Single and Hybrid Antimalarials Based on Artemisinin, Chloroquine and β-Lactams: Synthesis, Antiplasmodial Activity, Cytotoxicity and Effect of Selected Artemisinin-Chloroquine Hybrids on the Parasitic Endocytosis Pathway

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µg	Microgram(s)
µM	Micromolar
ACT	Artemisinin combination therapies
ADP	Adenosine diphosphate
Ar	Aromatic
Art	Artemisinin
Artes	Artesunate
ATP	Adenosine triphosphate
$BF_3 \cdot Et_2O$	Boron trifluoride etherate
br s	Broad singlet (in ^{1}H NMR)
^{13}C NMR	Carbon Nuclear Magnetic Resonance
$CDCl_3$	Deuterochloroform
CD_3OD	Deuteromethanol
CH_3CN	Acetonitrile
CNS	Central nervous system
COSY	Correlation spectroscopy (in NMR)
CQ	Chloroquine
Cys	Cysteine
d	Doublet (in ^{1}H NMR)
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
dd	Doublet of doublet (in ^{1}H NMR)
ddd	Doublet of doublet of doublets (in ^{1}H NMR)
dH_2O	Distilled water
D_2O	Deuterowater
DEA	Diethylamine
DHA	Dihydroartemisinin
DMF	*N*,*N*'-Dimethylformamide
DML	Designed Multiple Ligand
DMSO	Dimethylsulfoxide
DMSO-d_6	Deuterodimethylsulfoxide
DNA	Deoxyribonucleic acid
EtOAc	Ethyl acetate
EtOH	Ethanol

Et_3N	Triethylamine
eq	Equivalent(s)
Fe[III]PPIX	Ferriprotoporphyrin IX
FV	Food vacuole
GLA	Glutaraldehyde
GR	Glutathione reductase
h	Hour(s)
^{1}H NMR	Proton Nuclear Magnetic Resonance
HAP	Histo-aspartic protease
Hb	Haemoglobin
Hex	Hexane
His	Histidine
H_2O	Water
HSQC	Heteronuclear single quantum coherence (in NMR)
Hz	Hertz
IC_{50}	50% Inhibitory concentration
IUPAC	International Union of Pure and Applied Chemistry
J	Coupling constant
K_2CO_3	Potassium carbonate
KOH	Potassium hydroxide
Leu	Leucine
m	Multiplet (in ^{1}H NMR)
M	Molar
mA	MilliAmpere(s)
MCR	Multi-component reactions
MDR	Multidrug resistance
MeOH	Methanol
min	Minute(s)
ml	Millilitre
mM	Millimolar
m.p.	Melting point
m/z	Mass to charge ratio
$NaBH_4$	Sodium borohydride
$NaCNBH_3$	Sodium cyanoborohydride
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH	Sodium hydride

NaOMe	Sodium methoxide
Na_2SO_4	Sodium sulfate
nM	Nanomolar
NMP	*N*-Methyl-2-pyrrolidine
PBP	Penicillin-binding proteins
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
pfcrt	*P. falciparum* chloroquine resistance transporter gene
PfCRT	*P. falciparum* chloroquine resistance transporter protein
pfmdr1	*P. falciparum* multidrug resistance 1
Pgh 1	P-glycoprotein homologue 1
Pgp	P-glycoprotein
Ph	Phenyl
PHA	Phytohaemagglutinin
Phe	Phenylalanine
PM	Plasmepsin(s)
ppm	Parts per million
PPh_3	Triphenyl phosphine
q	Quartet (in ^{1}H NMR)
QSAR	Quantitative structure-activity relationship
RBC	Red blood cell(s)
R_f	Retention factor
RI	Resistance Index
s	Singlet (in ^{1}H NMR)
SAR	Structure-activity relationship
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
$SO_3 \cdot Pyr$	Sulfur trioxide/pyridine complex
t	Triplet (in ^{1}H NMR)
td	Triple of doublets (in ^{1}H NMR)
THF	Tetrahydrofuran
TI	Therapeutic index
TLC	Thin layer chromatography
TMSCl	Tetramethylsilyl chloride
U-4CC	Ugi four-component condensation
Ugi 3C-4CC	Ugi 3-component 4-centre condensation
V	Volt(s)
WHO	World Health Organization

ABSTRACT

Malaria remains to be one of the leading causes of morbidity and mortality throughout recorded history. It is caused by protozoan parasites of the genus *Plasmodium*, where *P. falciparum* is the most lethal. Current estimates are that over 500 million people are afflicted, while 3 million people die annually. With the emergence of resistance to antimalarial drugs in the malaria parasite, it is critical to develop new chemotherapeutic agents that can combat the disease and/or overcome resistance. This may be achieved by identifying molecules that target or interfere with unique parasitic pathways such as haemoglobin degradation or parasitic endocytosis.

This thesis describes the design and synthesis of novel antimalarial agents based on the 'Designed Multiple Ligand' approach. Compounds were synthesized via conjugate addition or multi-component condensation reaction. 4-Aminoquinolines were hybridized with artemisinin or 1,4-naphthoquinone derivatives; selected hybrids were further investigated for their effect on the parasitic endocytosis pathway and compared to the effect of chloroquine and artemisinin on the same pathway. The effects of drug treatment on the morphology and haemoglobin levels in the parasites as well as localization of transport vesicles via immunofluorescence microscopy were determined.

A series of β-lactam derivatives containing a terminal acetylene moiety were synthesized via the Staudinger and Ugi 3-component 4-centre condensation reactions. The compound with the best activity from the series was used to couple these reactions to post-condensation chemical modifications via the Mannich reaction, another multi-component reaction, to

create a more diversified library. A small series of 4-aminoquinoline analogues, including amodiaquine-like compounds and bisquinoline derivatives, was also prepared in an attempt to elucidate their structure-activity relationships.

The antiplasmodial and cytotoxic activities were determined for all compounds; where applicable, assays on β-haematin inhibitory activity were also carried out.

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CHAPTER 1

INTRODUCTION TO MALARIA

Malaria or a disease resembling malaria has infected humans for over 50 000 years,[1] where references of the unique periodic fevers were found throughout recorded history, as far back as 2700 BC in China;[2] early documentations were also found in Greece and ancient Egypt. Due to its association with swamps and wetlands, malaria was termed "*mala aria*" – which translates to 'bad air' in Medieval Italian. In 1880, Charles Louis Alphonse Laveran was the first to discover that the cause of malaria is a protozoan, and was awarded the Nobel Prize in 1907.[3] The discovery that mosquitoes transmit malaria was made in 1897 by Ronald Ross, a British officer in the Indian Medical Service, where he demonstrated that mosquitoes could transmit malaria parasites from bird to bird, and established the complete parasite life cycle. He was awarded the Nobel Prize in 1902 for his pioneering work.[4]

1.1 World Distribution

Of all the parasitic tropical diseases occurring in man, malaria is the most dangerous and widespread disease, and is the fifth deadliest infectious disease worldwide. About 350–500 million cases arise annually, and contributing to approximately 1 million deaths,[5] especially among children and pregnant women.[6] Malaria is transmitted to humans by four species of parasitic protozoa, *Plasmodium falciparum*, *vivax*, *malariae* and *ovale*.[7] Of these, *P. falciparum* is the most important, as it causes almost all the deaths associated with malaria. *P. falciparum* exists in over 100 countries, but is mainly confined to poorer tropical areas of Africa, Asia and Latin America (Fig. 1.1).[6,7] *P. vivax* is distributed worldwide in tropical and

some temperate zones, *P. ovale* is mainly confined in tropical West Africa, and *P. malariae* is found worldwide but very locally distributed.[8] More than 90% of malaria cases and the great majority of malaria deaths occur in tropical Africa.[7] It also poses risk to travellers and immigrants, with imported cases increasing in non-endemic areas.[6] The spreading of the disease to these areas may also be a result of changing land utilization, especially plantation agriculture, which led to increasing cases in highland areas of East Africa.[7]

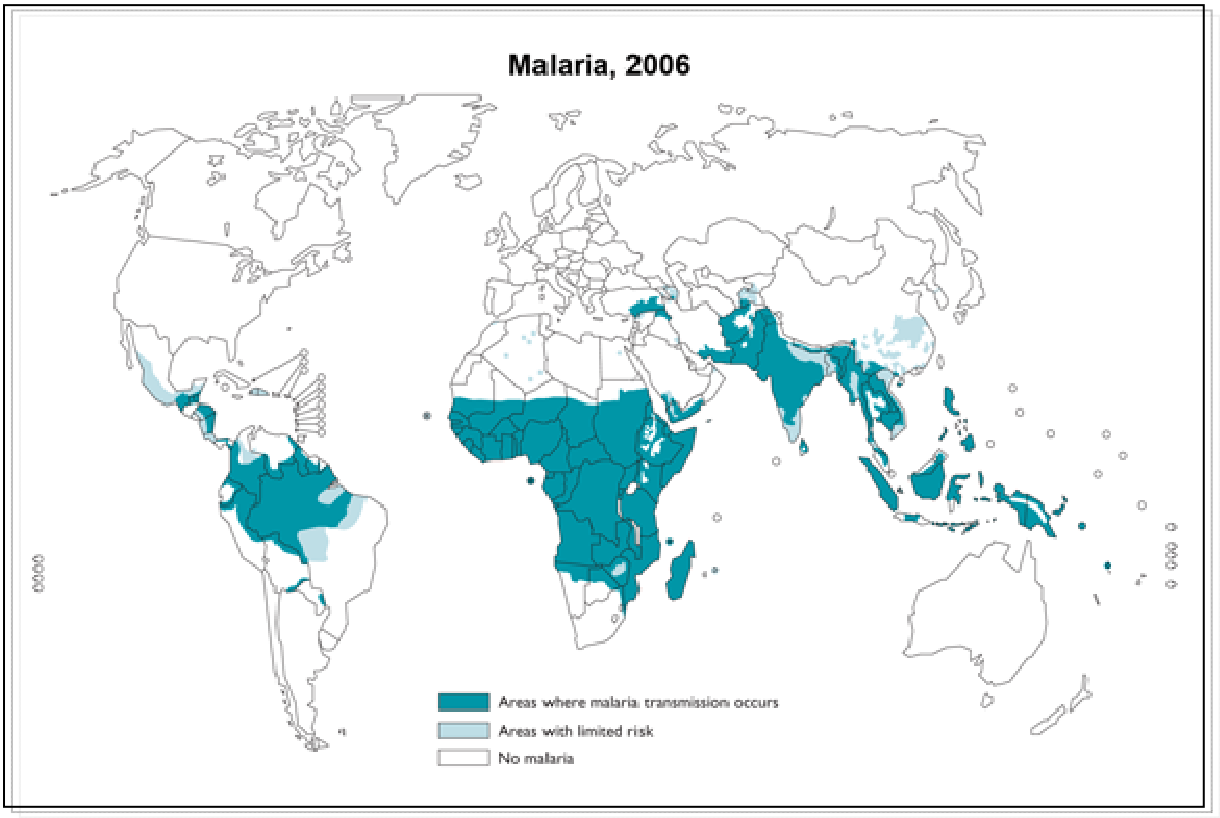


Figure 1.1. World distribution of malaria infections.[9]

1.2 Life Cycle of the Malaria Parasite

The human malaria parasite has a complex life cycle (Fig. 1.2) that requires both human host (carrier) and an insect vector. The parasite reproduces sexually in the Anopheles mosquito.

When in people, they reproduce asexually (by cell division) first in the liver cells (hepatocytes), then repeatedly in the red blood cells (erythrocytes). A person is infected with malaria when bitten by an infected female Anopheles mosquito. The mosquito sucks blood to nourish her eggs, and at the same time injects saliva that contains sporozoites, the infectious form of the parasite, into the person's bloodstream. The sporozoite promptly invades liver cells, developing into tissue schizonts. The schizont is a structure that contains thousands of tiny rounded merozoites. Upon maturation of the schizonts, merozoites are released into the bloodstream of the human host. These merozoites rapidly invade red blood cells, where they develop into ring forms and trophozoites, enlarging in the process, and eventually become schizonts. During the trophozoite stage, the parasite is actively metabolizing the oxygen-carrying haemoglobin by ingestion from the host cytoplasm, and proteolysis of haemoglobin into amino acids. Within a few days in a non-immune host, the parasitaemia may rise up to 10^{11}. They then go through further rounds of asexual reproduction, forming more schizonts and merozoites. Every 48-78 hours, the red blood cells rupture synchronously, dispersing more parasites in the merozoite stage along with waste products and toxins into the bloodstream. This produces the clinical signs of malaria: fever, chills and anemia. The newly released merozoites invade more erythrocytes, and the infection continues its cycle until it is brought under control by medicine or the body's immune defenses, or the patient dies. The parasite is pathogenic only in the blood stage; erythrocytes that are infected by malaria at the blood stage adhere to endothelial cells and sequester in the microvasculature of vital organs, including the brain, heart and lungs. Sequestration in the brain often leads to cerebral malaria.

Some of the merozoites that penetrate red blood cells do not change into schizonts, but into sexual forms known as gametocytes that circulate in the bloodstream. When a female

anopheles mosquito bites an infected person, she sucks up these gametocytes along with blood. These gametocytes then develop into sperm-like male gametes or large, egg-like female gametes. Upon fertilization of the male and female gametes, an oocyst filled with infectious sporozoites is produced. When the oocyst matures, it ruptures, and thousands of sporozoites migrate to the salivary gland of the mosquito, ready to recommence the cycle when the mosquito feeds again.

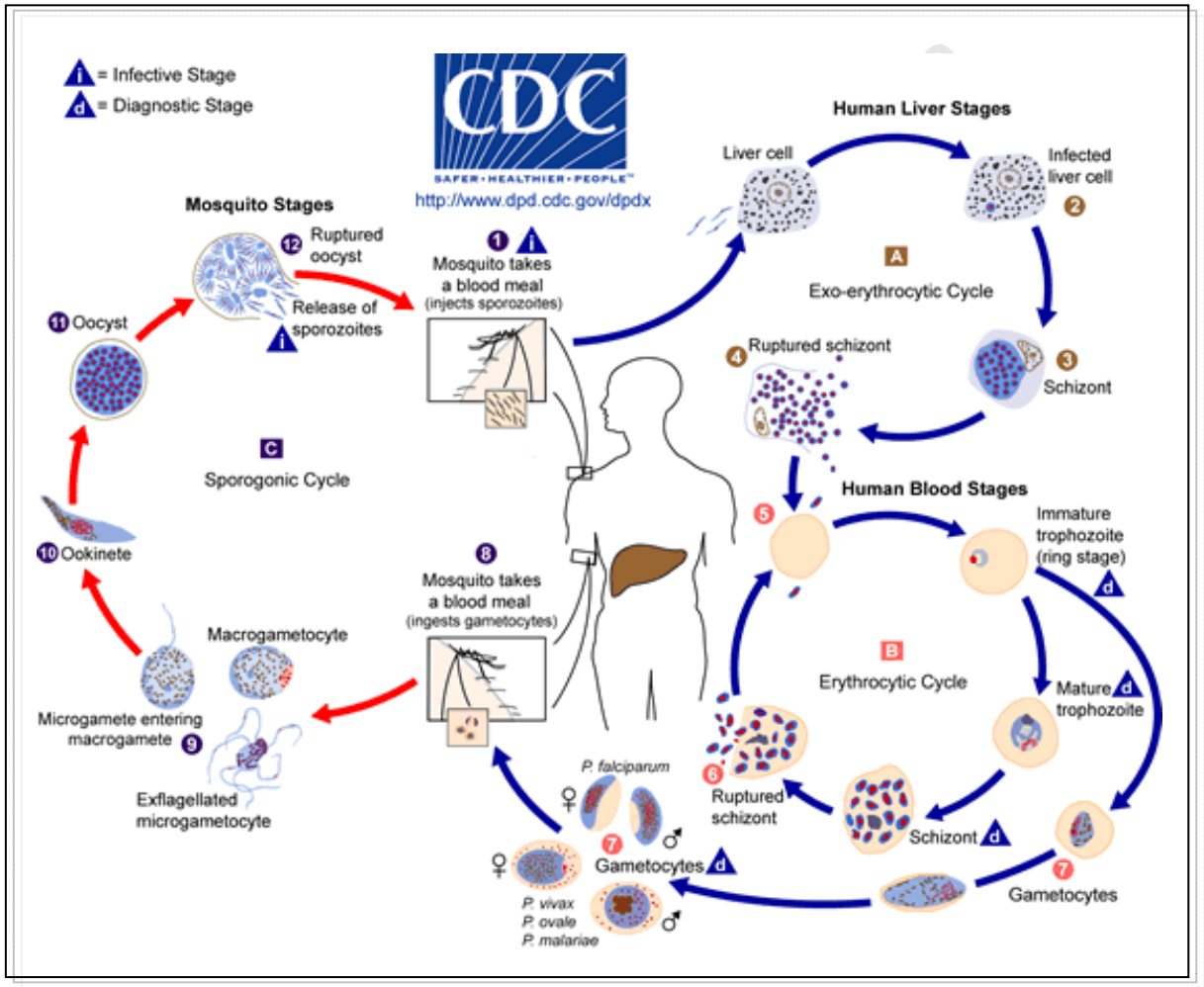


Figure 1.2. Life cycle of the malaria parasite.[10]

1.3 Treatment and prevention of malaria

Chemotherapy of malaria can specifically target the parasites. As the liver stage is the longest single stage in the parasite's life cycle, studies have shown that the liver is the ideal target for drugs and vaccines to treat and prevent malaria.[11] When the parasites enter the liver, they remain there for at least five and a half days before being released into the blood stream. During this time, the patient displays no symptoms of the disease. This period is hence a tremendous window to attack the parasites, but it is, however, extremely difficult to study the liver stages of the parasites, and most of the curative drugs act against the blood stages of the parasites.

1.4 Classes of Antimalarial Drugs

1.4.1 Clinically Established Drugs

Antimalarial drugs can be categorized by (a) the stage of the malaria parasite at which it affects and the corresponding clinical objective,[12] and (b) the mode of action through which they act.[13]

1.4.1.1 Based on the stage of the life cycle

Antimalarial drugs can be classified as blood schizonticides, tissue schizonticides, gametocides or sporontocides, according to the specific stage of the malaria cycle they target:[12]

Blood schizonticides - active against the asexual intra-erythrocytic stages of the parasite. They act by suppressing the replication of *Plasmodia* species within the erythrocyte.

Tissue schizonticides - prevent the development of hepatic schizonts. They are more of prophylactic drugs than curative.

Gametocides - prevent transmission between hosts by destroying the intra-erythrocytic sexual forms of the parasite.

Sporontocides - block the development of oocysts and sporozoites in the mosquito.

1.4.1.2 Based on the Mode of Action

Antimalarial drugs can also be classified according to the mode action by which they act in the parasite. Subclasses to which most of the currently marketed drugs belong are summarized below.

1.4.1.2.1 Compounds Acting on Haem Detoxification

Drugs in this group act by interfering with the formation of haemozoin, a detoxification process specific to the malaria parasites. These drugs include chloroquine and amodiaquine, belonging to the class of 4-aminoquinolines. Other drugs in this group include quinine and quinidine, which are alkaloids isolated from the bark of *Cinchona ledgenaria* ('fever tree'). The bark was used as an ancient remedy for the treatment of malaria, by mixing the ground bark with sweetened water to offset the bark's bitter taste.

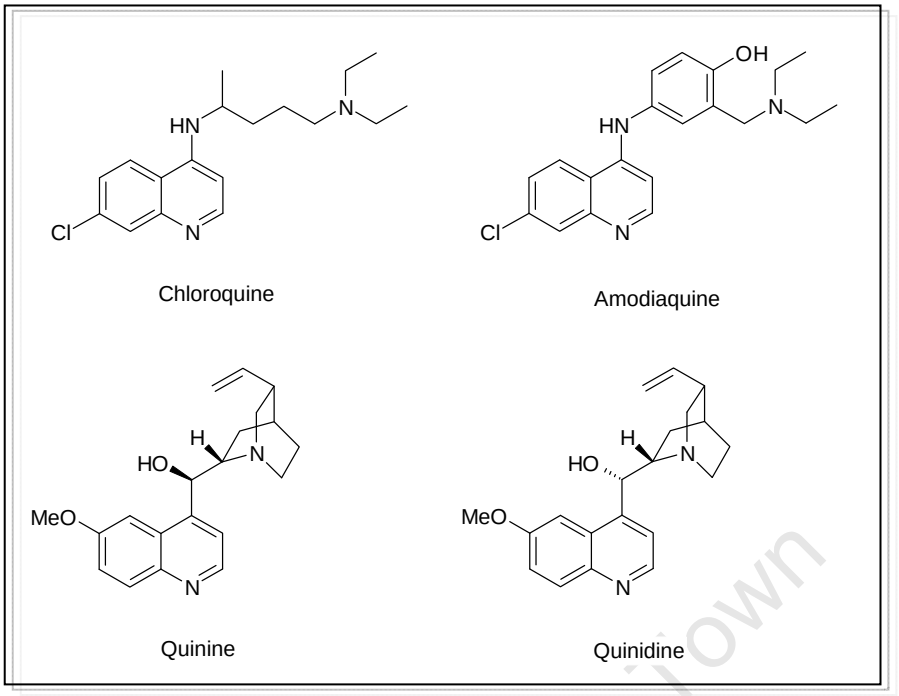


Figure 1.3. Chemical structures of chloroquine, amodiaquine, quinine and quinidine.

Chloroquine has been the drug of choice in malaria treatment for many years, until the emergence of widespread chloroquine resistance where malaria is endemic. A more detailed discussion on the mode of action and resistance of chloroquine is presented in Chapter 2. Due to the widespread of resistance to chloroquine, WHO initiated efforts to synthesize new antimalarials, which ultimately resulted in the development of mefloquine and halofantrine.[14]

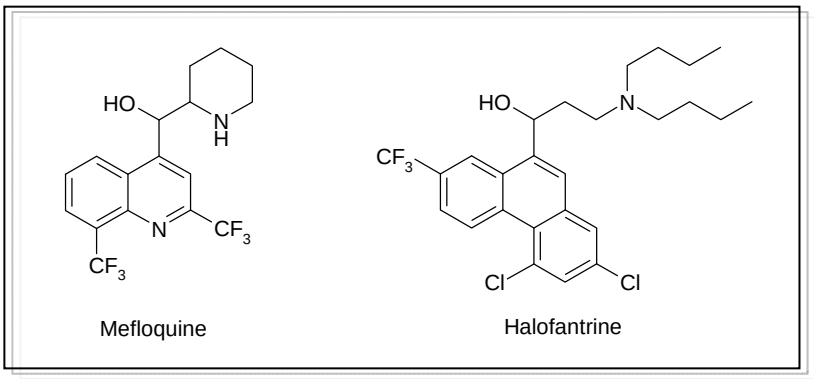


Figure 1.4. Chemical structures of mefloquine and halofantrine.

1.4.1.2.2 Inhibitors of Nucleic Acid Synthesis

Inhibitors in this group include sulfonamides, antifolates and naphthoquinones. Sulfonamides act by mimicking *para*-aminobenzoic acid (PABA) to compete for the active site of dihydropteroate synthase, hence preventing the formation of dihydropteroate from hydroxymethyldihydropterin. This leads to reduced dihydropyrimidine synthesis, which affects DNA, serine and methionine formation. Resistance to sulfonamides arises from point mutations in the gene that encodes for dihydropteroate synthase.[15] An example of a sulfonamide is Sulfadoxine, which is used in combination with pyrimethamine (see below) as Fansidar® to treat and prevent malaria.

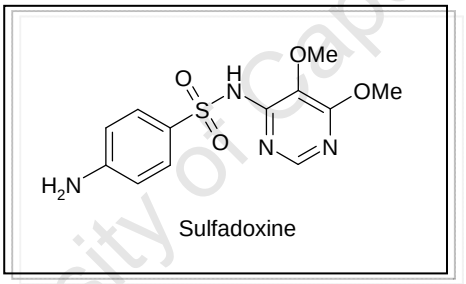


Figure 1.5. Chemical structure of sulfadoxine.

Antifolates inhibit dihydrofolate reductase, causing a depletion of tetrahydrofolate and consequently DNA synthesis. Similar to sulfonamides, resistance to antifolates may be from point mutations in the gene encoding for dihydrofolate reductase.[15] Examples of antifolate inhibitors include pyrimethamine and proguanil.

Atovaquone is the most successful drug in the class of 1,4-naphthoquinones, and is administered in a fixed preparation with proguanil as Malarone®. Malarone has fewer side effects when compared to other antimalarials, and can be used both to treat and prevent

malaria. This combination is more efficacious than either drug alone, has a favourable safety margin, and also effective against strains that are resistant to a variety of other antimalarials. The mode of action of atovaquone is generally agreed to be acting on the mitochondrial electron transfer chain, and interfering with mitochondrial membrane potential.[15]

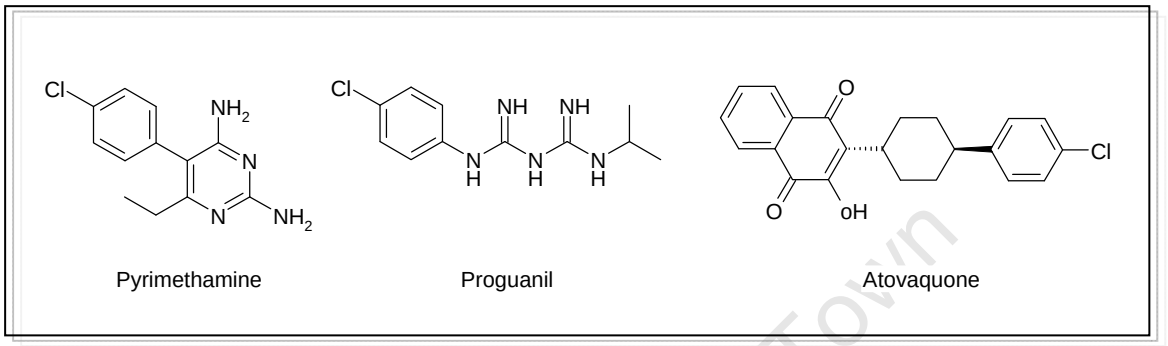


Figure 1.6. Chemical structures of pyrimethamine, proguanil and atovaquone.

1.4.1.2.3 Inhibitors of Protein Metabolism

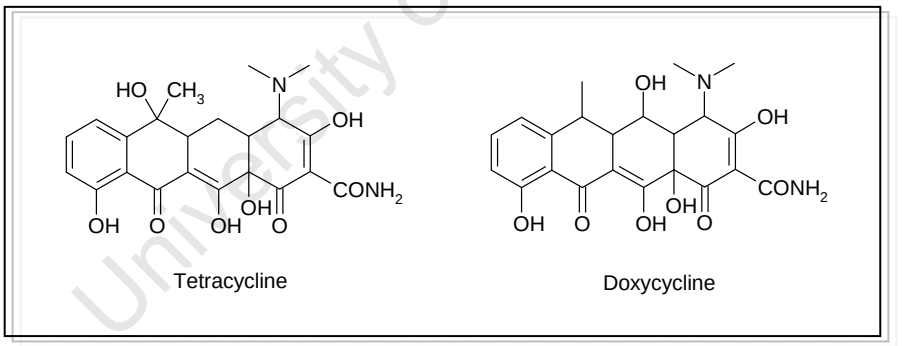


Figure 1.7. Chemical structures of tetracycline and doxycycline.

In this group are antibiotics, which have a long history in the treatment of malaria. With the emergence of chloroquine-resistant and multidrug resistant strains of *P. falciparum*, there has been a renewed interest in the use of antibiotics to treat malaria. Antibiotics such as tetracycline and doxycycline inhibit parasite protein synthesis in the mitochondria and/or apicoplast.[16]

1.4.1.2.4 Drugs Generating an Oxidative Stress

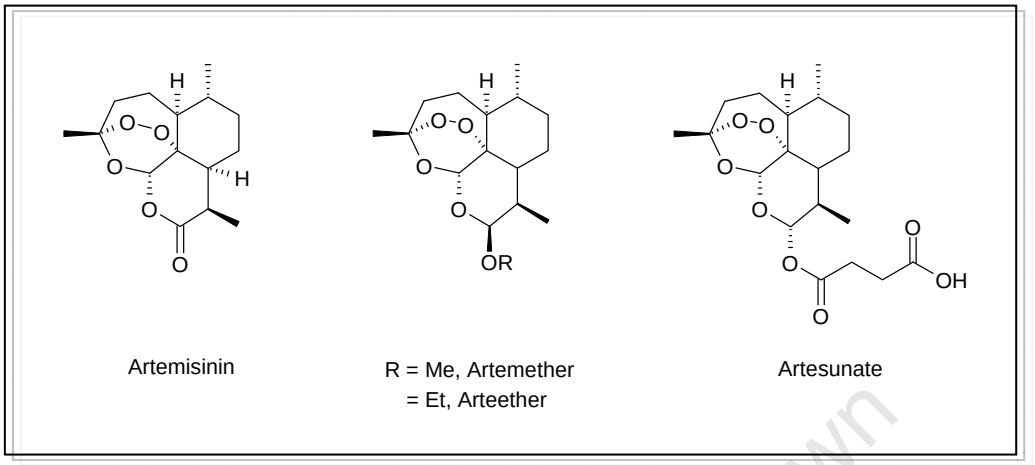


Figure 1.8. Chemical structures of artemisinin, artemether, arteether and artesunate.

Peroxides such as artemisinin and its family of derivatives, which include artemether, arteether, artesunate and many others fall under this category. They are believed to generate oxidative stress within the parasite via the endoperoxide group, which is important for antimalarial activity.[17] Other mechanisms of action include the inhibition of the plasmodial sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA).[18] SERCA is responsible for the maintenance of calcium ion concentrations, which is important for the generation of calcium-medicated signaling and the correct folding and post-translational processing of proteins. To date, the mechanism(s) of action is widely debated, and is discussed in more detail in Chapter 2.

1.4.1.2.5 Iron chelating agents

Iron chelators such as desferrioxamine enhance the clearance of parasites in cases of mild malaria. In severe *falciparum* malaria, administration of desferrioxamine in conjunction with

quinine and Fansidar® can hasten the recovery from deep coma.[19] The mechanism(s) of action of these iron chelators has been widely debated, but several hypotheses have been proposed. These include formation of free radicals, iron binding and inhibition of iron-dependent enzymes, such as ribonucleotide reductase, phosphoenol pyruvate carboxykinase, dihydroorotate dehydrogen-ase, cytochrome c oxidase, and superoxide dismutase.[20]

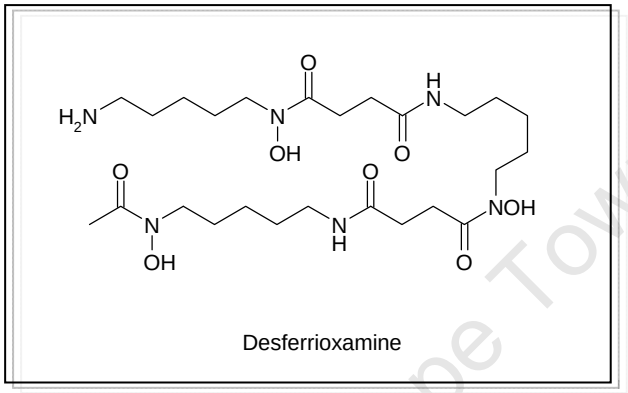


Figure 1.9. Chemical structure of desferrioxamine.

1.4.2 Novel Inhibitors of Parasitic Pathways and Enzymes

In light of the rapid development of resistance to antimalarial drugs, there is a pressing need for new chemical entities with no cross-resistance to existing antimalarials. Identification of new biological targets will be useful to base new treatment strategies on. A number of biosynthetic processes are known to be occurring within the malaria parasite, some of which are unique to the organism. As some of these processes are essential for parasite survival, their inhibition could result in parasite death. This strategy is currently under exploitation, and several targets have been identified and validated. Some of these targets include host cell proteins, the mitochondrion, membrane biosynthesis, cell cycle control, the plastid organelle and parasite transporters.[21]

1.5 Endocytosis of the malaria parasite

Endocytosis is a fundamental process occurring in all eukaryotic cells. Cells rely on endocytosis to uptake external macromolecules (such as proteins), particles and fluids as nutrient sources to sustain their survival.[22] The malaria parasite has only a limited capacity to synthesize amino acids *de novo* or scavenge them exogenously, thus it must obtain them from the host[23] by degrading host haemoglobin (Hb). The parasites can degrade up to 75% of the Hb once they enter the red blood cell.[24] These Hb are ingested via the endocytosis pathway. They do so by invaginations of the parasitophorous vacuolar membrane and the parasite plasma membrane known as the cytostome, enclosing the red blood cell cytoplasm (rich in Hb) in double-membraned endocytotic vesicles called transport vesicles.[25] Some proteolytic digestion of the enclosed Hb cytoplasm may have already occurred at this stage.[26] These transport vesicles deliver the ingested Hb to the food vacuole by fusing their outer membrane with the single membrane-delimited food vacuole. Fusion releases a single membraned-vesicle,[27,28] which is then lysed to release the haemoglobin.[29] The endocytosis pathway is illustrated in Fig. 1.10. The food (or digestive) vacuole, where Hb catabolism occurs, is a specialized acidic lysosomal organelle with pH 5.2 – 5.6.[30] The Hb proteolysis yields not only the needed amino acids, but also releases toxic free haem into the food vacuole.[23] Here, the iron of the haem moiety is oxidized from the predominantly ferrous (II) to the ferric (III) state, forming haematin.

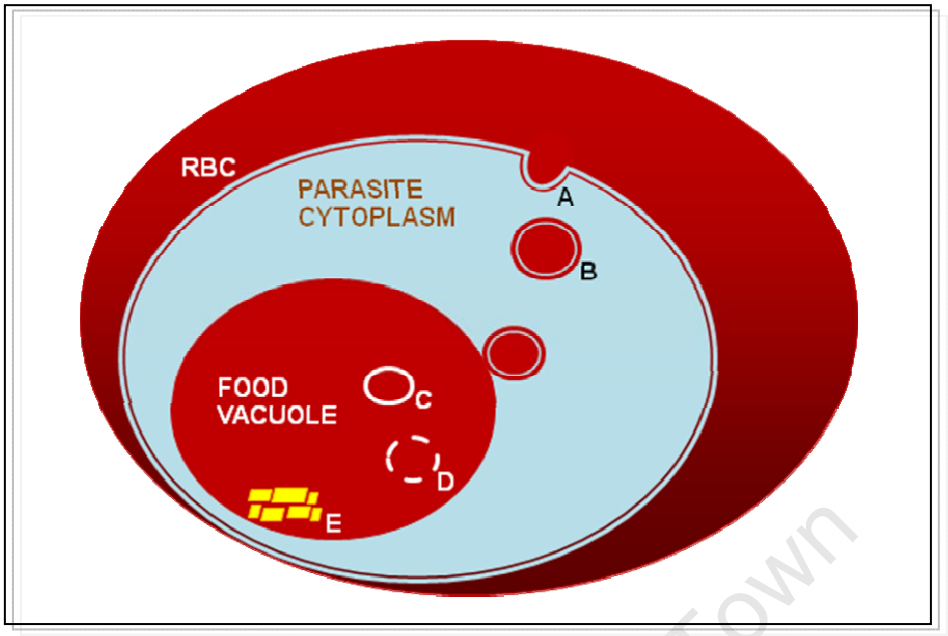


Figure 1.10. Schematic drawing showing the parasite in RBC, and the endocytosed haemoglobin transported to the FV via transport vesicles. A, cytostome; B, double-membraned transport vesicle; C, single membraned-transport vesicle; D, lysed transport vesicle; E, haemozoin crystals.

The oxidation of haem to haematin leads to the production of hydrogen peroxide from superoxide generated through the one electron oxidation of Fe (II). Furthermore, the concentration of haematin released into the food vacuole can reach as high as 0.4 M, which is potentially toxic.[31,32] Thus the parasite requires a detoxification mechanism. This involves the conversion of haematin to a crystalline haem aggregate known as haemozoin (malaria pigment).[33] The chemical nature and structure of haemozoin has been elucidated only in the past decade. It is now known to be a cyclic dimer of ferriprotoporphyrin IX (Fe[III]PPIX) (see Fig. 1.11). By converting haematin to hemozoin, the parasite removes it permanently from solution, and deposits the iron porphyrin in a relatively harmless solid form.[34]

Ginsburg and co-workers have observed that the parasite generates more amino acids from haemoglobin breakdown than are used for protein synthesis.[35] As a result, some

haemoglobin-derived amino acids are diffused into the host cell. This has raised the possibility that there may be additional functions to haemoglobin catabolism. One hypothesis is that the parasite prevents premature red cell lysis by digesting host cell cytosol, as parasite growth needs to be compensated by reduced host cell volume.[35,36]

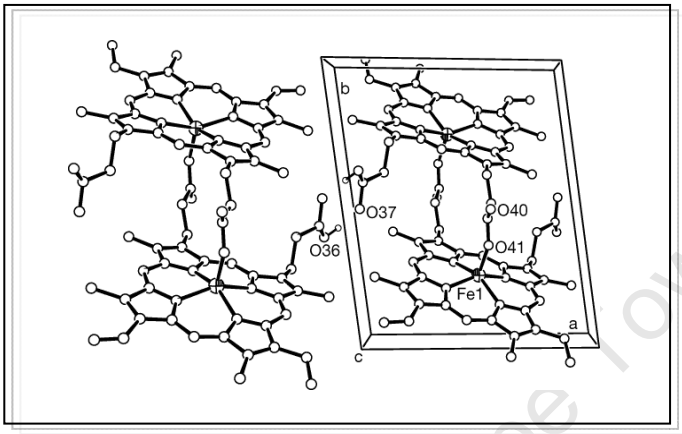


Figure 1.11. Structure of the malaria pigment, haemozoin.[34] Dimers are formed through the Fe1 – O41 bonds between stacking ferriprotoporphyrins, and are linked to other dimers by hydrogen bonds through O36 and O37.

1.6 Haem degradation pathway

The Hb is degraded in the food vacuole by the cooperative process involving a number of proteases, such as aspartic (plasmepsins),[37, 38] cysteine (falcipains)[39, 40] and metalloproteases,[41] and at least one cytosolic metalloaminopeptidase.[42] When the lysate of the food vacuole was added to denatured globin and incubated at acidic pH, activities of both the aspartic and cysteine proteases were detected. Aspartic proteases account for 60-80%, and cysteine proteases account for 20-40% of the haemoglobin-degrading activity in purified digestive vacuoles.[43, 38]

The availability of the full *P. falciparum* genome sequence and biochemical studies have rendered the identification of additional members of the above-mentioned protease families. Four plasmepsins were proposed to be involved in the onset of haemoglobin degradation;[44] these include plasmepsins (PM's) I, II, IV and histo-aspartic protease (HAP),[45] the third gene encoding an aspartic protease that was identified.[46] HAP and PM IV are homologous to PM I and PM II.[47] Falcipain 1, -2 and -3 were identified in the cysteine protease family.[39,40] A fourth member has also been identified via the expanded analysis of the complete *P. falciparum* genome, and is 96% and 93.4% identical at the nucleotide and amino acid level, respectively, to falcipain-2.[48] The original gene (PF11-0165) is referred to as falcipain 2A, and upstream homologue (PF11-0161) is referred to as falcipain 2B. Another gene homologous to falcipain-2 and falcipain-2' has been reported recently by Singh *et al*;[49] they have shown that falcipain-2 and falcipain-2', differing by only 3 amino acids, share more similarity in comparison to falcipain-2 and falcipain-3.

1.6.1 Order of Action

Data has suggested that the Hb degradation process is ordered, and that it requires an aspartic protease-mediated initial cleavage, followed by secondary aspartic protease and cysteine protease cleavages.[37,43] Hb degradation assays have implicated that two aspartic proteases, plasmepsins I and II, are involved in the early stages of the degradative process, and cleave the native globin at acidic pH.[38] Both of these enzymes, with 75% homology, can cleave the Phe33-Leu34 bond in the hinge region of the haemoglobin, the domain responsible for holding the tetramer together when oxygen is bound.[50,51] This region is also highly conserved among vertebrate species.[52] This is thought to be the crucial first step in Hb degradation, after which the Hb unravels, and susceptible to further degradation by

plasmepsins and other proteases.[44]

Studies have shown that HAP and PM IV act later in the haemoglobin degradation pathway, as they prefer to cleave denatured globin rather than native haemoglobin at a site known to be the essential primary cleavage site in the haemoglobin hydrolysis pathway.[45] Studies have shown that haemoglobin-degrading plasmepsins may not be promising targets, as minimal effect on parasite growth was observed when plasmepsin was treated with pepstatin A, a potent protease inhibitor.[53] Hanspal *et al*[54] have proposed that falcipain-2 may have a second role, in that the enzyme assists in the cleavage of specific components of the erythrocyte skeleton at the inner surface of the parasite membrane.

Once the tertiary structure of Hb has been lost, falcipains-2 and -3, belonging to the class of cysteine proteases, degrade the larger fragments into smaller peptides (Fig 1.12). This is consistent with the notion of ordered proteolysis, as falcipains did not cleave haemoglobin unless it has been first denatured by reducing conditions, acid-acetone treatment, or cleavage by plasmepsins.[38,43] These two proteases both require a reducing environment and acidic pH for optimal activity.[55] The two enzymes share sequence homology (68%) and substrate specificity, but have markedly different activities.[56] Falcipain-2', which was recently discovered, has similar biochemical properties to those of falcipain-2. These include sequence homology (99%), pH optima (5.5 – 7.0), reducing requirements, substrate preference and both readily hydrolyze haemoglobin. When compared to falcipain-2, falcipain-2' displayed similar inhibitory activity against cysteine protease inhibitors. This suggests that falcipain-2' is also an active haemoglobinase.[49]

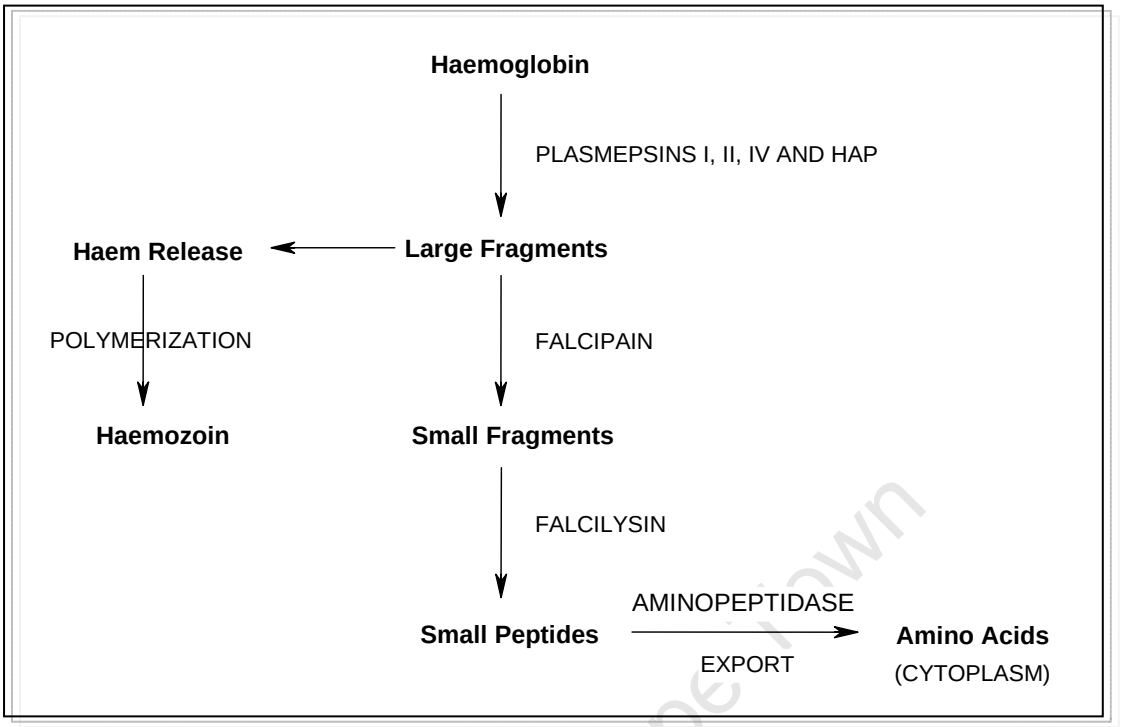


Figure 1.12. Proposed pathway for haemoglobin degradation in *P. falciparum*. Adapted from [25].

The zinc metalloprotease falcilysin[57] cleaves relatively smaller peptides (up to 20 amino acids) at polar residues of the Hb fragment under an acidic pH (optimum of 5.2),[41] and is unable to cleave either haemoglobin or denatured globin. These small peptide residues are then transported from the food vacuole into the parasite cytosol by aminopeptidases, where the ultimate steps in haemoglobin degradation takes place.[58] Falcilysin cleaves sites along the α- and β-chains of haemoglobin and are polar in character.[41] It was also demonstrated that the enzyme is robustly active at neutral pH, but with a substantially different substrate specificity. This hypothesis is supported by imaging studies, which suggest that falcilysin may have an extended role beyond globin catabolism and may function as two different proteases in two locations.[57]

1.7 Erythrocyte Rupture and Invasion

Plasmodial proteases are not only involved in haem degradation, but also play a role in erythrocyte invasion[59,60] and rupture.[61,62] Early observations have suggested that the transit through the erythrocyte cytoskeleton is made possible by plasmodial proteases, as protease inhibitors blocked both erythrocyte rupture and invasion by malaria parasites.[63] Studies to characterize the specific proteases involved in these processes have found that some of the same proteases that degrade haemoglobin also degrade cytoskeletal proteins, and are discussed below.

1.7.1 Erythrocyte Rupture

At the completion of each asexual cycle, erythrocytes must be ruptured to release free merozoites. The cytoskeletal proteins of the erythrocyte must be hydrolyzed by plasmodial proteases to allow parasite egress.[64] Cysteine and serine proteases seem to play a role in this process; when leupeptin and chymostatin (cysteine and serine protease inhibitors, respectively) were added to the parasite culture, an accumulation of mature schizonts was observed.[61,62] Two studies have provided improved insight in this process. Salmon *et al*[65] have shown that the addition of E-64, a cysteine protease inhibitor, to mature schizonts could block the lysis of the parasitophorous vacuole membrane, which surrounds the intraerythrocytic parasite. This suggests that cysteine protease activity is required for the hydrolysis of parasitophorous vacuole membrane-associated proteins to mediate merozoite release. Studies by Wickham *et al*[66] have demonstrated that the lysis of the erythrocyte membrane is blocked by leupeptin and antipain, an oligopeptide cysteine protease inhibitor of bacterial origin. These results suggest that the release of merozoites is a two-step process,

firstly, the hydrolysis of proteins associated with the parasitophorous vacuole, and secondly, the hydrolysis of erythrocyte membranes. However, the sequence of these two steps is in dispute.[67]

Although there has been no report on aspartic protease activities in erythrocyte rupture, a study by Le Bonniec *et al*[68] that showed plasmepsin II may be involved. Plasmepsin II was also shown to be localized both inside and outside the erythrocytic schizont, which suggests it has activities against more than one target. Additionally, Le Bonniec *et al* have also demonstrated that plasmepsin II and the cysteine protease falcipain-2 cleave haemoglobin at acidic pH in the food vacuole, and cytoskeletal proteins at neutral pH.[68] Hence, it is likely that the proteases are transported to the food vacuole for haemoglobin degradation (and not synthesized in the food vacuole), and also capable of exiting the parasite to act against host cytoskeletal proteins.

1.7.2 Erythrocyte Invasion

During the invasion of the erythrocyte by merozoites, the cytoskeletal proteins of the erythrocytes are once again hydrolyzed by parasite proteases released from merozoite secretory organelles.[69] The proteolysis of the complex cytoskeletal network is necessary, as this allows movement of the parasite into the erythrocyte.

The invasion of erythrocytes by merozoites takes place in a sequence of three discreet steps, involving (1) the reversible attachment of the merozoite to erythrocyte receptors, (2) realignment of the merozoite such that its apical end apposes the erythrocyte membrane to form an irreversible tight junction, and (3) entry to the erythrocyte within the

parasitophorous vacuole.[70] During this process, the contents of three different types of secretory organelles (rhoptries, micronemes and dense granules) are released from the apical end of merozoites, which are believed to include proteases that facilitate erythrocyte entry.[69]

Protease activities involved in erythrocyte invasion by merozoites have been shown to involve chymotrypsin-like serine proteases.[59,60] Two plasmodial serine proteases have been well-described, they are Pfsub-1[71] and Pfsub-2[72], and are both subtilisin-like proteases. Studies that evaluated invasion of isolated merozoites have showed that there has been no inhibition of invasion by the cysteine and trypsin-like protease inhibitor leupeptin,[61] the cysteine protease inhibitor E-64,[73] or the aspartic protease inhibitor pepstatin.[61,73]

Proteases in *P. falciparum* are summarized in Table 1.1 below.

Table 1.1. Proteases of *Plasmodium falciparum*

Protease	Class	Substrate(s)	Putative Role(s)
Falcipain	cysteine	haemoglobin, ankyrin	haemoglobin hydrolysis, erythrocyte rupture
Plasmepsins	aspartic	haemoglobin, spectrin	haemoglobin hydrolysis, erythrocyte rupture
Falcilysin	metalloprotease	globin fragments	haemoglobin hydrolysis
Aminopeptidase	metalloaminopeptidase	globin fragments	haemoglobin hydrolysis
Pfsub-1 and Pfsub-2	serine	? parasite proteins	erythrocyte invasion

Well-characterized enzymes are shown. ? denotes incomplete knowledge of processes involved.[74]

1.8 Cysteine Proteases

Cysteine proteases, one of the main catalytic proteases involved in the haemoglobin degradation pathway, have aroused interest to many scientists as they are very widespread in nature, and play a vital role in the metabolism of both eukaryotic and prokaryotic organisms. In humans, cysteine proteases have been implicated in cardiovascular, inflammatory, neurological, respiratory, viral, musculoskeletal, immunological, CNS disorders and cancer.[75] Cysteine proteases are also essential for the life cycle and pathogenicity of several parasites, such as cruzain from *Trypanosoma cruzi*, the causing agent of Chagas' disease,[76,77] falcipain from *Plasmodium falciparum*, the causing agent of malaria,[78] and rhodesain from *T. brucei*, the causing agent of the African sleeping sickness.[79]

1.8.1 Cysteine Protease Superfamilies

In 1879, the first cysteine protease was purified and characterized from the papaya fruit *Carica papaya*, and was thus named papain.[80] Two amino acid residues in the parasitic cysteine protease, namely Cys 25 and His 159, are actively involved in the hydrolysis of peptide bonds. The cysteine thiol functionality is responsible for the catalytic activity of cysteine proteases Proteases that possess sequences in common with papain have been referred to as 'papain-like'. Cysteine proteases are categorized into three structurally distinct groups based on their sequence homology; these are papain-like (clan CA), ICE-like (clan CD) and picomain-like (clan PA(C)).[81] Clan CA and clan CD contain the majority of cysteine proteases in which the catalytic residues (Cys 25 and His 159) are highly conserved among all the members. Clan CA are well-characterized for many eukaryotic organisms and these are among the best characterized cysteine proteases of *Plasmodium*.[77,82] Clan CA

comprises of cathepsins, calpains, and parasite cysteine proteases. Clan CD includes several important enzymes including caspases, clostripain, gingipains, legumain and separase. The human rhinoviruses (HRVs), which are the single most significant cause of the common cold, belong to Clan PA(C).[81]

1.8.2 Protease Binding and Classification of Binding Pockets

A protease must bind the protein or peptide substrate in its active site before hydrolysis can take place. The binding efficiency is determined by the respective chemical environments that the protease subsites create, and the chemical nature of the substrate that interacts with the active site groove. Other important factors that affect interactions between the active site and a substrate include size, charge, polarity, hydrophobicity and accessibility.[80]

A nomenclature to designate substrate residues and the corresponding enzyme subsites have been established by Schechter and Berger,[83] and are labeled based on their relative position to the scissile peptide bond (Fig. 1.13). 'P' denotes amino acid residues from the peptide backbone, and the subsites that interact with these residues are denoted 'S'. Amino acid residues and subsites at the carboxyl side are conventionally referred to as P_1', P_2', S_1', S_2', etc, while those at the amino side are designated P_1, P_2, S_1, S_2, etc.

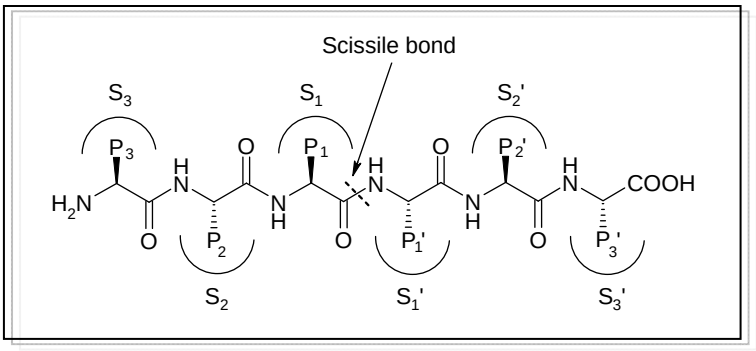


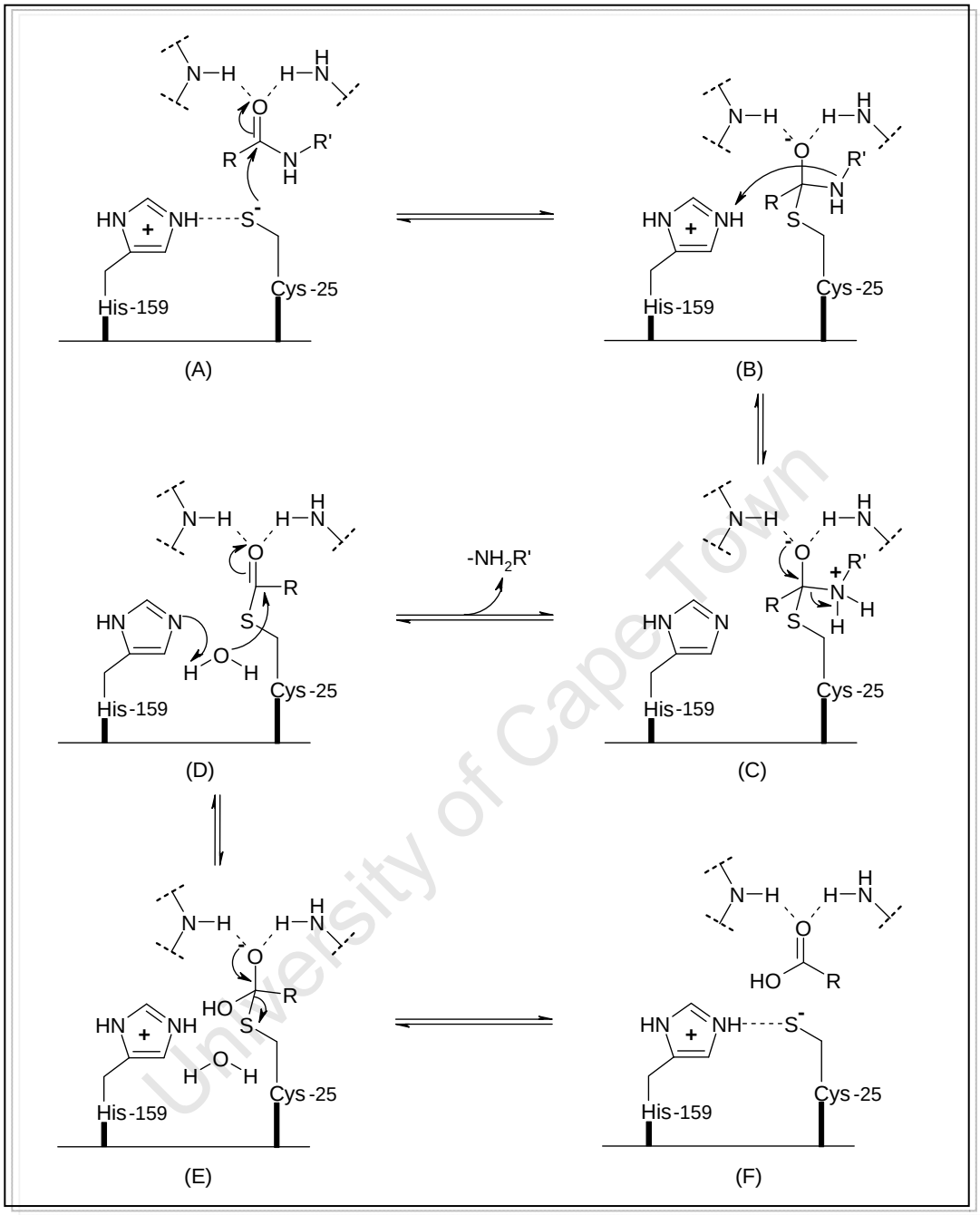
Figure 1.13. Nomenclature of substrate residues and corresponding binding sites.[83]

1.8.3 Mechanism of Proteolysis

The proposed mechanism of peptide hydrolysis of cysteine proteases is illustrated in Scheme 1.1, in which a cysteine thiol in the active site is involved in the hydrolysis. The close proximity of His-159 to Cys-25 polarizes the thiol group of the cysteine, allowing the generation of a highly nucleophilic thiolate/imidazolium ion pair by deprotonation of the thiol group to be possible at near neutral pH (Scheme 1.1A).[84] The strongly nucleophilic thiolate ion attacks the carbonyl carbon of the scissile amide bond to form a tetrahedral intermediate (Scheme 1.1B). The oxyanion intermediate is stabilized via H-bonding to the NH backbone of Cys-25 and to the NH_2 group of the Gln-19 side chain. This is followed by acylation of the enzyme and the concomitant release of the C-terminal portion of the substrate (Scheme 1.1C). A base-catalyzed hydrolysis of the acyl-enzyme takes place, forming another tetrahedral intermediate (Scheme 1.1D). The final step in the hydrolysis is where this intermediate collapses (Scheme 1.1E) to regenerate the free enzyme and releasing the N-terminal portion of the substrate (Scheme 1.1F).

1.8.4 Inhibitors of Cysteine Proteases

The development in cysteine protease inhibitors has rapidly increased owing to advances in knowledge, computer-aided design and the use of combinatorial syntheses. To date, a wide range of cysteine protease inhibitors exist, and are broadly categorized into three distinct classes depending on their mechanism of inhibition.[85,86]



Scheme 1.1. Mechanism of substrate hydrolysis by the papain superfamily of cysteine proteases.

1.8.4.1 Irreversible Inhibitors

Inhibitors belonging to this class bind covalently to the active site of the enzyme with a high affinity, modifying the enzyme and thus irreversibly inactivating its catalytic activities.

These inhibitors stay in the active site for a longer time which disables the enzyme permanently. Examples include epoxide **1.1**,[87] α,β-unsaturated ester and vinyl sulfone Michael acceptors **1.2** and **1.3** (Fig. 1.14).[88]

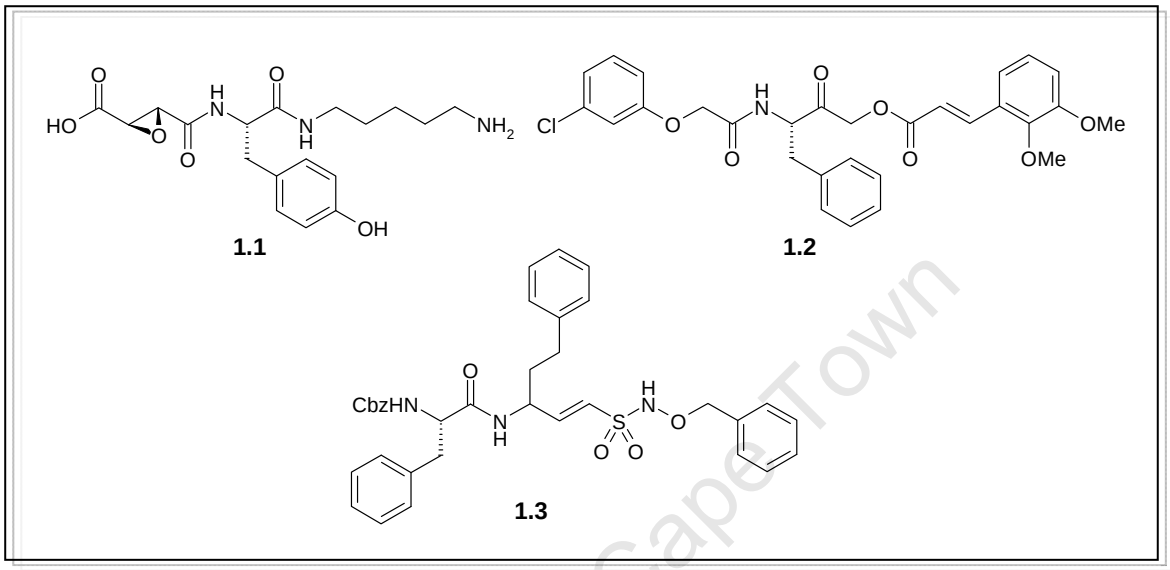


Figure 1.14. Epoxide **1.1**, α,β-unsaturated ester and vinyl sulfone Michael acceptors **1.2** and **1.3** as irreversible cysteine protease inhibitors

1.8.4.2 Reversible Inhibitors

Inhibitors that interact reversibly via covalent or non-covalent bond formations with the active site of the protease are termed reversible inhibitors. The bonds that form are weak and do not permanently disable the enzyme. Examples of such inhibitors include methylisatin-5-carboxamide **1.4**,[89] cyclic ketones **1.5**,[90] ureas **1.6**,[91] nitriles **1.7**,[92] thio-semicarbazones **1.8** and pyrazolines **1.9** (Fig. 1.15).[93]

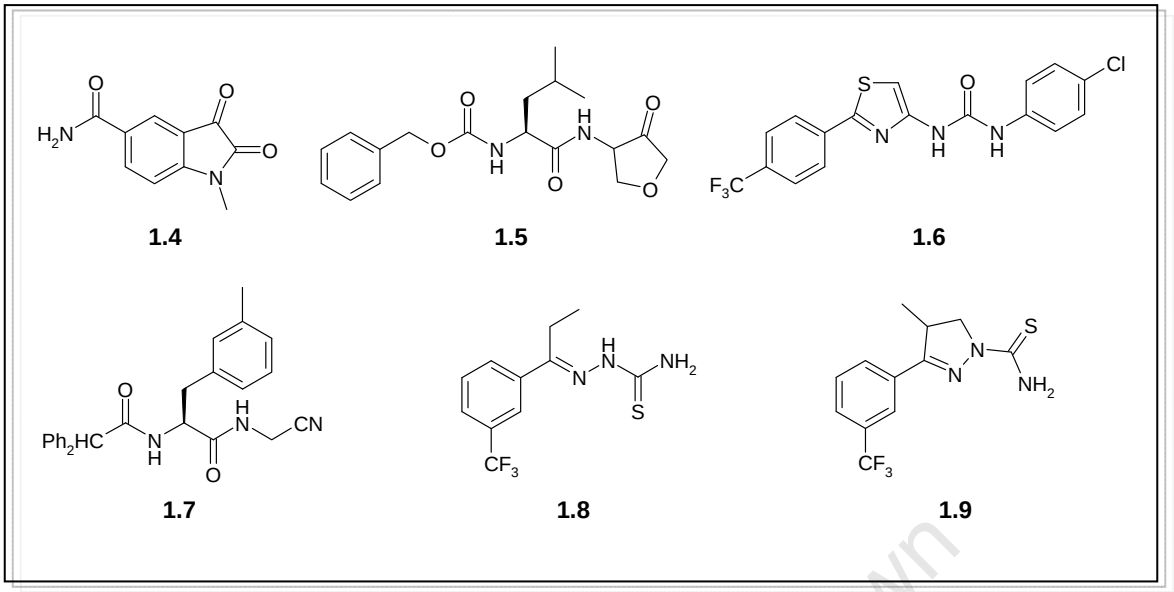


Figure 1.15. Methylisatin-5-carboxamide **1.4**, cyclic ketones **1.5**, ureas **1.6**, nitriles **1.7**, thiosemicarbazones **1.8** and pyrazolines **1.9** as reversible cysteine protease inhibitors.

1.8.4.3 Slow Turnover Inhibitors

Slow turnover inhibitors are compounds that act by forming a stable thioacyl-enzyme complex that is slow to hydrolyze. Principal examples of inhibitors belonging to this class include aza-substituted peptides such as peptidyl carbazate ester **1.10**[94] and diacyl hydrazine derivative **1.11** (Fig. 1.16).[95]

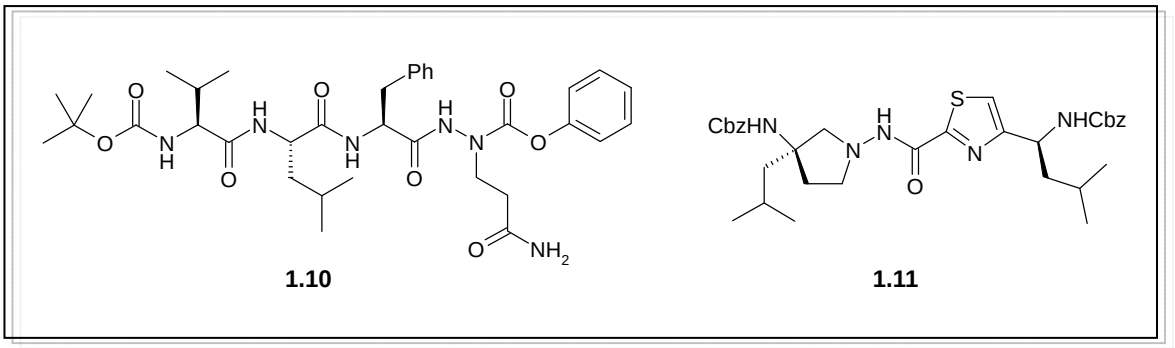


Figure 1.16. Peptidyl carbazate ester **6.10** and diacyl hydrazine **6.11** as slow turnover inhibitors of cysteine proteases.

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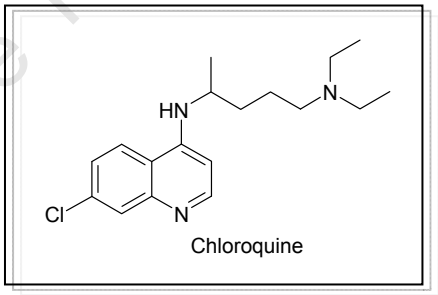
CHAPTER 2

CHLOROQUINE AND ARTEMISININ AS ANTIMALARIALS

There has been a long history for the use of chloroquine and artemisinin as antimalarials. This chapter presents their background, mode of action and resistance to the causative agents for malaria, as well as their application in cancer.

2.1 Chloroquine

Chloroquine, belonging to the class of 4-aminoquinolines, was synthesized by Hans Andersag in Germany in 1934, and was first named as Resochin by its founder.



Chloroquine

2.1.1 Mode of Action

Despite its many years of field use and study, the mode of action of chloroquine remains disputed. In 1964, Cohen *et al*[1] showed that chloroquine associates with haematin, and in 1980, Fitch and co-workers proposed that haematin is the target of chloroquine.[2] It wasn't until 1992 when it was proposed by Slater and Cerami that chloroquine inhibits an enzyme responsible for malaria pigment formation.[3] It is widely believed that chloroquine is selectively taken up by the parasite into the food vacuole, where the parasite actively digests haemoglobin.[4] Chloroquine, at neutral pH, diffuses across the membrane against a pH gradient using energy. Once it reaches the food vacuole, the basic nitrogen atoms present in chloroquine assists with its accumulation within the acidic food vacuole.[5] This phenomenon

is often referred to as 'pH trapping'. Here, only the free base diffuses across the food vacuole membrane, and the increased protonation of the drug at low pH in the vacuole results in inflow of more drug until free base concentrations are equal on both sides of the membrane.

