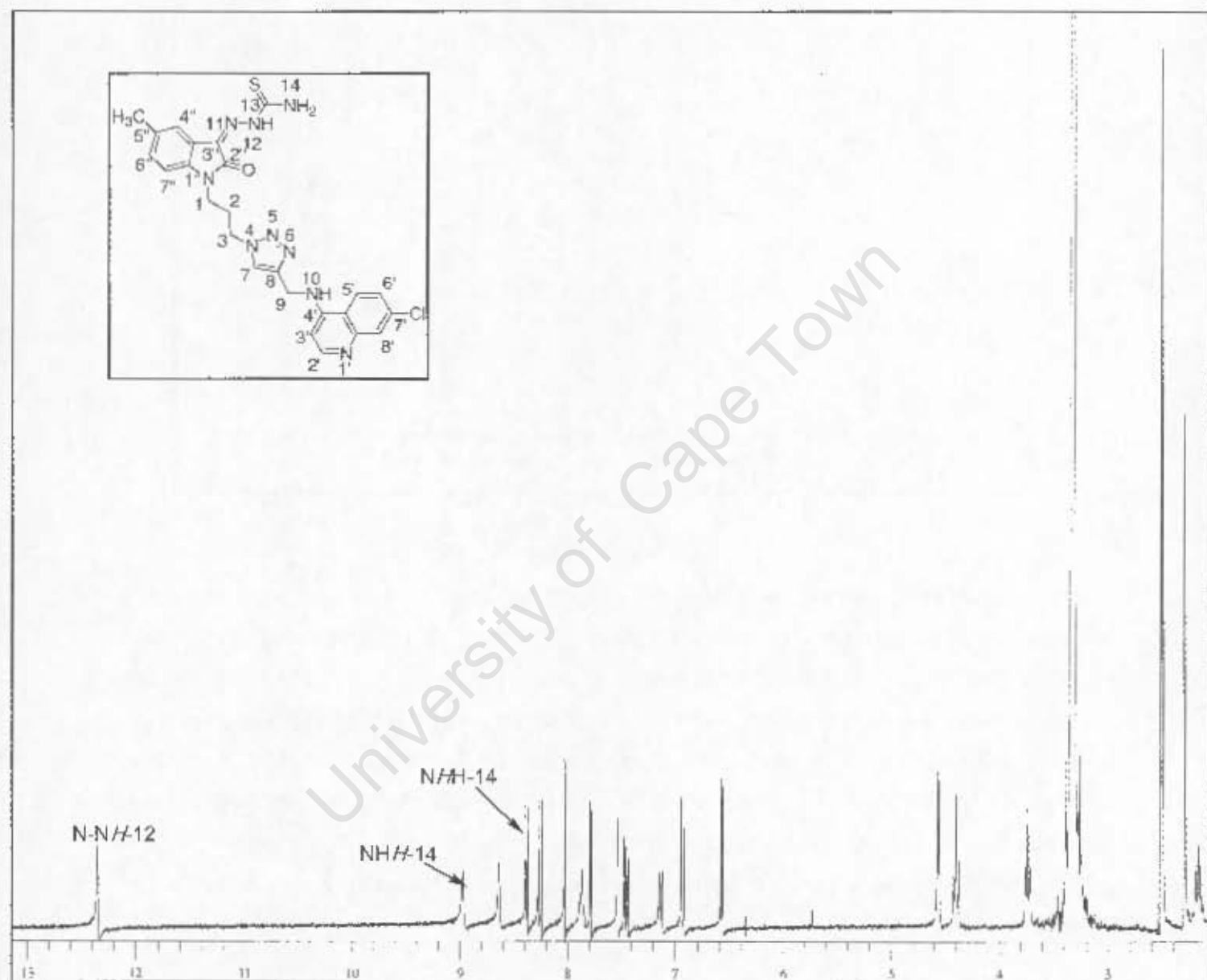


Figure 4.72: ^1H NMR of (4.159) in $\text{DMSO}-d_6$

4.5.4.11 Conclusion

Three methods were used to design and synthesize novel libraries of compounds based on the 'dual/double' drug approach. The first class of compounds was synthesized using peptide coupling as the key reaction steps. The peptidyl scaffolds were preformed *via* a one-pot synthesis and then attached to various antimalarial pharmacophores *via* peptide coupling. The precursors and target compounds were successfully synthesized in reasonable yields.

In the second class of compounds, 4-aminoquinoline- containing pharmacophores were appended to presynthesized dihydropyrimidine (DHPM) scaffolds, which were formed *via* the Biginelli three multicomponent reaction. The target molecules were purified with no complication by column chromatography and were obtained in reasonable to excellent yields.

In the third class of compounds, bi-functional isatin-derived triazoles were synthesized *via* "click" chemistry. Most of the target triazoles were synthesized using a one-pot synthesis which involve the *in situ* generation of the azide-derived pharmacophore. Alternatively, in cases where this procedure was unsuccessful, the azides were presynthesized before subjecting it to "click" conditions. Despite the low yields obtained in some cases, all the envisaged compounds were successfully synthesized.

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CHAPTER 5

BIOLOGICAL RESULTS AND DISCUSSION

5.1 Introduction

The effective interpretation of results of target compounds is achieved by determining the IC_{50} and ED_{50} values. The 50% inhibitory concentration (IC_{50}) is defined as the concentration at which a measured parameter falls to 50% of its initial value. The 50% effective dose, (ED_{50}) is the drug concentration required to kill 50% of the population of the parasites or to inhibit 50% of enzyme activity. The efficacy of the drug is determined by the IC_{50} or ED_{50} value, thus the lower IC_{50} or ED_{50} value, the more potent the drug. For a drug to be classified as a good drug it should show selective cytotoxicity towards the parasite and not towards the host although it shows a low IC_{50} or ED_{50} value. This is determined by the Therapeutic Index (TI), which is defined as the ratio of a drug's undesirable effects with respect to its desirable effects and is therefore a measure of how safe the drug is. Usually this involves comparing the dose levels leading to a toxic effect with respect to the dose levels leading to a therapeutic effect. The larger the therapeutic index, the safer the drug. Mathematically the TI is expressed as,

$$TI = IC_{50} \text{ or } ED_{50} \text{ (host cell)} / IC_{50} \text{ or } ED_{50} \text{ (parasite cell)}$$

A high TI value shows cytotoxicity towards the parasite and not towards the host cell. The IC_{50} values for the compounds screened can also be obtained from dose-response curves. The dose-response curve is a plot of the log of concentration of the drug against the plots of response (enzyme activity) expressed in percentage. The IC_{50} values are simply the concentration of drug required to cause a response halfway between the Y-axis. Depending on which test, dose-response curve can have almost any shape.

Resistance index is defined as the ratios of the IC₅₀ against resistant strains (W2 or K1 in this case) to the IC₅₀ of the same compound against sensitive strains (e.g. D10). The ratio measures the potential of any new compound against chloroquine-resistant strains relative to chloroquine (CQ). Mathematically the Resistance Index (RI) is expressed as,

$$RI = IC_{50} \text{ of compound in resistant strain (W2 or K1)} / IC_{50} \text{ of compound in a sensitive strain (D10 or 3D7)}$$

For example, relative to CQ, RI greater than CQ indicates a weaker activity against the chloroquine-resistant strains (K1 or W2), while RI less than that of CQ indicates a better activity of the compound against the chloroquine-resistant strains (K1 or W2).

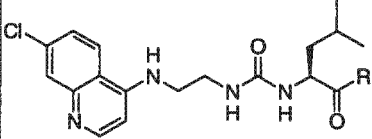
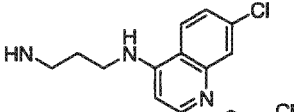
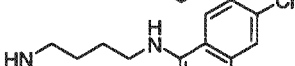
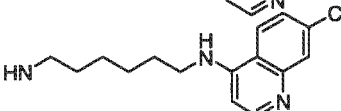
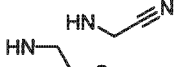
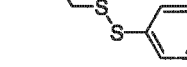
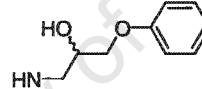
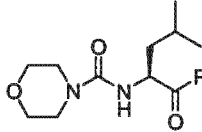
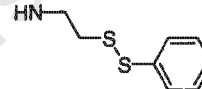
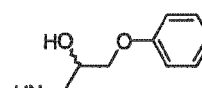
5.2 Results and Discussion of 4-Aminoquinoline-and-Morpholine-Derived Target Molecules

The target hybrid molecules were exposed to different *in vitro* assays to assess their inhibition activities. In the first test, the compounds were screened against the plasmodial cysteine proteases, falcipain-2 and falcipain-3 and the chloroquine-resistant W2 *P. falciparum* strain, using E64 (natural product cysteine protease inhibitor) as the control in all the tests. The antiplasmodial and enzyme inhibition evaluations were performed at the Department of Medicine, San Francisco General Hospital, University of California at San Francisco (UCSF), in collaboration with Professor P. J. Rosenthal. The antiprotozoal activities of the compounds against the chloroquine-sensitive (3D7) and chloroquine-resistant (K1) *P. falciparum* strains, as well as antitrypanosomal activities against *T. brucei brucei* and *T. b. rhodesiense* parasite cultures were evaluated at the London School of Hygiene and Tropical Medicine (LSHTM), University of London in collaboration with Dr. V. Yardley. Finally, the compounds were screened for activity against β -haematin formation, at the University of Cape Town, Department of Chemistry in collaboration with Professor T. J. Egan.

5.2.1 *In Vitro* Antiplasmodial Activity of 4-Aminoquinoline-and-Morpholine-Derived Target Molecules

The inhibitory activities for target molecules (4.46–4.51, 4.53 and 4.54) against plasmodial cysteine proteases, FP-2 and FP-3 and the chloroquine -resistant strain (CQR), W2 are presented in Table 5.1. Compounds (4.46), (4.47) and (4.48), distinguished by the variable length of their respective amino alkyl chain spacer were derived from the same 4-aminoquinoline precursor. Figure 5.1 clearly shows that compounds (4.46), (4.47) and (4.48) inhibit the growth of CQR (W2) strain more significantly compared to the plasmodial cysteine proteases, FP-2 and FP-3. Compounds (4.46), (4.47) and (4.48) exhibited inhibitory activities above 30 μM against FP-2 and FP-3, (Fig. 5.1 a, b). Nevertheless, compound (4.48) with the 6-carbon spacer was 11 fold more potent ($\text{IC}_{50} = 0.2313 \mu\text{M}$) than the reference drug, E64 ($\text{IC}_{50} = 2.482 \mu\text{M}$) while the shorter carbon chained compounds, (4.46) [(n = 3, $\text{IC}_{50} = 0.9601 \mu\text{M}$)] and (4.47) [(n = 4, $\text{IC}_{50} = 1.089 \mu\text{M}$)] revealed analogous antiplasmodial activities against the W2 strain, (Fig. 5.1 a).

Table 5.1: Inhibition of plasmodial cysteine proteases, FP-2, FP-3 and chloroquine-resistant strain, W2

General Structure	R	Compound (Code)	IC ₅₀ (μM)		
			FP2	FP3	W2
		Control:E64	0.0095	0.056	2.482
		4.46(NAT48)	46.60	34.60	0.960
		4.47(NAT46)	49.33	46.64	1.089
		4.48(NAT47)	58.03	35.07	0.2313
		4.49(NAT50)	2.592	1.20	5.425
		4.50(NAT43)	16.40	15.65	1.40
		4.51(NAT56)	74.35	46.63	4.38
		4.53(NAT42)	19.4	17.81	5.21
		4.54(NAT60)	89.91	>100	>20

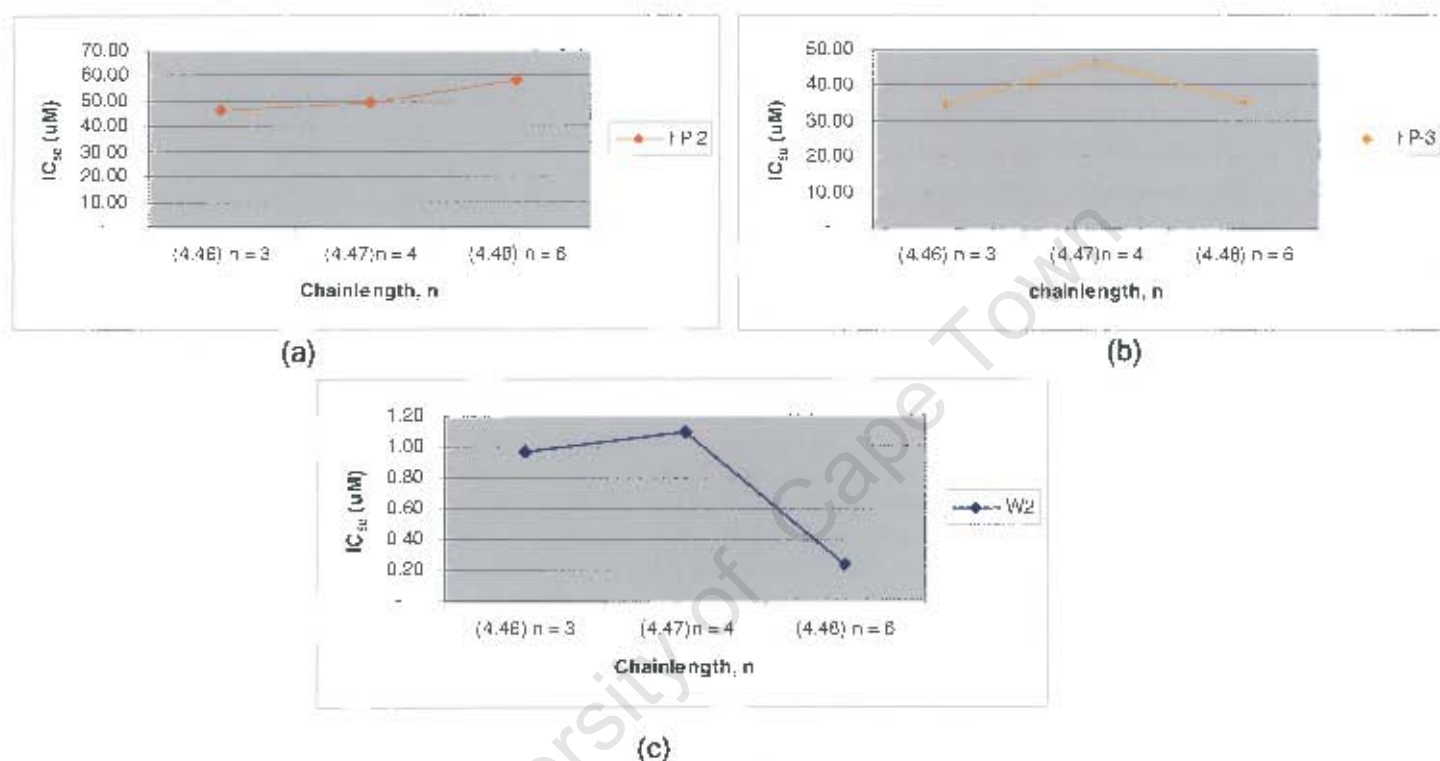
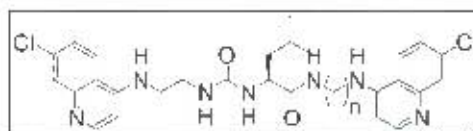


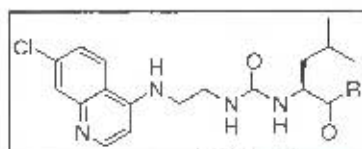
Figure 5.1: Correlation of chain length, n to the inhibitory activity of compounds, (4.46-4.48) against plasmodial cysteine proteases, (a) FP2 and (b) FP3 and the chloroquine-resistant strain, (c) W2

The most active compound, not belonging to the homologous series (4.46-4.48) was compound [(4.50) ($IC_{50} = 1.40 \mu M$)], which was 2 times more efficacious than E64 ($IC_{50} = 2.482 \mu M$) against the W2 strain, Table 5.1. Compounds that exhibited low IC_{50} values against FP-2 and FP-3 were the nitrile-containing compound (4.49) with IC_{50} values of 2.592 and 1.20 μM respectively and the two disulfide-containing compounds (4.50) and (4.53), which revealed consistent IC_{50} values against FP-2 and FP-3. Generally, nearly all the compounds revealed their lowest antiplasmodial activities against W2, except the nitrile-containing compound, (4.49), which demonstrated its lowest activity against the

FP-3 enzyme. Analysis of the data displayed in Table 5.1 concludes that compounds [(4.46-4.50) (IC_{50} values ranging from 0.2313 to 1.4 μM)] disclosed greater antiplasmodial activities than E64, against the W2 strain.

5.2.2 *In Vitro* Antiplasmodial Activity of 4-Aminoquinoline-and-Morpholine-Derived Target Molecules against K1 and 3D7 strains

An assessment on selected compounds against the CQR (K1) and chloroquine-sensitive (CQS), 3D7 *P. falciparum* strains were performed with chloroquine (CQ) and artesunate as the control drugs. Two independent assays were conducted against the CQR (K1) strain, (Table 5.2 and Table 5.3) and this is the reason for having two separate tables. As it can be seen from Table 5.2, compounds (4.45) and (4.50) with IC_{50} s of 0.26 μM and 0.31 μM respectively, revealed a 2-3-fold greater inhibitory activity than CQ (IC_{50} = 0.69 μM) against the K1 strain. However, these compounds displayed lower inhibitory activities than artesunate (IC_{50} = 0.002 μM) against the same K1 strain. Compounds (4.20) and (4.52) did not show significant inhibition against K1 with IC_{50} values ranging from 8.44-22.33 μM . Notably, the 4-aminoquinoline-derived compounds (4.45) and (4.50) were more active against the K1 strain than the morpholine-derived compound (4.52). Generally, all the compounds revealed low therapeutic indices, even though they exhibit low inhibitory activities, except compound (4.45), which revealed the highest therapeutic index (265.60) and lowest inhibitory activity within the selected series.

Table 5.2: *In vitro* inhibition of K1 strain

Compound (Code)	R	IC ₅₀ μg/ml	IC ₅₀ μM	Cyt. μg/ml	Cyt. μM	TI
Chloroquine		0.22	0.69	-	-	-
Artesunate		0.001	0.002	-	-	-
4.20(NAT32)	OH	8.44	22.33	90.35	239.02	10.70
4.45(NAT34)		0.15	0.26	39.85	68.47	265.60
4.50(NAT43)		0.17	0.31	5.84	0.70	4.35
4.52(NAT24)		8.47	18.90	203.00	371.80	23.96

Table 5.3 reveals the results obtained for compounds (4.45-4.49) from independent experiments against K1 and 3D7. Even though compounds (4.45-4.49) generally showed good activities against the K1 strain, the most active compounds (4.46) and (4.48) within the series with IC₅₀s of 0.003 and 0.019 μM respectively, 3-19 times less active than the artesunate (IC₅₀ of 0.001 μM). However, for a chloroquine derivative, the activity of compound (4.46) is highly noteworthy, being 284 times more effective than chloroquine (IC₅₀ of 0.853 μM) against the K1 strain. Compounds (4.45) [(IC₅₀ of 0.129 μM)] and (4.48) [(IC₅₀ of 0.035 μM)] revealed the best antiplasmodial activities among the compounds (4.45-4.49) assayed against the 3D7 strain; however they were less active than chloroquine (IC₅₀ of 0.014 μM) in the same strain. Figure 5.2 shows the correlation between the IC₅₀s with respect to the variation in chain length, n in

compounds (4.45-4.48) against K1 and 3D7 strains. The short carbon chain ($n = 3$) compound (4.45) and the longer carbon chain ($n = 6$) compound (4.46) were the most active compounds against the K1 strain, (Fig. 5.2). Compounds tested against 3D7 demonstrated a similar trend, with the 2- and 6-carbon spacer compounds (4.45) and (4.46) showing the lowest inhibitory activities within the homologous series.

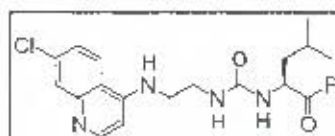


Table 5.3: *In vitro* inhibition of K1 and 3D7 strains

Compound (Code)	R	K1 IC ₅₀ (μ M)	3D7 IC ₅₀ (μ M)	Cytotoxicity IC ₅₀ (μ M)	TI ^a K1	TI ^b 3D7
Chloroquine		0.853	0.014	-	-	-
Artesunate		0.001	-	-	-	-
Podophyllotoxin		-	-	0.004	-	-
4.45(NAT45)		0.200	0.129	30.3	152	235
4.46(NAT48)		0.003	0.202	23.80	7933	119
4.47(NAT46)		0.200	0.479	78.33	392	164
4.48(NAT47)		0.019	0.035	33.50	1763	957
4.49(NAT50)		<0.123	1.077	209.7	>1705	195

TI^a = Therapeutic Index a = Cytotoxicity/K1 IC₅₀. TI^b = Therapeutic Index b = Cytotoxicity/3D7 IC₅₀

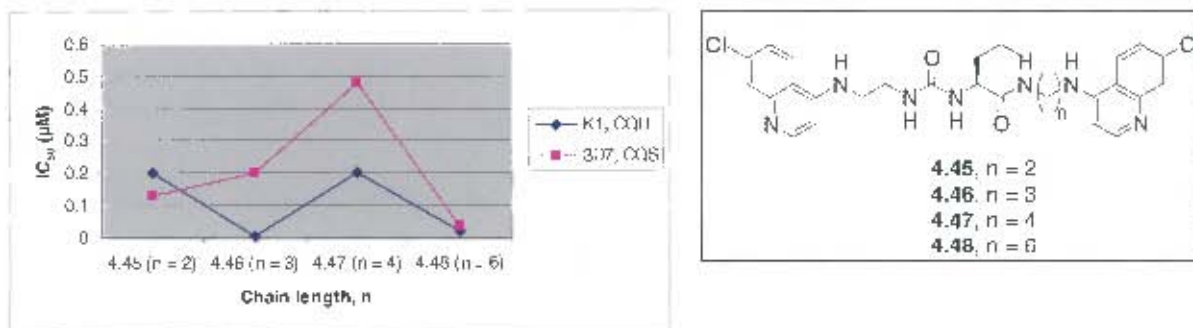


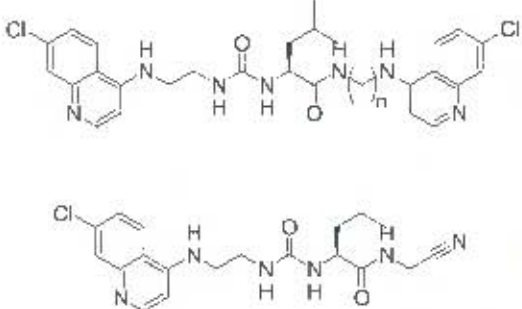
Figure 5.2: Correlation of the IC_{50} s to the variation in chain length, n in the K1, (CQR) and 3D7 (CQS) strains respectively for compounds (4.45-4.48)

With the exception of compound (4.46), most of the compounds displayed low therapeutic indices. Compound (4.46) was nearly 8000 times more cytotoxic towards the parasite than towards the mammalian cell line and was also the most active compound against the K1 strain, making it a potential candidate for further drug development.

5.2.3 *In vitro* β -haematin inhibition of compounds 4.45, 4.46, 4.48 and 4.49

From the results displayed in Table 5.4, it can be seen that all the compounds, (4.45, 4.46, 4.48 and 4.49) showed great promise as β -haematin formation inhibitors as they exhibit inhibitory activities that were more superior to CQ. Figure 5.3 indicates that the most active compound (4.46) with a 3 carbon spacer ($IC_{50} = 0.083 \pm 0.007$ equiv.), was 24 times more efficacious than chloroquine ($IC_{50} = 1.91 \pm 0.3$ equiv.), whereas the least active compound (4.48) with a 6 carbon spacer ($IC_{50} = 0.15 \pm 4.00$ equiv.), was 13 times more potent than CQ. The structurally related compounds (4.45, 4.46 and 4.48) were more active than the nitrile-containing compound (4.49) [$IC_{50} = 0.89 \pm 0.05$ equiv.], which was 2 times more effective than CQ. The higher inhibitory activities exhibited by these compounds may be due to the presence of two 4-aminoquinoline moieties which may lead to enhanced activity. Overall the superior activities (relative to CQ) in all of these compounds may be attributed to the presence the 4-aminoquinoline moiety which is essential for β -haematin inhibition.¹

Table 5.4: IC₅₀ value for β -haematin formation inhibition by amines 4.45, 4.46, 4.48 and 4.49

General Structure	n	Compound (Code)	IC ₅₀ (equiv.) ^a
	2	Chloroquine	1.91 ± 0.3
	2	4.45(NAT34)	0.27 ± 0.04
	3	4.46(NAT48)	0.08 ± 0.007
	6	4.48(NAT47)	0.15 ± 4.00
	-	4.49(NAT50)	0.89 ± 0.05

^aThis study ± standard error of the mean on three determinations

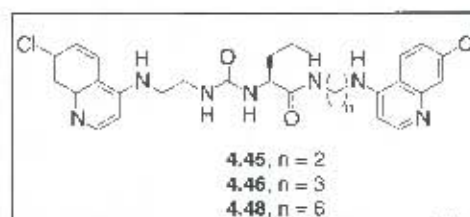
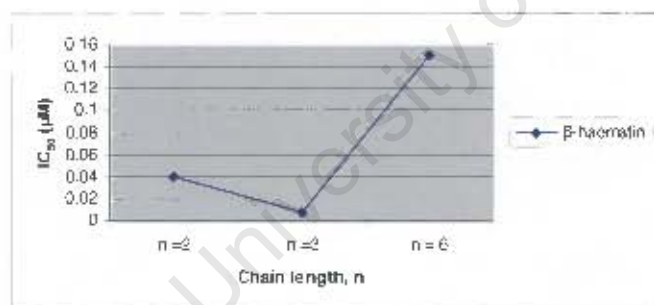


Figure 5.3: Correlation of the IC₅₀s to the variation in chain length, n against β -haematin formation of compounds (4.45, 4.46 and 4.48)

5.2.4 Discussion

Prior work done by De *et al.* have established that the length of the alkylamine side chain in chloroquine plays a major role in determining the antiplasmodial activity against chloroquine-resistant *P. falciparum*.² Based on this finding, corresponding data was revealed for the homologous series (4.45-4.48) against the two CQR strains, W2 and K1. The results demonstrated that the short alkyl chain compound, (4.46) and long alkyl chained compound, (4.48) exhibited the greatest antiplasmodial activities within the homologous series against the CQR strains. This is consistent with earlier findings.²

Like chloroquine and other antimalarial bisquinolines, bisquinolines (4.45-4.48) may exert their antimalarial properties by inhibiting haemozoin (β -haematin) formation. They may bind to haematin μ -oxo dimer, shifting the equilibrium between haematin monomer and haematin μ -oxo dimer, thereby decreasing the concentration of haematin monomer incorporated into haemozoin.³ However, it should be noted that bisquinoline-haematin μ -oxo binding may have other consequences which may or may not contribute to their antimalarial properties. Examples include the inhibition of both glutathione- and glutathione/hydrogen peroxide-mediated iron release from haematin and inhibition of haematin-dependent lipid peroxidation⁴ and inhibition of polyamine transport.⁵ Since, chloroquine resistance parasites show a reduced accumulation of the drug in the cell compared to chloroquine sensitive parasites, it is proposed that this reduced accumulation is due to a diminished uptake of the drug or an enhanced drug efflux from the infected cells. In this case, the bulkiness of the bisquinoline structure may render it to be less efficiently extruded by the chloroquine resistant plasmodia and hence making the drug more effective than chloroquine.¹

The disulfide (4.50), derived from the 4-aminoquinoline-containing precursor, also exhibited an increased activity against the W2 and K1 strains compared to the disulfide (4.53) which was derived from the morpholine-containing precursor. This suggests that the presence of the 4-aminoquinoline subunit is essential for enhancing the antiplasmodial activity.

Table 5.5 shows the relationship between *C* logP (partition coefficient) and 4-aminoquinoline-containing derivatives. The quinoline moiety may improve the antiplasmodial activity by revealing an increased lipophilicity, reflected by high *C* logP

values. The *C* logP values are a measure of the partition coefficient of a drug in a water/octanol solution and are estimated using ChemDraw® v.6.01, which gives results for comparative lipophilicity studies. For the drug to reach the acidic food vacuole it must be able to cross the membranes of the parasitized erythrocytes and accumulate through the protonatable nitrogens. It can also improve the antiplasmodial activity through the inhibition of β -haematin formation by the 7-chloro aminoquinoline moiety as mentioned before. Table 5.5 shows that compounds (4.46-4.48 and 4.45) have higher *C*logP values than chloroquine, suggesting that they will be transported more efficiently across the cell membrane. The *C* logp values in Table 5.5, also demonstrate that the bisquinolines (4.46-4.48) are more lipophilic than the monoquinoline, (4.50) which will ultimately lead to enhanced antimalarial activities, as previously noted against the CQR, W2 strain (Table 5.1).

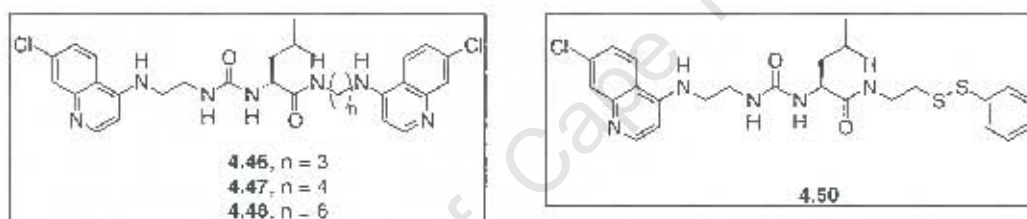


Table 5.5: *C*logp values obtain for compounds (4.46-4.50)

Compounds	<i>C</i> logp
Choroquine	5.1
4.46(NAT48)	7.8
4.47(NAT46)	7.6
4.48(NAT47)	8.7
4.50(NAT43)	6.5

None of the tested compounds exhibited promising activities against the malarial cysteine proteases, FP-2 and FP-3, except compound (4.49), (Table 5.1) which displayed IC_{50} s higher than the E64 but noticeably low within the selected series. The lack of activity of these compounds against FP-2 and FP-3, may suggest that the antimalarial effects are not as a result of inhibition of these enzymes. The high inhibitory activity observed for compound (4.49) against FP-2/FP-3 enzymes and β -haematin formation may be credited to the presence of the electrophilic warhead and the 4-amino-

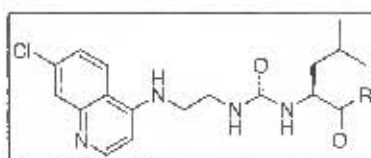
7-chloroquinoline subunits respectively. The electrophilic warhead may present the site of attack by the thiolate anion of the cysteine protease and the 4-amino-7-chloroquinoline subunits may bind to haeme as discussed earlier. Therefore, it is proposed that compound (4.49) may demonstrate dual antimalarial activities through inhibiting β -haematin formation and the enzymes, F-2 and FP-3. Given the poor inhibitory activity of the most potent compound (4.46) against the falcipain enzymes, FP-2 and FP-3 (IC_{50} = 46.60 and 34.60 μ M respectively) coupled with the high inhibitory activity (IC_{50} = 0.08 ± 0.007 equiv.) displayed by compound (4.46) in the haemozoin formation assay, it is likely that the latter mechanism is largely responsible for the observed antimalarial activity.

5.2.5 *In Vitro* Inhibition against *T. brucei brucei*

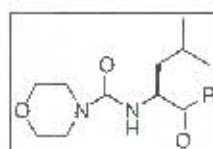
The inhibitory activities of selected compounds within the series were evaluated against *T. brucei brucei*, which is responsible for African sleeping sickness or African trypanosomiasis. The activities of these compounds were determined at different concentrations (30.00, 10.00, 3.33, 1.11 μ g/ml). The data for these assays are presented in Table 5.6.

As it is apparent from the table, no significant inhibition of the compounds was revealed against *T. brucei brucei*. Although, compounds (4.45) and (4.50) exhibited the lowest IC_{50} s among the screened compounds (IC_{50} s, 21.90 and 6.17 μ M respectively), they also showed high toxicities towards the human KB cell line used in this assay. This is evident from their low therapeutic indices presented in Table 5.6. All the other compounds showed low antitrypanosomal activities and high toxicities towards the mammalian cells. Notably, the most active compounds (4.45) and (4.50) within the series were derived from the 4-aminoquinoline-containing precursor while the least active compound, (4.52) was derived from the morpholine-containing precursor

General Structures:



4.20, 4.45, 4.50



4.52

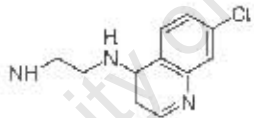
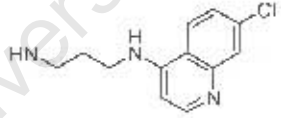
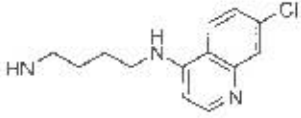
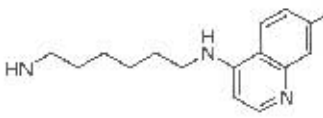
Table 5.6: *In vitro* inhibition against *T. brucei brucei*

Compound (Code)	R	IC ₅₀ μg/ml	IC ₅₀ μM	Cyt. μg/ml	Cyt. μM	TI
Pentamidine		0.006	0.0018	-	-	-
Podophyllotoxin		-	-	0.00024	-	-
4.20(NAT32)	OH	>30	>79.37	90.60	239.8	3.02
4.45(NAT34)		12.75	21.90	39.9	68.56	3.13
4.50(NAT43)		3.37	6.17	5.80	10.28	1.72
4.52(NAT24)		19.23	42.92	203.0	453.2	10.5

5.2.6 *In Vitro* Inhibition against *T. b. rhodesiense*

From the results shown in Table 5.7, it is apparent that almost all the compounds failed to exhibit significant inhibition against parasite growth. Although compounds (4.45) and (4.47) revealed the lowest IC_{50} s within the series, their activities were still significant lower than pentamidine. Furthermore, the low therapeutic indices demonstrated by all the compounds suggested that they were generally not at all that selective.

Table 5.7: *In vitro* inhibition against *T. b. rhodesiense*

Compound (Code)	R	IC_{50} (μ M)	Cytotoxicity IC_{50} (μ M)	TI
Pentamidine		0.009, 0.008	-	-
Podophyllotoxin		-	0.004	-
4.45(NAT34)		2.00	30.3	15
4.46(NAT48)		24.94	23.80	1
4.47(NAT46)		4.10	78.33	19
4.48(NAT47)		14.18	33.50	2
4.49(NAT50)		>30	209.7	7

5.3 Biological Results and Discussion of Dihydropyrimidinones (DHPMs)

The biological activities of the compounds synthesized in this part of the project were determined in the laboratories of the respective collaborators. All the antiplasmodial activities against the CQS (3D7), CQR (K1) *P. falciparum* strains, *T.b. rhodesiense* as well as *T. brucei brucei* antitrypanosomal and cytotoxicity assays were conducted in the laboratories of Dr. V Yardley (LSHTM). β -Haematin inhibition assays were performed in the laboratories of Professor TJ Egan, Department of Chemistry, UCT.

5.3.1 *In vitro* Antiplasmodial Activities of Quinoline-Based Compounds (4.105-4.107) and their Citrate Salts (4.109-4.112) against CQS (3D7) and CQR (K1) *P. falciparum* Strains

Table 5.8 displays the data for the antiplasmodial screening of compounds (4.105-4.107) and citrate salts (4.109-4.112) against the CQS (3D7) and CQR (K1) *P. falciparum* strains. CQ and artemisinin (ART) were used as controls. All compounds were tested for percentage inhibition at decreasing concentrations, after which the actual IC_{50} values were determined. Compared to CQ, almost all compounds in the CQS, 3D7 *P. falciparum* strain exhibited IC_{50} values comparable to CQ, with the exception of compound (4.112) [IC_{50} = 0.007 μ M] which revealed a 2 fold greater activity than that of CQ (IC_{50} = 0.014 μ M) and compound (4.106) [IC_{50} = 0.1 μ M] which was less superior, Table 5.8. In the K1 *P. falciparum* strain, all compounds were several orders of magnitude more active than CQ, with the the least active compounds (4.105, 4.106 and 4.109-4.112) [IC_{50} = 0.1-0.14 μ M] within the series being ~ 9 times more potent than CQ (IC_{50} = 0.853 μ M), while compound (4.107) [IC_{50} = 0.004 μ M], the most potent compound being 200 times more efficacious than CQ. According to Fig. 5.4 (a), the 6-carbon chain compound (4.112) [IC_{50} = 0.007 μ M] exhibited the greatest activity whereas Fig. 5.4 (b) shows that the 4-carbon chain compound (4.107) [IC_{50} = 0.004 μ M] revealed the best activity among the compounds. Compounds with shorter (n = 2) and longer (n = 6) alkyl chain lengths were generally more active against *P. falciparum* strains 3D7 and K1, (Fig. 5.4). With the exception of compounds (4.105) and (4.107), nearly all the compounds showed modest toxicity towards the mammalian KB cell line

employed in the assay in both strains (3D7 and K1) as demonstrated by their lower therapeutic indices presented in Table 5.8.

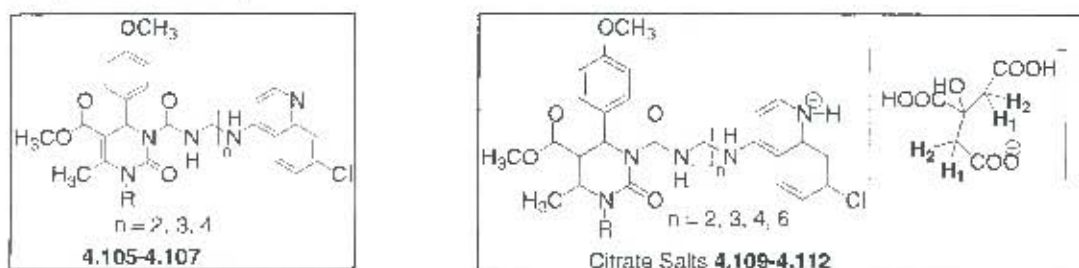
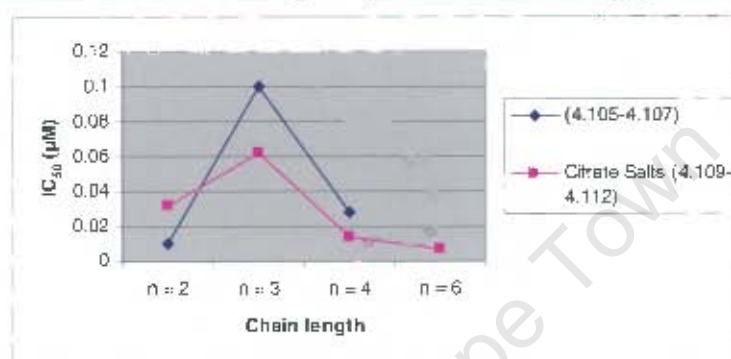


Table 5.8: Antiplasmodial activities of compounds (4.105-4.108) and citrate salts (4.109-4.112)

Compound (Code)	R	n	IC ₅₀ (μM)		Cytotoxicity (μM)	TI	
			3D7	K1		3D7	K1
CQ	-	-	0.014	0.853	-	-	-
ART	-	-	0.005	0.001	-	-	-
Podophyllotoxin	-	-	-	-	0.004	-	-
4.105 (NAT1)	CH ₃	2	0.01	0.1	56.86	5686	569
4.106 (NAT2)	CH ₃	3	0.1	0.1	21.66	217	217
4.107 (NAT3)	CH ₃	4	0.028	0.004	8.89	318	2223
4.109 (NAT1CS)	CH ₃	2	0.032	0.131	31.40	981	240
4.110 (NAT2CS)	CH ₃	3	0.062	0.144	26.97	435	187
4.111 (NAT3CS)	CH ₃	4	0.014	<0.123	4.97	355	nd ^a
4.112 (NAT4CS)	CH ₃	6	0.007	<0.123	11.60	1657	nd ^a



(a)



(b)

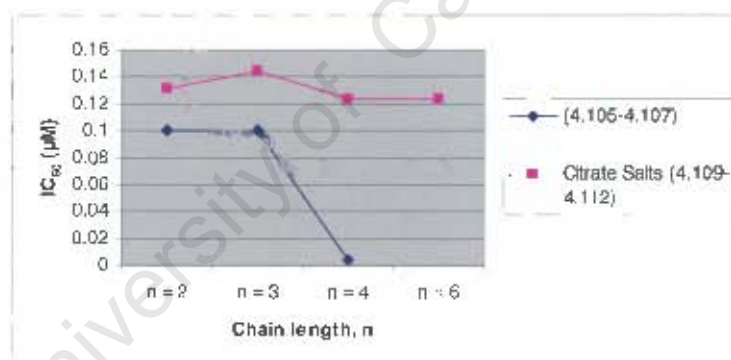


Figure 5.4: Correlation of chain length, n , to antiplasmodial activity in (a) CQS, 3D7 and (b) CQR, K1 strains

As it can be seen from Table 5.9, all compounds revealed RI values less than CQ, suggesting a more favorable activity of the compounds against the CQR strain, K1.

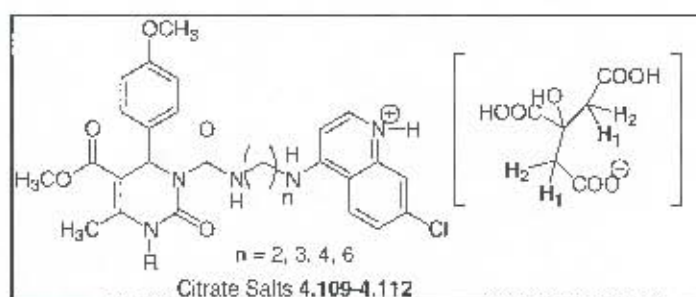
Table 5.9: Resistance Indices of DHPMs (4.105-4.107, 4.109-4.112)

Compound (Code)	R	n	RI ^a (K1)
Chloroquine	-	-	60.9
4.105 (NAT1)	CH ₃	2	10
4.106 (NAT2)	CH ₃	3	1
4.107(NAT3)	CH ₃	4	0.14
4.109(NAT1CS)	CH ₃	2	4.19
4.110(NAT2CS)	CH ₃	3	2.32
4.111(NAT3CS)	CH ₃	4	nd ^b
4.112(NAT4CS)	CH ₃	6	nd ^b

RI^a = Resistance Index = IC₅₀ of compound in resistant strain/ IC₅₀ of compound in sensitive strain; Nd^b = not determined

5.3.2 *In vitro* β -haematin inhibition of Citrate Salts (4.109-4.112)

Table 5.10, clearly shows that all the citrate salts (4.109-4.112) were several folds more active than CQ. The IC₅₀ values for β -haematin inhibition ranged from 0.388-0.870 equiv. and the most potent compound, (4.112) with an IC₅₀ of 0.388 ± 0.02 equiv. was almost 5 times more active than CQ (IC₅₀ = 1.91 ± 0.3 equiv.). The suitability of the compounds for this test was based on the presence of the quinoline moiety, necessary for binding to haematin/haeme. As already indicated, formation of β -haematin is a process believed to be essential to the survival of the malaria parasite in the bloodstream. Chloroquine is believed to inhibit this process, and any compound demonstrating similar activity represents a possible antimalarial drug candidate.

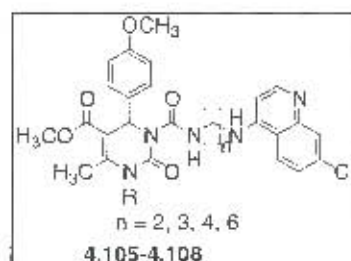
Table 5.10: IC_{50} values for β -haematin inhibition of citrate salts (4.109-4.112)

Compound (Code)	R	n	IC_{50} (equiv.) ^a
Chloroquine	-		1.91 ± 0.3
4.109(NAT1CS)	CH ₃	2	0.870 ± 0.08
4.110(NAT2CS)	CH ₃	3	0.862 ± 0.03
4.111(NAT3CS)	CH ₃	4	0.464 ± 0.02
4.112 (NAT4CS)	CH ₃	6	0.388 ± 0.02

^aThis study $\pm$ standard error of the mean on three determinations

5.3.3 *In vitro* Antiprotozoal Activities of 4-Aminoquinoline DHPMs (4.105-4.108) against *T. brucei brucei*

The antiprotozoal activities of 4-aminoquinoline DHPMs (4.105-4.108) against *T. brucei brucei* were determined at four different concentrations (30, 10, 3 and 1 $\mu\text{g/ml}$) from which the IC_{50} -values were obtained as presented by Table 5.11. Data for the standard control drugs pentamidine (*T. brucei brucei*) and Podophyllotoxin (cytotoxicity) is included for comparative purposes. It is evident from Table 5.11, none of the compounds exhibited significant antiprotozoal activities when compared to pentamidine. Although compound (4.108) exhibited the best activity within the homologous series, its efficacy was several hundred-fold less potent towards pentamidine. Their low therapeutic indices, also clearly revealed that all compounds exhibited unfavorable cytotoxicities towards the human KB cell line. The unexpected increase in activity of compound (4.108) could be credited to the increase in the amino alkyl chain length of the quinoline derivative.

Table 5.11: *In vitro* antiprotozoal activities of 4-aminoquinoline DHPMs (4.105-4.108)

Compound (Code)	R	n	IC ₅₀ (µg/ml)	IC ₅₀ (µM)	Cyt. ED ₅₀ (µg/ml)	Cyt. ED ₅₀ (µM)	TI
Pentamidine	-	-	0.00007	0.0002	-	-	-
Podophyllotoxin	-	-	-	-	0.00024	-	-
4.105 (NAT1)	CH ₃	2	3.2	5.9	40.4	75	13
4.106 (NAT2)	CH ₃	3	3.29	6.0	11.9	21.5	4
4.107 (NAT3)	CH ₃	4	5.28	9.3	5.9	10.4	1
4.108 (NAT4)	CH ₃	6	0.357	0.6	11.3	19.0	32

5.3.4 *In vitro* antiprotozoal activity of 4-Aminoquinoline DHPMs (4.105-4.108) and citrate salts (4.109-4.112) against *T.b. rhodesiense*

The *in vitro* antiprotozoal activity of 4-aminoquinoline DHPMs (4.105-4.108) and citrate salts (4.109-4.112) against *T.b. rhodesiense* were determined at different compound concentrations of 10.00, 3.33, 1.11, 0.37 and 0.12 µg/ml (data not shown). All the compounds within the homologous series were several magnitudes less effective than pentamidine (IC₅₀ = 0.009 µM). Nevertheless, compound (4.112) displaying promising antiprotozoal activity at one of the lowest concentrations tested (1.11 µg/ml, IC₅₀ = 2.12 µM), Table 5.12.

Table 5.12: *In vitro* antiprotozoal activities of 4-Aminoquinoline and citrate salt DHPMs against *T. b. rhodesiense*

Compound (Code)	R	n	IC ₅₀ (μM)
Pentamidine	-	-	0.009
4.105 (NAT1)	CH ₃	2	4.04
4.106 (NAT2)	CH ₃	3	8.56
4.107 (NAT3)	CH ₃	4	8.16
4.109 (NAT1CS)	CH ₃	2	8.31
4.110 (NAT2CS)	CH ₃	3	8.08
4.110 (NAT3CS)	CH ₃	4	5.91
4.112 (NAT4CS)	CH ₃	6	2.12

5.3.5 Discussion

Chloroquine resistance in *P. falciparum* malaria has been found to be strongly associated with mutations in the digestive vacuole (DV) membrane protein termed PfCRT.⁶⁻⁹ Excessive export of the CQ from its site of action in the DV is thought to result from these mutations. Many structurally diverse molecules, termed reversal agents (RA), have been identified that are known to inhibit *P. falciparum* chloroquine resistance transporter (PfCRT)-associated CQ export from the DV in CQ resistant parasites.¹⁰⁻¹³ Examples of such molecules include the Ca²⁺-channel blocker verapamil, 4-aryl-1, 4-dihydropyridines (DHPs), dihydropyrimidinones (DHPMs) (see section 4.4.9, page 109) and tricyclic antidepressants among many others, (see section 4.4.2, page 96). Burgess *et al.*¹⁴ demonstrated by linking a CQ-like moiety to a RA can yield highly effective drugs against CQS and CQR strains of *P. falciparum*. For these types of drugs to function effectively they should accumulate efficiently in the DV which is accomplished *via* pH trapping of the protonatable nitrogens and binding of the CQ-like moiety to haeme and/or haematin, (discussed above). In addition to promoting drug accumulation in the DV it was speculated that the RA component would interfere with the export from the DV of the CQ moiety by the mutated PfCRT in chloroquine resistant parasites. Together,

these effects could allow a curative dose against CQR strains to be much lower than that of its components given separately, with resultant diminished cost and toxicity. Compounds (4.109-4.112) were found to be potent against the CQR K1 strain. These compounds may exert their antimalarial effects in a similar manner as proposed by Burgess *et al.*¹⁴ since they were designed on the same basis.

In terms of SAR studies, no direct linear correlation between the chain length and the antiplasmodial activity was observed for the structurally related compounds (4.105-4.108). However, and consistent with earlier findings, the short and long amino alkyl chain compounds exhibited the highest inhibitory activities as illustrated by Fig. 5.4.² In most assays the citrate salt derivatives were more potent than their corresponding analogues, suggesting that these salts may possess physico-chemical properties that will enable them to be transported more efficiently across the cell membranes and hence accumulate more readily in the DV, where they are able to exert their antimalarial effects.

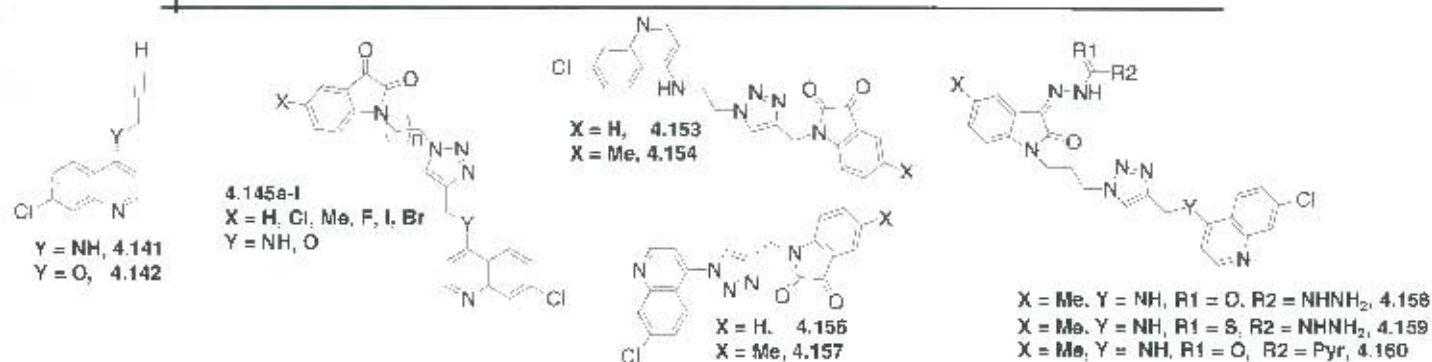
There is fairly a good correlation between the antimalarial activity against the chloroquine resistant strain and inhibition of β -haematin formation. The observed correlation may be a result of similar pKa values and thus comparable vacuolar accumulation of the dihydropyrimidinones derivatives without regard to the chloroquine resistance mechanism. Slight deviations in the correlation between the activity and β -haematin inhibition may be a result of the small differences in pKa values. The correlation/lack of correlation between the biological activity and β -haematin inhibition is normally considered in the chloroquine sensitive strains due to other factors in play in chloroquine resistant strains that may disallow an accurate comparison.

5.4 Biological Results and Discussion Isatin-Derived Triazoles

The antiplasmodial and antiprotozoal *in vitro* activities of the synthesized compounds were evaluated at various laboratories. The antiplasmodial activities against chloroquine-sensitive (D10) *P. falciparum* were conducted at the Division of Pharmacology, in the laboratories of Prof. PJ Smith (UCT). The antiplasmodial evaluation against chloroquine-resistant (W2) *P. falciparum* strain was performed at the Department of Medicine, San Francisco General Hospital, University of California at San Francisco (UCSF), in collaboration with Professor P. J. Rosenthal. Assessment against CQS (3D7) and CQR (K1) *P. falciparum* strains as well as *T. brucei brucei* and cytotoxicity assays were conducted in the laboratories of Dr. V Yardley (LSHTM). Lastly, testing against β -haematin formation was performed at the Chemistry Department (UCT), in the laboratories of Prof. TJ Egan.

5.4.1 *In vitro* Antiplasmodial Activities of Isatin-Derived Triazoles against Chloroquine-sensitive (D10) and Chloroquine-resistant (K1) *P. falciparum* strains

The antiplasmodial activities of structurally related compounds (4.15a-4.145e) against D10 and W2 respectively were determined at two different laboratories, Table 5.13. As it is evident from Fig. 5.5 (a) and (b), compounds (4.145a-e) were less active than the control drug, CQ against both D10 and W2 strains. The most active compound (4.145c) [$IC_{50} = 0.393 \mu M$] within the selected series (4.145a-e) was 26 times less effective than CQ ($IC_{50} = 0.0153 \mu M$) against the D10 strain, (Fig. 5.5 a) while compound [(4.145b) ($IC_{50} = 0.920 \mu M$)] was found to be 6 times less active than CQ ($IC_{50} = 0.1525 \mu M$) against the W2 strains, (Fig. 5.5 b). Overall, the unsubstituted isatin triazole (4.145a) (where, X = H) displayed lower inhibitory than the substituted isatin triazoles (4.145b-e) (where X = Cl, Me and F). Another interesting observation was the notable difference in the antiplasmodial activities exhibited by regioisomers, (4.145d) and (4.145e); one regioisomer, (4.145e) was found to be more potent than the other regioisomer, (4.145d) against D10. Regioisomer, (4.145e) was about 2 times more active than (4.145d) against the D10 strain while comparable antiplasmodial activities were revealed against the W2 strain.

Table 5.13: IC₅₀ values obtained for CQ-sensitive strain, D10

Compound	X	n	Y	R1	R2	IC ₅₀ (μM)	
						D10	W2
CQ	-	-	-	-	-	0.0153	0.1525
4.141 (Nat 78)	-	-	NH	-	-	0.4513	0.846
4.142 (Nat 113)	-	-	O	-	-	44.268	>20
4.145a (Nat 100)	H	3	NH	-	-	0.678	6.820
4.145b (Nat 124)	Cl	3	NH	-	-	0.509	0.920
4.145c (Nat 97)	CH ₃	3	NH	-	-	0.393	2.479
4.145d (Nat 101 FR1)	F(regioisomer)	3	NH	-	-	0.819	2.567
4.145e (Nat 101 FR2)	F(regioisomer)	3	NH	-	-	0.482	2.471
4.145f (Nat 115)	Cl	2	O	-	-	2.368	8.228
4.145g (Nat 129)	Cl	3	O	-	-	5.119	>20
4.145h (Nat 116)	Cl	4	O	-	-	5.706	>20
4.145i (Nat 117)	Cl	5	O	-	-	3.789	>20
4.145j (Nat 118)	Cl	6	O	-	-	1.932	8.897
4.145k (Nat 119)	I	3	O	-	-	2.551	8.340
4.145l (Nat 121)	Br	3	O	-	-	4.946	>20
4.153 (Nat 141)	H	2	-	-	-	1.187	5.845
4.154 (Nat 142)	CH ₃	2	-	-	-	2.057	7.276
4.156 (Nat 150)	H	-	-	-	-	3.007	4.763
4.157 (Nat 151)	CH ₃	-	-	-	-	1.381	>20
4.158 (Nat 138)	CH ₃	3	NH	O	NH ₂	0.103	0.435
4.159 (Nat 130)	CH ₃	3	NH	S	NH ₂	0.0614	0.395
4.160 (Nat 139)	CH ₃	3	NH	O	pyr	2.262	0.903

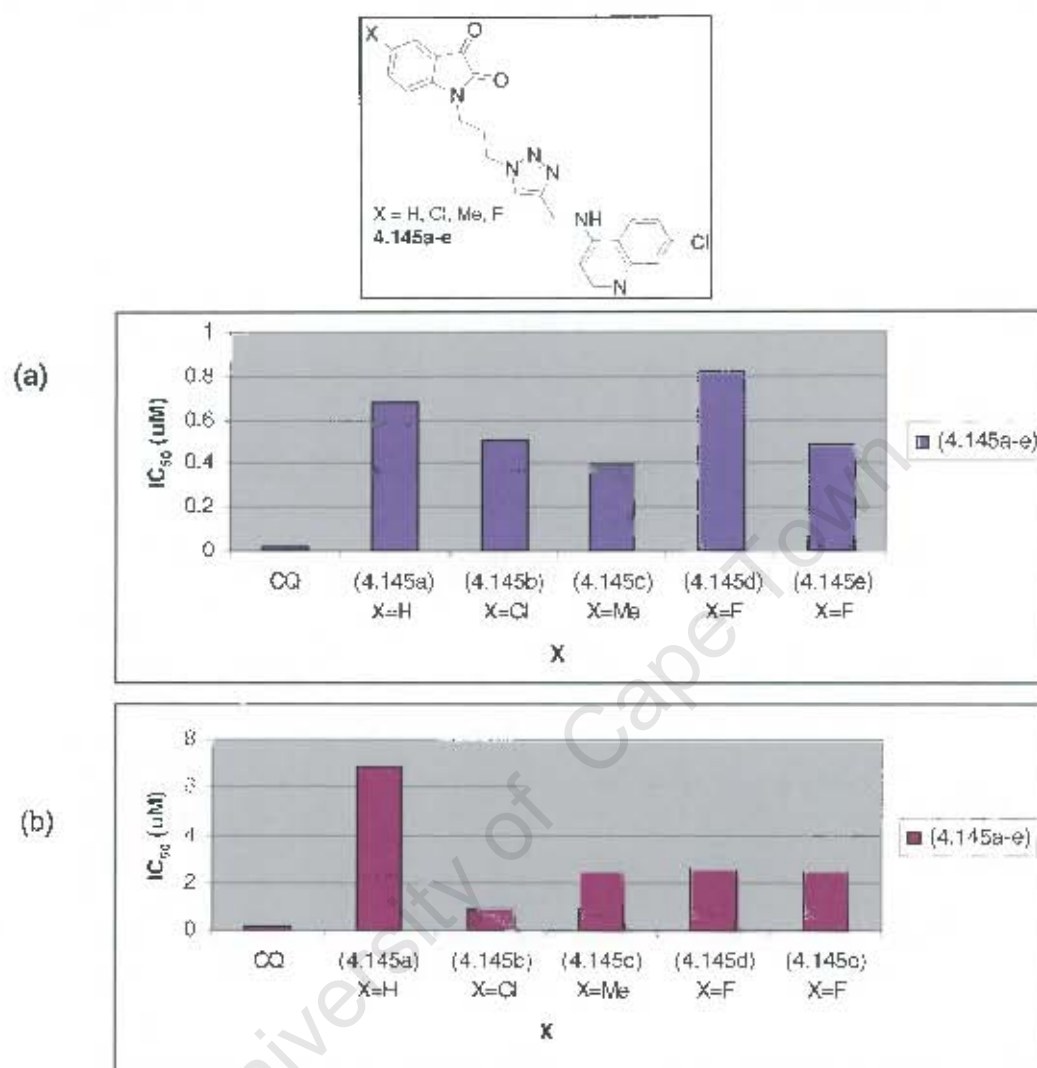


Figure 5.5: A plot of isatin-derived triazoles against (a) CQS, D10 and (b) CQR, W2 *P. falciparum* strains

Fig. 5.6 demonstrates the relationship between the variable chain lengths and antiplasmodial activities of compounds (4.145f-j) against both D10 and W2 strains. The 2 carbon chain compound (4.145f), and the 6 carbon chain compound (4.145j) exhibited the best antiplasmodial activity within the homologous series against the D10 strain. However, all the compounds exhibited activities of several magnitudes less than CQ against the D10 strain. The compounds (4.145f-j) showed a similar trend against the W2

strain with all the compounds exhibiting activities less active than their respective reference drug CQ.

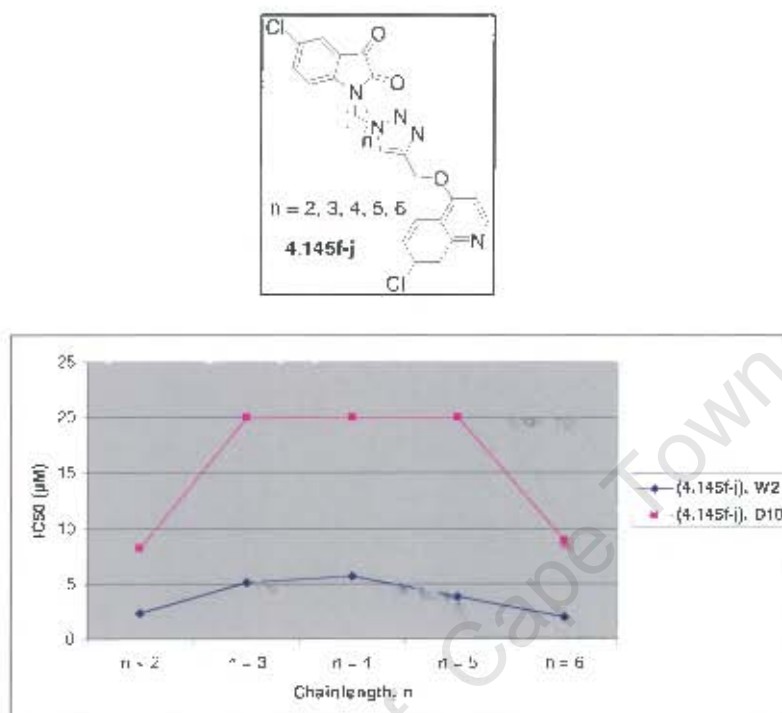


Figure 5.6: Correlation of chain length, n to antiparasmodial activities in the D10 and W2 strains respectively

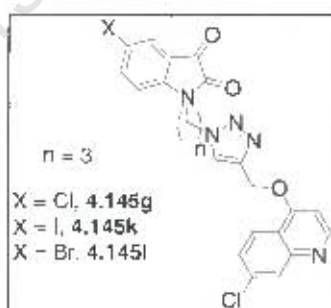


Figure 5.7: Structurally related compounds 4.145g, 4.145k and 4.145l

The influence of the different halogen substituents at position-5 of the aromatic isatin derivatives (4.145g, $X = Cl$), (4.145k, $X = I$) and (4.145l, $X = Br$) on the antiparasmodial activities were investigated, (Fig. 5.7). Compounds (4.145g) and (4.145l) showed comparable inhibitory activities against both D10 and W2 strains, while compound

(4.145k, X = I), $IC_{50} = 2.551$ against D10 and $8.340 \mu\text{M}$ against W2 respectively was the most active compound within the selected series against both D10 and W2 strains, even though their activities were less significant against CQ.

Fig. 5.8 (a), (b) shows that methyl substituted compound (4.154, X = Me, n = 2) was less active than the unsubstituted compound (4.153, X = H, n = 2) against both the D10 and W2 strains. However, the removal of the 2 carbon spacer between the isatin and triazole moieties of compounds (4.153) and (4.154) have demonstrated the substituted compound (4.157) [$IC_{50} = 1.381 \mu\text{M}$], to be 3 times more active than the unsubstituted derivative (4.156) [$IC_{50} = 3.007 \mu\text{M}$] against the D10 strain. On the contrary the compound (4.156) [$IC_{50} = 4.763 \mu\text{M}$] was found to be more active than compound (4.157) [$IC_{50} = >20 \mu\text{M}$] against the W2 strain.

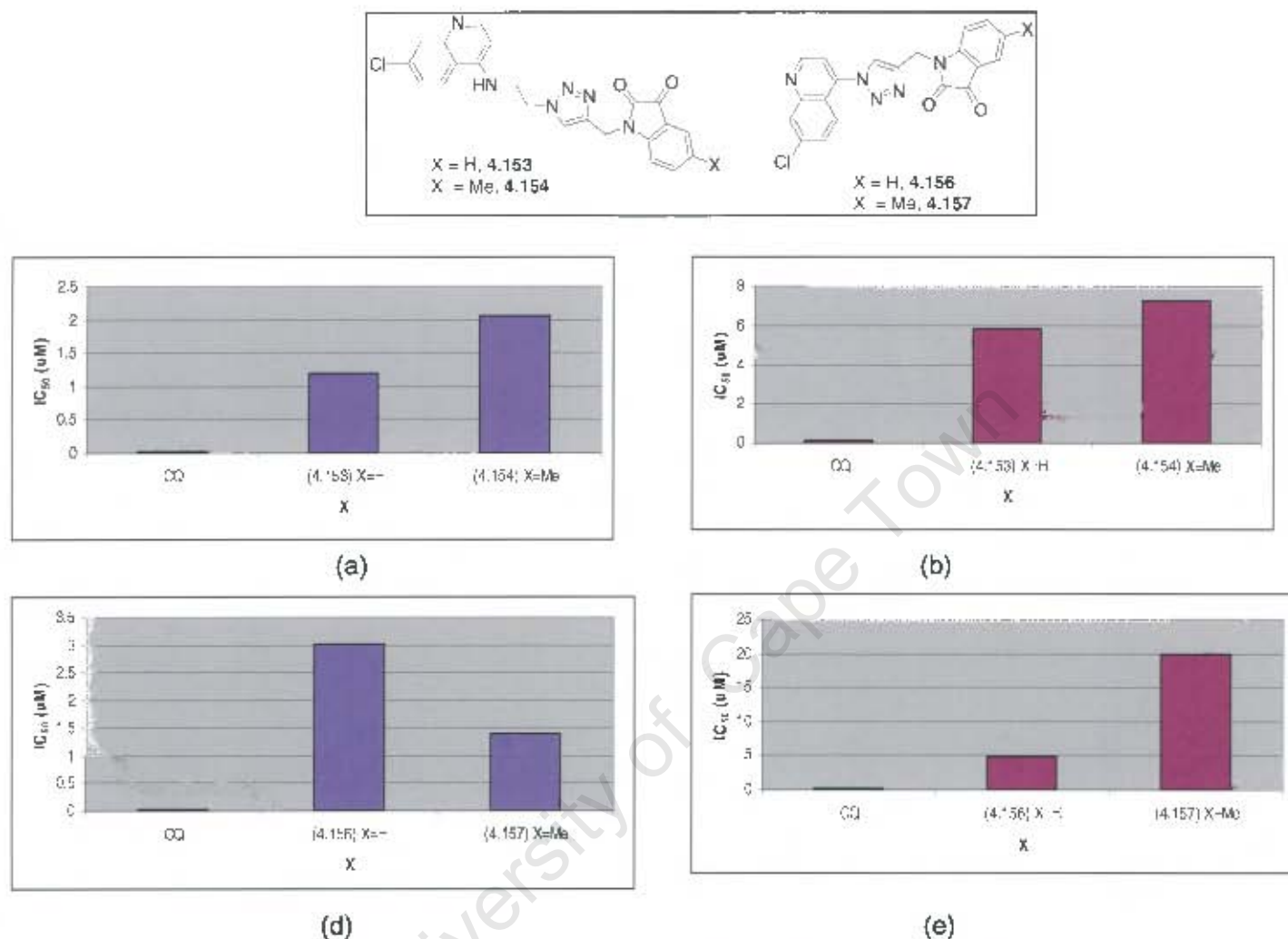
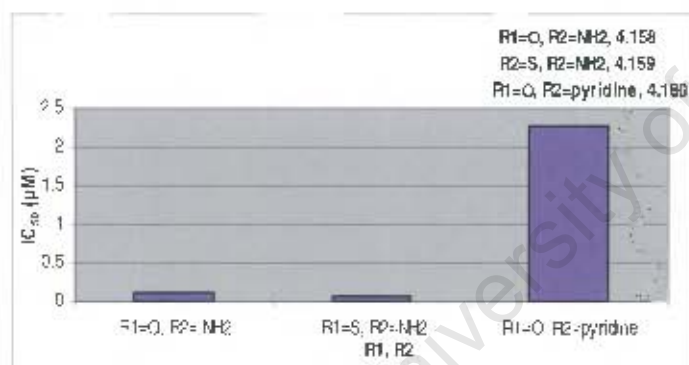
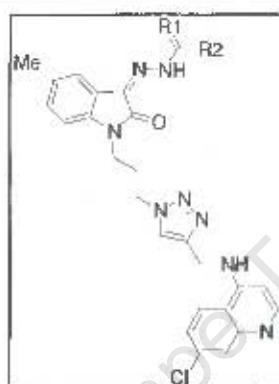


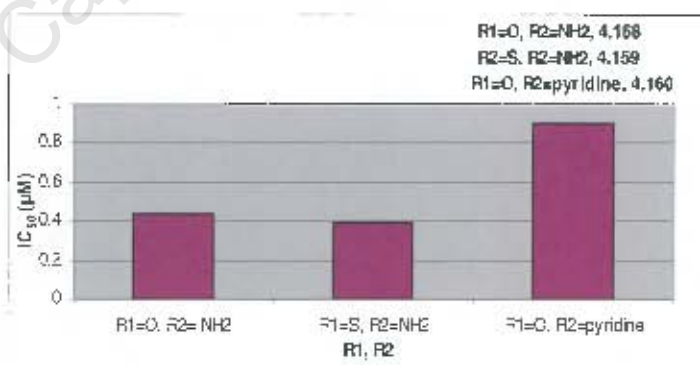
Figure 5.8: A plot of isatin-derived triazoles (4.153, 4.154) against (a) CQS, D10 and (b) CQR, W2 and isatin-derived triazoles (4.156, 4.157) against (c) CQS, D10 and (d) CQR, W2 *P. falciparum* strains

Fig. 5.9 shows that among the three compounds, semicarbazone (4.158) thiosemicarbazone (4.159) and isonicotinic acid-derived (4.160), thiosemicarbazone (4.159) [(IC_{50} = 0.0614 against D10 and 0.395 μM against W2 respectively)] exhibited the best antiparasitic activities against both the D10 and W2 strains, Table 5.13. Regardless of the high activity associated with the thiosemicarbazone (4.159) [(IC_{50} = 0.0614 against D10 and 0.395 μM against W2 respectively)] they were unable to inhibit the parasite to the same extent as CQ (IC_{50} = 0.0153 against D10 and 0.1525 μM

against W2 respectively), which was 4 times more active against D10 and 3 times more active against the W2 strain. Semicarbazone (4.158), the second most potent compound within this series, revealed nearly analogous activities in both D10 and W2 strains, (Fig. 5.10). The semicarbazone (4.159) with IC_{50} s 0.0614 and 0.395 μ M respectively exhibited the lowest activities among the compounds tested against the D10 and W2 strains.



(a)



(b)

Figure 5.9: A plot of isatin-derived triazoles (4.158, 4.159 and 4.160) against (a) CQS, D10 and (b) CQR, W2

5.4.2 *In vitro* Antiplasmodial Activities of Isatin-Derived Triazoles against CQS (3D7) and CQR (K1) *P. falciparum* strains

The isatin-derived triazoles were screened against two different strains, chloroquine-sensitive (3D7) and chloroquine-resistant (K1) of *P. falciparum*, (Table 5.14). Table 5.14 shows that unsubstituted compound (**4.145a**, X = H, Fig. 5.10), (IC_{50} = 0.288 μ M) is more potent than the substituted analogues (**4.145b**, X = Cl, Fig. 5.11) (IC_{50} = 0.697 μ M) and (**4.145c**, X = CH₃, Fig. 5.10) (IC_{50} = 0.333 μ M) against the 3D7 strain. However, the chloro substituted analogue, (**4.145b**) (IC_{50} = 0.02 μ M) was more effective than both compounds (**4.145c**) (IC_{50} = 0.1 μ M) and (**4.145a**) (IC_{50} = 2.01 μ M) against K1 strain. Despite the high antiplasmodial activities revealed by (**4.145a**) (in 3D7 strain) and (**4.145b**) (in K1 strain) within the selected series, their respective control drugs, CQ and artesunate, demonstrated to be stronger inhibitors against both strains.