Inside the food vacuole, chloroquine may form a complex with haematin via π-π stacking with the porphyrin system (Fig. 2.1),[6] which may further enhance drug accumulation.[7] The incorporation of haematin into haemozoin is thus inhibited, resulting in a buildup of toxic haematin and chloroquine-haematin complexes.[8] The accumulation of haematin and chloroquine-haematin adduct may potentially inhibit key enzymes, leading to parasite death, as well as bind to membranes, compromising membrane integrity and resulting in erythrocyte lysis.[9]

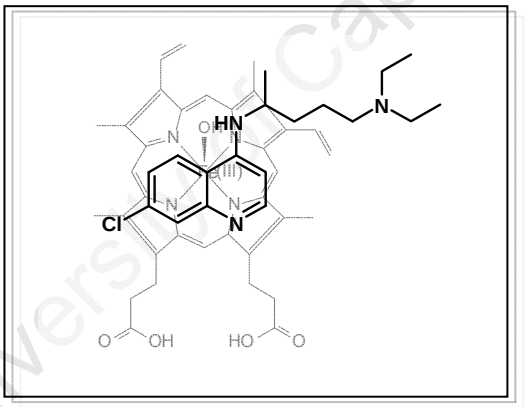


Figure 2.1. Schematic representation of the proposed π-π stacking between chloroquine and haematin.

However, another mode of action of chloroquine and related drugs has been proposed by Ginsburg and co-workers.[10] Haematin in concert with reactive oxygen species is thought to catalyze oxidation reactions and protein damage. Haematin that is released from the food vacuole in the cytosol may be rapidly reduced back into haem by glutathione and other reducing agents, which then enter the Fenton reaction (Fig. 2.2, eq 1) to form hydroxyl radical or some higher oxidation state transition metal species (Fig. 2.2, eq 2). These reactive

species remain bound to the haem iron, and can initiate attack on the porphyrin ring to cause protoporphyrin protein destruction.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O \quad (2)$$

Figure 2.2. Formation of hydroxyl radical and higher oxidation state transition metal species initiated via the Fenton reaction.

This reaction is well-known in cytochrome *c* chemistry,[11] and investigations of the autoxidation of haemoglobin in the presence of glutathione at the molecular level have been carried out by Beuzard and co-workers.[12] It has been postulated that glutathione indirectly contributes to haem degradation, via the Fenton reaction, in the cytosol by redox-cycling the haematin (Fe III) to haem (Fe II).[13] The inhibitory effect in *Plasmodium* parasites displayed by chloroquine and the related 4-aminoquinolines may also be the result of the inhibition of the glutathione-dependent haem degradation, by slowing the overall rate of the haem redox cycling.[10]

Stucture-activity relationship studies by Egan *et al*[14] have shown that the following are important for biological activity (see Fig. 2.3):

(a) The aminoquinoline ring – involved in π-π stacking with the porphyrin system, important for haem binding;

(b) An electron withdrawing group at C7 – plays an important role in inhibition of haemozoin formation;

(c) Basic nitrogen atoms – assist in vacuolar accumulation via pH trapping.

(d) Aminoalkyl side chain – required for antiplasmodial activity. Additionally, Krogstad and co-workers have found that changes in length of the side chain alter activities in chloroquine-resistant strains but not in chloroquine-sensitive strains.[15]

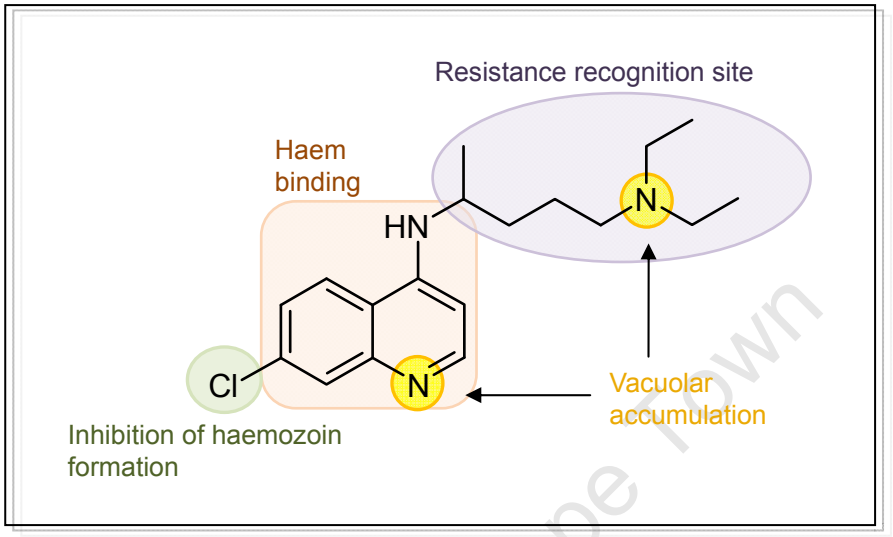


Figure 2.3. Representation of important groups for activity in chloroquine.

2.1.2 Mechanism of chloroquine resistance

The development of resistance to chloroquine was gradual, suggesting that multiple mutations may be required to produce the resistant phenotype. Resistance to chloroquine was localized to South America and South East Asia in the early stage resistance development, but to date the resistance has spread to virtually all areas where malaria is endemic.[16]

To understand the occurrence of resistance towards quinoline drugs in *P. falciparum*, three fundamental points needs to be considered: (a) the cellular mechanism(s) by which the parasites escape the toxic action of the drugs, (b) possible changes in gene expression or genetic mutations that may lead to development of resistance, and (c) reversal of resistance by changes to existing quinoline drugs.

2.1.2.1 P-glycoprotein

It was found that when compared to non-resistant strains of *P. falciparum*, the resistant strains have exhibited a reduction in cellular accumulation of chloroquine.[17,18] This reduction may arise from either a reduced uptake of the drug, or an enhanced drug efflux from the infected cell.[19] In cancer, multidrug resistance (MDR) arises from the presence of an ATP-dependent (ATP – adenosine triphosphate, an energy source in cells), membrane-bound protein called P-glycoprotein (Pgp),[19,20] belonging to the family of ATP-binding cassette (ABC) transporters. The simultaneous binding of the drug substrate and ATP to Pgp is followed by ATP hydrolysis, which shifts the substrate into a position ready to be excreted from the cell. The release of a phosphate from ATP (forming ADP – adenosine diphosphate) is accompanied by substrate excretion. ADP is released, and a new molecule of ATP binds to the secondary ATP-binding site. The hydrolysis of this ATP resets the protein, ready for another round of drug substrate binding.

Chemosensitizers are compounds typically without any inherent cytotoxicity, but are able to completely reverse the resistance of cells to the cytotoxic action of drugs.[20] Reversal of resistance to MDR in cancer was observed when chemosensitizers such as verapamil were combined with chloroquine. Verapamil was believed to compete with anticancer drugs for Pgp binding, thereby preventing efflux from the cell compartments. The similarities between MDR and chloroquine resistance in malaria parasites suggest that a similar energy-dependent efflux protein may be present in the malaria parasite.[17,21] It was found that the *P. falciparum* multidrug resistance 1 (*pfmdr1)* gene codes for a trans-membrane protein called P-glycoprotein homologue 1 (Pgh 1), resembling the mammalian Pgp.[22]

2.1.2.2 *Plasmodium falciparum* Chloroquine Resistance Transporter

Recent studies have shown that chloroquine resistance may be associated with a second gene, called *Plasmodium falciparum* chloroquine resistance transporter *(pfcrt)* gene,[23,24] encoding the protein PfCRT. PfCRT, a member of the drug metabolite/transporter superfamily, is located on the membrane of the food vacuole in the parasite, and has been directly linked with chloroquine resistance. Studies have found that this protein functions as a transporter that directly mediates the efflux of chloroquine from the food vacuole.[24] The cause of resistance seems to be a common mutation (K76T) across the different chloroquine resistant strains.

2.1.2.3 Other Hypotheses of Chloroquine Resistance

The inhibition of glutathione-dependent haem modification by chloroquine implicated the participation of glutathione in the developing resistance to chloroquine. Studies have shown that when the level of glutathione was elevated in the malaria parasite, increase in resistance to chloroquine was observed, while depletion of the glutathione content restored the chloroquine-sensitivity in the chloroquine-resistant strains.[13,25]

Other hypotheses include reduced Na^+/H^+ exchanger (NHE),[26] increased vacuolar pH[27] and reduced access to haematin.[7]

2.1.3 Application in Cancer

The anticancer activities of chloroquine against various cancer cell lines have also been widely studied. It has been found that at lower concentrations, chloroquine is able to inhibit A549 lung cancer cell growth by increasing the volume of lysosomes, while at higher concentrations, apoptosis (self-killing) and necrosis are induced, using a mechanism different to growth inhibition.[28]

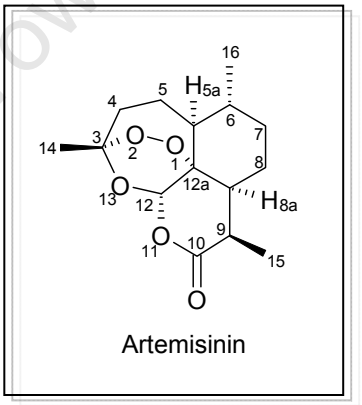
It was also observed that chloroquine may improve mid-term survival (the median survival was double in the chloroquine group compared with controls) for glioblastoma multiforme, the most common and most aggressive type of primary brain tumour, when it was given in addition to conventional therapies.[29] Loehberg *et al* have demonstrated that chloroquine exhibits a breast-cancer protective effect in an animal model, indicating that a short prior exposure to chloroquine may have a preventative application for mammary carcinogenesis.[30]

Transferrin-CRM107 (Tf-CRM107) is a conjugate of transferrin and a point mutant of diphtheria toxin that selectively kills cells expressing high levels of the transferring receptor, such as brain tumour cells. However, it is also toxic against central nervous system in the brain. A study by Hagihara *et al* has shown that chloroquine was able to reduce the toxicity of Tf-CRM107 and widen its therapeutic window for malignant brain tumour therapy.[31] The mechanism by which chloroquine acts may be via blocking the lysosomal processing of the endocytosed toxin in the tumour cells.[32]

Very recently, chloroquine has been shown to be able to inhibit autophagy (self-ingestion) in cancer cells, which leads to the inhibition of tumorigenesis, specifically *Myc*-induced

lymphoma in mice.[33] Autophagy, like apoptosis, is also a mode of self-destruction in cells.[34] However, when cells experience nutrition deprivation, autophagy is activated, which degrades damaged organelles, and recycles them as an alternate energy source.[35] Hence the inhibition of autophagy by chloroquine does not prevent the cell from eating itself to death, but rather causes cell death by provoking bio-energetic failure that triggers necrosis.

2.2 Artemisinin



Artemisinin

Artemisinin (Qinghaosu; Chinese 青蒿素) and its derivatives are endoperoxide-containing natural products, isolated from Qinghao (*Artemisia annua* L, or sweet wormwood; Chinese 青蒿, Fig. 2.4). It belongs to the Compositae family, grows abundantly and is indigenous all over China. It has been used for about 2 000 years to treat hemorrhoids and fever in Chinese folk medicine. Chemically, artemisinin is a sesquiterpene lactone, the first and last naturally occurring 1,2,4-trioxane.[36] This unusual compound has been of interest to many researchers due to the interesting chemical structure, and also the lack of a nitrogen-containing hetero-cyclic ring, which is found in most antimalarials.

Artemisinins exhibit a quick onset of action and high efficacy against the blood stages of *Plasmodium.* They have been shown to act against even the early stages (ring forms) of the parasite,[37] and have been shown to reduce the number of gametocytes in the blood,[38] owing to their activity against both the precursors of the sexual stages and early (stage I – III) gametocytes. Additionally, artemisinins also decrease the infectivity of the surviving gametocytes,[39] which may help diminish transmission rates in areas of low transmission.[40]

This effect may not be evident in high transmission areas, as rapidly re-infected individuals will continue to maintain a considerable number of transmissible parasites in the blood stream.

Figure 2.4. Flower and leaves of *Artemisia annua* L, sweet wormwood.

2.2.1 Discovery of Qinghaosu

The earliest written reference for qinghao discovered so far was documented in the "Recipes for 52 kinds of diseases", which was unearthed from the Mawangdui Tomb of the West Han Dynasty (168 BC) in Changsha, Hunan Province. In "The Handbook of Prescriptions for Emergency Treatments", Ge Hong (AD 281 – 340, Eastern Jin Dynasty) recommended the taking of an aqueous infusion of the plant to alleviate the chills and fever of malaria.[41] In 1596, Li Shizhen of the Ming Dynasty included this remedy as the treatment of agues in his

famous work "Compendium of Medical Herbs".[42] In the now modern countryside of Southeast China, people believed that patients will recover soon after drinking the aqueous extraction of qinghao. This led to great interest in qinghao by many research groups, but the isolation of the active ingredient has suffered repeated setbacks, as water or alcohol extracts of qinghao never showed antimalarial activity. It was only until the early 1970's when three research groups in Beijing, Yunnan Province and Shandong Province almost simultaneously obtained the active ingredient by extracting with ethyl ether, petroleum ether or acetone respectively.[43] The neutral isolations possessed good antimalarial activity in mice infected with *Plasmodium berghei* and in monkeys infected with *Plasmodium cynomolgi*,[41,42] and was soon found that the component was very effective in clinical trials. The colourless needle crystal was hence named quinghaosu (active essence of qinghao) in Chinese, and artemisinin (sometimes called arteannuin) in English. Its structure and relative configuration was proved by X-ray crystal structural analysis, and the absolute configuration was obtained by X-ray diffraction crystal analysis.[44]

2.2.2 Setbacks

The effectiveness of artemisinin and its derivatives as antimalarials has received increasing attention in recent years for their treatment of chloroquine, mefloquine, or multidrug resistant malaria. However, the usefulness of artemisinin has been impaired by its (a) high rate of recrudescence,[45] (b) poor solubility in oil and water,[46] (c) short plasma half-life[47,48] and (d) poor oral activity (active only at very high dosage).[45] Dihydroartemisinin, obtained by sodium borohydride reduction of artemisinin, was reported to be therapeutically more active than the parent compound.[46] However, it is also associated with toxicity[49] and short plasma half-life.[50] Analogues of artemisinin with improved efficacy and increased solubility

in oil (i.e. arteether and artemether[46,51]) and water (i.e. sodium artelinate[52] and sodium artesunate[53]) were synthesized from dihydroartemisinin. These are the most well-known analogues of the so-called first-generation derivatives of artemisinin. Although showing higher potency than artemisinin *in vitro*, the oil-soluble artemether and arteether have poor bioavailability principally as a result of the metabolic instability of the acetal function. For example, one of the principal routes of metabolism of artemether involves oxidative dealkylation to the toxic DHA, which produced fatal central nervous system (CNS) toxicity in chronically dosed rats and dogs.[54,55,56]

The desire for a water-soluble derivative of artemisinin for the treatment of advanced cases of *P. falciparum* malaria led to the design and synthesis of sodium artesunate. Water-soluble derivatives can be injected intravenously (iv), and thus drugs can be delivered more quickly than by intramuscular (im) injection.[57] Due to its high water-solubility, treatment of comatose cerebral malaria patients with sodium artesunate had resulted in a rapid diminish in parasitaemia and restoration of consciousness.[57] However, the usefulness of sodium artesunate was offset due to its lability to chemical hydrolysis in aqueous solution,[52] high rate of recrudescence and the extremely short plasma half-life (20 – 30 min).[58] Due to its high recrudescence rate, sodium artesunate is normally administered in combination therapy, most often with mefloquine.[59] Out of all the first generation analogues of artemisinin, sodium artesunate is currently the drug of choice.[60]

To overcome the hydrolytic instability of artesunate, sodium artelinate was synthesized. It turned out to be not only more stable than artemether and arteether in aqueous solution, but also has a much longer half-life (1.5 – 3 h). Literature has also suggested that sodium artelinate is substantially less CNS-toxic in rats and dogs than artemether and arteether.[61]

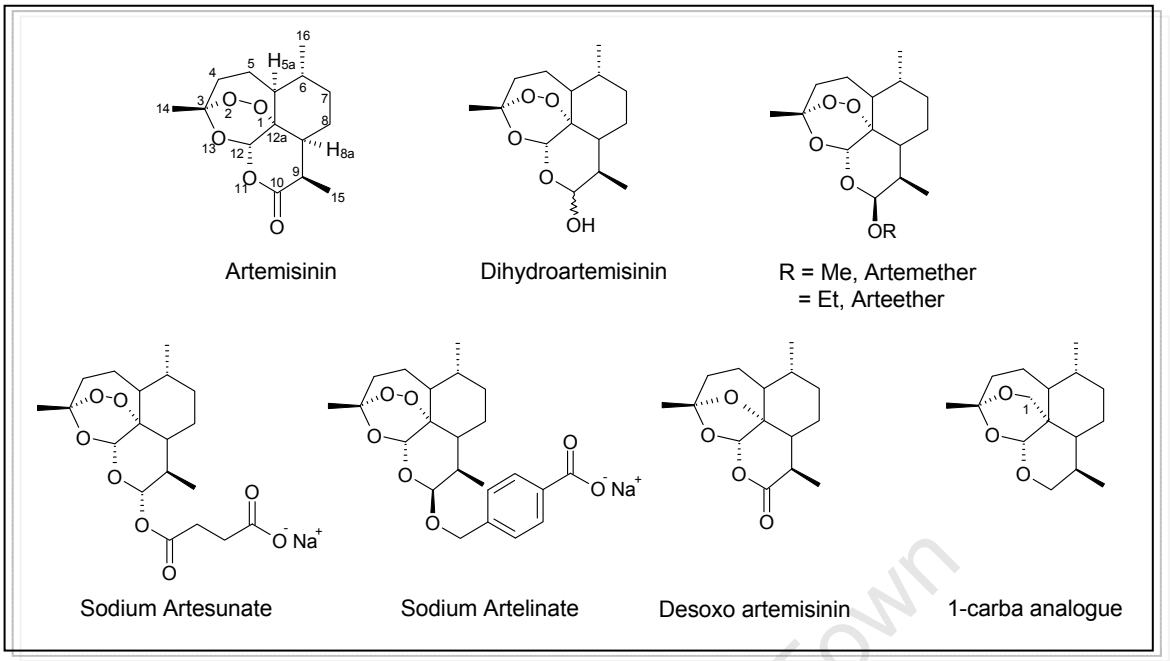


Figure 2.5. Artemisinin and its derivatives.

2.2.3 Activity against multidrug resistant parasites

The phenomenon of drug resistance is most common in southeast Asia. On the borders of Thailand, this phenomenon has been studied intensively where mefloquine-resistant parasites emerged after only a few years of monotherapy.[62] Efficacy can be restored in some areas when artemisinins are combined with drugs that no longer work well enough. The combination of artesunate and mefloquine was able to consistently cure more than 95% of patients, where mefloquine alone only worked for about 50-60% of cases.[62,63]

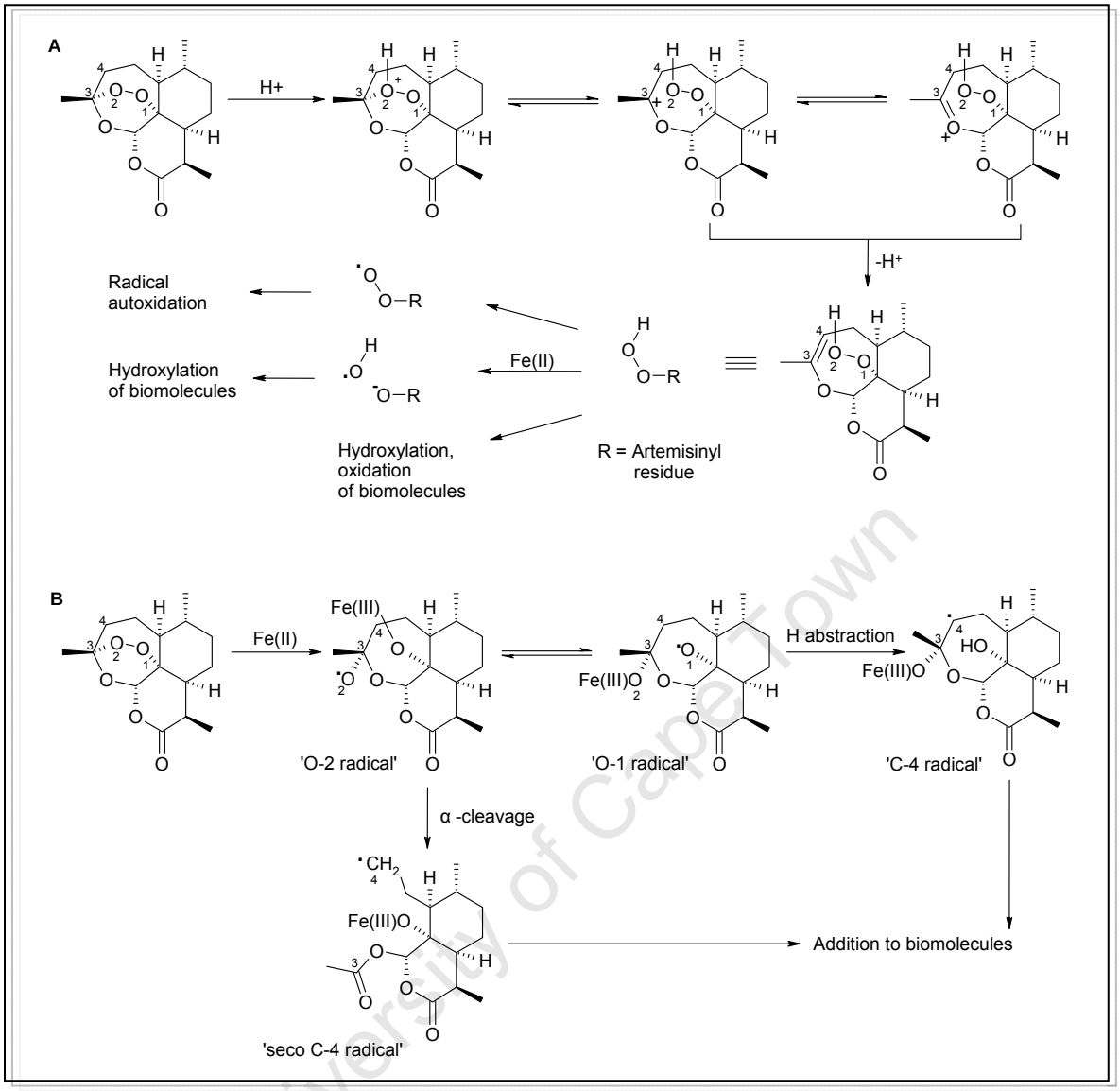
These findings have given rise to the idea that antimalarial drugs should be combined for an effective treatment of patients, and that such a combination should always include an artemisinin derivative.[64] The advantages of using artemisinin in combination is that (a) cure rates can be raised when combined with antimalarials that are taken from different classes, and (b) development of drug resistance by the parasite can be delayed.

The use of artemisinin in combination is also beneficial in terms of the metabolism of artemisinins, as treatment with artemisinin drugs causes reduction of parasitaemia below detectable levels without eliminating all parasites, resulting in a higher risk of recrudescence.[65,66] Additionally, a fraction of the parasites exposed to the drug are thought to become dormant and unsusceptible to further dosing until reactivation.[67] Therefore, in order to completely eliminate the parasites, combinations with other longer-acting drugs are necessary.[68,69] The World Health Organization (WHO) has currently recommended artemisinin combination therapies (ACT) as the first-line antimalarial treatment for *P. falciparum* malaria.[70] Several ACTs have been developed, including Coartem® (artemether + lumefantrine), and combination of artesunate with amodiaquine, mefloquine or sulfadoxine-pyrimethamine.[68,70]

2.2.4 Theories on Mechanism of action

2.2.4.1 Non-specific proposed mechanisms of action

Artemisinin and its derivatives kill malaria parasites at nanomolar concentrations, but they are only toxic to mammalian cells at micromolar concentrations. Meshnick *et al* have found that this selectivity is due to the enhanced uptake of artemisinin by the malaria parasite.[71] They have shown that the concentration of [^{3}H]-dihydroartemisinin and [^{14}C]-artemisinin in *P. falciparum* infected erythrocytes is >100 times more concentrated than that of uninfected erythrocytes. Desoxo derivatives[72] which lack the endoperoxide bridge, and the 1-carba analogue of artemisinin,[73] in which one of the peroxide oxygen atoms is replaced by a carbon atom (Fig. 2.5), are inactive towards malaria. This indicates that the peroxide functionality is vital for the biological activity of these trioxanes.



Scheme 2.1. Pathways of radical formation

2.2.4.1.1 Generation of reactive oxygen species from the peroxide

It has been proposed that the peroxide entity of artemisinin may generate reactive oxygen species, such as hydroxyl, alkoxyl, superoxide or peroxyl radicals, within the parasitized erythrocyte. The formation of these reactive oxygen species can be enhanced through the Fe^{2+}-dependent Fenton reaction, as illustrated in pathway A in Scheme 2.1. Addition of an iron (Fe^{2+}) chelator inhibits the parasiticidal activity of peroxides,[74]

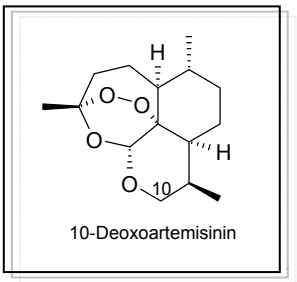
supporting the hypothesis that Fe^{2+} plays an important role. These reactive oxygen species may overwhelm the parasite anti-oxidant defense system, leading to parasite death.[75]

2.2.4.1.2 Activation by "free" ferrous iron

The reductive cleavage of a peroxide by Fe^{2+}, via the Fenton reaction, to generate O-centred or alkoxyl radicals, which rearranges to C-centred radicals and finally neutral products has been well understood and thoroughly examined before the advent of the artemisinins.[76] The peroxide entity is cleaved and generates two different alkoxyl radicals, labeled 'O-1' and 'O-2' radicals, as illustrated in pathway B in Scheme 2.1. The 'O-2' radical is adjacent to an oxygen atom, and is predisposed to undergo 'α-cleavage', which is the cleavage of the C3–C4 bond, to generate the 'seco' C-4 radical. On the other hand, the 'O-1' radical abstracts the nearby α-disposed hydrogen atom on C-4 to generate the C-4 radical. The anticipated targets of the carbon-centered radicals include haem-binding proteins and proteases involved in haemoglobin degradation.[77,78]

2.2.4.1.3 Inconsistencies in the iron-activation theory

Eckstein-Ludwig *et al* have later shown that the Fe^{2+}-dependent activation of artemisinin is independent of haem.[79] The use of a protease inhibitor inhibited the activity of chloroquine, as was widely accepted that chloroquine acts by binding to haem. In contrast, no antagonism or synergism was observed between the protease inhibitor and artemisinin activity. Furthermore, the fluorescent labeling of artemisinin derivatives has shown that instead of being localized in the parasite FV, as would be expected in the case of haem-dependent activation, they were distributed throughout the parasite.[79]



10-Deoxoartemisinin

Artemisinin derivatives that are chemically unable to react with haem, but are very potent antimalarials have been found by Haynes *et al*.[80] 10-Deoxoartemisinin, possessing superior *in vitro* activity against *P. falciparum* and is approximately equipotent *in vivo* against *P. berghei* compared to artemisinin, is hydrolytically more stable than artemisinin and dihydroartemisinin, and was found to not interfere at all with haemozoin formation. This shows that binding of artemisinin to Fe(III)PPIX is unnecessary for antimalarial activity, as the inhibition of β-haematin does not relate to biological activity.[81]

Several workers have proposed that the parasite death in the presence of artemisinin may involve high affinity binding of the intact artemisinin to an active site, prior to any "activation", as opposed to non-specific or random cell damages caused by freely diffusing oxygen radical species.

2.2.4.2 Parasite-specific proposed mechanisms of action

Results by Avery *et al*[82] and Golenser *et al*[83] have shown that for various analogs of artemisinin, the IC_{50} for *Plasmodium* is less than that of *L. major* and other eukaryotic cells by several orders of magnitude (0.10 nM and 0.63 μM respectively for DHA), suggesting a specific mechanism of action of the artemisinin class of antimalarials.

2.2.4.2.1 Inhibition of plasmodial sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA)

Research done by Krishna and Haynes[65,78] have suggested that artemisinin undergoes activation after binding to a specific site, as they have found that the *in vitro* antimalarial

activity of artemisinin is sensitive to steric effects. For example, the replacement of the methyl group at C3 of artemisinin and 10-deoxodihydro-artemisinin by much larger groups (e.g. phenylethyl) results in a decrease in activity. As artemisinin is structurally similar to thapsigargin (extracted from the plant *Thapsia gargania)*, also a sesquiterpene lactone, which is a highly specific inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), it was hypothesized by Eckstein-Ludwig *et al* that artemisinins specifically inhibit *P. falciparum* SERCA.[79]

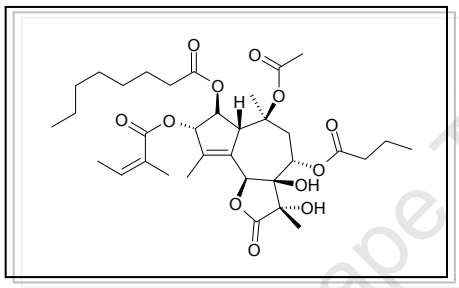


Figure 2.6. Chemical structure of thapsigargin.

SERCA is responsible for the maintenance of calcium ion concentrations, which is important for the generation of calcium-mediated signaling and the correct folding and post-translational processing of proteins. Eckstein-Ludwig *et al* have found that artemisinin completely inhibited PfATP6, the only SERCA-type Ca^{2+}-ATPase in *P. falciparum*, with high specificity. Addition of thapsigargin and artemisinin to *P. falciparum* cultures showed antagonism, indicating that both drugs have a common target. Further tests done by Eckstein-Ludwig *et al* have also suggested that artemisinin binds to the same region in the protein that binds thapsigargin.[79]

Artemisinins may bind to PfATP6 by hydrophobic interactions while leaving the peroxide bonds exposed, as demonstrated by three-dimensional modeling of the PfATP6 amino acid sequence and subsequent docking simulation.[84] This exposure of the peroxide bond may

allow its cleavage by iron to generate carbon-centered radicals (as discussed earlier), leading to enzyme inactivation and parasite death. This theory is supported by the fact that a single amino acid mutation in the plasmodial SERCA modulates sensitivity to artemisinins *in vitro,*[85] and is also the most widely accepted as the possible mechanism of action.

2.2.4.2.2 Research contradicting protein-binding as a mode of action

Research conducted by various groups has produced results that contradict the findings of Eckstein-Ludwig *et al*.[86,87] del Pilar Crespo *et al*[86] have found that thapsigargin caused a marked contraction and re-organization of the endoplasmic reticulum; however, this was not observed for artemisinin. Additionally, they have also found that there was no antagonism between thapsigargin and artemisinin based on their isobolograms. These results thus do not support the reported antagonism between artemisinin and thapsigargin by Eckstein-Ludwig *et al*.

Hartwig *et al*[87] have studied the distribution of fluorescent dansyl trioxane derivatives in living, intraerythrocytic-stage *P. falcipaum* parasites using microscopic imaging. The fluorescent trioxanes rapidly accumulate in parasitized erythrocytes, and localizes within food vacuole-associated neutral lipid bodies of trophozoites and schizonts, and surrounding the developing merozoite membranes. They have found that artemisinin pre-treatment significantly reduced fluorescent labeling of neutral lipid bodies. However, thapsigargin pre-treatment resulted in no such effect. Moreover, staining of thapsigargin-treated parasites with Nile Red has demonstrated that neutral lipid bodies formation was unaffected by thapsigargin pre-treatment, thus once-again suggesting that artemisinin and thapsigargin act via different mode(s) of action.

The PfATPase-dependent model of activity and the differences in the antimalarial activity of enantiomeric forms of artemisinins suggest that the highly specific, noncovalent interaction between artemisinins and a protein target could result in stereoselectivity. However, results from O'Neill *et al* have shown otherwise.[88] They have found that the enantiomers of trioxanes structurally related to artemisinin showed equivalent levels of activity against chloroquine-sensitive, chloroquine-resistant and multidrug-resistant strains of *P. falciparum*. This implies that the activation of artemisinin does not depend on stereospecific interaction with a protein. Additionally, introduction of bulky side groups to artemisinin may cause either an increase or decrease in activity, depending on the residues added.[89] Both points described here weaken the theory that a protein-binding site is crucial for artemisinin activity.

2.2.4.2.3 Interference with mitochondrial electron transport

Li *et al* have shown that the addition of artemisinin causes depolarization of the mitochondrial membrane, using genetic manipulation in yeast models.[90] They have found that two yeast genes that encode NADH dehydrogenases are vital for artemisinin sensitivity or resistance, and a gene homologous to one of the two are found in the *P. falciparum* database. This suggests that artemisinin and its derivatives are activated by the transition metals, including iron, in the mitochondria, and subsequently cause local production of reactive oxygen species and depolarization of the mitochondrial membrane. Artemisinin has also been shown to inhibit the respiratory chain of both sexual and asexual stages of *P. falciparum*.[91] The loss of membrane potential in the parasite affects pyrimidine biosynthesis,[90,92] a key metabolic process for nucleic acid production, and may lead to parasite death.

Artemisinin derivatives may be active via several mechanisms. At this point, there is no decisive evidence as to the absolute target(s) that they act against.

2.2.5 Semisynthetic and synthetic endoperoxide antimalarial drugs

Semisynthetic and synthetic endoperoxide derivatives have been synthesized by various research groups, and found to possess potent antimalarial activities. These include the semisynthetic artemisone[93] and synthetic trioxolanes (ozonides)[94] and tetroxanes (Fig. 2.7).[95]

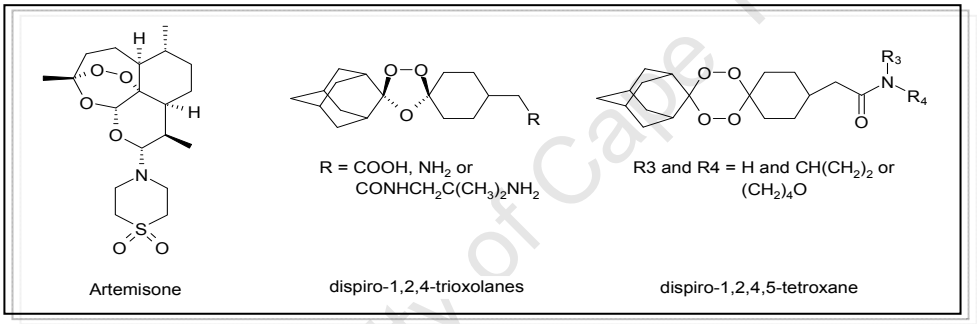


Figure 2.7. Structures of representative semisynthetic and synthetic endoperoxide derivatives.

2.2.6 Artemisinins as anti-cancer drugs

Anti-cancer properties of artemisinins have been assayed *in vitro* since the late 1980's. It has been found that apart from anti-malarial activities, artemisinins such as artesunate has also been found to be active against a variety of unrelated tumour cell lines, being the most effective against leukemia and colon cancer, while inhibitory effects on cancer cell growth such as lung, melanomas, breast, ovarian, renal, prostate, CNS cancer cells including many drug resistant cancer cells have been reported.[96,97] Other studies have also shown that artemisinins can suppress the growth of human tumour xenografts in rats and mice.[98,99]

2.2.6.1 Possible mechanisms of action

The potential general mechanism against these tumour cells has been identified to be the inhibition of angiogenesis[100] (formation of new blood vessels from existing host capillaries), and the normalization of the upregulated Wnt/b-catenin pathway in colorectal cancer.[101] Artemisinins also inhibit the proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC), as well as vascular endothelial growth factor (VEGF) binding inhibition to surface receptors on HUVEC, and a reduction in the expression of VEGF receptors Flt-1 and KDR/flk-1 on HUVECs.[100 f-h] The reduction in the expression of the VEGF receptor KDR/flk-1 was also observed in tumour and endothelial cells.[100 b-d]

Artemisinins may function in different ways to classical cancer chemotherapeutic drugs, as when genes that encode for transporters that mediate drug resistance, such as MDR gene 1, MDR associated protein 1 and breast cancer resistance protein, were over-expressed, a change in susceptibility was not observed. These studies have also shown that the delivery of iron by the use of holotransferrin enhances the anticancer properties of artemisinins in some cell lines.[102,103]

2.2.6.2 Development of Artesunate as a drug candidate

Artesunate acts at targets different to those of many current cancer chemotherapeutics, and hence unlikely to pose antagonistic effects to existing anticancer agents.[104] A study in which an animal model carries a human colorectal cancer cell line have confirmed that artesunate has independent antitumour activity, and can decrease the size of primary tumours and reduce the risk of the development of hepatic metastases.[101] In addition to this, individual

cases of human studies[105] and a recent Phase II study of lung cancer[106] both support the idea that artesunate should be forthwith studied as a primary or adjunct antitumour agent, particularly for leukaemia and colorectal cancers. The relatively low toxicity on normal human cells,[102] ease of administration and good oral bioavailability of artesunate also makes it an ideal drug candidate.

2.3 Aims and objectives

Overall aim:

- To design and synthesize new antimalarial agents that target or interfere with unique parasitic pathways such as haemoglobin degradation or parasitic endocytosis.

Objectives:

- To synthesize antimalarials based on the "Designed Multiple Ligand" approach, in which quinoline inhibitors of β-haematin formation are hybridized with artemisinin, which is believed to act via the inhibition of the plasmodial sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), or 1,4-naphthoquinones, which are inhibitors of glutathione redutase.
- To synthesize β-lactams via the Staudinger reaction or the Ugi 3-component 4-centre condensation, and subsequent post-condensation modifications via the Mannich reaction.
- To synthesize amodiaquine-like and bisquinoline compounds.
- To pharmacologically evaluate the synthetic compounds in order to identify potential new antimalarials, and the exploration of their effects on plasmodial endocytosis, and/or inhibitory effects on β-haematin formation and/or mammalian cells, including tumour cells.

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CHAPTER 3

DESIGNED MULTIPLE LIGANDS VIA THE AZA-MICHAEL ADDITION REACTION

3.1 Introduction

This chapter describes the synthesis, characterization and biological evaluations of novel artemisinin derivatives, some of which contain a 4-aminoquinoline group, the pharmacophore responsible for antiplasmodial activity in chloroquine. These compounds were designed and synthesized primarily to determine their antiplasmodial and anticancer activities.

3.2 Definition of Pharmacophore

The definition of a pharmacophore was first described by Paul Ehrlich as "a molecular framework that carries (phoros) the essential features responsible for a drug's (pharmacon) biological activity".[1] This definition remained unperturbed for over 90 years, until the currently widely used definition was presented in 1977 by Peter Gund as "a set of structural features in a molecule that is recognized at a receptor site and is responsible for that molecule's biological activity".[2] The IUPAC definition of a pharmacophore is "an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response".[3]

3.2.1 Pharmacophore of Artemisinin

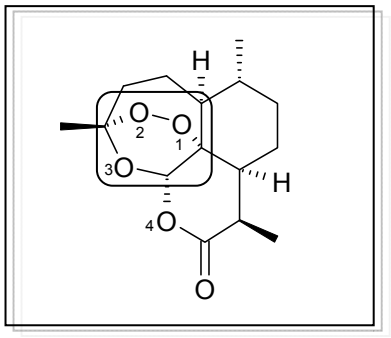


Figure 3.1 Pharmacophore of artemisinin.

The pharmacophore of artemisinin has been identified as the 1,2,4-trioxane (6-membered ring containing 3 oxygen atoms) moiety.[4,5] Studies have shown that, when compared to artemisinin, *in vitro* activities have vastly decreased for artemisinin analog without the endoperoxide bridge,4 or carba-analog where O-3 is replaced by a carbon atom.5

3.2.2 Pharmacophore of Chloroquine

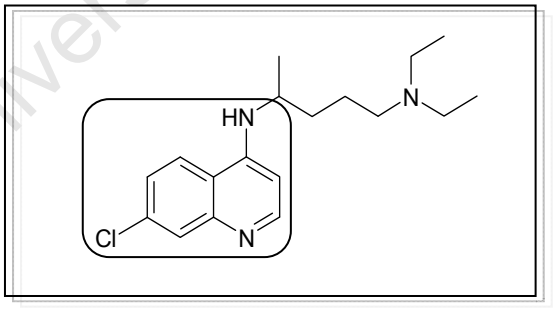


Figure 3.2 Pharmacophore of chloroquine.

As discussed in Chapter 2, the various components responsible for biological activity of chloroquine have been identified. In general, the pharmacophore of chloroquine has been generally accepted as the 4-amino-7-chloroquinoline plane.[6]

3.3 Rationale of Pharmacophore Hybridization

The antimalarial activities of artemisinin and chloroquine have been long established. Here, these two pharmacophores are combined into a single agent, and their antiplasmodial activities assessed compared to their parent drugs (Fig. 3.3). This combination is based on the idea of designed multiple ligands (DML),[7] and is analogous to combination therapy whereby two or more drugs are administered at the same time.[8] These DMLs hold potential to serve as a single drug and simultaneously modulate multiple targets.

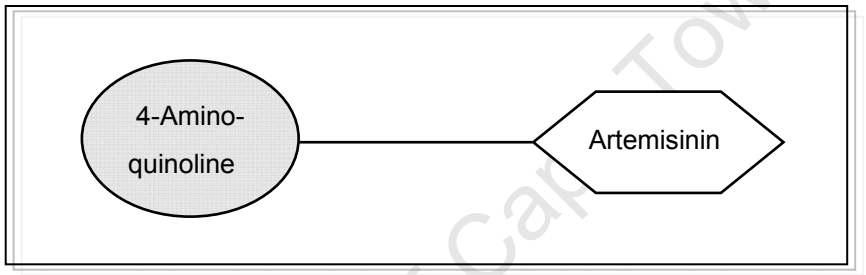


Figure 3.3. A DML containing chloroquine and artemisinin pharmacophores.

The antimalarial properties of such hybrid molecules have previously been investigated. Examples include trioxane-aminoquinoline chimeras ('trioxaquines'),[9] 1,2,4,5-tetraoxa-cycloalkane-aminoquinoline chimeras ('tetraoxaquines'),[10] artemisinin-quinine hybrids[11] and acridine-endoperoxide derivatives.[12] Structures of selected examples are shown in Fig. 3.4. Studies have demonstrated that the trioxaquines and artemisinin-quinine hybrids possessed improved antimalarial activity in comparison to parent drugs;[9,11] acridine-endoperoxide derivatives have also displayed low nanomolar *in vitro* activities.[12] In particular, a few trioxaquines were able to alkylate haem[13] and inhibit β-haematin formation better than chloroquine,[14] and a few tetraoxaquines cured mice in a modified Thompson test for antimalarial blood stage activity.[10]

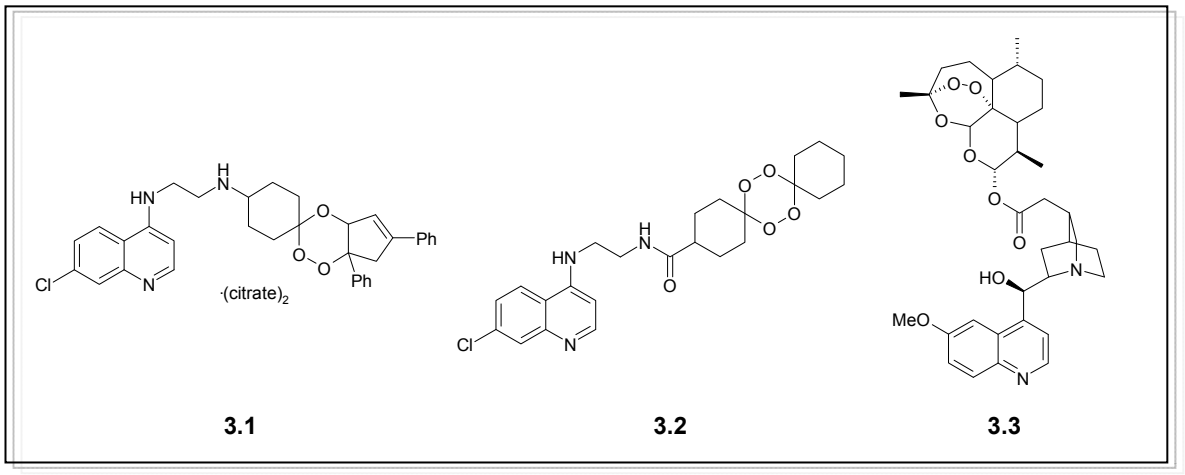


Figure 3.4. Chemical structures of trioxaquine **3.1** (**Du-1102**), tetraoxaquine **3.2** and artemisinin-quinine hybrid **3.3**.

3.4 Designed Multiple Ligands in Drug Discovery

The pharmaceutical industry today is dominated by the 'one-target, one-disease' approach, where many successful drugs have emerged from this strategy. However, this approach has been readily challenged, and strategies to develop agents that can modulate multiple targets simultaneously (polypharmacology) were designed. The aim of this strategy was to enhance the efficacy or improve the safety relative to drugs that only focus on a single target.[15,16,17]

There are three approaches to polypharmacology (Figure 3.5):

A. Drug cocktail, administered in the form of two or more individual tablets;

B. Multicomponent drugs, whereby two or more agents are coformulated into a single tablet;

C. Multiple ligands, whereby a single chemical compound is able to simultaneously modulate multiple targets.

A	B	C
		
Drug Cocktail	Multicomponent drug	Multiple ligand
2 tablets	1 tablet	1 tablet
2 agents	2 agents	1 active agent

Figure 3.5. Three main approaches to polypharmacology. Adapted from reference7

3.4.1 Advantages and disadvantages of these approaches

Drug cocktail therapies are often accompanied by poor patient compliance.[18] However, it offers greater dose flexibility when compared to multicomponent and multiple ligand drugs. The pharmacokinetic/pharmacodynamic relationships for multicomponent drugs may be highly complex, as the relative rates of metabolism may be very different between patients. This leads to unpredictable variability between patients, hence compelling extensive and expensive clinical studies.

The multiple ligand approach has a different risk-benefit profile when compared to multicomponent drugs, as summarized in Table 3.1. The increased complexity in the design and optimization of such ligands is shifted toward the earlier stages of the drug discovery process. The clinical development of multiple ligand drugs is in principle no different from the development of any other single entity, in terms of the risks and costs involved. Drug-drug interactions are also lower when compared to the other two approaches.[19] However, a major disadvantage of multiple ligand drugs is that it is difficult to adjust the ratio of activities at the different targets.

Table 3.1. Advantages and disadvantages for the three approaches

	Drug Cocktail	**Multicomponent drug**	**Multiple ligand**
Patient compliance	Bad	Good	Good
Dose flexibility	Yes	No	No
Ease of clinical development	Yes	No	Yes
Possible drug-drug interactions	Yes	Yes	No

Many of the drugs that are currently marketed are in essence multiple ligands, while very few were rationally designed to be so. Their mechanisms of action were conventionally elucidated retrospectively. An increase in the number relevant publications indicates the growing interest in the rational design of ligands acting specifically on multiple targets. However, DMLs should not be confused with 'nonselective' ligands that possess significant activity at irrelevant targets, with undesirable cross-reactivity that may lead to deleterious side effects.

3.4.2 Pharmacophore Combination Approach

The current predominant technique used for the generation of multiple ligands is the methodical combination of pharmacophores from selective ligands. A 'conjugate' is a multiple ligand where the underlying pharmacophore element for each target is separated by a distinct linker group that is not found in either of the selective ligands. A 'cleavable conjugate' is designed to release two ligands that interact independently with each target when metabolized. As the size of the linker decreases, a point is reached where the two pharmacophores are in such close proximity that they are touching each other. DMLs like

these are regarded as 'fused'. The pharmacophores can also be 'merged' by integrating common structural motifs that are present in both ligands.7

The degree of overlap of these pharmacophores forms a continuum, with high-molecular weight conjugates at the one end, and simple, low-molecular weight but highly integrated (or 'merged') pharmacophores at the other, as illustrated in Fig 3.6.

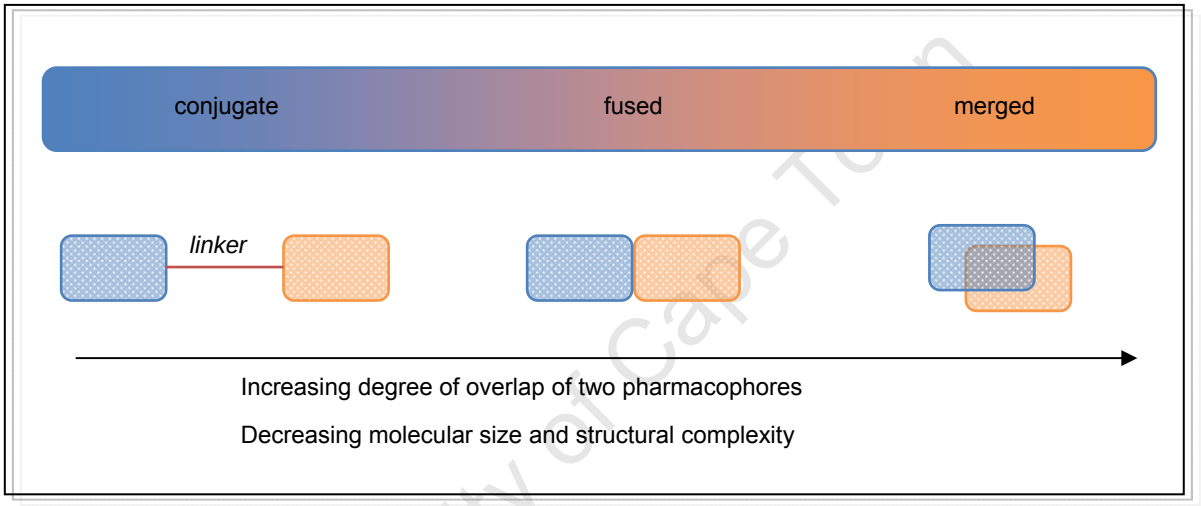


Figure 3.6. DML continuum. Adapted from references 7and [18].

3.5 Rationale for Drug Design

The rationale for the design of drug compounds in this chapter is based on the idea of DML as illustrated in section 3.3. In principle, biological activities are envisaged to be maximized by the incorporation of the artemisinin core and 4-amino-7-chloroquinoline moiety into a single molecule, through modification on C-10 of the artemisinin core (Fig. 3.7). This concept was first proposed by Vennerstrom.[9c] Additionally, the emergence of drug resistance could potentially be overcome or delayed when a drug acts against multiple targets.

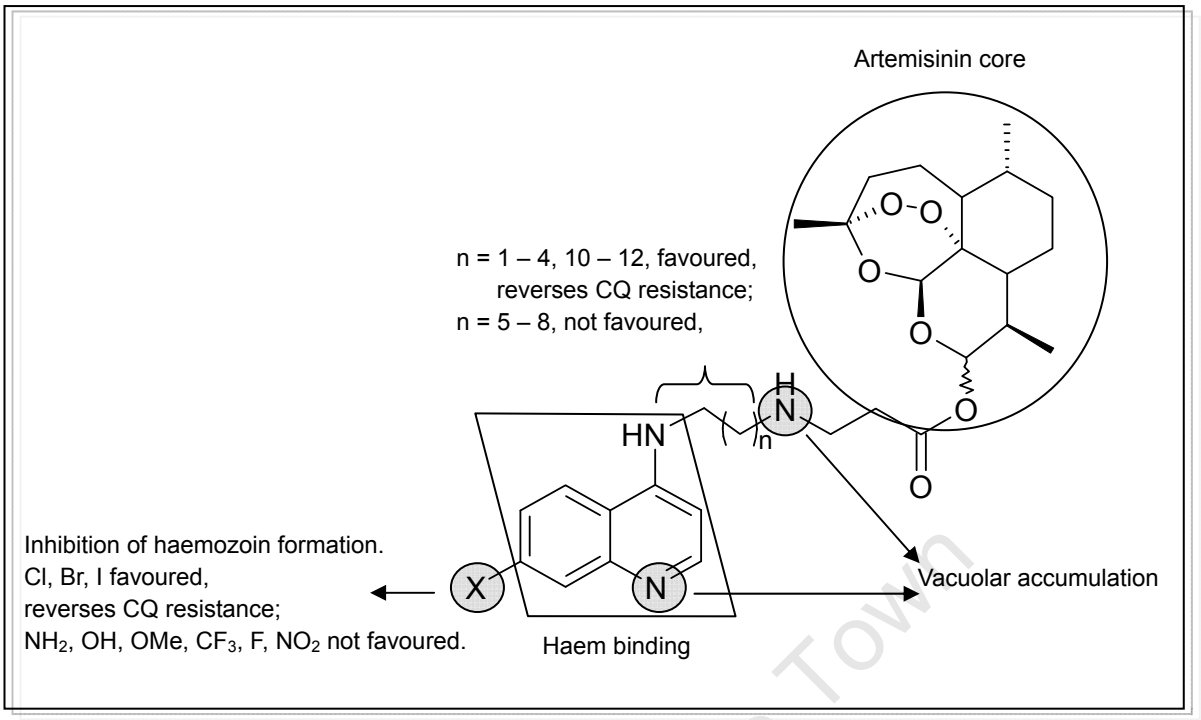


Figure 3.7. Rationale for design of target molecules.

In addition to what was discussed in section 2.1, it has also been found that the length of the alkyl group between the two nitrogen atoms on the 4-amino side chain may play a role in the degree of biological activity of chloroquine. Chloroquine analogs where this alkyl chain is shortened to 2 or 4 atoms between the nitrogen atoms, or lengthened to 10 or 12 atoms, retain their activities against chloroquine resistant strains; while those with chain lengths similar to chloroquine (5 – 8 atoms) have intermediate activities.[20] The level of accumulation of CQ depends upon the p*K*a of the quinoline side chain nitrogen.[6,21]

It has been found that having a chloro-, bromo- or iodo- group at the C-7 position of the quinoline ring had enhanced activities, and that 7-iodo- and 7-bromoquinolines had activities superior to 7-chloroquinolines, although not to a significant degree. Studies by Egan and co-workers on β-haematin (synthetic haemozoin) inhibition of quinolines established that the association of β-haematin depends on the nature of the substituent at C-7 of the quinoline

moiety, although this does not translate directly to good antiplasmodial activity.[22] They have found that strongly electron-donating groups at C-7 exerted the most effect in β-haematin inhibition, but moderately electron-withdrawing groups displayed better antiplasmodial activities. Inhibition of β-haematin formation was found to correlate with good antiplasmodial activity, as the *in vitro* IC_{50} values had a linear dependence on the inhibition of β-haematin formation.

Before discussing the synthesis of the target compounds, the general reactivity and behavior of quinoline towards nucleo- and electrophiles are described in the next section.

3.6 General Reactivity of the Quinoline Ring

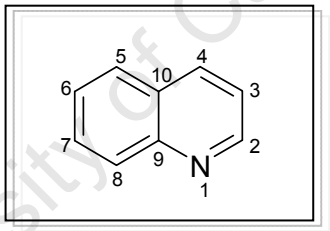


Figure 3.8. Structure of Quinoline

A quinoline is a fused benzo[b]pyridine heterocycle, derived from the fusion of a benzene and pyridine ring. Its activity can also be viewed as similar to its parent rings, i.e. electrophilic reactions occur on the benzene ring, and nucleophilic reactions occur on the pyridine ring. The nitrogen atom in the ring causes quinoline to be more π-deficient through mesomeric and inductive effects, as illustrated in Fig. 3.9. The effect is most pronounced in C-2 and C-4 positions, which are in close proximity to the nitrogen atom. Reactivity at these positions can be further enhanced by the presence of electron-withdrawing substituents, such as halogens, on the ring.

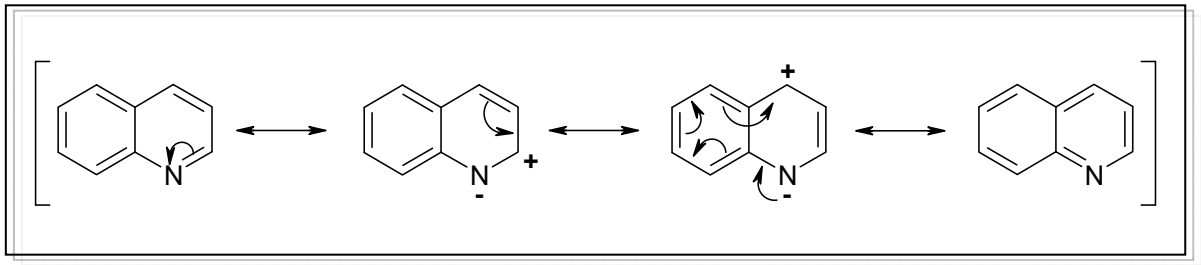


Figure 3.9. Canonical structures for the inductive and mesomeric effects of the nitrogen atom on quinoline ring.

Quinoline is more reactive towards electrophiles when compared to pyridine, as the reaction intermediate is stabilized by the adjacent benzene ring, as shown in Fig. 3.10 below.

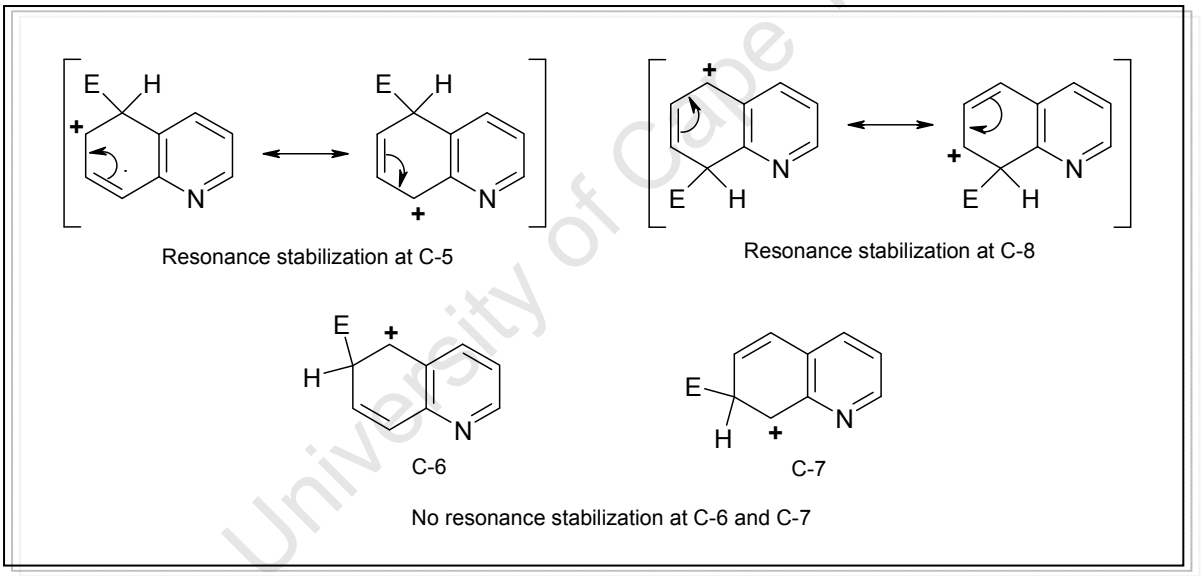


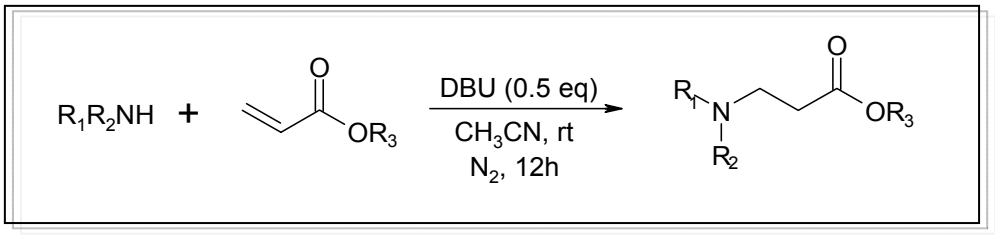
Figure 3.10. Sites of electrophilic substitution on quinoline ring, as depicted by mesomeric effects.

Electrophilic substitutions predominantly take place at C-5 and C-8, as these intermediates are resonance-stabilized, where as C-6 and C-7 are not stabilized by resonance.

3.7 Synthesis of Target Compounds via the aza-Michael Addition

Many nitrogen-containing biologically important compounds, such as β-amino alcohols, 1,2-diamines and β-lactams,[23] can be synthesized from the β-aminocarbonyl building block.[24] A number of efficient methods for the preparation of this unit have been developed, such as the Mannich reaction,[25] which has been the reaction of choice for many years. However, it often requires harsh reaction conditions. The Aza-Michael reaction has recently attracted much attention as an alternative route, owing to its mildness and operational simplicity. Most aza-Michael reactions employ Lewis acid catalysts, such as transition metals, or Brønsted acid catalysts, such as $PdCl_2(MeCN)_2$,[26] $InCl_3$,[27] $CeCl_3 \cdot 7H_2O$,[28] $Yb(OTf)_3$,[29] SmI_2,[30] $Cu(OTf)_2$,[31] $Bi(NO_3)$,[32] $Bi(OTf)_2$,[33] etc. These methods, however, are often associated with drawbacks, e.g. high price and toxicity of catalysts, harsh reaction conditions and unexpected side reactions such as polymerization of Michael acceptors.

Yeom *et al*[34] have since developed a protocol for the aza-Michael addition, employing 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a promoter, for the synthesis of β-aminocarbonyl compounds, and this mild alternative was chosen for the synthesis of our target compounds (Scheme 3.1).



Scheme 3.1. General reaction scheme for the DBU-promoted aza-Michael reaction.

3.7.1 Strategies for the synthesis of target compounds

The combination of artemisinin and chloroquine pharmacophores via the aza-Michael addition can be realized by placing the two pharmacophores on either ends of the β-aminocarbonyl unit, as illustrated in Fig. 3.11.

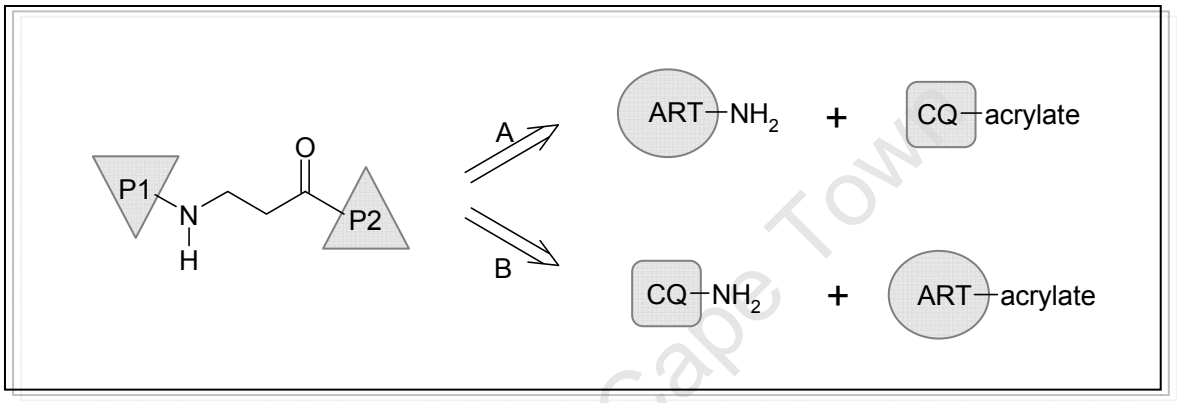
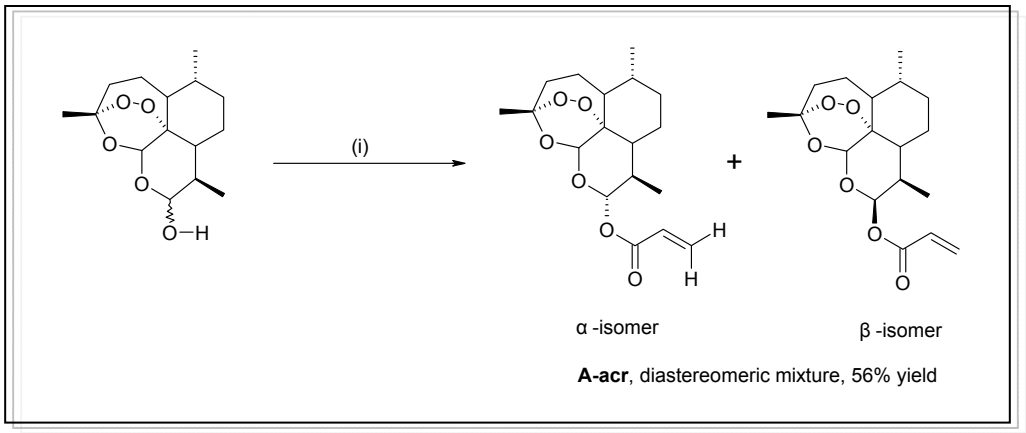


Figure 3.11. Rationale for the inputs of aza-Michael addition, where P1 and P2 represent various pharmacophores; ART and CQ represent the artemisinin and chloroquine pharmacophores, respectively.

We have chosen to synthesize target compounds via path A, i.e. conjugate addition of 7-chloro-4-aminoquinoline amines to artemisinin-acrylate.

3.7.2 Synthesis of Artemisinin Acrylate

To incorporate an α,β-unsaturated system to the artemisinin core, dihydroartemisinin (DHA) was reacted with acryloyl chloride in DCM, in the presence of triethylamine (Scheme 3.2.).



Scheme 3.2. Reagents and conditions: (i) acryloyl chloride (1.2 eq), Et_3N (1.2 eq), DCM, N_2, 0 °C to r.t., 12 h.

The requisitive acrylate was obtained as a diastereomeric (α and β) mixture.

3.7.2.1 Spectroscopic Analyses

^{1}H NMR of the crude material shows that the products are present in 4.7:5.3 ratio of α- and β-isomers (Fig. 3.12a), starting from a 1:1 mixture of α- and β-anomers of dihydroartemisinin, evident from the integration of H-10 and H-12 for the two isomers. Purification by column chromatography yielded pure stereoisomers (Fig. 3.12b, only 4.5 to 6.7 ppm region of the α-isomer ^{1}H NMR is shown).

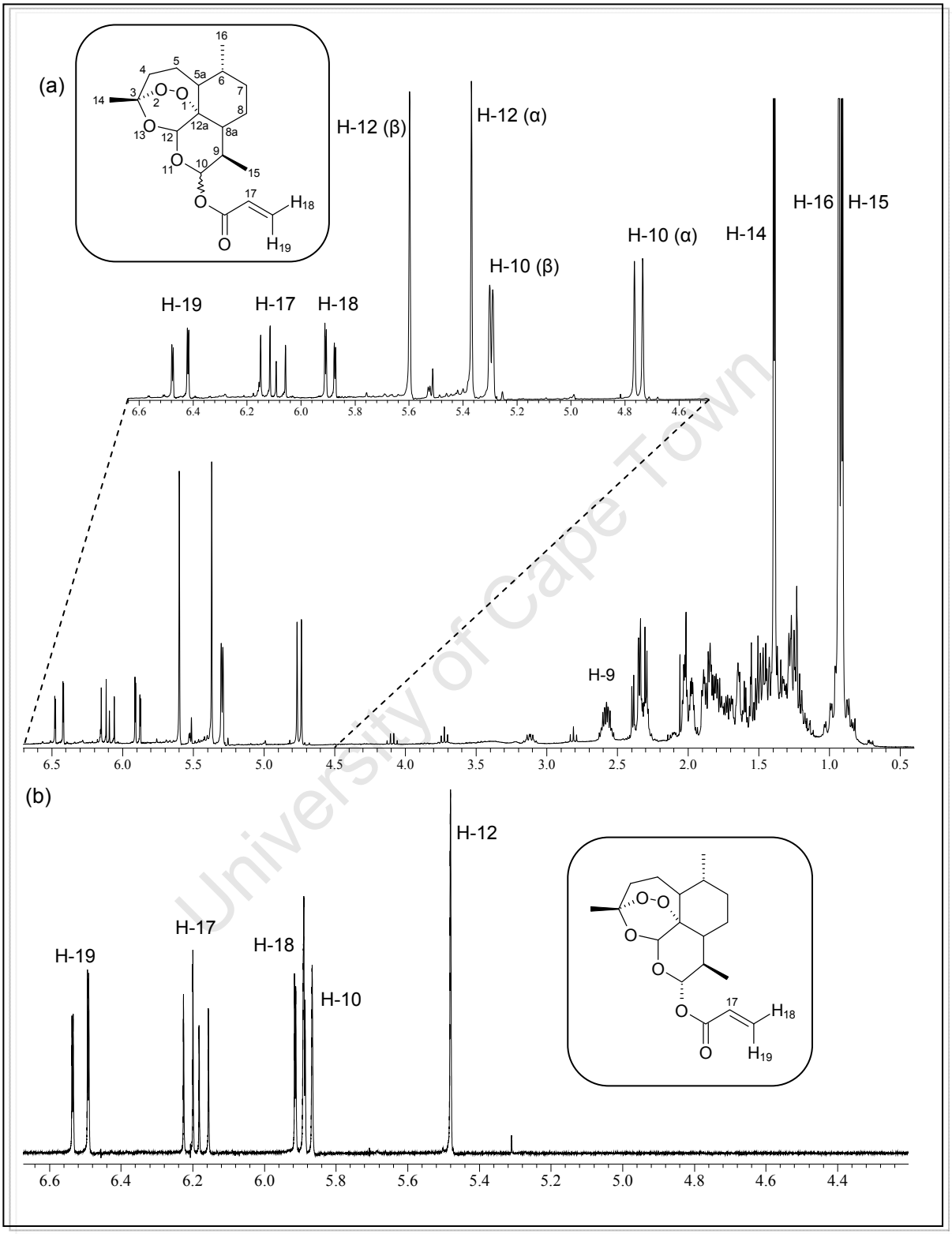


Figure 3.12. ^{1}H NMR spectra of (a) crude and (b) α-isomer of **Art-acr** (4.2 – 6.6 ppm).