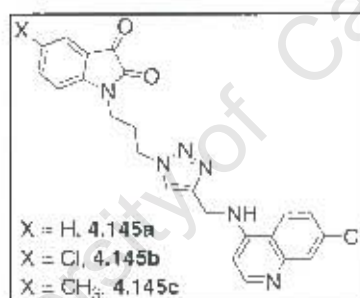
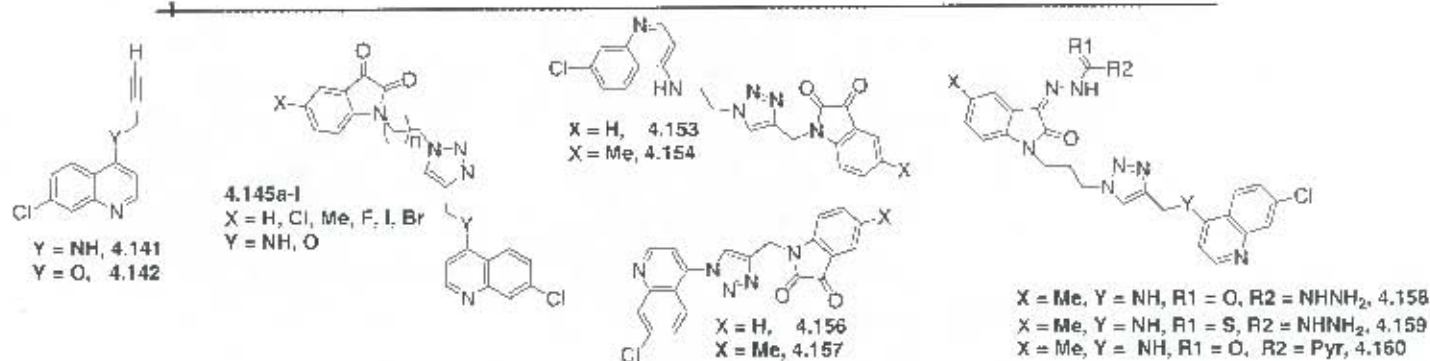


Figure 5.10: Chemical structures of compounds (**4.145a-c**)

Table 5.14: IC₅₀ values obtained for CQS strain, 3D7 and CQR strain, K1

Compound (Code)	X	n	Y	R1	R2	IC ₅₀ (μM)		Cytotoxicity (μM)	T _{1/2} (h)	
						3D7	K1		3D7 ^a	K1 ^b
Chloroquine	-	-	-	-	-	0.014	-	-	-	-
Artesunate	-	-	-	-	-	-	0.001	-	-	-
Podophyllotoxin	-	-	-	-	-	-	-	0.004	-	-
4.141 (Nat 78)	-	-	NH	-	-	0.440	1.2	170.49	387	142
4.142 (Nat 113)	-	-	O	-	-	0.027	>30	172.68	6396	>6
4.145a (Nat 100)	H	3	NH	-	-	0.288	2.01	295.11	1025	147
4.145b (Nat 124)	Cl	3	NH	-	-	0.697	0.02	220.118	316	11006
4.145c (Nat 97)	CH ₃	3	NH	-	-	0.333	0.1	82.32	247	832
4.145f (Nat 115)	Cl	2	O	-	-	1.516	<0.12	240.29	159	2002
4.145g (Nat 129)	Cl	3	O	-	-	0.697	0.02	235.29	338	11765
4.145h (Nat 116)	Cl	4	O	-	-	1.769	0.65	76.63	43	118
4.145i (Nat 117)	Cl	5	O	-	-	4.163	2.80	61.21	15	22
4.145j (Nat 118)	Cl	6	O	-	-	0.055	3.32	51.21	931	15
4.145k (Nat 119)	I	3	O	-	-	5.43	0.82	56.28	10	69
4.145l (Nat 121)	Br	3	O	-	-	1.020	0.68	47.64	47	70
4.153 (Nat 141)	H	2	-	-	-	1.314	1.77	222.56	169	126
4.154 (Nat 42)	CH ₃	2	-	-	-	1.73	5.51	>300	>173	54
4.158 (Nat 138)	CH ₃	3	NH	O	NH ₂	0.156	0.09	73.3	470	814
4.159 (Nat 130)	CH ₃	3	NH	S	NH ₂	0.022	0.2	5.17	235	26
4.160 (Nat 139)	CH ₃	3	NH	O	Pyr	0.333	0.4	5.80	17	15

All compounds were assayed against CQS (3D7) and CQR (K1) *P. falciparum* strains. Fig. 5.11 indicates that members of the homologous series, compounds (4.145f-j), broadly exhibited low to moderate activities against both strains. Different trends in the inhibitory activities with respect to the various chain lengths of these compounds were observed in the respective strains. In the 3D7 strain, it was found that compound (4.145g) [(n = 3)], with the shorter chain length and compound (4.145j) [(n = 6)], with the longer chain length exhibited greater antiparasmodial activities within the homologous series. The most active compound in 3D7, (4.145j) [(IC₅₀ = 0.055 μ M)] within the series was 4 times less active than CQ (IC₅₀ = 0.014 μ M). On the contrary, in the K1 strain the compound with the longer carbon spacer, compound (4.145j) [(n = 6)] was found to be the least active compound while the shorter 2- and 3- carbon spacer compounds (4.145f) and (4.145g) were the most potent compounds among the series. Varying the substituents at position 5 in compound (4.145g) with other halogens, for example, I and Br, (Fig. 5.7, page 218) resulted in a decrease in the antiparasmodial activity against both 3D7 and K1 strains.

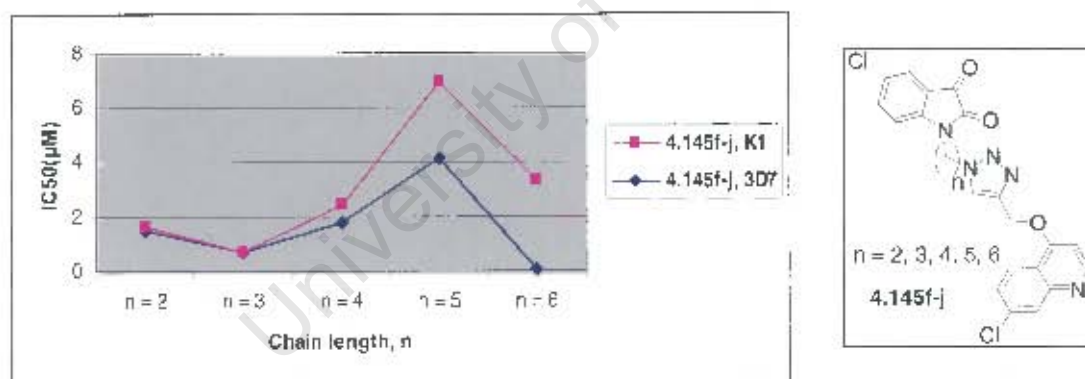


Figure 5.11: A plot of isatin-derived triazoles (4.145f-j) against the K1 and 3D7 strains

Within the series presented in Fig. 5.12, it is clear that the thiosemicarbazone 4.159, R1= S, R2 =NH₂) [(IC₅₀ = 0.022 μ M)] exhibited the greatest antiparasmodial activity against the 3D7 strain even though its activity was almost 2 times less efficacious than CQ (IC₅₀ = 0.014 μ M). However, in the K1 strain, the semicarbazone (4.158) [(R1= O, R2 = NH₂)] with an IC₅₀ of 0.09 μ M was found to be the most active compound. The isonicotinic

acid derivative, (4.160) [(R1= O, R2 = pyr)], did not show significant antiplasmodial activities and was the least active compound among the semicarbazone and thiosemicarbazone series in both strains. Furthermore, Table 5.14 revealed that the majority of the compounds also displayed undesirable toxicity towards the mammalian KB cell line, as displayed by their low therapeutic indices. The compounds with the most striking therapeutic indices are compounds (4.142), (4.145b) and (4.145g), which displayed the highest therapeutic indices, suggesting that these compounds were significantly more toxic towards the parasite than towards the human cell line. From Table 5.14, it is clear that compounds (4.142), (4.145b) and (4.145g) also exhibited excellent antiplasmodial activities against the respective strains, which implies that these compounds may be suitable candidates for further drug development.

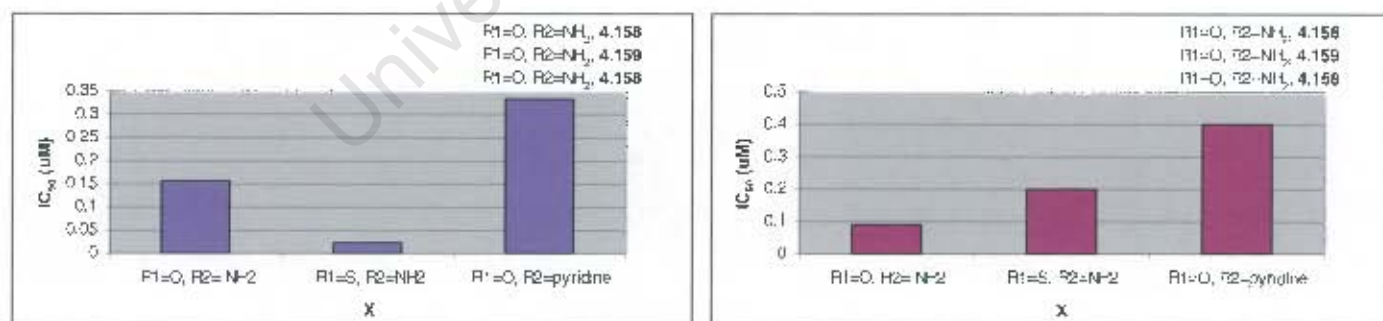
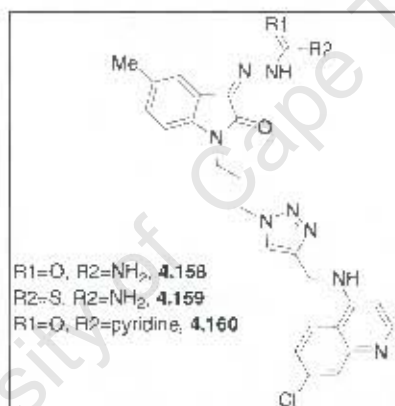
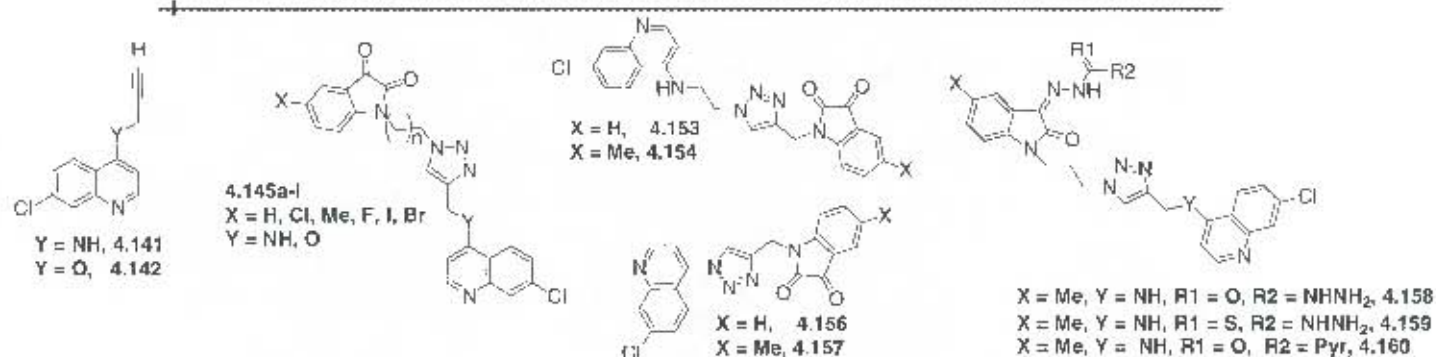


Figure 5.12: A plot of isatin-derived triazoles (4.158, 4.159 and 4.160) against (a) CQS, 3D7 and (b) CQR, and K1

5.4.3 *In vitro* β -haematin inhibition of Isatin-Derived Triazoles

The data in Table 5.15 revealed that all the compounds exhibit potential as inhibitors of β -haematin formation as most have activities comparable or superior to CQ, except compounds (4.141), (4.142), (4.156), (4.157) and (4.158-4.160) which have shown no activity ($IC_{50} = > 4.00$ equiv.).

University of Cape Town

Table 5.15: Inhibition of β -haematin formation by Isatin-Derived Triazoles

Compound (Code)	X	n	Y	R1	R2	IC ₅₀ (equiv.) ^a
CQ	-	-	-	-	-	1.91 ± 0.3
4.141 (Nat 78)	-	-	NH	-	-	Not active ^b
4.142 (Nat 113)	-	-	O	-	-	Not active ^b
4.145a (Nat 100)	H	3	NH	-	-	1.004 ± 0.09
4.145b (Nat 124)	Cl	3	NH	-	-	0.351 ± 0.1
4.145c (Nat 97)	CH ₃	3	NH	-	-	0.880 ± 0.2
4.145d (Nat 101 FR1)	F (regioisomer)	3	NH	-	-	0.809 ± 0.3
4.145e (Nat 101 FR2)	F (regioisomer)	3	NH	-	-	0.883 ± 0.1
4.145f (Nat 115)	Cl	2	O	-	-	0.473 ± 0.06
4.145g (Nat 129)	Cl	3	O	-	-	0.471 ± 0.05
4.145h (Nat 116)	Cl	4	O	-	-	0.495 ± 0.09
4.145i (Nat 117)	Cl	5	O	-	-	0.791 ± 0.2
4.145j (Nat 118)	Cl	6	O	-	-	0.502 ± 0.04
4.145k (Nat 119)	I	3	O	-	-	0.451 ± 0.05
4.145l (Nat 121)	Br	3	O	-	-	0.466 ± 0.09
4.153 (Nat 141)	H	2	-	-	-	1.380 ± 0.3
4.154 (Nat 142)	CH ₃	2	-	-	-	1.730 ± 0.1
4.156 (Nat 150)	H	-	-	-	-	Not active ^b
4.157 (Nat 151)	CH ₃	-	-	-	-	Not active ^b
4.158 (Nat 138)	CH ₃	3	NH	O	NH ₂	Not active ^b
4.159 (Nat 130)	CH ₃	3	NH	S	NH ₂	Not active ^b
4.160 (Nat 139)	CH ₃	3	NH	O	-	Not active ^b

^aThis study ± standard error of the mean on three determinations; ^b At (IC₅₀ = >4.00 equiv.)

Fig. 5.13 demonstrates the effect of variation of the halogen substituent on β -haematin inhibition. Compound (4.145b), where X = Cl, which was 5 times more efficacious than CQ, was the most active among the selected series. Compound (4.145c) and the regioisomers (4.145d) and (4.145e), broadly revealed comparable activities and were about 2 times more active than CQ. The least active within the series, the unsubstituted isatin derivative (4.145a), was more potent than CQ.

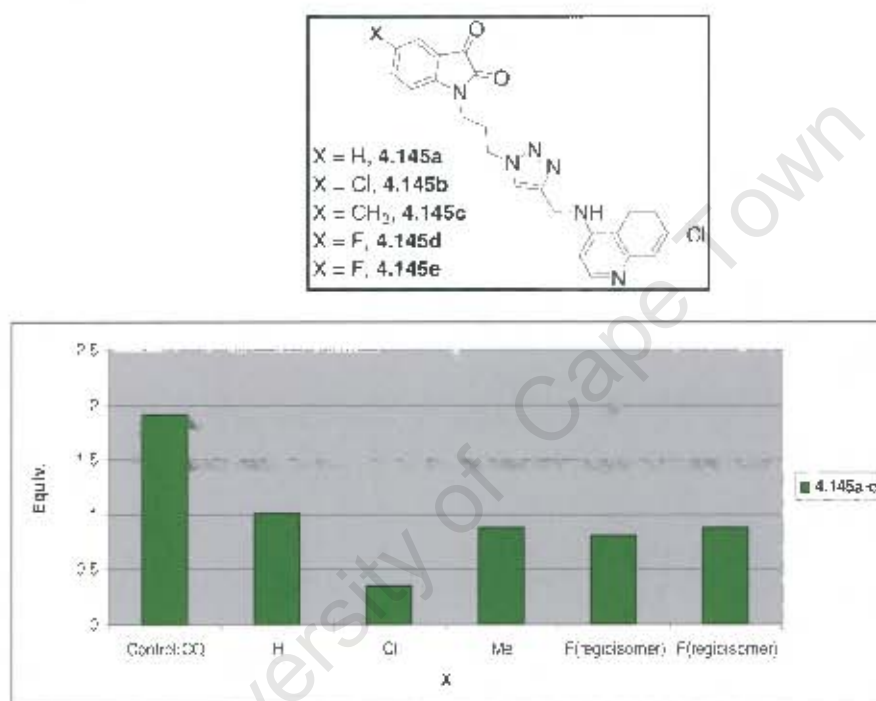


Figure 5.13: A plot of isatin-derived triazoles (4.145a-e) against β -haematin formation

From Fig. 5.14 it can be seen that with the exception of compound (4.145i), almost all the compounds showed comparable activities of 3-4 fold greater than CQ against β -haematin formation. Even though compound (4.145i) [(where n = 5)] exhibited the lowest activity within the selected series but was 2 times more active than CQ.

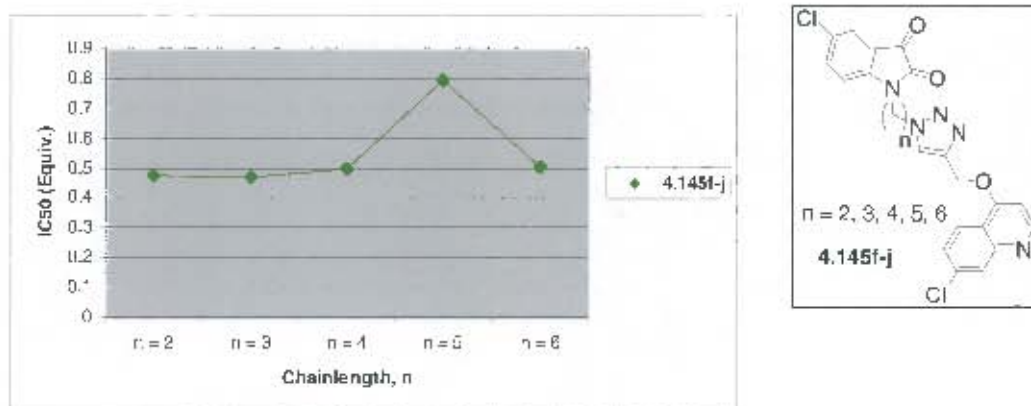


Figure 5.14: A plot of Isatin-derived triazoles (4.145f-j) against β -haematin formation

5.4.4 *In vitro* antiprotozoal activity of Isatin-Derived Triazoles against *T.b. rhodesiense*

The antiprotozoal activities of the isatin-derived triazoles were determined at various descending concentrations (30, 10, 3, 1, 0.3, 0.1, 0.041, 0.014, 0.0046, 0.0015, 0.0005 and 0.0002 $\mu\text{g/ml}$) to obtain the IC_{50} values. Nearly all the compounds, except compound (4.159) were several thousand folds less active than the reference drug, pentamidine, (Table 5.16). The most active compound [(4.159) ($\text{IC}_{50} = 2.97 \mu\text{M}$)] among the tested compounds, was more than 300 times less efficacious than pentamidine ($\text{IC}_{50} = 0.009, 0.008 \mu\text{M}$). The remaining compounds had IC_{50} s greater than 10.00 μM . Despite the lack of significant antiprotozoal activity exhibited by compound (4.159) relative to pentamidine, its activity revealed among the selected 16 compounds dictates the necessity for further investigation.

Table 5.16: Antiprotozoal Activities of Isatin-Derived Triazoles

Compound ^a	R	n	Y	X	Z	IC ₅₀ (μ M)
Pentamidine	-	-	-	-	-	0.009, 0.008
4.141 (Nat 78)	-	-	NH	-	-	>30
4.142(Nat 113)	-	-	O	-	-	>30
4.145a (Nat 100)	H	3	NH	-	-	16.26
4.145b (Nat 124)	Cl	3	NH	-	-	11.28
4.145c (Nat 97)	CH ₃	3	NH	-	-	16.14
4.145f (Nat 115)	Cl	2	O	-	-	>30
4.145g (Nat 129)	Cl	3	O	-	-	12.71
4.145h (Nat 116)	Cl	4	O	-	-	24.66
4.145i (Nat 117)	Cl	5	O	-	-	24.49
4.145j (Nat 118)	Cl	6	O	-	-	25.15
4.145k (Nat 119)	I	3	NH	-	-	13.97
4.145l (Nat 121)	Br	3	NH	-	-	14.16
4.153(Nat 141)	H	2	-	-	-	>30
4.154(Nat 142)	CH ₃	2	-	-	-	>30
4.158(Nat 138)	CH ₃	3	NH	O	NH ₂	>30
4.159(Nat 130)	CH ₃	3	NH	S	NH ₂	2.97
4.160 (Nat 139)	CH ₃	3	NH	O	-	25.59

5.4.5 Discussion: Effects of the Different Moieties on the Antimalarial Activities

5.4.5.1 Effect of the Semicarbazone and Thiosemicarbazone Moieties

The superior activities associated with the semicarbazone- and thiosemicarbazone - based compounds (4.158) and (4.159) may be attributed to three potential features:

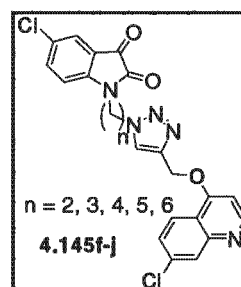
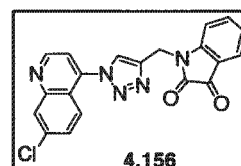
- (i) Thiosemicarbazones are potential iron-chelators in *P. falciparum*, which may exhibit biological activity either by withholding iron from the plasmodia. It has been demonstrated that withholding iron can lead to the malfunctioning of iron dependent enzymes such as δ -aminolevinate synthase, an enzyme involved in the haeme biosynthetic pathway and ribonucleotide reductase (RR), an enzyme essential for DNA synthesis.¹⁵⁻¹⁷ Disruption of these vital mechanisms may result in parasite death. Iron chelators such as desferrioxamine (DFO), have been shown to inhibit ribonucleotide reductase activity by binding to the iron.^{18, 19}
- (ii) Metal chelators are also known to form complexes that subsequently enter the erythrocytes to produce a lethal free radical-mediated reaction. A study done by Movrin *et al.*²⁰ have demonstrated that copper complexes of thiosemicarbazones produce oxidative stress upon binding to glutathione, a known reducing agent.
- (iii) The thiosemicarbazone moiety contains a protonatable hydrazinic nitrogen atom (see Fig. 4.70, page 176) which possibly increases accumulation in the acidic food vacuole of the parasite *via* the pH trapping principle.²¹
- (iv) Lastly, the thiosemicarbazone and semicarbazone possess an imine (C=N) and a (thiol) carbonyl (C=S) and (C=O) groups which serve as potential sites of attack by a cysteine thiolate in the active site of the cysteine proteases (see Schemes 4.45 and 4.46, pages 176 and 177).³⁴

5.4.5.2 Effect of the Chain Length

Table 5.17 demonstrates the effect of the chain length of compounds (4.145f-j) on the lipophilicity. As discussed earlier, *C* logP measures the lipophilicity characteristic of a molecule that determines whether the molecule can penetrate and transverse many cell membranes to reach the site of action. The *C* logP values, estimated using ChemDraw® v.6.01, give values for comparative lipophilicity studies. As it is apparent from Table 5.17, the lipophilicity of compounds (4.145f-j) increases with chain length. A correlation between the antimalarial activity of the 6-carbon chain compound (4.145j) and its *C* logP value was observed. The long carbon chain compound (4.145j) exhibited the best antimalarial activity among the compounds (4.145f-j) against most of the *P. falciparum* strains and also the highest *C* logP value, which implies that the lipophilicity may play an important role in the efficient passage of this compound across the parasite membranes to reach the acidic food vacuole. Also, the introduction of the ethylene linker increases the lipophilicity in compound (4.145f) compared to compound (4.150) that do not possess one. If these compounds are also exerting their antimalarial effects *via* chelation of iron in *P. falciparum*, then the high lipophilicity is an essential parameter. Lipophilicity is a principal physical property of iron chelators since the iron withheld by chelators from *P. falciparum* most likely resides within the parasitic compartment of the malaria infected red blood cell. An effective antimalarial iron chelator should have the ability to cross lipid membranes and display high selective affinity for iron (II) or iron (III) compared to other endogenous transition metals. A correlation between the degree of lipophilicity of an iron chelator and its antiplasmodial activity has been demonstrated.²²

Table 5.17: *C* logP values of compounds (4.156) and (4.145f-j)

Compound	Chainlength, n	<i>C</i> logP
4.156	0	3.21
4.145f	2	3.66
4.145g	3	3.98
4.145h	4	4.10
4.145i	5	4.63
4.145j	6	5.16



5.4.5.3 Effect of the Quinoline Moiety in the Molecule

Chloroquine and related 4-aminoquinoline compounds have been demonstrated to inhibit synthetic haemozoin (β -haematin) formation²³⁻²⁶ and are believed to inhibit haemozoin formation in the food vacuole (FV).²⁷ During this process it is proposed that 4-aminoquinolines is selectively taken up inside the parasite food vacuole where the parasite actively digests haemoglobin. The weak base properties of chloroquine ($pK_{a1}=8.1$; $pK_{a2}=10.2$) would explain its selective accumulation in the acidic food vacuole. At neutral pH, chloroquine is uncharged and can diffuse freely across the membrane against a pH gradient using energy and once inside the acidic vacuole, the protonated drug becomes trapped.²⁸ Upon proteolysis of haemoglobin, toxic haeme is released that is polymerized into an inert, nontoxic crystal called malarial pigment or haemozoin. Chloroquine would thus interfere with polymerization by binding to haeme and thus forming a haeme-chloroquine complex, thereby causing an accumulation of haeme, which is toxic to the parasite. Given that most compounds in this PhD thesis are 4-aminoquinolines, the same mechanism of antimalarial activity as chloroquine is presumably contributing to the potency of the compounds as antimalarials.

This target of the 4-aminoquinoline antimalarials is completely specific to the parasite and resistance appears to arise from changes in a FV membrane protein, PfCRT,²⁹ rather than changes in the haeme detoxification pathway. It therefore remains uniquely suitable for antimalarial chemotherapy.^{30, 31}

5.4.5.4 Effect of substituents at position 5 in the Isatin moiety

The antiplasmodial activity increased with the size of the halogen atom. The antiplasmodial activity for compound (4.145k) [(X = I)] is greater than that of compound (4.145l) [(X = Br)], which suggests that bigger groups might be favored for binding to the target.

The effects of the different moieties on the antimalarial activities were demonstrated by Chiyanzu *et al.*³² who designed 4-aminoquinoline isatin derived compounds based on a multi-therapeutic strategy that were effective against a chloroquine-sensitive (D10) and two resistant (K1 and W2) strains of *P. falciparum*. In this strategy antimalarial

bioactophores containing electrophilic sites were hybridized with the goal to target the nucleophilic thiolate anion of cysteine residues which permits S-alkylation/arylation and S-acylation/sulfonylation. To achieve this approach the privileged natural product isatin was utilized as a scaffold onto which bioactophores were appended. The thiosemicarbazone moiety was selected as the bioactophore to provide the reactive sites (imine and thiol carbonyl) for alkylation of the enzyme cysteine thiolate.^{33, 34} The hydrazinic nitrogen could assist in the accumulating within the acidic food vacuole through binding to endogenous iron within *P. falciparum*. The 7-chloroquinoline moiety has been proposed from earlier findings to bind to haematin in the parasite's acidic food vacuole, thus inhibiting haemazoin formation, and also increases accumulation of the drug due to the protonatable quinoline nitrogen.³¹ In this way the drug is able to exert its antimalarial activities within the acidic food by targeting the different mechanisms.

The compounds in this class were designed as an extension to the work reported by Chiyanzu *et al.*³² Compound (4.145b) may exert its dual antimalarial effects in the same way as discussed above since it was found to be effective against the chloroquine-resistant strain (K1) strain and β -haematin formation. However, the compounds with the electrophilic reactive sites (imine and thiol carbonyl), the semicarbazone (4.158) and the thiosemithiocarbazone (4.159) were surprisingly not active against β -haematin formation ($IC_{50} = > 4.00$ equiv.) but they were active against the chloroquine-sensitive strains D10 and 3D7 and chloroquine-resistant strains (W2 and K1). These compounds may exert their dual antimalarial effects *via* different pathways, for example they may bind to the falcipains and bind to iron within the acidic food vacuole.

5.5 Conclusion

A series of novel peptidyl based compounds with promising inhibitory activities against various plasmodia strains and enzymes were identified. Several compounds have revealed potency against the chloroquine-resistant strain of *Plasmodium falciparum*. The most potent peptidyl based compounds, (4.46, 4.47, 4.50, and 4.48) have IC_{50} values less than 2.48 μ M against chloroquine-resistant (W2) strain. Overall, no significant inhibitions were shown against the plasmodial enzymes, FP-2 and FP-3, with the lowest IC_{50} s ranging from 1.0-20 μ M and the highest IC_{50} s ranging from 30->100 μ M. Evidently, the data for these compounds have suggested a significant enhanced activity, which

was governed by the presence of the 4-aminoquinoline derived substrate. The consistent moderate activities revealed by peptidyl based compounds (4.49), (4.50) and (4.53) against both enzymes and W2 strain compared to the other compounds in the series, suggests that these compounds may exert dual antimalarial activities. The less active compounds comprised mostly of compounds devoid of the 4 -aminoquinoline moiety while the 4-aminoquinoline pharmacophore in the active compounds, suggests that it may largely contribute to the promising inhibitory activities.

No significant inhibitory activities of the 4-aminoquinoline-containing dihydropyrimidinones (DHPMs) and their salt derivatives were revealed against chloroquine-sensitive, D10 strain. On the other hand, moderate to high inhibitory activities were shown against chloroquine-sensitive, 3D7 and chloroquine-resistant, K1 strains. Within the series, DHPMs (4.112) [(IC₅₀ = 0.007 µM)] and (4.107) [(IC₅₀ = 0.004 µM)] were the most potent compounds in the respective 3D7 and K1 strains. All the compounds were more active than chloroquine against β-haematin formation, especially the DHPM (4.112) which was 5 times more active than chloroquine. No significant *in vitro* antiprotozoal activity of the DHPMs was revealed against *T.b. brucei*. The most active DHPM (4.72) within the series, having an IC₅₀ value of 0.6 µM, was several magnitudes less active than pentamidine. The heterocyclic scaffold of the DHPMs serves as a point of diversity that provides the opportunity to explore meaningful SAR studies. Furthermore, the rich chemistry offered at the various positions on the heterocyclic scaffold creates accessibility to the synthesis of potentially more active compounds with the hope of generating lead compounds.

From the analog libraries evaluated, the thiosemicarbazone derivative (4.159) exhibited the best antiplasmodial activity with IC₅₀ values of 0.0614, 0.395 and 0.022 µM against the respective *P. falciparum* strains, D10, W2 and 3D7. The isatin-derived triazoles (4.145b) and (4.145g) were the most active compounds against the K1 strain with comparable IC₅₀ values of 0.02 µM. Interestingly, in terms of SAR studies, isatin-derived triazoles (4.145b) [(Y = NH)] and (4.145g) [(Y = O)] displayed similar inhibitory activities, even though they solely differ in their respective substituents at position Y, (see Table 5.14, page 223). The thiosemicarbazone derivative (4.159) [(IC₅₀ = 2.97 µM)] was also trypanocidal towards *T. b. rhodesiense*. With the exception of thiosemicarbazone (4.159), semicarbazones (4.158) and (4.160), isatin-derived triazoles (4.156) and (4.157) and

acetylene precursor (4.142), all the Isatin-derived triazoles displayed more superior inhibitory activity than chloroquine against β -haematin formation. In this assay, the most active compound (4.145b) [(IC₅₀ = 0.351 $\pm$ 0.1 equiv.)] was found to be 5 times more potent than chloroquine (IC₅₀ = 1.91 $\pm$ 0.3 equiv.).

There is fairly a good correlation between the antimalarial activity against CQR strain and inhibition of β -haematin formation. The observed correlation may be a result of similar pKa values and thus comparable vacuolar accumulation of the synthesized compounds with regard to the chloroquine resistance mechanism. Slight deviations in the correlation between the activity and β -haematin inhibition may be a result of the small differences in pKa values. The correlation/lack of correlation between the biological activity and β -haematin inhibition is normally considered in the chloroquine sensitive strains due to other factors in play in chloroquine resistant strains that may disallow an accurate comparison.

The varying in the *in vitro* inhibitory activities obtained for the assayed compounds across the respective *P. falciparum* strains, e.g. D10 vs 3D7 and W2 vs K1, suggest that the antimalarial activity is both compound and strain specific. However, the majority of the compounds exhibit excellent antiplasmodial activities against the chloroquine-resistant strains and β -haematin formation. In particular the activity of DHPMs suggests that these potentially active compounds may exert dual antimalarial effects through acting as reversal agents or chemosensitizers and through inhibition of β -haematin formation. Therefore the dual drug approach is a worthwhile strategy for identifying compounds with promising antimalarial activities. Drug development strategies that rely on known parasite-specific targets and build on the existing battery of antimalarial compounds are more likely to yield an effective antimalarial therapy.

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CHAPTER 6**EXPERIMENT****Purification of Solvents**

All solvents used for reactions and preparative and column chromatography were distilled prior to use. Anhydrous solvents were used in all experiments. Other reagents obtained from commercial sources were used without further purification.

Chromatographic Separations

Column chromatography and preparative column chromatography were performed using Merck Kieselgel 60: 70-230 mesh. Reactions were monitored by thin layer chromatography using Merck F₂₅₄ aluminium-backed precoated silica gel plates and were visualized by a combination of ultraviolet light, iodine vapour and staining with 2% ninhydrin solution. Melting points were determined on a Reichert-Jung Thermovar hot-stage microscope. Infrared spectra were recorded on a Thermo Nicolet FTIR instrument in the 4000-700 cm⁻¹ range as chloroform, methylene chloride solutions or nujol mulls. Microanalyses were determined using a Fisons EA 1108 CHNO-S instrument. Mass spectra were recorded on a VG micromass 16F spectrometer operating at 70eV with an accelerating voltage of 4kV and a variable temperature source. At the University of Potchefstroom, SA, mass spectra were determined using a VG11-250J Data System, micromass Autospec-TOF double focusing magnetic mass spectrometer, and at University of Witwatersrand, SA, VG70-SEQ (FAB, operating at 7kV) and VG70-SEQ (EI, operating at 8kV) instruments were used.

Physical and Spectroscopic Data

¹H-NMR (nuclear magnetic resonance) spectra were recorded on a Varian Mercury spectrometer at 300 MHz or a Varian Unity Spectrometer at 400 MHz. Spectra were recorded in deuterated solvents: CDCl₃, DMSO-*d*₆ and CD₃OD and the chemical shifts are reported in parts per million downfield from tetramethylsilane, the internal standard;

coupling constants are given in Hertz. ^1H NMR coupling constants are rounded off to one decimal place, resulting in exactly the same coupling constants for coupling partners. In cases where deuterated methanol was used as ^1H NMR solvent, H-bonding between compounds containing NH_2 or OH groups and the deuterated methanol solvent resulted in a reduction of hydrogen signals observed in ^1H NMR spectra.

^{13}C -NMR (nuclear magnetic resonance) spectra were recorded on a Varian Mercury spectrometer at 75 Hz or Varian Unity Spectrometer at 100 Hz. The format used for recording ^{13}C NMR data is that accepted by most international journals (including American Chemical Society journals). In this format chemical shift values are simply listed without specific assignments to carbon atoms.