The α- and β-isomers of **A-acr** can be differentiated from their coupling constants; $^{3}J_{HH}$ between C-9 and C-10 for α-isomer is expected to be much larger than that of the β-isomer, arising from axial-axial coupling of the two protons on α-isomer as opposed to the axial-equatorial coupling on the β-isomer. This is illustrated in Fig. 3.13. The typical coupling constants for H-10 were observed to be 9.6 and 3.2 Hz for α- and β-isomers respectively.

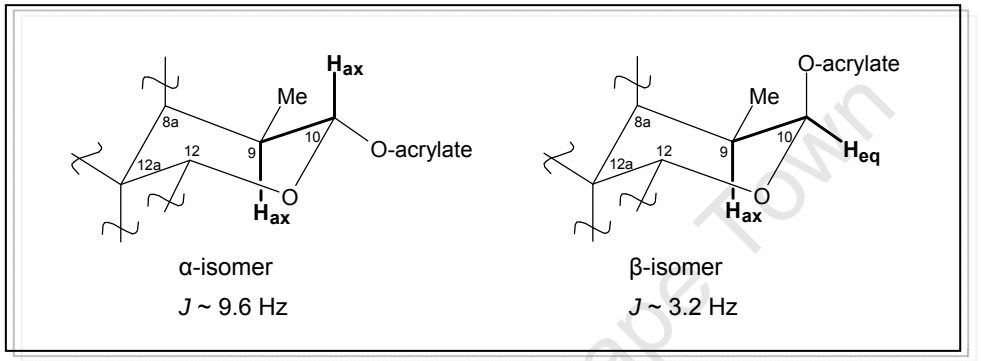


Figure 3.13. Coupling between C-9 and C-10 for the two isomers.

Protons on the alkene system can also be easily assigned by looking at the magnitude of the coupling constants. Protons that are *trans* across a double bond typically show larger vicinal coupling (13 – 18 Hz) than *cis*-protons (7 – 12 Hz), hence $^{3}J_{HH}$ between H-17 and H-19 is expected to be larger than that of H-17 and H-18. This was indeed observed in ^{1}H NMR. Although geminal couplings are typically large for saturated carbons (12 – 15 Hz), they are generally quite small in alkenes (0 – 3 Hz). The observed coupling constants for the alkene system in **A-acr** were: H-17, 17.2 and 10.4 Hz; H-18, 10.4 and 1.6 Hz, and H-19, 17.2 and 1.6 Hz (Fig. 3.14).

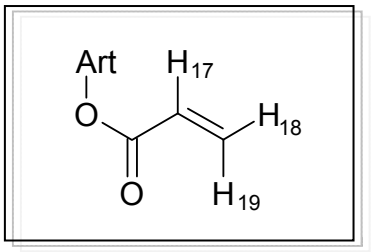


Figure 3.14. Protons across the alkene system of **A-acr**.

^{13}C NMR of **A-acr** can be assigned via 2D NMR techniques such as HSQC. For artemisinin derivatives, the most distinguishable peaks are the four signals between 80 and 105 ppm, which are carbons 12a, 12, 10 and 3. They are away from the high field region, where numerous peaks are present from the alkyl carbons (Fig. 3.15).

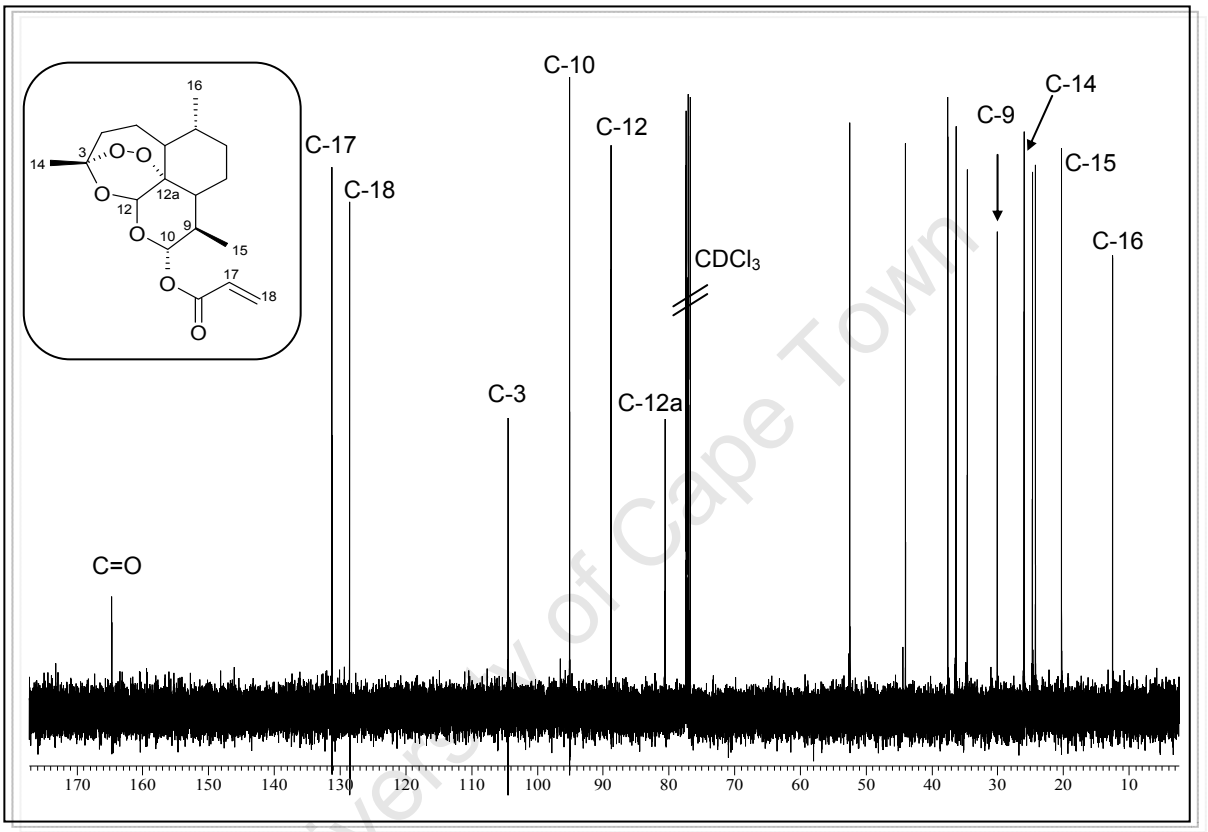


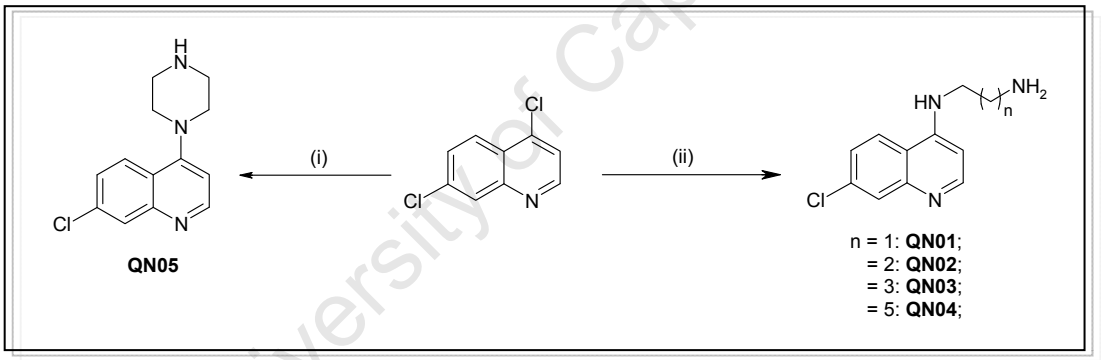
Figure 3.15. ^{13}C NMR of **A-acr**.

3.7.3 Synthesis of Quinoline Amines

A series of 7-chloro-4-aminoquinolines were synthesized using standard protocols that have been widely used.[35,36] Amines **QN01** – **QN04** were chosen such that the number of atoms between the two nitrogen atoms on the side chain span across the optimal and intermediate biological activities (except for long carbon chains, such as 10 or more carbon atoms), as described in section 3.4. The amine with five carbons between the nitrogens was not

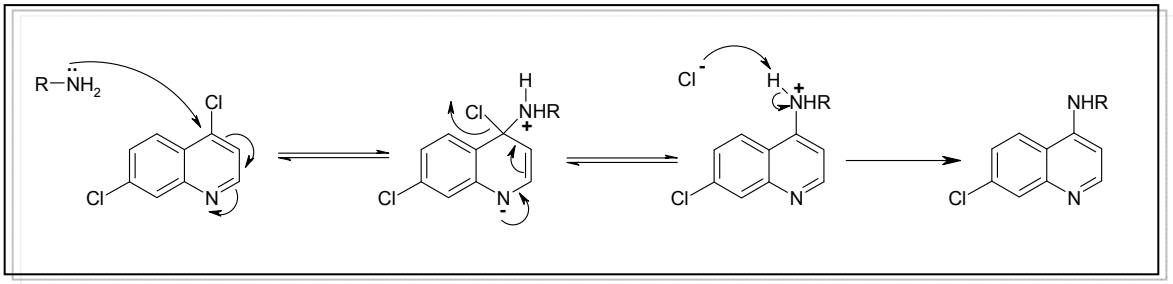
synthesized, as the corresponding starting diamine was not commercially available at the time of synthesis. **QN05**, incorporating a piperazine moiety at the 4' position, was also synthesized.

Using the protocol described by De *et al*[37], **QN01** – **QN04** were synthesized in neat from 4,7-dichloroquinoline and an excess (approximately 20 eq) of the corresponding alkyl diamines. The large excess (no less than 4 eq) was necessary to avoid the formation of terminally disubstituted amines. The synthesis of **QN05** was achieved by, again, an excess of piperazine, using *N*-methylpyrrolidinone (NMP) as the solvent, with Et_3N as the base and a catalytic amount of K_2CO_3 (Scheme 3.3).[38]



Scheme 3.3. Reagents and conditions: (i) piperazine (5.0 eq), Et_3N (1.2 eq), K_2CO_3 (0.5 eq), NMP, N_2, 150 °C, 4 h. (ii) $H_2N(CH_2)_nNH_2$ (20 eq), 110 – 150 °C, 4 h.

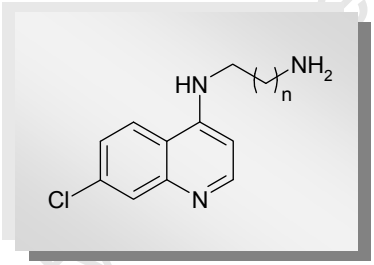
As indicated earlier, quinoline is particularly susceptible to nucleophilic attack when electronegative groups are present at the C-2 and C-4 positions. The quinoline nitrogen acts as an "electron sink", which aids in directing the incoming nucleophile to the C-4 position and displacing the chloride. Hence the regioselectivity is driven by resonance stability (Scheme 3.4).



Scheme 3.4. Mechanism of formation of 1-(7'-chloro-4'-amino)-diamines.

The isolated yields and melting points of the 7-chloro-4-aminoquinoline amines are tabulated in Table 3.2.

Table 3.2. Isolated yields and melting points of 1-(7'-chloro-4'-amino)-diamines.



Compound	n	Yield (%)	Melting point (°C)	
			observed	literature
QN01	1	64	136 – 139	137 – 139[35]
QN02	2	88	123 – 126	124 – 147[35]
QN03	3	87	43 – 46	43 – 47[35]
QN04	5	95	135 – 138	136 – 138[35]
QN05	-	89	113 – 116	113 – 115[36]

3.7.3.1 Spectroscopic Analyses

Quinolines exhibit a unique pattern in ^{1}H and ^{13}C NMR spectra, and can be easily recognized. The ^{1}H NMR spectrum of **QN05** is used as an example for the discussion on their coupling constants (Fig. 3.16). Coupling constant is typically 5.4 Hz between H-2 and H-3, and

9.0 Hz between H-5 and H-6. H-6 is often seen as a doublet of doublet (dd), due to additional long-range coupling to H-8, with a typical *J* value of 2.1 Hz. The piperazine N-H (H-11) appeared as a pentet at 3.31 ppm, with a coupling constant of 1.8 Hz. Piperazine protons H-9 and H-10 appeared as multiplets at 3.24 and 3.11 ppm.

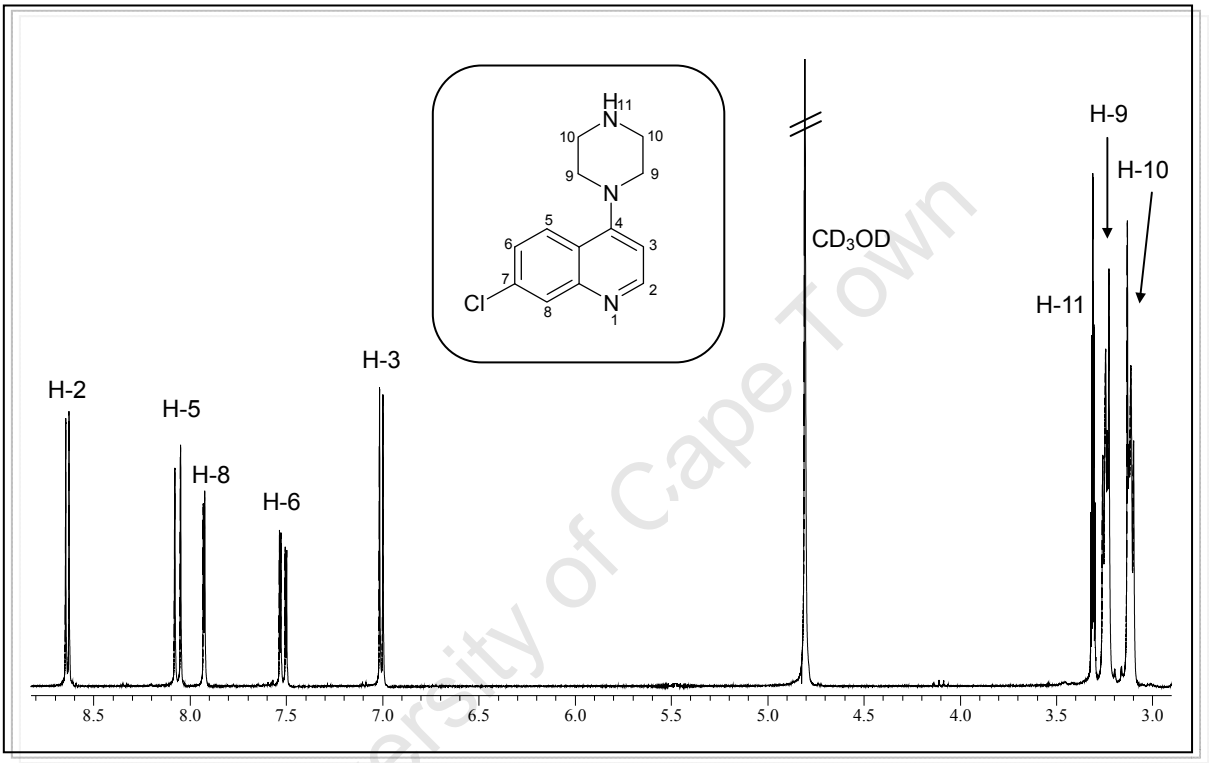


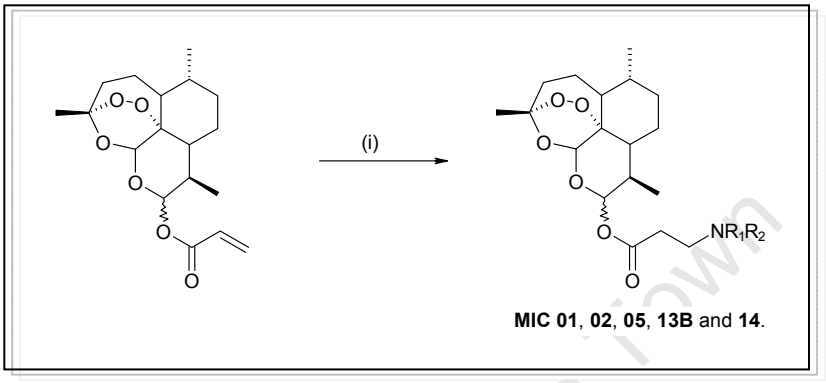
Figure 3.16. ^{1}H NMR spectrum of **QN05**.

3.7.4 Syntheses of Target Compounds

3.7.4.1 Using 7-chloro-4-aminoquinoline amines

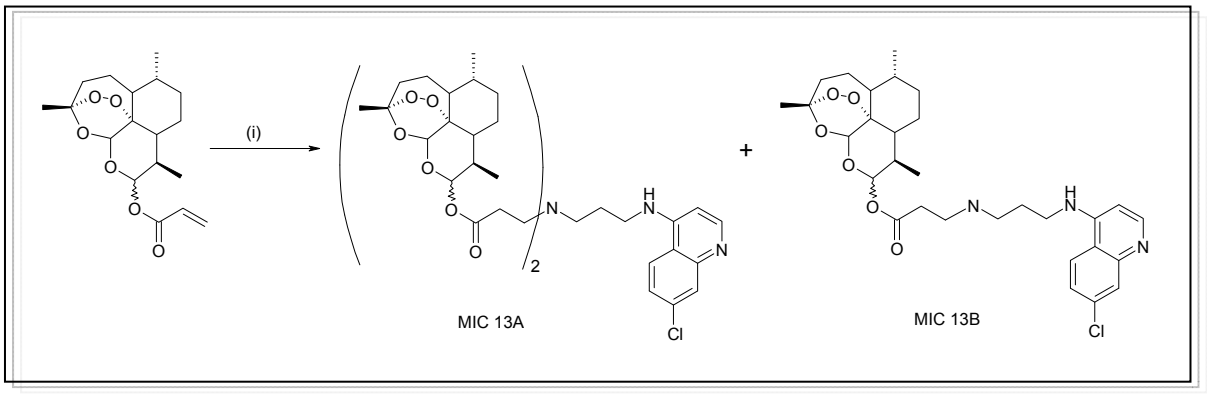
The target compounds were synthesized via the DBU-promoted aza-Michael addition, as described earlier (Scheme 3.5). In order to prevent over-alkylation of the amine, 1.5 equivalents of the amine were used. Additionally, choosing a solvent that is able to dissolve

both starting materials (especially the quinoline amines in this case) is crucial, as a solvent that is unable to fully dissolve the quinoline amine, due to its extreme polarity, creates an environment where there is more artemisinin acrylate than quinoline amine in the solution phase, which may encourage over-alkylation.



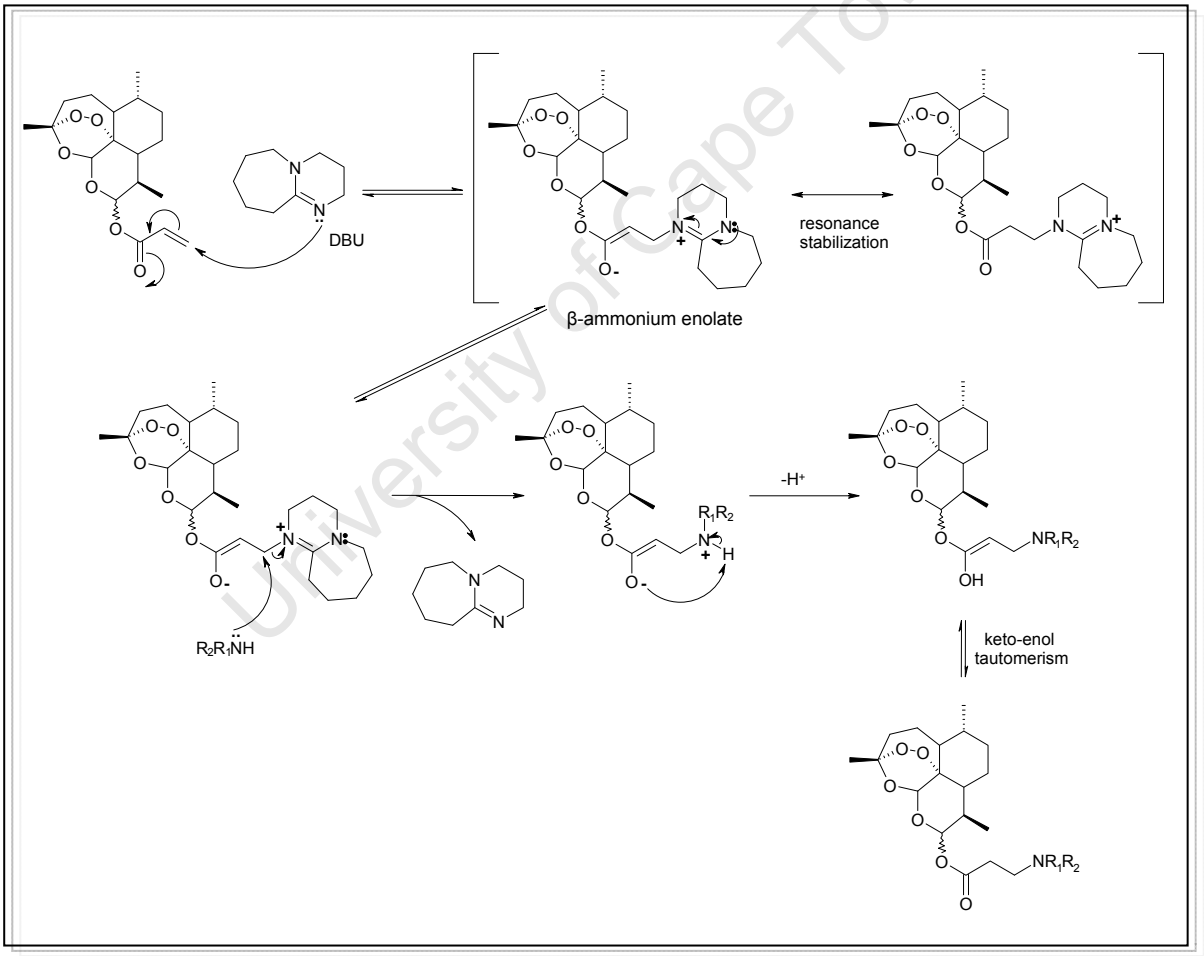
Scheme 3.5. Reagents and conditions: (i) **QN01**-**05** (1.5 eq), DBU (0.5 eq), DMF, N_2, r.t., 12 h.

For example, an attempt at the synthesis of **MIC 13B** using acetonitrile alone as the solvent yielded not only the desired product, but also the bis-Michael adduct, **MIC 13A**, as the quinoline amines are only partially soluble in acetonitrile (Scheme 3.6). Hence acetonitrile alone is not an ideal solvent for these starting materials, and a more polar solvent, such as *N,N'*-dimethylformamide (DMF), was chosen as the solvent for the syntheses of quinoline-containing conjugate adducts.



Scheme 3.6. Reagents and conditions: (i) **QN02** (1.5 eq), DBU (0.5 eq), CH_3CN, N_2, r.t., 12 h.

Mechanistically, it may be speculative that the aza-Michael addition commences via the nucleophilic attack of DBU at the β-carbon of the acrylate, forming a resonance-stabilized β-ammonium enolate. Although DBU is normally regarded as a hindered and non-nucleophilic base, relative to secondary amines, the nucleophilicity of the amine is apparently less important than the resonance stabilization of the β-ammonium enolate as is the case with DBU. This stabilization increases the equilibrium concentration of the β-ammonium enolate which results in a higher rate of formation. Enhanced reaction rates have been observed by Aggarwal in the Bayliss-Hillman reaction.[39]

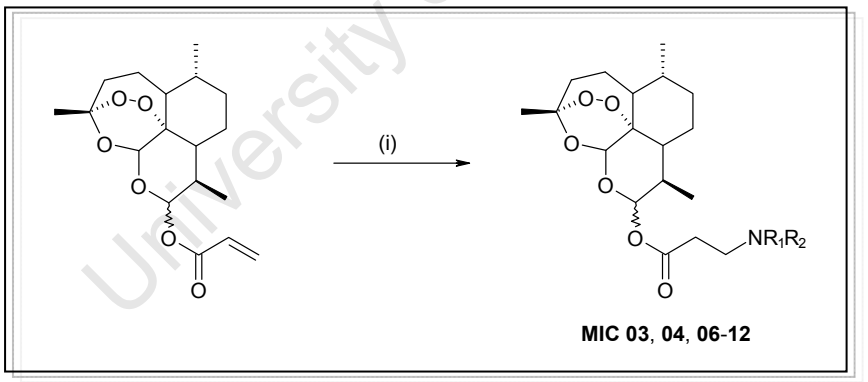


Scheme 3.7. Proposed mechanism for the aza-Michael addition.

After the Michael addition by DBU, the resulting β-enolate ungergoes attack by the secondary amine, regenerating DBU promoter at the same time. The enolate then picks up a proton to form an enol, yielding the desired product after tautomerization to the keto tautomer (Scheme 3.7).

3.7.4.2 Using commercial amines

Apart from the target compounds where 7-chloro-4-aminoquinoline amines were added to the artemisinin acrylate, commercially available amines were also used in the conjugate addition. Acetonitrile was used as the solvent, as there were no solubility problems. After the necessary reaction time, no work-up was required, as the solvent was simply removed under reduced pressure, and the crude material chromatographed.



Scheme 3.8. Reagents and conditions: (i) Amine (1.5 eq), DBU (0.5 eq), CH_3CN, N_2, r.t., 12h.

3.7.4.3 Spectroscopic Analyses

The ^{1}H NMR spectra of **MIC 13B** and **MIC 06** are presented in Fig. 3.17 and 3.18. In the case of **MIC 13B**, the five quinoline protons were, as discussed earlier, easily distinguished in the lower field region. H-10 appeared at 6.32 ppm with J = 3.2 Hz, confirming the stereo orientation at C-10 (β-isomer). The propylene protons from the quinoline amine appeared at 3.43, 2.92 and 1.94 ppm, each with a coupling constant of 5.6 Hz. Protons H-17 and H-18, which were previously on an alkene system, have transformed from doublet of doublets to triplets, each with J of 6.4 Hz. The three methyl groups on C-14, 15 and 16 remain unchanged compared with the starting acrylate, with H-14 being a singlet, while H-15 and H-16 both being doublets (Fig. 3.17).

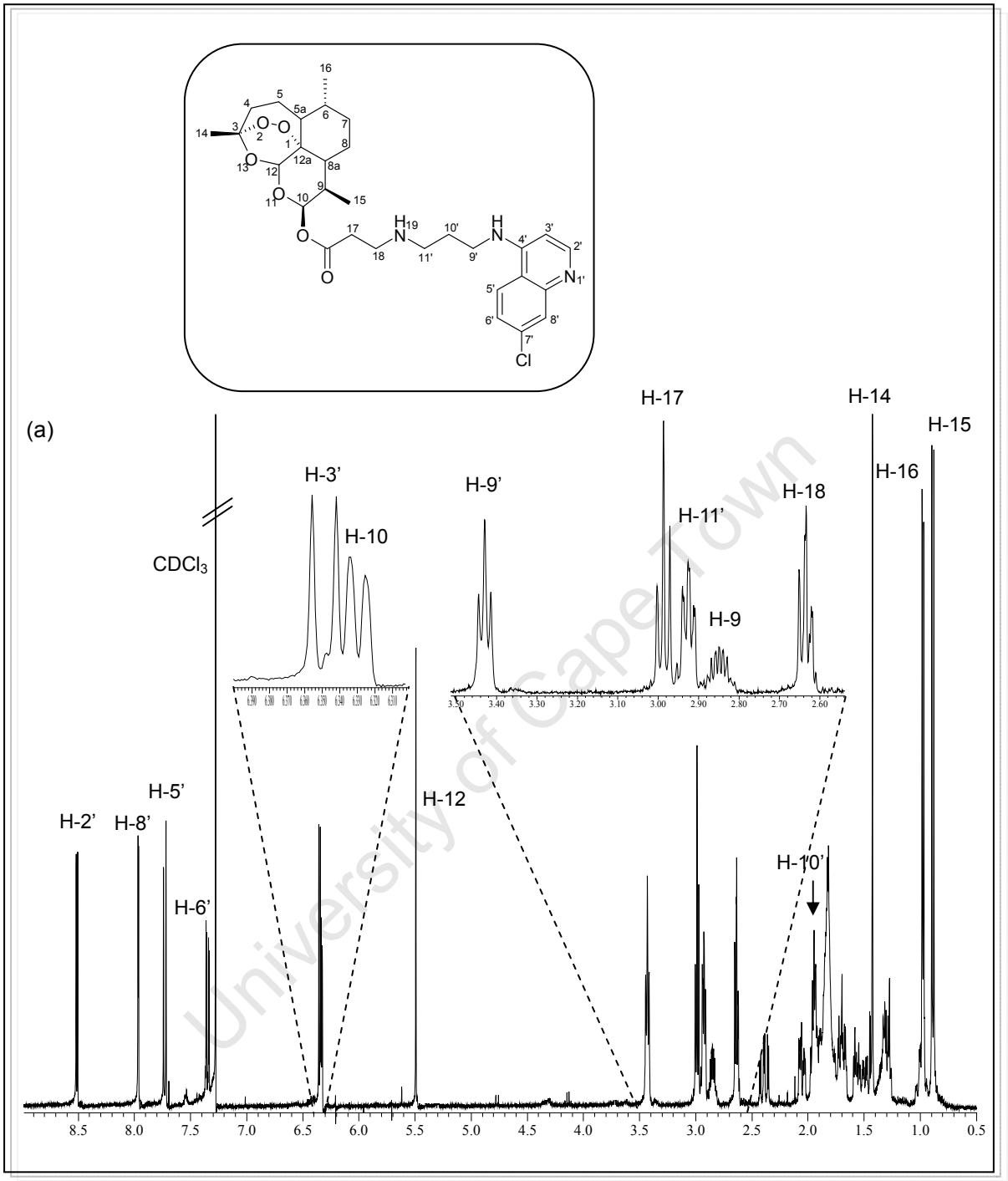


Figure 3.17 ^{1}H NMR spectrum of **MIC 13B**.

The ^{1}H NMR spectrum of **MIC 06** is similar to that of **MIC 13B**, but with no proton signals after 6.0 ppm. Proton signals from the morpholine appeared at 3.70 and 2.47 ppm, with J = 4.8 Hz (Fig. 3.18).

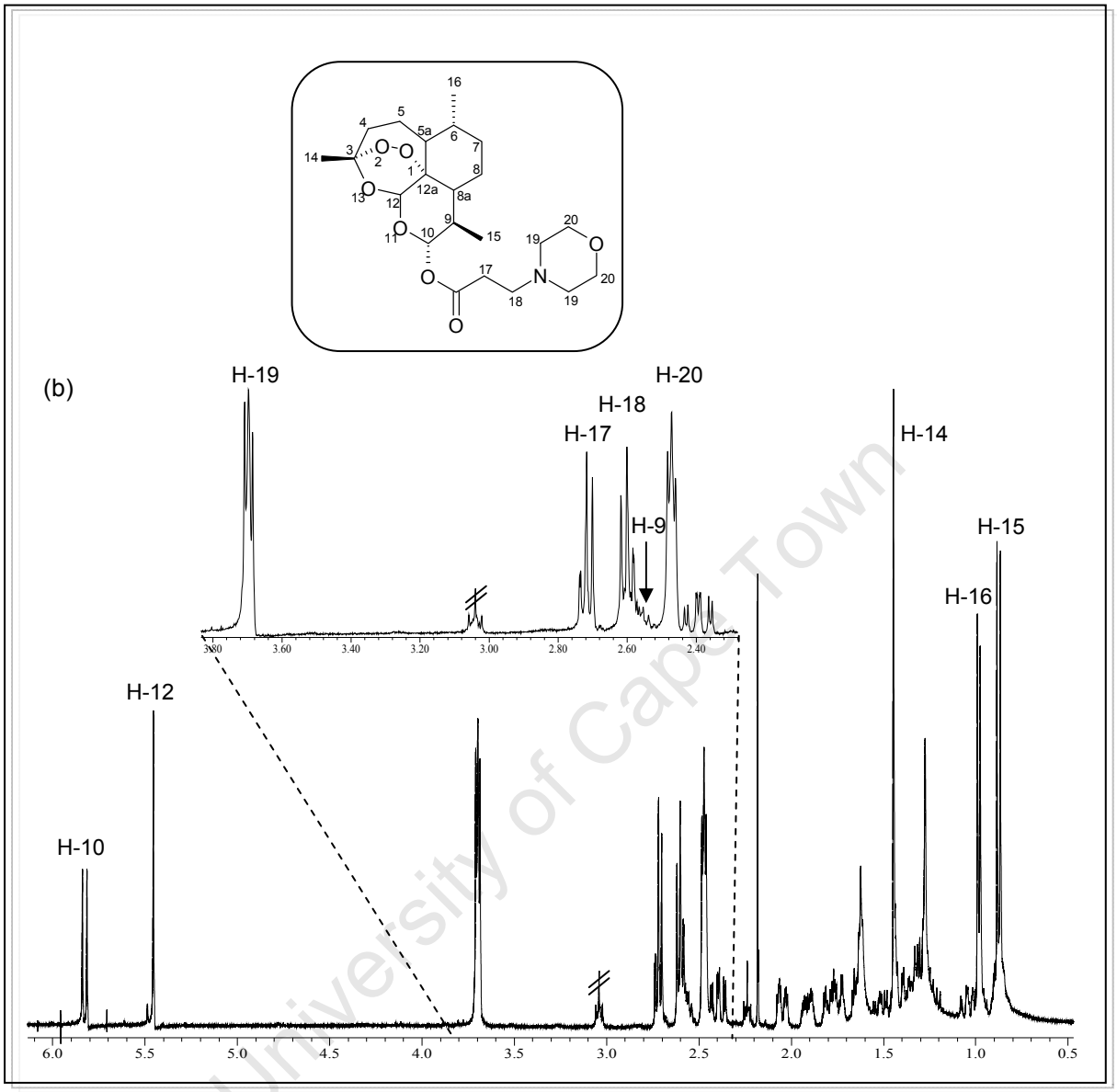


Figure 3.18 ^{1}H NMR spectrum of **MIC 06**.

^{13}C NMR spectra of **MIC 05** and **MIC 06** are used here as representatives (Fig. 3.19). The confirmation for the successful syntheses of the desired products is the shifting of the two peaks at 131 and 129 ppm to 53.4 and 32.8 ppm, corresponding to C-17 and C-18 respectively.

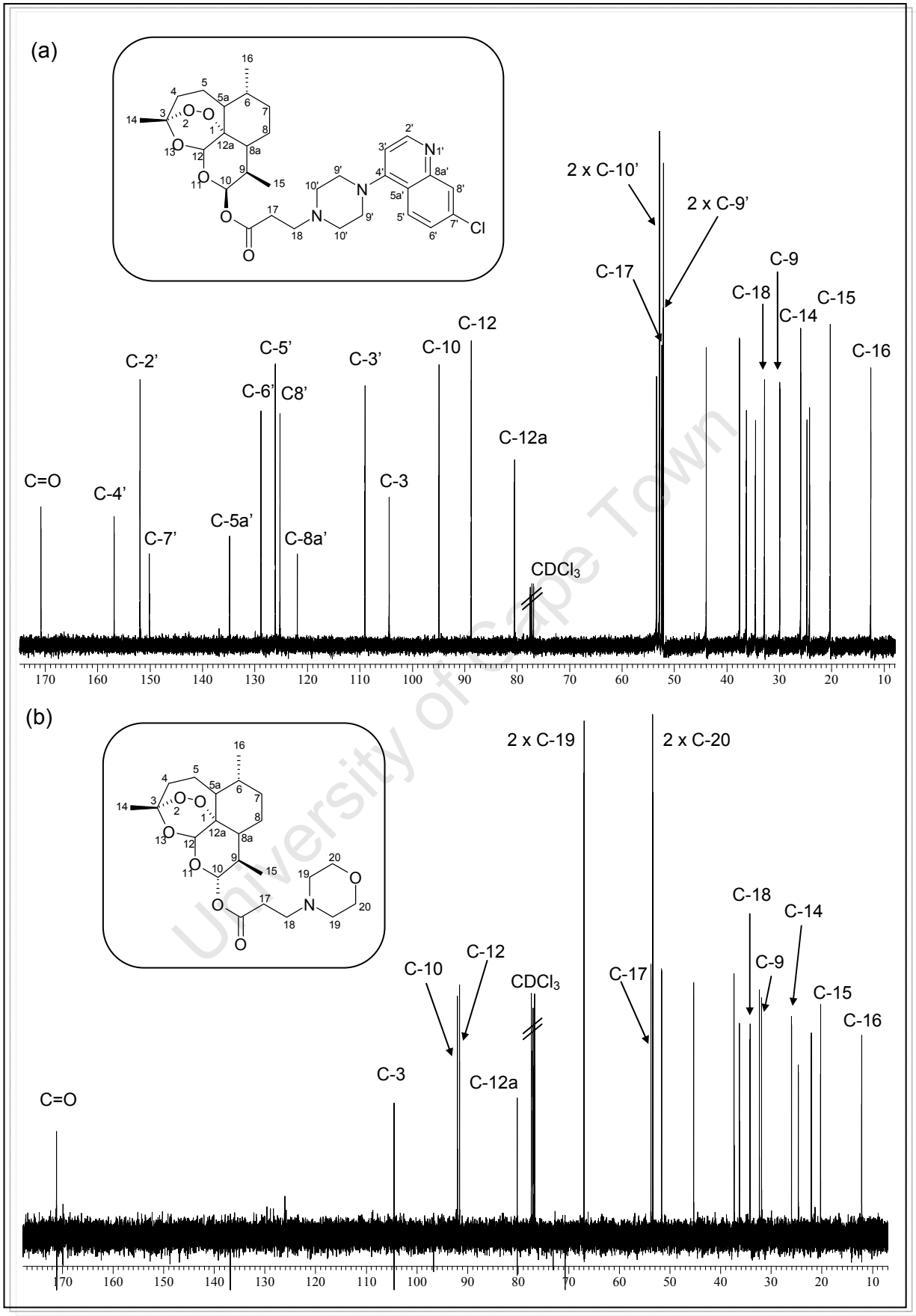
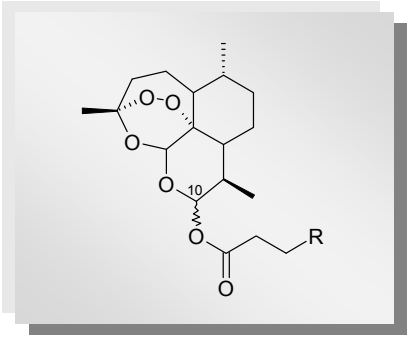


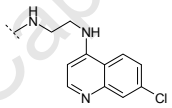
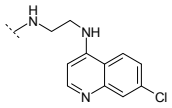
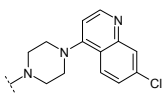
Figure 3.19. ^{13}C NMR spectrum of (a) **MIC 05** and (b) **MIC 06**.

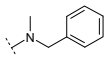
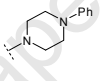
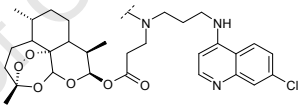
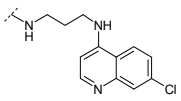
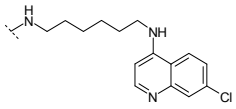
Structures of the target compounds and their yields are tabulated in Table 3.3 below.

Table 3.3. Structure and yields of the artemisinin conjugates



General structure of artemisinin conjugates

Compound	Stereochemistry at C-10	R	Yield (%)
MIC 01	β		73
MIC 02	β		71
MIC 03	β		72
MIC 04	α		69
MIC 05	β		80
MIC 06	α		75
MIC 07	α		54

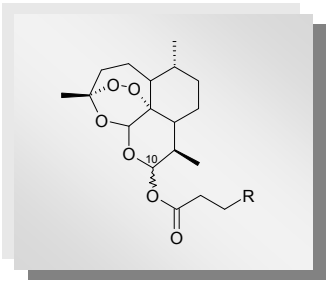
Compound	Stereochemistry at C-10	R	Yield (%)
MIC 08	α		62
MIC 09	α		80
MIC 10	α		61
MIC 11	α		63
MIC 12	α		65
MIC 13A	β		41
MIC 13B	β		37
MIC 14	β		62

3.8 Results and Discussion

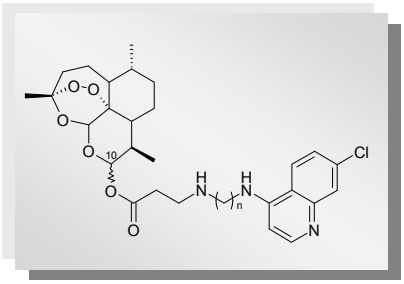
3.8.1 Antimalarial Activities

The target compounds, along with their parent compounds, were tested for their *in vitro* activities against malaria. Results are tabulated in Table 3.4 below.

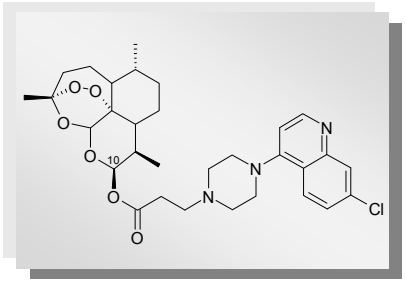
Table 3.4. Antiplasmodial activities of the target molecules and their parent compounds.



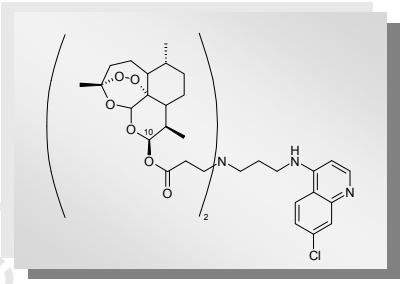
MIC 03, **MIC 04**,
MIC 06 – **MIC 12**



MIC 01, **MIC 02**,
MIC 13B, MIC 14



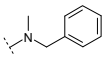
MIC 05



MIC 13A

General structure of artemisinin conjugates

Cmpd	C-10	R	n	*P. falciparum* IC_{50} (nM)		RI[a]	HeLa IC_{50} (μM)	TI[b]
				D10	Dd2			
A-acr alpha	α	-	-	5.85	9.27	1.6	6.37	687
A-acr-beta	β	-	-	7.54	12.43	1.6	2.88	232
QN01	-	-	-	231	1142	4.9	7.22	6
QN02	-	-	-	240	1251	5.2	9.83	7
QN03	-	-	-	482	2266	4.7	11.24	5
QN04	-	-	-	2164	1861	0.9	15.69	8
QN05	-	-	-	1463	1639	1.1	27.58	17
MIC 01	β	-	2	20.60	20.14	1.0	9.47	470
MIC 02	β	-	4	24.04	27.48	1.1	10.13	368

Cmpd	C-10	R	n	*P. falciparum* IC_{50} (nM)		RI[a]	HeLa IC_{50} (μM)	TI[b]
				D10	Dd2			
MIC 03	β		-	7.74	11.33	1.5	4.56	402
MIC 04	α		-	11.33	11.36	1.0	ND[c]	-
MIC 05	β	-	-	10.10	10.03	1.0	6.58	656
MIC 06	α		-	6.23	9.02	1.4	4.80	532
MIC 07	α		-	12.98	13.62	1.0	6.02	442
MIC 08	α		-	5.27	6.16	1.2	4.48	727
MIC 09	α		-	16.88	18.68	1.1	13.04	698
MIC 10	α		-	9.22	16.03	1.7	8.91	556
MIC 11	α		-	7.16	12.56	1.8	6.55	521
MIC 12	α		-	5.59	7.27	1.3	5.76	792
MIC 13A	β	-	3	6.98	4.24	0.6	9.13	2153
MIC 13B	β	-	3	18.67	24.39	1.3	5.58	229
MIC 14	β	-	6	55.63	53.55	0.9	0.37	7
ART	-	-	-	25.50	25.36	1.0	ND	-
CQ	-	-	-	30.61	145.3	4.7	9.67	66
Cisplatin	-	-	-	ND[c]	ND[c]	-	0.142	-

[a] RI, resistance index, calculated as [IC_{50} (Dd2)] / [IC_{50} (D10)]; [b] TI, therapeutic index, calculated as [IC_{50} (HeLa)] / [IC_{50} (Dd2)]; [c] ND, not determined.

Antiplasmodial activities are expressed as the 50% inhibitory concentration (IC_{50}) of the compounds against a chloroquine sensitive (D10) and resistant (Dd2) strain of *Plasmodium falciparum*, using a standard parasite viability assay which measures parasite lactate dehydrogenase activity after 48 hours of drug exposure. Cytotoxicities were obtained by determining the IC_{50}s of the compounds against a human ovarian cancer cell line (HeLa) using a standard MTT assay which measures the redox activity of live cells after 48 hours of drug exposure. Antiplasmodial results against chloroquine-sensitive (D10) and resistant (Dd2) strains of malaria have shown that almost all of the target compounds, including the artemisinin-acrylate precursor, displayed better activities than their parent compounds, artemisinin and chloroquine. It also seems that the orientation at C-10 does not have an impact on biological activity, as both α- and β-stereoisomers display activities to the same extent. This is not unexpected, as artesunate and artelinic acid have different orientations at C-10, but are both active antimalarials.

The activities of the quinoline amine precursors in Dd2 are much lower than those in the chloroquine-sensitive D10 strain (as indicated by the high resistance index, RI), which is expected, as these are chloroquine-like compounds. Their activities are also at least 12 times lower than their corresponding conjugates. Additionally, the gradual decrease in *in vitro* activities for the quinoline amines with increasing length of side-chains, is also observed in the corresponding conjugates (**MIC 01**, **13B, 02** and **14**, in the order of increasing side-chain length). Out of the five intended quinoline-containing conjugates, compound **MIC 05** is the most active, with IC_{50} values of 10 nM in both D10 and Dd2. Interestingly, the bis-Michael adduct **MIC 13A** displayed activities far better than the other five quinoline-containing conjugates, and is almost 6 times more active than the intended conjugate adduct **MIC 13B** in the Dd2 strain.

In general, conjugate adducts with commercial amines exhibited better activities than those containing quinoline moieties. The best compounds out of this series are **MIC 08, 12** and **13A**, with the latter being the most active in the Dd2 strain. The side-chains of **MIC 08** and **12** are *N*-methylbenzylamine and phenylpiperazine respectively, which do not bear structural similarities, except maybe the phenyl ring that is present in both **MIC 08** and **12**. However, the phenylpiperazine unit has been identified as a 'privileged' structure, which, according to Evans, is a substructure that confers activities to multiple proteinaceous receptor surfaces.[40] Unsubstituted phenylpiperazines have been shown to exhibit antiplasmodial activities, displaying mefloquine-type antimalarial behavior in being more potent against chloroquine-resistant than chloroquine-sensitive strains in *P. falciparum*.[41] Thus the enhanced activity of **MIC 12** may partly be due to the inherent activities of the phenylpiperazine unit.

It is worth noting that although the improvements in activities of the quinoline-containing conjugates were not to a great extent, when compared to artemisinin and chloroquine, resistance in Dd2 strain was abolished (RI values typically around 1). This suggests that the incorporation of artemisinin can aid 4-aminoquinolines in circumventing chloroquine-resistance.

Cytotoxic activities of these compounds were assessed in HeLa cells (human adenocarcinoma of the cervix), using cisplatin as the control. All compounds, with the exception of **MIC 14**, displayed cytotoxic activities in the low micromolar range. This renders large therapeutic windows, and the therapeutic indices (TI) with respect to the Dd2 strain are about 500 for the target compounds. The specificity towards plasmodial cells makes these target compounds good leads for further antimalarial development.

3.8.2. Anticancer Activities

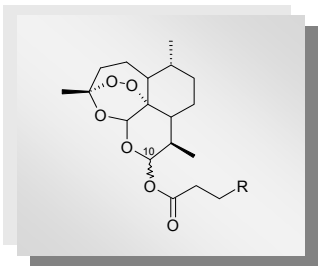
The low micromolar activities of the target compounds against HeLa cells have prompted further tests against non-cancerous cells. Selected compounds (based on cytotoxicity activities in HeLa cells) were tested against primary resting and phytohaemagglutinin (PHA)-stimulated lymphocytes.

Primary resting lymphocytes are cells that grow naturally in the body, undergoing normal cell cycles. When these cells are stimulated by PHA, the speed of cell cycles was increased, mimicking that of cancerous cells. It is important to note that PHA-stimulated cells will only replicate to a point, while cancerous cells will divide indefinitely.

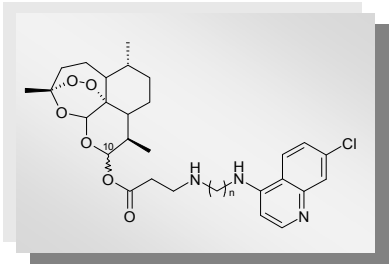
The activities of the selected compounds are tabulated in Table 3.5. The tumour specificity of these compounds were also shown, calculated by taking the average of the IC_{50} in resting and PHA-stimulated primary lymphocytes, and dividing it by the IC_{50} in HeLa cells.

Here, we have found that all of the selected compounds displayed low toxicities towards non-cancerous mammalian cells, but also with low tumour specificity. Only one compound, **MIC 14**, displayed good potential, with tumour specificity of 31. This high tumour specificity renders **MIC 14** to be a good potential anticancer lead, and should be further tested in animal models.

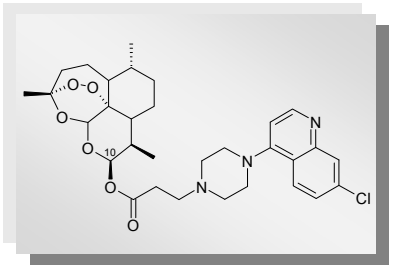
Table 3.5. Anticancer activities of the selected potential compounds.



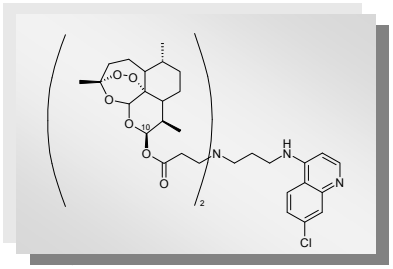
MIC 03, **MIC 04**,



MIC 01, **MIC 02**,

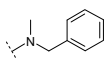


MIC 05



MIC 13A

General structure of artemisinin conjugates

Cmpd	C-10	R	n	HeLa IC_{50} (μM)	Primary lymphocytes IC_{50} (μM)		Tumour specificity
					resting	PHA-stimulated	
A-acr alpha	α	-	-	6.37	39.58	11.47	4.0
A-acr-beta	β	-	-	2.88	30.53	9.22	6.9
MIC 01	-	-	-	9.47	> 50	16.94	1.8
MIC 02	-	-	-	10.13	32.40	8.99	2.0
MIC 03	-		-	4.56	> 50	38.16	8.4
MIC 05	-	-	-	6.58	44.61	14.04	4.5
MIC 06	-		-	4.80	> 50	27.27	5.7
MIC 07	β		2	6.02	> 50	30.45	5.0
MIC 08	β		4	4.48	> 50	25.00	5.6

Cmpd	C-10	R	n	HeLa IC_{50} (μM)	Primary lymphocytes IC_{50} (μM)		Tumour specificity
					resting	PHA-stimulated	
MIC 10	β		-	8.91	> 50	30.98	3.5
MIC 11	α		-	6.55	> 50	27.18	4.1
MIC 12	β		-	5.76	> 50	27.56	4.8
MIC 13A	α	-	-	9.13	32.44	1.44	1.9
MIC 13B	α	-	-	5.58	21.52	4.71	2.4
MIC 14	α	-	-	0.37	19.03	3.40	30.7
Cisplatin	α	-	-	0.142	> 100	21.69	-

3.9 Conclusion and Future Work

Biological results of the artemisinin-based compounds against D10 and Dd2 strains of *P. falciparum* have shown that four compounds, **MIC 06**, **08**, **12** and **13A** displayed most potent activities. As artemisinins are well-known for their short plasma half-lives, as described in section 2.2.2, it would be useful to assess the half-lives and metabolites of these compounds in comparison to those of artemisinin or artesunate. Additionally, it would also be worthwhile to modify the phenyl ring on the piperazine unit in **MIC 12** for quantitative structure-activity relationship (QSAR) studies, which may aid in generating further compounds with even more potent activities than the ones reported here.

In terms of development as possible anticancer agents, only **MIC 14** was found to be a good potential lead. Further *in vivo* studies have been planned, and will be under way in 2009.

3.10 References

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CHAPTER 4

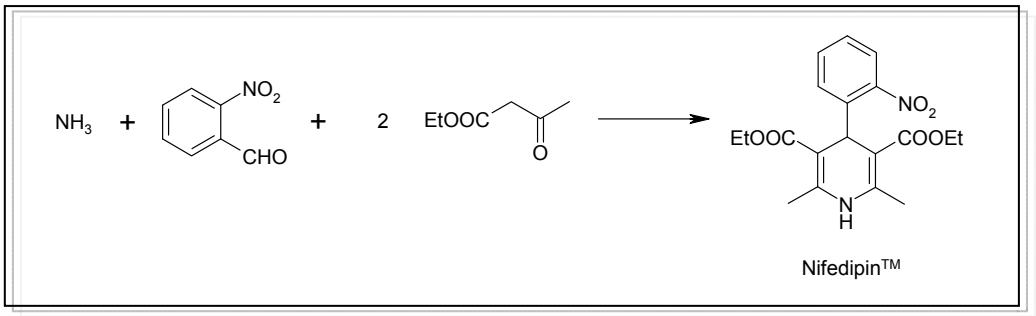
DESIGNED MULTIPLE LIGANDS VIA MULTI-COMPONENT REACTIONS

4.1 Introduction

Continuing on the idea of designed multiple ligands, pharmacophores can also be integrated into a single molecule by employing multi-component reactions. This chapter describes the synthesis, characterization and biological evaluation of hybrid molecules of artemisinin and 1,4-naphthoquinones with 4-aminoquinolines, and their effect on plasmodial endocytosis.

4.2 Multi-Component Reactions

Multi-component reactions (MCR) are chemical reactions that utilize three or more different starting materials as chemical structure inputs in a one-step-one-pot synthesis to yield one single product that contains features of all inputs. Such reactions are highly efficient, as they create molecular complexity by generating more than two chemical bonds per operation.[1] The Strecker synthesis of α-amino acids via α-amino cyanides, first published in 1850, is generally considered to be the first MCR[2]. Other well-known MCR's include Hantzsch's dihydropyridine synthesis (1882),[3] Biginelli's dihydropyrimidine synthesis (1891),[4] Mannich Reaction (1912),[5] Passerini reaction (1921)[6] and the Ugi Reaction (1958),[7] just to name a few. Nifedipin™, a cardiovascular drug, is one of the early examples of a drug synthesized using the multi-component Hantzsch synthesis (Scheme 4.1).



Scheme 4.1. Hantzsch synthesis of Nifedipin.

The MCR has been widely used to study structure-activity relationships and also to create libraries of organic molecules.[8] The various inputs do not come together simultaneously, but rather a series of ordered steps, where two inputs condense first to form an intermediate, which then condenses with the third input, and so on. MCRs can be grouped into three basic types, as presented in Table 4.1 below.

Table 4.1. The basic types of MCRs.[8]

MCR Type	General reaction scheme
I	A + B ⇌ C ⇌ D ⇌ … O ⇌ P
II	A + B ⇌ C ⇌ D ⇌ … O → P
III	A + B → C → D → … O → P

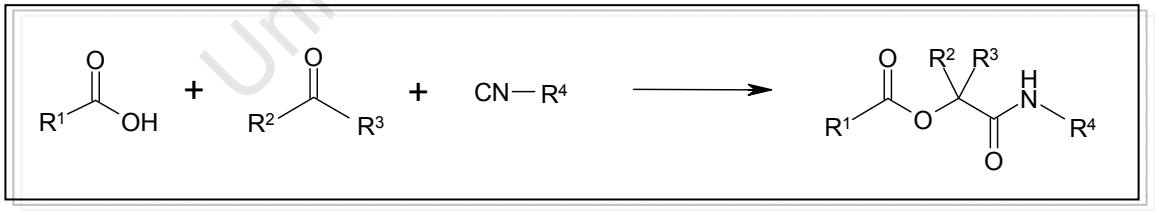
Type I: Starting materials, intermediates and products are all in a mobile equilibrium. Yield ranges between 0 and 100%, depending on the state of balance that prevails in the reaction. This is not the ideal type of MCR, as in most cases, the products occur as mixtures with the intermediates and/or starting materials, and are difficult to isolate. Further impurities may arise from incomplete reactions that lead to side reactions.

Type II: All reactions are in reversible equilibra, while only the product forming step is irreversible. This is advantageous for preparative chemistry as the total equilibrium is shifted to the side of the products by the last irreversible step. Reactions belonging to this type therefore produce products with higher yield and purity when compared to conventional sequential reactions of forming the same product.

Type III: They consist of a sequence of irreversible reactions that seldom occur in preparative chemistry, but mostly in biochemical reactions. Many of these reactions are actually irreversible partial reactions, either due to the thermodynamic circumstances or due to the combination of endothermal with exothermal reactions.

4.2.1 Isocyanide Based Multi-Component Reactions

Isocyanide based multi-component reactions (IMCR) have been around for almost 90 years. The first of these IMCR's was described by the Italian Passerini in 1921 (Scheme 4.2), and was named after its founder.[6]



Scheme 4.2. The Passerini Reaction.

While most of the classical MCRs have been found by chance, new MCRs are being discovered by using novel methods such as rational and combinatorial approaches.[9,10] Up and until 2002, about 300 – 400 different MCRs are known, 20-25% of which are isocyanide-based.[11]

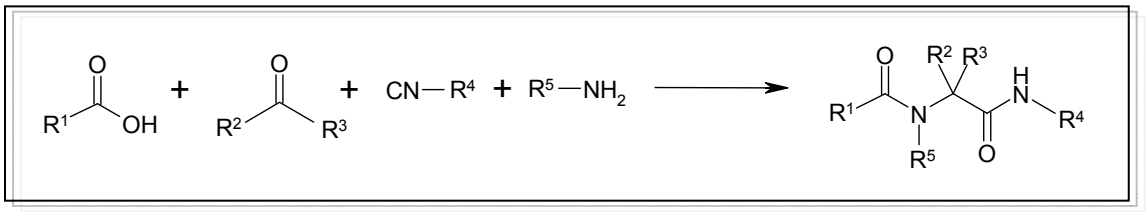
4.2.1.1 Isocyanides

Isocyanides, formerly known as isonitriles, have been discussed for over one and a half centuries due to their unusual valence structure and reactivity. They are the only class of stable organic molecules with a formally divalent carbon.[8] Isocyanides were first synthesized in 1859 by Lieke,[12] who did not recognize them as such, but believed them to be nitriles. Like many chemists today, he was struck by their pungent odour. Ivar Ugi, who described the four-component Ugi reaction, once stated "*The development of the chemistry of isocyanides has probably suffered ... through the characteristic odor of volatile isonitriles, which has been described by Hofmann and Gautier as 'highly specific, almost overpowering', 'horrible', and 'extremely distressing'. It is true that many potential workers in this field have been turned away by the odor.*"[13] Almost all commercial isocyanides carry this strong odour, but majority of the isocyanides with higher molecular weights are solid and odourless. Fortunately, despite the repulsive odour, isocyanides are only slightly toxic, and many natural isocyanides display a strong antibiotic, fungicidal or antineoplastic effect.[14]

4.2.1.2 The Ugi Reaction

Ivar Ugi *et al*[15] described the Ugi four-component condensation (U-4CC) in 1959, which was the most elegant and important variant of the four-component condensations. Most of the condensation types known today were discovered after this publication.

The U-4CC utilizes an aldehyde, an amine, and acid and an isocyanide, and converts them into an α-acetamidoamide in one step with good yields (Scheme 4.3).



Scheme 4.3. The Ugi four-component condensation.

4.2.1.3 Solvent effects

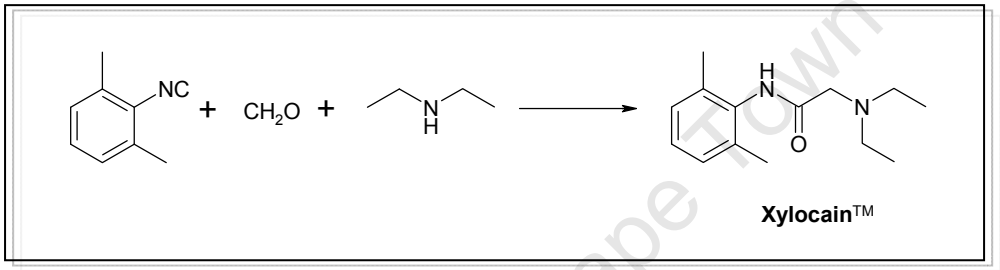
The U-4CC is mainly influenced by inductive, mesomeric and steric effects, the latter to a lesser extent, as established from the reactivity series of the reaction as a function of solvent and concentration. It has been found that the concentration of the reactants plays a more important role than the properties of the solvent in which the reactants are dissolved in.[16] Solvents that are suitable for solution-phase U-4CC include: (a) low molecular weight alcohols such as MeOH, EtOH or trifluoroethanol,[17] (b) polar aprotic solvents such as DMF, chloroform, dichloromethane, THF and dioxane, (c) biphasic aqueous media and (d) aqueous media, with rate-accelerating effects on the Ugi and Passerini reactions, discovered in 2004 by Pirrung and Sarma.[18]

Other approaches to optimize the yield include: (a) keeping the concentration of the reactants between 0.5 to 2 M, (b) pre-condensation of the amine and aldehyde or ketone, and (c) addition of a Lewis acid. The reaction rate can be accelerated by the use of a microwave.[19]

4.2.2 Application of multi-component reactions in drug discovery

A short time after the U-4CC was discovered by Ugi, the first medicinal chemistry related application of this reaction was realized, with the one step preparation of the local anesthetic

Xylocain (Scheme 4.4).[20] He also recognized that the reaction is well-suited to probe structure-activity relationships by synthesizing libraries of compounds.[8] With the emergence of combinatorial chemistry and high-speed parallel synthesis today, the MCR is widely employed for the rapid assembly of arrays with high molecular diversity.[21] The power of these reactions is increased even further, when coupled with post-condensation modifications, giving rise to a plethora of complex, pharmacologically relevant templates for screening purposes.



Scheme 4.4. Synthesis of Xylocain™ using the Ugi reaction

MCR synthesis is advantageous over an equivalent linear synthesis, in that the size of a linear derived library is a function of the number of steps and individual inputs. For example, a four step synthesis with 10 inputs at each step would create 10^4 compounds, whereas a four component MCR would have the same size by having 10 inputs per component, yet achieving this in a single chemical operation.[22]

Combinatorial chemistry derived libraries may be split into three categories:

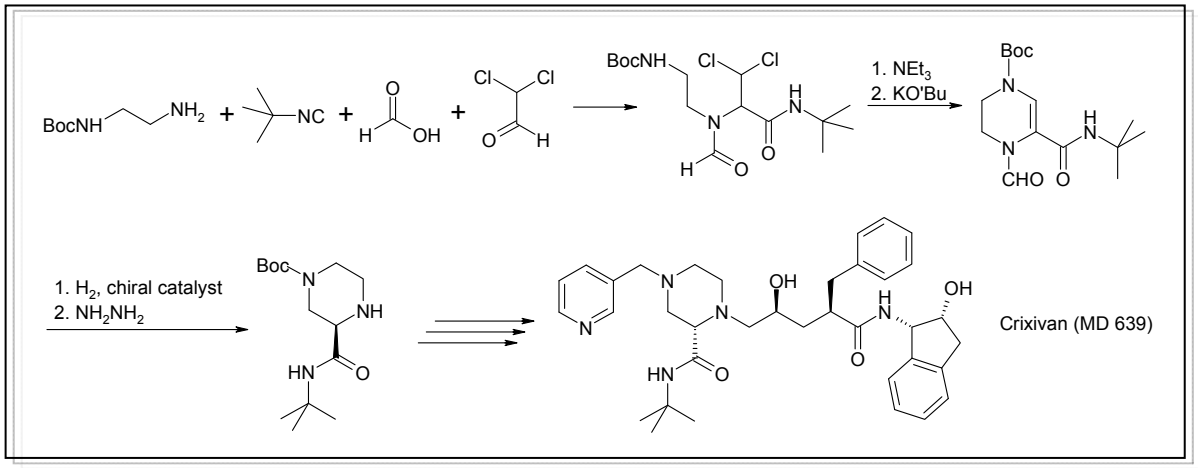
1. General libraries. These are specifically designed to fill diversity voids in corporate collections, and are typically in the $10^3 – 10^4$ compound range.
2. Gene family targeted libraries. These utilize privileged chemical motifs[23] or public domain structures, and are often seen in ligands that bind to several member targets

of the gene family. They are typically in the $10^2 – 10^3$ compound range.

3. Focused libraries. A library such as this is designed for a specific target and is SAR driven, typically in the $10 – 10^3$ compound range.

Additional advantages that the MCR methodologies bring to drug discovery,[24] listed in chronological order from early to late stage discovery:

1. Chemistry development is dramatically shortened, as more compounds can be made in the same amount of time taken for linear synthesis.
2. Average library purity should be higher than linear synthesis, as impurities will not have the opportunity to build up as in sequential synthesis.
3. MCRs are often compatible with a solution phase approach. This enables simpler reaction monitoring and reduces automation needs.
4. The 'hit-lead transitions' are often dramatically shorter and relatively simple. As fast protocols are already in place, this is a major advantage in hit validation.
5. MCR methodologies can produce quality drug-like hits for further optimization, by defining drug-likeness qualities such as log P, molecular weight, number of H-bond donors and acceptors, molar refractivity, rotatable bonds and polar surface area.
6. Beyond lead optimization, the cost savings for final marketed drug production may be dramatic if the final compound is accessible via MCR methodology, as opposed to a sequential route. Multi-step chemical process development is often a bottleneck in drug discovery. Crixivan, a HIV protease inhibitor, requires at least 15 synthetic steps that render the production at the high cost end. Merck&Co has developed a synthesis,[25] where a Ugi-type MCR is a central step (Scheme 4.5).



Scheme 4.5. Ugi MCR synthesis of Crixivan.

4.3 Rationale for Drug Design

Based on its versatility, we have chosen the Ugi four-component condensation as the approach towards the synthesis of the target compounds. In principle, 1,4-naphthoquinone and artemisinin were chosen to be hybridized with 4-aminoquinolines to form 'dual-drugs' via the Ugi four-component condensation (Fig. 4.1).