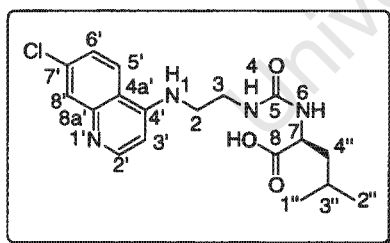
All solvents were dried prior to use. Tetrahydrofuran, toluene and diethyl ether were dried over lithium aluminium hydride and subsequently distilled over sodium wire, using benzophenone as indicator. Triethylamine was dried over and distilled from calcium hydride and stored over potassium hydroxide pellets. Methylene chloride was dried over phosphorous pentoxide and distilled prior to use.

A. General Method for the preparation of compounds 4.20(NAT32) and 4.32(NAT18)

Trimethylsilyl chloride (3 mL, 2.65 g, 24.40 mmol) was added to a solution of amino acid 4.29 (2.00g, 15.25 mmol) in dry DCM (50 mL) and CH₃CN (11 mL) under N₂ at 25 °C. The heterogeneous reaction mixture was stirred at 25 °C 12 h. DIEA (6 mL, 4.33 g, 33.54 mmol) was then added dropwise. The resultant clear solution was then added using an addition funnel over a period of 1h at 15-22 °C to a stirred solution of triphosgene (1.67 g, 5.64 mmol) in dry DCM (20 mL). After stirring for 20 min, a solution made from amine (15.25 mmol, 1.0 eq.), DIEA (4 mL) in dry DCM (15 mL) was added in one portion. After stirring for 30 min, MeOH (3 mL) was added. The solution was concentrated to an oil which was partitioned between EtOAc (100 mL) and NaOH (0.5 M, 200 mL). The aqueous phase was washed with EtOAc (50 mL), acidified with 10% KHSO₄, and product was extracted with DCM (5 x 50 mL). The DCM extract was washed with brine (2 x 50 mL), water (2 x 50 mL), dried over MgSO₄, filtered and concentrated to give the acid.

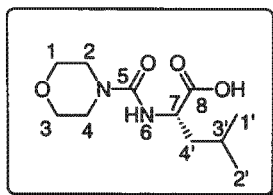
The method employed for the synthesis of 4.20 and 4.32 was that described in general Method A, using 15.25 mmol of the amino acid.

2-[3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-ureido]-4-methyl-pentanoic-acid 4.20



Light brown powder (3.00 g, 52%), m.p. 206-208 °C (from methanol); *R_f* (MeOH:DCM, 1:19) 0.14; IR ν_{max} (CH₂Cl₂)/cm⁻¹ 3500 (O-H), 3261(N-H), 3052 (C-H), 1722 (C=O) and 1645 (C=O); δ_{H} (300 MHz, CD₃OD) 8.28 (1H, d, *J* 6.6, H-2'), 8.14 (1H, d, *J* 8.7, H-5'), 7.78 (1H, d, *J* 1.8, H-8'), 7.51 (1H, dd, *J* 1.8 and 8.7, H-6'), 6.65 (1H, d, *J* 6.6, H-3'), 4.20 (1H, m, H-7), 3.53 (4H, m, CH₂-2 and CH₂-3), 1.58 (3H, m, CH₂-3'' and CH-4''), 0.93 (3H, d, *J* 6.2, CH₃-1'') and 0.91 (3H, d, *J* 6.2, CH₃-2''); δ_{C} (75 MHz, CD₃OD) 174.0 (C=O), 153.7 (C=O), 151.9, 148.8, 134.6, 127.3, 125.1, 124.5, 123.7, 113.8, 99.2, 51.7, 44.3, 41.6, 38.5, 24.9, 23.4 and 22.2; LRMS (EI) *m/z* 378 (60%, M⁺); Found: C, 57.08; H, 6.10; N, 14.70%; Anal. Calc.: C, 57.07; H, 6.12; N, 14.79% for C₁₈H₂₃ClN₄O₃.

4-Methyl-2-[(morpholine-4-carbonyl)-amino]-pentanoic acid 4.32



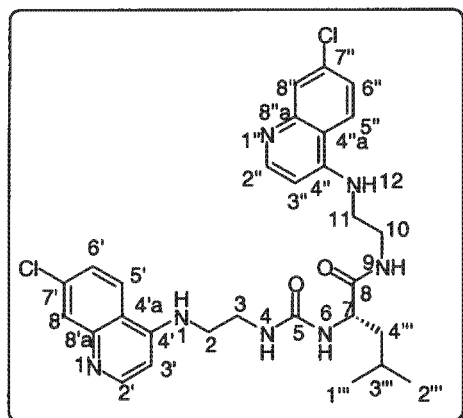
Thick brown oil (2.50g, 67%), R_f (MeOH:DCM, 1:9) 0.13; IR $\nu_{\max}$ (CH₂Cl₂) / cm⁻¹ 3500 (O-H), 3359 (N-H), 3052 (C-H), 1720 (C=O) and 1627 (C=O); δ_H (400 MHz, CDCl₃) 7.63 (1H, bs, O-H), 5.10 (1H, d, J 7.6, NH), 4.45 – 4.40 (1H, m, H-7), 3.68 (4H, t, J 4.8, NC H_2 -2, and NC H_2 -4), 3.40 (4H, t, J 4.8, OC H_2 -3 and OC H_2 -4), 1.77-1.65 (2H, m, H-4'), 1.60-1.50 (1H, m, H-3'), 0.95 (3H, d, J 6.2, CH₃-1') and 0.93 (3H, d, J 6.2, CH₃-2'); δ_C (100 MHz, CDCl₃) 177.0 (C=O), 158.1 (C=O), 66.8 (2C), 61.5, 52.5 (2C), 44.2, 25.1, 23.0 and 22.0.

B. General Method for the preparation of compounds 4.45-4.49 (NAT34-NAT50)

To a solution of acid (200 mg, 0.53 mmol) in DMF (10 mL), amine (120 mg, 0.53 mmol), HOBT (71 mg, 0.53 mmol), EDC-HCl (68 mg, 0.53 mmol) and DIEA (68.2mg, 92 μ L, 0.53 mmol) were added. The mixture was stirred for 16h at 25 °C. Complete consumption of starting material was determined by TLC analysis. The reaction mixture was diluted with water (50 mL) and then extracted with EtOAc (5 x 50 mL). The combined organic extracts were washed with NaHCO₃ (2 x 50 mL), brine (50 mL), water (5 x 50 mL), dried over MgSO₄ and evaporated *in vacuo* to afford a residue which was purified by column chromatography on SiO₂ gel using MeOH/DCM (1:9) eluent to yield the desired product in moderate yield.

The method employed to synthesize 4.45-4.49 was that described in general Method B, using 0.53 mmol of the quinoline carboxylic acid.

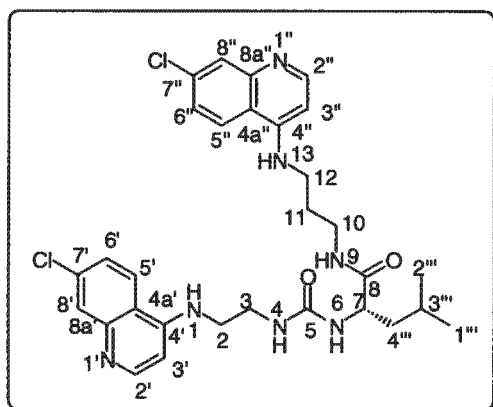
2-{3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-ureido}-4-methyl-pentanoic acid [2-(7-chloro-quinolin-4-ylamino)-ethyl]-amide, 4.45 (NAT34)



Pale brown powder (140 mg, 45%), m.p. 207-209 °C (from methanol); R_f (MeOH:DCM, 1:9) 0.21; IR $\nu_{\max}$ (CH₂Cl₂) / cm⁻¹ 3268 (N-H), 3052 (C-H), 1615 (C=O) and 1576 (C=O); δ_H (300 MHz, CD₃OD) 8.28 (2H, d, J 6.0, H-2' and H-2''), 7.96 (1H, d, J 8.7, H-5'), 7.93 (1H, d, J 8.7, H-5''), 7.71 (1H, d, J 2.4, H-8'), 7.70 (1H, d, J 2.4, H-8''), 7.33 (2H, dd, J 2.4 and 8.7, H-6' and H-6''), 6.43 (1H, d, J 6.0, H-3'), 6.41 (1H, d, J 6.0, H-3''), 4.23 (1H,

m, H-7), 3.60-3.37 (8H, m, HNCCH₂-2, CH₂-3, HNCCH₂-10 and CH₂-11), 1.63 - 1.35 (3H, m, CH₂-3''' and H-4'''), 0.83 (3H, d, J 6.6, CH₃-1''') and 0.84 (3H, d, J 6.6, CH₃-2'''); δ_C (75 MHz, CD₃OD) 174.5 (C=O), 159.1 (C=O), 152.5 (2C), 150.8, 150.7, 149.7 (2C), 134.1 (2C), 128.2 (2C), 124.7 (2C), 124.4 (2C), 118.1 (2C), 99.2 (2C), 52.5, 44.6, 42.9, 42.7, 41.1, 38.0, 24.9, 23.5 and 22.5; LRMS (EI) m/z 582 (30%, M⁺); Found: C, 59.80; H, 5.62; N, 16.75%; Anal. Calc.: C, 59.79; H, 5.71 N, 16.83% for C₂₉H₃₃Cl₂N₇O₂.

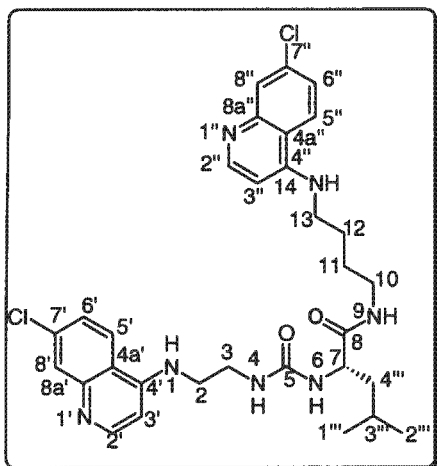
2-[3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-ureido]-4-methyl-pentanoic acid]-[3-(7-Chloro-quinolin-4-ylamino)-propyl]-amide, 4.46 (NAT48)



Off-white powder (131 mg, 41%), m.p. 150-151 °C (from methanol); R_f (MeOH:DCM, 1:9) 0.20; IR $\nu_{\max}$ (CH₂Cl₂)/cm⁻¹ 3281(N-H), 3052 (C-H), 1648 (C=O) and 1583 (C=O); δ_H (400 MHz, DMSO-*d*₆) 8.37 (1H, d, J 5.4, H-2'), 8.36 (1H, d, J 5.4, H-2''), 8.24 (1H, d, J 8.7, H-5'), 8.16 (1H, d, J 8.7, H-5''), 8.07 (1H, bt, J 5.4, NH), 7.77 (2H, d, J 2.1, H-8' and H-8''), 7.34 (2H, dd, J 2.1 and 8.7, H-6' and H-6''), 7.26

(1H, bt, J 5.7, NH), 6.50 (1H, d, J 5.4, H-3'), 6.44 (1H, d, J 5.4, H-3''), 6.31-6.24 (2H, m, (2 x) NH), 4.05 (2H, m, H-7 and NH), 3.29-3.27 (6H, m, CH₂-2, CH₂-3 and CH₂-12), 3.17 (2H, m, CH₂-10), 1.70 (2H, quint, J 7.4, CH₂-11), 1.50-1.20 (3H, m, CH₃-3''' and CH₂-4'''), 0.75 (3H, d, J 6.6, CH₃-1''') and 0.74 (3H, d, J 6.6, CH₃-2''); δ_C (100 MHz, DMSO-*d*₆) 173.8 (C=O), 158.2 (C=O), 151.9 (2C), 151.3, 151.5, 148.1 (2C), 134.0 (2C), 127.1, 126.4, 124.3 (2C), 123.6 (2C), 117.0 (2C), 100.3, 98.6, 51.7, 43.7, 41.4, 41.2, 35.7, 28.4, 27.7, 24.0, 22.7 and 21.2; LRMS (EI) m/z 596 (57%, M⁺); Found: C, 60.50; H, 5.90; N, 16.40%; Anal. Calc.: C, 60.40; H, 5.91; N, 16.44% for C₃₀H₃₅Cl₂N₇O₂.

2-{3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-ureido}-4-methyl-pentanoic acid [4-(7-chloro-quinolin-4-ylamino)-butyl]-amide, 4.47 (NAT46)



Off-white solid (71 mg, 22%), m.p. 146-148 °C (from methanol); R_f (MeOH:DCM, 1:9) 0.15; IR $\nu_{\max}$ (CH₂Cl₂)/cm⁻¹ 3281(N-H), 3052 (C-H), 1648 (C=O) and 1583 (C=O); δ_H (400 MHz, DMSO-*d*₆) 8.36 (2H, d, J 5.6, H-2' and H-2''), 8.27 (1H, d, J 9.2, H-5'), 8.14 (1H, d, J 9.2, H-5''), 7.95 (1H, t, J 5.6, NH), 7.75 (2H, dd, J 9.2 and 2.1, H-6' and H-6''), 7.55 (1H, bt, J 4.0, NH), 7.40 (3H, m, H-8', H-8'' and NH), 6.50 (1H, d, J 5.6, H-3'), 6.44 (1H, d, J 5.6, H-3''), 6.30 (1H, bt, J 5.0, NH), 6.20 (1H, d, J 8.3, NH-6), 5.51 (1H, m, H-7),

3.32-3.0 (8H, m, CH₂-2, CH₂-3, CH₂-10 and CH₂-13), 1.62 (2H, quint, J 6.6, CH₂-12), 1.54-1.44 (3H, m, CH₃-3''' and CH₂-4'''), 1.32 (2H, quint, J 6.6, CH₂-11) and 0.80 (3H, d, J 7.0, CH₃-1''') and 0.78 (3H, d, J 7.0, CH₃-2'''); δ_C (100 MHz, DMSO-*d*₆) 173.2 (C=O), 158.6 (C=O), 151.5, 151.4, 150.8, 150.7, 148.3 (2C), 134.0 (2C), 127.0, 126.8, 124.6, 124.5, 124.3, 124.0, 117.4 (2C), 98.8 (2C), 51.9, 43.9, 42.3, 42.1, 40.3, 38.1, 26.8, 25.2, 24.4, 23.1 and 22.0; LRMS (EI) m/z 611 (50%, M⁺); Found: C, 60.90; H, 6.10; N, 15.96%; Anal. Calc.: C, 60.98; H, 6.11; N, 16.06% for C₃₁H₃₇Cl₂N₇O₂.

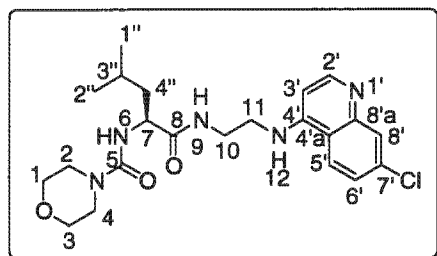
6.0, *NH*), 6.18 (1H, d, *J* 8.5, *NH*-6), 4.13 (1H, m, H-7), 3.28 (6H, m, *CH*₂-2, *CH*₂-3 and *CH*₂-15), 3.00 (2H, m, *CH*₂-10), 1.53-1.37 (3H, m, *CH*^{3'''} and *CH*₂-4'''), 1.34-1.08 (8H, m, *CH*₂-11, *CH*₂-12, *CH*₂-13 and *CH*₂-14), 0.75 (3H, d, *J* 6.6, *CH*₃-1''') and 0.70 (3H, d, *J* 6.6, *CH*₃-2'''); δ_c(100 MHz, DMSO-*d*₆) 173.8 (C=O), 158.2 (C=O), 151.4, 151.3, 150.2 (2C), 148.3, 147.9, 133.9 (2C), 126.5, 126.3, 124.4, 124.2, 123.7, 123.4, 117.5, 117.1, 100.3, 98.6, 51.9, 44.0, 44.5, 42.1, 41.5, 37.1, 28.5, 27.4, 25.9, 25.7, 24.1, 22.6 and 21.5; LRMS (FAB) *m/z* 638 (55%, M⁺); Found: C, 62.00; H, 6.41; N, 15.43%; Anal. Calc.: C, 62.06; H, 6.47; N, 15.35% for C₃₃H₄₁Cl₂N₇O₂.

and 9.0, H-6'), 6.60 (1H, d, J 5.7, H-3'), 4.25 (1H, m, H-7), 4.10 (2H, s, CH₂-10), 3.52-

3.44 (4H, m, CH_2 -2 and CH_2 -3), 2.60-1.44 (3H, m, CH -4'' and CH_2 -3'''), 0.90 (3H, d, J 6.6, CH_3 -1'') and 0.89 (3H, d, J 6.6, CH_3 -2''); δ_{c} (100 MHz, CD_3OD) 176.5 (C=O), 161.1(C=O), 153.5, 151.4, 148.4, 136.9, 126.7, 126.4, 124.5, 118.5, 117.5, 99.6, 53.7, 45.3, 42.6, 39.8, 28.0, 25.9, 23.4 and 22.0; LRMS (EI) m/z 417 (100%, M^+); Found: C, 57.50; H, 6.00; N, 20.01%; Anal. Calc.: C, 57.62; H, 6.04; N, 20.16% for $\text{C}_{20}\text{H}_{25}\text{ClN}_6\text{O}_2$.

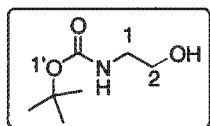
The method employed for the synthesis of **4.52** was that described in general Method B, using 0.82 mmol of the morpholine carboxylic acid **4.32**.

Morpholine-4-carboxylic acid[1-[2-(7-chloro-quinolin-4-ylamino)-ethylcarbamoyl]-3-methyl-butyl]-amide, 4.52

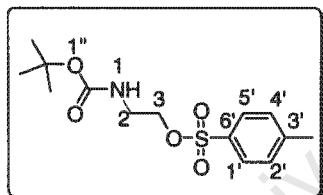


Pale brown powder (200 mg, 54%), m.p. 100-103 °C (from methanol); R_f (MeOH:DCM, 1:9) 0.32; IR ν_{max} (CH_2Cl_2)/ cm^{-1} 3352 (N-H), 3052 (C-H), 1631(C=O) and 1550 (C=O); δ_{H} (400 MHz, CDCl_3) 8.39 (1H, d, J 5.2, H-2'), 8.15 (1H, d, J 9.2, H-5'), 8.04 (1H, bt, J 5.2, NH-12), 7.77 (1H, d, J 2.4, H-8'),

7.42 (1H, dd, J 2.4 and 9.2, H-6'), 7.28 (1H, bt, J 5.2, NH-9), 6.55 (1H, d, J 5.2, H-3'), 6.43 (1H, d, J 7.6, NH-6), 4.14 - 4.08 (1H, m, H-7), 3.47 (4H, t, J 5.6, NCH_2 -2, and NCH_2 -4), 3.88-3.33 (8H, m, OCH_2 -1, OCH_2 -3, CH_2 -10, CH_2 -11), 1.59 -1.36 (3H, CH_2 -4'' and H-3''), 0.82 (3H, d, J 6.4, CH_3 -1'') and 0.78 (3H, d, J 6.4, CH_3 -2''); δ_{c} (100 MHz, CDCl_3) 174.8 (C=O), 158.0 (C=O), 152.5, 150.8, 149.7, 134.1, 128.1, 124.8, 124.6, 118.0, 99.4, 66.6 (2C), 53.5, 44.7 (2C), 43.0, 41.5, 38.0, 25.0, 22.7 and 22.3; LRMS (EI) m/z 448 (20%, M^+); Found: C, 58.80; H, 6.70; N, 15.45%; Anal. Calc.: C, 58.99; H, 6.75 N, 15.63% for $\text{C}_{22}\text{H}_{30}\text{ClN}_5\text{O}_3$.

(2-Hydroxy-ethyl)-carbamic acid tert-butyl ester, 4.37² (NAT35)

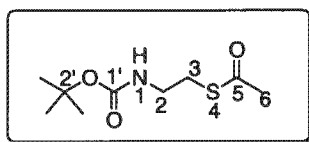
A solution of ethanolamine 4.36 (2.00 g, 33 mmol) in dry DCM (30 mL) was cooled to 0 °C in an ice-water bath. A solution of boc-anhydride (7.15g, 33 mmol) was added *via* an addition funnel over a period of 20 min. The reaction mixture was stirred for an additional 30 min at 0 °C and then for 18 h at 25 °C. TLC analysis indicated complete conversion of starting material to product. The reaction mixture was washed with Na₂CO₃ (20 mL), water (20 mL) dried over MgSO₄ and filtered. The filtrate was concentrated to afford the product 4.37 (5.28g, 100%) as a colourless oil; *R*_f (MeOH:DCM, 1:9) 0.55; δ_H(400 MHz, CDCl₃) 5.02 (1H, bs, N-H), 3.67 (2H, t, *J* 5.2, CH₂-2), 3.26 (2H, q, *J* 5.2, CH₂-1), 2.72 (1H, bs, OH) and 1.43 (9H, s, (3x) (CH₃-1')); δ_C(100 MHz, CDCl₃) 157.5 (C=O), 79.6, 62.4, 43.2 and 28.3 (3C) (CH₃-1').

Toluene-4-sulfonic acid 2-tert-butoxyamino-ethyl ester, 4.38 (NAT36)

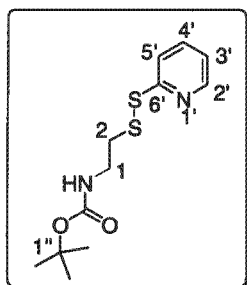
To a suspension of *p*-toluenesulfonylchloride (4.26 g, 22.33 mmol) in DCM (20 mL) at 0 °C, Et₃N (9.42 g, 12.97 mL, 93.05 mmol) was added. After stirring for 5 min, a solution of the alcohol 4.37 (3.00 g, 18.61 mmol) in DCM (20 mL) was added dropwise to give a clear solution which was stirred for an additional 6 h at 0 °C. After this time, the reaction was warmed to 25 °C and stirred at this temperature for 6 h. After complete conversion of starting material to product (TLC), the reaction mixture was quenched with water (100 mL), washed with brine (2 x 100 mL) and water (2 x 100 mL). The organic phase was dried over MgSO₄, filtered and evaporated *in vacuo* to afford a colourless residue which was purified by column chromatography on SiO₂ gel using EtOAc/Hexane (1:9) followed by EtOAc/Hexane (1:4) as eluent to yield the tosylate 4.38 (4.45 g, 76%) as a white solid, m.p. 31-34 °C (from CH₂Cl₂-hexane); *R*_f (EtOAc:Hexane, 1:4) 0.10; IR ν_{max} (CH₂Cl₂)/cm⁻¹ 3320 (N-H), 3052 (C-H) and 1690 (C=O); δ_H(300MHz, CDCl₃) 7.72 (2H, d, *J* 8.4, H-1' and H-5'), 7.28 (2H, d, *J* 8.4, H-2' and H-4'), 4.55 (1H, bs,

NH -1), 4.00 (2H, t, J 5.1, CH_2 -3), 3.31 (2H, q, J 5.1, CH_2 -2), 2.38 (3H, s, CH_3 -3') and 1.34 (9H, s, (3x) (CH_3 -1'')); δ_c (75 MHz, $CDCl_3$) 155.5 (C=O), 145.3, 132.8, 129.9 (2C), 127.9 (2C), 79.6, 69.4, 39.7, 28.1 (3C) (CH_3 -1'') and 21.6 ($ArCH_3$ -3'); LRMS (EI) m/z 315 (8%, M^+); Found: C, 53.41; H, 6.75; N, 4.51; S, 10.15%; Anal. Calc.: C, 53.32; H, 6.71; N, 4.44; S, 10.17% for $C_{14}H_{21}NO_5S$.

Thioacetic acid *S*-(2-*tert*-butoxycarbonylamino-ethyl) ester, 4.39 (NAT37)

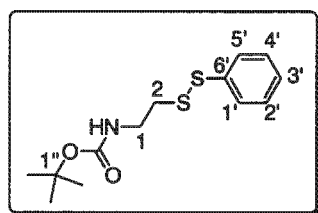


To 20 mL of DMF cooled in an ice-water bath was added (1.08 g, 19.02 mmol) of KOH. After slow addition of thioacetic acid (1.70 g, 22.19 mmol), the mixture was stirred for 15 min. The bath was removed and the mixture stirred until all the KOH dissolved giving an orange solution. The ice bath was returned and a solution of 4.38 (2.00 g, 6.34 mmol) in DMF (15 mL) added dropwise. The reaction was allowed to warm to ambient temperature 12 h. The mixture was then diluted with DCM (300 mL) and 5% aqueous citric acid (75 mL). The organic layer was separated and was washed again with 5% aqueous citric acid (75 mL), saturated $NaHCO_3$ (75 mL) and saturated brine (75 mL). The combined extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford a red brown residue which was chromatographed (SiO_2 gel) using EtOAc/Hexane (1:9) as eluent to give the thioacetate 4.39 (1.00 g, 72%) as a brown oil; R_f (EtOAc:hexane, 1:1) 0.57; IR ν_{max} (CH_2Cl_2)/ cm^{-1} 3444 (N-H), 3052 (C-H), 1707 (C=O) and 1166 (C=S); δ_H (300 MHz, $CDCl_3$) 4.78 (1H, bs, NH -1), 3.29 (2H, q, J 6.8, CH_2 -2), 2.99 (2H, t, J 6.8, CH_2 -3), 2.33 (3H, s, CH_3 -6) and 1.43 (9H, s, (3x) (CH_3 -2')); δ_c (75 MHz, $CDCl_3$) 196.5 (C=O), 157.5 (C=O), 70.3, 62.4, 40.0, 29.3 (CH_3 -6) and 28.2 (3C) (CH_3 -2'); LRMS (EI) m/z 219 (25%, M^+); Found: C, 49.28; H, 7.85; N, 6.42; S, 21.92%; Anal. Calc.: C, 49.29; H, 7.81; N, 6.39; S, 21.89% for $C_9H_{17}NO_3S$.

[2-(Pyridin-2-yldisulfanyl)-ethyl]-carbamic acid tert-butyl ester, 4.40 (NAT38)

A solution of the thioacetate 4.39 (1.00g, 4.56 mmol) in 20 mL of methanol was degassed by subjecting it to a vacuum and nitrogen purge. Addition of 2,2'-dithiodipyridine (1.31 g, 6.00 mmol) was followed by addition of a solution of LiOH·H₂O (0.29 g, 6.84 mmol) in water (5 mL). The reaction mixture was stirred for 4 h at 25 °C after which methanol was removed under reduced pressure. The

residue obtained was diluted with water (50 mL), extracted with EtOAc (3 x 50 mL), dried over MgSO₄ and concentrated *in vacuo*. Purification by column chromatography on SiO₂ gel eluting with EtOAc/Hexane (1:1) afforded 4.40 (1.02g, 78%) as a brown oil; *R_f* (EtOAc:hexane, 1:9) 0.21; IR ν_{max} (CH₂Cl₂)/cm⁻¹ 3431(N-H), 3052 (C-H) and 1576 (C=O); δ_{H} (300 MHz, CDCl₃) 8.44 (1H, d, *J* 4.8, H-2'), 7.57 (2H, m, H-4' and H-5'), 7.04 (1H, m, H-3'), 5.78 (1H, bs, NH), 3.35 (2H, q, *J* 6.0, CH₂-1), 2.86 (2H, t, *J* 6.0, CH₂-2) and 1.34 (9H, s, (3x) (CH₃-1'')); δ_{C} (75 MHz, CDCl₃) 178.0 159.3 (C=O), 149.8, 136.7, 121.0, 120., 69.3, 39.4, 38.9 and 28.4 (3C) (CH₃-1''); LRMS (EI) *m/z* 287 (3%, M⁺); Found: C, 50.28; H, 6.41; N, 9.82; S, 22.35%; Anal. Calc.: C, 50.32; H, 6.33; N, 9.78; S, 22.39% for C₁₂H₁₈N₂O₂S₂.

(2-Phenyldisulfanyl-ethyl)-carbamic acid tert-butyl ester, 4.41 (NAT41)

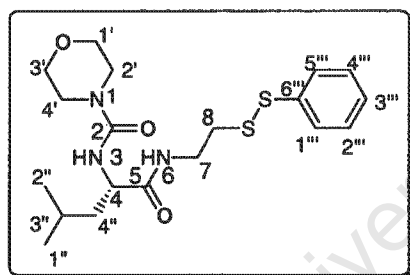
Thiophenol (0.58 g, 0.54 mL, 5.24 mmol) was added to a solution of 4.40 (1.00 g, 3.49 mmol) in DCM (20 mL). The resultant reaction mixture was stirred for 3 days at 25 °C. TLC analysis indicated complete consumption of starting material. The reaction mixture was diluted with EtOAc (50

mL), washed with saturated aqueous NaHCO₃ (3 x 50 mL) and brine (50 mL). The organic solution was dried over Na₂SO₄, filtered and concentrated *in vacuo* to give a residue which was purified by column chromatography on SiO₂ gel using EtOAc/Hexane

(1:9) as eluent. Purification of the residue yielded the phenyl disulfide **4.41** (0.54 g, 54%) as white crystals, m.p. 51-54 °C (from CH₂Cl₂-hexane); *R*_f (EtOAc:Hexane, 1:1) 0.78; IR $\nu_{\max}$ (CH₂Cl₂)/cm⁻¹ 3268 (N-H), 2973 (C-H) and 1530 (C=O); δ_{H} (300 MHz, CDCl₃) 7.55-7.22 (5H, m, ArH), 4.78 (1H, bs, NH), 3.14 (2H, q, *J* 5.6, CH₂-1), 2.83 (2H, t, *J* 5.6, CH₂-2), and 1.43 (9H, s, (3x) (CH₃-1'')); δ_{C} (75 MHz, CDCl₃) 159.3 (C=O), 137.0, 129.1 (2C), 127.9 (2C), 127.1 (C-3'), 69.1, 56.0, 38.4 and 28.2 (3C) (CH₃-1''); LRMS (EI) *m/z* 285 (30%, M⁺); Found: C, 54.29; H, 6.57; N, 5.02; S, 22.29%; Anal. Calc.: C, 54.70; H, 6.71; N, 4.91; S, 22.47% for C₁₃H₁₉NO₂S₂.

Under similar experimental conditions described in General Method B, **4.53** were prepared, using 0.82 mmol of the morpholine carboxylic acid **4.32**.

Morpholine-4-carboxylic-acid[3-methyl-1-(2-phenyldisulfanyl-ethylcarbamoyl)-butyl]-amide, **4.53 (NAT42)**



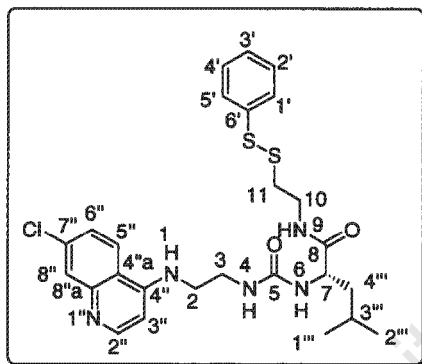
N-deprotection was accomplished by dissolving the phenyl disulfide **4.41** (0.30 g, 1.05 mmol) in 20% trifluoroacetic acid in DCM (15 mL). After stirring for 45 min at 25 °C, TLC analysis indicated the formation of free amine, *R*_f (MeOH:DCM, 1:9) 0.1. The reaction mixture was concentrated *in vacuo*, diluted with

EtOAc (30 ml) and washed with 10% NaHCO₃ (50 mL), dried over MgSO₄ and concentrated *in vacuo*. The crude amine was used in the next reaction step without further purification. As mentioned above, conditions employed for the preparation of **4.53** were those described in General Method B to yield the *morpholine disulfide*, **4.53** as a yellow solid (150 mg, 44%), m.p. 121-125 °C (from CH₂Cl₂-hexane); *R*_f(MeOH:DCM, 1:19) 0.70; IR $\nu_{\max}$ (CH₂Cl₂)/cm⁻¹ 3431(N-H), 3052 (C-H), 1658 (C=O) and 1583 (C=O); δ_{H} (300MHz, CDCl₃) 7.45-7.15 (5H, m, ArH''), 6.56 (1H, bt, *J* 5.1, NH-6), 4.94 (1H, d, *J* 7.8, NH-3), 4.26 - 4.19 (1H, m, H-4), 3.60 (4H, t, *J* 5.1, NCH₂-2' and NCH₂-4'), 3.52-3.40 (2H, m, CH₂-7), 3.30-3.26 (4H, t, *J* 5.1, OCH₂-1' and OCH₂-3'), 2.81-2.75 (2H, m, CH₂-8), 1.66-1.39 (3H, m, CH₂-4'' and H-3''), 0.88 (3H, d, *J* 6.4, CH₃-1'')

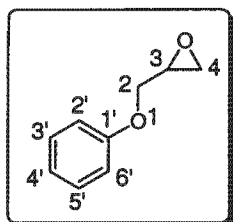
and 0.85 (3H, d, J 6.4, $\text{CH}_3\text{-2''}$); δ_{C} (75 MHz, CDCl_3) 174.0 (C=O), 164.2 (C=O), 130.4, 129.2 (2C), 128.0 (2C), 127.3, 66.4 (2C), 52.9, 44.0 (2C), 41.6, 37.9, 26.4, 24.9, 23.0 and 22.9; LRMS (EI) m/z 412.0 (5%, M^+); Found: C, 55.45; H, 7.08; N, 10.21; S, 15.50%; Anal. Calc.: C, 55.45; H, 7.10; N, 10.21; S, 15.58% for $\text{C}_{19}\text{H}_{29}\text{N}_3\text{O}_3\text{S}_2$.

Under similar experimental conditions described in General Method B, **4.50** were prepared, using 0.53 mmol of the quinoline carboxylic acid **4.20**.

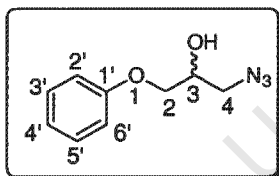
2-[3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-ureido]-4-methyl-pentanoicacid (phenyldisulfanyl-ethyl)-amide, 4.50 (NAT43)



Off- white powder (100 mg, 35%), m.p. 99-102 °C (from CH_2Cl_2 -hexane); R_f (MeOH:DCM, 1:19) 0.55; IR ν_{max} (CH_2Cl_2)/ cm^{-1} 3281(N-H), 3052 (C-H), 1648 (C=O) and 1583 (C=O); δ_{H} (400 MHz, CD_3OD) 8.33 (1H, d, J 5.6, H-2''), 8.04 (1H, J 8.8, H-5''), 7.77 (1H, d, J 2.0, H-8''), 7.49 (1H, dd, J 8.8 and 2.0, H-6''), 7.42 - 7.19 (5H, m, ArH), 6.55 (1H, d, J 5.6, H-3''), 4.23 - 4.19 (1H, m, H-7), 3.54 - 3.35 (6H, m, $\text{CH}_2\text{-2}$, $\text{CH}_2\text{-3}$ and $\text{CH}_2\text{-10}$), 2.77 (2H, t, J 6.8, $\text{CH}_2\text{-11}$), 1.65 -1.41 (3H, m, H-3''' and $\text{CH}_2\text{-4''}$), 0.91 (3H, d, J 6.4, $\text{CH}_3\text{-1'''}$) and 0.88 (3H, d, J 6.4, $\text{CH}_3\text{-2'''}$); δ_{C} (100 MHz, CD_3OD) 175.0 (C=O), 160.0 (C=O), 152.2, 150.3, 147.3, 139.2, 137.03, 137.0 (2C), 135.7, 129.1 (2C), 128.9, 127.7, 127.0, 113.4, 98.3, 52.8, 44.4, 41.6, 38.6, 38.2, 37.2, 24.8, 22.2 and 20.9; LRMS (EI) m/z 546 (75%, M^+); Found: C, 57.10; H, 5.87; N, 12.85, S, 11.70%; Anal. Calc.: C, 57.18; H, 5.91; N, 12.82; S, 11.74% for $\text{C}_{26}\text{H}_{32}\text{ClN}_5\text{O}_2\text{S}_2$.

2-Phenoxyethyl-oxirane, 4.43 (NAT51)

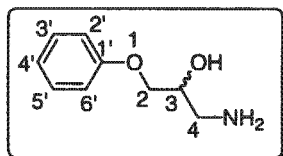
Epichlorohydrin (2.54 g, 17 mL, 27.50 mmol) was added to phenol (2.00 g, 25.00 mmol) and refluxed at 60 °C for 12 h, after which excess epichlorohydrin was removed under reduced pressure to yield a colourless oil. The oily residue was dissolved in DCM (100 mL), washed with 1M NaOH (3 x 50 mL), water (50 mL), dried over Na₂SO₄ and concentrated *in vacuo* to yield a colourless oil as the product, **4.43** (2.90 g, 91%), *R_f* (MeOH:DCM, 1:49) 0.40; IR $\nu_{\max}$ (CH₂Cl₂)/cm⁻¹ 3052 (C-H), 1045 (COC); δ_{H} (400 MHz, CDCl₃) 7.31-7.26 (2H, m, *ArH*), 6.99-6.92 (3H, m, *ArH*), 4.22 (1H, dd, *J* 3.3 and 11.2, H-2 α), 3.97 (1H, dd, *J* 5.5 and 11.2, H-2 β), 3.36 (1H, m, H-3), 2.90 (1H, dd, *J* 4.0 and 4.9, H-4 α) and 2.75 (1H, dd, *J* 2.6 and 4.9, H-4 β); δ_{C} (100 MHz; DMSO-*d*₆) 158.5, 129.5 (2C), 121.2, 114.2 (2C), 68.7, 50.1 and 44.7; LRMS (EI) *m/z* 150 (5%, M⁺); Found: C, 71.84; H, 6.76%; Anal. Calc.: C, 71.98; H, 6.71 for C₉H₁₀O₂.

1-Azido-3-phenoxy-propan-2-ol, 4.44 (NAT54)

NaN₃ (1.30 g, 19.98 mmol), NH₄Cl (0.64 g, 11.99 mmol) and epoxide **4.43** (0.60 g, 4.00 mmol) in 28 mL of 8:1 MeOH:H₂O were heated at 80 °C for 21 h. The reaction mixture was cooled to room temperature and partitioned between diethyl ether and 10% aqueous NaHCO₃. The aqueous phase was extracted with diethyl ether (3 x 50 mL) and the combined extracts were washed with brine (50 mL) and dried over Na₂SO₄. The solvent was removed under reduced pressure to afford the azide, **4.44** as a colourless oil (0.75 g, 97%), *R_f* (DCM) 0.30; IR $\nu_{\max}$ (CH₂Cl₂)/cm⁻¹ 3453 (OH), 3052 (C-H); δ_{H} (400 MHz, CDCl₃) 7.33-7.30 (2H, m, *ArH*), 6.99-6.90 (3H, m, *ArH*), 4.18 (1H, m, H-3), 4.01 (2H, m, CH₂-2), 3.52 (2H, m, CH₂-4) and 2.38 (1H, bs, OH); δ_{C} (100 MHz, CDCl₃) 158.5, 129.8 (2C), 121.7, 114.8 (2C), 70.0, 69.3 and 53.7; LRMS (EI) *m/z* 193 (50%, M⁺);

Found: C, 55.91; H, 5.79; N, 21.82%; Anal. Calc.: C, 55.95; H, 5.74; N, 21.75% for $C_9H_{11}N_3O_2$.

1-Amino-2-phenoxy-propan-2-ol, 4.26 (NAT55)

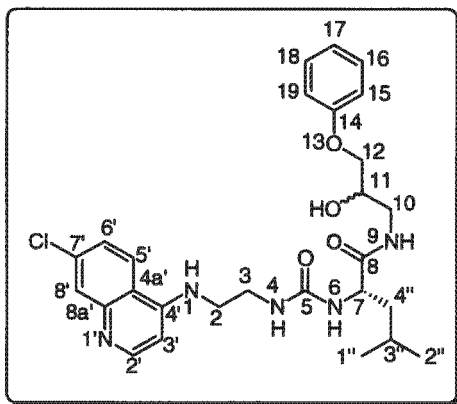


To a stirred solution of azide **4.44** (0.50 g, 2.59 mmol) in THF:H₂O (20:0.1 mL) was added Ph₃P (1.36 g, 5.18 mmol) at room temperature. The reaction mixture was heated at 65 °C for 3 h. The solvent was removed *in vacuo* to give a residue

which was purified by column chromatography on SiO₂ gel using 20% MeOH:DCM as eluent to yield the pure product, **4.26** as a white solid, (0.40 g, 93%), m.p. 82-85 °C; *R*_f (MeOH:DCM, 2:8) 0.12; IR $\nu_{\max}$ (CH₂Cl₂)/cm⁻¹ 3450 (O-H), 3281(N-H), 3052 (C-H); δ_H (400 MHz, CDCl₃) 7.30-7.26 (2H, m, ArH), 6.98-6.90 (3H, m, ArH), 4.00 (3H, m, H-3 and CH₂-4), 2.98 (2H, m, CH₂-2) and 2.00 (3H, s, OH and NH₂); δ_C (100 MHz, CDCl₃) 158.6, 129.5 (2C), 121.1, 114.6 (2C), 70.5, 70.0 and 44.0; LRMS (EI) *m/z* 167 (100%, M⁺); Found: C, 64.60; H, 7.90; N, 8.35%; Anal. Calc.: C, 64.65; H, 7.84; N, 8.38% for $C_9H_{13}NO_2$.

Under similar experimental conditions described in General Method B, compounds **4.51** and **4.53** were prepared, using 0.53 mmol of the quinoline carboxylic acid **4.20**.

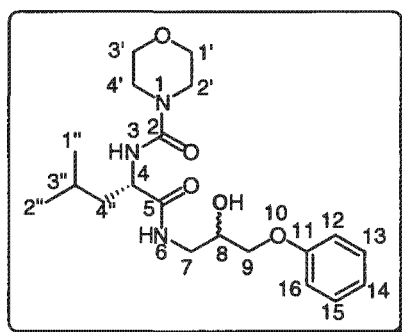
2-{3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-ureido}-4-methyl-pentanoic acid (2-hydroxy-3-phenoxy-propyl)-amide, 4.51 (NAT56)



Yellow powder (0.24 g, 89%), m.p. 139-142 °C (from CH₂Cl₂-hexane); *R*_f (MeOH:DCM, 1:9) 0.34; IR $\nu_{\max}$ (CH₂Cl₂)/cm⁻¹ 3525 (OH), 3281(N-H), 3052 (C-H), 1648 (C=O) and 1583 (C=O); δ_H (300 MHz, CD₃OD) 8.33 (1H, d, *J* 5.8, H-2'), 8.23 (1H, d, *J* 8.9, H-5'), 7.77 (1H, d, *J* 2.1, H-8'), 7.42 (1H, dd,

J 2.1 and 8.9, H-6'), 7.22-7.23 (2H, m, ArH), 6.91-6.89 (3H, m, ArH), 6.54 (1H, d, J 5.8, H-3'), 4.25 (1H, m, H-7), 4.10 (1H, m, H-11), 3.92 (2H, m, CH₂-12), 3.53-3.43 (6H, m, CH₂-10, CH₂-2 and CH₂-3), 1.75-1.42 (3H, m, CH₄''' and CH₂-3''), 0.90 (3H, d, J 6.0, CH₃-1'') and 0.88 (3H, d, J 6.0, CH₃-2''); δ_c (75 MHz, CD₃OD) 176.6 (C=O), 160.3 (C=O), 152.1, 151.6 (2C), 149.2, 136.8, 130.4 (2C), 126.9, 126.4, 124.4, 121.9, 118.5, 115.7 (2C), 99.5, 71.1, 69.8, 54.1, 45.4, 43.5, 42.7, 39.7, 25.9, 23.4 and 22.0; LRMS (EI) m/z 528 (30%, M⁺); Found: C, 61.43; H, 6.55; N, 13.28%; Anal. Calc.: C, 61.41; H, 6.49; N, 13.26% for C₂₇H₃₄ClN₅O₄.

Morpholine-4-carboxylic acid [1-(2-hydroxy-3-phenoxy-propylcarbamoyl)-3-methyl-butyl]-amide, 4.54 (NAT60)



Colourless oil (0.15g, 71%); R_f (MeOH:DCM, 1:9) 0.34; IR ν_{max} (CH₂Cl₂)/cm⁻¹ 3500 (OH), 3270 (N-H), 3052 (C-H), 1648 (C=O) and 1583 (C=O); δ_H (300 MHz, CDCl₃) 7.29-7.25 (2H, m, ArH), 6.97-6.88 (3H, m, ArH), 5.04 (1H, bs, NH), 4.31 (1H, m, H-4), 4.12 (1H, m, H-8), 3.96 (2H, m, CH₂-9), 3.64 (4H, t, J 4.8, NCH₂-2', and NCH₂-4'), 3.38-3.28 (6H, m, OCH₂-1', OCH₂-3' and CH₂-7), 2.19 (2H, bs, NH and OH), 1.72-

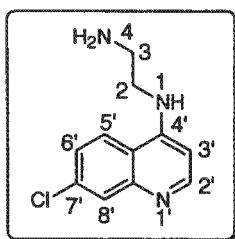
1.53 (3H, m, CH₄'' and CH₂-3''), 0.94 (3H, d, J 5.7, CH₃-1'') and 0.93 (3H, d, J 5.7, CH₃-2''); δ_c (75 MHz, CDCl₃) 174.8 (C=O), 158.2 (C=O), 130.4, 129.8 (2C), 121.7, 114.8 (2C), 69.4, 66.6 (2C), 53.5, 44.7 (2C), 43.0, 41.5, 38.0, 25.0, 22.7 and 22.3; LRMS (EI) m/z 393 (30%, M⁺); Found: C, 61.12; H, 7.81; N, 10.75%; Anal. Calc.: C, 61.05; H, 7.94; N, 10.68% for C₂₀H₃₁N₃O₅.

C. General Method for the preparation of compounds 4.21-4.23

A mixture of 4,7-dichloroquinoline (1.00 g, 5.05 mmol) and diamine (35.35 mmol) was stirred under reflux at 80 °C for 1 h and subsequently at 135-145 °C for 3 h. After this time, the reaction mixture was cooled to room temperature. NaOH (1M, 20 mL) was added and the organic product was extracted with EtOAc (3 x 100 mL), washed with

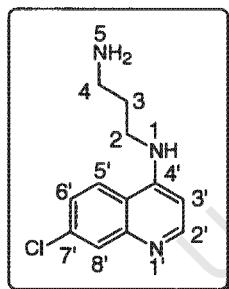
water (3 X 50 mL) and dried over MgSO_4 . After filtration the solvent was evaporated *in vacuo* to yield the products in good yield.

N1-(7-Chloro-quinolin-4-yl)-ethane-1,2-diamine, 4.21



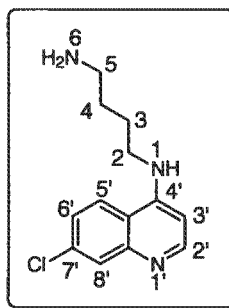
Pale yellow solid (1.05 g, 94%), m.p. 92-95 °C, lit.³ m.p. 94-97 °C (from methanol); R_f (MeOH:DCM, 1:9) 0.2; δ_H (400 MHz, $\text{DMSO}-d_6$) 8.37 (1H, d, J 5.6, H-2'), 8.26 (1H, d, J 9.2, H-5'), 7.76 (1H, d, J 2.4, H-8'), 7.41 (1H, dd, J 2.4 and 9.2, H-6'), 7.19 (1H, bs, NH-1), 6.47 (1H, d, J 5.6, H-3'), 3.24 (2H, q, J 6.4, CH_2 -2), 3.11 (2H, q, J 6.4, CH_2 -3) and 1.39 (2H, bs, NH-4); δ_C (100 MHz, $\text{DMSO}-d_6$) 152.0, 151.4, 148.7, 134.5, 128.7, 125.2, 121.1, 116.8, 99.2, 44.2 and 40.2.

N1-(7-Chloro-quinolin-4-yl)-propane-1,3-diamine, 4.22



Pale yellow solid (1.00 g, 83%), m.p. 58-59 °C, lit.³ m.p. 55-57 °C (from methanol); R_f (MeOH:DCM, 1:9) 0.2; δ_H (400 MHz, $\text{DMSO}-d_6$) 8.65 (1H, d, J 5.2, H-2'), 7.95 (1H, d, J 2.0, H-8'), 7.72 (1H, d, J 8.8, H-5'), 7.35 (1H, dd, J 2.0 and 8.8, H-6'), 6.40 (1H, d, J 5.2, H-3'), 5.77 (1H, bs, NH-1), 3.40 (2H, t, J 6.6, CH_2 -2), 2.71 (2H, t, J 6.6, CH_2 -4), 1.72 (2H, quint, J 6.6, CH_2 -3) and 1.39 (2H, bs, NH-5); δ_C (100 MHz, $\text{DMSO}-d_6$) 151.9, 150.1, 149.1, 133.3, 127.5, 124.0, 123.9, 117.4, 98.5, 40.6, 40.3 and 40.2

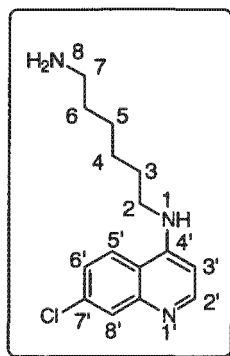
N1-(7-Chloro-quinolin-4-yl)-butane-1,4-diamine, 4.23



A pale yellow solid (1.20 g, 95%), m.p. 76-78 °C, lit.³ m.p. 75-80 °C (from methanol); R_f (MeOH:DCM, 1:9) 0.2; δ_H (300 MHz, CD_3OD) 8.39 (1H, d, J 5.4, H-2'), 8.28 (1H, d, J 8.7, H-5'), 7.78 (1H, d, J 2.1,

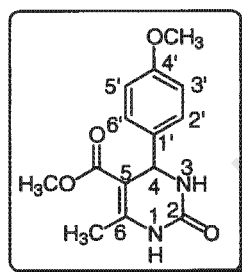
H-8'), 7.42 (1H, dd, J 2.1 and 8.7, H-6'), 6.47 (1H, d, J 5.4, H-3'), 3.29 (2H, t, J 6.0, CH_2 -2), 2.61 (2H, t, J 6.0, CH_2 -5), 1.74- 1.64 (2H, m, CH_2 -3) and 1.52-1.43 (2H, m, CH_2 -4); δ_c (75 MHz, CD_3OD) 151.9, 150.2, 149.1, 133.3, 127.4, 124.1, 123.9, 117.5, 98.5, 42.3, 41.2, 30.5 and 25.3.

N1-(7-Chloro-quinolin-4-yl)-hexane-1,6-diamine, 4.24



Pale yellow solid (1.30 g, 93%), m.p. 139-141 °C, lit.³ m.p. 137-138 °C (from methanol); R_f (MeOH:DCM, 1:9) 0.2; δ_H (300 MHz, CD_3OD) 8.35 (1H, d, J 5.7, H-2'), 8.29 (1H, d, J 9.0, H-5'), 7.78 (1H, d, J 2.4, H-8'), 7.43 (1H, dd, J 2.4 and 9.0, H-6'), 6.47 (1H, d, J 5.7, H-3'), 3.26 (2H, t, J 6.0, CH_2 -2), 2.51 (2H, t, J 6.0, CH_2 -7), 1.74-1.64 (2H, m, CH_2 -3) and 1.45-1.38 (6H, m, CH_2 -4, CH_2 -5 and CH_2 -6); δ_c (75 MHz, CD_3OD) 151.9, 150.1, 149.1, 133.3, 127.4, 124.1, 123.9, 117.5, 98.5, 42.3, 41.5, 33.1, 27.8, 26.6 and 26.2.

4-(4-Methoxy-phenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylicacid methyl ester, 4.98 (NAT75)



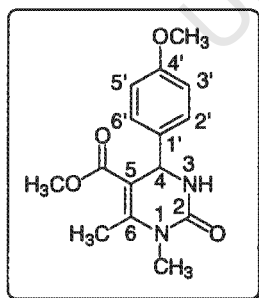
A solution of methyl acetoacetate 4.97 (1.16 g, 10.00 mmol), 4-methoxybenzaldehyde 4.96 (1.36g, 10 mmol) and urea 4.66 (1.80 g, 30.00 mmol) in ethanol (15 mL) was heated under reflux in the presence of $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ (931 mg, 25.00 mmol %) for 3 h. After complete conversion of starting material to product (monitored by TLC), the reaction mixture was cooled to ambient temperature, poured into crushed ice (30 g) and stirred for 15 min which resulted in the formation of a white precipitate. The precipitate was filtered by suction, washed with ice-cold water (50 mL) and recrystallized from hot ethanol to afford the pure product, 4.98 as white needles (2.62g, 94%), m.p. 202-204 °C (lit.⁴ m.p. 201-203 °C); δ_H (400 MHz, CD_3OD) 7.21 (2H, d, J 8.6, H-2' and H-6'), 6.86 (2H, d, J 8.6, H-3' and H-5'), 5.25 (1H, s, H-4), 3.79 (3H, s, OCOCH_3), 3.75 (3H, s, ArOCH_3) and 2.32 (3H, s, 6- CH_3); δ_c (100 MHz, CD_3OD) 166.9

(C=O), 159.5, 154.0 (C=O), 147.5, 136.7, 127.6 (2C), 113.8 (2C), 101.1, 54.5, 48.7, 50.3 and 16.9.

4-(4-Methoxy-phenyl)-1, 6-dimethyl-2-oxo-1,2,3, 4-tetrahydro-pyrimidine-5-carboxylic acid, 4.102 and **4-(4-Methoxy-phenyl)-1,3,6-trimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester, 4.103**

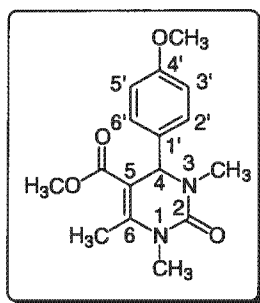
To a suspension of a 60% oil dispersion of NaH (0.478 g (60%), 11.99 mmol) in DMF (10 mL) at 0 °C was added a solution of **4.98** (3.0 g, 10.90 mmol) in DMF (5 mL) at 0 °C. After the mixture was stirred for 30 min at 0 °C, a solution of methyl iodide (1.70 g, 11.99 mmol) in DMF (1 mL) was added to the reaction mixture. The reaction mixture was stirred at 25 °C for 45 min. TLC analysis indicated complete conversion of starting material to product. The reaction mixture was diluted with water (50 mL) and extracted with EtOAc (3 x 50 mL). The organic extracts were combined, washed with brine (50 mL), water (5 x 50 mL), dried over MgSO₄, filtered and concentrated *in vacuo* to yield a white residue. The desired pure product, **4.102** and a byproduct, **4.103** were separated by column chromatography on SiO₂ gel using Et₂O/DCM (1:9) as eluent.

4-(4-Methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid, 4.102 (NAT79)



White solid (0.40 g, 76%), m.p. 149-151 °C (from CH₂Cl₂-hexane); *R_f* (Et₂O:DCM, 1:9) 0.56; IR *v*_{max} (CH₂Cl₂)/cm⁻¹ 3300 (N-H), 3052 (C-H), 1696 (C=O) and 1628 (C=O); δ_H(400 MHz, CDCl₃) 7.18 (2H, d, *J* 8.8, H-2' and H-6'), 6.82 (2H, d, *J* 8.8, H-3' and H-5'), 5.60 (1H, s, NH), 5.38 (1H, s, H-4), 3.77 (3H, s, OCOCCH₃), 3.64 (3H, s, ArOCCH₃), 3.21 (3H, s, NCH₃-1), and 2.50 (3H, s, CH₃-6); δ_C(100 MHz, CDCl₃) 167.0 (C=O), 159.0, 154.0 (C=O), 149.5, 135.0, 127.5 (2C), 114.3 (2C), 11.0, 55.5, 53.5, 51.5, 30.5 and 16.8; LRMS (EI) *m/z* 290 (60%, M⁺); Found: C, 62.15; H, 6.16; N, 9.57%; Anal. Calc.: C, 62.06; H, 6.25; N, 9.65% for C₁₅H₁₈N₂O₄.

4-(4-Methoxy-phenyl)-1,3,6-trimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester, 4.103 (NAT78)



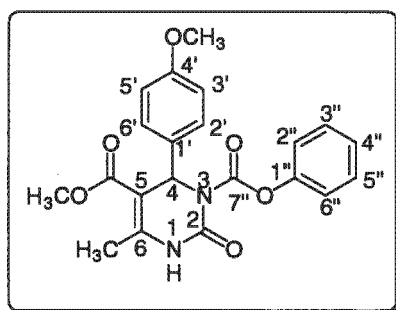
White solid (0.19 g, 6%), m.p. 86-87 °C (from CH₂Cl₂-hexane); *R*_f (Et₂O:DCM, 1:9) 0.18; IR ν_{max} (CH₂Cl₂)/cm⁻¹ 3398 (N-H), 3052 (C-H), 1778 (C=O) and 1638 (C=O); δ_{H} (400 MHz, CDCl₃) 7.18 (2H, d, *J* 8.8, H-2' and H-6'), 6.82 (2H, d, *J* 8.8, H-3' and H-5'), 5.20 (1H, s, H-4), 3.78 (3H, s, OCOC*H*₃), 3.67 (3H, s, ArOC*H*₃), 3.26 (3H, s, NC*H*₃-1), 2.90 (3H, s, NC*H*₃-3) and 2.73 (3H, s, C*H*₃-6); δ_{C} (100 MHz, CDCl₃) 167.0 (C=O), 159.0, 154.0 (C=O), 149.5, 135.0, 127.9 (2C), 114.2 (2C), 110.0, 55.5, 53.5, 51.5, 32.0, 30.0 and 16.8; LRMS (EI) *m/z* 304 (75%, M⁺); Found: C, 63.25; H, 6.58; N, 9.15%; Anal. Calc.: C, 63.14; H, 6.62; N, 9.20% for C₁₆H₂₀N₂O₄.

D. General Method for the preparation of compounds 4.99 and 4.104

Phenyl chloroformate (10.28 g, 8.293 mL, 65.67 mmol) was added dropwise to a mixture of DHPM, 4.102 (1.90 g, 6.58 mmol) and a 60 % oil suspension of NaH (2.89 g (60%), 72.24 mmol) in THF (100 mL) at 0 °C. The mixture was allowed to stir for 12 h at 60 °C. Thereafter, excess NaH was quenched by adding water drop-wise until effervescence stopped. The product was then extracted with EtOAc (3 x 100 mL), washed with water (3 x 50 mL), dried over anhydrous MgSO₄, and the solvent was evaporated under vacuum. The product was purified on a silica gel column using a mobile phase of 10-50 % EtOAc in hexane.

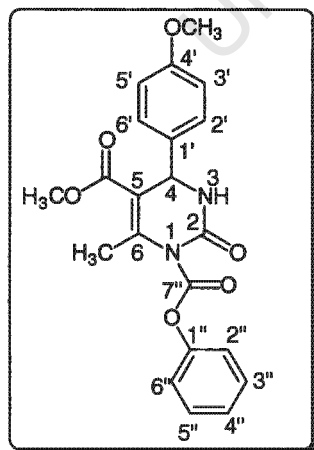
Under similar experimental conditions described in the General Method D, 4.99 and a byproduct 4.100 were prepared, using 6.88 mmol of the DHPM, 4.98.

6-(4-Methoxy-phenyl)-4-methyl-2-oxo-3,6-dihydro-2H-pyrimidine-1,5-dicarboxylic acid 5-methyl ester 1-phenyl ester, 4.99 (NAT76)



Light yellow solid (2.34 g, 86%), m.p. 176-178 °C (from CH₂Cl₂-hexane); *R*_f (MeOH:DCM, 1:19) 0.52; IR *v*_{max} (CH₂Cl₂)/cm⁻¹; 3687 (N-H), 1780 (C=O), 1734 (C=O) and 1638 (C=O); δ_{H} (400 MHz, CDCl₃) 8.60 (1H, bs, *NH*), 7.42-6.87 (9H, m, (4x) *ArH* and (5x) *ArH'*), 6.45 (1H, s, H-4), 3.80 (6H, s, OCOC*H*₃ and ArOC*H*₃) and 2.68 (3H, s, C*H*₃-6); δ_{C} (100 MHz, CDCl₃) 164.9 (C=O), 159.6, 151.9 (C=O), 150.7 (C=O), 149.8, 145.0, 131.5, 129.4 (2C), 128.3 (2C), 126.2, 121.4 (2C), 114.0 (2C), 105.6, 56.6, 55.3, 53.4 and 18.0; HRMS (FAB) Found *m/z* 396.13213 [*M*+1]⁺, C₂₁H₂₀N₂O₆ requires 396.13214].

4-(4-methoxy-phenyl)-6-methyl-2-oxo-3,4-dihydro-2H-pyrimidine-1,5-dicarboxylic acid 5-methyl ester 1-phenyl ester, 4.100 (NAT77)

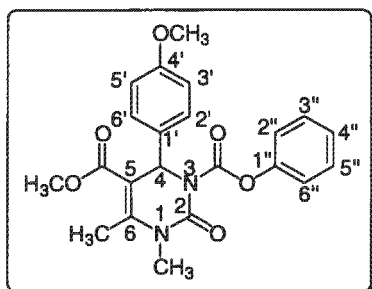


A yellow solid (0.1 g, 4%), m.p. 112-116 °C; (from CH₂Cl₂-hexane); *R*_f (MeOH:DCM, 1:19) 0.50; IR *v*_{max} (CH₂Cl₂)/cm⁻¹; 3687 (N-H), 1780 (C=O), 1734 (C=O) and 1638 (C=O); δ_{H} (400 MHz, CDCl₃) 7.60 (1H, bs, *NH*), 7.41-6.80 (9H, m, (4x) *ArH* and (5x) *ArH'*), 6.40 (1H, s, H-4), 3.78 (3H, s, ArOC*H*₃), 3.71 (3H, s, OCOC*H*₃) and 2.40 (3H, s, C*H*₃-6); δ_{C} (100 MHz, CDCl₃) 165.4 (C=O), 159.5, 151.8 (C=O), 150.6, 150.3 (C=O), 145.8, 131.3, 29.4 (2C), 128.2 (2C), 126.2, 121.4 (2C), 114.0 (2C), 105.1, 56.4, 55.2, 51.5 and 14.0; HRMS (FAB) Found *m/z* 396.13213 [*M*+1]⁺, C₂₁H₂₀N₂O₆

requires 396.13214.

The conditions employed for the preparation of compounds 4.104 were those described in General Method A, using 6.58 mmol DHPM, 4.102.

6-(4-Methoxy-phenyl)-3,4-dimethyl-2-oxo-3,6-dihydro-2H-pyrimidine-1,5-icarboxylic acid 5-methyl ester 1-phenyl ester, 4.104



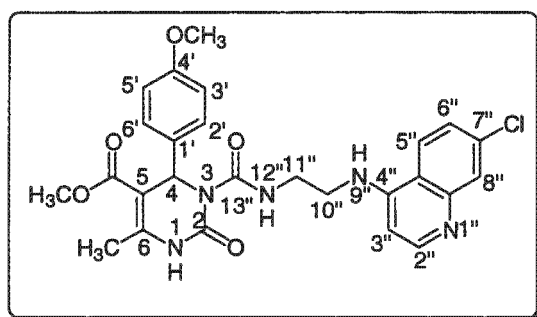
White foam (2.30 g, 85%), m.p. 176-178 °C (from CH₂Cl₂-hexane); *R*_f (EtOAc:Hexane, 1:4) 0.12; IR ν_{max} (CH₂Cl₂)/cm⁻¹ 3398 (N-H), 3052 (C-H), 1778 (C=O), 1730 (C=O) and 1648 (C=O); δ_{H} (400 MHz, CDCl₃) 7.40 (2H, d, *J* 8.7, H-2' and H-6'), 7.25-7.22 (5H, m, ArH'), 6.83 (2H, d, *J* 8.7, H-3' and H-5'), 6.40 (1H, s, H-4), 3.78 (3H, s, OCOCH₃), 3.74 (3H, s, ArOCH₃), 3.23 (3H, s, NCH₃-1) and 2.59 (3H, s, CH₃-6); δ_{C} (100 MHz, CDCl₃) 166.0 (C=O), 160.0, 153.0 (C=O), 151.0 (C=O), 150.3, 149.4, 131.2, 129.6 (2C), 128.0 (2C), 126.3, 121.7 (2C), 114.2 (2C), 109.5, 55.5, 55.4, 52.0, 31.5 and 16.5; LRMS (EI) *m/z* 410 (25%, M⁺); Found: C, 64.48; H, 5.31; N, 6.40%; Anal. Calc.: C, 64.38; H, 5.40; N, 6.38% for C₂₂H₂₂N₂O₆.

E. General Method for the preparation of compounds 4.101 ((NAT145), 4.105-4.108 (NAT1-4))

To a stirred mixture of 4.99 (0.20 g, 0.50 mmol) and K₂CO₃ in THF (10 mL) at 25 °C, under an argon atmosphere was added a solution of the diamine, 4.21 (0.50 mmol) in THF (15 mL) and stirring was continued for 2 h. TLC analysis indicated complete conversion of starting material to product. The solvent was removed under reduced pressure and the residue obtained was redissolved in DCM (50 mL). The solution was washed with 5% NaHCO₃ (3 x 25 mL), brine (50 mL), dried over MgSO₄ and filtered. The solvent was reduced in vacuo and the residue obtained was purified by column chromatography using MeOH: DCM (1:9) as eluent to give the desired product.

The conditions employed for the preparation of compound 4.101 were those described in General Method E, using 0.50 mmol of 4.99.

3-[2-(7-Chloro-quinolin-4-ylamino)-ethylcarbamoyl]-4-(4-methoxy-phenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester, 4.101 (NAT145)

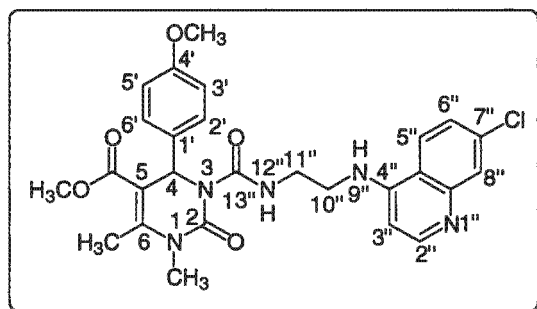


A white solid (30 mg, 12%), m.p. 72-74 °C (from CH₂Cl₂-hexane); *R*_f (MeOH:DCM, 1:9) 0.31; IR *v*_{max} (CH₂Cl₂)/cm⁻¹ 3409 (N-H), 1750 (C=O), 1703 (C=O) and 1638 (C=O); δ _H(400 MHz, CDCl₃) 9.26 (1H, t, *J* 5.4, NH-12''), 8.45 (1H, d, *J* 5.4, H-2''), 7.93 (1H, d, *J* 2.0, H-8''), 7.68 (1H, d, *J* 9.0, H-5''), 7.30

(1H, dd, *J* 2.0 and 9.0, H-6''), 7.22 (2H, d, *J* 8.8, H-2' and H-6'), 7.08 (2H, d, *J* 8.8, H-3' and H-5'), 6.74 (1H, s, H-4), 6.49 (1H, s, NH-1), 6.27 (1H, d, *J* 5.4, H-3''), 5.90 (1H, bt, *J* 5.4, NH-9''), 3.75 (3H, s, OCOCH₃), 3.65 (3H, s, ArOCH₃), 3.51 (2H, q, *J* 5.4, CH₂-11'), 3.41 (2H, q, *J* 5.4, CH₂-10'') and 2.38 (3H, s, CH₃-6); δ _C(100 MHz, CDCl₃) 165.3 (C=O), 159.3 (C=O), 154.1, 151.5 (C=O), 150.2, 148.5, 144.5, 135.0, 132.2, 129.4, 127.8 (2C) 125.8, 125.4, 121.9, 121.5, 113.9 (2C), 105.0, 98.3, 55.1, 53.8, 51.7, 45.4, 39.8, and 17.9; HRMS (FAB) Found *m/z* 523.16212 [M+1]⁺ C₂₆H₂₆ClN₅O₅ requires 523.16225; Found C, 59.57; H, 5.39; N, 13.08%; Anal. Calc.: C, 59.60; H, 5.00; N, 13.37% for C₂₆H₂₆ClN₅O₅.

The conditions employed for the preparation of compounds 4.105-4.108 were those described in General Method B, using 0.48 mmol 4.104.

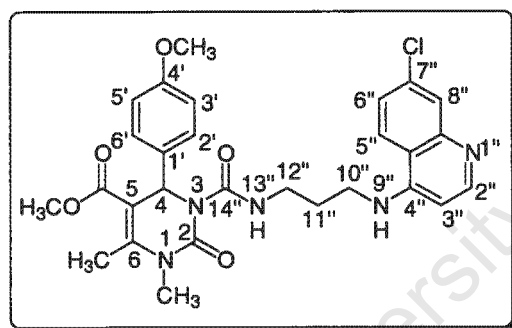
3-[2-(7-Chloro-quinolin-4-ylamino)-ethylcarbamoyl]-4-(4-methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester, 4.105 (NAT1)



White foam (0.25 g, 97%), m.p. 98-100 °C (from CH₂Cl₂-hexane); *R*_f (MeOH:DCM, 1:19) 0.35; IR *v*_{max} (CH₂Cl₂)/cm⁻¹ 3320 (N-H), 2987 (C-H), 1765 (C=O), 1693 (C=O) and 1638 (C=O); δ _H(400 MHz, CDCl₃) 9.20

(1H, bt, J 6.4, NH -12''), 8.40 (1H, d, J 5.6, H-2''), 7.88 (1H, d, J 2.0, H-8''), 7.67 (1H, d, J 9.2, H-5''), 7.22 (1H, dd, J 2.0 and 9.2, H-6''), 7.19 (1H, s, NH -9''), 7.02 (2H, d, J 9.2, H-2' and H-6'), 6.76 (1H, s, H-4), 6.54 (2H, d, J 9.2, H-3' and H-5'), 6.24 (1H, d, J 5.6, H-3''), 3.80 (2H, q, J 5.2, CH_2 -11''), 3.77 (3H, s, $OCOCH_3$), 3.66 (3H, s, $ArOCH_3$), 3.38 (2H, q, J 5.2, CH_2 -10''), 3.14 (3H, s, NCH_3), 2.51 (3H, s, CH_3 -6); δ_c (100 MHz, $CDCl_3$) 165.8 (C=O), 159.0 (C=O), 156.8, 154.4 (C=O), 151.4, 150.7, 148.6, 148.5, 135.4, 131.6, 128.0, 127.8, 127.7, 125.7, 122.2, 117.3, 114.2 (2C), 108.2, 98.4, 55.3, 52.1, 51.5, 46.0, 39.7, 31.6 and 16.2; HRMS (FAB) Found m/z 537.17355 $[M+1]^+$ $C_{27}H_{28}ClN_5O_5$ requires 537.17790; Found: C, 60.08; H, 5.20; N, 12.98%; Anal. Calc.: C, 60.28; H, 5.25; N, 13.02% for $C_{27}H_{28}ClN_5O_5$.