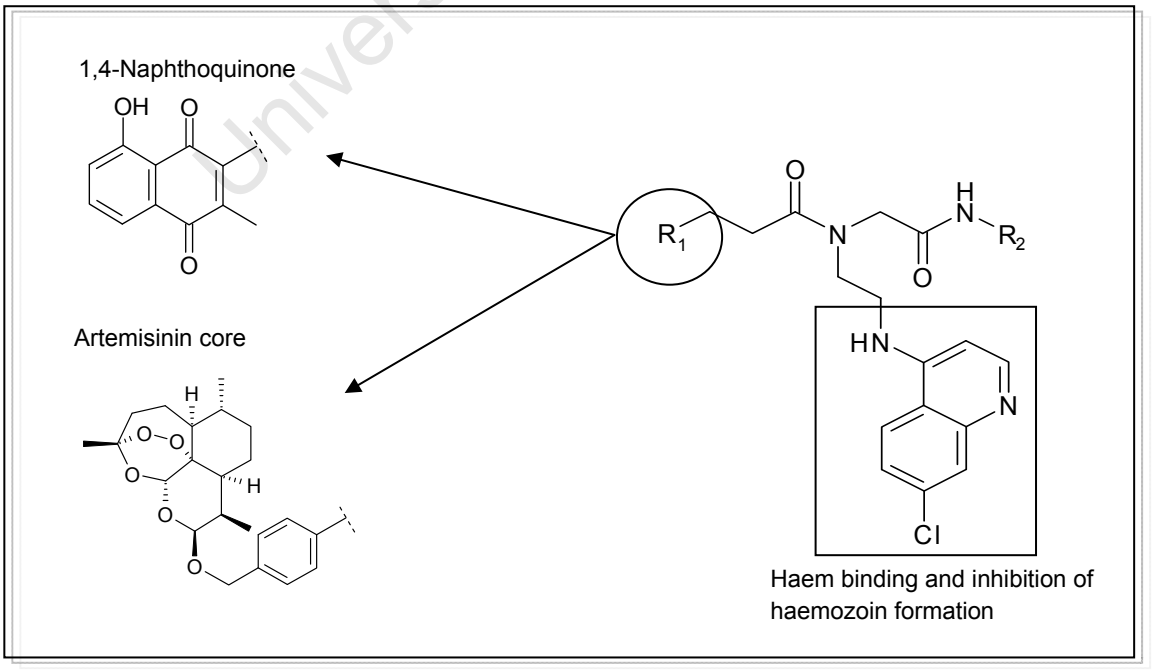


Figure 4.1 Rationale for the design of target molecules.

The rationale for the incorporation of artemisinin and 4-aminoquinolines has already been discussed in the previous chapter. In this section, the rationale for the choice of 1,4-naphthoquinones is described.

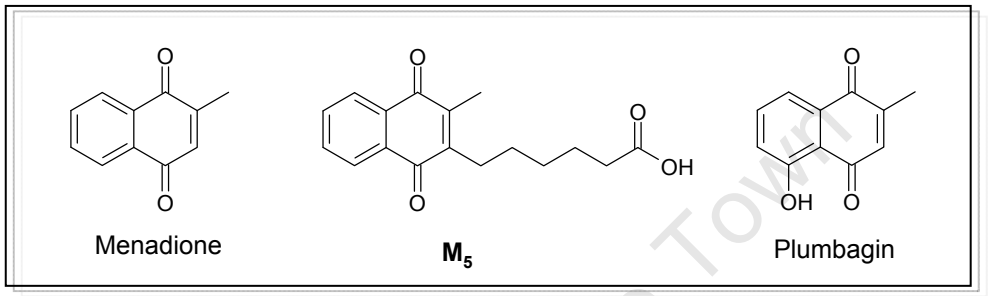
4.3.1 1,4-Naphthoquinones

4.3.1.1 As Antimalarial Agents

Glutathione (GSH) is a major thiol responsible for the detoxification of reactive oxygen radicals both in man and the malaria parasite,[26] to prevent oxidative damage. The level of GSH present depends on the efficiency of glutathione reductase (GR) in *P. falciparum* and in man, and thioredoxin reductase in *P. falciparum*,[27] to reduce glutathione disulfide (GSSG) to GSH. Thus glutathione reductase is often the target when designing new antimalarials[28] and chloroquine-resistance reversal agents.[29,30] It has also been shown to be involved in the degradation of toxic haem released during haemoglobin digestion,[26] as described in section 2.1.1 in Chapter 2.

Parasites of the *Plasmodium* family are exposed to higher fluxes of reactive oxygen species, as a result of host immune response to infection and from haemoglobin digestion, hence high activities of intracellular antioxidant systems are needed. Studies have shown that antioxidant systems are important for parasite survival, as they do not develop in glucose-6-phosphate dehydrogenase deficient red blood cells, or in erythrocytes depleted in GR activity.[31] Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme in the pentose phosphate pathway that converts glucose-6-phosphate into 6-phosphoglucono-δ-lactone. It supplies reducing energy to cells by maintaining the level of the co-enzyme, nicotinamide

adenine dinucleotide phosphate (NADPH), which in turn maintains the supply of reduced GSH in the cells. 1,4-Naphthaquinones are an important class of inhibitors for glutathione and thioredoxin reductases, with plumbagin, menadione and its hexanoic acid derivative **M_5** being representatives (Scheme 4.6),[29, 32] and they have been demonstrated to be uncompetitive inhibitors of GR with respect to both NADPH and glutathione disulfide.



Scheme 4.6. Structures of 1,4-naphthoquinones as glutathione and thioredoxin reductase inhibitors.

The biological activity of the 1,4-naphthoquinones arises from the ability of the quinone to accept one and/or two electrons to form the corresponding anion or dianion radical species. 1,4-Naphthoquinones can be reduced to semiquinone radicals, which, upon reduction of oxygen to superoxide anion radicals, regenerates the quinone.[33] This redox cycling and oxygen activation leads to increased levels of hydrogen peroxide and GSSG. The cytotoxicity of 1,4-naphthoquinones may also be attributed to thiol and amine depletion by alkylation, DNA modification and oxidation of essential protein thiols by activated oxygen species and/or GSSG..[33]

4.3.1.2 As Anticancer, Antifungal and Antimicrobial Agents

The 1,4-naphthoquinone pharmacophore is common in numerous natural products that display antifungal, antibacterial, antiviral or antitumour activities.[33] Anticancer drugs, such

as streptonigrin,[34] actinomycins,[35] mitomycins,[36] alkannins,[37] 2-hydroxynaphthoquinone derivatives [38] and 1,4-furanonaphthoquinones [39] contain the 1,4-naphthoquinone or 1,4-quinone pharmacophore. Recent studies have also found that 1,4-naphthoquinone derivatives were active against Walker 256 carcinoma cell lines,[40] *Lymphoid Leukaemia P 388*, [41] prostate cancer [42] and many others. [43] A number of sulfur-containing 1,4-naphthoquinone derivatives have been shown to possess antifungal activity. [44] Additionally, significant antimicrobial activities have also been observed.[45]

4.3.1.3 Design of 1,4-Naphthoquinones and 4-Aminoquinolines as 'Dual-Drugs'

Double headed prodrugs based on 1,4-naphthoquinones and 4-aminoquinolines have previously been developed, and proved to be potent antimalarials as glutathione reductase inhibitors, both *in vitro* and *in vivo*.[29,46] Here, we propose to hybridize them via the Ugi reaction, assess their biological activities, hoping to gain more insight to their possible mode(s) of action with respect to inhibition of endocytosis.

4.3.2 Effects of Artemisinin and Chloroquine on Plasmodial Endocytosis

Research conducted by H. Hoppe *et al* has shown that chloroquine interferes with the parasite endocytic pathway.[47] Incubation of malaria parasites with chloroquine for 12 hours caused a striking increase in haemoglobin levels when compared to the control parasites, indicative of haemoglobin degradation inhibition. Immunofluorescence assays showed that haemoglobin was located prominently in the food vacuole of control parasites. By contrast, in chloroquine-treated parasites no haemoglobin-associated fluorescence was seen in the food vacuole, but was found instead to be concentrated in cytoplasmic punctate structures

representing endocytic transport vesicles. This suggests that chloroquine causes a block in transport vesicle-vacuole fusion, resulting in an inability to correctly deliver the endocytosed haemoglobin to the food vacuole and a consequent accumulation of undigested haemoglobin in transport vesicles.

Similar assays with artemisinin showed that artemisinin treatment also increased haemoglobin levels within the parasite, but to a much lesser extent than chloroquine, and that artemisinin does not inhibit transport vesicle-vacuole fusion. By contrast, artemisinin was found to more significantly inhibit the endocytosis of red blood cell haemolobin by the parasite. The interpretation of the combined results of the study by Hoppe et al. and others was that the principal effect of chloroquine is a block in vesicle-vacuole fusion, resulting in a marked increase in parasite haemoglobin and transport vesicle content. By contrast, artemisinin inhibits both the proteolytic digestion of haemoglobin in the food vacuole as well as haemoglobin endocytosis itself, and the combination of these effects produces a small increase in total parasite haemoglobin levels compared to controls.[47]

Based on these findings, it would be informative to investigate the effects on endocytosis of the artemisinin-chloroquine hybrid molecules, since each of the parent compounds have been shown to exert different effects. The results could indicate whether these hybrid molecules preferentially display the effects of one of the parent molecules, a combination of the effects, or a new and independent mode of action within the parasite.

4.4 Synthesis of Target Compounds via the Ugi Four-Component Condensation

4.4.1 Approaches for the Synthesis of Target Compounds

The Ugi adducts were synthesized using 1,4-naphthoquinone-acid and artelinic acid as the acid component; 4-aminoquinoline amines as the amine component; paraformaldehyde as the aldehyde input; and cyclohexyl- and *tert*-butyl isocyanides as the isocyanide component (Figure 4.2).

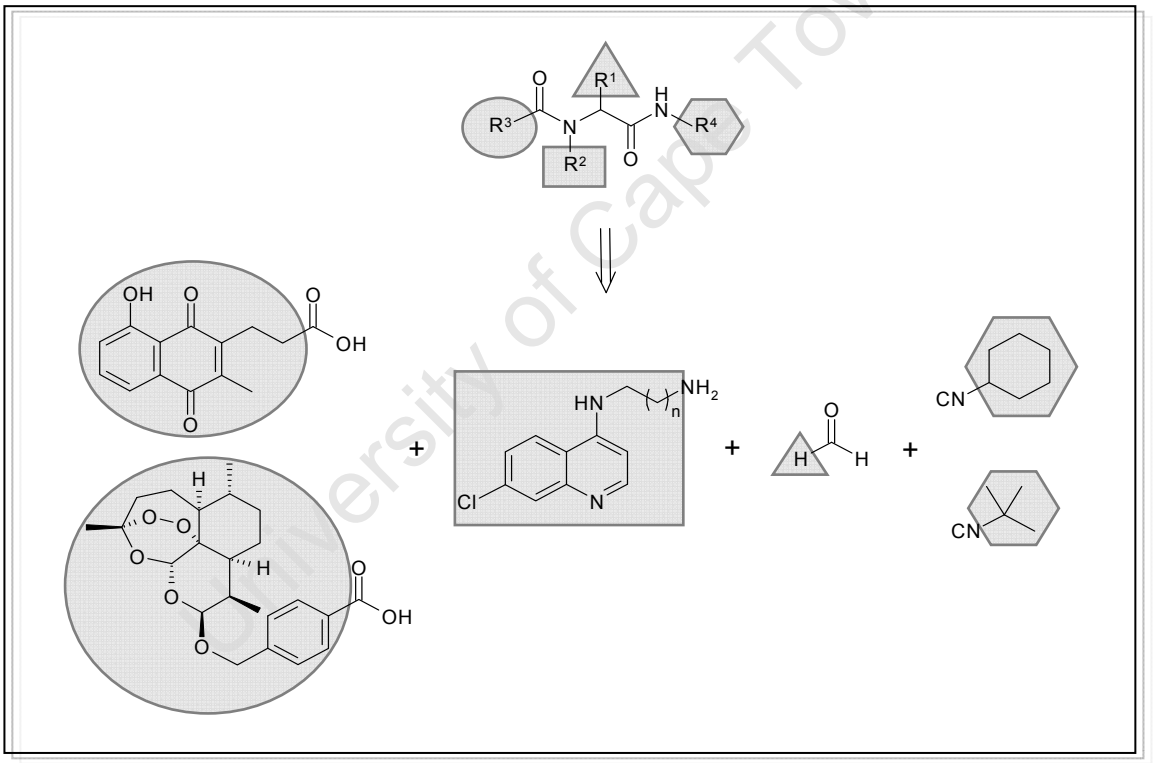
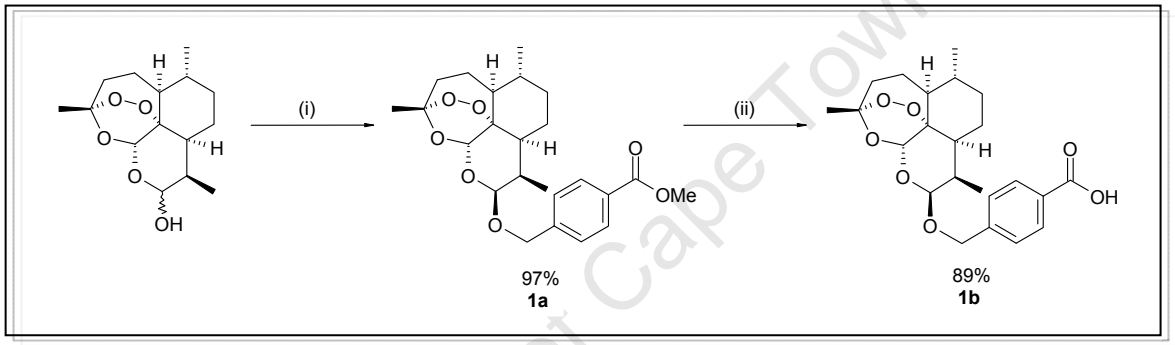


Figure 4.2. Various inputs for the Ugi 4-CC.

4.4.2 Synthesis of Acid Components

4.4.2.1 Artelinic acid

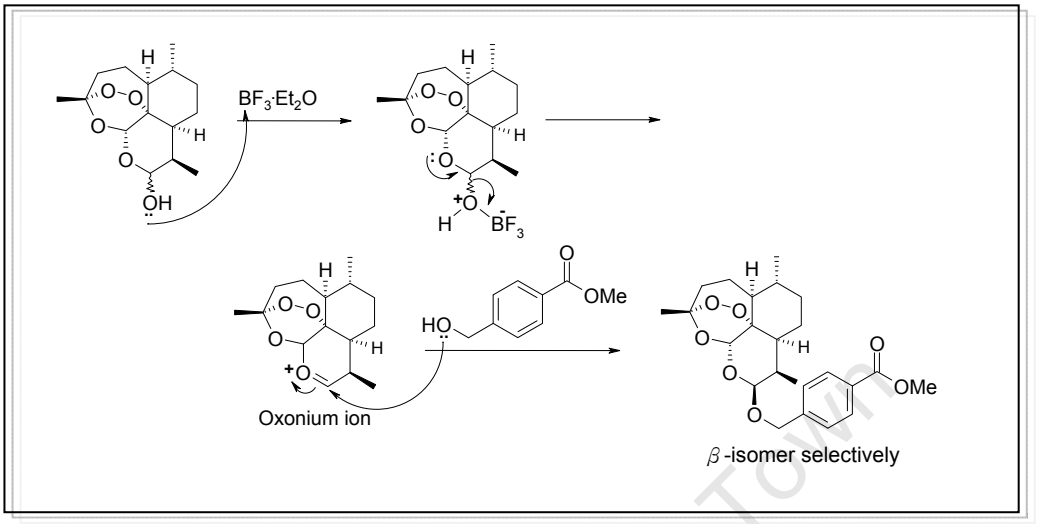
Artelinic acid was yielded after a two-step synthesis starting with dihydroartemisinin. Dihydroartemisinin was coupled with methyl 4-(hydroxymethyl)-benzoate in the presence of boron trifluoride etherate to form **1a**, which was then hydrolyzed by KOH to give artelinic acid **1b** (Scheme 4.7).



Scheme 4.7 Reagents and conditions: (i) methyl 4-(hydroxymethyl)-benzoate (3.4 eq), $BF_3{\cdot}Et_3O$ (1.13 eq), Et_3O, N_2, r.t., 24 h; (ii) 2.5% KOH/MeOH, r.t., 3 days.

The β-isomer was the major product (diastereomeric ratio α:β 2:98), and can be understood from the reaction mechanism shown in Scheme 4.8. The Lewis acid $BF_3{\cdot}Et_3O$ coordinates to the hydroxyl group of dihydroartemisinin, and is displaced with the formation of the oxonium (or oxocarbenium) ion. At this point, the hydroxyl of the benzoate can attack either from the α or β face. Dihydroartemisinin can be seen as a pyranose ring, and the electrophilic carbon centre as the anomeric centre. When coupling to the oxocarbenium ion, axial attack from the β face is favoured, as this result in the more stable chair conformation. Even though the incoming benzoate is now at the higher energy axial position, this is favourable as it is stabilized through the anomeric effect, hence β-selectivity. The anomeric effect is widely

observed in carbohydrate chemistry, where there is orbital alignment between the oxygen atom in the ring and the oxygen atom on the anomeric carbon. This is illustrated in Fig. 4.3.



Scheme 4.8. Mechanistic details for the formation of **1a**.

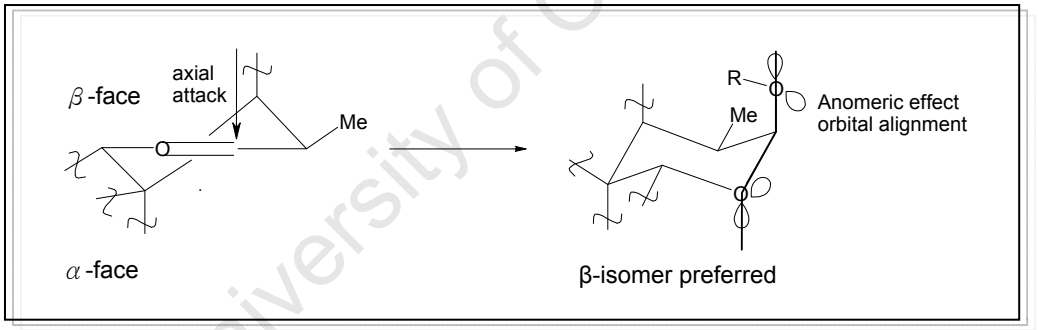


Figure 4.3. Axial attack at the anomeric centre and anomeric effect.

The conformations of the products were also confirmed via ^{1}H NMR spectroscopy. As previously mentioned, $^{3}J_{HH}$ between H-9 and H-10 is typically 3.2 Hz for β-isomer (equatorial-axial coupling) and 9.6 Hz for α-isomer (axial-axial coupling).[48]

4.4.2.1.1 Spectroscopic Analyses

The ^{1}H NMR of **1b** is presented below in Fig. 4.4. The AB system of the protons on the aromatic ring was easily distinguished in the aromatic region. Benzyl protons (H-17A and H-17B) were found between 4.5 and 5.0 ppm, displaying a large geminal coupling of 13.5 Hz. H-10 was found at 4.9 ppm with the expected coupling constant of 3.0 Hz for the β-isomer. The singlet at 3.9 ppm corresponding to the OMe group was no longer seen in the spectrum, indicating a successful hydrolysis. Proton assignments were also confirmed via 2D NMR techniques such as COSY and HSQC; all ^{1}H and ^{13}C NMR data corresponded to those given in the literature.[49]

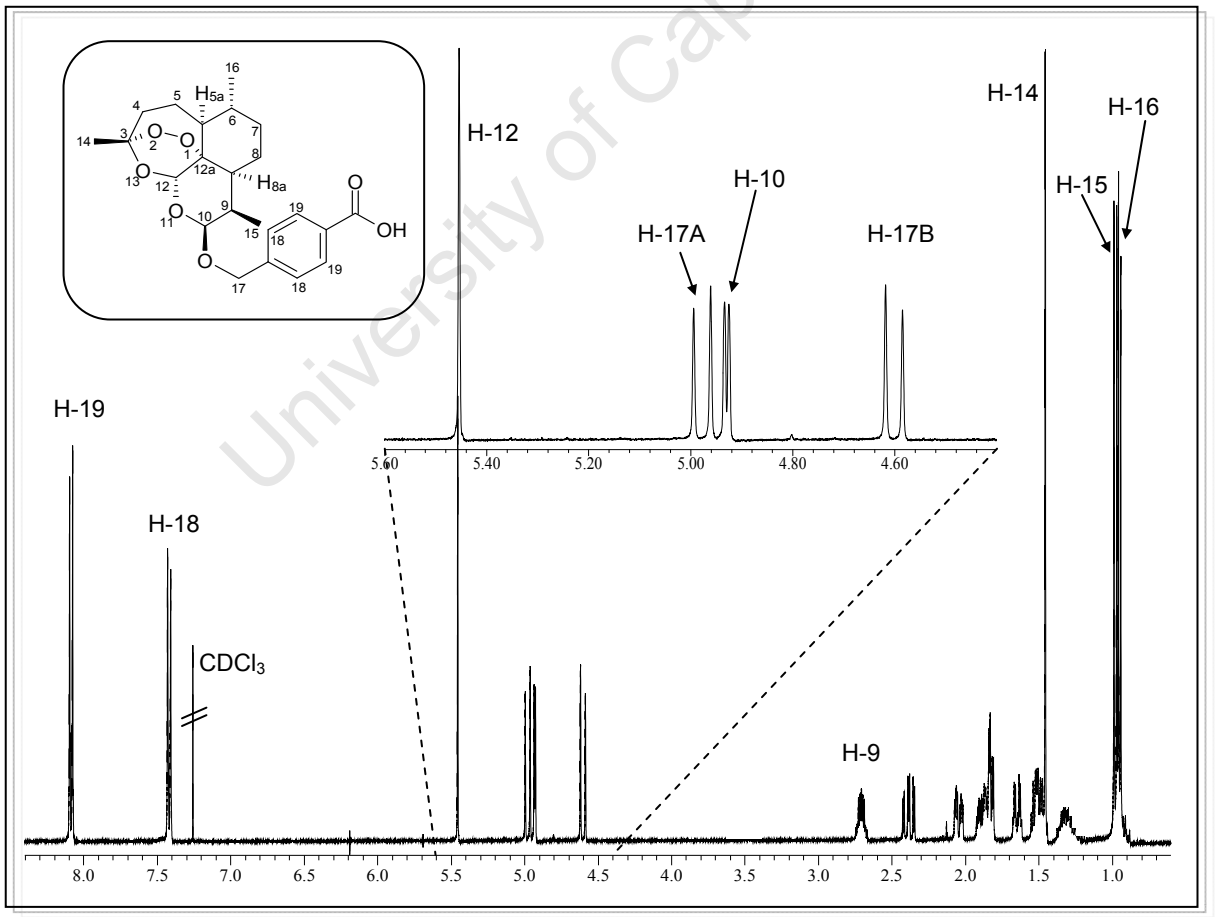
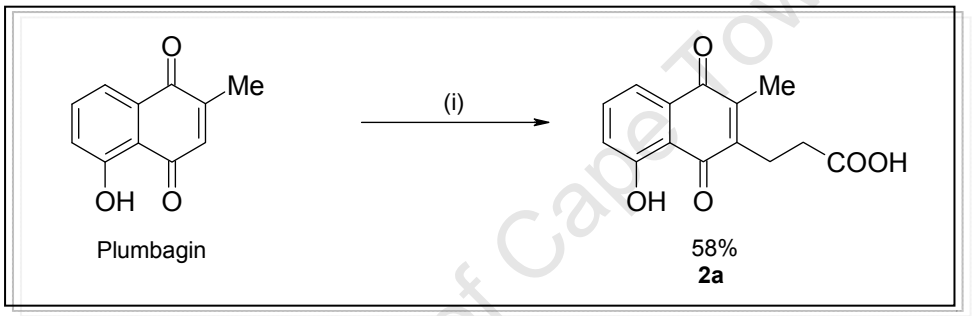


Figure 4.4. ^{1}H NMR of artelinic aicd **1b**.

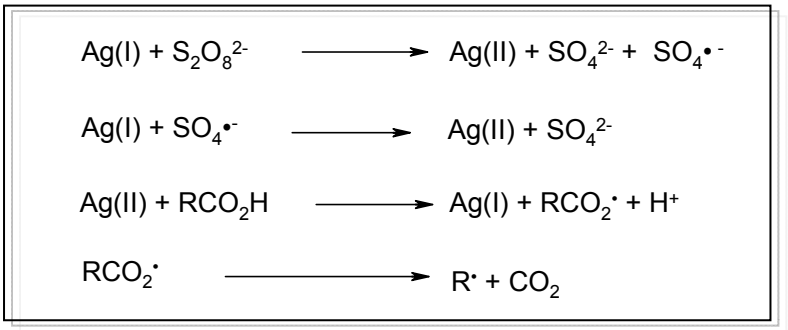
4.4.2.2 1,4-Naphthoquinone Acid

The 1,4-naphthoquinone acid was synthesized by coupling plumbagin and succinic acid ($HOOCCH_2CH_2COOH$) via oxidative decarboxylation of acids, catalysed by silver nitrate in the presence of ammonium persulfate (Scheme 4.9).[50,51] The yield was fairly modest; this may be due to the redox chemistry, or decomposition of the product during the reaction at elevated temperature, as plumbagins are heat-sensitive.



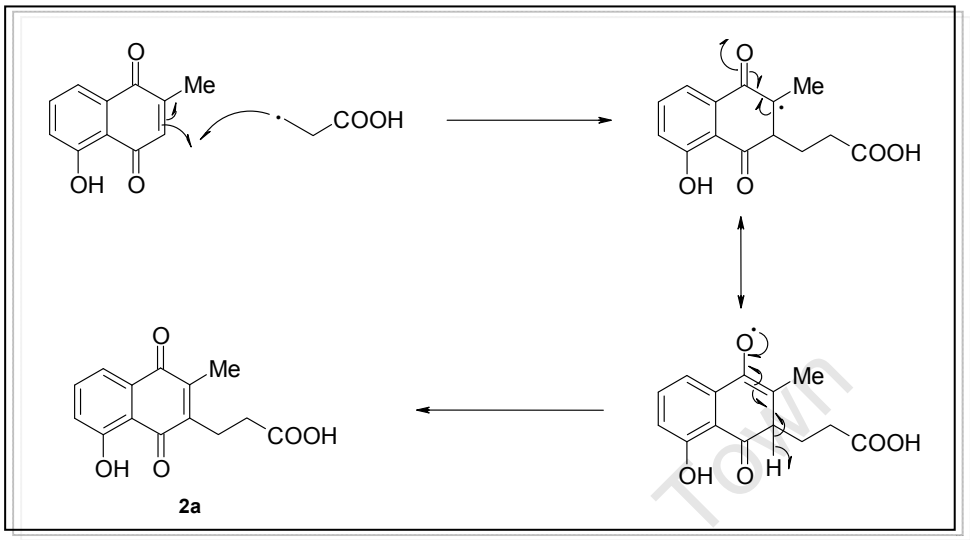
Scheme 4.9. Reagents and conditions: (i) succinic acid (3.0 eq), silver nitrate (0.5 eq), ammonium persulfate (1.3 eq), 30% aq. CH_3N, 65 – 70 °C, 3 h.

The reaction starts with a series of redox reactions, shown in Scheme 4.10, after which the diacid looses one of its carboxylic acids to become a nucleophilic alkyl radical,[52] at the same time giving off CO_2.



Scheme 4.10. Redox reaction between silver nitrate and ammonium persulfate.

The nucleophilic alkyl radical then attacks the plumbagin, forming the naphthoquinone derivative **2a** after a series of radical rearrangements (Scheme 4.11).



Scheme 4.11. Nucleophilic attack of the alkyl radical, and subsequent radical rearrangements to form **2a**.

4.4.2.2.1 Spectroscopic Analyses

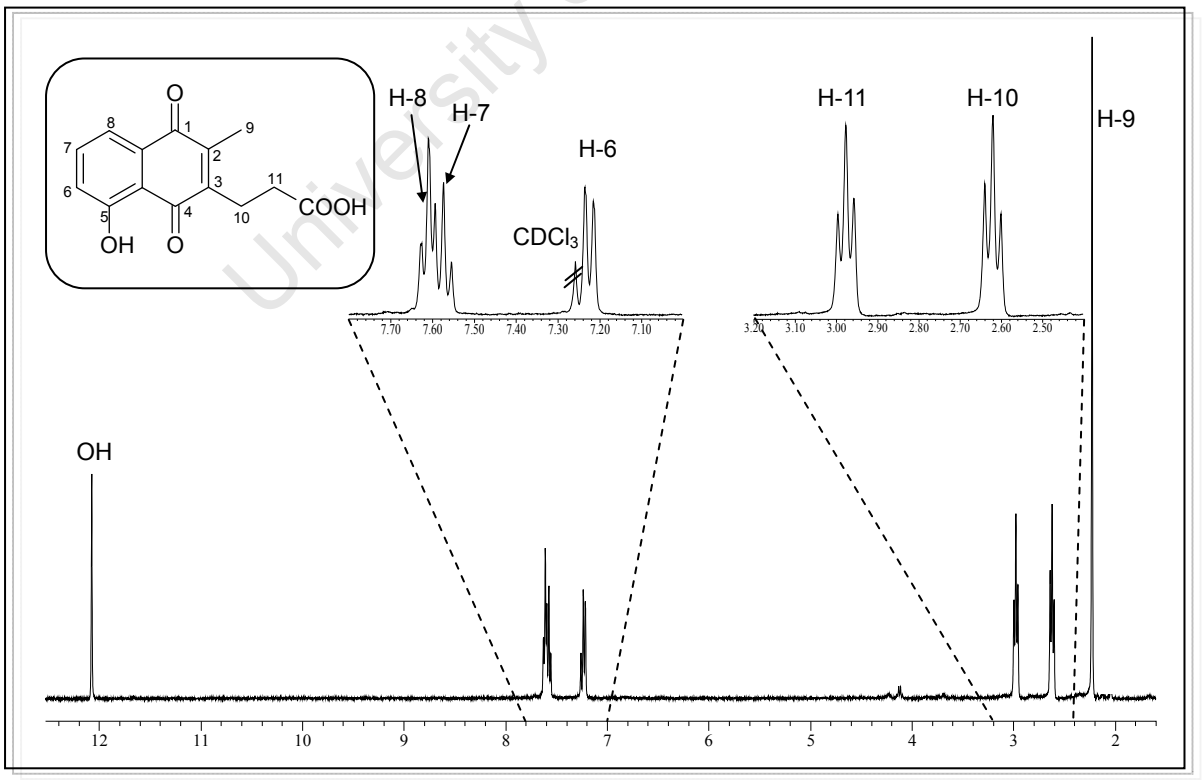


Figure 4.5. ^{1}H NMR of **2a**.

When compared to the ^{1}H NMR spectrum of plumbagin, two additional triplets, each integrating for 2 protons (J = 7.6 Hz), were evident at 2.62 and 2.98 ppm (Fig. 4.5). Additionally, a singlet integrating for one proton at 12 ppm indicated the presence of the carboxylic acid. These signals confirmed the presence of the 1,4-naphthoquinone-acid **2a**, and corresponded to data given in literature.[50]

4.4.3 Synthesis of Amine Components

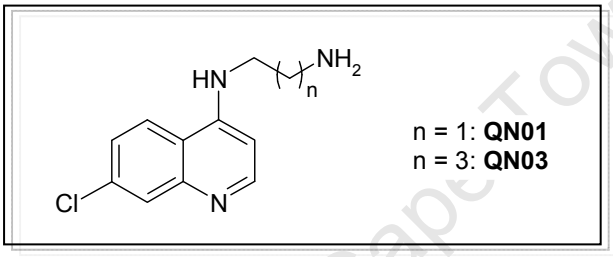


Figure 4.6. Amine components for the Ugi reaction.

4-Aminoquinoline amines **QN01** and **QN03** (Figure 4.6) were used as the amine inputs for the synthesis of the Ugi adducts. As their syntheses have already been described in the previous chapter, they will not be discussed again here.

4.4.4 Aldehyde and Isocyanide Components

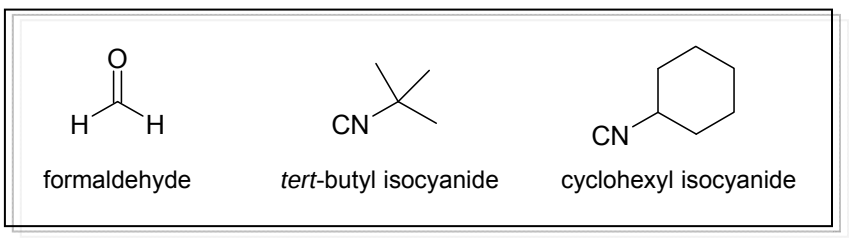


Figure 4.7. Aldehyde and isocyanide inputs for the Ugi reaction.

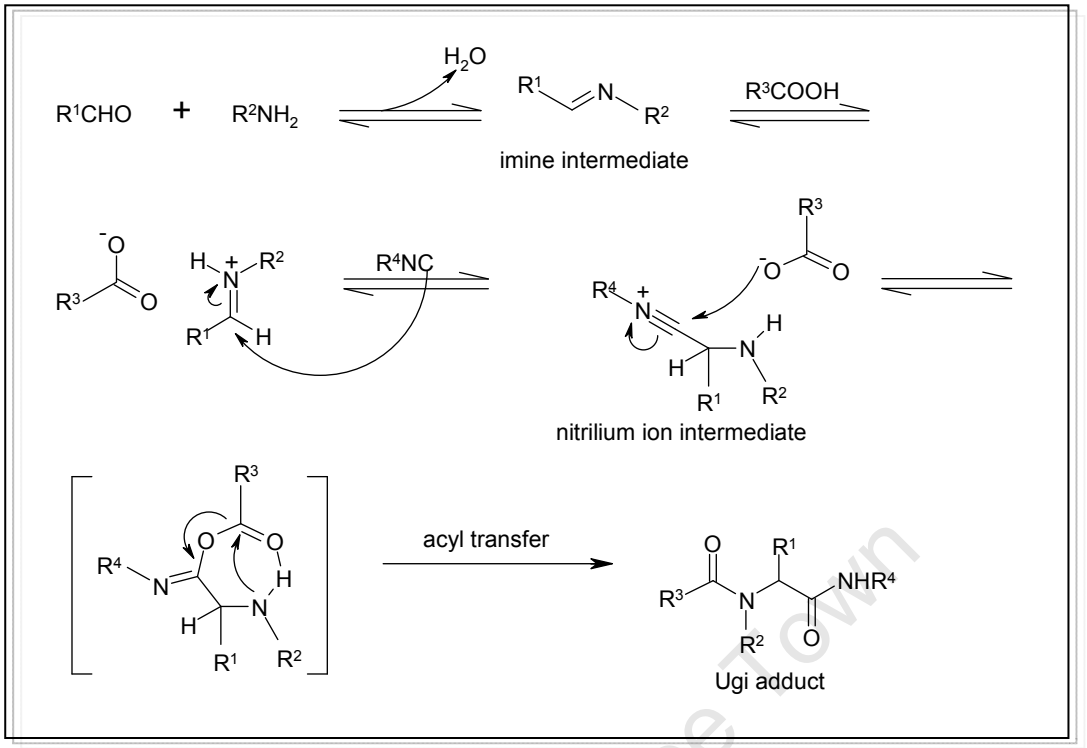
For simplicity reasons, commercially available *tert*-butyl and cyclohexyl isocyanides were used as the isocyanide inputs (Figure 4.7); formaldehyde was used as the aldehyde input to deliberately avoid creating stereoisomers.

4.4.5 Synthesis of Target Compounds

Scheme 4.12. Reagents and conditions: Carboxylic acid (1.0 eq), amine (1.2 eq), aldehyde (1.0 eq), isocyanide (1.0 eq), MeOH, r.t., 3 – 5 days.

All reagents were stirred in methanol at room temperature, and the reaction monitored by thin layer chromatography (TLC) until completion, which is typically 3-5 days. Solvent was removed under reduced pressure, and crude materials chromatographed to yield pure products.

This one-step-one-pot MCR involves a sequence of steps, illustrated in Scheme 4.13 below. Firstly, the imine is formed by the condensation of the amine with the ketone or aldehyde. Upon protonation by the acid, the activated iminium ion is formed, with increased electrophilicity of the C=N bond, and combines with the isocyanide to form the nitrilium ion intermediate. The carboxylate then adds to the nitrilium ion intermediate, followed by an irreversible intramolecular acyl transfer that drives the reaction forward to give the classical Ugi skeleton. The driving force for the irreversible reaction is the oxidation of the isocyanide C^{II} to the amide C^{IV} atom.



Scheme 4.13. Reaction mechanism of the Ugi 4-CC.

Yields for the artelinic acid-containing Ugi adducts were very good (80 – 90%), but not for the 1,4-naphthoquinone-containing ones. Their low yields may be the result of heat-sensitivity of the 1,4-naphthoquinone acid **2a**, and its subsequent decomposition in the reaction vessel during the 3 day reaction period at room temperature.

Compound **6c** was also made into a citrate salt, to determine whether or not the salt derivative would enhance biological activities by virtue of being more soluble.

The target compounds that were synthesized are illustrated in Fig. 4.8:

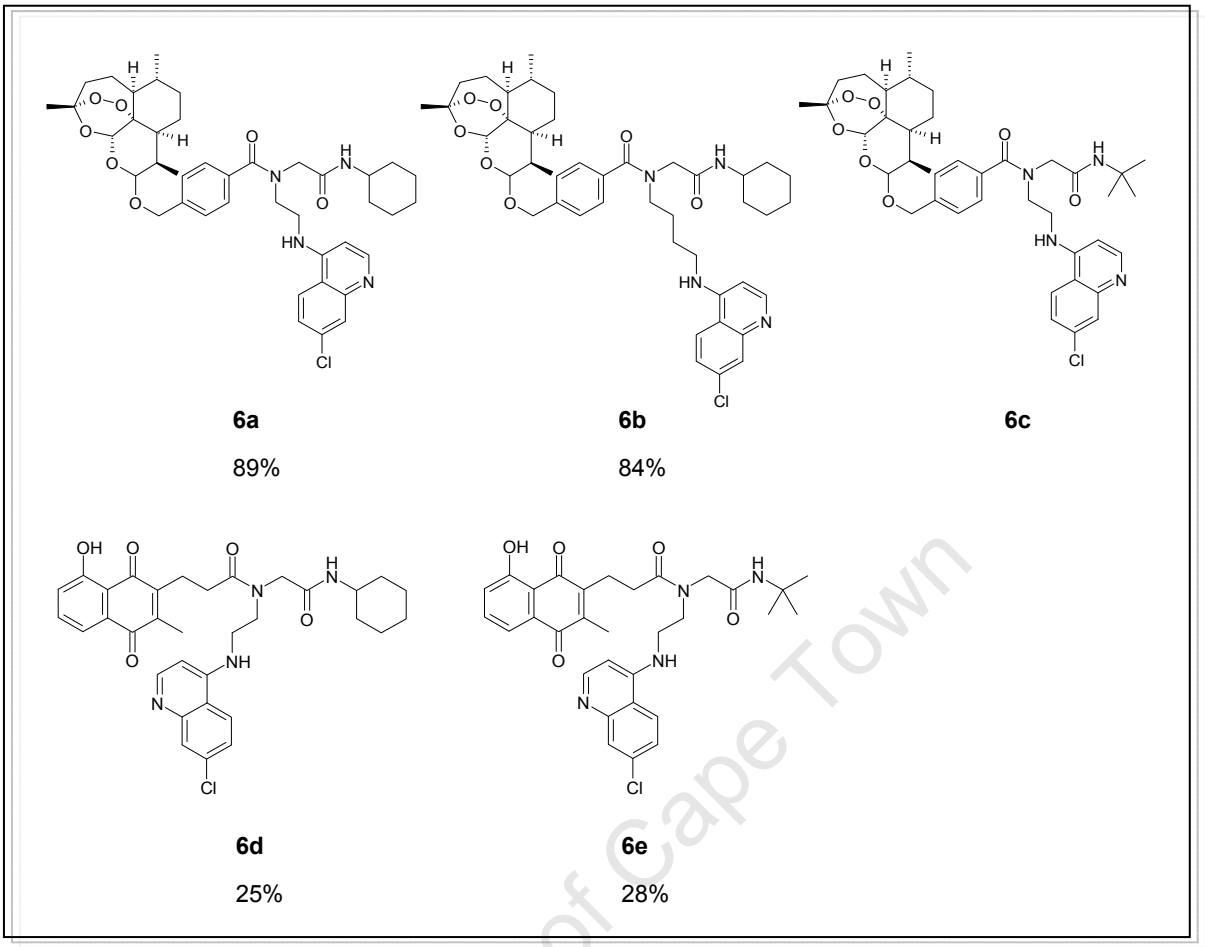


Figure 4.8. Target compounds synthesized via the Ugi 4-CC.

4.4.5.1 Spectroscopic Analyses

Both ^{1}H and ^{13}C NMR spectra of **6d** and **6e** showed duplication of individual peaks, an observation attributed to the slow interconversion between the *cis* and *trans* isomers about the tertiary amide bond.[53] When the samples were run at variable temperature between 60 and 90 °C, peaks started to broaden and coalesce, owing to the increase in the rate of interconversion at elevated temperature. Here, we present the ^{1}H NMR spectrum of **6e** and the duplication of peaks (Fig. 4.9). ^{1}H NMR spectra of **6a**, **6b** and **6e**, however, did not show duplication of peaks, but rather peak broadening of each signal (see Fig. 4.10 for ^{1}H NMR spectrum of **6b**). Mass spectroscopy, elemental analyses both indicated that the desired

products were formed, and running the samples in other solvents such as CD_3OH or DMSO could not eliminate peak broadening. Hence the peak broadening may be due to a faster interconversion rate at room temperature for these compounds, and proton assignments were made with the aid of COSY spectra.

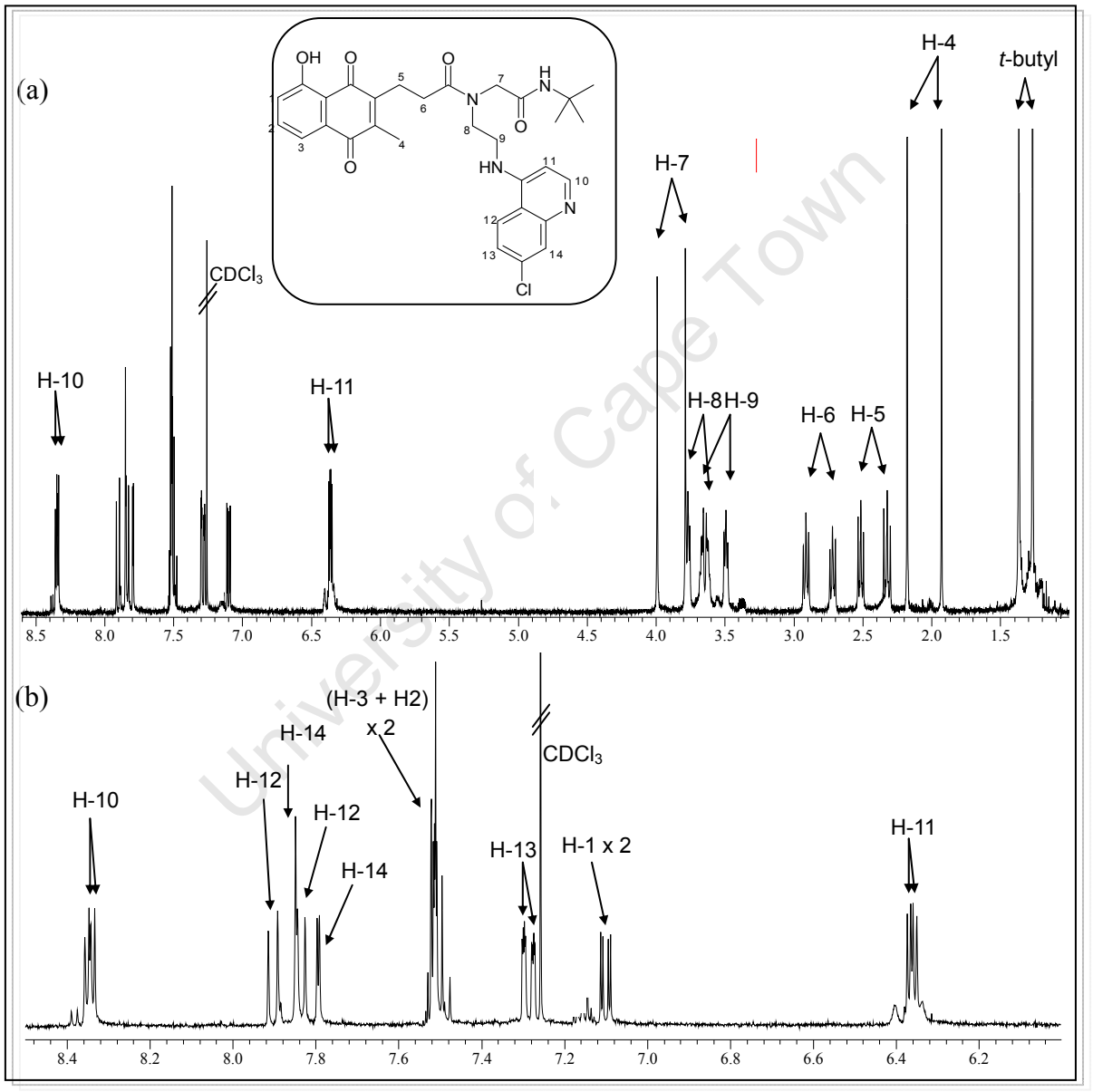


Figure 4.9. ^{1}H NMR spectrum of **6e** with doubling of signals. (a) full spectrum; (b) expansion of 6.0 – 8.5 ppm region.

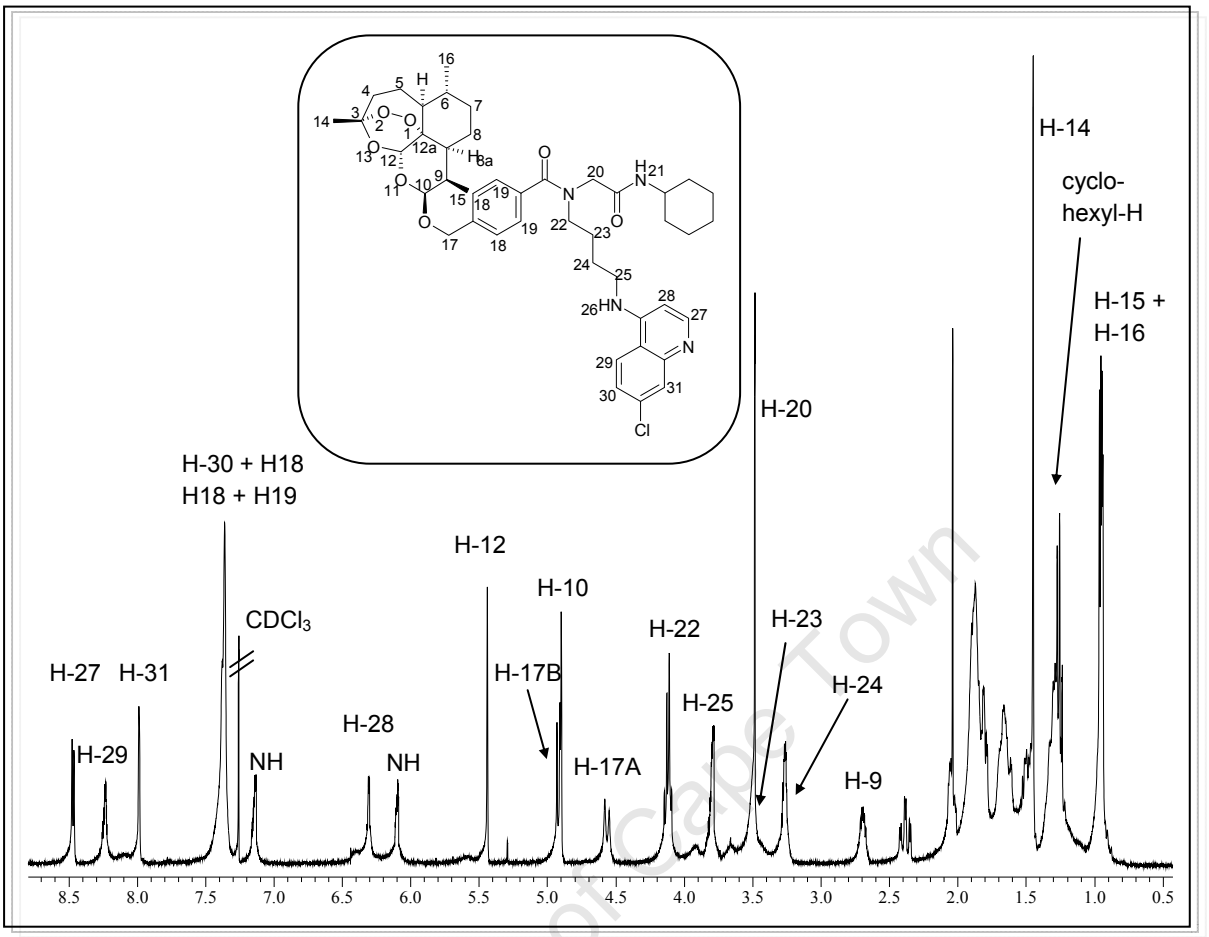


Figure 4.10. ^{1}H NMR spectrum of **6b** with peak broadening.

4.5 Biological Assays and Discussion

4.5.1 Antiplasmodial Activities

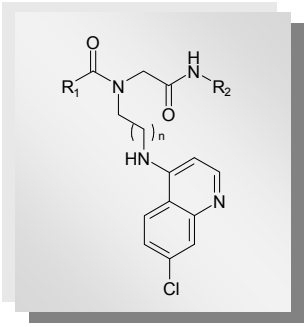
Antiplasmodial activities and cytotoxicities were determined for the target compounds, and are tabulated in Table 4.2 below. Therapeutic indices were calculated by dividing the IC_{50} obtained for HeLa cells with those obtained for the K1 parasite strain. Chloroquine was used as the control for both antiplasmodial and cytotoxicity assays.

All three of the artemisinin-containing Ugi adducts displayed good *in vitro* activities comparable to chloroquine against the chloroquine-sensitive D10 strain (26, 35 and 27 nM

for **6a**, **6b** and **6c** respectively, compared to 20 nM for CQ). The salt derivative of **6c**, with its increased solubility, was expected to exhibit a significant enhancement of *in vitro* activity; however, this was not observed. As was observed for the artemisinin-containing conjugate addition adducts described in Chapter 3, the chloroquine-resistant K1 strain did not display cross-resistance to the chloroquine-artemisinin hybrids. This is shown in the resistance index (RI), calculated by dividing the IC_{50} obtained for the chloroquine-resistant strain (K1) over that of the chloroquine-sensitive strain (D10). A compound with low RI indicates that the parasite is less likely to experience cross-resistance to it, unlike chloroquine, which comparatively has a very high RI of 11. Although hybrid molecules that contain the 1,4-naphthoquinone pharmacophore only exhibited moderate antiplasmodial activities, they also displayed low RI.

The cytotoxicity assays suggest that these hybrid molecules possess a narrow therapeutic window, as indicated by the therapeutic indices. The IC_{50} for mammalian cells should ideally be at least two orders of magnitude higher than that for parasites, as this indicates that the compound acts more selectively against the parasites than mammalian cells, which would ultimately make dosing easier to control. Drugs with low therapeutic indices will need to be taken under constant monitoring to achieve therapeutic levels while minimizing toxicity. Hence these hybrid molecules in their current form would not be good drug candidates, unless their antiplasmodial activities could be selectively enhanced.

Table 4.2. Biological activities of target compounds.



General structure of Ugi adducts

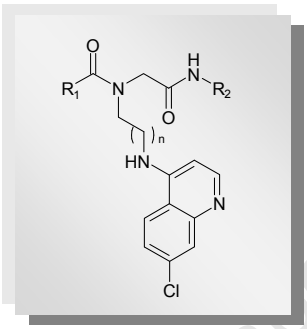
Cmpd	R_1	R_2	n	IC_{50} *P. falciparum* (μM)		RI[a]	HeLa IC_{50} (μM)	TI[b]
				D10	K1			
CQ	-	-	-	0.020	0.219	11	8.54	39
ART	-	-	-	0.023	0.014	0.61	ND	-
DHA	-	-	-	0.004	0.003	0.75	ND	-
1b	-	-	-	0.006	0.006	1	ND	-
2a	-	-	-	38.35	20.29	0.53	ND	-
QN01	-	-	-	1.37	2.56	1.87	ND	-
QN03	-	-	-	2.15	2.74	1.27	ND	-
6a	ART[c]		1	0.026	0.023	0.88	0.286	12
6b	ART[c]		3	0.035	0.021	0.60	0.169	8
6c	ART[c]		1	0.027	0.019	0.70	0.496	26
6c·citrate salt	ART[c]		1	0.024	0.018	0.75	0.356	20
6d	NQ[d]		1	0.638	0.467	0.73	5.211	11
6e	NQ[d]		1	0.569	0.454	0.80	6.259	14

[a] RI, resistance index, calculated as [IC_{50} (K1)] / [IC_{50} (D10)]; [b] TI, therapeutic index, calculated as [IC_{50} (HeLa)] / [IC_{50} (K1)]; [c] ART = artelinic acid; [d] NQ = naphthoquinone.

4.5.2 β–Haematin Inhibition Assays

The hybrid compounds were tested for their ability to inhibit the polymerization of haematin *in vitro* using the colorimetric β–haematin inhibition screening assay,[54] and the results are tabulated in Table 4.3.

Table 4.3. Results for β–haematin inhibition.



General structure of Ugi adducts

Compound	R_1	R_2	n	β-Haematin Inhibition IC_{50} (equiv.)[a]
CQ	-	-	-	1.91 ± 0.3
ART	-	-	-	0.66 ± 0.1
6a	ART		1	0.45 ± 0.04
6b	ART		3	0.31 ± 0.01
6c	ART		1	0.44 ± 0.04
6c·citrate salt	ART		1	0.40 ± 0.01
6d	NQ		1	Not active[b]
6e	NQ		1	Not active[b]

[a] This study ± standard error of the mean on three determinations.

[b] At IC_{50} > 10.0 equiv.

Chloroquine was used as the control, with an IC_{50} value of 1.91 equivalents. Artemisinin exhibited an IC_{50} of 0.66 equivalents. All three hybrid molecules that contain the artelinic acid moiety displayed enhanced β–haematin inhibitory activity when compared to CQ and artemisinin, with IC_{50} values of 0.45, 0.31 and 0.44 equivalents for **6a**, **6b** and **6c** respectively. The salt derivative of **6c** displayed a similar activity as the free base; this was also observed for chloroquine and its diphosphate salt. Compounds **6d** and **6e**, which contain the 1,4-naphthoquinone pharmacophore, displayed no inhibitory activity at 10 equivalents. This suggests that the 1,4-naphthoquinone moiety may interfere with the inhibitory mechanism of the 4-aminoquinoline ring.

4.5.3 Morphological Changes in the Parasites

The first 24 hours of the 48 h life cycle of the parasite is known as the ring stage. The nomenclature derives from the fact that the parasites morphologically resemble rings at this stage when stained with Giemsa and viewed by light microscopy (Fig. 4.11A). The ring stage is followed by the trophozoite stage, where the parasites actively endocytose and degrade haemoglobin, accompanied by a rapid increase in size (Fig. 4.11B). General morphological changes in the parasites across their life-cycle were visualized for untreated and drug-treated parasites. After the addition of various drugs at 0 h to ring and trophozoite stage cultures (at concentrations five times their respective IC_{50}s), parasites were smeared onto microscope slides, fixed with methanol and stained with Giemsa before viewing under light microscope. Light microscope images of parasites at 0 h of drug addition are shown in Fig. 4.11. Each panel shows a single parasite within a larger surrounding red blood cell.

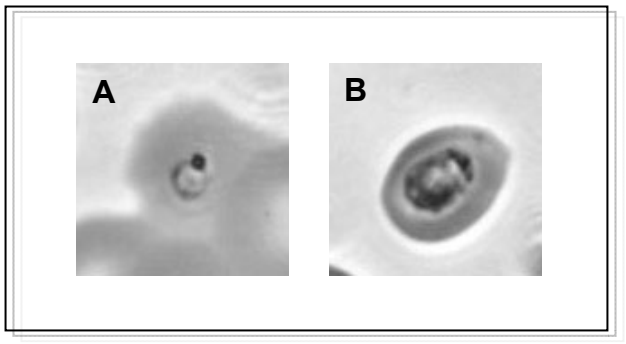


Figure 4.11. Light microscope images of A, ring stage and B, trophozoite stage parasites, at 0 h of drug addition.

Microscope images for untreated and drug-treated rings are shown in Fig. 4.12. Control untreated ring stage parasites remained in the ring stage after 6 h, but had developed into prominent trophozoites by 24 and 30 h. At 48 h, parasites had progressed through a round of asexual reproduction to produce new rings. When parasites were treated with chloroquine, no morphological changes were observed after 6 h. This was expected, as chloroquine has been widely accepted to target parasites at the actively metabolizing trophozoite stage.[55] However, subtle morphological changes in the ring stage parasites are difficult to discern by light microscopy due to their small size and heterogeneity, hence the presence of parasites that were already under stress but could not be distinguished from normal rings cannot be ruled out. However, after 24 and 30 h of drug treatment, the majority of the parasites in the cell culture remained in the ring stage, as opposed to developing into trophozoites. This suggests that chloroquine action had retarded or parasite life-cycle progression. After 48 h, parasites had developed into trophozoites with an insubstantial 'hollow' appearance that seem not to be able to undergo nuclear division and form schizonts.

Artemisinin appears to be much faster-acting than chloroquine, as dying parasites were already observed in the drug-treated culture at 24 h, indicated by the decrease in size and

contracted, pyknotic appearance of the rings. Smears of the culture at 30 and 48 h indicated that the culture predominantly consisted of dead rings, marked by the tiny, dense dots. Dead rings in the culture suggest that artemisinin displays marked parasiticidal activity in the ring stage, completely preventing survival into the trophozoite stage. This is in accordance with the finding that artemisinins cause a rapid reduction of parasitaemia below detectable levels, without eliminating all parasites.[56]

Morphological changes in parasites treated with hybrid molecules **6a**, **6b**, **6c** and its salt all followed the same pattern as that of artemisinin. Cultures also mainly consisted of dead rings and a small percentage of dead early trophozoites after 24 h of drug treatment.

Light microscope images of untreated and drug-treated trophozoites are shown in Fig. 4.13. After 6 h, untreated trophozoites started undergoing nuclear division (the multiple nuclei produce a marked punctated appearance in the parasite cytoplasm) to become schizonts. Each of the daughter nuclei in the schizont develops into a small, invasive merozoite, thus completing the asexual reproduction of the parasite. By 24h, these merozoites had invaded fresh red blood cells and developed into rings, staying in this stage until 48 h when trophozoites predominate again.

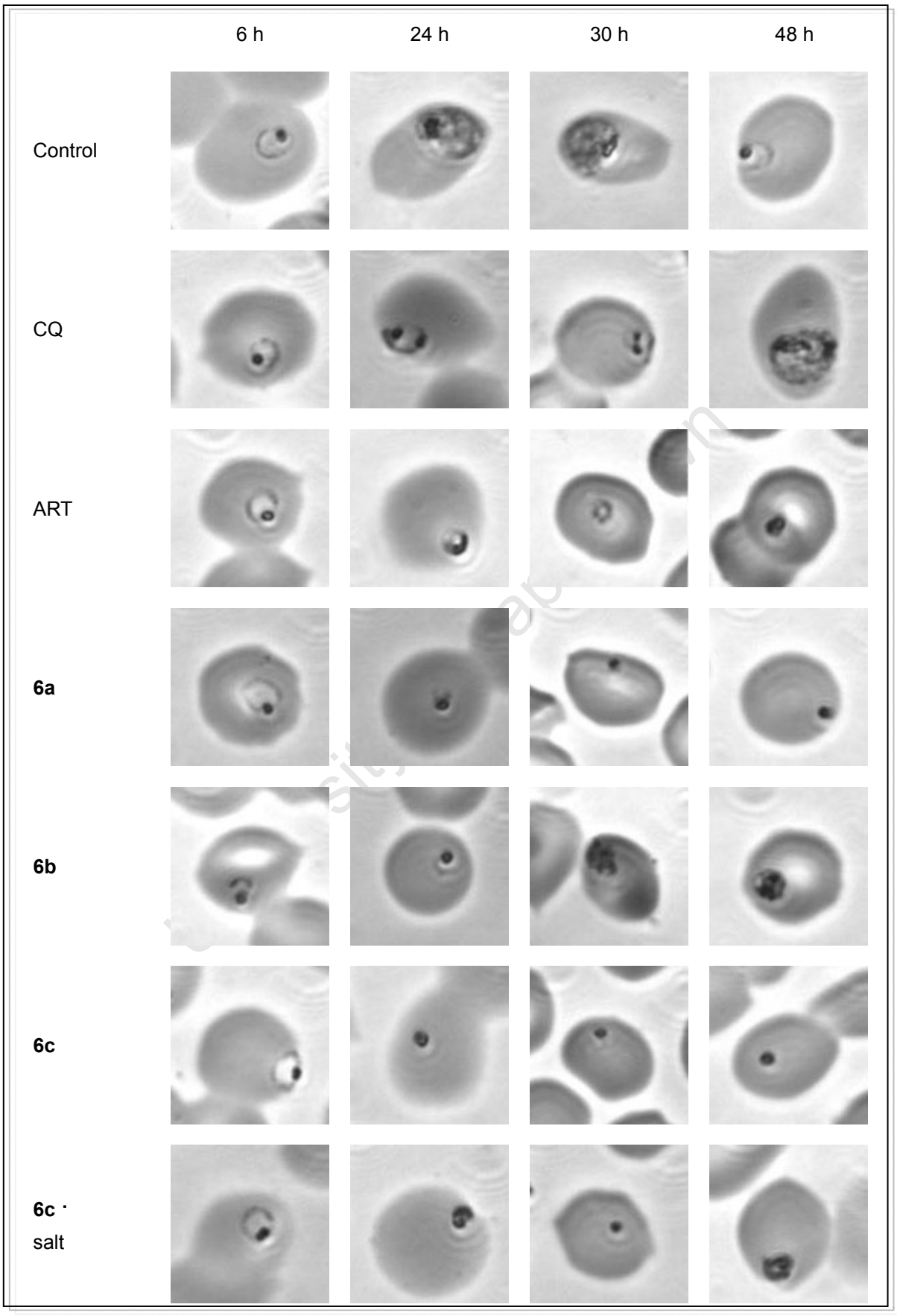


Figure 4.12. Phase images of parasites at various times after the addition of drugs, starting at ring stage.

Effects of chloroquine treatment in trophozoites were much more pronounced than in the ring stage. Morphological changes after 6 h demonstrated that chloroquine had already exerted considerable effects on these parasites, as the trophozoites were observed to be smaller and denser, a clear indication of stress in the parasites. After 24 h of drug treatment, deformed schizonts were observed, and this morphology was maintained for a further 24 h before the parasites eventually disappeared. Healthy schizonts typically contain between 10 and 30 daughter nuclei, which are evenly distributed within the parasite. However, schizonts in the chloroquine-treated culture had very different morphologies: (i) the shape was not round as healthy schizonts typically appear to be, and (ii) nuclei were not evenly distributed within the schizonts, (iii) individual schizonts contained less nuclei on average than healthy control schizonts. Distortion in shape may have arisen from the smearing technique, where cells could have been flattened and hence distorted during smearing. However, these distorted schizonts were enclosed in round RBCs, indicating that the distortions were not from poor smearing techniques.

Morphological changes in artemisinin-treated parasites were somewhat different to those incubated with chloroquine. After 6 h, parasites seemed to be initiating nucleation, entering the schizont stage. After 24 h of artemisinin treatment, parasites were still predominantly in this stage but had also started to deteriorate and die, and mainly dead parasites (detectable as small, darkly stained parasites) were observed after 30 and 48 h of drug treatment.

These hybrid compounds seemed to act faster than chloroquine or artemisinin. A small percentage of the parasites had already taken on the contracted appearance of dead/dying parasites after 6 h of drug treatment, while the majority of the parasites were developing into schizonts. After 24 h of drug treatment, most of the parasites appeared dead.

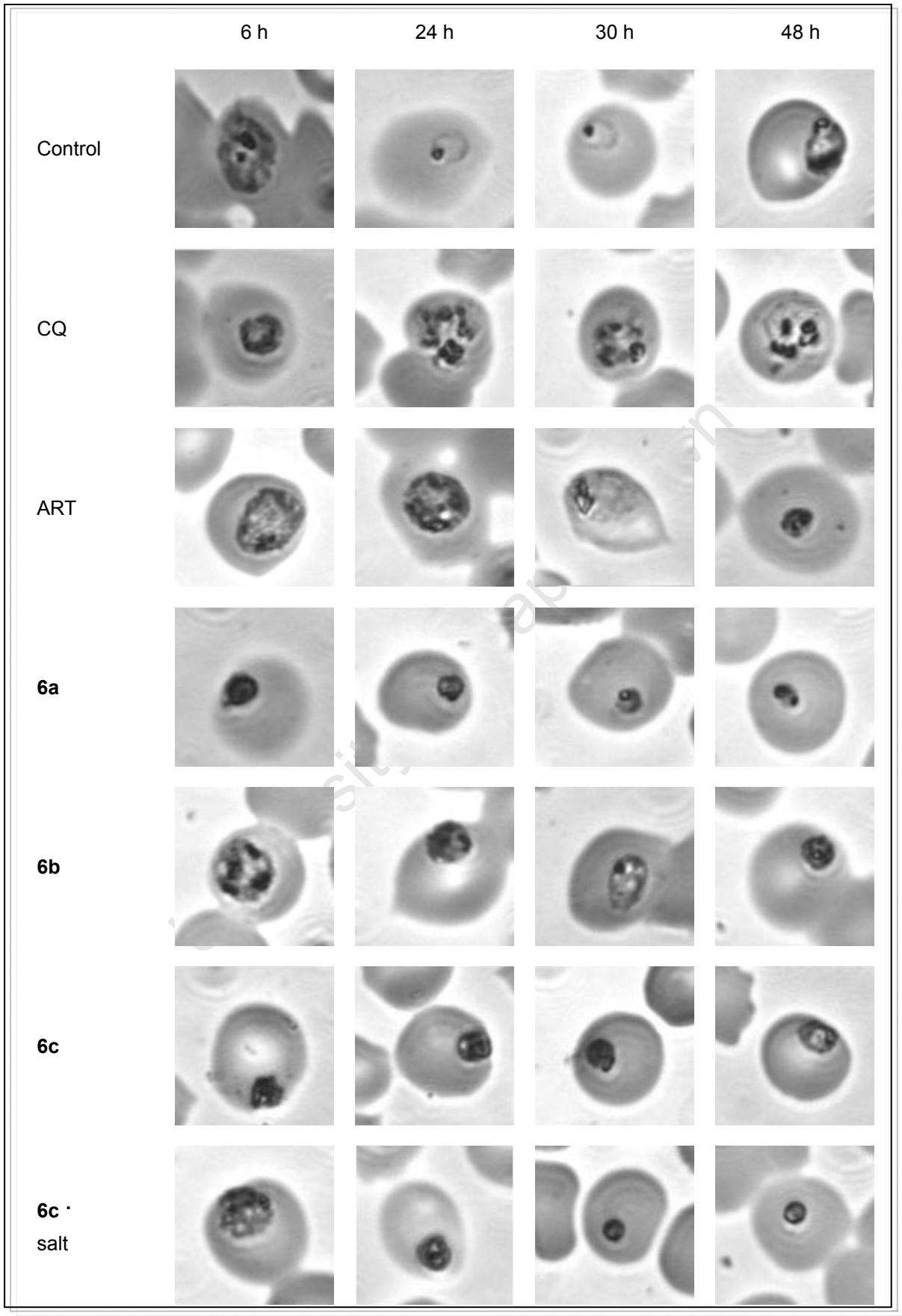


Figure 4.13. Phase images of parasites at various times after the addition of drugs, starting at trophozoite stage.

From the study of morphological changes of the drug-treated parasites, it was clear that chloroquine's parasiticidal action is mainly directed against trophozoite stage parasites, while its effect on ring stage parasites is to slow down development into trophozoites. By marked contrast, artemisinin is parasiticidal against all stages of parasites and has a more rapid action. These findings corresponded to findings published in literature.[55,57] Importantly, the blood smears of cultures treated with the hybrid molecules indicate that these compounds share the rapid parasiticidal action and wide stage-specificity of artemisinin and produce similar morphological changes.

4.5.4 Effect on Endocytosis

Various assays were conducted to determine the effects of the hybrid molecules on the endocytosis pathway in the D10 strain of *P. falciparum*.

4.5.4.1 Effect of Drug Treatment on Haemoglobin Levels

It has previously been shown by independent studies that chloroquine blocks haemoglobin degradation,[58,59] hence an accumulation of undigested haemoglobin is expected in chloroquine-treated parasites. Based on their pronounced antiplasmodial activities (Table 4.2), compounds **6a**, **6b**, **6c** and its salt were selected for the investigation of their effects on haemoglobin levels in the parasites by Western blotting.

Trophozoite stage parasites were incubated with chloroquine, artesunate and the hybrid compounds for 5 h (at five times the IC_{50} of individual drugs), released from the red blood cells by saponin treatment and washed extensively to remove extraneous haemoglobin,

following the protocol by Hoppe *et al*.[47] Artesunate was used instead of artemisinin in the investigation on effects on endocytosis, which is expected to penetrate the cells more efficiently *in vitro* due to its enhanced water-solubility.[60] Haemoglobin levels in the parasites were determined by running the parasite pellets on SDS-polyacrylamide gels and subsequent Western blotting with anti-haemoglobin antiserum (Fig. 4.14). To ensure that the different intensities of the bands were not the result of uneven loading of parasite pellets, gels were also stained with Coomassie dye to visualize protein bands in the gels; there were no variations in the intensities of the major non-globin proteins across the different samples.

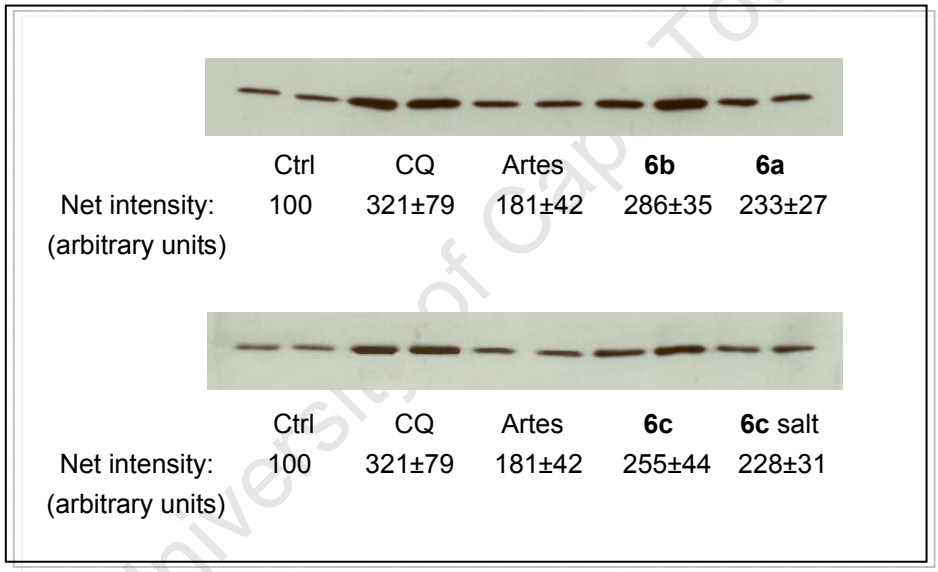


Figure 4.14. Western blots of haemoglobin levels in parasites. Parasites were left untreated (ctrl), or incubated with chloroquine (CQ), artesunate (Artes), or hybrid compounds **6a**, **6b**, **6c** and its salt at 37 °C for 5 h. Duplicate samples are shown for each treatment. Net intensities of individual bands were determined with Kodak 1D image analysis software, version 3.5. Normalized values ± SD obtained from five independent experiments are shown.

It was evident from the Western blots that both chloroquine and artesunate treatment caused an increase in haemoglobin level in the parasites, although the increase associated with artesunate was considerably less than that obtained with chloroquine. These results are in

agreement with published results.[47] The hybrid molecules also produced an increase in parasite haemoglobin levels compared to controls, and the effects were intermediate between those of chloroquine and artesunate.

To further investigate the effects of the chloroquine-artemisinin hybrid molecules on the endocytic pathway, the subcelluar location of haemoglobin within parasites was determined by immunofluorescence microscopy using anti-haemoglobin antiserum. As all four hybrid compounds exhibited similar patterns of haemoglobin accumulation in parasites by Western blotting, compound **6b** was selected for the immunofluorescence assay.

4.5.4 2 Localization of Transport Vesicles by Immunofluorescence Microscopy

Parasite-associated haemoglobin is predominently located in the food vacuole. Ultrastructural studies have suggested that the increase in haemoglobin level in chloroquine-treated parasites may be due to the presence of endocytic vesicles (or transport vesicles) filled with undigested haemoglobin found either in the food vacuole,[61] or throughout the parasite cytoplasm.[47] To investigate the effects of the drugs on endocytic trafficking within the parasite, subcellular localization of haemoglobin was visualized by immunofluorescence microscopy, with particular emphasis on the amount of haemoglobin in the food vacuole and the numbers and distribution of endocytic vesicles.

Parasites were incubated with chloroquine, artesunate and **6b** at five times their respective IC_{50} values, after which they were released from the RBCs by a brief saponin treatment to remove all extraparasitic haemoglobin (in order to reduce background levels and improve the quality of immunofluorescence signals). The parasites were subsequently fixed onto

poly-lysine-coated glass cover slips with paraformaldehyde-glutaraldehyde, permeabilzed with Triton and parasite-associated haemoglobin labeled by incubation with anti-Hb antiserum and TRITC-conjugated secondary antibodies. These cover slips were viewed by fluorescence microscopy to examine the localization of haemoglobin in the parasites. Representative images are shown in Fig. 4.15 below.

In the control parasites, haemoglobin was predominantly located in the food vacuole. The location of the FV is readily determined by referring to the corresponding phase-contrast images, where the shiny haemozoin crystals, formed from haemoglobin degradation in the FV, are prominently visible. The food vacuoles are further indicated by large arrows in the fluorescence images. Punctate endocytic vesicles, filled with haemoglobin, were also visible. These are indicated by small arrows. For chloroquine treated parasites, there was little fluorescence (or in some cases, no fluorescence) in the FV, and haemoglobin was mostly found enclosed in endocytic vesicles. Treatment with artesunate and **6b** resulted in decreased intensity of haemoglobin in the FV and fewer endocytic vesicles when compared to control parasites.

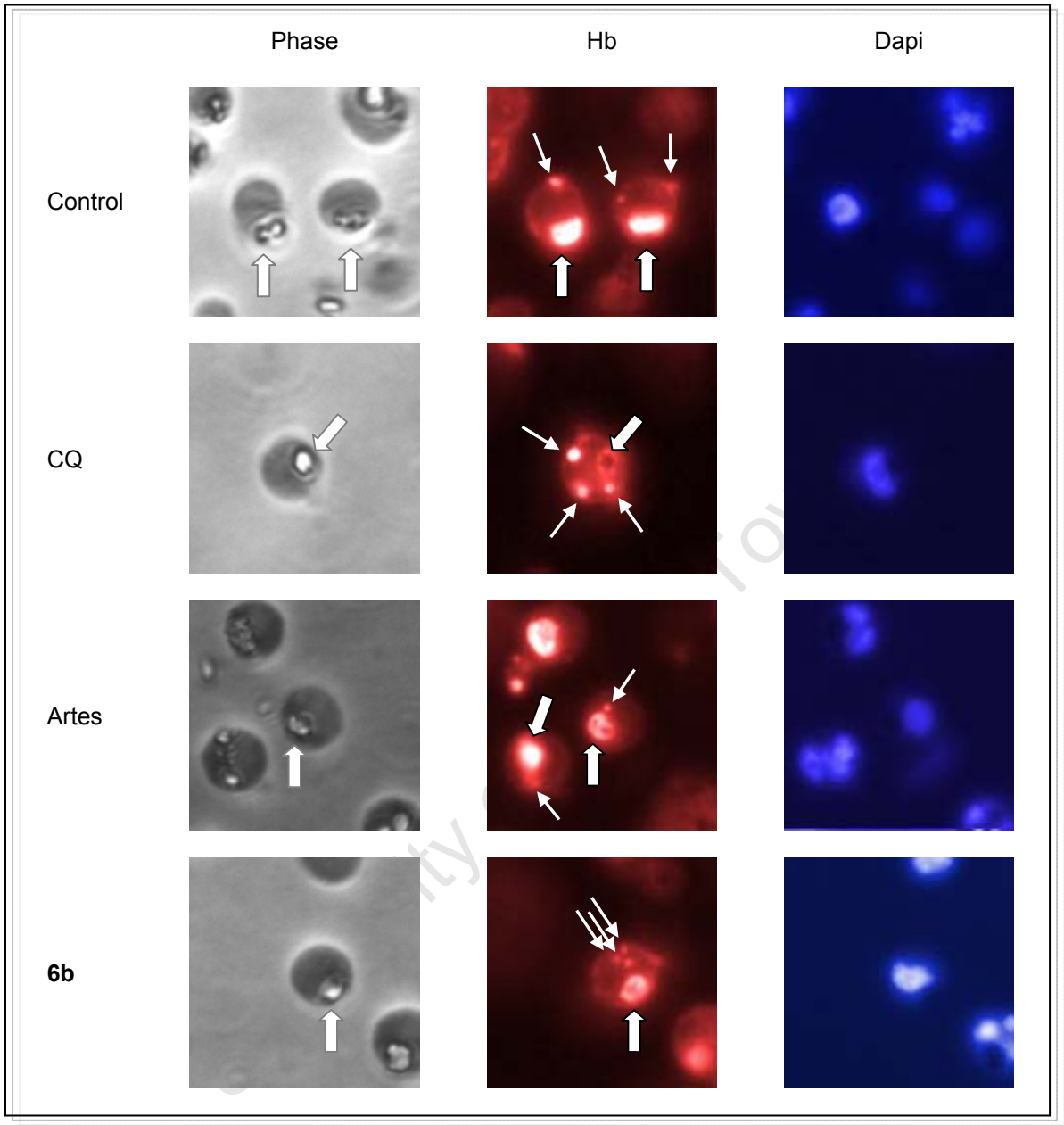


Figure 4.15. Immunofluorescence microscopy localization of haemoglobin in parasites following incubation with various drugs. The position of the FV is indicated by large arrows, whereas those of the haemoglobin-containing transport vesicles are indicated by small arrows. Control, untreated parasites; CQ, chloroquine; Artes, artesunate; Phase, phase-contrast images, Hb, localization of haemoglobin; DAPI, nucleic acids were stained using DAPI in order to determine the number of nuclei, to avoid including multi-nucleated schizonts in the analyses.

The average number of transport vesicles per parasite was enumerated by counting the amount of visible vesicles visible in 100 - 400 parasites per treatment, and it was found that the number of transport vesicles increased significantly in chloroquine-treated parasites, but decreased in parasites treated with artesunate and **6b** (Fig. 4.16).

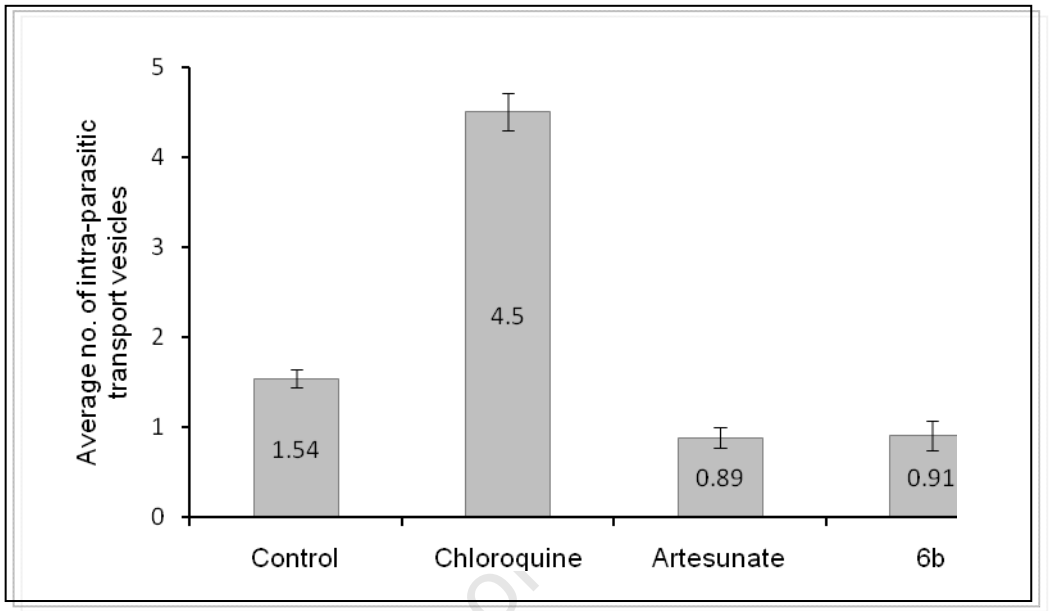


Figure 4.16. Average number of transport vesicles in drug-treated and untreated parasites. Quantification consisted of counting the number of fluorescent foci separate from the food vacuole. Error bars represent standard error of the mean. Comparisons were made using the Mann-Whitney U test, significance $\alpha = 0.05$.

The results obtained for chloroquine and artesunate agreed with those reported in literature.[47] As discussed in section 4.3.2, Hoppe *et al* have suggested that the increase in haemoglobin accumulation and the number of transport vesicles in chloroquine-treated parasites may be the result of a block in transport vesicle-vacuole fusion, so that haemoglobin cannot be delivered to the FV for digestion. In addition, the decrease in endocytic vesicle counts in artesunate-treated parasites agrees with the reported inhibition of endocytosis by this drug – decreased haemoglobin endocytosis should decrease the rate of endocytic vesicle formation and hence vesicle content per parasite.

The morphology and immunofluorescence assays suggest that the mode of action of the hybrid molecules more closely resembles that of the artemisinin parent drug, rather than that of chloroquine. The hybrid molecules and artemisinin rapidly kill parasites in the ring stage, as opposed to the parasiticidal action of chloroquine which is more prolonged and prevalent in the trophozoite stage. In addition, the hybrid molecules and artemisinin reduced the amount of haemoglobin-filled endocytic vesicles in the parasite, likely due to an inhibition of endocytosis, in contrast to the vesicle-vacuole fusion block and marked increase in vesicles caused by chloroquine. The only result which suggests that an element of chloroquine's mode of action may be maintained in the hybrid molecules is the fact that the increase in overall haemoglobin levels in the parasite obtained with the hybrids, as judged by Western blotting, is intermediate between the increase observed with artesunate and chloroquine respectively. As discussed earlier, the moderate increase in overall parasite haemoglobin levels caused by artemisinin has been interpreted as a combination of decreased haemoglobin uptake due to an endocytosis block on the one hand, balanced by increased haemoglobin content due to an inhibition of haemoglobin proteolysis in the food vacuole on the other.[47] The β-haematin inhibition assays suggest that the hybrid compounds are even more effective than chloroquine at inhibiting the formation of haemozoin crystals in the food vacuole. Vacuole damage caused by heme accumulation may result in more extensive haemoglobin proteolysis inhibition by the hybrids than with the artemisinin parent drug, thus explaining the greater extent of haemoglobin accumulation observed by Western blotting.

4.6 Conclusion and Future Work

4-Aminoquinolines were hybridized with artemisinin and 1,4-naphthoquinone derivatives via the Ugi 4-component condensation and their biological activities were investigated. No cross-resistance was observed for these hybrid compounds in chloroquine resistant parasites, with the artemisinin-containing compounds **6a**, **6b**, **6c** and its salt being the most active in antiplasmodial assays (26, 35, 27 and 24 nM respectively). However, despite the potent *in vitro* activities, they also displayed cytotoxic properties and therapeutic indices of approximately 15. Hence, the challenge would be to lower the cytotoxity of these compounds, whilst retaining their potent antiplasmodial activity. One of the places to start would be the incorporation of new side chains into the molecule through the aldehyde and/or isocyanide inputs, or alternatively by changing the positions of the artemisinin and 4-aminoquinoline pharmacophore by making, for example, artemisinin-amines, 4-aminoquinoline-isocyanides, etc.

Morphological changes in parasites treated with compounds **6a**, **6b**, **6c** and its salt have shown that they may act by mechanism(s) similar to artemisinin, as both ring and trophozoite stage parasites were affected by the drug treatment. Additionally, the potent β-haematin inhibitory activities of these hybrids (0.45, 0.31, 0.44 and 0.40 equivalents for **6a**, **6b**, **6c** and **6c**·salt, respectively) may have contributed to their inhibitory mechanism(s) against trophozoite stage parasites.

The effects of the artemisinin-containing hybrids on endocytosis were also investigated, and were found to result in an increase in accumulation of haemoglobin within the parasites. Subsequent immunofluorescence studies have shown that, for the representative compound

6b, there was a decrease in the number of transport vesicles in the parasites. These hybrid molecules may act by inhibition of endocytosis, suggested by the immunofluorescence results, and inhibition of haemoglobin degradation, from the observed increase in haemoglobin levels in the parasites in Western blots, supported by the potent activities of these hybrids to inhibit β-haematin formation.

It is also possible that these hybrid compounds do not act via the hypothesized artemisinin and chloroquine-related mechanisms, but on targets and/or pathways totally different to the two parent drugs that also impinge on the endocytic pathway. To further investigate the effects on endocytosis of these compounds, more extensive studies would be needed. For example, parasites could be incubated with the hybrid compound in the presence of protease inhibitors. This could eliminate haemoglobin degradation in the food vacuole, and solely examine the effect of the compounds on the degree of endocytosis. Alternatively, horseradish peroxidase could be used as a quantitative fluid-phase marker of endocytosis as it is indigestible by parasites. Ultimately, comparing the transcriptomic and/or proteomic profiles of parasites treated with artemisinin, chloroquine and the hybrid compounds should provide the most powerful means for assessing the commonality and nature of their respective modes of action.

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CHAPTER 5

SYNTHESIS AND BIOLOGICAL EVALUATION OF β-LACTAMS

5.1 Introduction

This chapter focuses on the synthesis and characterization of a library of small molecules containing the β-lactam pharmacophore, using the Staudinger reaction and the Ugi 3-component 4-centre condensation, and subsequent post-condensation modifications. Their antiplasmodial and cytotoxic activities are also presented. The diversities in the molecules would hopefully provide some insight into structure-activity relationships (SARs) for the β-lactams against malaria.

5.2 β-Lactams Antibiotics

β-Lactam antibiotics are potent, broad-spectrum agents of low toxicity to eukaryotes, and have served as a powerful line of defense against bacterial infection for over 60 years.[1] The β-lactam ring is vital for their antibacterial activities, as activities were lost if the ring was broken. Penicillin, derived from *Penicillium notatum*, is one of the earliest discovered antibiotics, and contained the β-lactam pharmacophore. It was first discovered by Alexander Fleming in 1928,[2] who received the Nobel Prize in 1945 for this great contribution.[3] Following the initial introduction of penicillin during the World War II, a variety of classes of β-lactam antibiotics were identified. These include, for example, the classical or traditional penicillins and cephalosporins, and the non-classical or non-traditional carbapenems and monobactams such as nocardicins and aztreonam.[4] Their structures are illustrated in Fig. 5.1.

The term 'monobactam' (monocyclic β-lactam) was first used by Sykes *et al* to describe a group of monocyclic, bacterially produced β-lactam antibiotics isolated from soil bacteria.[5]

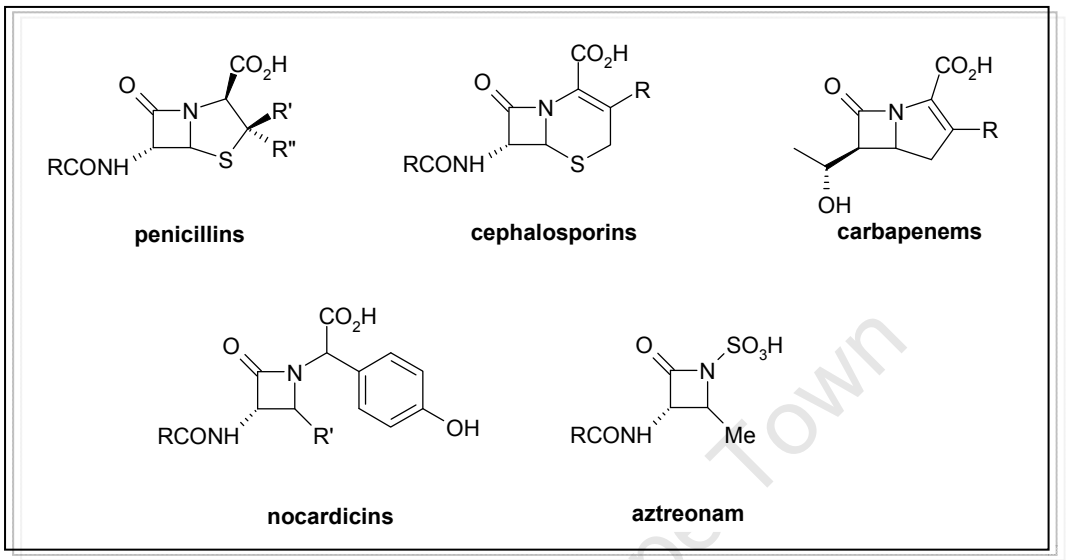


Figure 5.1. Examples of clinically important classes of β-lactam antibiotics.

5.2.1 Mode of Action

The growth and division of bacterial cells necessitate production of new cell wall material. The cell wall is important for the preservation of cell shape and rigidity. The final step of the cell wall material production is the cross-linking of specific peptide chains to form a peptidoglycan matrix. β-Lactam antibiotics inhibit bacterial cell wall synthesis by interacting with enzymes responsible for the cross-linking of the these peptides, thereby causing bacteria death. The target proteins were identified by their ability to covalently bind isotopically labeled penicillin, and were termed penicillin-binding proteins (PBPs).[6] PBPs are localized non-randomly on the outer face of the cytoplasmic membrane of the bacterium (hence relatively accessible by β-lactam antibiotics), and their major activities believed to be the essential peptidoglycan transpeptidase, and the dispensable D,D-carboxypeptidase. All

enzymes were believed to contain a serine residue at the active site. These enzymes are specific to bacteria, with no functional or structural counterpart in the human host, which is advantageous as this minimizes side-effects from antibiotic treatments.

The key step in the cross-linking process is the acylation of the serine hydroxyl group. β-Lactam antibiotics mimic the *D*-alanine-*D*-alanine portion of the peptide substrate, acylating the serine hydroxyl group with the opening of the β-lactam ring. This interference prevents the transpeptidases from completing the peptide cross-linkages in the cell wall, resulting in weakening of the cell wall as the bacterium grows, leading to its death.[6,7]

5.2.2 Bacterial Resistance Against β-Lactam Antibiotics

5.2.2.1 β-Lactamases

The heavy use of antibiotics in the past half-century has resulted in an increasing number of bacteria being resistant to the drugs available today.[8] The most common and clinically relevant bacterial mechanism of resistance to β-lactam antibiotics is the production of β-lactamases. β-Lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolyzing the β-lactam ring. A large number of these enzymes have already been described,[9] but new members are discovered every year.[10] Other means of resistance include the modification or overproduction of PBPs,[11] and the prevention of access of the antibiotic to the target by way of altered permeability or forced efflux.[12] The development of β-lactam antibiotics has thus been a continuous battle of the design of new compounds to withstand inactivation by the ever-increasing diversity of β-lactamases.

5.2.2.2 β-Lactams as β-Lactamase Inhibitors

Clavulanic acid, the first β-lactam inhibitor of β-lactamase, was isolated from *Streptomyces clavuligerus* and its structure elucidated in 1976.[13] After the isolation of clavulanic acid, many semisynthetic β-lactamase inhibitors, such as sulbactam, tazobactam and brobactam, have been further developed, and their comparative β-lactamase inhibitory activities studied against clinically important β-lactamases (Fig. 5.2).[14]

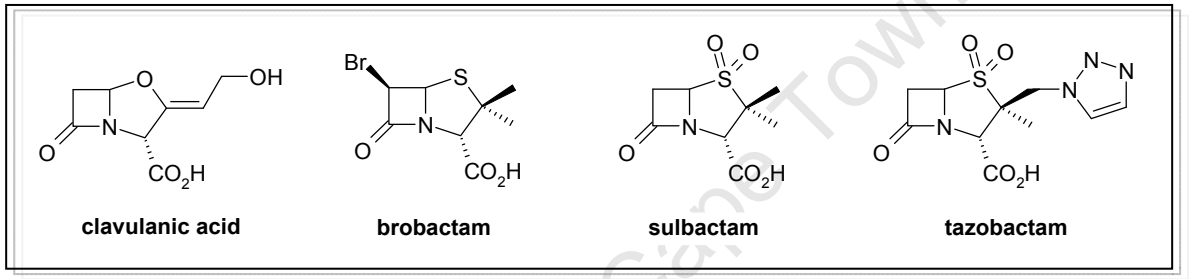


Figure 5.2. Representative β-Lactam Inhibitors of β-Lactamases

In combination with β-lactamase-susceptible antiobiotics, these β-lactamase inhibitors protect the β-lactam antibiotic from inactivation by β-lactamases, thereby extending the spectrum of activity of the antibiotic.

5.2.2.3 Novel Targets against Resistance

Due to the increasing number of bacteria being resistant to available drugs, antibacterial agents against novel targets in the microorganism have been developed. An example is inhibiting the formation of pili. Pili are a family of extracellular, supramolecular protein organelles that mediate attachment to host epithelial cells. Pilus assembly is performed by periplasmic chaperones that fold and transport the subunits to the outer cell membranes

where they are incorporated into a growing pilus.[15] Colonization of host tissue cannot occur if the chaperone subunit complex formation is prevented.[16] Almqvist and co-workers have designed a library of bicyclic β-lactam carboxylic acids that could potentially inhibit pili formation.[17]

5.3 Other Medically Important Uses

Apart from their use as potent antibacterial agents, β-lactams were also reported to display antimalarial,[18] anticancer[19] and anti-HIV[20] activities. They have also been found to be able to inhibit cholesterol absorption[21] and target the human leukocyte elastase,[22] whose degradative effect on lung elastin has been implicated as the major causative factor for serious degenerative diseases such as chronic bronchitis, cystic fibrosis and rheumatoid arthritis. Activities of β-lactams against thrombin,[23] prostate specific antigen[23, 24] and tryptase[25] have also been reported.

5.4 β-Lactams as Antimalarials

Although there have been no studies on their mode(s) of action in the malaria parasite *Plasmodium falciparum*, β-lactams may exert their antimalarial effects by inhibiting important protease activities in the parasite. In humans, the human leukocyte elastase, thrombin, prostate specific antigen and tryptase are all serine or serine-like enzymes.[26] Bacterial enzymes such as the *D*-alanyl-*D*-alanine transpeptidases/carboxypeptidases and β-lactamases are also serine enzymes.[27] The ability of β-lactams to react irreversibly with nucleophilic serine residues make them good serine protease inhibitors, and the interaction of β-lactam analogues with a serine protease have been studied.[28]

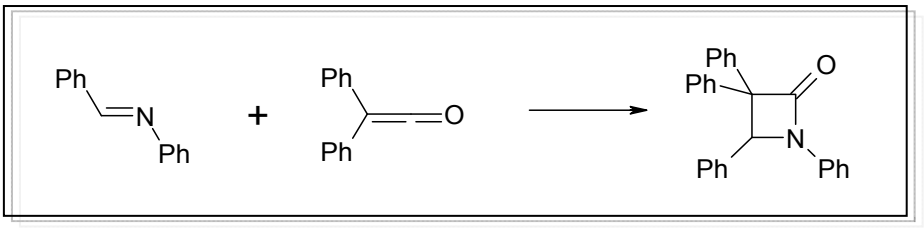
Studies by Singh *et al* have shown that β-lactams also act as cysteine protease inhibitors.[29] Various classes of β-lactam analogs that inhibit cysteine proteases such as the cathepsins have been synthesized. Hence the antimalarial activities of β-lactams may be associated with their ability to interact with serine and/or cysteine proteases in the malaria parasite.

5.5 Syntheses of β-Lactams

After the landmark discovery of penicillin by Fleming,[2] interest in β-lactam compounds has led to intensive research which generated numerous methods of synthesizing the β-lactam backbone. Here we present a few of the most well-known protocols.

5.5.1 Staudinger Reaction

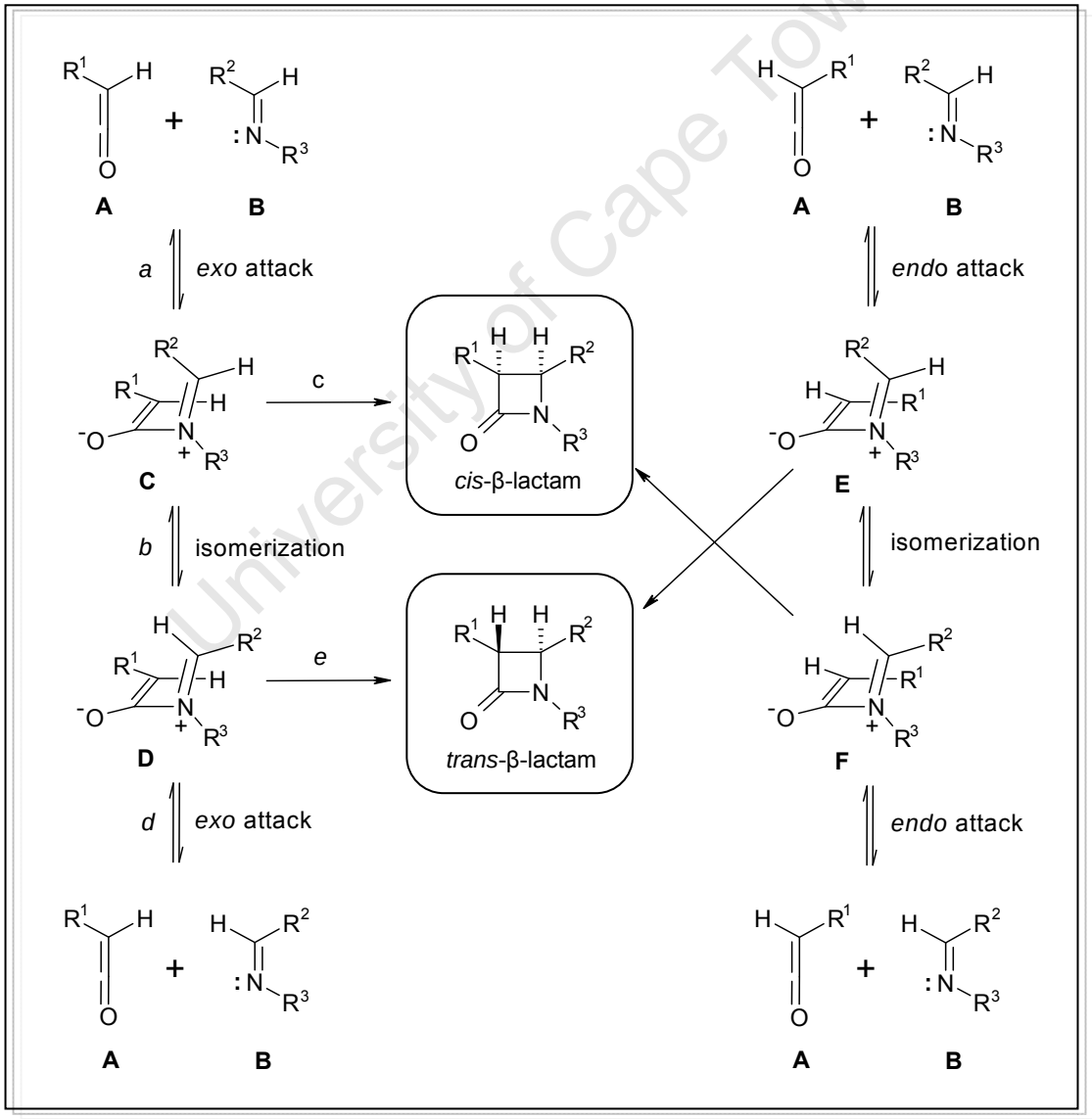
The β-lactam, a four-membered cyclic amide, was first synthesized in 1907 by Hermann Staudinger, using the [2+2] cycloaddition of the Schiff base of aniline and benzaldehyde (benzalaniline) with diphenylketene (Scheme 5.1).[30] This is also the most common approach towards β-lactam synthesis, and was chosen as one of the methods for the synthesis of the target compounds in this PhD study.



Scheme 5.1. Reaction of the first β-lactam via the Staudinger reaction.

5.5.1.1 Reaction Pathways and Relative Stereoselectivity

The most widely accepted reaction mechanism for the Staudinger reaction is the stepwise ketene-imine cycloaddition reaction, which involves (a) the nucleophilic attack of the imine to the ketene, giving rise to a zwitterionic intermediate and (b) a conrotatory electrocyclic ring closure of the zwitterion to produce the final β-lactam product.[31] When a monosubstituted ketene reacts with an acyclic imine, two new stereocentres are produced, giving rise to *cis*- or *trans*-isomers, or a mixture of both.



Scheme 5.2. Pathways for the formation of *cis*- and *trans*-β-lactams.[32] Only one enantiomer is shown.

Pathways for the formation of *cis*- or *trans*-isomers are illustrated in Scheme 5.2. The relative stereochemistry of the β-lactam product is decided by the competition between direct ring closure (*exo* or *endo* attack) and the isomerization of the imine moiety in the zwitterionic intermediate. Jiao *et al* have proposed a model that explains the relative stereoselectivity based on kinetic analysis of the *cis*/*trans* ratios of reaction products.[32] Based on their results, they have proposed that the origin of the relative stereoselectivity can be described as follows:

(i) The stereoselectivity is generated as a result of the competition between the direct ring closure and the isomerization of the imine moiety in the zwitterionic intermediate;

(ii) The ring closure step is most likely an intramolecular nucleophilic addition of the enolate to the imine moiety, which would be affected by the electronic effect of the ketene and imine substituents;

(iii) Electron-donating ketene substituents and electron-withdrawing imine substituents accelerate the direct ring closure, leading to a preference for *cis*-β-lactam formation, while electron-withdrawing ketene substituents and electron-donating imine substituents slow the direct ring closure, leading to a preference for *trans*-β-lactam formation, and

(iv) The electronic effect of the substituents on the isomerization is a minor factor in influencing the stereoselectivity.

The relationship between the relative stereoselectivity and the structural features are presented in Fig. 5.3.

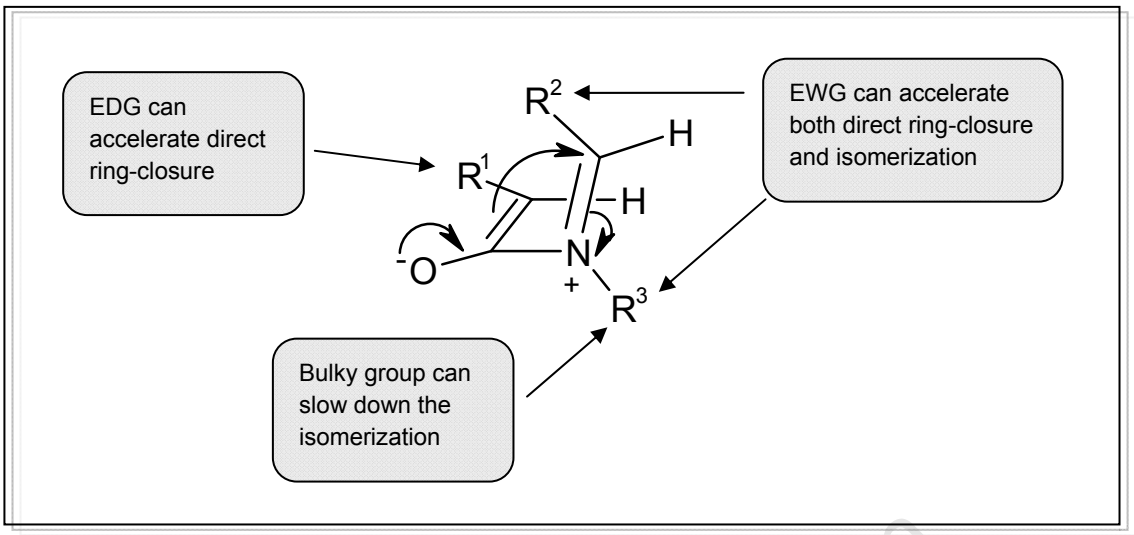
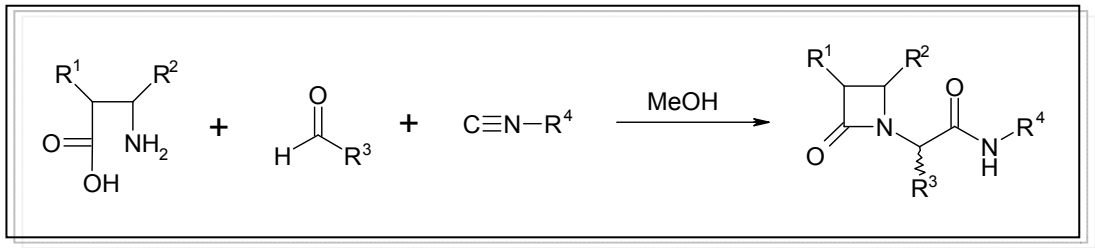


Figure 5.3. Influence of the structural features on the relative stereoselectivity in the Staudinger reaction.[32]

5.5.2 Ugi 3-Component 4-Centre Condensation (Ugi 3C-4CC)

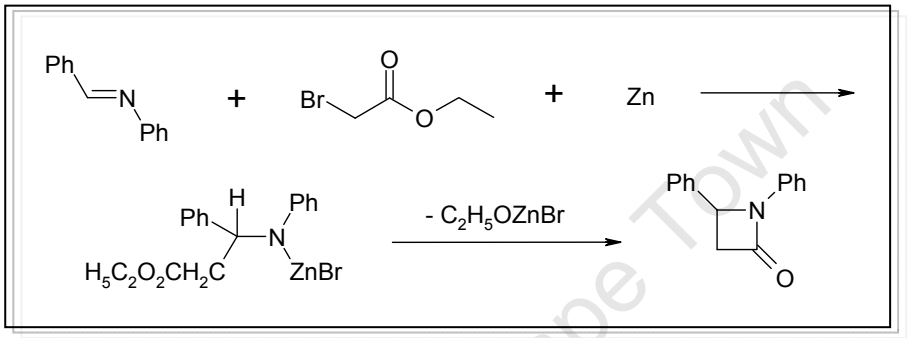
This condensation is a variant of the Ugi 4-component condensation. Here, two of the functional groups that take part in the reaction were tethered onto one starting material. This method has also been used to synthesize γ- and δ-lactams.[33] For the synthesis of β-lactams, β-amino acids (containing the amine and carboxylic acid components) were condensed with aldehydes and isocyanides to yield β-lactam derivatives.[34] Bicyclic β-lactams can be synthesized using cyclic β-amino acids.[35] This reaction was also chosen for the synthesis of the target compounds.



Scheme 5.3. The Ugi 3-Component 4-Centre Reaction.

5.5.3 Gilman-Speeter Reaction

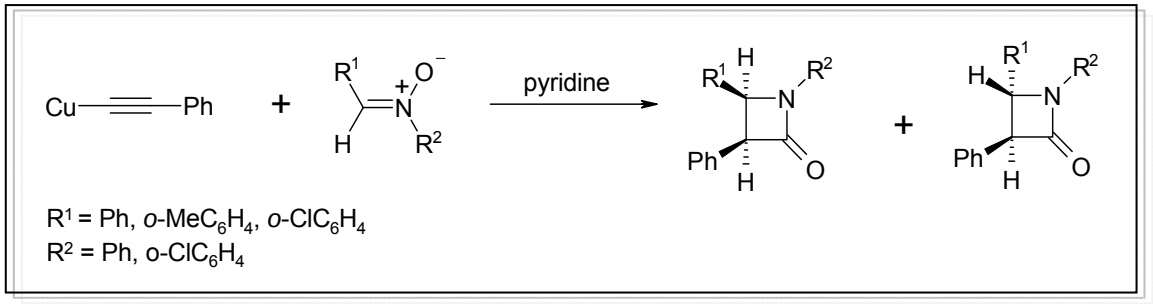
The Gilman-Speeter was discovered by Henry Gilman and Merrill Speeter in 1943.[36] This reaction involves the ester enolate-imine condensation between ethyl bromoacetate and benzalaniline in the presence of zinc. The first Gilman-Speeter synthesis is illustrated in Scheme 5.4.



Scheme 5.4. The Gilman-Speeter Reaction

5.5.4 Kinugasa Reaction

The first description of the Kinugasa reaction in 1972 involved the reaction of copper (I) phenylacetylide with nitrones to produce β-lactams (Scheme 5.5).[37] The reaction was performed in anhydrous pyridine at room temperature under nitrogen atmosphere. The use of copper (I) phenylacetylide as the reagent was critical, as reaction of alkynes with nitrones afforded pyrrolinediones or isoxazolines.[38]



Scheme 5.5. The Kinugasa Reaction.

5.5.5 Other Methods

Other notable methods employed towards the synthesis of the β-lactam backbone include photo-induced rearrangements[39] and radical cyclizations.[40]

5.6 Rationale for Drug Design - Series I

5.6.1 Design of Target Compounds

The Staudinger reaction and the Ugi 3-component 4-centre condensation have been chosen towards the synthesis of the β-lactam backbone due to their simplicity and versatility.

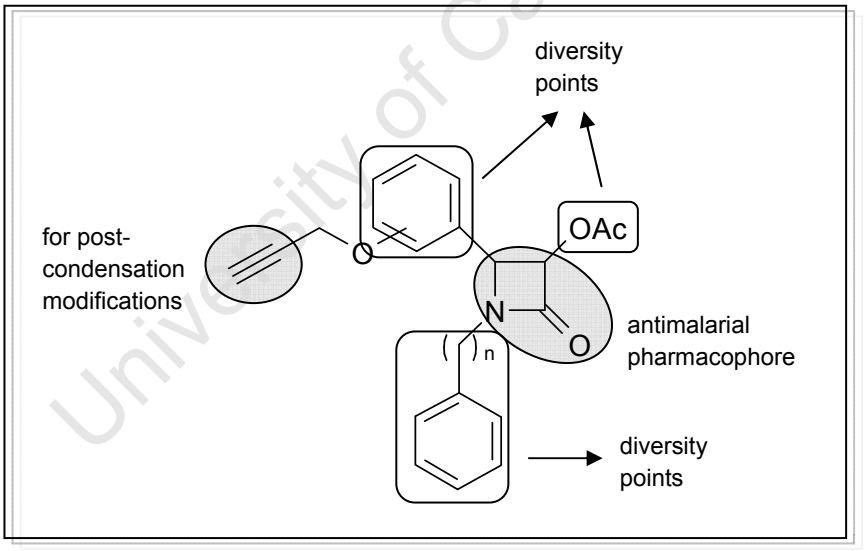
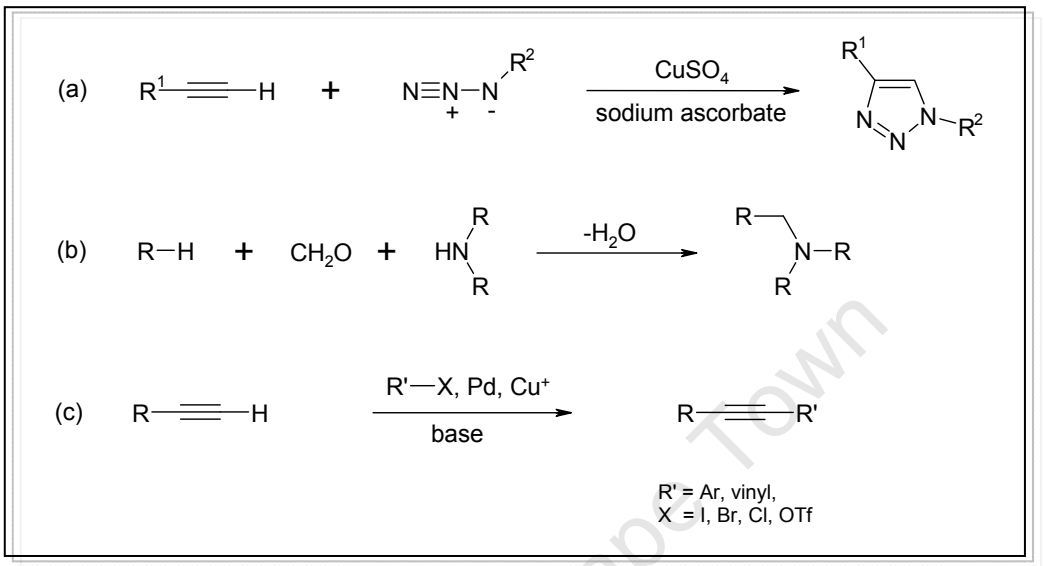


Figure 5.4. Rationale for design of target molecules.

The terminal acetylene moiety is present for possible post-condensation modifications, and is placed at different positions on the aromatic ring or directly on the β-lactam ring. This may be advantageous for structure-activity relationship studies. The acetylene moiety could be utilized in various reactions, for example, (a) the Click chemistry,[41] a 1,3-dipolar

cycloaddition (Scheme 5.6a), (b) the Mannich reaction,[42] an aminomethylation reaction (Scheme 5.6b) or (c) the Sonogashira coupling reaction[43] of aryl or vinyl halides to the terminal alkyne (Scheme 5.6c).



Scheme 5.6. (a) Click chemistry, (b) the Mannich reaction and (c) Sonogashira coupling reaction.

The compound with the most potent *in vitro* antiplasmodial activity within the exploratory series was chosen to be further modified to study whether modifications would enhance its activities. The Mannich reaction was chosen for this modification, as the inclusion of a tertiary amine may enhance biological activities due to, for example, vacuolar accumulation.

5.6.2 The Mannich Reaction

The Mannich reaction, also a multi-component condensation reaction, was first described by van Marle and Tollens in 1906.[44] The three components for this reaction are: (i) ammonia, a primary or secondary amine, (ii) a non-enolizable aldehyde (usually formaldehyde) and (iii) a compound with an active hydrogen atom or a reactive nucleophilic site (Scheme 5.5b). When these components condense, water is released concomitantly to produce a new base, known

as a 'Mannich base". The Mannich reaction is one of the most important carbon-carbon and carbon-nitrogen bond-forming reactions in organic chemistry.[42,45]

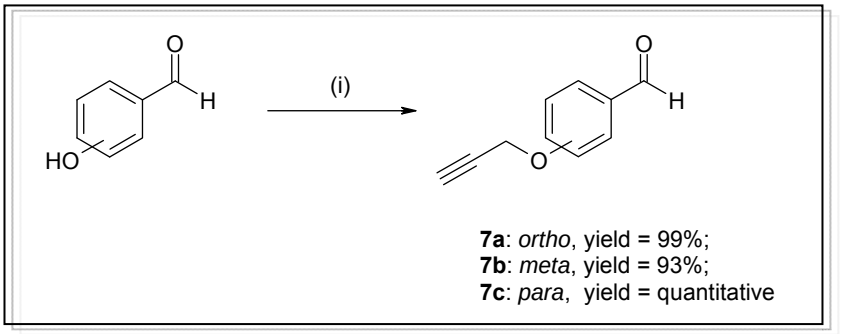
5.6.2.1 Classification of Mannich Bases

The active hydrogen component employed in the Mannich reaction may be a ketone, ester, nitrile, nitro, α-alkylpyridine, hydrogen cyanide, phenol, thiol, tertiary aromatic amine, enamines, alkyne, indole, heteroaromatic or heterocyclic compounds. The amine component used here cannot be a tertiary amine, as it lacks the N-H proton that is needed to form the imine intermediate. The amine component is usually commercially available or readily accessible.[42] The general classification of Mannich bases are presented in Table 5.1.

5.7 Synthesis of Target Molecules – Series I

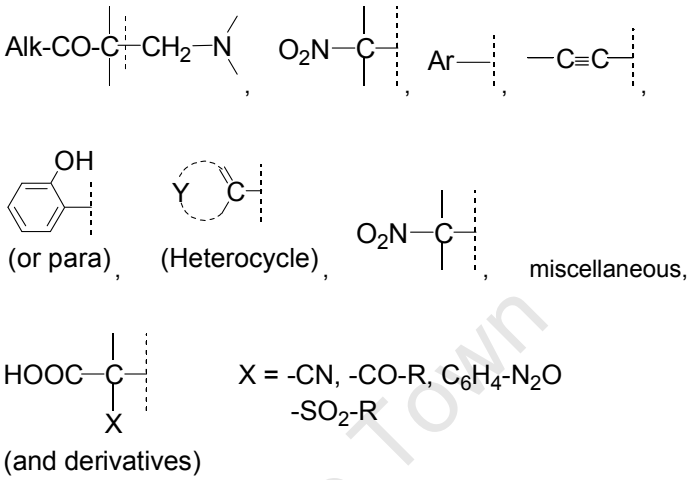
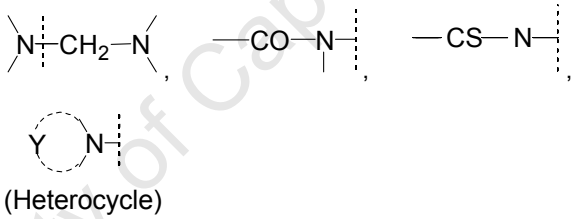
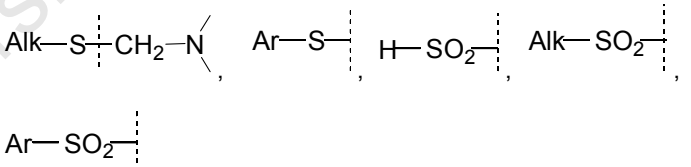
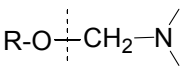
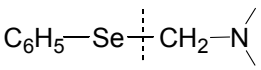
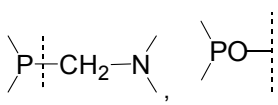
5.7.1 Via the Staudinger Reaction

Propargyloxy benzaldehydes were synthesized by reacting various hydroxy benzaldehydes with propargyl bromide (Scheme 5.7).

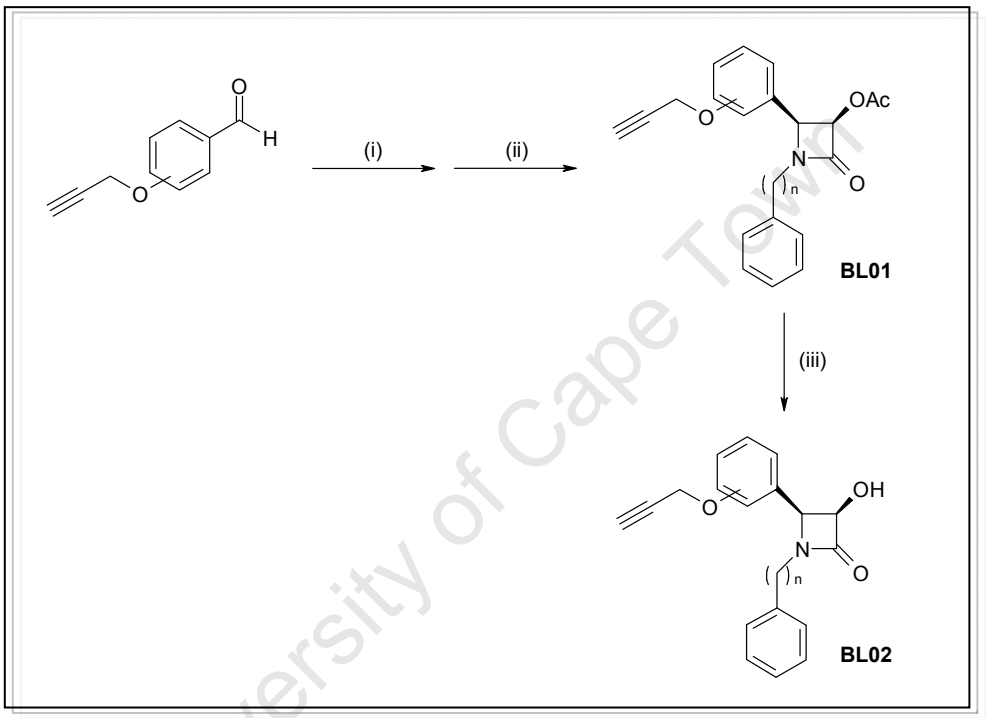


Scheme 5.7. Reagents and conditions: (i) Propargyl bromide (1.2 eq), K_2CO_3 (1.5 eq), anhydrous DMF, r.t., 17 h.

Table 5.1. General classification of Mannich bases.

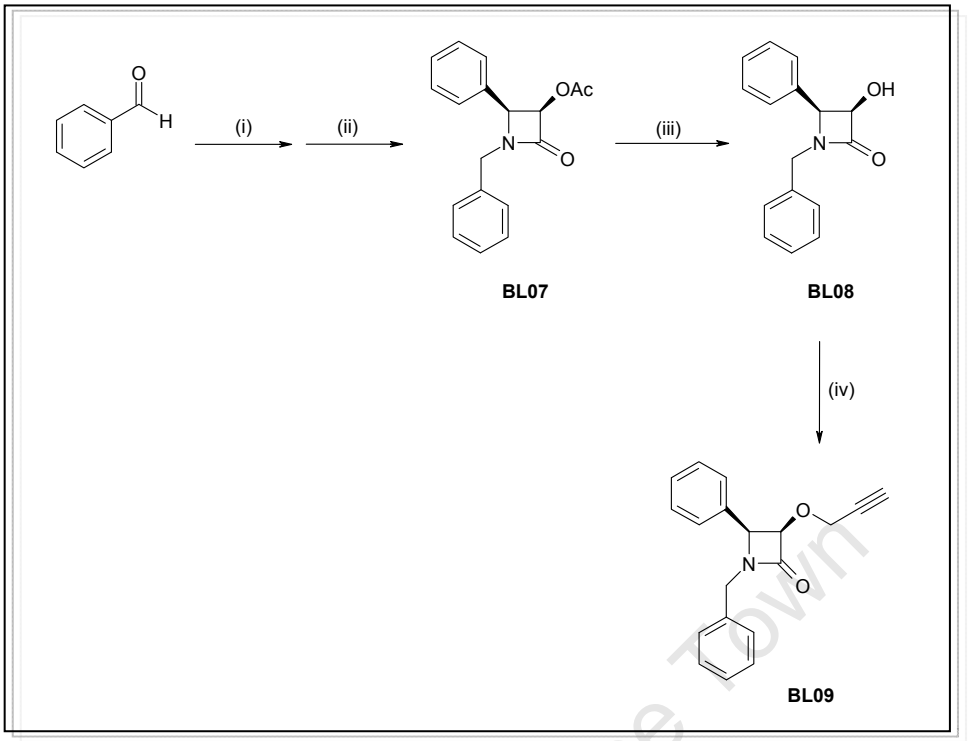
Types of Mannich Bases	Structures of Mannich Bases
C-Mannich bases	
N-Mannich bases	
S-Mannich bases	
O-Mannich bases	
Se-Mannich bases	
P-Mannich bases	

The benzaldehyde was then reacted with either benzylamine or aniline to form the imine intermediate, followed by cycloaddition with acetoxy acetyl chloride to form the β-lactam. For selected compounds, the acetate group was hydrolyzed using K_2CO_3 to yield the free alcohol (Scheme 5.8). Please note that only the one enantiomer of the products is shown here, and for subsequent sections in this chapter.



Scheme 5.8. Reagents and conditions: (i) amine (1.2 eq), methanol, r.t., 24 h. (ii) Acetoxy acetyl chloride (1.5 eq), Et_3N (3.0 eq), DCM, N_2, -78 °C to r.t., 14 h. (iii) K_2CO_3 (1.3 eq), methanol/H_2O (5:2), r.t., 18 h.

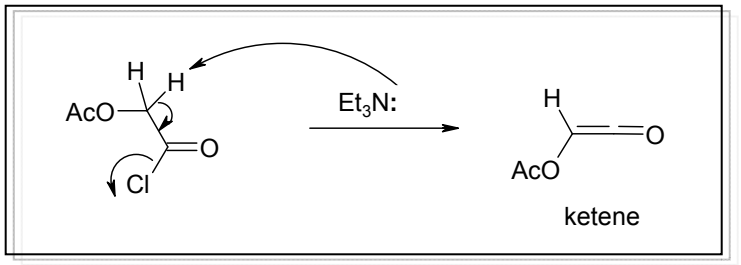
To install the propargyloxy group directly on the β-lactam ring, compound **BL09** was synthesized in a similar way as illustrated in Scheme 5.9. After the cycloaddition of acetoxy acetyl chloride to the Schiff base of benzaldehyde and benzylamine, the compound was hydrolyzed to yield the free hydroxyl group, which was then reacted with propargyl bromide using sodium hydride as the base (Scheme 5.9).



Scheme 5.9. Reagents and conditions: (i) benzylamine (1.2 eq), methanol, r.t., 24 h. (ii) Acetoxy acetyl chloride (1.5 eq), Et_3N (3.0 eq), DCM, N_2, -78 °C to r.t., 14 h. (iii) K_2CO_3 (1.3 eq), methanol/H_2O (5:2), r.t., 18 h. (iv) Propargyl bromide (1.2 eq), NaH (1.2 eq), anhydrous DMF, 0 °C, 1 h.

5.7.1.1. Mechanistic Details

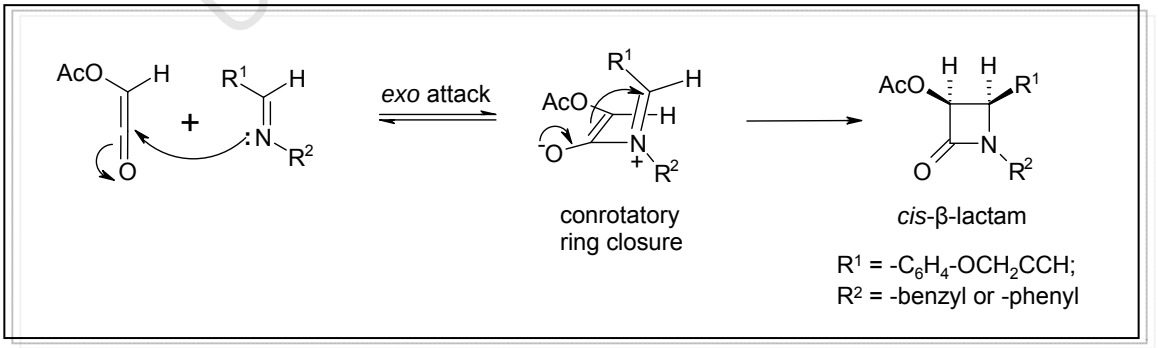
The imine that is formed by reacting the aldehyde and the amine undergoes a [2 + 2] cycloaddition with the ketene that is generated *in situ* from acetoxy acetyl chloride. The formation of the ketene from acetoxy acetyl chloride is illustrated in Scheme 5.10, which entails the abstraction of the acidic proton α to the carbonyl carbon, followed by the elimination of the chloride ion to generate the ketene.



Scheme 5.10. Formation of acetoxy ketene from acetoxy acetyl chloride.

The final β-lactam products are exclusively of the *cis*-configuration (confirmed by ^{1}H NMR spectroscopy), suggesting that the reaction proceeds via direct ring closure, where isomerization of the imine moiety of the zwitterionic intermediate was minimal (pathway *a*→ *c* in Fig. 5.2, section 5.5.1.1).

The imine that is formed is assumed to be of the thermodynamically stable *E* (*trans*) configuration. The initial step of the Staudinger reaction may take place through the less hindered side of the ketene, i.e. via *exo* attack. Hence, the preferential *exo* attack during this step would result in lower energy conrotatory transition structures,[46] giving rise exclusively or predominantly to *cis*-β-lactam products (Scheme 5.11).



Scheme 5.11. Proposed reaction pathway for the Staudinger products.

It is also possible that the *cis*-β-lactam products may form via *exo* attack of the *Z*-imine to the ketene, and undergoing a fast isomerization of the imine moiety of the zwitterionic intermediate to form the *cis*-β-lactam product (pathway $d \rightarrow b \rightarrow c$ in Fig. 5.2). The *cis*-β-lactam product could be exclusively formed if the rate of isomerization was faster than the rate of cyclization. However, from a geometric viewpoint, intermediate **D** is more favourable than **C** (Fig. 5.2), hence it is unlikely, but not impossible, that this is the reaction pathway for the Staudinger products synthesized.

5.7.1.2 Synthesized Products

Compounds containing a terminal acetylene moiety, synthesized via the Staudinger reaction, are presented in Figure 5.5, along with their respective yields.

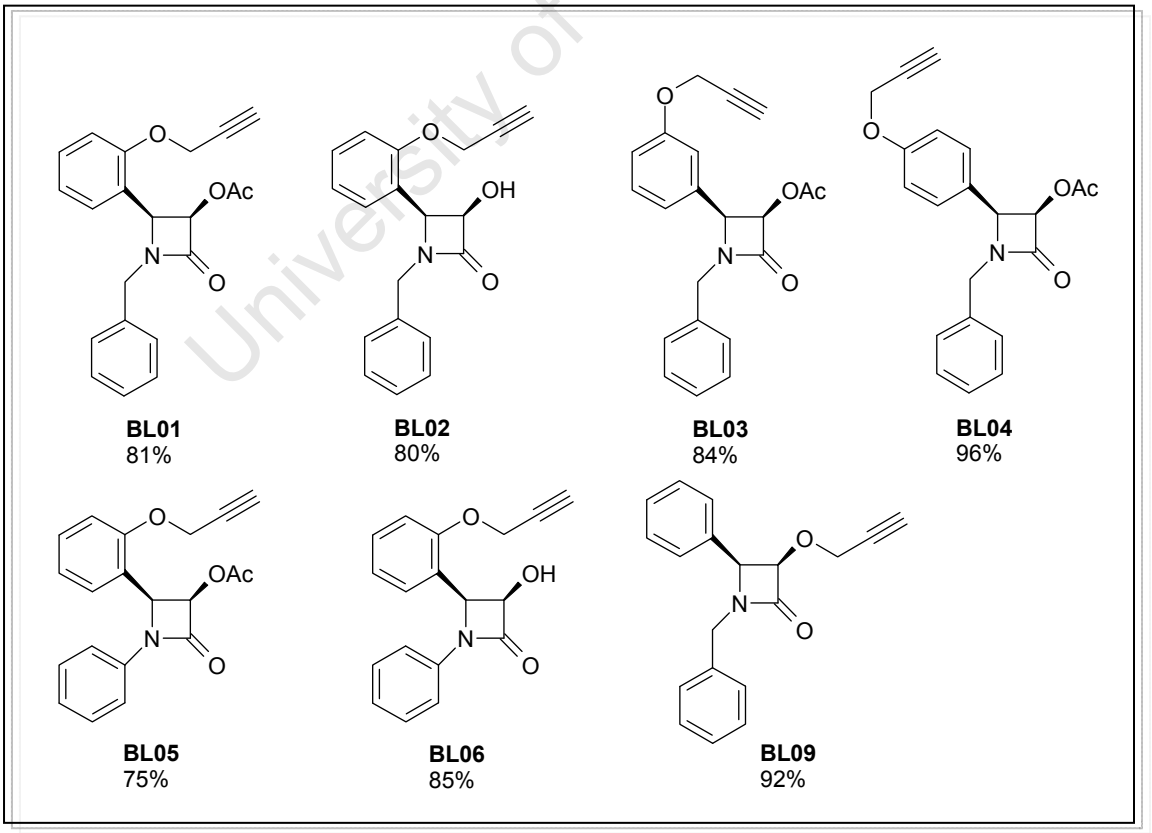


Figure 5.5. Acetylene-containing β-lactams via the Staudinger reaction

5.7.1.3 Spectroscopic Analyses

The syntheses of the β-lactams were confirmed by their respective ^{1}H and ^{13}C NMR spectra. Using the ^{1}H NMR spectrum of compound **BL01** as an example (Fig. 5.6), the presence of a sharp signal at 1.70 ppm, integrating for 3 protons, indicated the presence of the acetyl group. This signal disappeared after the compound was hydrolyzed to form the free alcohol **BL02**.

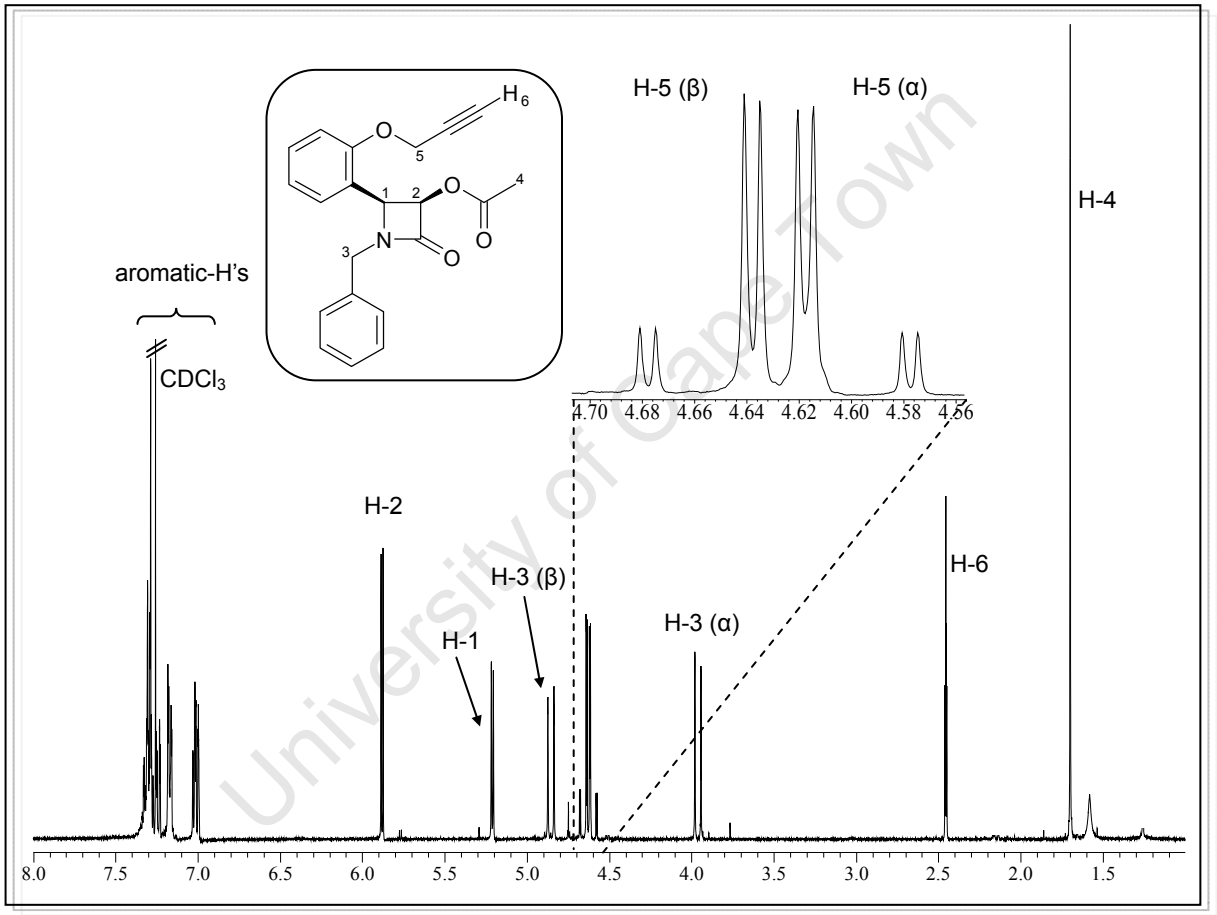


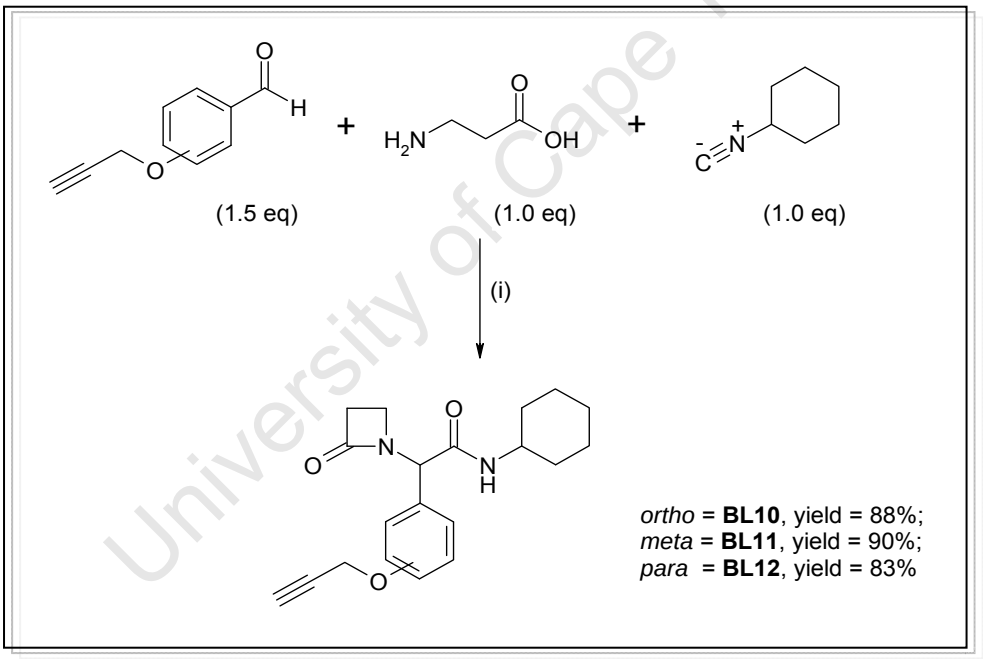
Figure 5.6. ^{1}H NMR spectrum of **BL01**.