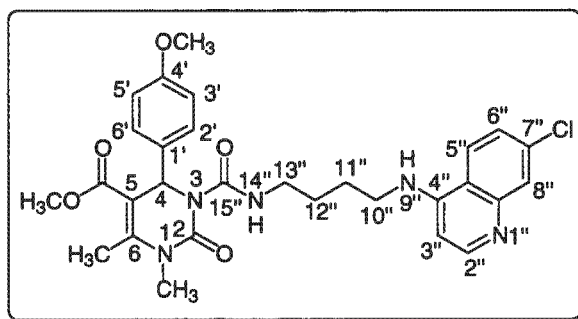
3-[3-(7-Chloro-quinolin-4-ylamino)-propylcarbamoyl]-4-(4-methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester, 4.106 (NAT2)



White foam (0.21g, 78%), m.p. 81-83 °C (from CH_2Cl_2 -hexane); R_f (MeOH:DCM, 1:19) 0.35; IR ν_{max} (CH_2Cl_2)/ cm^{-1} 3320 (N-H), 2941 (C-H), 1730 (C=O), 1697 (C=O) and 1635 (C=O); δ_H (400 MHz, $CDCl_3$) 9.08 (1H, t, J 5.6, NH -13''), 8.48 (1H, d, J 5.6, H-2''), 7.95 (1H, d, J 2.4, H-8''), 7.87 (1H, d, J 9.2, H-5''), 7.35 (1H,

dd, J 2.4 and 9.2, H-6''), 7.15 - 6.74 (5H, m, (4x) Ar-H and H-4), 6.39 (1H, d, J 5.6, H-3''), 6.11 (1H, bt, J 5.6, NH -9''), 3.69 (3H, s, $OCOCH_3$), 3.67 (3H, s, $ArOCH_3$), 3.50-3.24 (4H, m, CH_2 -10' and CH_2 -12'), 3.16 (3H, s, NCH_3), 2.54 (3H, s, CH_3 -6) and 1.97-1.86 (2H, m, CH_2 -11''); δ_c (100 MHz, $CDCl_3$) 164.7 (C=O), 158.3 (C=O), 153.9, 153.2 (C=O), 150.6, 149.1, 147.9, 147.5, 134.0, 130.8, 127.25, 126.5 (2C), 124.3, 120.7, 116.4, 113.3 (2C), 106.9, 97.6, 54.2, 50.8, 50.1, 38.7, 36.6, 30.3, 27.6 and 15.0; HRMS (FAB) Found m/z 551.19219 $[M+1]^+$, $C_{28}H_{30}ClN_5O_5$ requires 551.19355; Found: C, 60.80; H, 5.41; N, 12.63%, Anal. Calc.: C, 60.92; H, 5.48; N, 12.69% for $C_{28}H_{30}ClN_5O_5$.

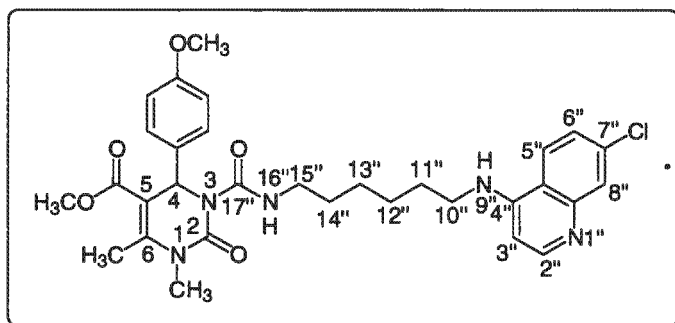
3-[4-(7-Chloro-quinolin-4-ylamino)-butylcarbamoyl]-4-(4-methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester, 4.107 (NAT3)



White solid (0.19, 70%), m.p. 81-83 °C (from CH₂Cl₂-hexane); *R*_f (MeOH:DCM, 1:19) 0.26; IR ν_{max} (CH₂Cl₂)/cm⁻¹ 3320 (N-H), 3057 (C-H), 1770 (C=O), 1703 (C=O) and 1635 (C=O); δ_{H} (400 MHz, CDCl₃) 8.90 (1H, bt, *J* 6.0, NH-14''), 8.49 (1H, d, *J* 5.2, H-2''), 7.94 (1H, d, *J*

2.0, H-8''), 7.79 (1H, d, *J* 8.8, H-5''), 7.30 (1H, dd, *J* 2.0 and 8.8, H-6''), 7.15-6.76 (5H, m, (4x) ArH and H-4), 6.38 (1H, d, *J* 5.2, H-3''), 5.51 (1H, bs, NH-9''), 3.73 (3H, s, OCOCH₃), 3.72 (3H, s, ArOCH₃), 3.60-3.33 (4H, m, CH₂-10'' and CH₂-13''), 3.16 (3H, s, NCH₃), 2.53 (3H, s, CH₃-6) and 1.81-1.72 (4H, m CH₂-11'' and CH₂-12''); δ_{C} (100 MHz, CDCl₃) 166.0 (C=O), 159.5 (C=O), 154.5, 154.4 (C=O), 151.9 150.2, 149.0, 148.8, 135.1, 132.2, 128.6, 127.8 (2C), 125.5, 121.7, 117.0, 114.2 (2C), 108.0, 99.2, 55.4, 52.0, 51.2, 43.2, 40.3, 31.5, 27.8, 25.8 and 16.2; HRMS (FAB) Found *m/z* 565.20485 [M+1]⁺, C₂₉H₃₂ClN₅O₅ requires 565.20920; Found: C, 61.52; H, 5.65; N, 12.27%; Anal. Calc.: C, 61.53; H, 5.70; N, 12.37% for C₂₉H₃₂ClN₅O₅.

3-[6-(7-Chloro-quinolin-4-ylamino)-hexylcarbamoyl]-4-(4-methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester, 4.108 (NAT4)



White foam (0.20g, 70%), m.p. 73-74 °C (from CH₂Cl₂-hexane); *R*_f (MeOH:DCM, 1:19) 0.35; IR ν_{max} (CH₂Cl₂)/cm⁻¹ 3398 (N-H), 3057 (C-H), 1780 (C=O), 1696 (C=O) and 1635 (C=O); δ_{H} (400 MHz, CDCl₃) 8.74 (1H, bt, *J* 5.6, NH-16''), 8.43 (1H, d, *J* 5.6, H-

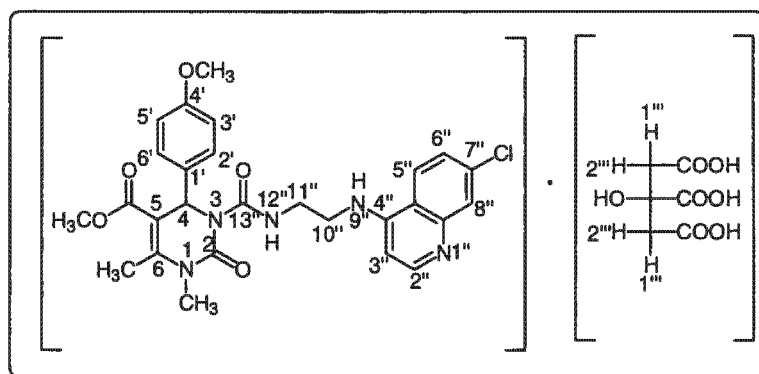
2''), 7.88 (1H, d, J 2.0, H-8''), 7.68 (1H, d, J 9.2, H-5''), 7.24 (1H, dd, J 2.0 and 9.2, H-6''), 7.07 (2H, d, J 8.8, H-2' and H-6'), 6.74 (1H, s, H-4), 6.71 (2H, d, J 8.8, H-3' and H-5'), 5.23 (1H, bs, NH-16''), 3.73 (3H, s, OCOCH_3), 3.68 (3H, s, ArOCH_3), 3.29-3.20 (4H, m, CH_2 -15'' and CH_2 -10''), 3.15 (3H, s, NCH_3), 2.46 (3H, s, CH_3 -6), 2.15 (1H, bs, NH-9'') and 1.72-1.27 (8H, m, CH_2 -14'', CH_2 -13'', CH_2 -12'' and CH_2 -11''); δ_c (100 MHz, CDCl_3) 166.0 (C=O), 159.4 (C=O), 154.6, 154.2 (C=O), 152.0, 150.1, 149.2, 148.9, 135.1, 132.3, 128.8, 127.7 (2C), 125.4, 121.4, 117.4, 114.4 (2C), 108.0, 99.1, 55.4, 52.0, 51.1, 43.0, 40.3, 31.5, 29.6, 28.8, 26.5, 26.3 and 16.1; HRMS (FAB) Found m/z 594.24832, $[\text{M}+1]^+$ $\text{C}_{31}\text{H}_{36}\text{ClN}_5\text{O}_5$ requires 594.24050; Found: C, 62.69; H, 6.05; N, 11.68%; Anal. Calc.: C, 62.67; H, 6.11; N, 11.79% for $\text{C}_{31}\text{H}_{36}\text{ClN}_5\text{O}_5$.

F. General procedure for the preparation for compounds 4.109-4.112 (NAT1CS-NAT4CS)

Citric acid (0.27 g, 1.301 mmol) dissolved in acetone (2 mL) was added dropwise to a 20 mL acetone solution of 4.105 (0.70 g, 1.301 mmol). The reaction mixture was allowed to stand for 24 h at 5 °C during which a precipitate formed. The precipitate was filtered, dissolved in CHCl_3 (50 mL), which was removed *in vacuo* to yield the product in moderate to good yield.

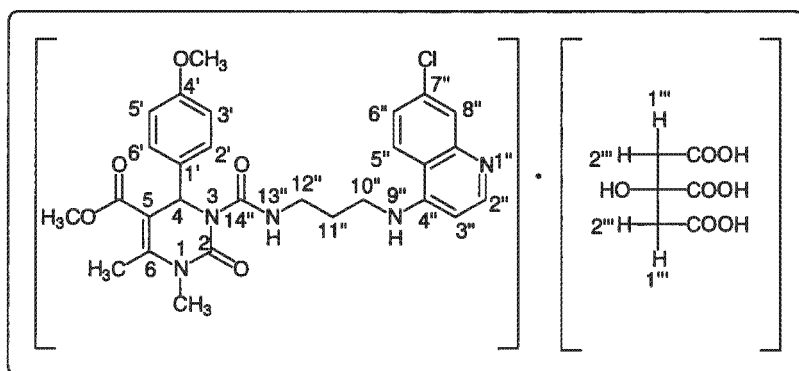
Due to the poor solubility of the salts, 4.109-4.112 in CDCl_3 and DMSO- d_6 the resolution of many signals was reduced, resulting in inaccurate descriptors (e. g. broad s instead of a d). However, the integration and position of the citrate protons, required for characterization of the salts remain determinable throughout.

3-[2-(7-Chloro-quinolin-4-ylamino)-ethylcarbamoyl]-4-(4-methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester; compound with 3-carboxy-3-hydroxy-pentanedioic acid, 4.109 (NAT1CS)



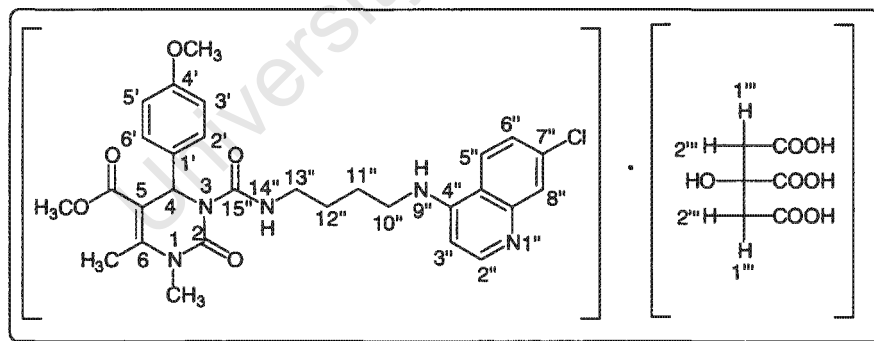
White powder (0.55 g, 57%), m.p. 102-108 °C, IR $\nu_{\max}$ (nujol)/cm⁻¹ 3275 (N-H), 1700 (C=O), 1606 (C=O); δ_{H} (300 MHz, CDCl₃) 12.34 (3H, bs, COOH), 9.22 (1H, bs, NH-12''), 8.75 (1H, bs, H-2''), 8.22 (1H, bs, H-8''), 7.87 (1H, bd, J 8.7, H-5''), 7.73 (1H, bs, H-3''), 7.18 (1H, bs, H-6''), 7.06 (2H, J 8.7, H-6' and H-2'), 6.71 (1H, s, H-4), 6.66 (2H, J 8.7, H-3' and H-5'), 6.43 (1H, bs, NH-9''), 3.66 (3H, bs, OCOCH₃-5), 3.71 (3H, bs, ArOCH₃-4'), 3.56 (4H, bs, CH₂-11'' and CH₂-10''), 3.11 (3H, s, NCH₃), 3.01 (2H, d, J 15.6, H-1''' and H-2'''), 2.90 (2H, d, J 15.3, H-1''' and H-2'''), 2.46 (3H, s, CH₃-6) and 2.00 (1H, bs, OH); δ_{C} (75 MHz, CDCl₃) 179.3, 174.3, 165.7, 159.2, 155.9, 155.1, 153.9, 148.5, 138.9, 138.6, 131.6, 127.4 (2C), 124.1, 119.7, 115.0, 114.0 (2C), 107.9, 97.9, 73.1, 55.2 (2C), 51.8, 51.3, 44.8 (2C), 39.1, 36.2, 31.5, 30.1, 28.3, 16.1; LRMS (FAB) m/z 730 (45%, M⁺); Found: C, 54.29; H, 4.97; N, 9.59%; Anal. Calc.: C, 53.63; H, 5.05; N, 9.48% for C₃₃H₃₆ClN₅O₁₂

3-[3-(7-Chloro-quinolin-4-ylamino)-propylcarbamoyl]-4-(4-methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester; compound with 3-carboxy-3-hydroxy-pentanedioic acid 4.110 (NAT2CS)



White powder (0.55 g, 83%), m.p. 75-79 °C, IR $\nu_{\max}$ (nujol)/cm⁻¹ 3275 (N-H), 1700 (C=O), 1606 (C=O); δ_{H} (400 MHz, CDCl₃) 12.30 (3H, bs, COOH), 9.04 (1H, bs, NH-13''), 8.68 (1H, bs, H-2''), 8.20 (1H, bs, H-8''), 7.98 (1H, bs, H-5''), 7.66 (1H, bs, H-6''), 7.19 (1H, bs, NH-9''), 7.01 (2H, *J* 8.8, H-6' and H-2'), 6.75 (2H, *J* 8.8, H-3' and H-5'), 6.72 (1H, s, H-4), 6.38 (1H, bs, H-3''), 3.71 (3H, bs, OCOC H_3 -5), 3.70 (3H, bs, Ar-OC H_3 -4'), 3.44 (4H, bs, CH₂-12'' and CH₂-10''), 3.13 (3H, s, N-CH₃), 3.00 (2H, d, *J* 14.8, H-1''' and H-2'''), 2.87 (2H, d, *J* 12.4, H-1''' and H-2'''), 2.49 (3H, s, CH₃-6), 1.95 (2H, bs, CH₂-11'') and 2.05 (1H, bs, OH); δ_{C} (100 MHz, CDCl₃) 178.7, 173.9 (2C), 165.9, 159.3, 155.1, 155.0, 154.1, 148.7, 143.0, 139.1, 138.2, 131.9, 127.5 (2C), 124.4, 119.6, 115.0, 114.1 (2C), 107.9, 97.9, 73.2, 55.3 (2C), 51.9, 51.3, 44.4, 41.2, 38.1, 31.5, 30.9, 28.1, 16.2; LRMS (FAB) *m/z* 744 (20%, M⁺); Found: C, 54.78; H, 5.41; N, 9.00%; Anal. Calc.: C, 54.88; H, 5.15; N, 9.41% for C₃₄H₃₈Cl N₅O₁₂.

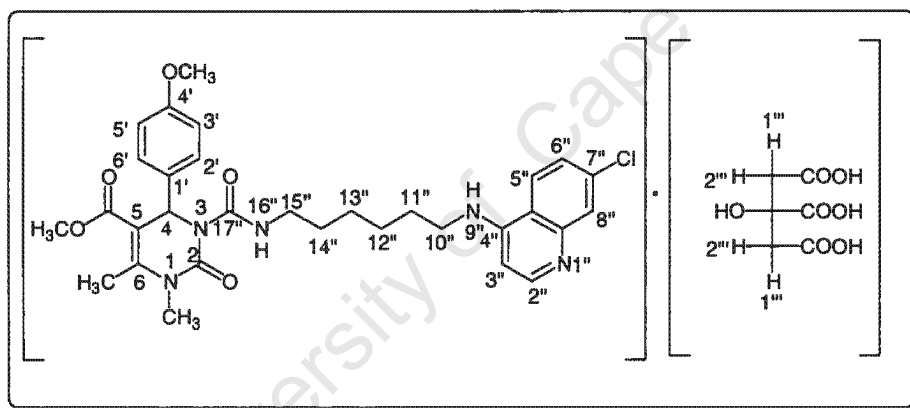
3-[4-(7-Chloro-quinolin-4-ylamino)-butylcarbamoyl]-4-(4-methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester, compound with 3-carboxy-3-hydroxy-pentanedioic acid, 4.111 (NAT3CS)



White powder (0.55 g, 95%), m.p. 71-73 °C, IR $\nu_{\max}$ (nujol)/cm⁻¹ 3275 (N-H), 1700 (C=O), 1606 (C=O); δ_{H} (400 MHz, CDCl₃) 12.22 (3H, bs, COOH), 8.95 (1H, bs, NH-14''), 8.78 (1H, bd, *J* 5.6, H-2''), 8.16 (1H, bs, H-8''), 8.07 (1H, bd, *J* 8.0, H-5''), 7.62 (1H, bs, H-6''), 7.13 (2H, *J* 8.8, H-6' and H-2'), 7.11 (1H, bs, NH-9''), 6.77 (1H, s, H-4), 6.76 (2H, *J* 8.8, H-3' and H-5'), 6.29 (1H, bd, *J* 5.6, H-3''), 3.72 (3H, bs, OCOC H_3 -5), 3.71

(3H, bs, Ar-OCH₃-4'), 3.33 (2H, bs, CH₂-10''), 3.15 (3H, s, NCH₃), 3.03 (2H, d, *J* 14.4, H-1''' and H-2'''), 2.92 (2H, d, *J* 15.2, H-1''' and H-2'''), 2.51 (3H, s, CH₃-6), 1.78 (2H, bs, CH₂-13''), 1.69 (4H, bs, CH₂-11'' and CH₂-12'') and 2.01 (1H, bs, OH); δ_c (100 MHz, CDCl₃) 179.3, 174.3, 165.9, 159.3, 155.0, 154.5, 154.3, 148.7, 142.8, 138.9, 138.4, 132.0, 127.5 (2C), 127.1, 124.8, 119.6, 115.0, 114.1 (2C), 107.9, 97.7, 73.2, 55.3 (2C), 51.9, 51.1, 44.9, 43.7, 40.1, 31.5, 30.9, 27.5, 25.0 and 16.1; LRMS (FAB) *m/z* 758 (70%, M⁺); Found: C, 55.51; H, 5.40; N, 9.00%; Anal. Calc.: C, 55.45; H, 5.32; N, 9.24% for C₃₅H₄₀ClN₅O₁₂.

3-[6-(7-Chloro-quinolin-4-ylamino)-hexylcarbamoyl]-4-(4-methoxy-phenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid methyl ester; compound with 3-carboxy-3-hydroxy-pentanedioic acid, 4.112 (NAT4CS)



White powder (0.55g, 52%), m.p. 75-80 °C, IR $\nu_{\max}$ (nujol)/cm⁻¹ 3275 (N-H), 1700 (C=O), 1606 (C=O); δ_H (400 MHz, CD₃OD) 8.35 (2H, m, H-2'' and H-5''), 7.88 (1H, bs, H-8''), 7.65 (1H, bs, H-6''), 7.09 (2H, *J* 8.7, H-6' and H-2'), 6.81 (1H, s, H-4), 6.79 (2H, *J* 8.7, H-3' and H-5'), 6.78 (1H, bs, H-3''), 3.73 (3H, bs, OCOCH₃-5), 3.72 (3H, bs, Ar-OCH₃-4'), 3.60-3.45 (4H, m, CH₂-15'' and CH₂-10''), 3.16 (3H, s, N-CH₃), 2.86 (2H, d, *J* 15.6, H-1''' and H-2'''), 2.76 (2H, d, *J* 15.3, H-1''' and H-2'''), 2.54 (3H, s, CH₃-6), 1.86-1.38 (8H, m, CH₂-14'', CH₂-11'', CH₂-13'' and CH₂-12''); δ_c (100 MHz, CD₃OD) 187.6, 178.9, 174.1, 167.4, 165.9, 164.9, 159.2, 155.1, 154.3, 154.2, 148.8, 142.7, 138.9, 138.2, 132.1, 127.5 (2C), 124.8, 119.6, 115.0, 114.1 (2C), 107.8, 97.7, 73.2, 55.3 (2C), 51.8, 51.0, 44.6, 43.8, 40.5, 31.4, 29.5, 27.8, 26.2 and 16.1; LRMS (FAB) *m/z* 786 (35%, M⁺); Found: C, 56.59; H, 5.67; N, 8.88 %; Anal. Calc.: C, 56.52; H, 5.64; N, 8.91% for C₃₇H₄₄ClN₅O₁₂.

Crystal structure determination

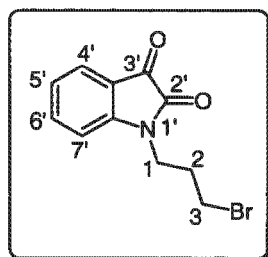
Crystals of 4.102, 4.103 and 4.105 were prepared by dissolving the compounds in heated methanol. Each solution was then filtered while hot through a 0.45 μm nylon microfilter into a glass vial, and left to stand semi-covered to allow the solvent to evaporate slowly and facilitate crystal formation. Crystals of each compound were obtained within 3 days.

A single crystal with good extinction properties, as determined on a polarizing microscope, was selected and cut into a cubic shape for X-ray diffraction data-collection. This was mounted on a glass fibre and coated with Paratone N oil to hold it in position. Unit cell parameters and probable crystal system were determined using a Nonius Kappa CCD diffractometer with 1.2 kW graphite-monochromated MoK_α -radiation ($\lambda = 0.71069 \text{ \AA}$) generated by a Nonius FR590 generator operated at 53 kW and 23 mA. Diffraction data were then collected on the same apparatus with the crystals cooled to 113 K by a steady stream of nitrogen gas in order to reduce atomic thermal motion and thus enhance diffraction quality. Denzo Scalepack ⁶ was used for data reduction and SADABS ⁷ used for absorption corrections.

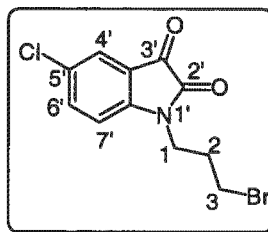
The crystal space group was determined by reading the intensity data and unit cell parameters into the program XPREP. ⁸ The correct space group was identified from characteristic systematic absences and intensity statistics. The structures were then solved by direct methods using SHELXS-86 ⁹ with full-matrix least-squares refinement on F^2 using SHELXL-97, ¹⁰ incorporated into the X-SEED interface. ¹¹ All H atoms were located in difference Fourier maps and were generally included in idealised positions in a riding model with isotropic thermal parameters 1.2 or 1.5 times those of their parent atoms. All non-hydrogen atoms were refined anisotropically. Crystal structure diagrams were created using ORTEP-3. ¹²

G. General Procedure for preparation of compounds 4.137a-f, 4.138a-d, 4.147, 4.148

NaH (60%) (0.27 g, 11.21 mmol) was added to a mixture of commercially available isatin or substituted isatin (0.50 g, 3.40 mmol) in anhydrous DMF (15 mL) at 0 °C. 1, 3-Dibromopropane (0.69 g, 1.38 mL, 13.60 mmol) was then added to this reaction mixture, allowing it to warm to ambient temperature with stirring over 1 h. The temperature was then raised to 60 °C and stirring continued for an additional 12 h. The reaction mixture was diluted with water (50 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were washed with water (5 x 50 mL) to remove traces of DMF. Drying (Na_2SO_4) and concentration under reduced pressure afforded a crude orange residue which was purified by column chromatography on SiO_2 gel using 20-40% EtOAc/Hexane as eluent to yield the desired product in moderate yield.

1-(3-Bromo-propyl)- 1H-indole-2,3-dione, 4.137a (NAT96)

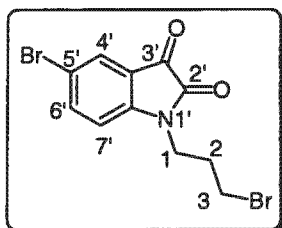
Yellow needles (0.62 g, 68%), m. p. 85-87 °C (from DCM:Hex); R_f (EtoAc:Hex 2:3) 0.65; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (300 MHz, CDCl_3) 7.63 (1H, dd, J 0.6, 8.3, H-4'), 7.59 (1H, ddd, J 0.6, 7.6 and 7.8, H-6'), 7.10 (1H, ddd, J 0.8, 7.6 and 8.3, H-5'), 7.02 (1H, dd, J 0.8 and 7.8, H-7'), 3.89 (2H, t, J 6.3, CH_2 -1), 3.46 (2H, t, J 6.3, CH_2 -3) and 2.28 (2H, quint, J 6.3, CH_2 -2); δ_{C} (75 MHz, CDCl_3) 183.0 (C=O), 158.3 (C=O), 150.7, 138.5, 125.5, 123.9, 117.6, 110.0, 38.8, 30.3 and 30.0; LRMS (EI) m/z 268 (25%, M^+); Found: C, 49.72; H, 3.73; N, 5.02%; Anal. Calc.: C, 49.28; H, 3.76; N, 5.22% for $\text{C}_{11}\text{H}_{10}\text{BrNO}_2$.

1-(3-Bromo-propyl)-5-chloro-1H-indole-2,3-dione, 4.137b (NAT80)

Orange needles (0.50 g, 60%), m. p. 143-145 °C (from DCM:Hex); R_f (EtoAc:Hex 1:9) 0.58; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (NC=O); δ_{H} (300 MHz, CDCl_3) 7.58 (2H, m, H-6' and H-4'), 7.02 (1H, d, J 9.0, H-7'), 3.90 (2H, t, J 6.3, CH_2 -1), 3.46 (2H, t, J 6.3, CH_2 -3) and 2.27 (2H, quint, J 6.3, CH_2 -2); δ_{C} (75

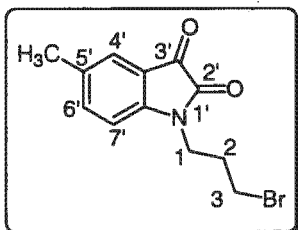
MHz, CDCl_3) 183.7 (C=O), 157.9 (C=O), 150.9, 137.8, 129.8, 125.5, 118.7, 111.3, 39.1, 30.2 and 29.7; LRMS (EI) m/z 303 (10%, M^+); Found: C, 43.87; H, 2.94; N, 4.33%; Anal. Calc.: C, 43.67; H, 3.00; N, 4.63% for $\text{C}_{11}\text{H}_9\text{BrClNO}_2$.

5-Bromo-1-(3-bromo-propyl)-1H-indole-2,3-dione, 4.137c (NAT91)



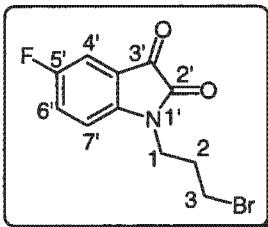
Orange needles (0.42 g, 55%), m. p. 149-151 °C (from DCM:Hex); R_f (EtoAc:Hex 2:8) 0.37; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (300 MHz, CDCl_3) 7.58 (1H, d, J 1.8, H-4'), 7.56 (1H, d, J 9.3, H-7'), 6.90 (1H, dd, J 1.8 and 9.3, H-6'), 3.89 (2H, t, J 6.3, CH_2 -1), 3.46 (2H, t, J 6.3, CH_2 -3), 2.27 (2H, quint, J 6.3, CH_2 -2); δ_{C} (75 MHz, CDCl_3) 182.3 (C=O), 157.6 (C=O), 149.0, 137.9, 129.5, 125.6, 118.4, 111.2, 39.4, 32.6 and 29.5; LRMS (EI) m/z 347 (25%, M^+); Found: C, 38.10; H, 2.61; N, 3.98%; Anal. Calc.: C, 38.07; H, 2.61; N, 4.04% for $\text{C}_{11}\text{H}_9\text{Br}_2\text{NO}_2$.

1-(3-bromo-propyl)-5-methyl-1H-indole-2,3-dione, 4.137d (NAT92)



Orange needles (0.46 g, 52%), m. p. 109-110 °C (from DCM:Hex); R_f (EtoAc:Hex 2:8) 0.32; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (300 MHz, CDCl_3) 7.58 (1H, d, J 2.1, H-4'), 7.56 (1H, d, J 8.3, H-7'), 6.90 (1H, dd, J 2.1 and 8.3, H-6'), 3.88 (2H, t, J 6.3, CH_2 -1), 3.46 (2H, t, J 6.3, CH_2 -3), 2.34 (3H, s, CH_3 -5') and 2.26 (2H, quint, J 6.3, CH_2 -2); δ_{C} (75 MHz, CDCl_3) 183.1 (C=O), 157.5 (C=O), 148.4, 138.8, 134.0, 125.9, 118.0, 109.8, 38.8, 30.4, 30.0 and 20.5; LRMS (EI) m/z 282 (50%, M^+); Found: C, 51.00; H, 4.10; N, 5.02%; Anal. Calc.: C, 51.09; H, 4.29; N, 4.96% for $\text{C}_{12}\text{H}_{12}\text{BrNO}_2$.

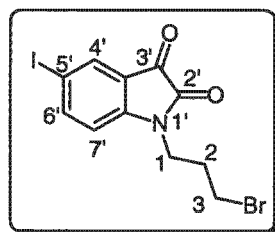
1-(3-bromo-propyl)-5-fluoro-1H-indole-2,3-dione, 4.137e (NAT93)



Orange needles (0.41 g, 47%), m. p. 117-118 °C (from DCM:Hex); R_f (EtoAc:Hex 2:3) 0.63; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (300 MHz, CDCl_3) 7.32 (1H, d, J 2.1, H-4'), 7.30 (1H, d, J 8.4, H-7'), 7.01 (1H, dd, J 2.1 and 8.4, H-6'),

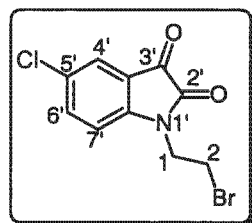
3.90 (2H, t, J 6.3, CH_2 -1), 3.47 (2H, t, J 6.3, CH_2 -3) and 2.28 (2H, quint, J 6.3, CH_2 -2); δ_{c} (75 MHz, CDCl_3) 182.5 (C=O), 160.5 (C=O), 158.1, 143.9, 135.2, 124.5, 117.6, 112.8, 39.0, 30.2 and 30.0; LRMS (EI) m/z 286 (45%, M^+); Found: C, 46.20; H, 3.17; N, 4.65%; Anal. Calc.: C, 46.18; H, 3.17; N, 4.90% for $\text{C}_{11}\text{H}_9\text{BrFNO}_2$.

1-(3-bromo-propyl)-5-iodo-1H-indole-2,3-dione, 4.137f (NAT94)



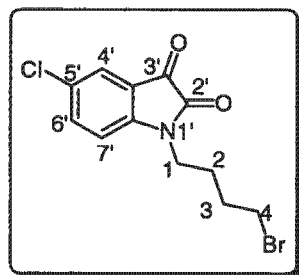
Orange needles (0.30 g, 42%), m. p. 159-161 °C (DCM:Hex); R_f (EtoAc:Hex 2:8) 0.37; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (300 MHz, CDCl_3) 7.91 (1H, d, J 2.1, H-4'), 7.90 (1H, d, J 7.2, H-7'), 6.84 (1H, dd, J 2.1 and 7.1, H-6'), 3.88 (2H, t, J 6.3, CH_2 -1), 3.46 (2H, t, J 6.3, CH_2 -3) and 2.26 (2H, quint, J 6.3, CH_2 -2); δ_{c} (75 MHz, CDCl_3) 182.3 (C=O), 157.0 (C=O), 150.0, 146.5, 134.1 (2C), 119.2, 112.2, 38.9, 30.2 and 29.8; LRMS (EI) m/z 394 (35%, M^+); Found: C, 33.64; H, 2.41; N, 3.60%; Anal. Calc.: C, 33.53; H, 2.30; N, 3.55% for $\text{C}_{11}\text{H}_9\text{BrINO}_2$.

1-(2-Bromo-ethyl)-5-chloro-1H-indole-2,3-dione, 4.138a (NAT109)



Orange needles (0.60 g, 80%), m. p. 115-117 °C (from DCM:Hex); R_f (EtoAc:Hex 2:8) 0.38; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (300 MHz, CDCl_3) 7.57 (1H, d, J 2.1, H-4'), 7.55 (1H, d, J 7.8, H-7'), 6.95 (1H, dd, J 2.1 and 7.8, H-6'), 4.14 (2H, t, J 6.6, CH_2 -1) and 3.61 (2H, t, J 6.6, CH_2 -2); δ_{c} (75 MHz, CDCl_3) 182.5 (C=O), 157.6 (C=O-), 149.1, 137.7, 129.9, 125.5, 118.4, 111.6, 42.2 and 27.1; LRMS (EI) m/z 289 (20%, M^+); Found: C, 41.82; H, 2.35; N, 4.79%; Anal. Calc.: C, 41.63; H, 2.45; N, 4.85% for $\text{C}_{10}\text{H}_7\text{BrClNO}_2$.

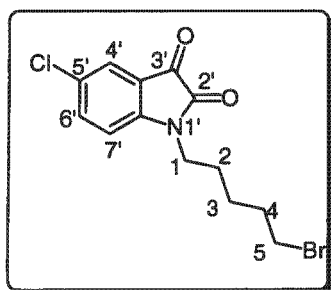
1-(6-Bromo-hexyl)-5-chloro-1H-indole-2,3-dione, 4.138b (NAT110)



Orange needles (0.54 g, 62%), m. p. 91-92 °C (from DCM:Hex); R_f (EtoAc:Hex 2:3) 0.37; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (400 MHz, CDCl_3) 7.58 (1H, d, J 1.6, H-4'), 7.56 (1H, d, J 7.3, H-7'), 6.90 (1H, dd, J 1.6 and 7.3, H-6'),

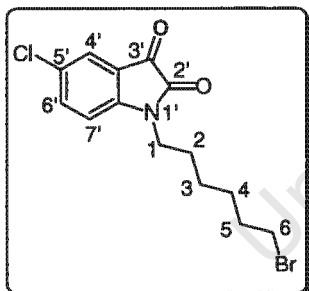
3.77 (2H, t, J 6.6, CH_2 -1), 3.47 (2H, t, J 6.6, CH_2 -4) and 1.99-1.85 (4H, m, CH_2 -2 and CH_2 -3); δ_{c} (100 MHz, CDCl_3) 182.3 (C=O), 157.6 (C=O), 149.0, 137.9, 129.5, 125.6, 118.4, 111.2, 39.4, 32.6, 29.5 and 25.6; LRMS (EI) m/z 316 (15%, M^+); Found: C, 45.57; H, 3.52; N, 4.08%; Anal. Calc.: C, 45.53; H, 3.50; N, 4.42% for $\text{C}_{12}\text{H}_{11}\text{BrClNO}_2$.

1-(5-Bromo-pentyl)-5-chloro-1H-indole-2,3-dione, 4.138c (NAT111)

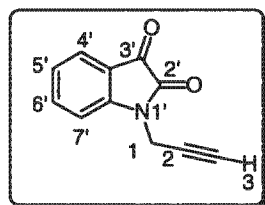


Orange needles (0.60 g, 66%), m. p. 98-99 °C (from DCM:Hex); R_f (EtoAc:Hex 3:7) 0.33; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (300 MHz, CDCl_3) 7.55 (1H, d, J 2.1, H-4'), 7.56 (1H, d, J 7.8, H-7'), 6.90 (1H, dd, J 2.1 and 7.8, H-6'), 3.74 (2H, t, J 7.2, CH_2 -1), 3.40 (2H, t, J 6.9, CH_2 -5) and 1.98-1.44 (6H, m, CH_2 -2, CH_2 -3 and CH_2 -4); δ_{c} (75 MHz, CDCl_3) 182.5 (C=O), 157.6 (C=O), 149.1, 137.7, 129.6, 125.4, 118.5, 111.3, 40.1, 33.1, 32.0, 26.4 and 25.3; LRMS (EI) m/z 331 (20%, M^+); Found: C, 46.82; H, 3.85; N, 4.10%; Anal. Calc.: C, 47.23; H, 3.96; N, 4.24% for $\text{C}_{13}\text{H}_{13}\text{BrClNO}_2$.