Two sets of doublet of doublets (dd) were found at 4.60 and 4.66 ppm, corresponding to the two geminal protons H-5. They couple to each other with a coupling constant of 16.0 Hz, and to the alkyne proton (H-6) with a smaller coupling of 2.4 Hz. Proton signal corresponding to H-6 appeared at 2.43 ppm as a triplet, with a corresponding coupling constant of 2.4 Hz.

Benzylic protons were found at 3.96 and 4.83 ppm, with a coupling constant of 14.8 Hz. The coupling constant between H-1 and H-2 was found to be 4.8 ppm (typically between 4 – 6 Hz for *cis* configuration and 2 – 3 ppm for *trans* configuration),[32] confirming the configuration of the β-lactam products to be *cis*.

5.7.2 Via the Ugi 4-Component 3-Centre Condensation

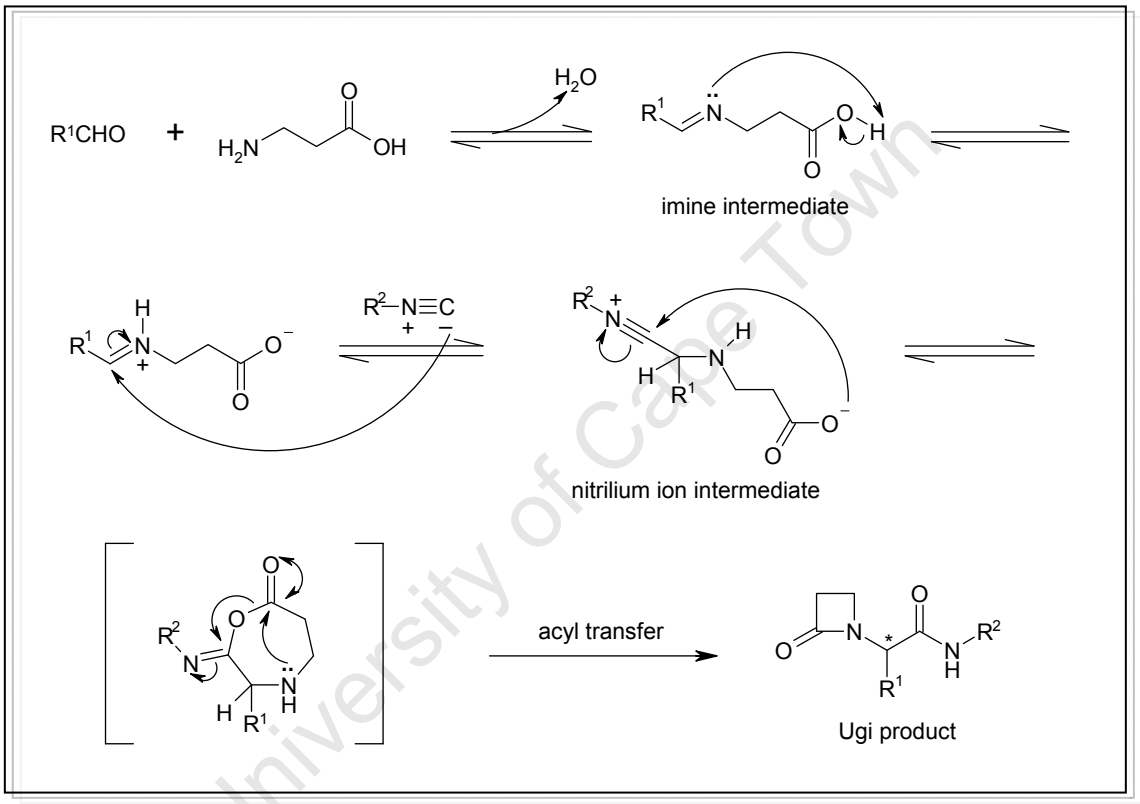
Three compounds were synthesized via the Ugi 3-component 4-centre condensation (Ugi 3C-4CC), namely **BL10**, **BL11** and **BL12** (Scheme 5.12).



Scheme 5.12. Reagents and conditions: (i) methanol, r.t., 72 h.

5.7.2.1 Reaction Mechanism

The reaction mechanism for the Ugi 3C-4CC is the same as the Ugi 4-component condensation, the only difference is that the amine and carboxylic acid components were tethered onto a single molecule. The reaction mechanism is illustrated in Scheme 5.13.



Scheme 5.13. Reaction mechanism of the Ugi 3C-4CC.

A new stereocentre was created in the β-lactam (marked by an asterisk). As R^1 is not a hydrogen atom as in the previous Ugi adducts, isolated products were racemic.

5.7.2.2 Spectroscopic Analyses

The ^{1}H NMR spectrum of **BL11** is presented in Figure 5.7. The region of particular interest is the 2.4 – 4.0 ppm region, where the protons on the β-lactam ring were found. Two sets of triplet of doublets (td, *J* 5.6 and 3.2 Hz, 1H, H-2) appeared between 3.2 – 3.6 ppm, while two sets of doublet of doublet of doublets (ddd, *J* 14.6, 5.6 and 3.2 Hz, 1H, H-1) appeared around 2.9 ppm. Interestingly, H-1 displayed a large geminal coupling of 14.6 Hz, while H-2 did not. The variation in the geminal coupling constants is striking (14.6 Hz for H-1 and 5.6 Hz for H-2). A *J* value of 14.6 Hz falls within the normal range for geminal couplings, while a *J* value of 5.6 Hz is considerably smaller than expected, and is close to values reported for substituted cyclopropanes.[47] This effect cannot be explained by the presence of the neighbouring electronegative nitrogen atom, as benzylic protons displayed a normal coupling constant of ~14.8 Hz (see Fig. 5.6, section 5.7.1.3). This phenomenon was also reported by Barrow and Spotswood,[48] and they have suggested that the ring strain in the β-lactam ring may have been accommodated by rehybridization of C-2, and hence the observed small geminal coupling of H-2 that is similar to values for substituted cyclopropanes. *J* values of 5.6 and 3.2 Hz for *cis* and *trans* couplings respectively were in agreement to those reported in literature.[32]

The coupling constant between H-6 and H-7 was found to be 2.4 Hz, typical of long-range coupling. However, no geminal coupling was observed between H-6's. The signal for H-3 appeared as a singlet at 5.3 ppm.

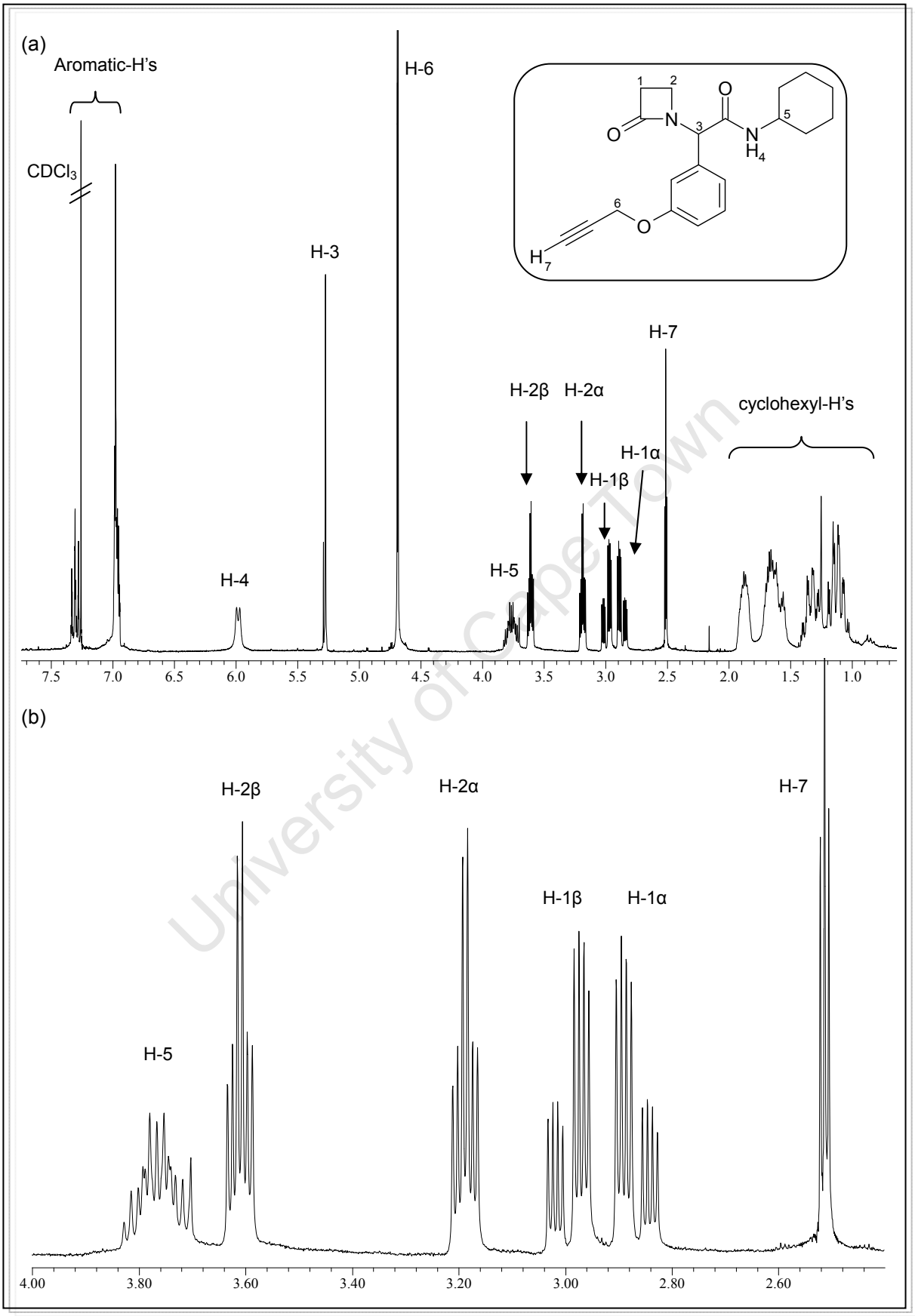
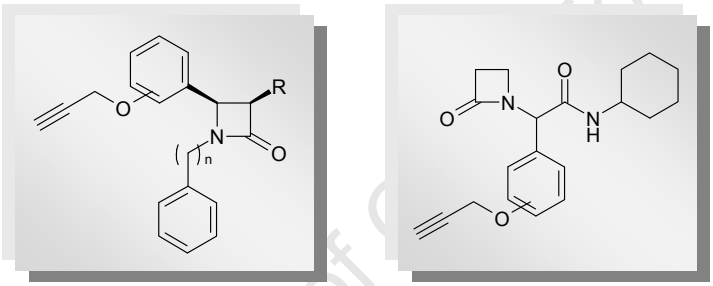


Figure 5.7. ^{1}H NMR spectrum of **BL11**. (a) full spectrum; (b) 2.4 – 4.0 pm region expansion.

5.7.3 Biological Activities of the Terminal Acetylene-Containing β-Lactams

Preliminary studies on *in vitro* antiplasmodial activity and cytotoxity were conducted to determine the compound with the lowest IC_{50} value out of these β-lactams for further modifications. It was found that **BL11** possessed the most potent activity against the chloroquine-sensitive 3D7 strain of *P. falciparum*, using a standard parasite viability assay that measures parasite luciferase activity after 60 hours of drug exposure (Table 5.2).

Table 5.2. Antiplasmodial activities of the terminal acetylene-containing β-lactams.



BL01 – BL09 **BL10 – BL11**

General structure of artemisinin conjugates

Compound	Position of *O*-propargyl group	R	n	*P. Falciparum* 3D7 IC_{50} (μM)	HeLa IC_{50} (μM)
BL01	*ortho*	OAc	1	100	> 50
BL02	*ortho*	OH	1	92	47.3
BL03	*meta*	OAc	1	100	> 50
BL04	*para*	OAc	1	55	> 50
BL05	*ortho*	OAc	0	27	> 50
BL06	*ortho*	OH	0	22	> 50
BL09	-	OCH2C≡CH	1	50	> 50
BL10	*ortho*	-	-	24	> 50
BL11	*meta*	-	-	3	> 50
BL12	*para*	-	-	47	> 50

The position of the *O*-propargyl group on the benzene ring did not seem to have an effect on the *in vitro* antiplasmodial activities against the 3D7 strain (compare **BL01**, **BL03** and **BL04**; **BL10**, **BL11** and **BL12**). For the Staudinger β-lactams, compounds bearing a free hydroxyl group (**BL02** and **BL06**) displayed slightly better activities when compared to the parent acetate (**BL01** and **BL05**).

Compound **BL05** displayed antiplasmodial activity almost 4 times higher than **BL01** (27 and 100 μM, respectively). The only difference between the two compounds is that **BL05** contains an *N*-aniline moiety, while **BL01** contains a *N*-benzyl moiety. This may suggest that the length/size of the group attached to the β-lactam nitrogen is important, however, more analogues with different *N*-substituents will need to be synthesized to investigate this effect.

5.8 Rationale for Post-Condensation Modifications – Series II

As described in section 5.6, the best compound from the first series of the β-lactam (**BL11**) was to be subjected to further post-condensation modifications via the Mannich reaction.

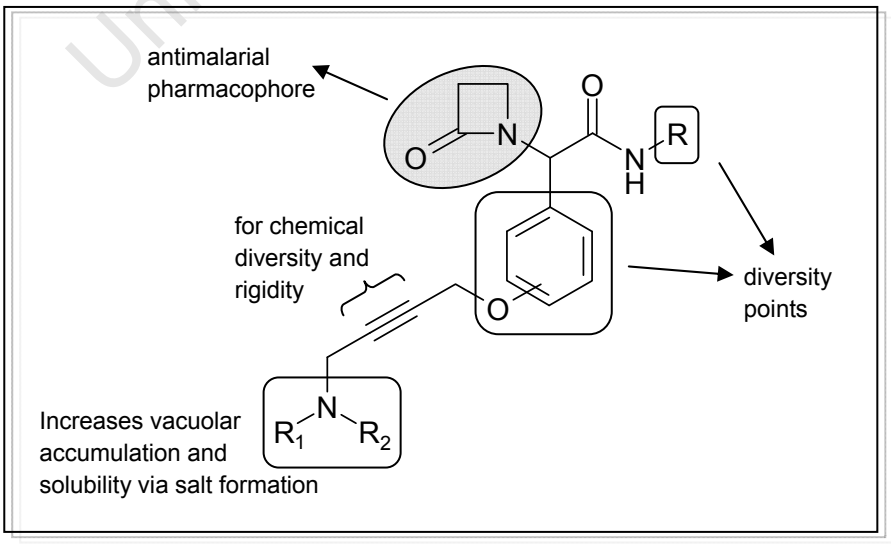


Figure 5.8. Rationale for design of target Mannich bases.

The acetylene moiety of the Mannich bases adds rigidity to the molecule, and is often present in natural products that possess interesting biological properties such as antibacterial,[49,50] antifungal,[49,51] pesticidal[52,53] and antitumour[53,54] activities. The molecular rigidity around the acetylene moiety is advantageous, as this may increase bioavailability by locking out access to clearance enzymes and reducing the number of rotatable bonds while retaining the inhibitory potency of the molecule.[55,56]

Chemical diversity can be introduced at the amino or the alkyne site, which would be useful for structure-activity studies.[57] The presence of a protonatable nitrogen would be beneficial to vacuolar accumulation, as discussed in previous chapters. Additionally, this protonatable nitrogen also provides a site for salt formation, which would aid in aqueous solubility. Commercially available amines were used in this investigation.

The R group adjacent to the amide bond can be diversified by utilizing various isocyanide components, and commercially available isocyanides were employed. The phenoxy moiety could also be replaced by groups such as biaryl or alkyl groups for structure-activity relationship studies, but is not investigated here.

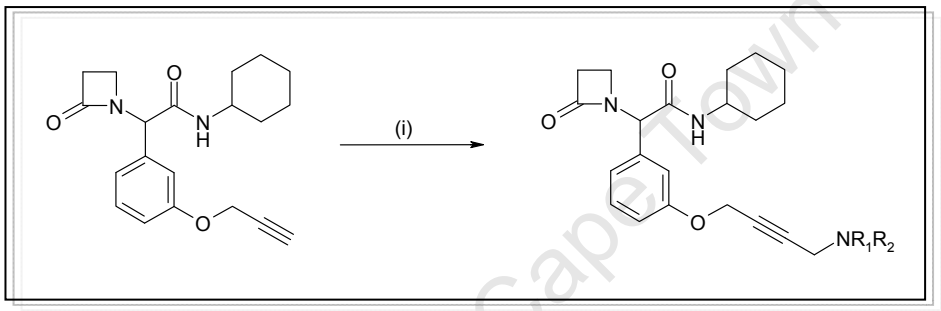
5.9 Synthesis of β-Lactam Mannich Bases – Series II

Mannich bases were synthesized according to the protocol described by Bieber and da Silva,[58] using 0.02 equivalents of copper iodide as the catalyst. Copper salts were frequently used in the Mannich reaction, as they have been found to increase the nucleophilicity of the acetylene.[42,59] Compared with other reported protocols for the Mannich reaction, which is often associated with harsh reaction conditions, the one described by Bieber and da Silva was

milder and the reaction could be conducted at room temperature and without the use of dry solvents.

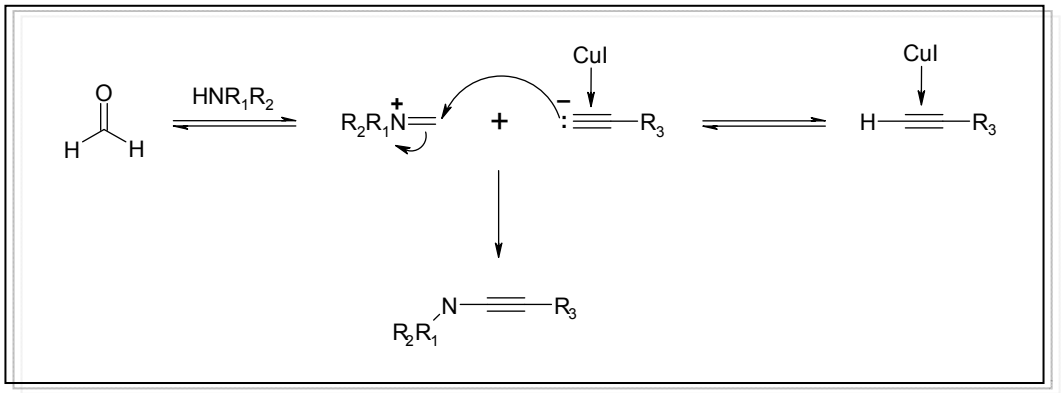
5.9.1 Altering the Amine Moiety of the β-Lactam Mannich Base

Compound **BL11** was reacted with various commercially available amines to yield various β-lactam Mannich bases (Scheme 5.14).



Scheme 5.14. Reagents and conditions: (i) aq. formaldehyde (5.0 eq), amines (1.2 eq), CuI (0.02 eq), DMSO, r.t., 3 h.

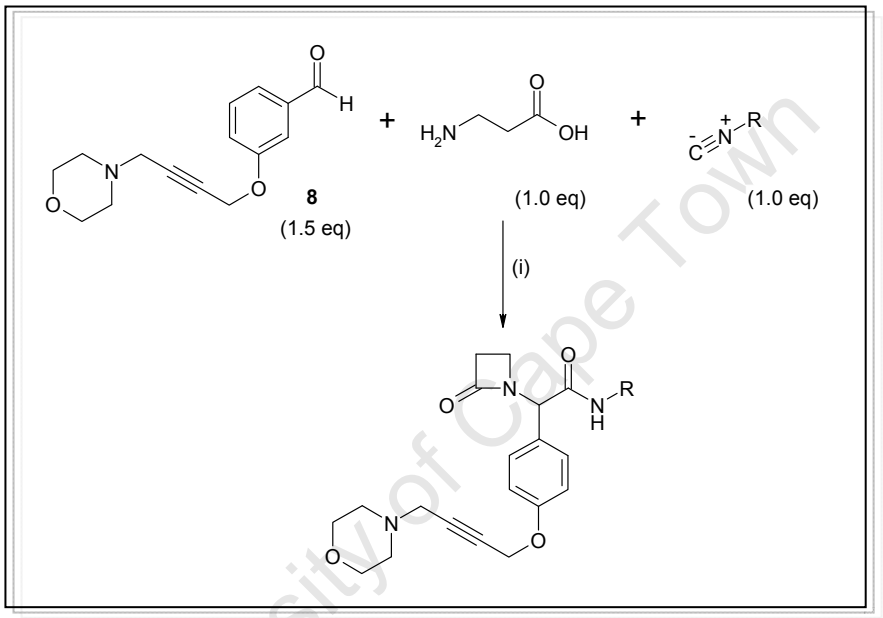
The mechanism of the Mannich reaction has been well investigated.[59] The iminium ion that is formed from the secondary amine and formaldehyde undergoes attack by the Cu(I)-complexed acetylenic anion to give the acetylenic Mannich base (Scheme 5.15).



Scheme 5.15. Reaction mechanism of the Mannich reaction.

5.9.2 Altering the Isocyanide Moiety of the β-Lactam Mannich Base

To create a more diversified library of β-lactam Mannich bases, a Mannich base-containing benzaldehyde **8** was synthesized and reacted with β-alanine and various isocyanides using the Ugi 4-C 3CC reaction as described in previous sections.



Scheme 5.16 Synthesis of β-lactam Mannich bases utilizing various isocyanides.

5.9.3 Spectroscopic Analyses

Using the ^{1}H NMR of **BL17** as an example (Fig. 5.9), the deciding evidence of successful synthesis of the Mannich base was the signal of H-6, which changed from a doublet into a triplet; the signal of the acetylenic proton disappeared, and was replaced by a new signal (H-7) that was found at 3.33 ppm. The presence of morpholine protons (H-8 and H-9) also confirmed the presence of the Mannich base.

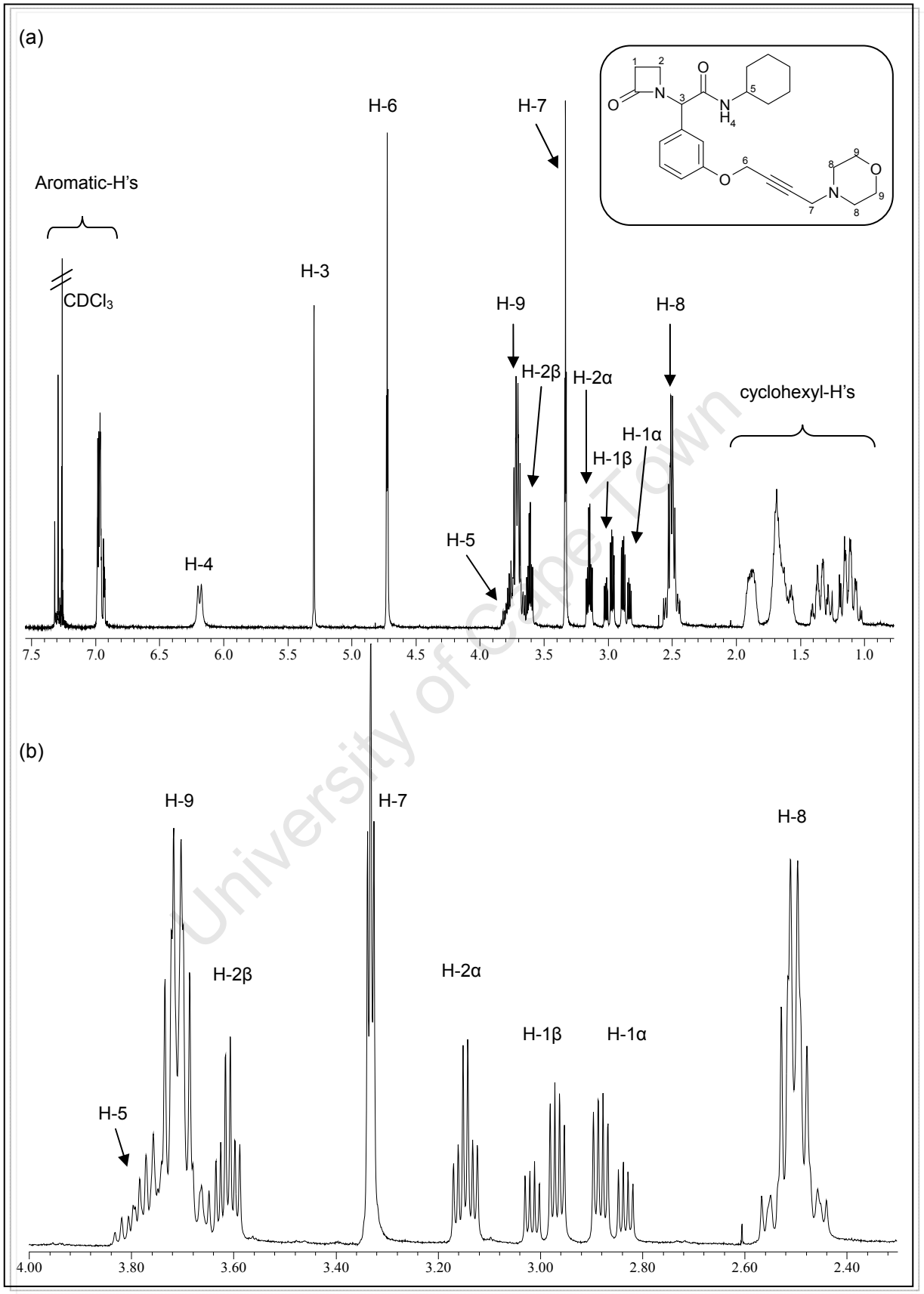
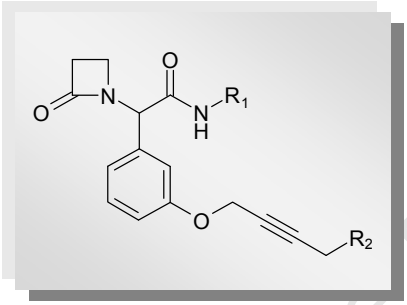


Figure 5.9. ^{1}H NMR spectrum of **ME** (a) full spectrum; (b) 2.3 – 4.0 pm region expansion.

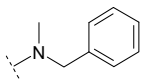
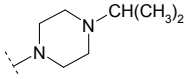
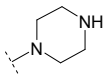
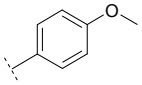
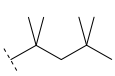
5.9.4 Products Synthesized

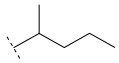
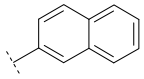
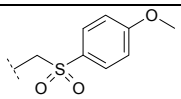
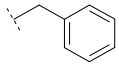
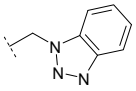
Structure and yields of the target β-lactam Mannich bases are presented in Table 5.3. Yields of the Mannich bases were generally very good.

Table 5.3. Structure and yields for the β-lactam Mannich bases.



General structure of β-lactam Mannich bases

Compound	R_1	R_2	Yield (%)
BL13			72
BL14			89
BL15			80
BL16			73
BL17			77
BL18			86
BL19			65
BL20			97
BL21			62

Compound	R_1	R_2	Yield (%)
BL22			91
BL23			82
BL24			69
BL25			99
BL26			60

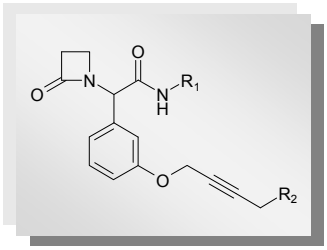
5.10 Results and Discussion

5.10.1 Antimalarial Activities

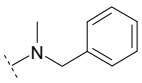
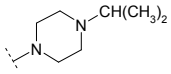
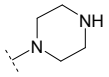
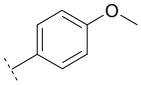
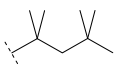
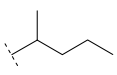
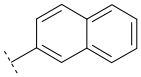
Antiplasmodial activities of the Mannich bases and their respective cytotoxicities are presented in Table 5.4.

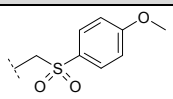
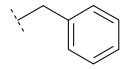
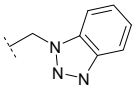
The antiplasmodial activities of the β-lactam Mannich bases were mostly in the low to mid micromolar range, either against the chloroquine-sensitive D10 or chloroquine–resistant Dd2 strain. Although *P. falciparum* parasites are unlikely to develop resistance against these compounds, as IC_{50} values against the Dd2 strain were either similar or lower than those against the D10 strain (indicated by the resistance index, with values not greater than 1.4), more modifications to these compounds would be needed to enhance their antiplasmodial activities.

Table 5.4. Antiplasmodial activities and cytotoxicity data for the β-lactam Mannich bases.



General structure of β-lactam Mannich bases

Cmpd	R_1	R_2	*P. falciparum* IC_{50} (uM)		HeLa IC_{50} (μM)	RI[a]	TI[b]
			D10	Dd2			
CQ	-	-	0.026	0.156	9.53	6.0	61
8	-	-	194.32	204.86	-	1.1	-
BL11			12.39	11.27	> 50	0.9	>4.4
BL13			15.68	13.42	44.9	0.9	3.3
BL14			39.50	14.92	> 50	0.4	>3.3
BL15			65.37	10.58	> 50	0.2	>10.7
BL16			26.17	17.66	> 50	0.7	>2.83
BL17			81.22	63.24	> 50	0.8	>0.79
BL18			28.07	10.66	> 50	0.4	>4.7
BL19			28.16	19.76	> 50	0.7	>2.5
BL20			82.35	96.98	> 50	1.2	>0.5
BL21			56.77	46.9	> 50	0.8	>1.7
BL22			103.61	71.64	> 50	0.7	>0.7
BL23			38.44	31.66	39.8	0.8	1.3

Cmpd	R_1	R_2	*P. falciparum* IC_{50} (uM)		HeLa IC_{50} (μM)	RI[a]	TI[b]
			D10	Dd2			
BL24			28.47	40.34	37.9	1.4	0.9
BL25			73.31	57.70	> 50	0.8	>0.9
BL26			15.06	13.74	> 50	0.9	>3.7

[a] Resistance index calculated as [IC_{50} (Dd2)] / [IC_{50} (D10)];

[b] Therapeutic index calculated as [IC_{50} (HeLa)] / [IC_{50} (Dd2)

It is worth noting that by converting compound **BL11** into various Mannich bases (**BL13** to **19**), antiplasmodial activities were not enhanced. The activities of most of the Mannich bases, except for compound **BL13**, were at least two times lower than the parent compound against the D10 strain. However, for activities against the Dd2 strain, most compounds displayed activities similar to the parent **BL11** (except for compound **BL17**, **BL20** and **BL22**), with compounds **BL15** and **BL18** (IC_{50}'s of 10.58 and 10.66 μM respectively) displaying the most potent activities.

By altering the isocyanide component (R_1) on the Ugi backbone of the β-lactam, antiplasmodial activities was enhanced (compare **BL20** – **BL26** with compound **BL17**) except for compounds **BL20**, **BL21** and **BL25**. Compounds **BL20**, **BL21** and **BL25** contained *N*-methoxyphenyl, *N*-1-methylbutyl and *N*-benzyl groups respectively, and it is possible that the activities of these compounds were lower as they contained a less bulky R_1 group when compared to the other Mannich bases. Compound **BL26**, which contained a benzotriazole moiety, has the best activity out of this series (15.06 and 13.74 μM against D10 and Dd2 strains respectively). Its enhanced activities may be attributed to the presence of the triazole moiety, but more studies would be required to confirm this hypothesis.

5.10.2 Cytotoxicity Studies

Cytotoxic activities of these compounds were studied on HeLa cells, using chloroquine as the control. These compounds did not display cytotoxicity against mammalian cells, as their IC_{50} values were mostly above 50 μM, irrespective of their antiplasmodial activities. These β-lactam Mannich bases could become good antimalarial drug candidates if their antiplasmodial activities could be enhanced without compromising the low cytotoxicity (i.e. increase the therapeutic index for these compounds) and/or lower the selective index.

5.11 Conclusion and Future Work

A series of β-lactams containing terminal acetylenes were synthesized via the Staudinger and Ugi 4-component 3-centre reactions, and tested against the chloroquine-sensitive 3D7 strain of *P. falciparum*. The compound with the best activity (**BL11**) was further modified by the Mannich reaction, and compounds **BL13** and **BL26** displayed the most potent activities against the D10 strain (15.68 and 15.06 μM respectively), while compounds **BL15** and **BL18** were the most active against the Dd2 strain (10.58 and 10.66 μM respectively). None of the β-lactams displayed cytotoxity against HeLa cells at 35μM.

The activities of the β-lactams that contains the terminal acetylene moiety were higher than those containing the Mannich base, while β-lactams with a bulky *N*-substituent on the Ugi backbone also displayed enhanced activities. This may suggest that the terminal acetylene moiety is important for activity. More diversified analogues would need to be prepared to fully investigate the structure-activity relationships.

5.12 References

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3. Fleming, A., **1945**, Nobel lecture, December 11.
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CHAPTER 6

SYNTHESIS AND BIOLOGICAL EVALUATION OF 4-AMINOQUINOLINE ANALOGUES

This chapter describes the synthesis, characterization and biological evaluation of 4-aminoquinoline analogues. These compounds were designed and synthesized to investigate whether their antiplasmodial activities could be enhanced, in comparison to chloroquine, and circumvent the development of cross-resistance in the malaria parasite.

6.1 4-Aminoquinoline Antimalarials

There has been a long history for the use of 4-aminoquinolines in the treatment of malaria. The most well-known antimalarials in this class are chloroquine and amodiaquine, which are both believed to act by inhibiting the formation of haemozoin in the food vacuole of the *Plasmodium* parasite.[1] The mode of action and the mechanism of resistance of chloroquine have been described in section 2.1.

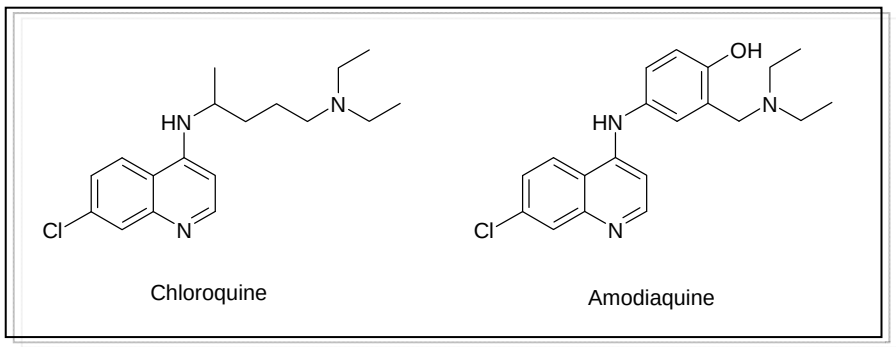


Figure 6.1. Structures of chloroquine and amodiaquine

6.1.1 Amodiaquine

Amodiaquine is a Mannich base derivative that was introduced in the late 1950s. It was developed during World War II by the US Army-sponsored program to develop alternatives to quinine.[2] It has been shown to be a superior alternative to chloroquine in areas of high chloroquine resistance.[3,4] In the mid-1980s, reports of agranulocytosis (an acute condition involving a severe and dangerous leucopenia – a drop in the number of white blood cells in the blood) and hepatitis on the use of amodiaquine in prophylaxis caused a rapid decline in the use of the drug.[5] However, detailed investigations have shown that when used to treat uncomplicated *P. falciparum* malaria, amodiaquine was no more toxic than chloroquine.[3,6] Results collated from 40 different clinical trials held in the late 1980s have suggested that adverse drug reactions to amodiaquine are likely to only occur during prophylaxis.[6] To date, there is no evidence for serious toxicity associated with amodiaquine therapy.[7]

6.1.1.1 Amodiaquine Resistance

Very little is known about the mechanism or epidemiology of amodiaquine resistance. A correlation between resistance to chloroquine and amodiaquine has been found from *in vitro* studies; however, chloroquine-resistant strains appeared to have lower levels of resistance to amodiaquine.[8] Studies have shown that amodiaquine is effective against chloroquine-resistant strains of malaria *in vivo*.[9] However, it has also been observed that amodiaquine resistance does occur in areas where it was regularly used.[10] Although some clinical evidence of amodiaquine sensitivity has been documented, cross-resistance between chloroquine and amodiaquine has been reported from laboratory analysis, which, in some settings, have transformed into clinical resistance.[11]

6.1.1.2 Amodiaquine in Combination Therapy

Artemisinin combination therapy (ACT), as described in section 2.2.3, has been recommended by the WHO as the first-line antimalarial treatment for *P. falciparum* malaria.[12] Although ACTs may have been extremely effective in Southeast Asia,[13] Meshnick and Alker believe that they may not be appropriate for sub-Saharan Africa.[14] There are two reasons for this: (i) as artemisinins have short half-lives, when they are co-administered with a long half-life drug, patients may have sub-therapeutic concentrations of the latter drug alone in their plasma for days or weeks. This would not be a problem in Southeast Asia, where the risk of new infection is rare. However, people in sub-Saharan Africa may be subjected to infectious mosquito bites on a daily basis. This would create an ideal scenario for the development of resistance, when parasites could be exposed to sub-therapeutic concentrations of single antimalarial agents; (ii) it has been found that artesunate causes fetal death and malformations in pregnant rodents.[15] In consideration of the fact that the average woman in sub-Saharan Africa gives birth 5.7 times,[16] women between the age of 15 and 45 may be ineligible for this treatment.

Zongo *et al* have suggested the use of amodiaquine in combination with other antimalarials such as sulfadoxine-pyrimethamine (SP).[17] This would be advantageous over artemisinins as the principal active metabolite of amodiaquine has a longer half-life of 9 to 18 days.[18] Thus, if amodiaquine was to be co-administered with another long half-life drug, there would be little chance that the parasite would be exposed to subcurative concentrations of a single antimalarial agent. The combination of amodiaquine with sulfadoxine-pyrimethamine has been evaluated by Zongo *et al* in a randomized placebo-controlled clinical trial; they have found that the combination performed better than SP alone. Various studies by this

group and by others have also shown an increased efficacy for this combination.[19]

6.1.2 Bis-, Tris- and Tetraquinolines

In the *P. falciparum* parasites, the emergence of resistance against chloroquine has eroded the efficacy of chloroquine and related drugs. Mechanisms of chloroquine resistance in the parasite has been discussed in section 2.1.2, which include the enhanced efflux of chloroquine from the parasite via the P-glycoprotein homologue 1 (Pgh 1)[20] or the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT)[21] protein.

Various efforts have been put into overcoming chloroquine-resistance, such as modifications to the side-chain of chloroquine or related 4-aminoquinolines,[22] the use of resistance reversal agents such as verapamil, desipramine and chlorpromazine,[20b, 23] tethering of 4-aminoquinolines to other pharmacophores such as chimeras of trioxane-aminoquinoline ('trioxaquines')[24] and tetraoxane-aminoquinolines[25]or the hybridization of artemisinin and quinine.[26]

Bisquinolines have been designed such that owing to their bulkiness, they may potentially overcome drug-resistance by escaping the recognition and subsequent extrusion by the proteins involved in drug efflux.[27] Bisquinolines have been synthesized, and found to inhibit the growth of both chloroquine-sensitive and chloroquine-resistant parasites with similar efficacy.[27,28] Bis-, tris- and tetraquinolines have been designed and synthesized by Girault *et al*,[29] and evaluations have shown that some of the bis- and tetraquinolines displayed potent antiplasmodial activities and at the same time noncytotoxic.

6.2 Rationale for Drug Design

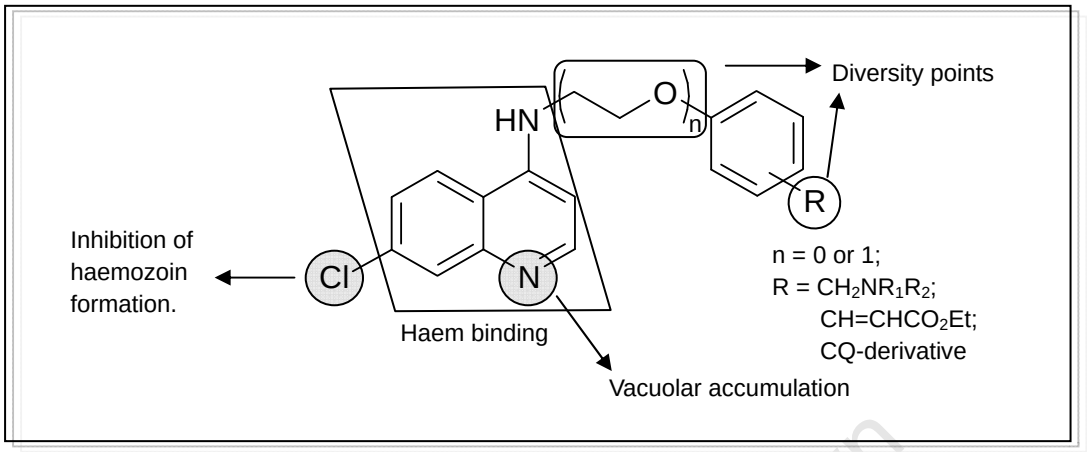


Figure 6.2. Rationale for the design of target compounds.

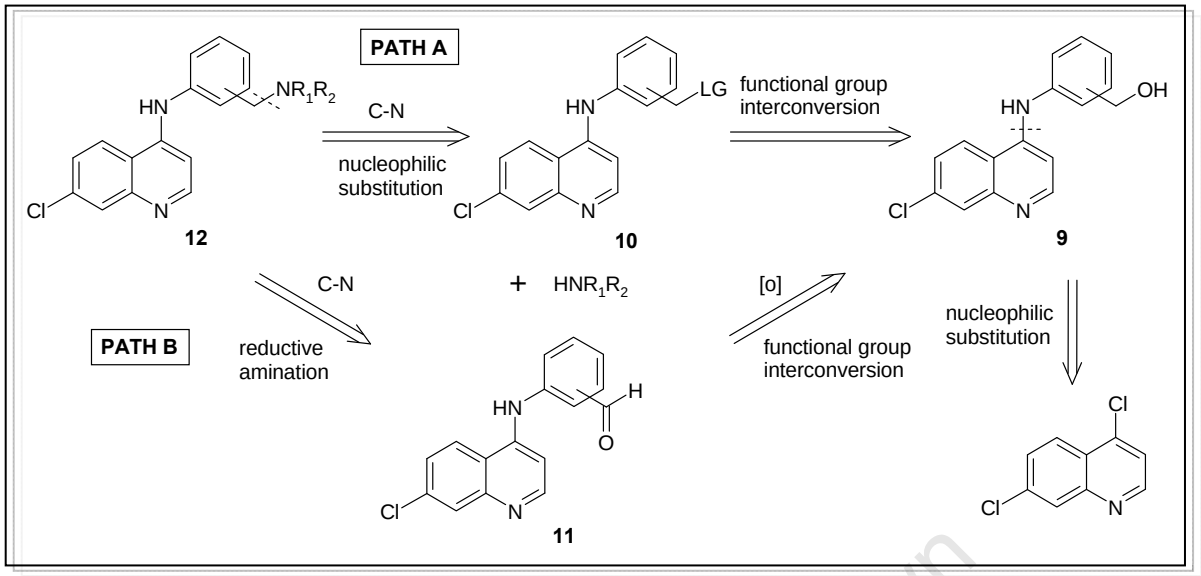
The rationale for the target compounds is summarized in Fig 6.2 above. These compounds could be either amodiaquine-like, or contain an α,β-unsaturated system. Aminoquinoline derivatives were also tethered to form a bisquinoline compound.

The general activity of the quinoline ring has been described in Chapter 3.5, and will not be repeated here.

6.3 Synthesis of Target Compounds

6.3.1 Retrosynthesis Analysis

Retrosynthesis was carried out for the amodiaquine-like compounds, and the retrosynthetic scheme is presented in Scheme 6.1.



Scheme 6.1. Retrosynthesis analysis of amodiaquine-like compounds.

The target compounds could be synthesized in two ways; Path A: starting from 4,7-dichloroquinoline, and undergoing a nucleophilic substitution by the aminobenzyl alcohol to form the intermediate **9**. The hydroxyl moiety of **9** could then be converted into a leaving group (intermediate **10**), and displaced by the amine to form the target compound **12**.

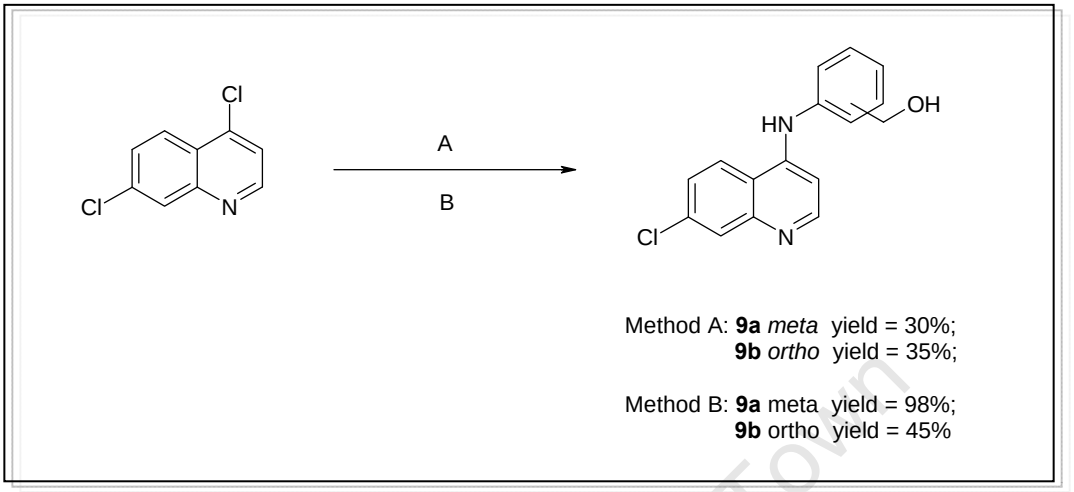
Path B: after the synthesis of the benzyl alcohol intermediate **9**, it could then be oxidized to form the aldehyde **11**, which, upon reductive amination of the aldehyde moiety, would yield the desired amodiaquine-like target compound **12**.

6.3.2 Synthesis of 4-Aminoquinoline Analogues

6.3.2.1 Amodiaquine-like Compounds

The synthesis of the benzyl alcohols **9a** and **9b** were explored using two methods (Scheme 6.2). The first method involved refluxing the starting materials and the bases in NMP under

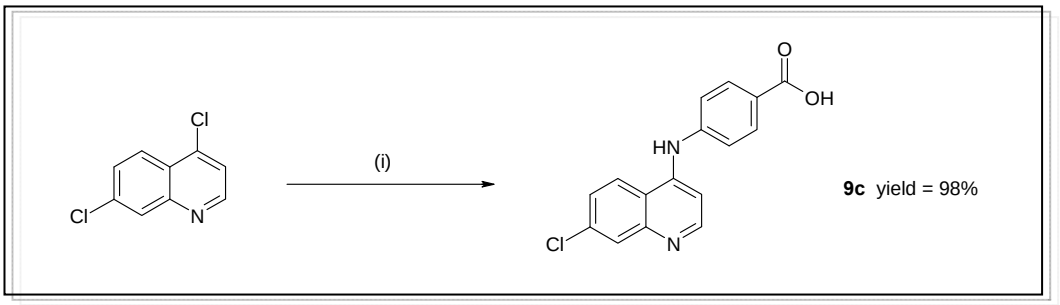
N_2 for 8 hours;[22b] the second method was simply refluxing the two starting materials in ethanol for 2 hours.[30]



Scheme 6.2. Reagents and conditions: (i) aminobenzyl alcohol (1.0 eq), Et_3N (8.0 eq), K_2CO_3 (1.5 eq), NMP, N_2, reflux, 18 h. (ii) aminobenzyl alcohol (1.0 eq), EtOH, reflux, 3 h.

After exploring both methods, the ethanol method was best suited for the synthesis of **9a** and **9b**, as better yields were achieved. In the case of **9a**, the pure product simply precipitated out from the reaction mixture. Evidence of the correct products was obtained from ^{1}H NMR, as the singlet at 4.5 ppm, integrating for two protons, confirmed the presence of benzylic protons in compounds **9a** and **9b**.

Acid **9c** was prepared similarly as described above (Scheme 6.).

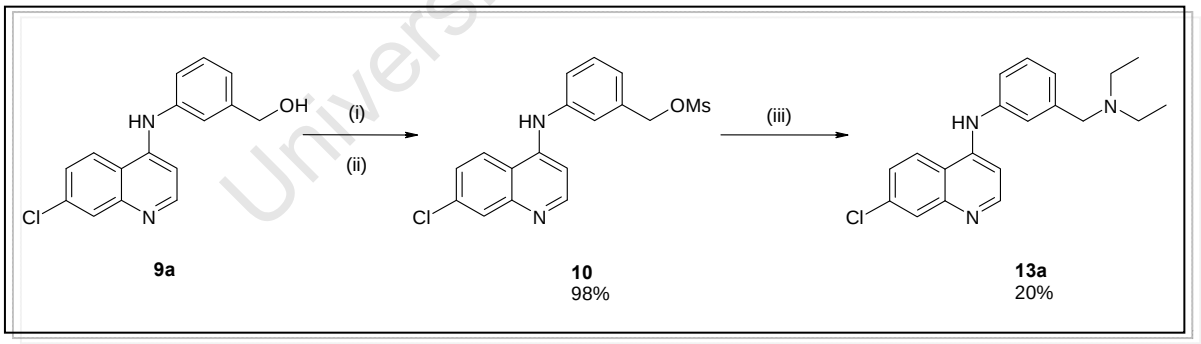


Scheme 6.3. Reagents and conditions: (i) aminobenzoic acid (1.0 eq), EtOH, reflux, 3 h.

Following the synthesis of the benzyl alcohols **9a** and **9b**, the target compound could be synthesized via pathway A or B, as described in section 6.3.1.

Path A

Path A entails the activation of the hydroxyl group, and its subsequent displacement by the amine to form the target compound. The reaction scheme is outlined in Scheme 6.4. Intermediate **9a** was converted into the mesylate **10** using methanesulfonyl chloride in the presence of pyridine. Mesylation is typically conducted in DCM/Et_3N,[31] but for the reaction with compound **9a**, pyridine was used as both the base and the solvent as 4-aminoquinoline compounds are generally poorly soluble in solvents such as DCM and THF. The mesylate intermediate **10** was then reacted with diethylamine, where the mesylate moiety was nucleophilically displaced by the amine to form compound **13a**.[32] The yield of this last step was unsatisfactory, and path B was investigated.

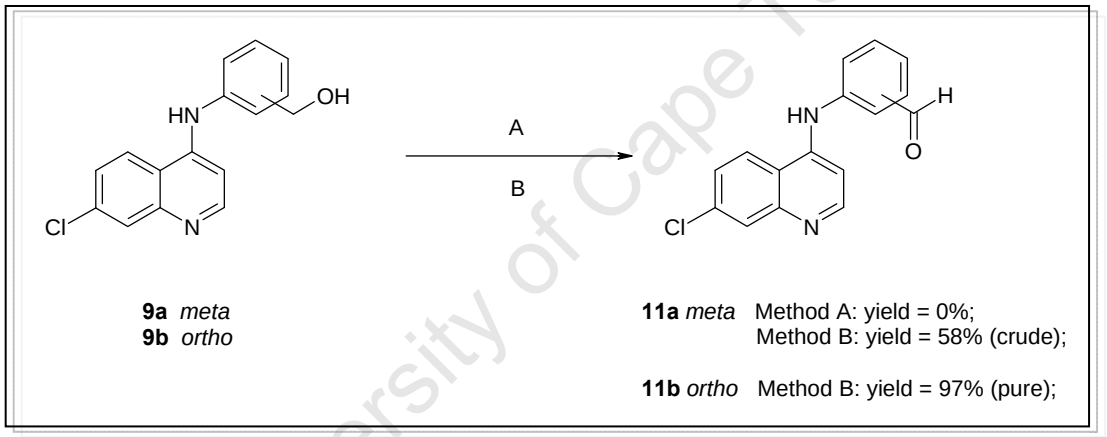


9a **10** 98% **13a** 20%

Scheme 6.4. Reagents and conditions: (i) pyridine, 0 °C, 30 min (ii) methanesulfonyl chloride (2.5 eq), 0 °C, 5 h. (iii) Et_2NH (1.1 eq), DEA (2.0 eq), acetonitrile, 50 °C, 22 h.

Path B

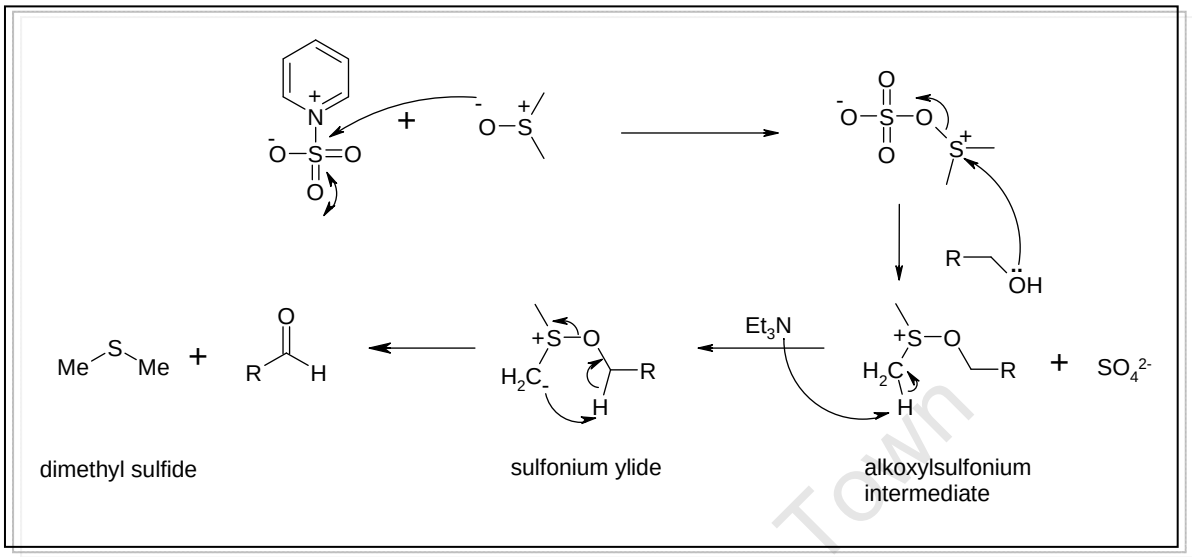
To prepare the aldehyde for reductive amination, oxidation of benzyl alcohol **9a** was initially carried out using pyridinium dichromate (PDC).[33] During the monitoring of the reaction using TLC, many spots were observed, indicating that PDC may have been too strong an oxidizing agent for the starting alcohol, and oxidized not only the benzyl alcohol, but also the quinoline nitrogen. Hence a milder oxidizing agent, sulfur trioxide/pyridine complex ($SO_3 \cdot Pyr$), was chosen (Scheme 6.5).[34] The disadvantage of this method was that the oxidation required a few days to go to completion.



Scheme 6.5. Reagents and conditions: (A) PDC (1.2 eq), DMF/THF (1:4), r.t., 4 h. (B) $SO_3 \cdot Pyr$ (2.0 eq), Et_3N (4.0 eq), DMSO, r.t., 2 days.

After stirring for 2 days, the oxidation went to completion for **11b**, but not for **11a**. ^1H NMR showed that the crude material was a mixture of the starting benzyl alcohol **9a** and the desired aldehyde **11a**, in a ratio of 42 : 58 (**9a** : **11a**). As the product was too polar, and could only be dissolved in large amounts of DMSO, chromatography was not practical. Hence the crude material was used for subsequent reactions. On the other hand, the oxidation worked well for **9b**; after washing the crude material with DCM, the crystals collected were pure and contained no traces of the starting material.

The mechanism of the oxidation is illustrated in Scheme 6.6 below.

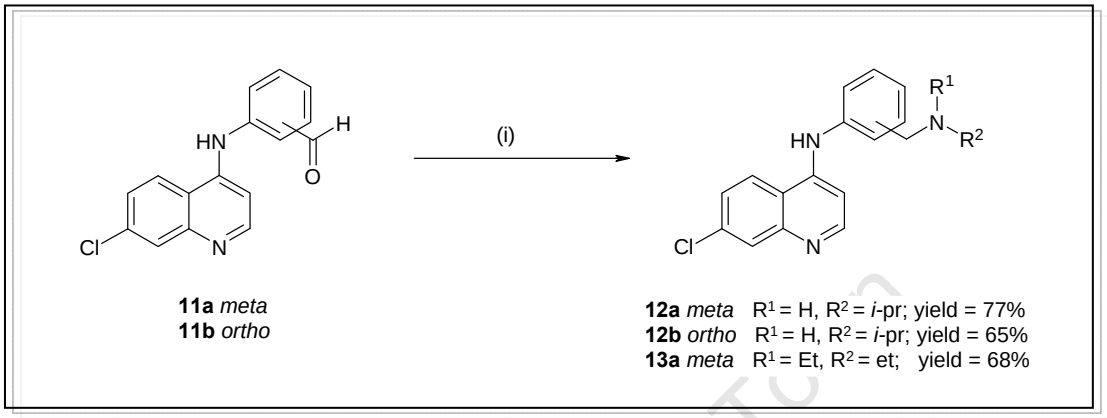


Scheme 6.6. Reaction mechanism for the oxidation using sulfutrioxide/pyridine.

DMSO is activated by the sulfur trioxide/pyridine complex, which, in the presence of triethylamine, converts primary and secondary alcohols to aldehydes and ketones at ambient temperature.[34] The alkoxylsulfonium intermediate that is formed here is the same as that formed in the Swern oxidation,[35] and, after oxidation, produces dimethyl sulfide as a side product, the causative agent in the well-known unpleasant odour that accompanies these oxidations. The advantage of utilizing the sulfur trioxide/pyridine complex rather than the more popular Swern oxidation is that the reaction could proceed at an ambient temperature, rather than at -60 °C for the Swern oxidation.

From the spectroscopic point of view, the disappearance of the benzylic protons at 4.5 ppm in ^{1}H NMR, and the appearance of a new signal at 10 ppm, corresponding to the aldehyde proton, both indicated that the benzyl alcohol **9b** has been successfully converted to the aldehyde **11b**.

The aldehydes **11a** and **11b** were then converted into various amines by reductive amination (Scheme 6.7), where sodium borohydride[36] was used as the reducing agent for primary amines. In the case of secondary amines, sodium cyanoborohydride[37] was used instead.



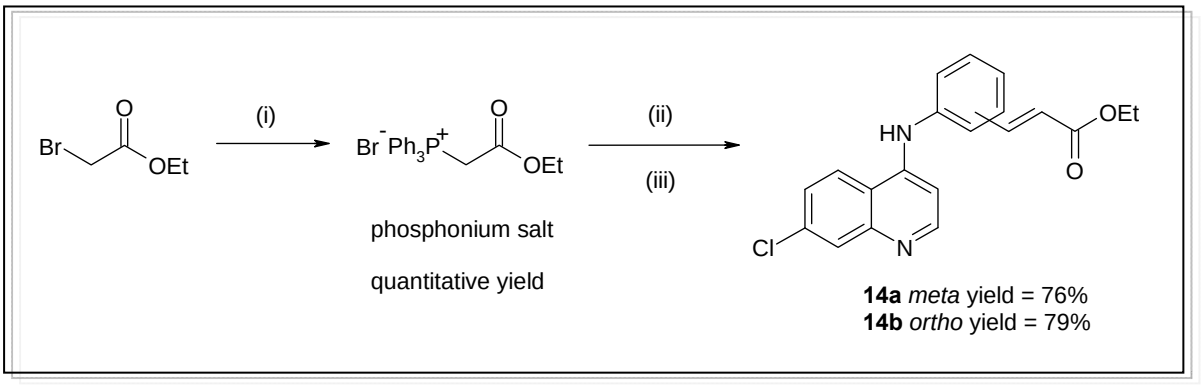
Scheme 6.7. Reagents and conditions: (i) amine (1.1 eq), $NaBH_4$/$NaCNBH_3$ (2.0 eq), r.t., 16 h.

The aldehydes were dissolved in a mixture of DMF and MeOH, as they were sparingly soluble in MeOH. As with all reductive aminations, the aldehyde and the amine were reacted first to form an imine (or iminium ions for secondary amines), which was then reduced to the amine by the borohydride. The yields were generally good.

Spectroscopic evidence from ^{1}H NMR was the reappearance of the benzylic protons at 3.8 ppm, and the disappearance of the aldehyde proton that was at 10 ppm.

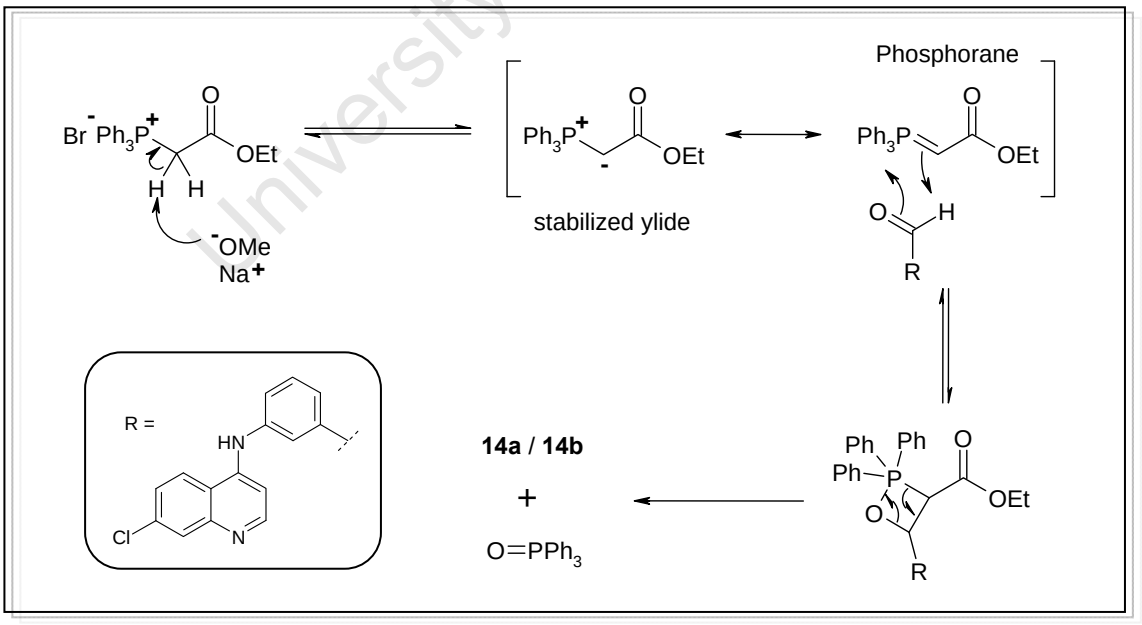
6.3.2.2 Wittig Products

Apart from reductive amination, the aldehydes **11a** and **11b** were also utilized in the Wittig reaction (Scheme 6.8) to create the α,β-unsaturated system.[38]



Scheme 6.8. Reagents and conditions: (i) PPh_3 (1.2 eq), toluene, N_2, r.t., 8 h. (ii) NaOMe (1.2 eq), MeOH, N_2, 0 °C, 20 min. (iii) **11a**/**11b** (0.9 eq), N_2, r.t., 18 h.

Ethyl bromoacetate and triphenylphosphine were stirred in toluene to form the phosphonium salt. The phosphorane was generated *in situ* using NaOMe as the base,[38c] followed by the addition of the aldehyde to afford the Wittig products **14a** and **14b**. The *E* isomer was the major product, as the phosphonium ylide that was generated was stabilized, and favoured the formation of the *E* isomer.



Scheme 6.9. Reaction mechanism for the Wittig reaction.

Mechanistically, the abstraction of the acidic α-proton by sodium methoxide generated the ylide/phosphorane. The phosphorane and the aldehyde form a 4-membered cyclic intermediate, which, after rearrangement and elimination of the triphenylphosphine oxide, yielded the Wittig product **14a** and **14b** (Scheme 6.9).

Using compound **14a** as an example, the key ^{1}H NMR signals for this Wittig product (Fig. 6.3) were the alkene protons from the α,β-unsaturated system, appearing as two doublets with a coupling constant of 15.6 Hz, situated at 6.4 and 7.9 ppm. Additionally, the presence of signals corresponding to the ethyl protons H-15 and H-16 at 4.2 and 1.3 ppm, respectively, also served as confirmation that the Wittig product was successfully synthesized.

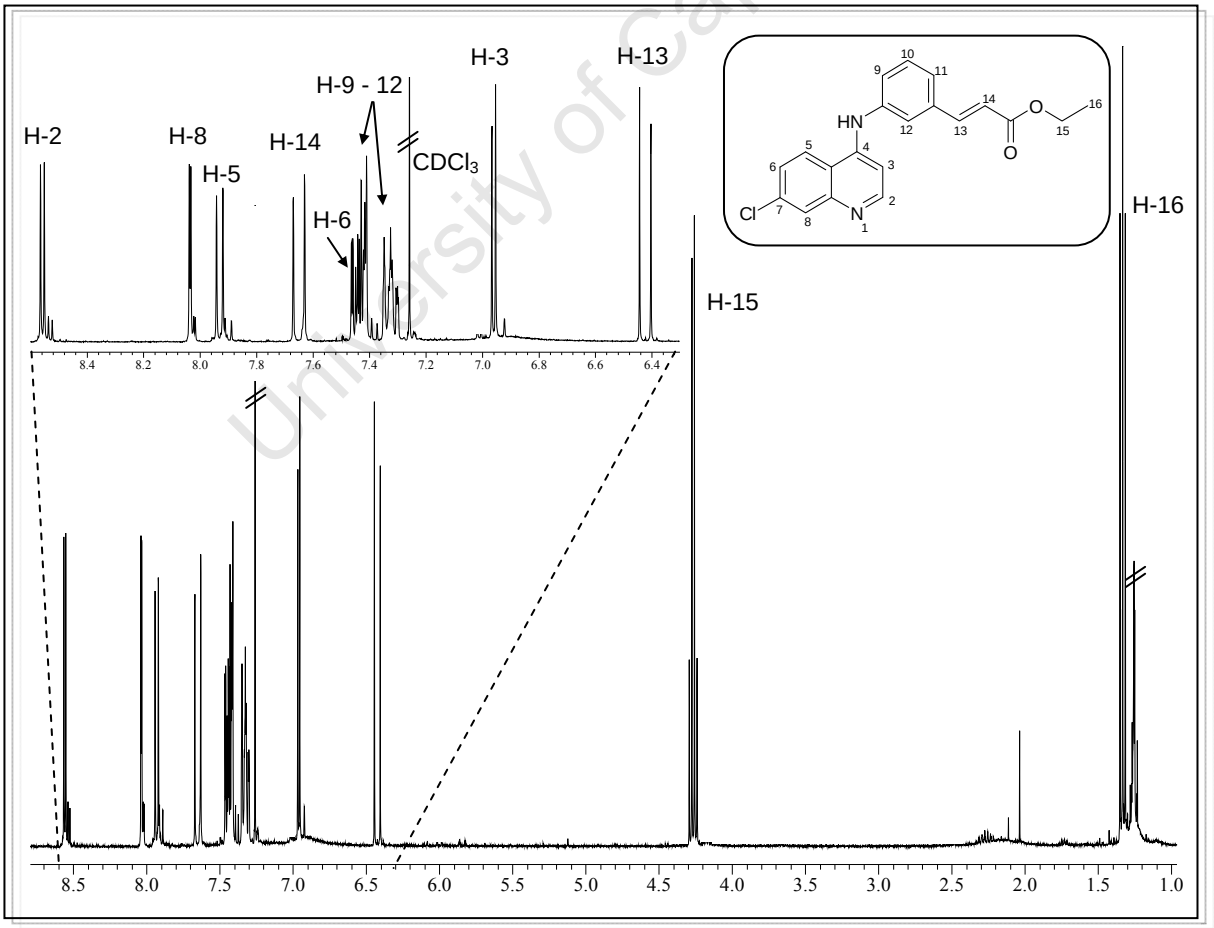
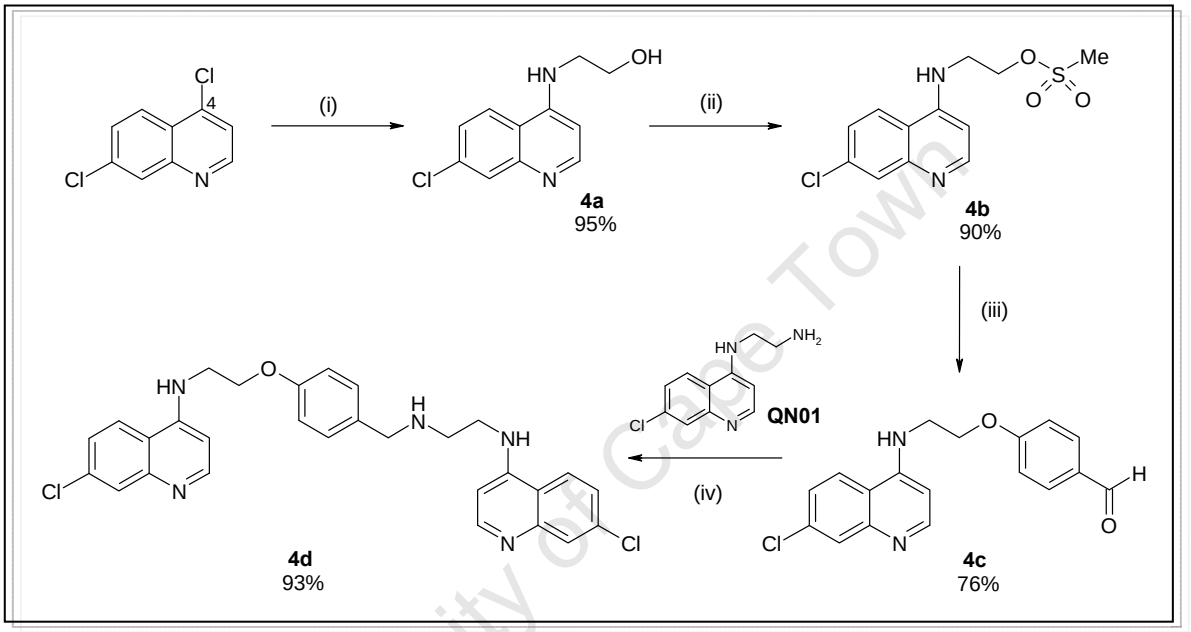


Figure 6.3. ^{1}H NMR of the Wittig product **14a**.