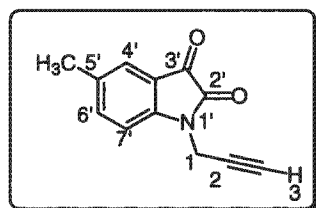
1-(6-Bromo-hexyl)-5-chloro-1H-indole-2,3-dione, 4.138d (NAT112)



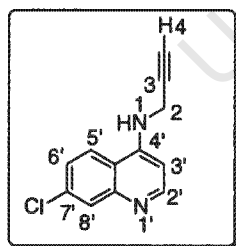
Orange needles (0.71 g, 75%), m. p. 60-62 °C from (DCM:Hex); R_f (EtoAc:Hex 2:8) 0.21; IR ν_{max} (nujol)/ cm^{-1} 1739 (C=O), 1601 (C=O); δ_{H} (400 MHz, CDCl_3) 7.57 (1H, d, J 8.1, H-7'), 7.54 (1H, d, J 2.1, H-4'), 6.86 (1H, dd, J 2.1 and 8.1, H-6'), 3.73 (2H, t, J 7.1, CH_2 -1), 3.40 (2H, t, J 6.6, CH_2 -6) and 1.86-1.41 (8H, m, CH_2 -2, CH_2 -3, CH_2 -4 and CH_2 -5); δ_{c} (100 MHz, CDCl_3) 182.4 (C=O), 157.7 (C=O), 149.2, 137.6, 129.8, 125.4, 118.5, 111.1, 40.2, 33.5, 32.4, 27.7, 27.0 and 26.0; LRMS (EI) m/z 345 (15%, M^+); Found: C, 48.97; H, 4.39; N, 4.02%; Anal. Calc.: C, 48.79; H, 4.39; N, 4.06% for $\text{C}_{14}\text{H}_{15}\text{BrClNO}_2$.

1-Prop-2-ynyl-1H-indole-2,3-dione, 4.147 (NAT83)

Orange needles (0.30 g, 48%), m. p. 120-123 °C (from DCM:Hex); R_f (EtoAc:Hex 2:8) 0.39; IR $\nu_{\max}$ (nujol)/cm⁻¹ 1739 (C=O), 1601 (C=O); δ_H (300 MHz, CDCl₃) 7.66-7.11 (4H, m, ArH), 4.53 (2H, d, J 2.4, H-1) and 2.30 (1H, t, J 2.4, CH-3); δ_C (75 MHz, CDCl₃) 183.7 (C=O), 157.9 (C=O), 138.3, 125.4 (2C), 124.2, 117.7, 111.0, 75.5, 73.3 and 29.4; LRMS (EI) m/z 185 (30%, M⁺); Found: C, 71.34; H, 3.69; N, 7.52%; Anal. Calc.: C, 71.35; H, 3.81; N, 7.56% for C₁₁H₇NO₂.

5-Methyl-1-Prop-2-ynyl-1H-indole-2,3-dione, 4.148 (NAT84)

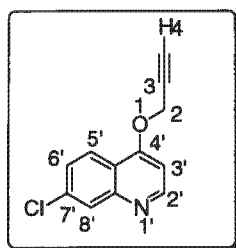
Orange needles (0.29 g, 47%), m. p. 139-142 °C (from DCM:Hex); R_f (EtoAc:Hex 2:8) 0.38; IR $\nu_{\max}$ (nujol)/cm⁻¹ 1739 (C=O) and 1601 (C=O); δ_H (300 MHz, CDCl₃) 7.45-7.01 (3H, m, ArH), 4.51 (2H, d, J 2.4, CH₂-1), 2.35 (3H, s, CH₃-5') and 2.29 (1H, t, J 2.4, H-3); δ_C (75 MHz, CDCl₃) 183.7 (C=O), 157.9 (C=O), 138.7 (2C), 125.7 (2C), 110.8 (2C), 77.3, 73.1, 29.4 and 20.7; LRMS (EI) m/z 199 (40%, M⁺); Found: C, 72.37; H, 4.50; N, 7.06%; Anal. Calc.: C, 72.35; H, 4.55; N, 7.03% for C₁₂H₉NO₂.

7-Chloro-quinolin-4-yl)-prop-2-ynyl-amine, 4.141 (NAT78)

A neat mixture of 4,7-dichloroquinoline (300 mg, 1.51 mmol) and propargyl amine (1.67 g, 2.08 mL, 30.20 mmol) was stirred under reflux at 140 °C for 24 h. After this time, the reaction mixture was cooled to room temperature. The reaction mixture was diluted with NaOH (1M, 20 mL), extracted with EtOAc (3 x 50 mL), washed with water (50 mL) and dried over MgSO₄. After filtration the solvent was evaporated *in vacuo* to yield the crude product which was purified by column chromatography on SiO₂ gel, using 5% MeOH/DCM as eluent to yield the product 4.141 as white needles (100 mg, 30%), m. p. 215-217 °C (from methanol); R_f (MeOH:DCM 1:19) 0.19; IR $\nu_{\max}$ (nujol)/cm⁻¹ 3455 (N-H), 2200 (C≡C); δ_H (300 MHz, CD₃OD) 8.43 (1H, d, J 5.7, H-2'), 8.03 (1H, d, J 9.3, H-5'), 7.81 (1H, d, J 2.4, H-8'), 7.42

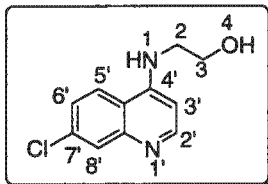
(1H, dd, J 2.4 and 9.3, H-6'), 6.86 (1H, d, J 5.7, H-3'), 4.18 (2H, d, J 2.4, CH_2 -2) and 2.64 (1H, t, J 2.4, H-4); δ_{c} (75 MHz, CD_3OD) 152.6, 149.8 (2C), 129.2, 127.9, 126.3, 124.3, 119.3, 101.0, 80.5, 72.9 and 33.2; LRMS (FAB) m/z 217 (100%, M^+); Found: C, 66.84; H, 4.19; N, 12.80%; Anal. Calc.: C, 66.52; H, 4.19; N, 12.93% for $\text{C}_{12}\text{H}_9\text{ClN}_2$.

7-Chloro-4-prop-2-ynyloxy-quinoline, 4.142 (NAT113)



NaH (0.073g, 3.03 mmol) was added to a solution of propargyl alcohol (0.17 g, 0.18 mL, 3.03 mmol) in THF (15 mL) at 0 °C. The reaction mixture was stirred for 10 min before addition of 4, 7-dichloroquinoline (0.50 g, 2.52 mmol) dissolved in THF (10 mL) was added dropwise. The resultant reaction mixture was allowed to warm to ambient temperature and subsequently heated to 50 °C. The reaction was stirred for 2 h, after which TLC indicated complete conversion into product. Water (50 mL) was added dropwise, and the mixture was extracted with EtOAc (3 x 50 mL) and dried over Na_2SO_4 . After evaporation of the solvent *in vacuo*, the residue was chromatographed on SiO_2 gel using 30% EtOAc/Hexane as eluent to afford the product 4.142 as white needles (2.20 g, 67%), m. p. 119-122 °C (from DCM:Hex); R_f (EtOAc:Hex 3:7) 0.68; IR ν_{max} (nujol)/ cm^{-1} 3100 (C-H) and 2260 ($\text{C}\equiv\text{C}$); δ_{H} (400 MHz, CDCl_3) 8.78 (1H, d, J 5.3, H-2'), 8.13 (1H, d, J 8.8, H-5'), 8.04 (1H, d, J 2.0, H-8'), 7.43 (1H, dd, J 2.0 and 8.8, H-6'), 6.86 (1H, d, J 5.3, H-3'), 4.94 (2H, d, J 2.4, CH_2 -2) and 2.62 (1H, t, J 2.4, H-4); δ_{c} (75 MHz, CDCl_3) 152.4, 150.9, 149.8, 136.2, 128.0, 127.1, 123.3, 115.6, 103.4, 90.3, 77.4 and 46.8; LRMS (FAB) m/z 218 (100%, M^+); Found: C, 66.03; H, 3.80; N, 6.39%; Anal. Calc.: C, 66.22; H, 3.70; N, 6.44% for $\text{C}_{12}\text{H}_8\text{ClNO}$.

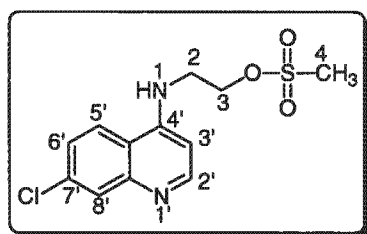
2-(7-Chloro-quinolin-4-ylamino)-ethanol, 4.150



A mixture of 4,7-dichloroquinoline (1.00 g, 5.05 mmol), ethanolamine 4.149 (6.20 g, 6.1 mL, 101.49 mmol), triethylamine (0.15 g, 0.2 mL, 1.51 mmol) and anhydrous K_2CO_3 (0.21 g, 1.51 mmol) was stirred under reflux at 120-140 °C for 5 hrs. The reaction mixture was then cooled to ambient temperature during which a precipitate formed. It was then filtered, washed with water (100 mL) and recrystallised from

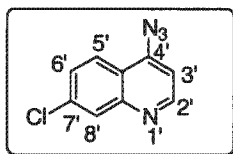
methanol to obtain the product **4.150** (1.09 g, 97 %) as pale white solid. mp: 216-218 °C (from methanol) (lit.⁴, m.p. 214 °C); R_f (MeOH:DCM, 1:9) 0.2; δ_H (300 MHz, DMSO- d_6) 8.38 (1H, d, J 5.7, H-2'), 8.23 (1H, d, J 8.7, H-5'), 7.78 (1H, d, J 2.1, H-8'), 7.43 (1H, dd, J 2.1 and 8.7, H-6'), 7.25 (1H, bs, N-H), 6.49 (1H, d, J 5.7, H-3'), 3.67 (4H, t, J 6.0, CH_2 -2 and CH_2 -3) and 2.60 (1H, bs, O-H); δ_C (75MHz, DMSO- d_6) 151.8, 150.2, 149.0, 133.3, 127.4, 124.1, 123.9, 117.4, 98.7, 58.7 and 45.1.

Methanesulfonic acid 2-(7-chloro-quinolin-4-ylamino)-ethyl ester, **4.151**



A solution of the alcohol **4.150** (0.50 g, 2.25 mmol) in pyridine (5 mL) was stirred at 0 °C for 30 min. Methanesulfonyl chloride dissolved in pyridine (5 mL) was added slowly at 0 °C and was stirred for a further 5 h. The reaction mixture was then diluted with 17 % aqueous NH_4OH (10 mL), extracted with DCM (3 x 50 mL), dried over $MgSO_4$, and concentrated *in vacuo* to form the mesylate **4.151** (0.55 g, 82%) as white crystals, m.p. 137-139 °C (from MeOH/ H_2O) (lit.⁵ 138-140 °C); R_f (MeOH:DCM 2:8) 0.76; IR ν_{max} (nujol)/ cm^{-1} 3218 (N-H); δ_H (300 MHz, $CDCl_3$) 8.57 (1H, d, J 5.4, H-2'), 7.97 (1H, d, J 1.8, H-8'), 7.73 (1H, d, J 9.3, H-5'), 7.40 (1H, dd, J 1.8 and 9.3, H-6'), 6.41 (1H, d, J 5.4, H-3'), 5.60 (1H, bs, N-H), 4.58 (2H, t, J 4.8, CH_2 -3), 3.69 (2H, q, J 4.8, CH_2 -2) and 3.08 (3H, s, CH_3 -4); δ_C (75 MHz, $CDCl_3$) 151.9, 149.2, 149.1, 133.2, 128.9, 125.9, 121.1, 117.3, 99.0, 66.8, 42.5 and 37.8.

4-Azido-7-chloro-quinoline, **4.155** (NAT153)



NaN_3 (98.48 mg; 1.51 mmol) was added to a stirred solution of 4,7-dichloroquinoline (0.20 g, 1.01 mmol) in DMSO (10 mL). The resultant reaction mixture was stirred for 12 h at 65 °C after which it was cooled to room temperature. The reaction mixture was diluted with water (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic extracts were washed with water (5 x 50 mL) to remove traces of DMSO, dried over Na_2SO_4 , filtered and concentrated *in vacuo* to afford the product as yellow-green needles (0.17 g, 80%), m.p. 109-111 °C (from DCM:Hex); R_f (MeOH:DCM 2:8) 0.76; IR

$\nu_{\max}$ (nujol)/ cm^{-1} 3100 (ArC-H); δ_{H} (300 MHz, CDCl_3) 8.80 (1H, d, J 4.9, H-2'), 8.05 (1H, d, J 2.0, H-8'), 7.99 (1H, d, J 9.0, H-5'), 7.40 (1H, dd, J 2.0 and 9.0, H-6') and 6.41 (1H, d, J 4.9, H-3'); δ_{C} (75 MHz, CDCl_3) 149.6, 146.4, 136.6, 128.2 (2C), 127.6 (2C), 123.7 and 108.7; LRMS (FAB) m/z 205 (100%, M^+); Found: C, 52.86; H, 2.46; N, 27.79%; Anal. Calc.: C, 52.83; H, 2.46; N, 27.38% for $\text{C}_9\text{H}_5\text{ClN}_4\text{O}$.

H. General procedures for the preparation of compounds 4.145a-l, 4.153, 4.154, 4.156, 4.157

Method (a)

To a mixture of propargyl quinoline (100 mg, 0.46 mmol) and isatin halide (120 mg, 0.46 mmol, 1.0 eq.) in DMSO/ H_2O (9:1, 10 mL) were added sodium ascorbate (11 mg, 0.055 mmol, 0.12 eq.), L-proline (11 mg, 0.092 mmol, 0.2 eq.), NaN_3 (36.03 mg, 0.55 mmol, 1.2 eq.), Na_2CO_3 (10 mg, 0.097 mmol, 0.21 eq.) and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (6.0 mg, 0.024 mmol, 0.0052 eq.). The resultant reaction mixture was stirred overnight at 65 °C. Upon completion of reaction (monitored by TLC), water (20 mL) was added to the crude reaction mixture and extracted with EtOAc (3 x 20 mL). The combined organic extracts were washed with diluted NH_4OH (20 mL) to remove traces of copper azide followed by water (5 x 10 mL). Drying (Na_2SO_4) and concentration under reduced pressure afforded a crude orange residue which was purified by column chromatography on SiO_2 gel using 5% MeOH/DCM as eluent to yield the desired product in low to moderate yield.

Method (b)

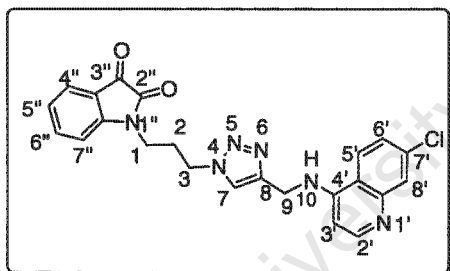
Mesylate 4.151 (0.15g, 0.50 mmol, 1.0 eq.) was added to a stirred anhydrous DMSO (5 mL) solution of NaN_3 (0.05 g, 0.75 mmol, 1.2 eq.) and the reaction mixture was stirred for 48 h at 50 °C. Water (2-3 mL) was added followed by solid sodium ascorbate (10% mmol), alkyne (0.50 mmol, 1.0 eq.) and aqueous $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.012 g, 0.050 mmol, 0.1 eq.). The resultant reaction mixture was stirred for 12-24 h (overnight) and then quenched with water (3 mL) at 65 °C. The reaction mixture was diluted with water (20 mL) and extracted with EtOAc (3 x 50 mL). The combined organic extracts were washed with water (5 x 20 mL) to remove traces of DMSO and then dried over Na_2SO_4 . After removal of the solvent under reduced pressure, the pure product was isolated by column chromatography on SiO_2 gel by eluting with 5% MeOH/DCM.

Method (c)

Isatin alkyne (0.20 g, 0.51 mmol) and quinoline azide (0.11 g, 0.51 mmol) were suspended in a 1:1 mixture of water and *tert*-butyl alcohol (3-5 mL). Sodium ascorbate (0.051 mmol, 51 μ L of freshly prepared 1 M solution in water) and aqueous $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (12.8 mg, 0.051 mmol, in 100 μ L of water) were added and the heterogeneous reaction mixture was vigorously stirred overnight. Upon completion of the reaction, indicated by TLC analysis, the reaction mixture was diluted with water, extracted with EtOAc (3 x 50 mL) and dried over Na_2SO_4 . Removal of the solvent under reduced pressure afforded a residue which was purified by column chromatography on SiO_2 gel using 5% MeOH /DCM as eluent.

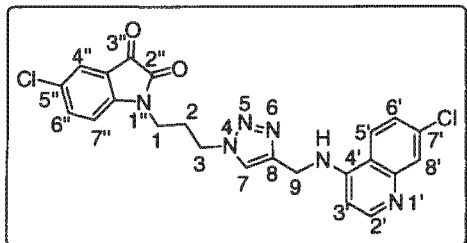
The conditions employed for the preparation of compounds 4.145a-l were those described in General Method H (a).

1-(3-{4-[(7-Chloro-quinolin-4-ylamino)-methyl]-[1,2,3]triazol-1-yl}-propyl)-1H-indole-2,3-dione, 4.145a (NAT100)



Yellow amorphous powder (80 mg, 40%), m. p. 229-233 $^{\circ}\text{C}$ (from CH_3CN); R_f (MeOH:DCM 1:19) 0.26; IR ν_{max} (nujol)/ cm^{-1} 3355 (N-H), 1725 (C=O), 1617 (C=O); δ_{H} (300 MHz, $\text{DMSO}-d_6$) 8.38 (1H, d, J 5.6, H-2'), 8.29 (1H, d, J 9.0, H-5'), 7.78 (1H, s, H-7), 7.79 (1H, d, J 2.2, H-8'), 7.87 (1H, bs, NH-10), 7.58 (1H, t, J 7.6, ArH-5''), 7.52 (1H, d, J 7.6, ArH-4''), 7.43 (1H, dd, J 2.2 and 9.0, H-6'), 7.12-7.06 (2H, m, ArH-6'' and ArH-7''), 6.61 (1H, d, J 5.6, H-3'), 4.58 (2H, s, CH_2 -9), 4.42 (2H, t, J 7.1, H-3), 3.71 (2H, t, J 7.1, H-1) and 2.19 (2H, quint, J 7.1, H-2); δ_{C} (75 MHz, $\text{DMSO}-d_6$) 183.0 (C=O), 160.1 (C=O), 150.8, 150.4, 150.3, 144.2, 144.0, 137.9, 136.3, 133.8, 126.6, 124.4, 124.3, 124.1, 123.1, 123.0, 117.6, 110.4, 99.3, 47.0, 38.2, 37.1 and 27.7; LRMS (FAB) m/z 447 (45%, M^+); Found: C, 61.91; H, 4.26; N, 18.90%; Anal. Calc.: C, 61.82; H, 4.29; N, 18.81% for $\text{C}_{23}\text{H}_{19}\text{ClN}_6\text{O}_2$.

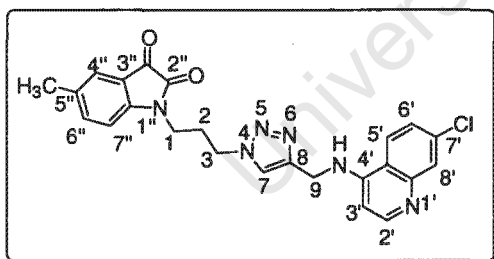
5-Chloro-1-(3-{4-[(7-Chloro-quinolin-4-ylamino)-methyl]-[1,2,3]triazol-1-yl}-propyl)-1H-indole-2,3-dione, 4.145b (NAT124)



Orange amorphous powder (100 mg, 45%), m. p. 106-109 °C (from CH₃CN); *R_f* (MeOH:DCM 1:19) 0.35; IR ν_{max} (nujol)/cm⁻¹ 3355 (N-H), 1725 (C=O), 1617 (C=O); δ_{H} (400 MHz, DMSO-*d*₆) 8.36 (1H, d, *J* 5.3, H-2'), 8.14 (1H, d, *J* 9.2, H-5'), 7.98 (1H, s, H-7), 7.89 (1H, bt, *J* 5.8, NH-10), 7.77

(1H, d, *J* 2.1, H-8'), 7.60 (1H, dd, *J* 2.1 and 8.3, ArH-6'), 7.56 (1H, d, *J* 2.1, ArH-4'), 7.45 (1H, dd, *J* 2.1 and 9.2, H-6'), 7.11 (1H, d, *J* 8.3, ArH-7'), 6.63 (1H, d, *J* 5.3, H-3'), 4.56 (2H, d, *J* 5.8, CH₂-9), 4.41 (2H, t, *J* 7.0, CH₂-3), 3.68 (2H, t, *J* 7.0, CH₂-1) and 2.13 (2H, quint, *J* 7.0, CH₂-2); δ_{C} (100 MHz, DMSO-*d*₆) 182.2 (C=O), 159.9 (C=O), 152.5, 150.0, 150.6, 145.2, 137.4, 134.4, 128.1 (2C), 125.0, 124.7, 124.6, 123.8, 119.8, 118.2, 112.8, 111.0, 100.0, 47.7, 38.6, 37.7 and 28.2; LRMS (FAB) *m/z* 481 (30%, M⁺); Found: C, 57.30; H, 3.51; N, 17.57%; Anal. Calc.: C, 57.39; H, 3.77; N, 17.46% for C₂₃H₁₈Cl₂N₆O₂.

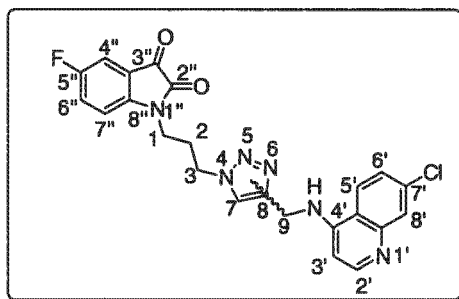
1-(3-{4-[(7-Chloro-quinolin-4-ylamino)-methyl]-[1,2,3]triazol-1-yl}-propyl)-5-methyl-1H-indole-2,3-dione, 4.145c (NAT97)



Orange amorphous powder (80 mg, 38%), m. p. 215-217 °C (from CH₃CN); *R_f* (MeOH:DCM 1:19) 0.19; IR ν_{max} (nujol)/cm⁻¹ 3355 (N-H), 1725 (C=O), 1617 (C=O); δ_{H} (300 MHz, DMSO-*d*₆) 8.37 (1H, d, *J* 5.2, H-2'), 8.26 (1H, d, *J* 9.0, H-5'), 7.96 (1H, s, H-7), 7.78 (1H, d, *J* 2.0, H-8'), 7.71 (1H, bs, NH-10), 7.43 (1H, dd, *J* 2.0 and 9.0, H-6'), 7.37-7.33 (2H, m, ArH-4' and ArH-6'), 6.92 (1H, d, *J* 8.0, ArH-7'), 6.57 (1H, d, *J* 5.2, H-3'), 4.56 (2H, s, CH₂-9), 4.41 (2H, t, *J* 6.8, H-3), 3.71 (2H, t, *J* 6.8, H-1), 2.25 (3H, s, ArCH₃-5') and 2.19 (2H, quint, *J* 6.8, H-2); δ_{C} (75 MHz, DMSO-*d*₆) 184.1 (C=O), 159.0 (C=O), 152.3, 150.5, 149.5, 148.9, 144.9, 138.9, 134.2, 133.2, 128.0, 125.3, 125.0, 124.7, 123.8, 118.3, 118.2, 110.9, 100.0, 47.7, 38.6, 37.6, 28.3 and 20.7; LRMS (FAB) *m/z* 461 (65%, M⁺); Found:

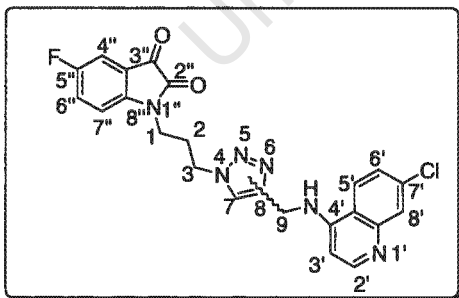
C, 62.52; H, 4.42; N, 18.32%; Anal. Calc.: C, 62.54; H, 4.59; N, 18.23% for $C_{24}H_{21}ClN_6O_2$.

1-(3-{4-[(7-Chloro-quinolin-4-ylamino)-methyl]-[1,2,3]triazol-1-yl}-propyl)-5-fluoro-1H-indole-2,3-dione, 4.145d (NAT101)



Orange amorphous powder (75 mg, 36%), m.p. 221-22 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.18; IR ν_{max} (nujol)/ cm^{-1} 3355 (N-H), 1725 (C=O), 1617 (C=O); δ_H (400 MHz, DMSO- d_6) 8.36 (1H, d, J 5.3, H-2'), 8.24 (1H, d, J 9.0, H-5'), 7.99 (1H, s, H-7), 7.86 (1H, bt, J 5.8, NH-10), 7.77 (1H, d, J 2.1, H-8'), 7.48-7.43 (2H, m, ArH-4'' and ArH-7''), 7.41 (1H, dd, J 2.1 and 9.0, H-6'), 7.11 (1H, m, ArH-6''), 6.53 (1H, d, J 5.3, H-3'), 4.56 (2H, d, J 5.8, H-9), 4.41 (2H, t, J 7.0, H-3), 3.68 (2H, t, J 7.0, H-1) and 2.13 (2H, quint, J 7.0, H-2); δ_C (100 MHz, DMSO- d_6) 182.9 (C=O), 159.1 (C=O), 150.9, 150.3, 150.4, 144.0 (2C), 136.9, 133.8, 126.7, 124.4, 124.1, 124.1, 123.9, 123.0, 117.4, 111.9, 111.4, 99.3, 47.0, 38.0, 37.1 and 27.4; LRMS (FAB) m/z 465 (75%, M^+); Found: C, 59.24; H, 4.01; N, 18.55%; Anal. Calc.: C, 59.42; H, 3.90; N, 18.08% for $C_{23}H_{18}ClFN_6O_2$.

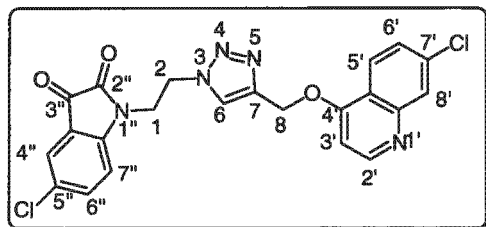
1-(3-{4-[(7-Chloro-quinolin-4-ylamino)-methyl]-[1,2,3]triazol-1-yl}-propyl)-5-fluoro-1H-indole-2,3-dione, 4.145e (NAT102)



Orange amorphous powder (30 mg, 15%), m. p. 118-120 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.18; IR ν_{max} (nujol)/ cm^{-1} 3355 (N-H), 1725 (C=O), 1617 (C=O); δ_H (400 MHz, DMSO- d_6) 8.37 (1H, d, J 5.6, H-2'), 8.27 (1H, d, J 9.0, H-5'), 7.99 (1H, s, H-7), 7.94 (1H, bt, J 5.6, NH-10), 7.77 (1H, d, J 2.1, H-8'), 7.48-7.43 (2H, m, ArH-4'' and ArH-7''), 7.41 (1H, dd, J 2.1 and 9.0, H-6'), 7.11 (1H, m, ArH-6''), 6.55 (1H, d, J 5.6, H-3'), 4.55 (2H, d, J 5.6, H-9), 4.41 (2H, t, J 7.0, H-3), 3.68 (2H, t, J 7.0, H-1) and 2.13 (2H, quint, J 7.0, H-2); δ_C (100 MHz, DMSO- d_6) 182.9 (C=O), 159.1 (C=O), 150.9, 150.3, 150.4, 144.7, 144.0, 136.9, 134.7, 126.7, 124.4,

124.1, 124.1, 123.7, 123.0, 117.4, 111.7, 111.2, 99.3, 47.7, 38.0, 37.1 and 27.4; LRMS (FAB) m/z 465 (75%, M^+); Found: C, 59.08; H, 4.02; N, 18.04%; Anal. Calc.: C, 59.42; H, 3.90; N, 18.08% for $C_{23}H_{18}ClFN_6O_2$.

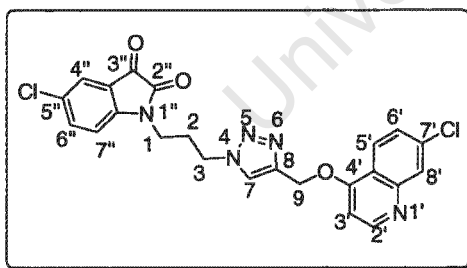
5-Chloro-1-{2-[4-(7-Chloro-quinolin-4-yloxymethyl)-[1,2,3]triazol-1yl]-ethyl}-1H-indole-2,3-dione, 4.145f (NAT115)



Brown amorphous powder (44 mg, 20%), m. p. 98-100 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.30; IR ν_{max} (nujol)/ cm^{-1} 1725 (C=O), 1617 (C=O); δ_H (400 MHz, $CDCl_3$) 8.70 (1H, d, J 5.3, H-2'), 8.00 (1H, d, J 2.0, H-8'), 7.98 (1H, d, J 9.0, H-5'), 7.79 (1H, s, H-6), 7.48 (1H, d, J 2.2, ArH-4''), 7.43 (1H, dd, J 2.0 and 9.0, H-6'), 7.35 (1H, dd, J 2.2 and 8.4, ArH-6''), 6.86 (1H, d, J 5.3, H-3'), 6.54 (1H, d, J 8.4, ArH-8''), 5.36 (2H, s, CH_2 -8), 4.75 (2H, t, J 6.8, CH_2 -2) and 4.26 (2H, t, J 6.8, CH_2 -1);

δ_C (100 MHz, $CDCl_3$) 183.3 (C=O), 160.6 (C=O), 152.4 (2C), 148.3 (2C), 143.6, 137.9, 136.2, 130.4, 127.9, 126.8, 125.6, 124.5, 123.3, 119.7, 118.3, 110.9, 101.4, 62.1, 47.8 and 40.8; LRMS (FAB) m/z 468 (15%, M^+); Found: C, 56.30; H, 3.37; N, 14.86%; Anal. Calc.: C, 56.43; H, 3.23; N, 14.96% for $C_{22}H_{15}Cl_2N_5O_3$.

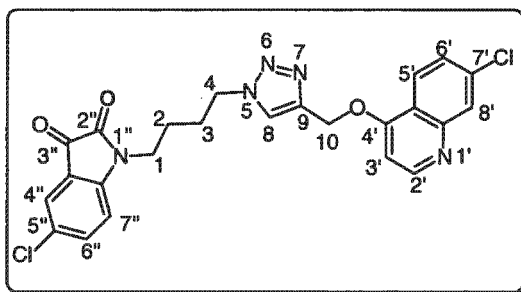
5-Chloro-1-{3-[4-(7-Chloro-quinolin-4-yloxymethyl)-[1,2,3]triazol-1yl]-propyl}-1H-indole-2,3-dione, 4.145g (NAT129)



Orange amorphous powder (80 mg, 36%), m. p. 106-109 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.45; IR ν_{max} (nujol)/ cm^{-1} 1725 (C=O), 1617 (C=O); δ_H (400 MHz, $CDCl_3$) 8.75 (1H, d, J 5.1, H-2'), 8.14 (1H, d, J 8.8, H-5'), 8.03 (1H, d, J 2.0, ArH-4''), 7.88 (1H, s, H-7), 7.70 (1H, dd, J 2.0 and 8.2, ArH-6''), 7.68 (1H, d, J 2.0, H-8'), 7.43 (1H, dd, J 2.0 and 8.8, H-6'), 6.96 (1H, d, J 5.1, H-3'), 6.83 (1H, d, J 8.2, ArH-7''), 5.45 (2H, s, CH_2 -9), 4.46 (2H, t, J 6.6, CH_2 -3), 3.81 (2H, t, J 6.6, CH_2 -1) and 2.42 (2H, quint, J 6.6, CH_2 -2); δ_C (100 MHz, $CDCl_3$) 180.0 (C=O), 166.5 (C=O), 151.6, 148.3, 145.7, 142.4, 137.9, 127.2 (2C), 127.1, 127.0, 125.6, 124.4, 123.6, 118.5, 115.6, 111.5, 108.9, 101.8, 62.8, 47.7, 37.6 and 27.3; LRMS (FAB)

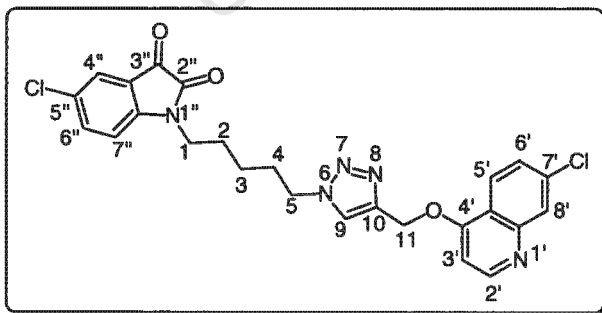
m/z 482 (30%, M^+); Found: C, 57.20; H, 3.54; N, 14.44%; Anal. Calc.: C, 57.27; H, 3.55; N, 14.52% for $C_{23}H_{17}Cl_2N_5O_3$.

5-Chloro-1-[4-[4-(7-Chloro-quinolin-4-yloxymethyl)-[1,2,3]triazol-1-yl]-butyl]-1H-indole-2,3-dione, 4.145h (NAT116)



Orange amorphous powder (97 mg, 44%), m. p. 176-177 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.33; IR ν_{max} (nujol)/ cm^{-1} 1725 (C=O), 1617 (C=O); δ_H (400 MHz, $CDCl_3$) 8.74 (1H, d, J 5.1, H-2'), 8.10 (1H, d, J 9.0, H-5'), 8.02 (1H, d, J 2.0, ArH-4''), 7.72 (1H, s, H-8), 7.54 (1H, dd, J 2.0 and 8.2, ArH-6''), 7.44 (1H, d, J 2.0, H-8'), 7.42 (1H, dd, J 2.0 and 9.0, H-6'), 6.95 (1H, d, J 5.1, H-3'), 6.85 (1H, d, J 8.2, ArH-7''), 5.44 (2H, s, CH_2 -10), 4.48 (2H, t, J 7.0, CH_2 -4), 3.75 (2H, t, J 7.0, CH_2 -1), 2.03 (2H, quint, J 7.0, CH_2 -3) and 1.75 (2H, quint, J 7.0, CH_2 -2); δ_C (100 MHz, $CDCl_3$) 183.0 (C=O), 157.9 (C=O), 152.5 (2C), 149.2, 148.7, 143.6, 140.0, 137.8 (2C), 128.0, 126.8, 125.6, 123.4, 123.2, 118.5, 118.0, 111.3, 101.6, 62.5, 49.5, 39.4, 27.3 and 24.1; LRMS (FAB) m/z 496 (7%, M^+); Found: C, 58.04; H, 3.80; N, 14.02%; Anal. Calc.: C, 58.08; H, 3.86; N, 14.11% for $C_{24}H_{19}Cl_2N_5O_3$.

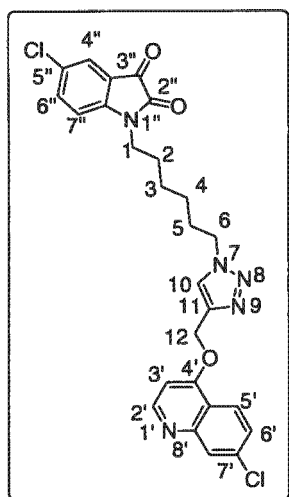
5-Chloro-1-[5-[4-(7-Chloro-quinolin-4-yloxymethyl)-[1,2,3]triazol-1-yl]-pentyl]-1H-indole-2,3-dione, 4.145i (NAT117)



Orange amorphous powder (93 mg, 42%), m. p. 57-59 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.20; IR ν_{max} (nujol)/ cm^{-1} 1725 (C=O), 1617 (C=O); δ_H (400 MHz, $CDCl_3$) 8.76 (1H, d, J 5.1, H-2'), 8.11 (1H, d, J 9.0, H-5'), 8.02 (1H, d, J 2.0, ArH-4''), 7.70 (1H, s, H-9), 7.55 (1H, dd, J 2.0 and 9.0, ArH-6''), 7.53 (1H, d, J 2.2, H-8'), 7.43 (1H, dd, J 2.2 and 9.0, H-6'), 6.95 (1H, d, J 5.1, H-3'), 6.81 (1H, d, J 9.0, ArH-7''), 5.44 (2H, s, CH_2 -11),

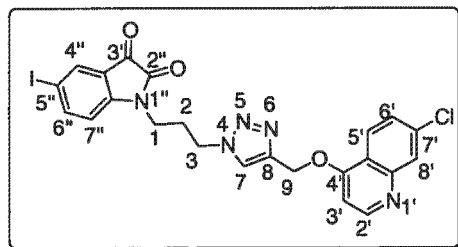
4.39 (2H, t, J 7.0, CH_2 -5), 3.69 (2H, t, J 7.0, CH_2 -1), 2.02 (2H, quint, J 7.0, CH_2 -4), 1.74 (2H, quint, J 7.0, CH_2 -2) and 1.39 (2H, quint, J 7.0, CH_2 -3); δ_{c} (100 MHz, CDCl_3) 183.0 (C=O), 161.5 (C=O), 152.5, 149.0 (2C), 142.9, 137.8, 136.0, 129.8, 128.1, 126.7, 125.6, 123.5, 123.1, 120.0, 118.6, 118.0, 111.4, 101.6, 62.4, 50.2, 39.8, 29.5, 26.5 and 23.6; LRMS (FAB) m/z 510 (3%, M^+); Found: C, 58.80; H, 4.17; N, 13.70%; Anal. Calc.: C, 58.83; H, 4.15; N, 13.72% for $\text{C}_{25}\text{H}_{21}\text{Cl}_2\text{N}_5\text{O}_3$.

5-Chloro-1-[6-[4-(7-Chloro-quinolin-4-yloxymethyl)-[1,2,3]triazol-1-yl]-hexyl]-1H-indole-2,3-dione, 4.145j (NAT118)



Orange needles (130 mg, 54%), m. p. 136-138 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.33; IR ν_{max} (nujol)/ cm^{-1} 1725 (C=O), 1617 (C=O); δ_{H} (400 MHz, CDCl_3) 8.76 (1H, d, J 5.1, H-2'), 8.34 (1H, s, H-10), 8.08 (1H, d, J 9.0, H-5'), 7.94 (1H, d, J 2.1, H-8'), 7.54 (1H, dd, J 2.1 and 8.3, ArH-6''), 7.55 (1H, d, J 2.1, ArH-4''), 7.52 (1H, dd, J 2.1 and 9.0, H-6'), 7.25 (1H, d, J 5.1, H-3'), 7.15 (1H, d, J 8.3, ArH-7'), 5.44 (2H, s, CH_2 -12), 4.36 (2H, t, J 7.0, CH_2 -6), 3.59 (2H, t, J 7.0, CH_2 -1), 1.81 (2H, quint, J 7.0, CH_2 -5), 1.54 (2H, quint, J 7.0, H-2), 1.32 (2H, quint, J 7.0, CH_2 -3) and 1.24 (2H, quint, J 7.0, CH_2 -4); δ_{c} (100 MHz, CDCl_3) 182.8 (C=O), 161.1 (C=O), 152.7, 150.0, 149.1, 143.0, 137.9, 136.1, 129.9, 128.1, 126.8, 125.7, 123.7, 123.2, 119.8, 118.7, 111.5, 111.0, 101.8, 62.6, 50.5, 40.0, 30.1, 27.0, 26.2 and 26.0; LRMS (FAB) m/z 524 (18%, M^+); Found: C, 59.50; H, 4.40; N, 13.30%; Anal. Calc.: C, 59.55; H, 4.42; N, 13.36% for $\text{C}_{26}\text{H}_{23}\text{Cl}_2\text{N}_5\text{O}_3$.

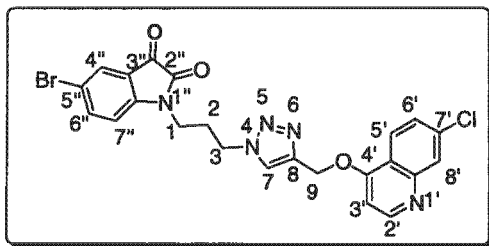
1-[3-[4-(7-Chloro-quinolin-4-yloxymethyl)-[1,2,3]triazol-1-yl]-propyl]-5-iodo-1H-indole-2,3-dione, 4.145k (NAT119)



Orange amorphous powder (80 mg, 31%), m. p. 81-84 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.20; IR ν_{max} (nujol)/ cm^{-1} 1725 (C=O), 1617 (C=O); δ_{H} (400 MHz, CDCl_3) 8.76 (1H, d, J 5.1, H-2'), 8.14

(1H, d, J 8.8, H-5'), 8.02 (1H, d, J 2.0, H-8'), 7.88 (1H, s, H-7), 7.87 (1H, dd, J 2.0 and 8.0, ArH-6''), 7.86 (1H, d, J 2.0, ArH-4''), 7.43 (1H, dd, J 2.0 and 8.8, H-6'), 6.96 (1H, d, J 5.1, H-3'), 6.71 (1H, d, J 8.0, ArH-7''), 5.45 (2H, s, CH₂-9), 4.46 (2H, t, J 6.6, CH₂-3), 3.80 (2H, t, J 6.6, CH₂-1) and 2.43 (2H, quint, J 6.6, CH₂-2); δ_c (100 MHz, CDCl₃) 181.4 (C=O), 160.8 (C=O), 152.4, 149.8, 149.4, 142.8, 138.6, 135.9, 134.2, 127.9, 126.7, 125.7, 124.2, 124.1, 123.4, 119.7, 119.1, 112.1, 110.0, 101.5, 62.2, 47.7 and 37.6; LRMS (FAB) m/z 574 (50%, M⁺); Found: C, 48.20; H, 3.03; N, 12.21%; Anal. Calc.: C, 48.15; H, 2.99; N, 12.21% for C₂₃H₁₇ClIN₅O₃.

5-Bromo-1-[3-[4-(7-Chloro-quinolin-4-yloxymethyl)-[1,2,3]triazol-1-yl]-propyl]-1H-indole-2,3-dione, 4.145I (NAT121)

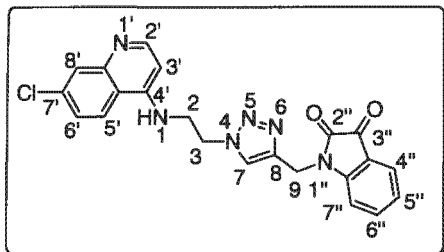


Orange amorphous powder (80 mg, 21%), m. p. 86-88 °C (from CH₃CN); R_f (MeOH:DCM 1:19) 0.43; IR ν_{max} (nujol)/cm⁻¹ 1725 (C=O), 1617 (C=O); δ_H (400 MHz, CDCl₃) 8.75 (1H, d, J 5.1, H-2'), 8.14 (1H, d, J 8.8, H-5'), 8.03 (1H, d, J

2.0, ArH-4''), 7.88 (1H, s, H-7), 7.70 (1H, dd, J 2.0 and 8.2, ArH-6''), 7.68 (1H, d, J 2.0, H-8'), 7.43 (1H, dd, J 2.0 and 8.8, H-6'), 6.96 (1H, d, J 5.1, H-3'), 6.83 (1H, d, J 8.2, ArH-7''), 5.45 (2H, s, CH₂-9), 4.46 (2H, t, J 6.6, CH₂-3), 3.81 (2H, t, J 6.6, CH₂-1) and 2.42 (2H, quint, J 6.6, CH₂-2); δ_c (100 MHz, CDCl₃) 183.4 (C=O), 161.4 (C=O), 152.4, 149.9, 145.9, 140.7 (2C), 135.8, 128.5, 127.9, 126.7, 125.7, 124.2 (2C), 123.4, 118.8, 117.1, 111.7, 109.9, 101.5, 62.2, 47.7 and 37.6; LRMS (FAB) m/z 527 (100%, M⁺); Found: C, 52.45; H, 3.23; N, 13.32%; Anal. Calc.: C, 52.44; H, 3.25; N, 13.29% for C₂₃H₁₇BrClIN₅O₃.

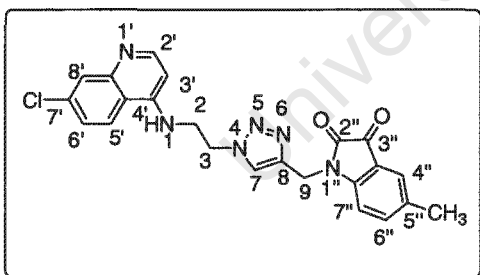
The conditions employed for the preparation of compounds 4.153 and 4.154 were those described in General Method H (b).

1-[1-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-1H-[1,2,3]triazol-4-ylmethyl]-1H-indole-2,3-dione, 4.153 (NAT141)



Orange amorphous powder (45 mg, 20%), m. p. 140-145 °C (from CH₃CN); *R_f* (MeOH:DCM 1:19) 0.35; IR ν_{max} (nujol)/cm⁻¹ 3355 (N-H), 1725 (C=O), 1617 (C=O); δ_{H} (300 MHz, DMSO-*d*₆) 8.34 (1H, d, *J* 5.4, H-2'), 8.09-8.06 (2H, m, Ar*H*), 8.01 (1H, d, *J* 8.7, H-5'), 7.78 (1H, d, *J* 2.4, H-8'), 7.54-7.51 (2H, m, Ar*H*), 7.42 (1H, dd, *J* 2.4 and 8.7, H-6'), 7.30 (1H, s, H-7), 7.14 (1H, bt, *J* 6.0, N*H*-1), 6.47 (1H, d, *J* 5.4, H-3'), 4.93 (2H, s, CH₂-9), 4.63 (2H, t, *J* 6.0, CH₂-3) and 3.81 (2H, q, *J* 6.0, CH₂-2); δ_{C} (75 MHz, DMSO-*d*₆) 183.0 (C=O), 157.6 (C=O), 151.4, 150.1, 149.8, 148.5, 141.3, 138.0, 133.6, 127.1, 124.4, 124.1, 123.8, 123.3, 117.4, 117.3, 111.1 (2C), 98.7, 48.0, 42.2 and 34.9; LRMS (FAB) *m/z* 433 (25%, M⁺); Found: C, 61.02; H, 4.02; N, 19.39%; Anal. Calc.: C, 61.04; H, 3.96; N, 19.42% for C₂₂H₁₇ClN₆O₂.

1-[1-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-1H-[1,2,3]triazol-4-ylmethyl]-5-methyl-1H-indole-2,3-dione, 4.154 (NAT142)

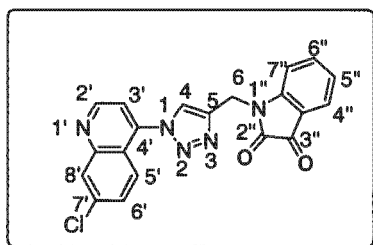


Orange amorphous powder (24 mg, 81%), m. p. 135-137 °C (from CH₃CN); *R_f* (MeOH:DCM 1:19) 0.38; IR ν_{max} (nujol)/cm⁻¹ 3355 (N-H), 1725 (C=O), 1617 (C=O); δ_{H} (300 MHz, DMSO-*d*₆) 8.31 (1H, d, *J* 5.4, H-2'), 8.09 (1H, bs, N*H*), 8.05 (1H, d, *J* 9.3, H-5'), 7.76 (1H, d, *J* 1.8, H-8'), 7.39 (1H, dd, *J* 1.8 and 9.3, H-6'), 7.35 (1H, s, H-7), 7.31-7.28 (2H, m, Ar*H*-4'' and Ar*H*-6''), 6.93 (1H, d, *J* 8.1, Ar*H*-7''), 6.44 (1H, d, *J* 5.4, H-3'), 4.88 (2H, s, CH₂-9), 4.58 (2H, t, *J* 6.0, CH₂-3), 3.74 (2H, t, *J* 6.6, CH₂-2) and 2.24 (3H, s, CH₃-5'); δ_{C} (75 MHz, DMSO-*d*₆) 183.2 (C=O), 157.7 (C=O), 150.8, 150.2, 147.9 (2C), 141.3, 138.6, 126.9, 124.6, 124.1, 123.9, 119.7, 117.4, 115.0, 112.7, 110.9, 103.2, 98.6, 63.1, 48.0, 45.6 and 20.0; LRMS

(FAB) m/z 447 (50%, M^+); Found: C, 61.80; H, 4.26; N, 18.80%; Anal. Calc.: C, 61.82; H, 4.29; N, 18.81% for $C_{23}H_{19}ClN_6O_2$

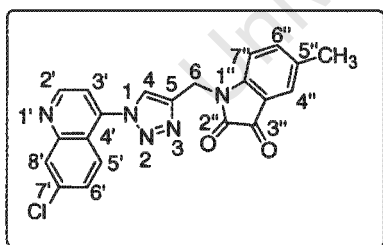
The conditions employed for the preparation of compounds 4.156 and 4.157 were those described in General Method H (c).

1-[1-7-Chloro-quinolin-4-yl]-1H-[1,2,3]triazol-4-ylmethyl]-1H-indole-2,3-dione, 4.156 (NAT150)



Pale orange amorphous powder (100 mg, 26%), m. p. 255-258 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.37; IR ν_{max} (nujol)/ cm^{-1} 1725 (C=O), 1617 (C=O); δ_H (400 MHz, DMSO- d_6) 9.11 (1H, d, J 4.7, H-2'), 8.87 (1H, s, H-4), 8.25 (1H, d, J 2.1, H-8'), 7.97 (1H, d, J 9.2, H-5'), 7.70 (1H, d, J 4.7, H-3'), 7.76 (1H, dd, J 2.1 and 9.2, H-6'), 7.63-7.14 (4H, m, ArH') and 5.14 (2H, s, CH_2 -6); δ_C (100 MHz, DMSO- d_6) 182.8 (C=O), 156.5 (C=O), 152.5, 150.4, 150.1, 142.3, 139.9, 138.4, 135.3, 128.9, 128.1, 125.9, 125.3, 124.5, 123.4, 120.1, 117.7, 116.8, 111.1 and 34.9; LRMS (FAB) m/z 390 (65%, M^+); Found: C, 61.63; H, 3.10; N, 17.95%; Anal. Calc.: C, 61.63; H, 3.10; N, 17.97% for $C_{20}H_{12}ClN_5O_2$.

1-[1-7-Chloro-quinolin-4-yl]-1H-[1,2,3]triazol-4-ylmethyl]-5-methyl-1H-indole-2,3-dione, 4.157 (NAT151)

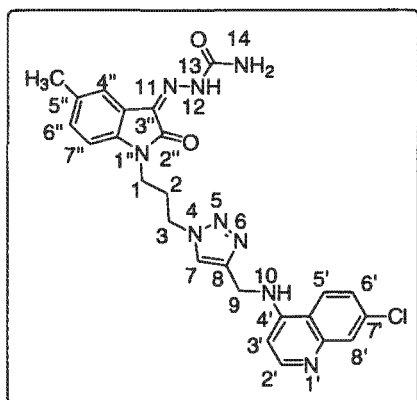


Orange amorphous powder (150 mg, 38%), m. p. 240-243 °C (from CH_3CN); R_f (MeOH:DCM 1:19) 0.37; IR ν_{max} (nujol)/ cm^{-1} 1725 (C=O), 1617 (C=O); δ_H (400 MHz, DMSO- d_6) 9.11 (1H, d, J 4.7, H-2'), 8.86 (1H, s, H-4), 8.25 (1H, d, J 2.1, H-8'), 7.97 (1H, d, J 9.2, H-5'), 7.74 (1H, d, J 4.7, H-3'), 7.76 (1H, dd, J 2.1 and 9.2, H-6'), 7.46-7.15 (3H, m, ArH'), 5.12 (2H, s, CH_2 -6) and 2.27 (3H, s, CH_3 -5''); δ_C (100 MHz, DMSO- d_6) 182.8 (C=O), 156.5 (C=O), 152.5, 150.4, 150.1, 141.9, 140.1, 138.4, 132.8, 128.9, 128.1, 125.9, 125.4, 124.8, 124.6, 122.1, 117.8, 116.8, 111.1, 34.9 and 20.1; LRMS (FAB) m/z 404 (5%, M^+); Found: C, 62.40; H, 3.48; N, 17.30%; Anal. Calc.: C, 62.46; H, 3.49; N, 17.34% for $C_{21}H_{14}ClN_5O_2$.

I. General procedure for the preparation of compounds 4.158, 4.159 and 4.160

A mixture of triazole isatin derivative (200 mg, 0.43 mmol) and thiosemicarbazide-HCl (40 mg, 0.43 mmol, 1.0 eq.) were dissolved in warm 99.99% ethanol. The heterogeneous mixture was vigorously stirred for 12 h at 45 °C. The reaction mixture was then cooled to 0 °C to assist formation of a yellow precipitate. The product was separated by filtration, vacuum dried and recrystallized from acetonitrile.

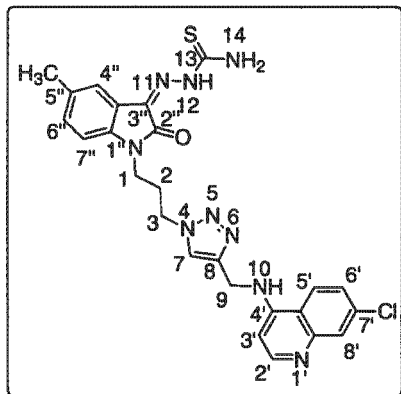
[1-(3-{4-[(7-Chloro-quinolin-4-ylamino)-methyl]-[1,2,3]triazol-1-yl}-propyl)-5-methyl-2-oxo-1,2-dihydro-indo-3-ylidene]-hydrazide, 3"-semicarbazide, 4.158 (NAT138)



Orange amorphous powder (95 mg, 43%), m. p. 224-227 °C (from CH₃CN); *R_f* (MeOH:DCM 1:9) 0.12 IR ν_{max} (nujol)/cm⁻¹ 3655 (N-H), 3410 (N-H), 1637 (C=O), 1517 (C=N); δ_{H} (300 MHz, DMSO-*d*₆) 11.58 (1H, s, N-H-12), 8.38 (1H, d, *J* 5.4, H-2'), 8.26 (1H, d, *J* 9.0, H-5'), 8.02 (1H, s, H-7), 7.87 (1H, bt, *J* 5.4, N-H-10), 7.78 (1H, d, *J* 2.4, H-8'), 7.54 (1H, d, *J* 2.4, ArH-4''), 7.45 (1H, dd, *J* 2.4 and 9.0, H-6'), 7.10 (1H, dd, *J* 2.4 and 8.0, ArH-6''), 7.05 (2H, bs, N-H₂), 6.94 (1H, d, *J*

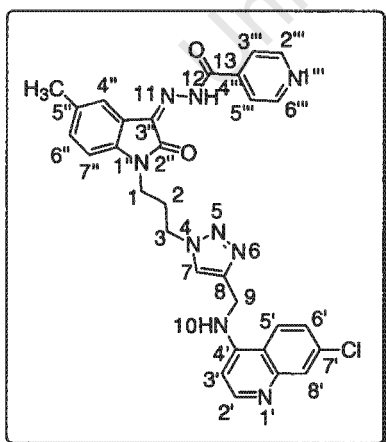
8.0, ArH-7'), 6.57 (1H, d, *J* 5.4, H-3'), 4.58 (2H, d, *J* 5.4, CH₂-9), 4.38 (2H, t, *J* 6.8, CH₂-3), 3.72 (2H, t, *J* 6.8, CH₂-1), 2.27 (3H, s, ArCH₃-5'') and 2.17 (2H, quint, *J* 6.8, CH₂-2); δ_{C} (75 MHz, DMSO-*d*₆) 179.4 (C=O), 161.8 (C=O), 151.9, 151.3, 150.3, 144.8, 141.4, 140.0, 134.7, 133.5, 133.2, 127.3, 124.4, 123.8, 123.2, 121.9, 119.6, 117.9, 110.4, 100.0, 47.9, 38.4, 37.6, 28.0 and 21.0; LRMS (FAB) *m/z* 518 (20%, M⁺); Found: C, 57.90; H, 4.75; N, 24.25; Anal. Calc.: C, 57.97; H, 4.67; N, 24.34% for C₂₅H₂₄ClN₉O₂.

[1-(3-{4-[(7-Chloro-quinolin-4-ylamino)-methyl]-[1,2,3]triazol-1-yl}-propyl)-5-methyl-2-oxo-1,2-dihydro-indo-3-ylidene]-hydrazide, 3"-thiosemicarbazide, 4.159 (NAT130)



Yellow amorphous powder (170 mg, 77%), m. p. 241-243 °C (from CH₃CN); *R_f* (MeOH:DCM 1:9) 0.13; IR $\nu_{\max}$ (nujol)/cm⁻¹ 3655 (N-H), 3410 (N-H), 1637 (C=O), 1517 (C=N), 1132 (C=S); δ_{H} (300 MHz, DMSO-*d*₆) 12.35 (1H, s, N-H-12), 8.98 (1H, s, N-H-14), 8.64 (1H, s, N-H-14), 8.38 (1H, d, *J* 5.4, H-2'), 8.26 (1H, d, *J* 9.0, H-5'), 8.03 (1H, s, H-7), 7.87 (1H, bt, *J* 5.6, N-H-10), 7.78 (1H, d, *J* 2.4, H-8'), 7.54 (1H, d, *J* 2.4, ArH-4''), 7.45 (1H, dd, *J* 2.4 and 9.0, H-6'), 7.14 (1H, dd, *J* 2.4 and 8.0, ArH-6''), 6.93 (1H, d, *J* 8.0, ArH-7''), 6.57 (1H, d, *J* 5.4, H-3'), 4.57 (2H, d, *J* 5.6, CH₂-9), 4.40 (2H, t, *J* 6.8, CH₂-3), 3.73 (2H, t, *J* 6.8, CH₂-1), 2.29 (3H, s, ArCH₃-5'') and 2.17 (2H, quint, *J* 6.8, CH₂-2); δ_{C} (75 MHz, DMSO-*d*₆) 179.4 (C=S), 160.8 (C=O), 152.2, 149.7, 149.7, 149.3, 144.6, 140.7, 132.1, 131.4, 127.4, 125.7, 124.2, 123.9, 123.0, 121.2, 119.5, 118.2, 117.5, 109.5, 47.0, 37.8, 36.6, 27.7 and 20.5; LRMS (FAB) *m/z* 534 (100%, M⁺); Found: C, 56.19; H, 4.44; N, 23.71; S, 5.98%; Anal. Calc.: C, 56.23; H, 4.53; N, 23.61; S, 6.00% for C₂₅H₂₄ClN₉OS.

Isonicotinic acid [1-(3-{4-[(7-Chloro-quinolin-4-ylamino)-methyl]-[1,2,3]triazol-1-yl}-propyl)-5-methyl-2-oxo-1,2-dihydro-indo-3-ylidene]-hydrazide, 4.160 (NAT139)



Yellow amorphous powder (150 mg, 60%), m. p. 180-182 °C (from CH₃CN); *R_f* (MeOH:DCM 1:9) 0.10 IR $\nu_{\max}$ (nujol)/cm⁻¹ 3655 (N-H), 3410 (N-H), 1637 (C=O), 1517 (C=N); δ_{H} (400 MHz, DMSO-*d*₆) 10.90 (1H, s, N-H-12), 8.76 (2H, d, *J* 5.1, H-2''' and H-6'''), 8.41 (1H, d, *J* 5.5, H-2'), 8.30 (1H, d, *J* 9.0, H-5'), 8.00 (1H, s, H-7), 7.93 (1H, bs, N-H-10), 7.84 (2H, d, *J* 5.1, H-3''' and H-5'''), 7.80 (1H, d, *J* 2.1, H-8'), 7.82 (1H, d, *J* 1.8, ArH-4''), 7.45 (1H, dd, *J* 2.1 and 9.0, H-6'), 7.15 (1H, dd, *J* 1.8 and 7.8, ArH-6''), 6.87 (1H, d, *J* 7.8, ArH-7''), 6.57

(1H, d, J 5.5, H-3'), 4.60 (2H, s, CH₂-9), 4.38 (2H, t, J 6.8, CH₂-3), 3.72 (2H, t, J 6.8, CH₂-1), 2.30 (3H, s, ArCH₃-5'') and 2.17 (2H, quint, J 6.8, CH₂-2); δ_c (100 MHz, DMSO-*d*₆) 163.8 (C=O) 161.1 (C=O), 150.8, 149.9, 143.8, 140.7, 139.1, 132.6, 132.3, 131.0, 127.1, 124.6, 124.2, 124.5, 124.2, 124.0, 123.2, 123.1, 122.3 (2C), 121.4, 119.0, 117.2 (2C), 109.8, 47.0, 36.3, 30.7, 27.1 and 20.5; LRMS (FAB) m/z 580 (10%, M⁺); Found: C, 62.07; H, 4.45; N, 21.68%; Anal. Calc.: C, 62.12; H, 4.52; N, 21.73% for C₃₀H₂₆ClN₉O₂.

Procedures – Biological Testing

In vitro Activities of compounds against β -haematin formation

Assay of β -haematin formation in 96-well plates

Serial dilutions of the drug solutions were carried out in triplicate in a 96-well plate using a multichannel pipette. Concentrations of drug solution corresponded to 0–10 equivalents relative to hematin in the final mixture. Each well contained 10.12 μ l of drug solution. Then 101.2 μ l of hematin stock solution (1.680 mM in 0.1 M NaOH) was added to each well. After mixing the solutions, 58.7 μ l of acetate solution (12.9 M, pH 5.0) preincubated at 60 °C was added and the plate was incubated for 60 min at 60 °C. Thereafter, 80 μ l of 30% (v/v) pyridine solution in 20 mM Hepes, pH 7.5, was added at room temperature. Solids were resuspended and allowed to settle for 15 min at ambient temperature. Then 38 μ l of supernatant was transferred to another plate and diluted to 250 μ l with 30% (v/v) pyridine solution (pH 7.5, 20 mM Hepes). Data were collected using a microplate reader and analyzed as described above.¹³

High-throughput screening

This was carried out using 1 and 5 equivalents of drugs or related compounds relative to haematin. Haematin stock solution (20.2 μ l) was mixed with 2.02 μ l of each drug dissolved in 1.0 M HCl or methanol. Acetate solution (11.74 μ l, 12.9 M, pH 5.0) prewarmed at 60 °C was added. After 60 min incubation, 250 μ l of 12% (v/v) pyridine solution (pH 7.5, 20 mM Hepes, ambient temperature) was added. Supernatants were carefully transferred to a flat-bottomed 96-well plate. Detection of β -haematin inhibition was by visual inspection and by absorbance measurement at 405 nm.¹³

In vitro* Activities of compounds against D10 *P.falciparum

All samples were tested in duplicate on a single occasion against a chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10). Continuous *in vitro* cultures of asexual erythrocyte stages of *P.falciparum* were maintained using a modified method of Trager and Jensen¹⁴. Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase (pLDH) assay using a modified method described by Makler (1993)¹⁵. The pLDH assay compares well with other available assays (Noedl, 2003)¹⁶. The average percentage parasite viability was determined at three different concentrations. Compounds, which show significant activity at three concentrations, were tested for their 50% inhibitory concentration (IC₅₀) value.

All compounds were dissolved in 10% methanol / DMSO. The samples were then diluted with water to a concentration of 20ug/ml. Samples were stored at – 20°C until use. All samples were further diluted in complete medium to their desired concentrations on the day of the experiment. Compounds in table 1 were tested at 250 ng/ml, 125ng/ml and 62.5 ng/ml. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The drugs were tested at different concentrations to determine which one show more promising results. Chloroquine was used as the positive control in all experiments. The starting concentration for a full dose-response of active compounds was 1000 ng/ml. The starting concentration of chloroquine in a full dose-response was 100 ng/ml. The 50% inhibitory concentration (IC₅₀) values were obtained using a non-linear dose-response curve fitting analysis via GraphPad Prism® v.4.0 software.

***In vitro* Activities of compounds against Falcipains -2 and-3 and Rhodesain**

IC₅₀ values against the falcipains were determined as described by Greenbaum *et al.*¹⁷ A equal molar of recombinant protein (~ 1 nM) was incubated with different concentrations of the inhibitors (added from 100x stock solutions in DMSO) in 100 mM sodium acetate (pH 5.5)-10 mM dithiothreitol for 30 min at room temperature before addition of the

substrate benzoxycarbonyl-Leu_Arg-7-amino-4-methyl-coumarin (final concentration = 25 μ M). Fluorescence was continuously monitored for 30 min at room temperature in a Labsystem Fluoroskan® II spectrofluorometer. IC₅₀ values were determined from plots of activity over inhibitor concentration with GraphPad Prism® software.

In vitro Activities of compounds against W2 *P.falciparum*

Effects of inhibitors on the parasites development were determined as described by Rosenthal *et al.*¹⁸ Synchronized W-2 strain *P. falciparum* parasites¹⁹ were cultured with compound derivatives (added from 1000x stocks in DMSO) for 48 h beginning at the ring stage. The medium was changed after 24 h, while maintaining appropriate inhibitor concentration. Giemsa-stained smears were made after 48 h, when control cultures contained nearly all ring-stage parasites. The number of new ring forms per 500 erythrocytes was counted, and compared with those of controls cultured in 0.1% DMSO. IC₅₀s for growth inhibition were determined with GraphPad Prism software from plots of percentages of the level of parasitemia of the control relative to inhibitor concentration.

In vitro Activities of compounds against 3D7 and K1 *P.falciparum*

Two clones of *P. falciparum* are used: the 3D7 clone of NF54 which is known to be sensitive to all antimalarials, (b) the K1 strain originating from Thailand that is resistant to cloroquine and pyrimethamine, but sensitive to mefloquine. The cultures are naturally asynchronous (65-75% ring stage) and are all maintained in continuous lod phase growth in RPMI1640 medium supplement with 5% washed human A+ erythrocytes, 25 mM Hepes, 32 nM NaHCO₃, and AlbuMAXII (lipid-rich bovine serum albumin) (GIBCO, Grand Island, NY) (CM). All cultures and assays are conducted at 37 °C under an atmosphere of 5% CO₂ and 5% O₂, with a balance of N₂.

Drug sensitivity assays

Stock drug solutions are prepared in 100% DMSO (dimethylsulfoxide) at 20 mg/ml unless otherwise suggested by the supplier. The compound is further diluted to the appropriate concentration using complete RPMI1640 supplement with 15 nM cold

hypoxanthine and AlbuMAXII. Assays were performed in sterile 96-well microtitre plates; each plate contains 100 μ l of parasite culture (0.5% parasitaemia, 2.5% hematocrit). Each drug tested in triplicate and parasite growth compared to control and blank (uninfected erythrocytes) wells. After 24 h of incubation at 37 °C, 3.7Bq of [^{3} H] hypoxanthine is added to each well. Cultures are incubated for a further 24 h before they are harvested onto glass-fiber filter mats. The radioactivity is counted using a Wallac Microbeta 1450 scintillation counter. The results are recorded as counts per minute (CPM) per well at each drug concentration, control and blank wells. Percentage inhibition is calculated from comparison to blank and control wells, and IC₅₀ values calculated using Microsoft XLFit line fitting software (IDBS, UK).

Primary Screening

The preliminary screen uses the 3D7 strain. The compounds are tested at 6 concentrations (30, 10, 3, 1, 0.3 and 0.1 μ g/ml). If the compound does not affect parasite growth at 10 μ g/ml it is classified as inactive, between 10 and 1 μ g/ml, the compound is designated as partially active, and if <1 μ g/ml, the compound is classified as active and is further evaluated by three-fold serial dilutions in a repeated test.

Secondary Screening

Both the 3D7 clone and the K1 line are used. The compound is diluted three-fold over at 12 different concentrations with the appropriate starting concentration based on preliminary screen. The IC₅₀ is determined by a sigmoidal dose response analysis using Microsoft XLFit™ (IDBS, UK). For each assay, the IC₅₀ and IC₉₀ values for each parasite line are determined against the known anti-malarial chloroquine, plus other standard compounds appropriate for the assay.

In vitro Activities of compounds against *Trypanosoma brucei brucei* S427

The bloodstream form trypanostigotes are maintained in MEM medium with Earle's salts supplemented with 25 mM HEPES, 1 g/l additional glucose, 10 ml/l MEM non-essential aminoacids (100x), 0.2 mM 2-mercaptoethanol, 2 mM Na-pyruvate, 0.1 mM

hypoxanthine, 0.05 mM bathocuprionedisulphonic acid, 0.15 mM L-cysteine and 15% heat inactivated, foetal calf serum. All cultures and assays are conducted at 37 °C under an atmosphere of 5% CO₂/95%air mixture.

Drug sensitivity assays

Stock drug solutions are prepared in 100% DMSO unless otherwise suggested by supplier at 20 mg/ml, and ball milled or sonicated if necessary. The stocks are kept at 4 °C. For the assays, the compound is further diluted to the appropriate concentration using complete medium. Assays are performed in sterile 96-well microtitre plates, each well containing 100 µl of parasite culture (1 x 10⁴ bloodstream forms) with or without serial drug dilutions at 37 °C for 72 h in 5% CO₂. The highest concentration for the test is 30 µg/ml. Each drug is tested in triplicate. A 3-fold serial dilution is performed down to a suitable concentration to obtain an IC₅₀ value. Initial testing is 30, 10, 3 and 0.1 µg/ml. Control drug is Pentamidine and is diluted down to 0.0001 (12 dilutions). Control wells are without drug, blank are medium only. After 72 h of incubation the plates are inspected under an inverted microscope to assure growth of the controls and to determine the minimum inhibitory concentration (MIC): this is the lowest drug concentration at which no trypanosomes with normal morphology and motility as compared to the control wells can be seen.

20 µl of Alamar Blue are added to each well and the plates incubated for another 2-4 h. Then the plates are read on a Gemini Plate Reader (Molecular Devices) using an excitation wave length of 530 nm and an emission wave length of 580 nm (cut-off 550 nm).

Primary Screening

The preliminary screen uses the *Trypanosoma b. brucei* strain. The compounds are tested at 7 concentrations (drug concentration range from 30 µg/ml to 0.1 µg/ml in 3-fold dilutions). In this assay pentamidine had an Ed₅₀ value of 0.1 to 0.02 µg/ml.

Secondary Screening

Active compounds (IC₅₀ <0.2 µg/ml) are tested again over the appropriate dose range against *Trypanosoma brucei rhodiense* STIB 900 to confirm IC₅₀ and IC₉₀ values in comparison to standard pentamidine and internal chemical group standard if available.

Toxicity Tests

Cell cultures

KB-cells-a cell line derived from a human carcinoma of the nasopharynx, typically used as an assay for antineoplastic agents.

KB cells are maintained as monolayers in RPMI1640 + 10% HIFCS.

All cultures and assays are conducted at 37 °C under an atmosphere of 5% CO₂/95% air mixture.

Drug toxicity

Stock drug solutions are prepared in 100% DMSO unless otherwise suggested by the suppliers at 20 µg/ml, and ball milled or sonicated if necessary. The stocks are kept at 4 °C. For the assays, the compound is further diluted to the appropriate concentration using complete medium.

Day1: KB cells harvested and washed in serum-free medium (2000 rpm, 10 mins. 4 °C) and resuspended in fresh medium (RPMI 1640 + 10% HIFC) at a concentration of 4×10^4 /ml. 100 µl is added to wells on a 96 well plate (4×10^3 /well). The plates are incubated overnight at 37 °C, 5% CO₂/air mix to allow the cells to adhere.

Day2: Test compounds are prepared in 100% DMSO 20mg/mg and diluted down to starting concentration of 600 µg/ml (2x top concentration) with RPMI 1640 + 10% HIFC. Control wells had no drug.

A 10-fold serial dilution is performed across the plate – 300, 30, 3 etc.

Podophyllotoxin is the control drug.

The plate is incubated for 72 h at 37 °C, 5% CO₂/air.

Day5: Each well is assessed by microscope observation. 20 µl Alamar Blue™ is then added to each well. Plates are incubated for a further 2-4 h before reading (Gemini), EX/EM 530/580, cut-off 550 nm.

IC₅₀ (IC₉₀) values calculated using sigmoidal regression analysis (MS *xfit*™)

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