6.3.3.3 Bisquinoline Derivative

Two aminoquinoline-containing compounds were tethered onto a single compound to investigate whether amtiplasmodial activity could be enhanced. To achieve this, reductive amination of aldehyde **4c** by amine **QN01** was carried out (Scheme 6.10)



Scheme 6.10. Reagents and conditions: (i) 1,2-ethanolamine (20 eq), Et_3N (0.3 eq), K_2CO_3 (0.5 eq), 140 °C, 4 h. (ii) methanesulfonyl chloride (2.5 eq), pyridine, 0 °C, 5 h. (iii) 4-hydroxybenzaldehyde (1.5 eq), K_2CO_3 (1.6 eq), DMF, reflux, 5 h. (iv) **QN01** (1.0 eq), $NaBH_4$ (2.0 eq), MeOH, N_2, r.t., 18 h.

The aminoquinoline derivative **4a** was prepared by refluxing 4,7-dichloroquinoline in 1,2-ethanolamine in the presence of bases K_2CO_3 and Et_3N. The hydroxyl group was activated by converting it into a mesylate. Spectroscopically, the product was confirmed by the appearance of a singlet, integrating for 3 protons, at 3.1 ppm in the ^1H NMR. The mesylated derivative **4b** was then reacted with 4-hydroxybenzaldehyde, where the hydroxyl of the benzaldehyde, after deprotonation by K_2CO_3, nucleophilically displaced the mesylate moiety to yield **4c**.

Reductive amination of aldehyde **4c** by amine **QN01** was carried out in a similar manner as described in 6.3.2.1, forming compound **4d** in good yield. The product was confirmed by ^{1}H NMR as two sets of quinoline protons were observed, along with two doublets from the phenyl ring. Additionally, the benzylic protons were observed as a distinct, sharp singlet, integrating for 2 protons, appearing at 5.7 ppm.

6.4 Biological Results and Discussion

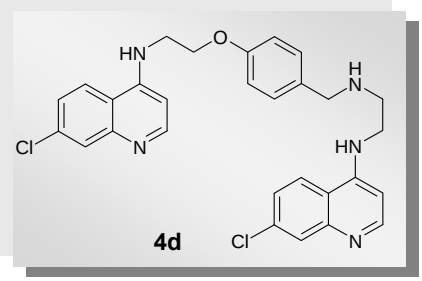
The *in vitro* antiplasmodial activities of these 4-aminoquinoline compounds were determined against the chloroquine-sensitive D10 and chloroquine-resistant K1 strains. Cytotoxicity and β-haematin inhibitory activities were also assessed. Results are presented in Table 6.1. Chloroquine was used as the control for all three assays.

The bisquinoline compound **4d** displayed good antiplasmodial activities of 53 and 43 nM against the D10 and K1 strains respectively. However, despite its equipotent activity against the two strains, it also displayed cytotoxicity against HeLa cells, with a therapeutic index of 26 with respect to the K1 strain. The cytotoxicity that is often associated with potent efficacy for the bisquinoline compounds has previously been observed,[28b, 39] hence more modifications would be necessary to lower the cytotoxicity.

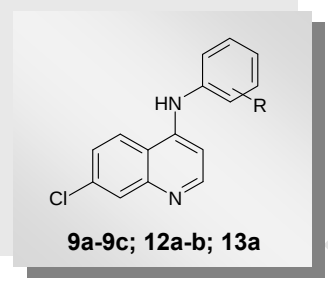
The benzyl alcohol intermediates **9a** and **9b** displayed modest activities against the two strains of *P. falciparum*, and **9c** was not active at the maximum concentration of 50 μM. When the hydroxyl moiety of **9a** and **9b** was converted into amino groups to form the amodiaquine-like compounds **12a**, **12b** and **13a**, their antiplasmodial activities increased by at least 27-fold. Although the activities against the K1 strain were slightly lower than against

the D10 strain for compounds **12a** and **13a**, this is not so for compound **12b**, which displayed superior activity in comparison to the other two analogues against the chloroquine-resistant K1 strain (8 nM). Cytotoxicity against HeLa cells was weak for these amodiaquine-like compounds, with therapeutic indices greater than 200.

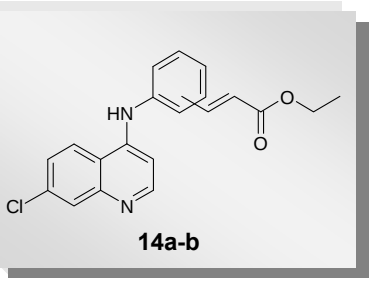
Table 6.1. Biological activities of 4-aminoquinoline target compounds



4d



9a-9c; 12a-b; 13a



14a-b

General structures of target compounds

Cmpd	Posi-tion	R	*P. falciparum* IC_{50} (μM)		HeLa IC_{50} (μM)	SI[a]	TI[b]	β-Haematin Inhibition (equiv.)[c]
			D10	K1				
CQ	-	-	0.017	0.219	8.54	12.8	39	1.91 ± 0.3
4c	-	-	1.47	2.20	7.0	1.5	3	Not active[d]
4d	-	-	0.053	0.043	1.1	0.8	26	Not active[d]
9a	*meta*	CH_2OH	2.16	5.52	>50	2.6	> 9	2.85 ± 0.1
9b	*ortho*	CH_2OH	1.90	2.54	23.6	1.3	9	2.32 ± 0.09
9c	*para*	COOH	>50	>50	>50	-	-	Not active[d]
12a	*meta*	$CH_2N(Me)_2$	0.034	0.052	25.6	1.5	438	1.92 ± 0.09
12b	*ortho*	$CH_2N(Me)_2$	0.068	0.008	35.8	118	4366	1.92 ± 0.1
13a	*meta*	$CH_2N(Et)_2$	0.061	0.081	18.8	1.3	229	2.4 ± 0.04
14a	*ortho*	-	1.00	0.89	2.7	0.9	3	Not active[d]
14b	*meta*	-	0.936	1.50	10.3	1.6	7	Not active[d]

[a] Resistance index calculated as [IC_{50} (K1)] / [IC_{50} (D10)]; [b] Therapeutic index calculated as [IC_{50} (HeLa)] / [IC_{50} (K1)]; [c] IC_{50} ± standard error of the mean on three determinations; [d] at IC_{50} >10.0 equivalents.

Wittig products **14a** and **14b** did not exhibit significant antiplasmodial activities (0.89 and 1.5 μM respectively against K1), and at the same time displayed cytotoxicity against HeLa cells (3 and 7μM respectively).

Additionally, the positions of the side chain on the phenyl ring for these compounds did not seem to play an important role in terms of biological activity, as no changes have been observed between activities of the *ortho* and *meta* analogues.

Inhibitory activities for the formation of β-haematin have also been determined for these aminoquinolines. It appears that there is no correlation between their ability to inhibit the formation of β-haematin and their antiplasmodial activities for these compounds, as the β-haematin inhibitory activities of the amodiaquine-like compounds **12a**, **12b** and **12c** were only slightly better than the benzyl alcohol intermediates **9a** and **9b**, which possessed only modest antiplasmodial activities. Surprisingly, bisquinoline compound **4d** also did not appear to be able to inhibit the formation of β-haematin. This may be due to the insolubility of the resulting complex with β-haematin.

6.5 Conclusion and Future Work

A series of 4-aminoquinoline analogues have been prepared, and their biological activities were assessed. These compounds contain either an aminomethyl moiety (amodiaquine-like), α,β-unsaturated system (Wittig product) or another aminoquinoline derivative (bisquinoline). The bisquinoline compound **4d** displayed good antiplasmodial activities against both the D10 and K1 strains of *P. falciparum*, but is also associated with cytotoxicity. Amodiaquine-like compounds **12a**, **12b** and **13a** all displayed good antiplasmodial activities,

and modest cytotoxicity against HeLa cells were observed. In particular, compound **12b** displayed potent antiplasmodial activity of 8 nM against the K1 strain. Hence compound **12b** would be a good candidate for further studies.

Wittig products **14a** and **14b**, on the other hand, displayed moderate antiplasmodial activities and cytotoxity against HeLa cells. The α,β-unsaturated system on these analogues could be further utilized by reacting with nucleophiles in chemical modifications in an effort to enhance their antiplasmodial activities and/or lower their cytotoxicity.

No clear correlation between the antiplasmodial activity and the inhibition of β-haematin has been observed. Although these compounds may exert their activity via inhibiting the formation of β-haematin, more analogues would need to be synthesized and tested to confirm this. Interestingly, bisquinoline **4d** was not active towards the inhibition of β-haematin formation, suggesting that alternative target(s) may be involved for this bisquinoline compound. Moreover, more bisquinoline compounds would also need to be synthesized and evaluated to confirm this.

Even though the antiplasmodial activities for the *ortho* and *meta* analogues do not seem to differ drastically, it would be useful to synthesize the *para* series for a more informative comparison.

6.6 References

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CHAPTER 7

SUMMARY AND CONCLUSION

Results from the various chapters are summarized below:

(i) A series of artemisinin based β-aminocarbonyl compounds, some of which contain a 4-aminoquinoline moiety, were synthesized via conjugate addition and evaluated for antiplasmodial and anticancer activities. Compounds **MIC 08**, **12** and **13A** have displayed the most potent antiplasmodial activities against D10 and Dd2 strains of *P. falciparum*, with activities at least 4 and 20 times higher than chloroquine against the D10 and Dd2 strains respectively. Compound **MIC 14** has been found to be the most active against HeLa cells, with an IC_{50} of 0.37 μM and tumour specificity of 31 in comparison to primary lymphocytes.

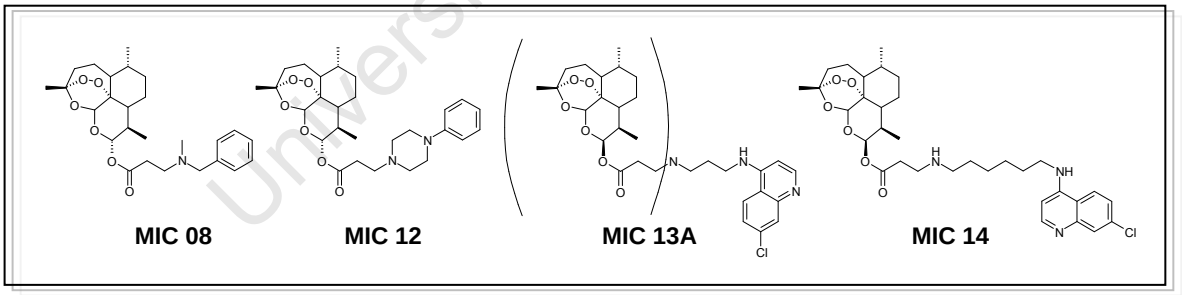


Figure 7.1. Structures of **MIC 08**, **MIC 12**, **MIC 13A** and **MIC 14**.

(ii) 4-Aminoquinolines were hybridized with artemisinin or 1,4-naphthoquinone derivatives via the Ugi 4-component condensation. The artemisinin-quinoline hybrids **6a**, **6b** and **6c** exhibited antiplasmodial activities 10 times more potent than chloroquine against the K1 strain, and displayed β-haematin inhibitory activities almost 5 times higher than chloroquine.

The influence of the artemisinin-quinoline hybrids on parasitic endocytosis was also investigated, by studying the effect of drug treatment on the morphology and haemoglobin levels in the parasites, as well as the localization of transport vesicles via immunofluorescence microscopy. Results have shown that the mode(s) of action of these hybrids closely resemble that of the artemisinin parent rather than that of chloroquine.

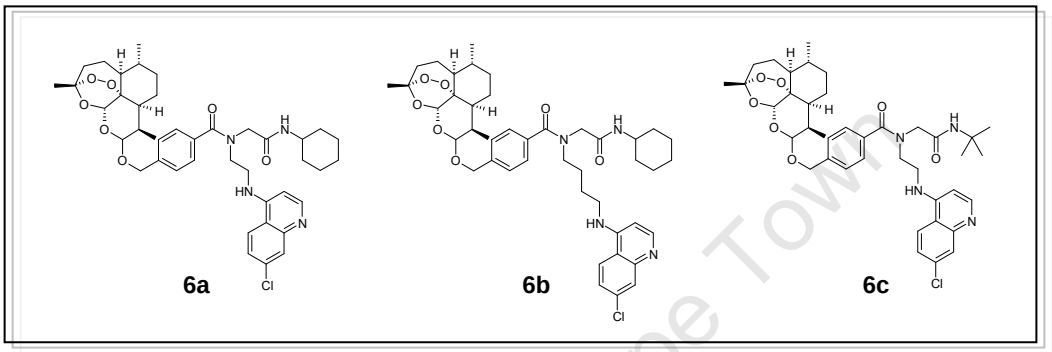


Figure 7.2. Structures of **6a**, **6b** and **6c**.

(iii) A series of β-lactams containing a terminal acetylene moiety was synthesized via the Staudinger reaction and the Ugi 4-component 3-centre condensation. The best compound was further diversified and modified by employing the Mannich reaction. None of these compounds exhibited significant antiplasmodial activities against the D10 and Dd2 strains of *P. falciparum*, nor did they display cytotoxic activities against HeLa cells.

(iv) 4-Aminoquinoline analogues containing an aminomethyl or α,β-unsaturated system were synthesized via reductive amination or Wittig reaction on the quinoline-aldehyde intermediate. A bisquinoline derivative was also synthesized. Compounds **12a**, **12b** and **13a** exhibited good antiplasmodial activities, with compound **12b** being the most potent, displaying an IC_{50} value of 8 nM against the K1 strain of *P. falciparum*, almost 30 times more potent than chloroquine. Weak cytotoxicity was observed for these three compounds**.**

The bisquinoline derivative **4d** displayed good antiplasmodial activities, but is also associated with cytotoxicity against HeLa cells, with a low therapeutic index of 26. Correlation between the antiplasmodial and β-haematin inhibitory activities has not been observed, suggesting that these compounds may act via targeting additional pathways in the parasite.

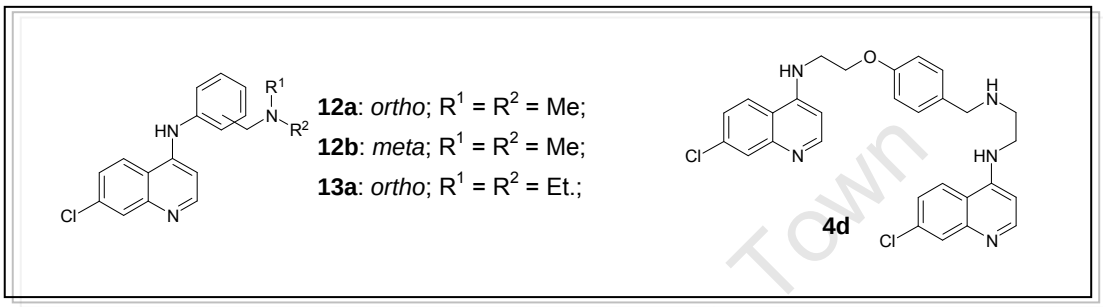


Figure 7.3. Structures of **12a**, **12b**, **13a** and**4d**.

In conclusion, novel antimalarial agents have been designed and synthesized, many of which are based on the 'Designed Multiple Ligand' strategy, with the aim of interfering with two or more targets at the same time. A number of compounds with potent antiplasmodial or anticancer activities have been identified, and further work would be needed to optimize their biological activities to warrant them as possible lead candidates.

CHAPTER 8

EXPERIMENTAL

8.1 Chemical Characterization

Purification of solvents

All solvents used for reactions, 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

Merck Kieselgel 60: 70 – 230 mesh was used for all preparative and column chromatography. Reactions were monitored by thin layer chromatography using Merck F_{254} aluminum-backed precoated silica gel plates and were visualized by a combination of ultraviolet light, iodine vapour and staining with 5% anisaldehyde solution. Melting points were determined on a Reichert-Jung Thermovar hot-stage microscope. Infrared spectra were recorded on a Thermo Nicolete FTIR instrument in the 4000-700 cm^{-1} range as methylene chloride solutions. 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 Witwatersrand, SA, VG70-SEQ (FAB, operating at 7kV) and VG70-SEQ (EI, operating at 8kV) instruments were used.

Physical and Spectroscopic Data

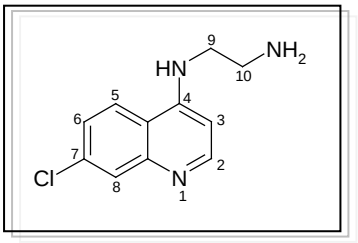
^{1}H NMR (nuclear magnetic resonance) spectra were recorded on a Varian Mercury spectrometer at 300 MHz or a Varian Unity Spectrometer at 400 MHz. Spectra were recorded in deuterated solvents: $CDCl_3$, DMSO-d_6 and CD_3OD and the chemical shifts are reported in parts per million downfield from the internal standard tetramethylsilane; coupling constants are given in Hertz. ^{1}H NMR coupling constants are rounded off to one decimal place, resulting in the same coupling constants for coupling partners. In cases where deuterated methanol was used as ^{1}H 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 ^{1}H NMR spectra.

^{13}C NMR spectra were recorded on a Varian Mercury spectrometer at 75 Hz or Varian Unity Spectrometer at 100 Hz. The format used for recording 13C 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.

General procedure for the synthesis of **QN01** – **QN04**.

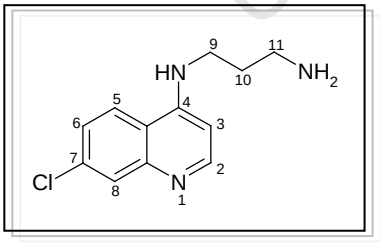
Et_3N (0.3 eq) was added drop-wise to a stirred solution of 4,7-dichloroquinoline (1.0 eq) and K_2CO_3 (0.5 eq) in respective diamines (20.0 eq) and refluxed at 110 °C for 4 hours. Products that precipitated on cooling were filtered and washed with brine and H_2O. For products that stayed in solution, NaOH was added, and the organic product was immediately basified with 10% NaOH (w/v) and extracted with EtOAc (5 times), washed with H_2O (2 times) and dried (Na_2SO_4). Recrystallisation from EtOAc afforded the amine in excellent yields.

***N*-(7-*Chloroquinolin*-4-*yl*)-*ethane*-1,2-*diamine*, QN01**



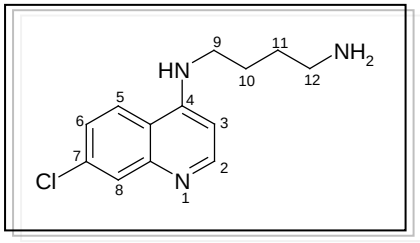
Pure product was recrystallised from EtOAc as white powder (0.4861 g, 64%). m.p. 136 – 138 °C (lit. 137 – 139 °C).[1] R_f (MeOH/DCM, 0.5:9.5) 0.32. δ_H (400 MHz; DMSO-d_6) 8.38 (d, *J* 5.6, 1H, H2), 8.10 (d, *J* 8.4 Hz, 1H, H5), 7.76 (d, *J* 2.0, 1H, H8), 7.40 (dd, *J* 8.4 and 2.0, 1H, H6), 7.24 (br s, 1H, NH), 6.48 (d, *J* 5.6, 1H, H3), 3.25 (t, *J* 5.6, 2H, H9), 2.82 (t, *J* 6.0, 2H, H10).

***N*-(7-*Chloroquinolin*-4-*yl*)-*propane*-1,3-*diamine*, QN02**



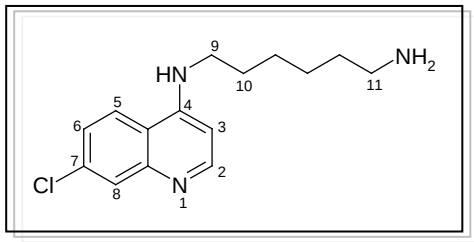
Pure product was recrystallised from EtOAc as a light-yellow solid (1.52 g, 88%). m.p. 123 – 126 °C (lit. 124 – 127 °C).[1] R_f (NH_3/MeOH, 1:49) 0.21. δ_H (300 MHz; CD_3OD) 8.36 (d, *J* 5.7, 1H, H2), 8.08 (d, *J* 9.0 Hz, 1H, H5), 7.78 (d, *J* 2.1, 1H, H8), 7.40 (dd, *J* 9.0 and 2.1, 1H, H6), 6.55 (d, *J* 5.7, 1H, H3), 3.44 (t, *J* 7.2, 2H, H9), 2.86 (t, *J* 7.2, 2H, H11), 1.94 (pentet, *J* 7.2, 2H, H10).

***N*-(7-*Chloroquinolin*-4-*yl*)-*butane*-1,4-*diamine*, QN03**



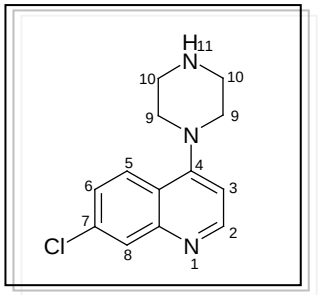
Pure product was recrystallised from EtOAc as a light-yellow solid (1.60 g, 87%). m.p. 43 – 46 °C (lit. 43 – 47 °C).[1] R_f (NH_3/MeOH, 1:49) 0.22. δ_H (300 MHz; CD_3OD) 8.48 (d, *J* 5.7, 1H, H2), 7.96 (d, *J* 9.0 Hz, 1H, H5), 7.69 (d, *J* 2.1, 1H, H8), 7.27 (dd, *J* 9.0 and 2.1, 1H, H6), 6.34 (d, *J* 5.7, 1H, H3), 3.40 (t, *J* 7.2, 2H, H9), 3.04 (t, *J* 7.2, 2H, H12), 1.88 (m, 2H, H10), 1.62 (m, 2H, H11).

***N*-(7-*Chloroquinolin*-4-*yl*)-*hexane*-1,6-*diamine*, QN04**



Pure product was recrystallised from EtOAc as yellow needles (1.94 g, 95%). m.p. 135 – 138 °C (lit. 136 – 138 °C).[1] R_f (NH_3/MeOH, 1:49) 0.22. δ_H (300 MHz; CD_3OD) 8.34 (d, *J* 5.7, 1H, H2), 8.09 (d, *J* 9.0 Hz, 1H, H5), 8.77 (d, *J* 2.1, 1H, H8), 7.39 (dd, *J* 9.0 and 2.1, 1H, H6), 6.50 (d, *J* 5.7, 1H, H3), 3.36 (t, *J* 7.2, 2H, H9), 3.15 (t, *J* 7.2, 2H, H11), 2.64 (m, 2H, H10), 1.8 – 1.40 (m, 6H, CH_2's).

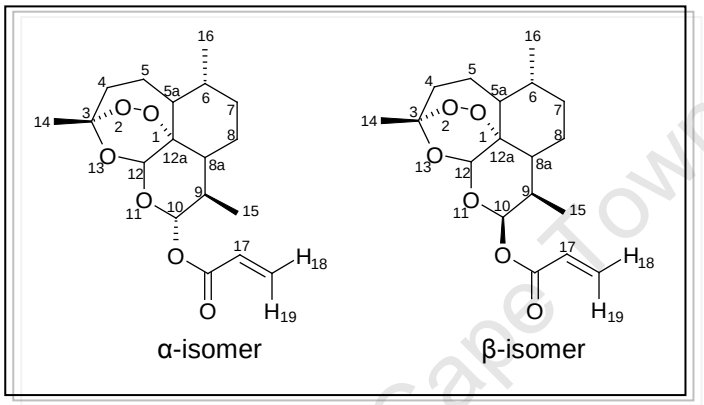
7-*Chloro*-4-*piperazin*-1-*ylquinoline*, QN05



Piperazine (3.20 g, 36.73 mmol), 4,7-dichloroquinoline (1.5 g, 7.35 mmol) and K_2CO_3 (0.52 g, 3.76 mmol) were refluxed in anhydrous NMP (10 ml) at 150 °C under N_2 and cooled to r.t. after 4 h. The reaction mixture was diluted with DCM (50 ml), and washed with H_2O (3 x 50 ml), brine (50 ml), dried (Na_2SO_4) and solvent reduced. The crude material was purified via column chromatography, eluting with MeOH/DCM (1:9) to yield the pure material as a white solid (1.62 g, 89%). m.p. 113 –

116 °C (lit. 113 – 115 °C)[2]. R_f (MeOH/DCM, 1:9) 0.13. δ_H (300 MHz; CD_3OD) 8.64 (d, *J* 5.4, 1H, H2), 8.07 (d, *J* 9.0 Hz, 1H, H5), 7.92 (d, *J* 2.1, 1H, H8), 7.52 (dd, *J* 9.0 and 2.1, 1H, H6), 7.01 (d, *J* 5.4, 1H, H3), 3.31 (pentet, *J* 1.8, 1H, H11), 3.24 (m, 4H, H9), 3.11 (m, 4H, H10).

A-acr-alpha and ***A-acr-beta***



Acroylyl chloride (1.78 ml, 21.0 mmol) was added drop-wise to a chilled (0 °C) solution of dihydroartemisinin (3.0 g, 10.5 mmol) and Et_3N (2.93 ml, 21.0 mmol) in anhydrous DCM (80 ml). The mixture was stirred for 3 h, whilst allowing it to slowly warm to r.t. (20 °C), after which the solvent was reduced, and column chromatographed to yield the pure isomers as white powders (2.03 g, 57%, d.e. α : β 4.7 : 5.3).

A-acr-alpha

m.p. 143 – 146 °C; R_f (EtOAc/Hex 3:7) 0.75; ν_{max} (DCM)/cm^{-1} 1724 (C=O), 1633 (C=C), 1048 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 6.51 (dd, *J* 17.6 and 1.6, 1H, H19), 6.19 (dd, *J* 17.6 and 10.4, 1H, H17), 5.90 (dd, *J* 10.4 and 1.6, 1H, H18), 5.88 (d, *J* 9.2, 1H, H10), 5.48 (s, 1H, H12), 2.64 (m, 1H, H9), 1.45 (s, 3H, H14), 0.99 (d, *J* 6.0, 3H, H16), 0.88 (d, *J* 7.2, 3H, H15); δc (100 MHz; $CDCl_3$) 164.8, 132.0, 128.2, 104.5, 92.2, 91.6, 80.2, 51.7, 45.3, 37.3, 36.3, 34.2, 32.0, 26.0, 24.6, 22.1, 20.2, 12.1; *m/z* 338.3997 (M^+); (Found: M^+,

338.3997. $C_{18}H_{26}O_6$ requires *M*, 338.4008) (Found: C, 63.87.; H, 7.77. $C_{18}H_{26}O_6$ requires C, 63.89; H, 7.74).

A-acr-beta

m.p. 134 – 137 °C; R_f (EtOAc/Hex 3:7) 0.77. ν_{max} (DCM)/cm^{-1} 1724 (C=O), 1635 (C=C), 1048 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 6.41 (dd, *J* 17.2 and 1.6, 1H, H19), 6.34 (d, *J* 3.6, 1H, H10), 6.12 (dd, *J* 17.2 and 10.4, 1H, H17), 5.88 (dd, *J* 10.4 and 1.6, 1H, H18), 5.50 (s, 1H, H12), 2.86 (m, 1H, H9), 1.44 (s, 3H, H14), 0.99 (d, *J* 6.0, 3H, H16), 0.90 (d, *J* 7.2, 3H, H15); δ_C (100 MHz; $CDCl_3$) 164.3, 131.2, 128.5, 104.4, 95.1, 88.8, 80.5, 52.4, 44.0, 37.6, 36.3, 34.6, 30.0, 25.9, 24.7, 24.2, 20.3, 12.5; *m/z* 338.3986 (M^+); (Found: M^+, 338.3986. $C_{18}H_{26}O_6$ requires *M*, 338.4008) (Found: C, 63.84.; H, 7.69. $C_{18}H_{26}O_6$ requires C, 63.89; H, 7.74).

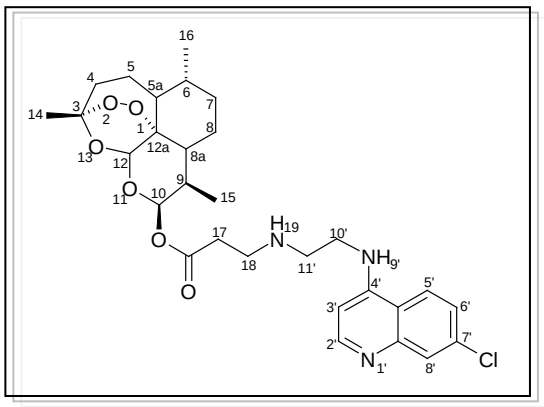
General procedure for Compounds **MIC 01**, **02**, **05**, **13** and **14**

A-acr-beta (1.0 eq), various amines (1.5 eq) and DBU (0.5 eq) were stirred in anhydrous DMF (2 ml) under N_2 for 18 h at r.t. (20 °C). H_2O (10 ml) was added to the reaction mixture, and extracted with EtOAc (3 x 10 ml), washed with H2O (3 x 20 ml), brine (30 ml), dried and solvent reduced to yield the crude products.

General procedure for Compounds **MIC 03**, **04**, **06** – **12**

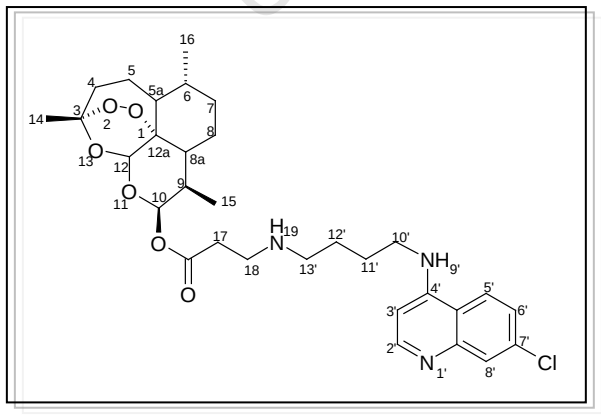
A-acr-alpha (or **A-acr-beta**) (1.0 eq), various amines (1.5 eq) and DBU (0.5 eq) were stirred in anhydrous acetonitrile (2 ml) under N_2 for 18 h at r.t. (20 °C). The solvent was reduced to yield the crude products.

MIC 01



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.5:9.5) to yield the product as a light brown oil (0.180 g, 73%); R_f (MeOH/DCM, 0.5:9.5) 0.12; ν_{max} (DCM)/cm^{-1} 1738 (C=O), 1451 (C=C (Ar)), 1265 (C–N), 1048 (C–O (ester)), 847 (O–O); δ_H (300 MHz; $CDCl_3$) 8.49 (d, *J* 5.4, 1H, H2'), 7.94 (d, *J* 2.1, 1H, H8'), 7.78 (d, *J* 8.7, 1H, H5'), 7.37 (dd, *J* 8.7 and 2.1, 1H, H6'), 6.36 (d, *J* 5.4, 1H, H3'), 6.29 (d, *J* 2.7, 1H, H10), 5.46 (s, 1H, H12), 3.34 (t, *J* 6.0, 2H, H10'), 3.02 (t, *J* 6.0, 2H, H11'), 2.97 (t, *J* 6.9, 2H, H17), 2.81 (m, 1H, H9), 2.56 (t, *J* 6.9, 2H, H18), 1.37 (s, 3H, H14), 0.94 (d, *J* 6.0, 3H, H16), 0.84 (d, *J* 7.2, 3H, H15); δc (75.5 MHz; $CDCl_3$) 171.4, 151.7, 130.9, 128.9, 128.3, 125.4, 121.7, 104.5, 99.1, 95.0, 88.8, 80.4, 52.3, 47.2, 44.2, 43.8, 41.9, 37.5, 36.2, 34.9, 34.4, 29.7, 25.8, 24.6, 24.1, 20.2, 12.5; *m/z* 560.2547 (MH^+); (Found: MH^+, 560.2547. $C_{29}H_{39}ClN_3O_6$ (MH^+) requires 560.2527).

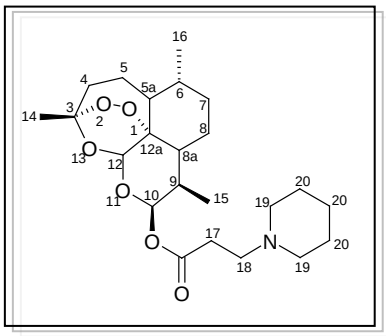
MIC 02



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.5:9.5) to yield the product as a light brown oil (0.185 g, 71%); R_f (MeOH/DCM, 0.5:9.5) 0.11; ν_{max} (DCM)/cm^{-1} 1740 (C=O), 1447 (C=C (Ar)), 1243 (C–N), 1048 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 8.47 (d, *J* 5.6, 1H, H2'), 7.95 (d, *J* 2.0, 1H, H8'), 7.81 (d, *J* 8.8, 1H, H5'), 7.35 (dd, *J* 8.8 and

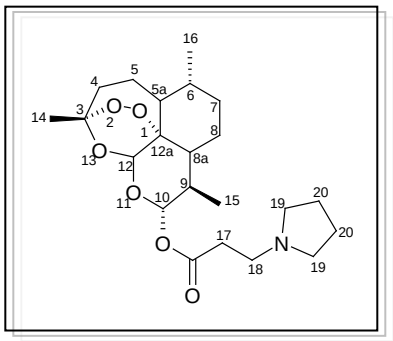
2.0, 1H, H6'), 6.36 (d, *J* 5.6, 1H, H3'), 6.28 (d, *J* 3.2, 1H, H10), 5.30 (s, 1H, H12), 3.33 (t, *J* 6.0, 2H, H10'), 2.96 (t, *J* 6.4, 2H, H17), 2.76 (t, *J* 6.0, 2H, H11'), 2.71 (m, 1H, H9), 2.61 (t, *J* 6.4, 2H, H18), 1.27 (s, 3H, H14), 0.96 (d, *J* 6.0, 3H, H16), 0.87 (d, *J* 7.2, 3H, H15); δ_C (100 MHz; $CDCl_3$) 151.1, 127.8, 125.3, 121.8, 104.5, 98.8, 95.1, 88.8, 80.5, 52.4, 44.7, 43.9, 43.1, 39.9, 37.6, 36.1, 34.5, 34.3, 29.8, 29.7, 27.1, 25.9, 24.6, 24.1, 22.1, 20.2, 12.5; *m/z* 588.2869 (MH^+); (Found: MH^+, 588.2869. $C_{31}H_{43}ClN_3O_6$ (MH^+) requires 588.2840).

MIC 03



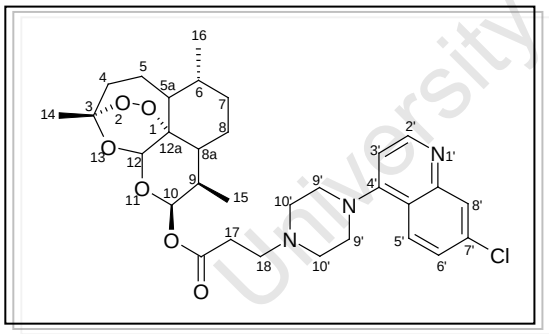
Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.2:9.8) to yield the product as a white powder (0.198 g, 72%); m.p. 102 – 105 °C. R_f (MeOH/DCM 1:9) 0.25; ν_{max} (DCM)/cm^{-1} 1743 (C=O), 1243 (C–N), 1048 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 6.27 (d, *J* 3.6, 1H, H10), 5.48 (s, 1H, H12), 2.82 (m, 1H, H9), 2.68 (m, 2H, H17), 2.55 (m, 2H, H18), 2.42 (m, 4H, H19), 1.58 (m, 6H, H20), 1.43 (s, 3H, H14), 0.98 (d, *J* 6.0, 3H, H16), 0.89 (d, *J* 7.2, 3H, H15); δ_C (100 MHz; $CDCl_3$) 171.1, 104.4, 94.7, 88.8, 80.6, 54.3, 54.1, 52.3, 44.0, 37.5, 36.3, 34.6, 32.7, 29.9, 25.9, 24.7, 24.3, 24.2, 20.3, 12.5; *m/z* 424.2720 (MH^+); (Found: MH^+, 424.2720. $C_{23}H_{38}NO_6$ (MH^+) requires 424.2699) (Found: C, 65.26.; H, 8.74; N, 3.11. $C_{23}H_{37}NO_6$ requires C, 65.22; H, 8.81; N, 3.31%).

MIC 04



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.2:9.8) to yield the product as a light-brown oil (0.183 g, 69%); R_f (MeOH/DCM 0.5:9.5) 0.24; ν_{max} (DCM)/cm^{-1} 1742 (C=O), 1240 (C–N), 1048 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 5.80 (d, *J* 9.6, 1H, H10), 5.44 (s, 1H, H12), 2.81 (m, 1H, H9), 2.80 (t, *J* 7.2, 2H, H17), 2.63 (t, *J* 7.2, 2H, H18), 2.54 (t, *J* 6.2, 4H, H19), 1.79 (t, *J* 6.2, 4H, H20), 1.44 (s, 3H, H14), 0.97 (d, *J* 6.0, 3H, H16), 0.86 (d, *J* 7.2, 3H, H15); δ_C (100 MHz; $CDCl_3$) 104.4, 91.9, 91.5, 80.1, 70.7, 66.9, 54.0, 53.9, 51.6, 45.3, 37.3, 36.3, 34.2, 33.9, 31.9, 26.0, 24.6, 23.5, 22.0, 20.2, 12.0; *m/z* 410.2543 (MH^+); (Found: MH^+, 410.2543. $C_{22}H_{36}NO_6$ (MH^+) requires 410.2558).

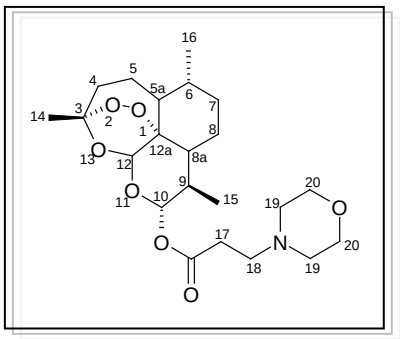
MIC 05



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.2:9.8) to yield the product as a white powder (0.304 g, 80%); m.p. 131 – 133 °C. R_f (MeOH/DCM 0.5:9.5) 0.25; ν_{max} (DCM)/cm^{-1} 1744 (C=O), 1451 (C=C (Ar)), 1243 (C–N), 1048 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 8.73 (d, *J* 5.1, 1H, H2'), 8.05 (d, *J* 1.6, 1H, H8'), 7.95 (d, *J* 9.2, 1H, H5'), 7.43 (dd, *J* 9.2 and 1.6, 1H, H6'), 6.84 (d, *J* 5.1, 1H, H3'), 6.32 (d, *J* 3.2, 1H, H10), 5.51 (s, 1H, H12), 3.20 (t, *J* 4.8, 4H, H9'), 2.85 (t, *J* 7.2, 2H, H17), 2.80 (m, 1H, H9),2.78 (t, *J* 4.8, 4H, H10'), 2.61 (t, *J* 7.2, 2H, H18), 1.44 (s, 3H, H14), 0.99 (d, *J* 6.0, 3H, H16), 0.91 (d, *J* 7.2, 3H, H15); δ_C (100 MHz; $CDCl_3$) 170.8, 156.8, 151.9, 150.1, 134.8, 128.8, 126.1, 125.2, 121.9, 109.0, 104.4, 94.9, 88.7, 80.5, 53.4, 52.8, 52.4, 52.1, 43.9, 37.6, 36.2, 34.5, 32.8, 29.9, 25.9, 24.7,

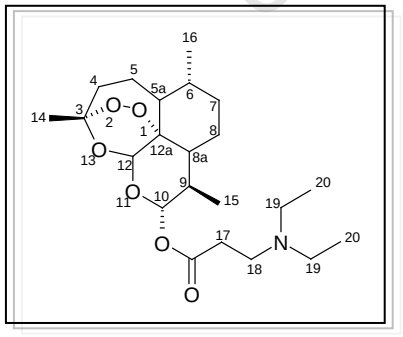
24.2, 20.2, 12.5; *m/z* 586.2699 (MH^+); (Found: MH^+, 586.2699. $C_{31}H_{41}ClN_3O_6$ (MH^+) requires 586.2684) (Found: C, 63.47; H, 6.69; N, 7.11. $C_{31}H_{40}ClN_3O_6$ requires C, 63.53; H, 6.88; N, 7.17%).

MIC 06



Crude residue was purified via column chromatography, eluting with EtOAc/Hex (1:1) to yield the product as a colourless oil (0.207 g, 75%); R_f (EtOAc/Hex 8:2) 0.21; ν_{max} (DCM)/cm^{-1} 1740 (C=O), 1264 (C–N), 1048 (C–O (ester)), 848 (O–O); δ_H (400 MHz; $CDCl_3$) 5.82 (d, *J* 9.6, 1H, H10), 5.45 (s, 1H, H12), 3.70 (t, *J* 4.8, 4H, H19), 2.72 (t, *J* 6.8, 2H, H17), 2.60 (t, *J* 6.8, 2H, H18), 2.58 (m, 1H, H9), 2.47 (t, *J* 4.8, 4H, H20), 1.44 (s, 3H, H14), 0.98 (d, *J* 6.0, 3H, H16), 0.87 (d, *J* 6.8, 3H, H15); δ_C (100 MHz; $CDCl_3$) 104.5, 92.2, 92.0, 91.5, 80.1, 70.7, 66.9, 53.7, 53.4, 51.6, 45.3, 37.3, 36.3, 34.1, 32.3, 31.9, 26.0, 24.6, 22.0, 20.2, 12.1; *m/z* 426.2488 (MH^+); (Found: MH^+, 426.2488. $C_{22}H_{36}NO_7$ (MH^+) requires 426.2492).

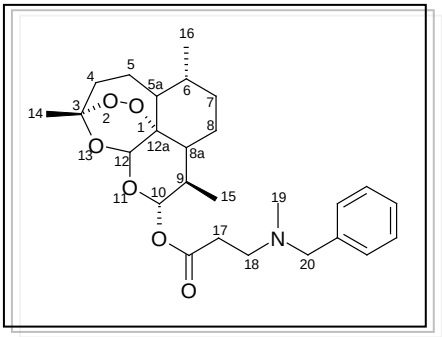
MIC 07



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.2:9.8) to yield the product as a light-brown oil (0.145 g, 54%); R_f (MeOH/DCM 0.5:9.5) 0.18; ν_{max} (DCM)/cm^{-1} 1737 (C=O), 1261 (C–N), 1049 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 5.81 (d, *J* 10.0, 1H, H10), 5.45 (s, 1H, H12), 2.84 (m, 3H, H9 + H17), 2.57 (m, 6H, H18 + H19), 1.44 (s, 3H, H14), 1.06 (t, *J* 7.6, 3H, H20), 1.05 (t, *J* 7.6, 4H, H20), 0.97 (d, *J* 6.0, 3H, H16), 0.88 (d, *J* 7.2, 3H, H15); δ_C (100 MHz; $CDCl_3$) 104.4, 91.9,

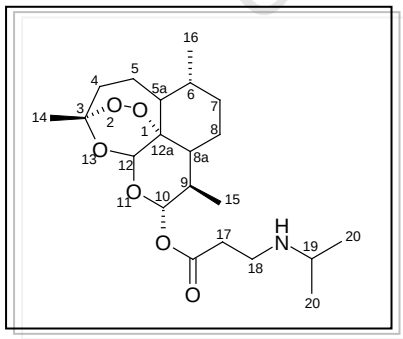
91.5, 80.1, 52.6, 51.6, 47.8, 46.7, 45.3, 37.3, 36.3, 34.1, 32.1, 31.8, 26.0, 24.6, 22.0, 20.2, 12.7; *m/z* 412.2714 (MH^+); (Found: MH^+, 412.2714. $C_{22}H_{38}NO_6$ (MH^+) requires 412.2699).

MIC 08



Crude residue was purified via column chromatography, eluting with EtOAc/Hex (1.5:8.5) to yield the product as a light-yellow oil (0.186 g, 62%); R_f (EtOAc/Hex 3:7) 0.13; ν_{max} (DCM)/cm^{-1} 1738 (C=O), 1510 (C=C (Ar)), 1265 (C–N), 1048 (C–O (ester)), 849 (O–O); δ_H (400 MHz; $CDCl_3$) 7.30 (s, 5H, Ar-H), 5.82 (d, *J* 9.6, 1H, H10), 5.46 (s, 1H, H12), 3.53 (s, 2H, H20), 2.80 (m, 1H, H9), 2.79 (t, *J* 6.8, 2H, H17), 2.63 (t, *J* 6.8, 2H, H18), 1.44 (s, 3H, H14), 0.98 (d, *J* 6.0, 3H, H16), 0.85 (d, *J* 7.2, 3H, H15); δ_C (100 MHz; $CDCl_3$) 171.1, 128.2, 127.0. 96.4, 94.7, 91.9, 91.5, 91.2, 90.0, 87.8, 80.1, 52.6, 45.3, 41.7, 37.5, 36.4, 34.1, 32.8, 31.8, 26.0, 24.6, 22.0, 12.1; *m/z* 460.3152 (MH^+); (Found: MH^+, 460.3152. $C_{26}H_{38}NO_6$ (MH^+) requires 460.3141).

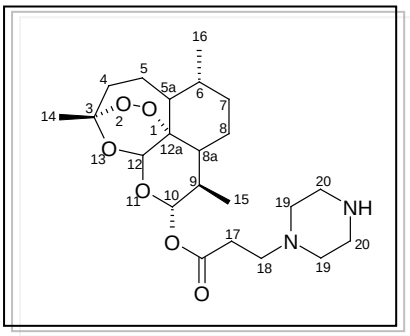
MIC 09



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.2:9.8) to yield the product as a light-yellow oil (0.207 g, 80%); R_f (MeOH.DCM 0.5:9.5) 0.25; ν_{max} (DCM)/cm^{-1} 1742 (C=O), 1265 (C–N), 1049 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 6.28 (d, *J* 9.6, 1H, H10), 5.48 (s, 1H, H12), 2.95 (t, *J* 6.4, 2H, H17), 2.91 (sept, *J* 6.0, 1H, H19), 2.68 (t, *J* 6.4, 2H, H18), 2.59 (m, 1H, H9), 1.44 (s, 3H, H14), 1.13 (d, *J* 6.0, 6H, H20), 0.99 (d, *J* 6.4, 3H, H16), 0.88 (d, *J* 7.2, 3H, H15); δ_C (100 MHz;

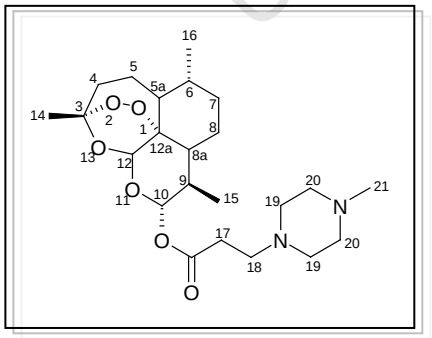
$CDCl_3$) 104.4, 94.6, 88.8, 80.5, 70.5, 52.6, 52.4, 51.6, 45.5, 37.5, 36.4, 34.1, 30.8, 29.8, 25.9, 24.7, 22.0, 20.4, 12.5; *m/z* 398.2445 (MH^+); (Found: MH^+, 398.2445. $C_{21}H_{36}NO_6$ (MH^+) requires 398.2431).

MIC 10



Crude residue was purified via column chromatography, eluting with EtOAc/Hex (6:4) to yield the product as a light-brown oil (0.168 g, 61%); R_f (EtOAc/Hex 1:1) 0.09; ν_{max} (DCM)/cm^{-1} 1740 (C=O), 1265 (C–N), 1051 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 5.81 (d, *J* 9.6, 1H, H10), 5.45 (s, 1H, H12), 2.92 (t, *J* 4.8, 4H, H19), 2.71 (t, *J* 7.0, 2H, H17), 2.59 (t, *J* 7.0, 4H, H18), 2.50 (m, 5H, H20 + H9), 1.44 (s, 3H, H14), 0.97 (d, *J* 6.0, 3H, H16), 0.87 (d, *J* 7.2, 3H, H15); δ_C (100 MHz; $CDCl_3$) 104.5, 91.9 ,91.5, 80.1, 53.8, 53.5, 51.6, 45.8, 45.5, 37.3, 36.3, 34.1, 34.3, 31.9, 26.0, 24.6, 22.0, 20.2, 12.0; *m/z* 425.2658 (MH^+); (Found: MH^+, 425.2658. $C_{22}H_{37}N_2O_6$ (MH^+) requires 425.2652).

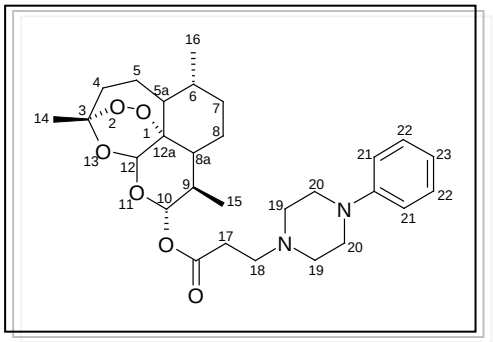
MIC 11



Crude residue was purified via column chromatography, eluting with EtOAc/Hex (3:7) to yield the product as a light-yellow oil (0.179 g, 63%); R_f (EtOAc/Hex 1:1) 0.37; ν_{max} (DCM)/cm^{-1} 1741 (C=O), 1265 (C–N), 1048 (C–O (ester)), 849 (O–O); δ_H (400 MHz; $CDCl_3$) 5.81 (d, *J* 9.6, 1H, H10), 5.44 (s, 1H, H12), 2.73 (t, *J* 6.8, 2H, H17), 2.59 (t, *J* 6.8, 2H, H18), 2.30 (s, 3H, H21), 2.56 – 2.44 (m, 9H, H9 + H19 + H20), 1.44 (s, 3H, H14), 0.98 (d, *J* 6.0, 3H, H16), 0.86 (d, *J* 7.2, 3H, H15); δc (100 MHz; $CDCl_3$) 171.2, 104.5, 91.9, 91.5, 55.0, 53.2, 52.7,

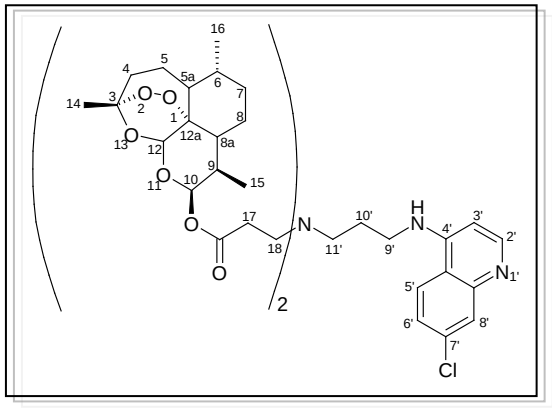
51.6, 45.9, 45.3, 37.3, 36.3, 34.1, 32.5, 31.9, 26.0, 24.6, 22.0, 20.2, 12.1; *m/z* 439.2821 (MH^+); (Found: MH^+, 439.2821. $C_{23}H_{39}N_2O_6$ (MH^+) requires 439.2808).

MIC 12



Crude residue was purified via column chromatography, eluting with EtOAc/Hex (4:6) to yield the product as a colourless oil (0.211 g, 65%); R_f (EtOAc/Hex 1:1) 0.28; ν_{max} (DCM)/cm^{-1} 1743 (C=O), 1551 (C=C (Ar)), 1257 (C–N), 1048 (C–O (ester)), 847 (O–O); δ_H (300 MHz; $CDCl_3$) 7.25 (dd, *J* 7.8 and 6.9, 2H, H22), 6.92 (d, *J* 7.8, 2H, H21), 6.84 (t, *J* 6.9, 1H, H23), 5.82 (d, *J* 9.6, 1H, H10), 5.44 (s, 1H, H12), 3.18 (d, *J* 4.8, 4H, H20), 2.77 (t, *J* 6.9, 2H, H17), 2.64 (t, *J* 6.9, 2H, H18), 2.63 (d, *J* 4.8, 4H, H19), 2.57 (m, 1H, H9), 1.43 (s, 3H, H14), 0.97 (d, *J* 6.0, 3H, H16), 0.86 (d, *J* 6.9, 3H, H15); δc (75.5 MHz; $CDCl_3$) 171.2, 151.3, 129.1, 119.6, 116.0, 104.4, 91.9, 91.5, 80.1, 53.3, 52.9, 51.6, 49.1, 45.3, 37.3, 36.2, 34.1, 32.5, 31.9, 26.0, 24.6, 22.0, 20.2, 12.1; *m/z* 502.2985 (MH^+); (Found: MH^+, 501.2985. $C_{28}H_{41}N_2O_6$ (MH^+) requires 501.2965).

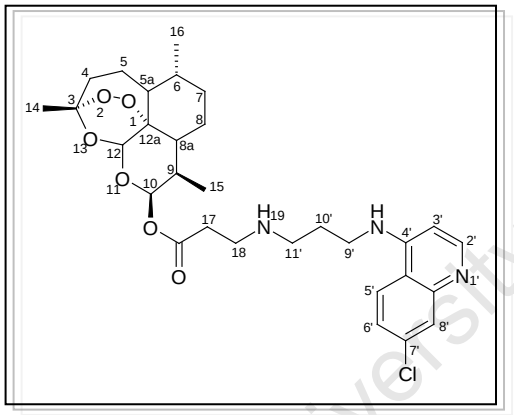
MIC 13A



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.1:9.9) to yield the product as a white powder (0.240 g, 41%); m.p. 82 – 84 °C. R_f (MeOH/DCM 0.5:9.5) 0.34; ν_{max} (DCM)/cm^{-1} 1741 (C=O), 1450 (C=C (Ar)), 1266 (C–N), 1048 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 8.51 (d, *J* 5.6, 1H, H2'), 7.96 (s, 1H,

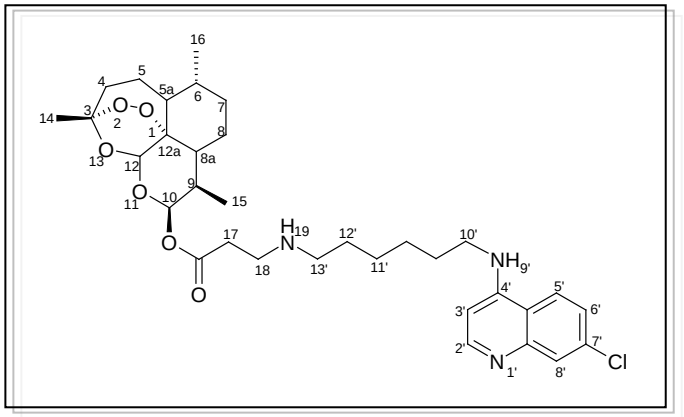
H8'), 7.81 (d, *J* 9.2, 1H, H5'), 7.38 (d, *J* 9.2, 1H, H6'), 6.38 (d, *J* 5.6, 1H, H3'), 6.28 (d, *J* 3.6, 2H, H10), 5.43 (s, 2H, H12), 3.42 (t, *J* 5.6, 2H, H9'), 2.91 (t, *J* 6.8, 4H, H17), 2.81 (m, 2H, H9), 2.64 (t, *J* 5.6, 2H, H11'), 2.53 (t, *J* 6.8, 4H, H18), 1.90 (t, *J* 5.6, 2H, H10'), 1.39 (s, 6H, H14), 0.97 (d, *J* 6.0, 6H, H16), 0.84 (d, *J* 7.2, 6H, H15); δc (100 MHz; $CDCl_3$) 151.8, 128.3, 125.1, 121.8, 104.5, 98.1, 95.1, 88.9, 80.0, 52.4, 48.9, 45.2, 43.8, 43.5, 39.9, 37.5, 36.2, 34.7, 34.6, 34.4, 29.8, 29.7, 27.3, 25.9, 24.7, 24.2, 20.2, 12.5; *m/z* 912.4432 (MH^+); (Found: MH^+, 912.4432. $C_{48}H_{67}ClN_3O_{12}$ (MH^+) requires 912.4413) (Found: C, 62.99; H, 7.18; N, 4.55. $C_{48}H_{66}ClN_3O_{12}$ requires C, 63.18; H, 7.29; N, 4.60%).

MIC 13B



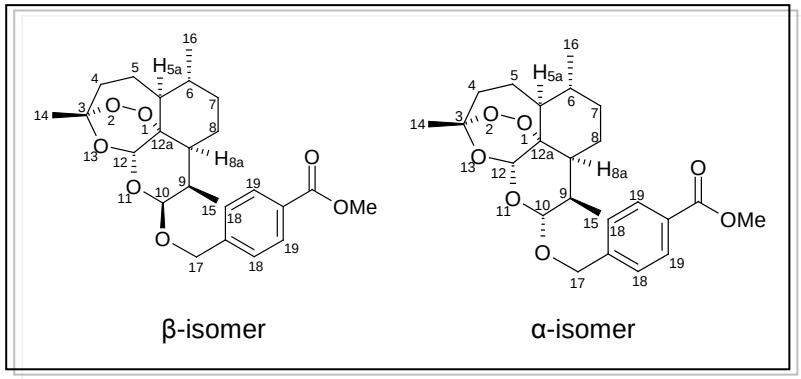
Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.1:9.9) to yield the product as a white powder (0.137 g, 37%); m.p. 62 – 64 °C. R_f (MeOH/DCM 0.5:9.5) 0.18; ν_{max} (DCM)/cm^{-1} 1741 (C=O), 1450 (C=C (Ar)), 1266 (C–N), 1048 (C–O (ester)), 847 (O–O); δ_H (400 MHz; $CDCl_3$) 8.51 (d, *J* 5.6, 1H, H2'), 7.96 (d, *J* 2.4, 1H, H8'), 7.72 (d, *J* 9.2, 1H, H5'), 7.34 (dd, *J* 9.2 and 2.4, 1H, H6'), 6.35 (d, *J* 5.6, 1H, H3'), 6.32 (d, *J* 3.6, 1H, H10), 5.49 (s, 1H, H12), 3.43 (t, *J* 5.6, 2H, H9'), 2.99 (t, *J* 6.4, 2H, H17), 2.92 (t, *J* 5.6, 2H, H11'), 2.85 (m, 1H, H9), 2.63 (t, *J* 6.4, 2H, H18), 1.94 (t, *J* 5.6, 2H, H10'), 1.42 (s, 3H, H14), 0.97 (d, *J* 6.0, 3H, H16), 0.88 (d, *J* 7.2, 3H, H15); δc (100 MHz; $CDCl_3$) 151.8, 128.4, 125.0, 121.9, 104.5, 98.4, 95.1, 88.8, 80.5, 52.4, 48.9, 45.1, 43.9, 43.5, 40.0, 37.6, 36.2, 34.7, 34.6, 34.4, 29.8, 29.7, 27.5, 25.9, 24.6, 24.2, 20.2, 12.5; *m/z* 574.2699 (MH^+); (Found: MH^+, 574.2699. $C_{30}H_{41}ClN_3O_6$ (MH^+) requires 574.2684) (Found: C, 62.66; H, 7.10; N, 7.41. $C_{30}H_{40}ClN_3O_6$ requires C, 62.76; H, 7.02; N, 7.32%).

MIC 14



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.5:9.5) to yield the product as a light-yellow oil (0.248 g, 62%);. R_f (MeOH/DCM 0.5:9.5) 0.17; ν_{max} (DCM)/cm^{-1} 1740 (C=O), 1451 (C=C (Ar)), 1266 (C–N), 1049 (C–O (ester)), 847 (O–O); δ_H (300 MHz; $CDCl_3$) 8.52 (d, *J* 5.4, 1H, H2'), 7.95 (d, *J* 2.1, 1H, H8'), 7.70 (d, *J* 9.0, 1H, H5'), 7.36 (dd, *J* 9.0 and 2.1, 1H, H6'), 6.40 (d, *J* 5.4, 1H, H3'), 6.26 (d, *J* 3.6, 1H, H10), 5.46 (s, 1H, H12), 3.33 (t, *J* 6.9, 2H, H17), 2.90 (t, *J* 6.6, 2H, H10'), 2.82 (m, 1H, H9), 2.65 (t, *J* 6.9, 2H, H18), 2.57 (t, *J* 6.6, 2H, H11'), 1.41 (s, 3H, H14), 0.97 (d, *J* 6.0, 3H, H16), 0.88 (d, *J* 7.5, 3H, H15); δc (75.5 MHz; $CDCl_3$) 171.2, 151.5, 128.2, 125.0, 121.4, 104.3, 98.8, 94.7, 88.6, 80.3, 52.2, 49.4, 44.8, 43.8, 43.0, 37.4, 36.1, 34.6, 34.3, 29.7, 29.6, 29.4, 28.5, 26.8, 25.8, 24.5, 24.0, 20.1, 12.4; *m/z* 616.3173 (MH^+); (Found: MH^+, 616.3173. $C_{33}H_{47}ClN_3O_6$ (MH^+) requires 616.3153).

***Methyl p-[(*10*-dihydroartemisininoxy)methyl]benzoate*, 1a**

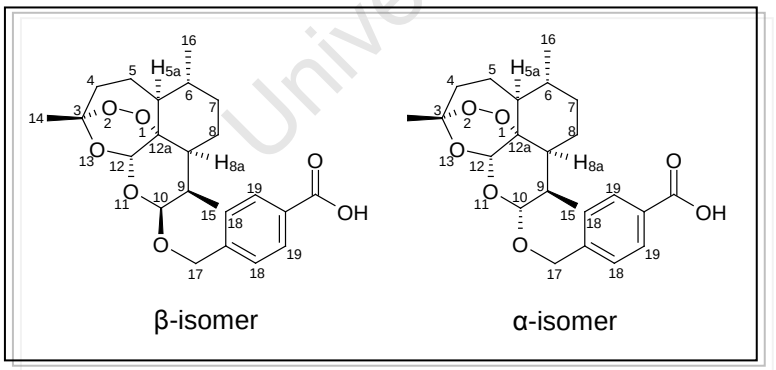


Dihydroartemisinin (1.00 g, 3.500 mmol, 1.0 eq) and 4-(hydroxylmethyl)-benzoa te (2.036 g, 12.006 mmol, 3.43 eq) were stirred in anhydrous Et_2O under N_2, where $BF_3{\cdot}Et_2O$ (0.50 ml, 3.955 mmol, 1.13 eq) was added drop-wise. The solution was

stirred under N_2 at room temperature for 23 hours, washed with 5% $NaHCO_3$ (until pH 8), H_2O (2 x 100 ml), dried (Na_2SO_4) and solvent evaporated. The crude product was purified via column chromatography (EtOAc/Hex, 1:9) to yield the pure product as colourless oil (1.460 g, 97% (both isomers)). R_f (EtOAc/Hex 1:9) 0.21. δ_H (400 MHz; $CDCl_3$) 8.01 (d, *J* 8.4, 2H, H19), 7.37 (d, *J* 8.4, 2H, H18), 5.44 (s, 1H, H12), 4.95 (d, *J* 13.2, 1H, H17), 4.91 (d, *J* 3.2, 1H, H10), 4.57 (d, *J* 13.2, 1H, H17), 3.91 (s, 3H, OCH_3), 2.69 (m, 1H, H9), 2.36 (ddd, *J* 14.0, 13.2 and 4.0, 1H, not known[a]), 2.20 – 1.50 (m, 4H, not known[a]), 1.45 (s, 3H, H14), 0.96 (d, *J* 7.6, 3H, H15), 0.94 (d, *J* 6.4, 3H, H16). α-Isomer: δ_H (400 MHz; $CDCl_3$) 8.00 (d, *J* 8.4, 2H, H19), 7.43 (d, *J* 8.8, 2H, H18), 5.34 (s, 1H, H12), 5.02 (d, *J* 14.2, 1H, H17), 4.68 (d, *J* 13.2, 1H, H17), 4.52 (d, *J* 9.2, 1H, H10), 3.91 (s, 3H, OCH_3), 2.53 (m, 1H, H9), 2.39 (ddd, *J* 14.4, 13.2 and 4.0, 1H, not known[a]), 2.20 – 1.50 (m, 4H, not known[a]), 1.45 (s, 3H, H14), 0.93 (d, *J* 5.6, 3H, H16), 0.93 (d, *J* 7.2, 3H, H15).

[a] Protons could not be assigned due to complexity.

***p-[(10-Dihydroartemisininoxy)methyl]benzoic acid (Artelinic Acid)*, 1b**

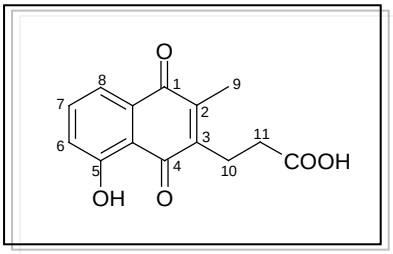


The methyl ether **1a** (1.272 g, 2.932 mmol) was stirred in 2.5% KOH/MeOH (25 ml) at room temperature for 3 days, and the solvent reduced under pressure. The residue was dissolved in H_2O (25 ml), and the pH adjusted to 8 via addition of acetic acid. The solution was extracted with Et_2O (3 x 50 ml), dried (Na_2SO_4) and solvent evaporated. The resulting oil was seeded and the pure compound crystallized out to yield white crystals (1.091 g, 89%). m.p. 141-144 °C (lit. 142-145 °C).[3] R_f (EtOAc/Hex 3.3:6.7) 0.18. β-isomer: δ_H (300 MHz;

$CDCl_3$) 8.01 (d, *J* 8.4, 2H, H19), 7.40 (d, *J* 8.4, 2H, H18), 5.45 (s, 1H, H12), 4.98 (d, *J* 13.5, 1H, H17), 4.93 (d, *J* 3.0, 1H, H10), 4.60 (d, *J* 13.5, 1H, H17), 2.70 (m, 1H, H9), 2.38 (ddd, *J* 14.0, 13.2 and 4.0, 1H, not known[b]), 2.20 – 1.50 (m, 4H, not known[b]), 1.45 (s, 3H, H14), 0.98 (d, *J* 7.2, 3H, H15), 0.95 (d, *J* 6.0, 3H, H16). α-isomer: δ_H (300 MHz; $CDCl_3$) 8.06 (d, *J* 8.4, 2H, H19), 7.46 (d, *J* 8.4, 2H, H18), 5.35 (s, 1H, H12), 5.05 (d, *J* 13.5, 1H, H17), 4.70 (d, *J* 13.2, 1H, H17), 4.53 (d, *J* 9.0, 1H, H10), 2.55 (m, 1H, H9), 2.39 (ddd, *J* 14.4, 13.2 and 4.2, 1H, not known[b]), 2.20 – 1.50 (m, 4H, not known[b]), 1.46 (s, 3H, H14), 0.95 (d, *J* 6.0, 3H, H16), 0.94 (d, *J* 7.2, 3H, H15).

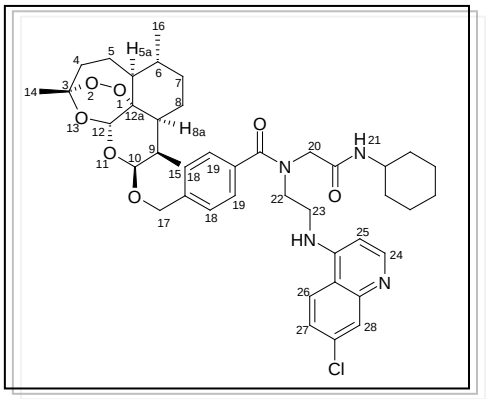
[a] Protons could not be assigned due to complexity.

3-*Carboxyethyl*-5-*hydroxy*-2-*methyl*-1,4-*naphthoquinone*, 2a



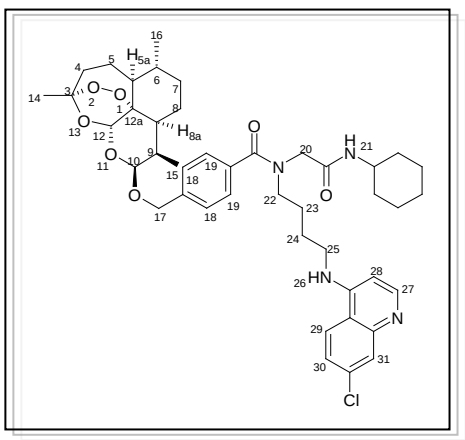
Plumbagin (1.00 g, 5.314 mmol, 1.0 eq) and succinic acid (1.902 g, 15.942 mmol, 3.0 eq) were stirred in 30% aqueous acetonitrile (40 ml) at 65 °C, followed by the addition of silver nitrate (0.456g, 2.657 mmol, 0.5 eq). A solution of ammonium persulfate (1.609 g, 6.908 mmol, 1.3 eq) in 30% aqueous acetonitrile (20 ml) was added drop-wise over 2 hours, and the resulting solution stirred at 65 – 70 °C for 3 hours. On cooling, the mixture was extracted with EtOAc (4 x 60 ml), and the organic layer washed with H_2O (3 x 20 ml), dried (Na_2SO_4) and solvent evaporated. The crude material was purified via column chromatography (DCM/MeOH 9.5:0.5) to yield the pure product as an orange powder (0.581 g, 58%). m.p. 155–159 °C.[4] R_f (DCM/MeOH, 9.5:0.5) 0.49. δ_H (400 MHz; $CDCl_3$) 7.62 (d, *J* 6.8, 1H, H8), 7.57 (t, *J* 7.6, 1H, H7), 7.23 (d, *J* 7.6, H6), 2.98 (t, *J* 7.6, 2H, H11), 2.62 (t, *J* 7.6, 2H, H10), 2.23 (s, 3H, CH3).

Ugi Adduct, 6a



Purified via column chromatography, first with EtOAC and then with EtOAC/MeOH 9.5:0.5 to yield the pure product as a white powder (0.5115 g, 89%). (m.p. 121 – 124 °C. R_f (MeOH/EtOAC, 1:9) 0.18; ν_{max} ($CHCl_3$)/cm^{-1} 3223 (N-H), 2920 (Ar-H), 1580 (C=O), 1216 (C-N), 781 (C-Cl); δ_H (400 MHz; $CDCl_3$) 8.45 (br s, 1H, H24), 8.28 (br s, 1H, H26), 7.96 (br s, 2H, H19), 7.41 (d, *J* 8.8, 2H, H18), 7.39 (br s, 1H, H27), 7.03 (br s, 1H, H27), 6.35 (br s, 1H, H25), 5.42 (s, 1H, H12), 4.88 (br s, 2H, H17 + H10), 4.51 (br s, 1H, H17), 4.09 (m, 2H, H22), 3.61 (br s, 2H, H23), 2.68 (m, 1H, H9), 2.38 (ddd, *J* 14.4 and 13.2, 4.2 Hz, 1H, not known), 1.45 – 1.93 (m, 15H), 1.44 (s, 3H, H14), 0.95 (d, *J* 6.0, 6H, H16 + H15); δc (75.5 MHz; DMSO-d_6) 171.6, 171.0, 170.2, 167.5, 151.9, 151.5, 150.1, 149.5, 149.1, 139.9, 139.2, 135.2, 134.9, 133.4, 127.5, 126.6, 126.3, 124.1, 123.7, 123.5, 117.5, 103.3, 100.6, 98.6, 87.0, 80.4, 52.0, 47.7, 36., 34.0, 32.1, 30.5, 25.6, 25.1, 24.4, 24.2, 20.0, 12.7; *m/z* 761.3596 (M^+); (Found: M^+, 761.3596. $C_{42}H_{53}ClN_4O_7$ requires *M*, 761.3584) (Found: C, 66.00, H, 7.26, N, 7.67 %; Requires C, 66.26, H, 7.02 N, 7.36 %).

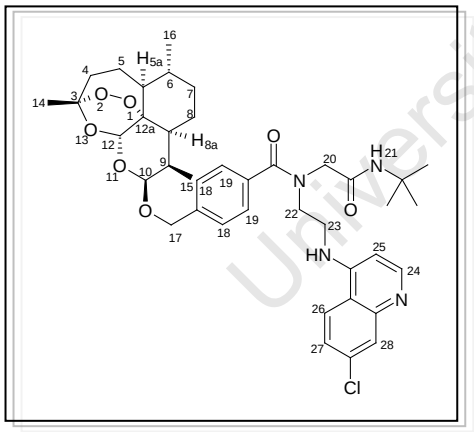
Ugi Adduct 6b



Purified via column chromatography, first with EtOAC and then with EtOAC/MeOH 9.5:0.5 to yield the pure product as a white powder (0.5009 g, 84%). (m.p. 121 – 123 °C. R_f (MeOH/EtOAC, 1:9) 0.48; ν_{max} ($CHCl_3$)/cm^{-1} 3333 (N-H), 2926 (Ar-H), 2894

(alkyls), 1666 (C=O), 1580 (Ar-C), 1010 (C-N), 753 (C-Cl); δ_H (400 MHz; $CDCl_3$) 8.45 (d, *J* 5.2, 1H, H24), 8.23 (br s, 1H, H29), 7.99 (s, 2H, H31), 7.36 (br s, 5H, H30+H18+H19), 7.13 (br s, 1H, NH), 6.31 (br s, 1H, H28), 6.10 (br s, 1H, NH), 5.44 (s, 1H, H12), 4.91 (d, *J* 12.4, 1H, H17), 4.90 (d, *J* 4.0, 1H, H10), 4.56 (d, *J* 12.4, 1H, H17), 4.11 (m, 2H, CH_2, H22), 3.81 (br s, 2H, CH_2, H25), 3.52 (br s, 2H, CH_2, H23), 3.48 (s, 2H, H20), 3.26 (br s, 2H, CH_2, H24), 2.52 (m, 1H, H9), 2.00 – 1.45 (m, 20H), 1.45 (s, 3H, H14), 0.96 (d, *J* 7.2, 3H, H15), 0.95 (d, *J* 5.6, 3H, H16); δc (100 MHz; DMSO-d_6) 170.9, 167.1, 151.8, 150.0, 149.0, 139.5, 135.7, 132.3, 127.4, 126.8, 126.6, 126.5, 124.4, 124.0, 123.9, 117.4, 103.3, 100.6, 98.6, 87.0, 80.4, 68.7, 52.0, 47.6, 45.8, 43.7, 42.3, 36.6, 36.0, 34., 32.4, 32.1, 30.5, 25.6, 24.7, 24.4, 20.0, 12.7; *m/z* 789.4125 (M^+); (Found: M^+, 789.4125. $C_{44}H_{57}ClN_4O_7$ requires *M*, 789.4122) (Found: C, 66.80, H, 6.91, N, 6.92 %; Requires C, 66.95, H, 7.28 N, 7.10 %).

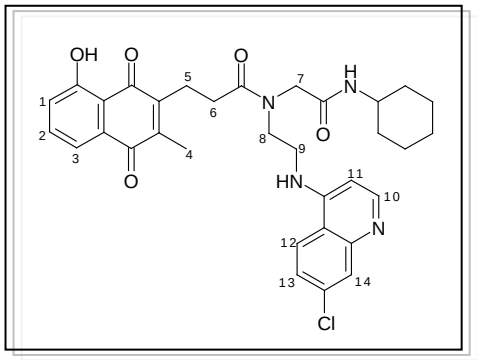
Ugi Adduct 6c



Purified via column chromatography, first with EtOAC and then with EtOAC/MeOH 9.5:0.5 to yield the pure product as a white powder (0.453 g, 82%). (m.p. 127 – 129 °C. R_f (MeOH/EtOAC, 1:9) 0.18; ν_{max} ($CHCl_3$)/cm^{-1} 3385 (N-H), 3017 (Ar-H), 1520 (C=O), 1215 (C-N), 756 (C-Cl); δ_H (400 MHz; $CDCl_3$) 8.47 (br s, 1H, H24), 8.24 (br s, 1H, H26), 8.12 (d, *J* 8.6, 2H, H19), 8.00 (s, 1H, H28), 7.40 (d, *J* 8.6, 2H, H18), 7.39 (br s, 1H, H27), 7.04 (br s, 1H, NH), 6.35 (br s, 1H, H25), 5.42 (s, 1H, H12), 4.87 (br s, 2H, H17 + H10), 4.51 (br s, 1H, H17), 3.93 (br s, 2H, H22), 3.64 (br s, 2H, H23), 2.68 (m, 1H, H9), 2.38 (ddd, *J* 14.4, 13.2 and 4.2, 1H, not known), 2.10 – 1.57 (m, 4H), 1.44 (s, 3H, H14), 1.35 (s, 9H, *t*-butyl), 0.95 (d, *J* 6.0, 6H, H16 + H15); δc (100 MHz; $CDCl_3$) 129.9, 127.2, 126.8, 125.9, 104.2, 104.2,

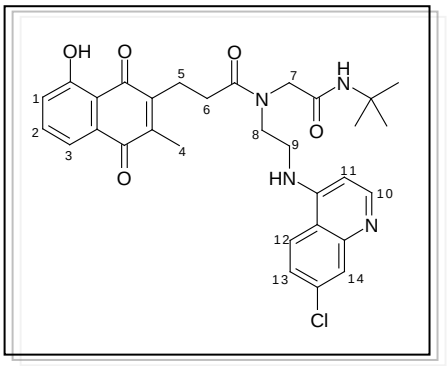
101.6, 101.6, 98.2, 88.1, 81.1, 70.7, 69.5, 69.1, 52.7, 52.6, 52.1, 44.5, 44.4, 37.5, 36.4, 34.6, 31.0, 30.9, 29.1, 28.7, 26.2, 24.7, 24.6, 20.3, 13.1; *m/z* 735.3211 (M^+); (Found: M^+, 735.3211. $C_{40}H_{51}ClN_4O_7$ requires *M*, 735.3205) (Found: C, 65.68, H, 7.28, N, 7.56 %; Requires C, 65.34, H, 6.99 N, 7.62 %).

***N*-(2-(7-chloroquinolin-4-ylamino)ethyl)-*N*-(2-(cyclohexylamino)-2-oxoethyl)-3-(3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)propanamide, 6d**



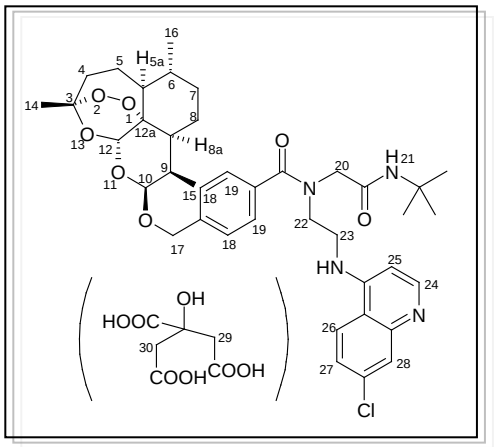
Purified via column chromatography, first with EtOAC and then with EtOAC/MeOH 9.5:0.5 to yield the pure product as an orange powder (0.1138 g, 25%). (m.p. 135 – 138 °C. R_f (MeOH/EtOAc, 1:9) 0.11; ν_{max} ($CHCl_3$)/cm^{-1} 3273 (N-H), 3020 (Ar-H), 2926 (alkyls), 1606 (C=O), 1430 (Ar-C), 1216 (C-N), 770 (C-Cl); δ_H (300 MHz; $CDCl_3$) 8.48 (d, *J* 5.7, 1H, H10), 8.44 (d, *J* 5.4, 1H, H10), 7.96 (d, *J* 1.8, 1H, H14), 7.88 (d, *J* 1.8, 1H, H14), 7.88 (d, *J* 9.0, 1H, H12), 7.74 (d, *J* 9.0, 1H, H12), 7.55 (m, 4H, (H2+H3)x2), 7.35 (dd, *J* 9.0 and 1.8, 1H, H13), 7.31 (dd, *J* 9.0 and 1.8, 1H, H13), 7.13 (d, *J* 7.5, 1H, H1), 7.13 (d, *J* 7.5, 1H, H1), 6.97 (br s, 1H, NH), 6.34 (d, *J* 5.4, 1H, H11), 6.33 (d, *J* 5.7, 1H, H11), 4.09 (s, 2H, H7),4.00 (s, 2H, H7), 3.88 (m, 2H, H8), 3.70 (br s, 4H, H8 + H9), 3.50 (m, 2H, H9), 2.97 (t, *J* 7.8, 2H, H6), 2.72 (t, *J* 8.1, 2H, H5), 2.57 (t, *J* 7.8, 2H, H6), 2.28 (t, *J* 8.1, 2H, H5), 2.24 (s, 3H, H4), 1.92 (s, 3H, H4), 1.75 (m, 11H, cyclohexyl-H), 1.22 (m, 11H, cyclohexyl-H); δc (100 MHz; $CDCl_3$) 174.9, 161.2, 151.6, 151.0, 137.3, 136.8, 136.2, 136.1, 132.0, 125.9, 125.8, 125.7, 123.9, 122.2, 122.0, 119.2, 119.1, 98.6, 98.0, 53.1, 43.0, 41.0, 32.9, 32.2, 31.5, 25.5, 25.2, 24.8, 24.7, 22.7, 22.4, 12.9; *m/z* 603.1182 (M^+); (Found: M^+, 603.1182. $C_{33}H_{35}ClN_4O_5$ requires *M*, 603.1177) (Found: C, 66.67, H, 7.07, N, 7.29 %; Requires C, 66.95, H, 7.28 N, 7.10 %).

***N*-(2-*(tert*-butylamino)-2-*oxoethyl*)-*N*-(2-(7-*chloroquinolin*-4-*ylamino*)*ethyl*)-3-(3-*methyl*-1,4-*dioxo*-1,4-*dihydronaphthalen*-2-*yl*)*propanamide*, 6e**



Purified via column chromatography, first with EtOAC and then with EtOAC/MeOH 9.5:0.5 to yield the pure product as an orange powder (0.123 g, 28%). m.p. 205 – 207 °C; R_f (EtOAc) 0.11; ν_{max} ($CHCl_3$)/cm^{-1} 3320 (N-H), 3026 (Ar-H), 1736 (C=O), 1430 (Ar-C), 1216 (C-N); 753 (C-Cl); δ_H (400 MHz; $CDCl_3$) 8.35 (d, *J* 5.2, 1H, H10), 8.34 (d, *J* 5.2, 1H, H10), 7.90 (d, *J* 9.2, 1H, H12), 7.85 (d, *J* 2.0, 1H, H14), 7.84 (d, *J* 9.2, 1H, H12), 7.79 (d, *J* 2.0, 1H, H14), 7.51 (m, 4H, (H2+H3)x2), 7.29 (dd, *J* 9.2 and 2.0, 1H, H13), 7.28 (dd, *J* 9.2 and 2.0, 1H, H13), 7.15 (d, *J* 7.2, 1H, H1), 7.14 (d, *J* 7.2, 1H, H1), 6.36 (d, *J* 5.6, 1H, H11), 6.35 (d, *J* 5.6, 1H, H11), 3.99 (s, 2H, H7), 3.79 (s, 2H, H7), 3.77 (d, *J* 5.6, 2H, H8), 3.66 (d, *J* 5.6, 2H, H9), 3.63 (d, *J* 5.6, 2H, H8), 3.49 (d, *J* 5.6, 2H, H9), 2.91 (t, *J* 8.0, 2H, H6), 2.72 (t, *J* 8.0, 2H, H6), 2.51 (t, *J* 8.0, 2H, H5), 2.30 (t, *J* 8.0, 2H, H5, 2.19 (s, 3H, H4), 1.93 (s, 3H, H4), 1.37 (s, 9H, *t*-butyl), 1.27 (s, 9H, *t*-butyl); δc (100 MHz; $CDCl_3$) 151.6, 150.3, 145.6, 136.1, 127.2, 126.4, 125.6, 123.7, 123.6, 122.5, 122.4, 119.0, 98.9, 98.2, 52.7, 52.0, 49.4, 14.2, 49.0, 48.7, 48.5, 47.7, 42.1, 40.8, 32.0, 31.4, 28.4, 22.5, 22.3, 12.6; *m/z* 577.0805 (M^+); (Found: M^+, 577.0805. $C_{31}H_{33}ClN_4O_5$ requires *M*, 577.0798) (Found: C, 66.80, H, 7.39, N, 7.48 %; Requires C, 66.95, H, 7.28 N, 7.10 %).

6c·Citrate salt

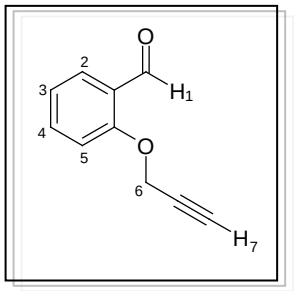


Citric acid (0.131 g, 0.680 mmol, 2.5 eq) in acetone (5 ml) was added to a solution of **6c** (0.200 g, 0.272 mmol, 1.0 eq) in acetone (5 ml). The product precipitated out over 2 days at 4°C to yield white solid (0.247 g, 98%). ν_{max} ($CHCl_3$)/cm^{-1} 3419 (OH (alcohol)), 3317 (N-H), 3017 (Ar-H), 2962 (OH (acid)), 1707 (C=O), 1612 (Ar-H), 1215 (C-N), 1007 (C-O), 752 (C-Cl); δ_H (300 MHz; CD_3OD) 8.51 (br s, 1H, H24), 8.43 (br s, 1H, H26), 7.91 (br s, 1H, H28), 7.61 (br s, 1H, H27), 7.39 (br s, 2H, H19), 6.99 (br s, 2H, H18), 6.42 (br s, 1H, H25), 5.44 (s, 1H, H12), 4.04 (br s, 2H, H22), 3.95 (br s, 2H, H23), 2.58 (m, 1H, H9), 2.33 (td, *J* 14.4 and 13.0, 4.2 Hz, 1H, not known), 2.20 – 1.50 (m, 4H, not known), 1.44 (br s, 4H, H29+H30), 1.37 (s, 3H, H14), 1.32 (s, 9H, *t*-butyl), 0.94 (d, *J* 6.0, 6H, H16 + H15); *m/z* 927.4471 (M^+); (Found: M^+, 927.4471. $C_{40}H_{51}N_4ClO_7$ requires *M*, 927.4459) (Found: C, 59.76, H, 6.63, N, 5.74 %; Requires C, 59.57, H, 6.41, N, 6.04 %).

General procedure for the synthesis of propargyl-benzaldehydes **7a**, **7b** and **7c**:

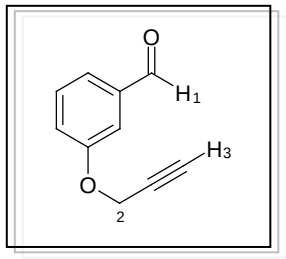
Propargyl bromide (3.2 ml, 80%w/w solution in toluene, 28.9 mmol) was added drop-wise to a mixture of the hydroxyl benzaldehyde (3.0 g, 24.1 mmol) and K_2CO_3 (4.99 g, 36.1 mmol) in anhydrous DMF (30 ml) at r.t., and stirred for 18 h. The reaction mixture was then diluted with H_2O (100 ml), and extracted with EtOAc (4 x 50 ml), washed with H_2O (4 x 200 ml) and brine (150 ml), dried (Na_2SO_4) and solvent removed to yield the crude products.

2-*(Prop-2-ynyloxy)benzaldehyde*, 7a



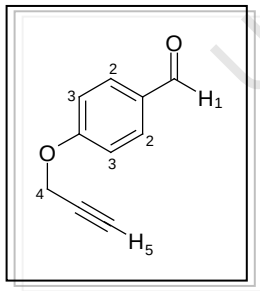
The crude product was purified via column chromatography, eluting with EtOAc/Hex (1:9) to yield the pure product as white solid (3.80 g, 99 %), m.p. 70 – 73 °C (lit. 69 – 72 °C).[5] Rf (EtOAc/Hex, 1:9) 0.20. δ_H (400 MHz; $CDCl_3$) 10.4 (s, 1H, H1), 7.81 (m, 1H, H2), 7.1 – 6.9 (m, 3H, H3, H4 and H5), 4.79 (d, *J* 2.4, 2H, H6), 2.56 (t, *J* 2.4, 1H, H7).

3-*(Prop-2-ynyloxy)benzaldehyde*, 7b



The crude product was purified via column chromatography, eluting with EtOAc/Hex (1:9) to yield the pure product as a yellow oil (3.54 g, 93 %).[6] Rf (EtOAc/Hex, 2:8) 0.48. δ_H (400 MHz; $CDCl_3$) 9.97 (s, 1H, H1), 7.49 (m, 3H, Ar-H), 7.25 (m, 1H, Ar-H), 4.75 (d, *J* 2.4, 2H, H2), 2.54 (t, *J* 2.4, 1H, H3).

4-*(Prop-2-ynyloxy)benzaldehyde*, 7c

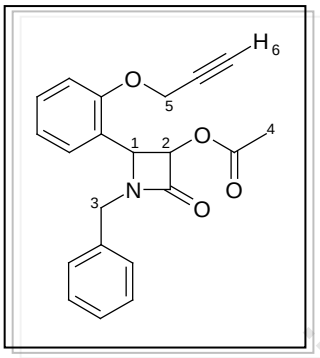


The crude product was purified via column chromatography, eluting with EtOAc/Hex (2:8) to yield the pure product as a white powder (3.75 g, quantitative), m.p. 77 – 80 °C (lit. 77 – 79 °C).[7] Rf (EtOAc/Hex, 3:7) 0.53. δ_H (400 MHz; $CDCl_3$) 9.87 (s, 1H, H1), 7.83 (d, *J* 8.4, 2H, H2), 7.07 (d, *J* 8.4, 2H, H3), 4.76 (d, *J* 2.4, 2H, H4), 2.56 (t, *J* 2.4, 1H, H5).

General procedures for Staudinger products

The aldehyde (1.0 eq) and amine (1.2 eq) were stirred in anhydrous MeOH (15 ml) at r.t., and stirred further for 18 h. The solvent was removed and the crude imine was dried on high-vacuum pump for 1 h. The dried imine was then stirred with Et_3N (3.0 eq) in anhydrous DCM (10 ml) at -78 °C and under N_2, followed by the drop-wise addition of acetoxy acetyl chloride (1.5 eq) in anhydrous DCM (6 ml). The mixture was allowed to slowly warm to r.t., and stirred further overnight. When the reaction has completed, the reaction mixture was washed with saturated $NaHCO_3$ (20 ml) and brine (20 ml), dried (Na_2SO_4) and solvent removed to yield the crude products.

1-*Benzyl*-2-*oxo*-4-*[2-(prop*-2-*ynyloxy)phenyl]azetidin*-3-*yl acetate,* BL01

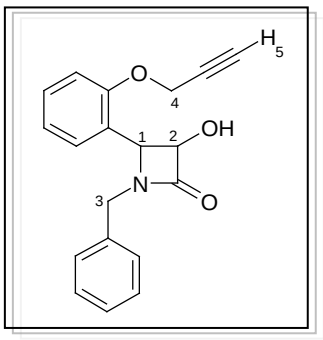


The crude product was purified via column chromatography, eluting with Hex/DCM (2:8) to yield the pure product as a white powder, (0.42 g, 81%); m.p. 89 – 91 °C; R_f (Hex/DCM, 2:8) 0.24; ν_{max} (DCM)/cm^{-1} 3275 (C≡C–H), 2116 (C≡C), 1758 (C=O), 1223 (C–O–C (ester)) and 1028 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.38 – 7.00 (m, 9H, Ar–H), 5.84 (d, *J* 4.8, 1H, H2), 5.21 (d, *J* 4.8, 1H, H1), 4.83 (d, *J* 14.8, 1H, H3), 4.66 (dd, *J* 16.0 and 2.4, 1H, H5), 4.60 (dd, *J* 16.0 and 2.4, 1H, H5), 3.96 (d, *J* 14.8, 1H, H3), 2.43 (t, *J* 2.4, 1H, H6), 1.70 (s, 3H, H4); δc (100 MHz; $CDCl_3$) 169.1, 164.6, 157.4, 134.5, 129.4, 128.4, 128.4, 128.0, 121.6, 115.2, 114.6, 78.0, 77.4, 75.7, 60.6, 56.1, 44.7 and 19.8; *m/z* 349.3872 (M^+); (Found: M^+, 349.3872. $C_{21}H_{19}NO_4$ requires *M*, 349.3862) (Found: C, 71.98; H, 5.61; N, 3.88 requires C, 72.19; H, 5.48; N, 4.01%).

General procedure for the free alcohol products **BL 02**, **BL 06** and **BL 08**.

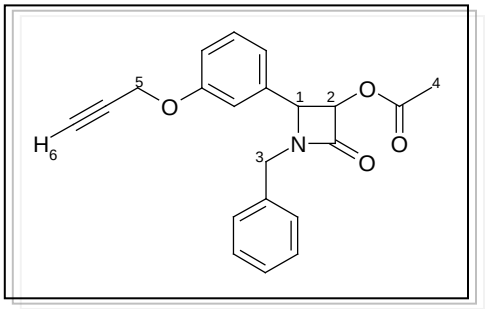
The acetates (1.0 eq) were stirred in a solution of K_2CO_3 (1.3 eq) in MeOH/H_2O (5:2 ml) at r.t. for 18 h. H_2O (5 ml) was added to the reaction mixture, and neutralized with 1 M HCl. The mixture was extracted with EtOAc (3 x 10 ml), washed with H_2O (15 ml) and brine (15 ml), dried (Na_2SO_4) and solvent removed *in vacuo* to yield the crude products.

1-*Benzyl*-3-*hydroxy*-4-*[2-(prop-2-ynyloxy)phenyl]azetidin*-2-*one*, BL02



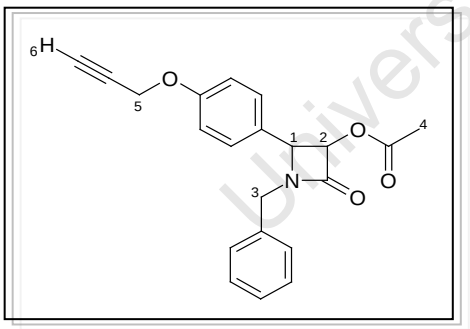
The crude product was purified via column chromatography, using EtOAc/Hex (1:1) as the eluent to yield the product as a white powder, (0.210 g, 80%); m.p. 105 – 108 °C; R_f (EtOAc/Hex, 1:1) 0.18; ν_{max} (DCM)/cm^{-1} 3470 (O–H), 3283 (C≡C–H), 2116 (C≡C), 1736 (C=O), 1223 (C–O–C (ester)) and 1021 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.4 – 7.0 (m, 9H, Ar–H), 5.07 (d, *J* 4.8, 1H, H2), 4.92 (d, *J* 4.8, 1H, H1), 4.80 (d, *J* 14.8, 1H, H3), 4.71 (dd, *J* 16.0 and 2.4, 1H, H4), 4.64 (dd, *J* 16.0 and 2.4, 1H, H4), 3.95 (d, *J* 14.8, 1H, H3), 2.51 (t, *J* 2.4, 1H, H5); δc (100 MHz; $CDCl_3$) 169.0, 155.8, 135.2, 129.7, 129.6, 128.7, 128.6, 127.8, 122.9, 122.1, 112.9, 78.7, 78.0, 76.2, 59.3, 56.2 and 44.6; *m/z* 307.3497 (M^+); (Found: M^+, 307.3497. $C_{19}H_{17}NO_3$ requires *M*, 307.3489) (Found: C, 74.51; H, 5.66; N, 4.28. requires C, 74.25; H, 5.58; N, 4.56%).

1-*Benzyl*-2-*oxo*-4-*[*3-*(prop*-2-*ynyloxy)phenyl]azetidin*-3-*yl acetate,* BL03



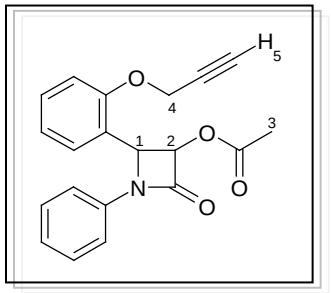
The crude product was purified via column chromatography, eluting with (EtOAc/Hex, 3:7) to yield the pure product as a light yellow oil (1.1 g, 84%); R_f (EtOAc/Hex, 3:7) 0.31; ν_{max} (DCM)/cm^{-1} 3275 (C≡C–H), 2116 (C≡C), 1762 (C=O), 1223 (C–O–C (ester)) and 1035 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.38 – 6.80 (m, 9H, Ar–H), 5.78 (d, *J* 4.4, 1H, H2), 5.29 (d, *J* 14.8, 1H, H3), 4.72 (d, *J* 4.4, 1H, H1), 4.65 (d, *J* 2.4, 2H, H5), 3.95 (d, *J* 14.8, 1H, H3), 2.61 (t, *J* 2.4, 1H, H6), 1.70 (s, 3H, H4); δc (100 MHz; $CDCl_3$) 169.0, 164.6, 157.5, 134.5, 134.3, 129.5, 128.4, 128.5, 128.0, 121.6, 115.4, 114.6, 78.3, 77.3, 75.7, 60.8, 55.9, 44.7 and 19.9; *m/z* 349.3871 (M^+); (Found: M^+, 349.3871. $C_{21}H_{19}NO_4$ requires *M*, 349.3862).

1-*Benzyl*-2-*oxo*-4-*[*4-*(prop*-2-*ynyloxy)phenyl]azetidin*-3-*yl acetate,* BL04



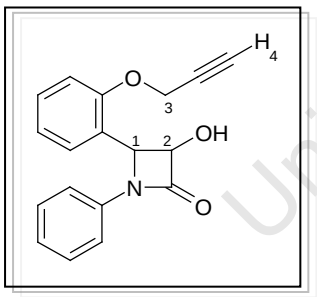
The crude product was purified via column chromatography, eluting with (EtOAc/Hex, 3:7) to yield the pure product as a light yellow oil (0.63 g, 96%); R_f (EtOAc/Hex, 3:7) 0.26; ν_{max} (DCM)/cm^{-1} 3275 (C≡C–H), 2116 (C≡C), 1765 (C=O), 1223 (C–O–C (ester)) and 1028 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.29 (m, 3H, Ar–H), 7.14 (m, 4H, Ar–H), 6.95 (m, 2H, Ar–H), 5.73 (d, *J* 4.8, 1H, H2), 4.84 (d, *J* 14.8, 1H, H3), 4.71 (d, *J* 4.8, 1H, H1), 4.70 (d, *J* 2.4, 2H, H5), 3.89 (d, *J* 14.4, 1H, H3), 2.52 (t, *J* 2.4, 1H, H6), 1.70 (s, 3H, H4); δc (100 MHz; $CDCl_3$) 169.1, 164.7, 157.9, 134.5, 129.7, 128.9, 128.0, 125.3, 114.9, 78.3, 77.5, 75.7, 60.5, 55.9, 44.5 and 19.9; *m/z* 349.3868 (M^+); (Found: M^+, 349.3868. $C_{21}H_{19}NO_4$ requires *M*, 349.3862).

2-*Oxo*-1-*phenyl*-4-*[2-(prop*-2-*ynyloxy)phenyl]azetidin*-3-*yl acetate*, BL05



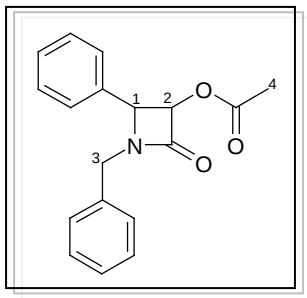
The crude product was purified via column chromatography, using EtOAc/Hex (2:8) as the eluent to yield the product as a cream-coloured powder, (0.49 g, 75%); m.p. 109 – 112 °C; R_f (EtOAc/Hex, 2:8) 0.25; ν_{max} (DCM)/cm^{-1} 3275 (C≡C–H), 2123 (C≡C), 1758 (C=O), 1223 (C–O–C (ester)) and 1020 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.40 – 6.93 (m, 9H, Ar–H), 6.11 (d, *J* 5.2, 1H, H2), 5.78 (d, *J* 5.2, 1H, H1), 4.79 (dd, *J* 16.0 and 2.4, 1H, H4), 4.73 (dd, *J* 16.0 and 2.4, 1H, H4), 2.54 (t, *J* 2.4, 1H, H5), 1.75 (s, 3H, H3); δc (100 MHz; $CDCl_3$) 168.7, 162.5, 155.7, 137.0, 129.6, 129.2, 128.4, 124.6, 121.4, 117.5, 112.3, 78.4, 75.8, 75.5, 58.9, 56.3, 56.1 and 20.0; *m/z* 335.3598 (M^+); (Found: M^+, 335.3598 $C_{20}H_{17}NO_4$ requires *M*, 335.3593) (Found: C, 71.89; H, 5.12; N, 4.55. requires C, 71.63; H, 5.11; N, 4.18%).

3-*Hydroxy*-1-*phenyl*-4-*[2-(prop*-2-*ynyloxy)phenyl]azetidin*-2-*one*, BL06



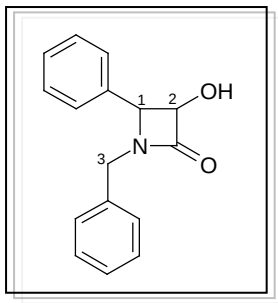
The crude product was purified via column chromatography, using EtOAc/Hex (2.5:7.5) as the eluent to yield the product as a yellow-brown powder, (0.23 g, 85%); m.p. 120 – 122 °C; R_f (EtOAc/Hex, 4:6) 0.42; ν_{max} (DCM)/cm^{-1} 3471 (O–H), 3275 (C≡C–H), 2122 (C≡C), 1736 (C=O) and 1023 (C–O (ether)); δ_H (300 MHz; $CDCl_3$) 7.3 – 6.5 (m, 9H, Ar–H), 5.39 (br s, 1H, H2), 4.84 (dd, *J* 16.0 and 2.4, 2H, H3), 4.65 (d, *J* 5.2, 1H, H1), 2.54 (t, *J* 2.4, 1H, H4), 1.59 (br s, 1H, OH); δc (100 MHz; $CDCl_3$) 174.0, 155.1, 146.2, 129.1, 128.5, 128.3, 127.9, 124.4, 119.9, 117.8, 113.9, 112.1, 78.5, 76.0, 72.4, 56.3, 53.0 and 52.9; *m/z* 293.3231 (M^+); (Found: M^+, 293.3231. $C_{18}H_{15}NO_3$ requires *M*, 293.3220) (Found: C, 73.93; H, 5.41; N, 4.45. requires C, 73.71; H, 5.15; N, 4.78%).

1-*Benzyl*-2-*oxo*-4-*phenylazetidin*-3-*yl acetate*, BL07



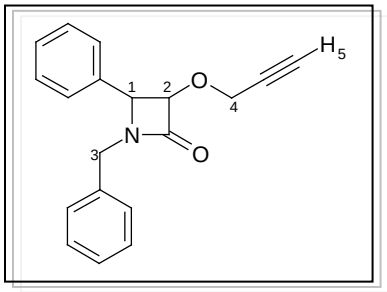
The crude product was purified via column chromatography, eluting with EtOAc/Hex (3:7) to yield the pure product as a colourless oil, (0.77 g, 78%); R_f (EtOAc/Hex, 3:7) 0.41; ν_{max} (DCM)/cm^{-1} 1751 (C=O), 1223 (C–O–C (ester)) and 1021 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.34 (m, 3H, Ar–H), 7.29 (m, 3H, Ar–H), 7.20 (m, 2H, Ar–H), 7.15 (m, 2H, Ar-H), 5.77 (d, *J* 4.4, 1H, H2), 4.87 (d, *J* 14.8, 1H, H3), 4.75 (d, *J* 4.4, 1H, H1), 4.91 (d, *J* 14.8, 1H, H3), 1.66 (s, 3H, H4); δc (100 MHz; $CDCl_3$) 169.1, 164.7, 134.5, 132.5, 128.9, 128.7, 128.5, 128.4, 128.1, 77.4, 60.9, 44.6 and 19.8; *m/z* 295.3381 (M^+); (Found: M^+, 295. 3381. $C_{18}H_{17}NO_3$ requires *M*, 295.3379).

1-*Benzyl*-3-*hydroxy*-4-*phenylazetidin*-2-*one*. BL08



The crude product was purified via column chromatography, using EtOAc/Hex (1:1) as the eluent to yield the product as a white powder, (0.55 g, 84%); m.p. 119 – 121 °C; R_f (EtOAc/Hex, 1:1) 0.35; ν_{max} (DCM)/cm^{-1} 3333 (O–H) 1733 (C=O); δ_H (400 MHz; $CDCl_3$) 7.39 (m, 3H, Ar–H), 7.27 (m, 5H, Ar-H), 7.13 (m, 2H, Ar–H), 5.03 (d, *J* 4.8, 1H, H2), 4.85 (d, *J* 14.8, 1H, H3), 4.66 (d, *J* 4.8, 1H, H1), 3.89 (d, *J* 14.8, 1H, H3), 1.63 (br s, 1H, OH); δc (100 MHz; $CDCl_3$) 168.9, 134.8, 133.5, 128.9, 128.8, 128.5, 127.9, 78.1, 61.9, 44.2; *m/z* 253.3014 (M^+); (Found: M^+, 253.3014 $C_{16}H_{15}NO_2$ requires *M*, 253.3006) (Found: C, 75.59; H, 6.02; N, 5.31. requires C, 75.87; H, 5.97; N, 5.53%).

1-*Benzyl*-4-*phenyl*-3-*(prop*-2-*ynyloxy)azetidin*-2-*one*, BL09

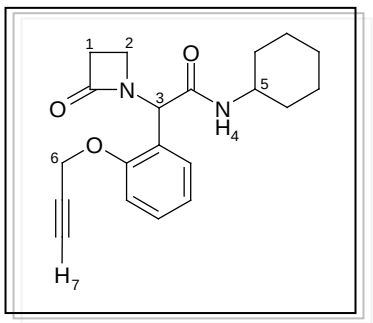


Propargyl bromide (0.13 ml, 80% w/w solution in toluene, 1.42 mmol) was added drop-wise to a stirred solution of NaH (60% suspension in oil, 0.057 g, 1.42 mmol) and **BL08** (0.30 g, 1.18 mmol) in anhydrous DMF (5 ml) at 0 °C , and stirred for 1 h. H_2O (20 ml) was added to the reaction mixture, and extracted with EtOAc (3 x 30 ml), washed with H_2O (4 x 30 ml) and brine (40 ml), dried (Na_2SO_4) and solvent removed *in vacuo*. The crude product was purified via column chromatography, using EtOAc/Hex (3:7) as the eluent to yield the product as a light yellow oil, (0.30 g, 92%); R_f (EtOAc/Hex, 3:7) 0.51; ν_{max} (DCM)/cm^{-1} 3289 (C≡C–H), 2115 (C≡C), 1751 (C=O) and 1021 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.36 (m, 3H, Ar–H), 7.28 (m, 5H, Ar–H), 7.12 (m, 2H, Ar–H), 5.07 (d, *J* 4.4, 1H, H2), 4.83 (d, *J* 14.8, 1H, H3), 4.59 (d, *J* 4.4, 1H, H1), 4.05 (dd, *J* 16.0 and 2.4, 1H, H4), 3.82 (d, *J* 14.8, 1H, H3), 3.78 (dd, *J* 16.0 and 2.4, 1H, H4), 2.35 (d, *J* 2.4, 1H, H5); δc (100 MHz; $CDCl_3$) 134.9, 133.3, 128.8, 128.7, 128.6, 128.5, 128.4, 127.9, 82.4, 78.2, 75.7, 75.4, 61.0, 57.4 and 44.0; *m/z* 291.3499 (M^+); (Found: M^+, 291.3499. $C_{19}H_{17}NO_2$ requires *M*, 291.3495).

General procedures for the Ugi adducts

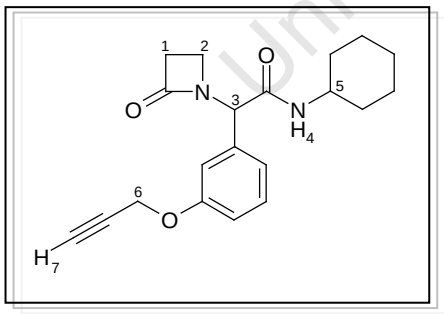
Propargyloxy-benzaldehyde (1.5 eq), β-alanine (1.0 eq) and isocyanide (1.0 eq) were stirred in MeOH (10 ml) at r.t. for 3 days. The solvent was removed *in vacuo* to yield the crude products.

***N*-Cyclohexyl-2-(2-oxoazetidin-1-yl)-2-[2-(prop-2-ynyloxy)phenyl]acetamide, BL10**



The crude product was purified via column chromatography, using EtOAc/Hex (6:4) as the eluent to yield the product as a white powder, (0.37 g, 88%); m.p. 152 – 154 °C; R_f (EtOAc/Hex, 1:1) 0.34; ν_{max} (DCM)/cm^{-1} 3289 (C≡C–H), 2116 (C≡C), 1740 (C=O), 1660 (C=O) and 1220 (C–N); δ_H (400 MHz; $CDCl_3$) 7.34 (m, 2H, Ar–H), 7.06 (m, 2H, Ar–H), 5.82 (d, 7.6, 1H, H4), 5.65 (s, 1H, H3), 4.76 (d, *J* 2.8, 2H, H6), 3.79 (m, 1H, H5), 3.67 (td, *J* 5.6 and 2.8, 1H, H1), 3.21 (td, *J* 5.6 and 2.8, 1H, H1), 3.00 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.88 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.54 (t, *J* 2.4, 1H, H7), 1.9 – 1.0 (m, 10H, cyclohexyl–H); δc (100 MHz; $CDCl_3$) 168.1, 167.7, 154.9, 129.9, 124.1, 121.9, 112.5, 78.2, 76.0, 64.5, 56.1, 54.8, 48.5, 39.6, 36.3, 32.8, 32.7, 25.5, 24.7 and 24.6; *m/z* 340.4230 (M^+); (Found: M^+, 340.4230. $C_{20}H_{24}N_2O_3$ requires *M*, 340.4222) (Found: C, 70.60; H, 7.08; N, 8.01. requires C, 70.57; H, 7.11; N, 8.23%).

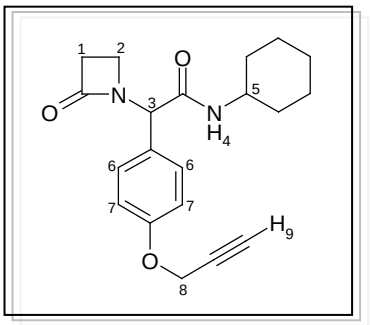
***N*-Cyclohexyl-2-(2-oxoazetidin-1-yl)-2-[3-(prop-2-ynyloxy)phenyl]acetamide, BL11**



The crude product was purified via column chromatography, using EtOAc/Hex (6:4) as the eluent to yield the product as a white powder, (0.38 g, 90%); m.p. 134 – 156 °C; R_f (EtOAc/Hex, 1.1) 0.24; ν_{max} (DCM)/cm^{-1} 3283 (C≡C–H), 2116 (C≡C), 1736 (C=O), 1653 (C=O) and 1248 (C–N); δ_H (300 MHz; $CDCl_3$) 7.30 (s, 1H, Ar–H), 6.96 (m, 3H, Ar–H), 6.02 (d, 8.0, 1H, H4), 5.28 (s, 1H, H3), 4.68 (d, *J* 2.4, 2H, H6), 3.78 (m, 1H, H5), 3.61 (td, *J* 5.6 and 3.2, 1H, H1), 3.19 (td, *J* 5.6 and 3.2, 1H, H1), 2.90 (ddd, *J* 14.6, 5.6 and 3.2, 1H, H2), 2.86 (ddd, *J* 14.6, 5.6 and 3.2, 1H, H2), 2.51 (t, *J* 2.4, 1H, H7), 1.9 – 1.0 (m,

10H, cyclohexyl–H); δc (100 MHz; $CDCl_3$) 167.9, 167.3, 158.0, 136.4, 130.2, 121.3, 115.1, 114.8, 78.3, 75.7, 61.2, 60.2, 55.9, 48.7, 39.0, 36.3, 32.8, 25.4 and 24.7; *m/z* 340.4228 (M^+); (Found: M^+, 340.4228. $C_{20}H_{24}N_2O_3$ requires *M*, 340.4222) (Found: C, 70.65; H, 7.07; N, 8.19. requires C, 70.57; H, 7.11; N, 8.23%).

***N-Cyclohexyl-2-(2-oxoazetidin-1-yl)-2-[4-(prop-2-ynyloxy)phenyl]acetamide*, BL12**

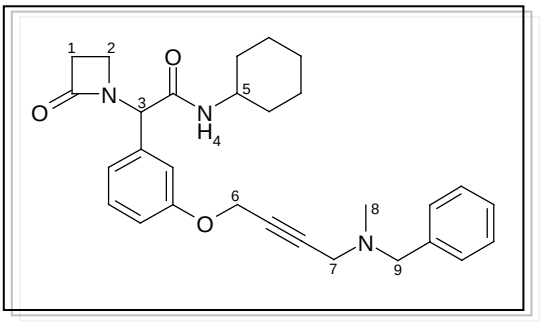


The crude product was purified via column chromatography, using EtOAc/Hex (7:3) as the eluent to yield the product as a light-yellow oil, (0.35 g, 83%); R_f (EtOAc/Hex, 1:1) 0.21; ν_{max} (DCM)/cm^{-1} 3290 (C≡C–H), 2116 (C≡C), 1730 (C=O), 1660 (C=O) and 1223 (C–N); δ_H (400 MHz; $CDCl_3$) 7.28 (d, *J* 8.8, 2H, H6), 6.97 (d, 2H, H7), 5.99 (d, 6.8, 1H, H4), 5.27 (s, 1H, H3), 4.69 (d, *J* 2.4, 2H, H8), 3.76 (m, 1H, H5), 3.58 (td, *J* 5.6 and 2.8, 1H, H1), 3.13 (td, *J* 5.6 and 2.8, 1H, H1), 2.98 (ddd, *J* 14.4, 5.6 and 2.8, 1H, H2), 2.84 (ddd, *J* 14.4, 5.6 and 2.8, 1H, H2), 2.52 (t, *J* 2.4, 1H, H9), 1.87 (m, 2H, cyclohexyl–H), 1.68 (m, 3H, cyclohexyl–H), 1.34 (m, 2H, cyclohexyl–H), 1.12 (m, 3H, cyclohexyl–H); δc (100 MHz; $CDCl_3$) 167.8, 167.7, 157.7, 129.5, 127.9, 115.4, 78.3, 75.8, 59.5, 55.9, 48.7, 38.8, 36.2, 32.8, 25.5, 24.7 and 24.6; *m/z* 340.4231 (M^+); (Found: M^+, 340.4231. $C_{20}H_{24}N_2O_3$ requires *M*, 340.4222).

General procedure for Mannich Reactions

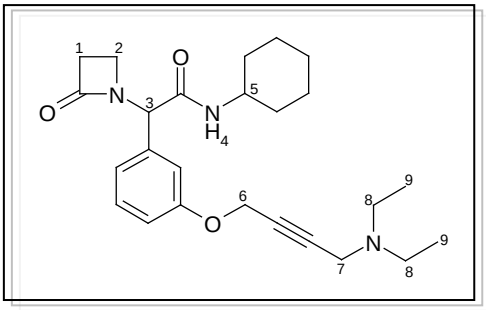
The terminal acetylene (1.0 eq), aqueous formaldehyde (5.0 eq), amine (1.2 eq) and copper (I) iodide (0.02 eq) were stirred in commercial DMSO at 25 °C for 2 h. H_2O was added to the reaction mixture, and basified to pH 8. The mixture was extracted with EtOAc, washed with brine, dried (Na_2SO_4) and solvent reduced to yield the crude products.

***N*-Cyclohexyl-2-*[*3-({4-[benzyl(methyl)amino]but-2-ynyl}oxy)phenyl]-2-*(*2-oxoazetidin-1-*yl)acetamide*, BL13**



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.5:9.5) to yield the product as a white powder, (75.1 mg g, 72%); m.p. 88 – 91 °C; R_f (MeOH/DCM, 0.5:9.5) 0.33; ν_{max} (DCM)/cm^{-1} 2312 (C≡C), 1742 (C=O), 1672 (C=O), 1249 (C–N) and 1025 (C–O (ether)); δ_H (300 MHz; $CDCl_3$) 7.26 (m, 6H, Ar–H), 6.98 (m, 3H, Ar–H), 5.90 (d, 7.5, 1H, H4), 5.24 (s, 1H, H3), 4.76 (t, *J* 1.8, 2H, H6), 3.73 (m, 1H, H5), 3.56 (td, *J* 5.7 and 2.7, 1H, H1), 3.50 (s, 2H, H9), 3.31 (t, *J* 1.8, 2H, H7), 3.11 (td, *J* 5.7 and 2.7, 1H, H1), 2.95 (ddd, *J* 14.7, 5.7 and 2.7, 1H, H2), 2.79 (ddd, *J* 14.7, 5.7 and 2.7, 1H, H2), 2.29 (s, *3*H, H8), 2.0 – 0.8 (m, 10H, cyclohexyl–H); δc (75.5 MHz; $CDCl_3$) 167.9, 167.4, 158.1, 138.1, 136.4, 130.0, 129.1, 128.3, 127.1, 121.0, 115.1, 114.9, 82.8, 80.0, 60.1, 59.9, 56.2, 48.6, 45.1, 41.9, 39.0, 36.2, 32.7, 29.6, 25.4, 24.7 and 24.6 *m/z* 473.6162 (M^+); (Found: M^+, 473.6162. $C_{29}H_{35}N_3O_3$ requires *M*, 473.6152) (Found: C, 73.24; H, 7.57; N, 8.80. requires C, 73.54; H, 7.45; N, 8.87%).

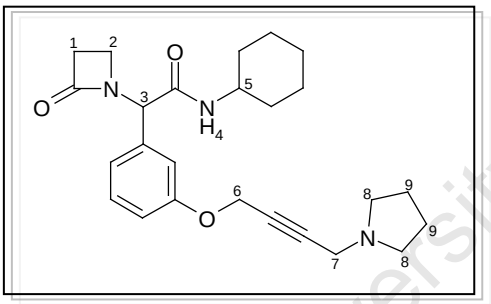
***N*-Cyclohexyl-2-*(*3-{[4-*(*diethylamino)but-2-ynyl]oxy}phenyl)-2-*(*2-oxoazetidin-1-*yl) *acetamide*, BL14**



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.1:9.9 to 1:9) to yield the product as a white powder, (83.0 mg, 89%); m.p. 102 – 105 °C; R_f (EtOAc) 0.12; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1741 (C=O), 1672 (C=O) and 1248 (C–N) and 1022 (C–O (ether)); δ_H (300 MHz; $CDCl_3$) 7.29 (m, 1H, Ar–H), 6.96 (m,

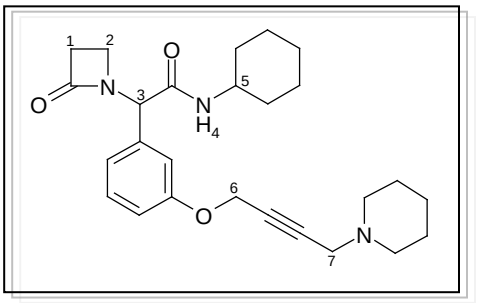
3H, Ar–H), 5.94 (d, 7.8, 1H, H4), 5.25 (s, 1H, H3), 4.71 (t, *J* 2.1, 2H, H6), 3.76 (m, 1H, H5), 3.60 (td, *J* 6.0 and 2.4, 1H, H1), 3.44 (t, *J* 2.1, 2H, H7), 3.17 (td, *J* 6.0 and 2.4, 1H, H1), 2.99 (ddd, *J* 14.7, 6.0 and 2.4, 1H, H2), 2.86 (ddd, *J* 14.7, 6.0 and 2.4, 1H, H2), 2.51 (q, *J* 7.5, 4H, H8), 2.0 – 1.1 (m, 10H, cyclohexyl–H), 1.03 (t, *J* 7.5, 6H, H9); δc (100 MHz; $CDCl_3$) 167.9, 167.4, 158.2, 136.3, 130.0, 120.9, 115.1, 114.8, 82.9, 79.2, 60.1, 56.3, 48.7, 47.2, 41.0, 39.0, 36.3, 32.8, 25.4, 24.7, 24.6 and 12.5; *m/z* 425.5715 (M^+); (Found: M^+, 425.5715. $C_{25}H_{35}N_3O_3$ requires *M*, 425.5712) (Found: C, 70.41; H, 7.98; N, 9.85. requires C, 70.56; H, 8.29; N, 9.87%).

***N*-Cyclohexyl-2-(2-oxoazetidin-1-yl)-2-{3-[(4-pyrrolidin-1-ylbut-2-ynyl)oxy]phenyl} acetamide, BL15**



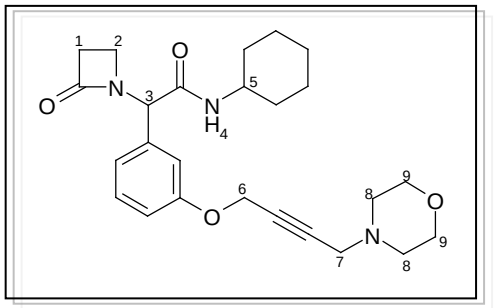
Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.3:9.7 to 1:9) to yield the product as a white powder, (74.2 mg, 80%); m.p. 89 – 91 °C; R_f (MeOH/EtOAc, 0.3:9.7) 0.11; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1742 (C=O), 1672 (C=O) and 1245 (C–N) and 1022 (C–O (ether)); δ_H (300 MHz; $CDCl_3$) 7.26 (m, 1H, Ar–H), 6.97 (m, 3H, Ar–H), 6.00 (d, 7.8, 1H, H4), 5.26 (s, 1H, H3), 4.71 (t, *J* 2.1, 2H, H6), 3.76 (m, 1H, H5), 3.61 (td, *J* 6.0 and 2.4, 1H, H1), 3.44 (t, *J* 2.1, 2H, H7) , 3.17 (td, *J* 6.0 and 2.4, 1H, H1), 2.99 (ddd, *J* 14.7, 6.0 and 2.4, 1H, H2), 2.86 (ddd, *J* 14.7, 6.0 and 2.4, 1H, H2), 2.59 (m, 4H, H8), 1.78 (m, 4H, H9), 2.1 – 1.0 (m, 10H, cyclohexyl–H); δc (100 MHz; $CDCl_3$) 167.9, 167.4, 158.2, 136.4, 130.0, 121.0, 120.9, 115.1, 114.9, 83.7, 78.8, 60.0, 56.3, 52.6, 48.7, 43.3, 39.0, 36.3, 32.7, 32.6, 25.4, 24.7, 24.6 and 23.8; *m/z* 423.5558 (M^+); (Found: M^+, 423.5558. $C_{25}H_{33}N_3O_3$ requires *M*, 423.5553) (Found: C, 70.67; H, 7.81; N, 9.75. requires C, 70.89; H, 7.85; N, 9.92%).

***N-Cyclohexyl-2-(2-oxoazetidin-1-yl)-2-{3-[(4-piperidin-1-ylbut-2-ynyl)oxy]phenyl} acetamide*, BL16**



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.3:9.7) to yield the product as a white powder, (70.8 mg, 73%); m.p. 107 – 109 °C; R_f (MeOH/EtOAc, 0.3:9.7) 0.24; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1741 (C=O), 1672 (C=O) and 1262 (C–N) and 1015 (C–O (ether)); δ_H (300 MHz; $CDCl_3$) 7.29 (m, 1H, Ar–H), 6.96 (m, 3H, Ar–H), 5.92 (d, 7.8, 1H, H4), 5.25 (s, 1H, H3), 4.71 (t, *J* 1.8, 2H, H6), 3.78 (m, 1H, H5), 3.60 (td, *J* 5.7 and 2.4, 1H, H1), 3.28 (t, *J* 1.8, 2H, H7), 3.17 (td, *J* 5.7 and 2.4, 1H, H1), 3.01 (ddd, *J* 14.7, 5.7 and 2.4, 1H, H2), 2.87 (ddd, *J* 14.7, 5.7 and 2.4, 1H, H2), 2.45 (m, 4H, CH_2's), 1.9 – 1.0 (m, 16H, CH_2's); δc (100 MHz; $CDCl_3$) 167.9, 167.4, 136.3, 130.1, 121.0, 115.1, 114.9, 83.5, 79.3, 60.1, 56.4, 53.4, 48.7, 47.9, 39.0, 36.3, 32.8, 25.9, 25.4, 24.7, 24.6 and 23.8; *m/z* 437.5827 (M^+); (Found: M^+, 437.5827. $C_{26}H_{35}N_3O_3$ requires *M*, 437.5822) (Found: C, 71.61; H, 8.14; N, 9.61. requires C, 71.37; H, 8.06; N, 9.60%).

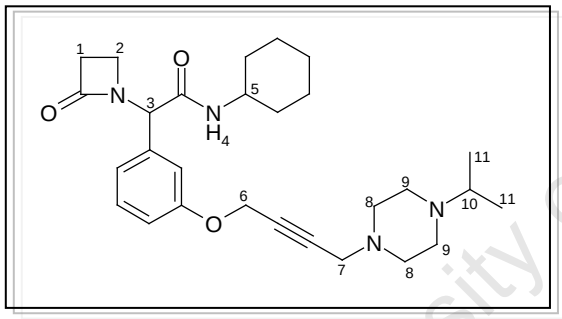
***N-Cyclohexyl-2-{3-[(4-morpholin-4-ylbut-2-ynyl)oxy]phenyl}-2-(2-oxoazetidin-1-yl)aceta mide*, BL17**



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.1:9.9 to 1:9) to yield the product as a white powder, (74.2 mg, 77%); m.p. 102 – 105 °C; R_f (EtOAc) 0.17; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1740 (C=O), 1682 (C=O) and 1263 (C–N) and 1031 (C–O (ether)) δ_H (300 MHz; $CDCl_3$) 7.29 (m, 1H, Ar–H), 6.96 (m, 3H, Ar–H), 6.18 (d, *J* 7.5, 1H, H4), 5.29 (s, 1H, H3), 4.72 (t, *J* 1.8, 2H,

H6), 3.71 (m, 4H, H9 + H5), 3.61 (td, *J* 5.4 and 2.4, 1H, H1), 3.33 (t, *J* 1.8, 2H, H7) , 3.15 (td, *J* 5.4 and 2.4, 1H, H1), 2.99 (ddd, *J* 14.7, 5.4 and 2.4, 1H, H2), 2.85 (ddd, *J* 14.7, 5.4 and 2.4, 1H, H2), 4.50 (m, 4H, H8), 1.9 – 1.0 (m, 10H, cyclohexyl–H); δc (100 MHz; $CDCl_3$) 167.8, 167.4, 158.1, 136.4, 130.0, 120.9, 115.4, 114.9, 82.5, 80.2, 66.8, 59.7, 56.2, 52.1, 48.7, 47.4, 38.9, 36.3, 32.8, 25.5, 24.8 and 24.7; *m/z* 439.5552 (M^+); (Found: M^+, 439.5552. $C_{25}H_{33}N_3O_4$ requires *M*, 439.5547) (Found: C, 68.45; H, 7.67; N, 9.40. requires C, 68.31; H, 7.57; N, 9.56%)

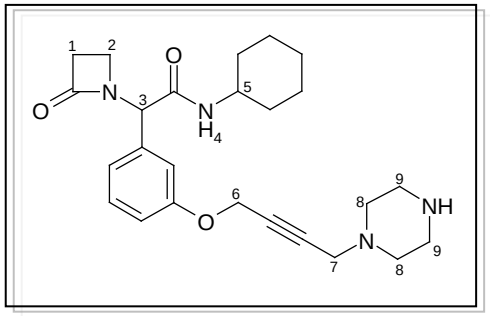
***N-Cyclohexyl-2-(2-oxoazetidin-1-yl)-2-(3-{[4-(4-isopropylpiperazin-1-yl)but-2-ynyl]oxy} phenyl)acetamide*, BL18**



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.5:9.5) to yield the product as a light yellow oil, (91.0 mg, 86%); R_f (MeOH/DCM, 0.5:9.5) 0.26; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1740 (C=O), 1675 (C=O) and 1265 (C–N) and 1022 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.26 (m, 1H, Ar–H), 6.93 (m, 3H, Ar–H), 6.06 (br s, 1H, H4), 5.25 (s, 1H, H3), 4.67 (t, *J* 2.0, 2H, H6), 3.73 (m, 1H, H5), 3.59 (td, *J* 5.6 and 2.8, 1H, H1), 3.29 (t, *J* 2.0, 2H, H7) , 3.16 (td, *J* 5.6 and 2.8, 1H, H1), 2.96 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.83 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.65 (septet, *J* 6.4, 1H, H10), 2.56 (br s, 8H, H8 + H9), 2.0 – 1.1 (m, 10H, cyclohexyl–H), 1.02 (d, *J* 6.4, 6H, H11); δc (100 MHz; $CDCl_3$) 167.9, 167.8, 167.4, 158.2, 136.4, 130.1, 120.9, 115.1, 115.0, 114.9, 83.0, 79.7, 59.9, 56.3, 52.3, 48.7, 48.4, 47.1, 39.0, 36.3, 32.8, 25.4, 24.7, 24.6 and 18.5; *m/z* 480.6514 (M^+); (Found: M^+, 480.6514. $C_{28}H_{40}N_4O_3$ requires *M*, 480.6506).

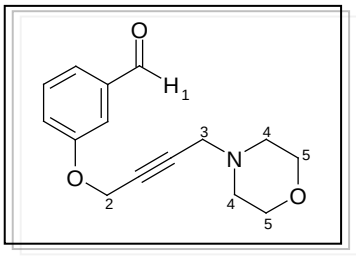
***N*-Cyclohexyl-2-*(*2-oxoazetidin-1-*yl)*-2-*{*3-*[(*4-piperazin-1-ylbut-2-ynyl)oxy]phenyl*}**

***acetamide*, BL19**



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.5:9.5) to yield the product as a white powder, (67.4 mg, 65%); m.p. 115 – 117 °C; R_f (MeOH/EtOAc, 0.3:9.7) 0.14; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1741 (C=O), 1672 (C=O) and 1265 (C–N) and 1025 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.25 (m, 1H, Ar–H), 6.92 (m, 3H, Ar–H), 6.18 (d, *J* 8.0, 1H, H4), 5.29 (s, 1H, H3), 4.67 (t, *J* 1.6, 2H, H6), 3.73 (m, 1H, H5), 3.59 (td, *J* 5.6 and 2.8, 1H, H1), 3.31 (t, *J* 1.6, 2H, H7), 3.17 (td, *J* 5.6 and 2.8, 1H, H1), 2.95 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.83 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.56 (br s, 4H, H8), 2.17 (br s, 2H, cyclohexyl–H), 1.84 (br s, 4H, H9), 1.7 – 1.1 (m, 8H, cyclohexyl–H); δc (100 MHz; $CDCl_3$) 167.9, 167.4, 158.1, 136.4, 130.1, 120.9, 115.0, 114.9, 114.8, 82.7, 80.0, 59.8, 59.7, 56.3, 51.7, 48.7, 47.0, 39.1, 36.3, 32.8, 25.5, 24.8, 24.7; *m/z* 438.5704 (M^+); (Found: M^+, 438.5704. $C_{25}H_{34}N_4O_3$ requires *M*, 438.5700) (Found: C, 68.20; H, 7.53; N, 12.59. requires C, 68.47; H, 7.81; N, 12.77%).

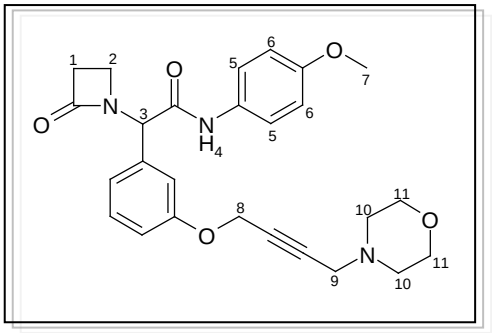
3-*[(*4-*Morpholin*-4-*ylbut*-2-*ynyl)oxy]benzaldehyde*, 8



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.3:9.7) to yield the product as a light-yellow oil, (1.57 g, 88%); R_f (Hex/EtOAc, 2:8) 0.26; ν_{max} (DCM)/cm^{-1} 3058 (C–H (aromatic)), 2862 and 2812 (C–H (aldehyde)), 2312 (C≡C), 1700 (C=O), 1586 (C=C (aromatic)), 1478 (C–C (aromatic)) and1266 (C–N); δ_H (300 MHz; $CDCl_3$) 9.97 (s, 1H, H1), 7.48 (m, 3H, Ar–H), 7.23 (m, 1H, Ar–H), 4.78 (t, *J* 2.0, 2H, H2), 3.69 (m, 4H, H5), 3.31 (t, *J* 2.0, 2H, H3), 2.49 (m, 4H, H4);

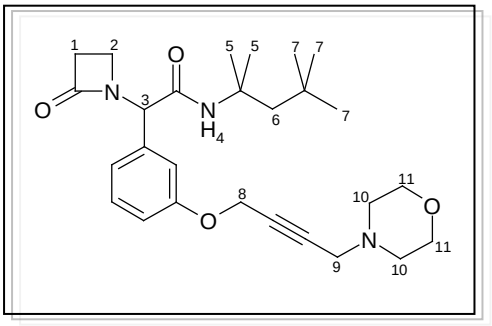
δc (100 MHz; $CDCl_3$) 191.8, 158.2, 137.8, 130.1, 124.1, 122.3, 113.5, 83.1, 79.6, 66.8, 56.3, 52.3 and 47.4; *m/z* 259.3053 (M^+); (Found: M^+, 259.3053. $C_{15}H_{17}NO_3$ requires *M*, 259.3049).

***N-(4-Methoxyphenyl)-2-{3-[(4-morpholin-4-ylbut-2-ynyl)oxy]phenyl}-2-(2-oxoazetidin-1-yl)acetamide*, BL20**



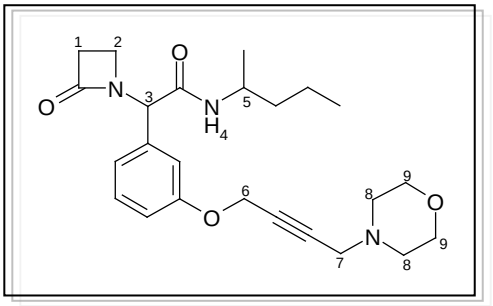
Crude residue was purified via column chromatography, eluting with EtOAc to yield the product as a light-brown oil, (0.172 g, 97%); R_f (EtOAc) 0.22; ν_{max} (DCM)/cm^{-1} 2304 (C≡C), 1743 (C=O), 1282 (C=O), 1262 (C–N) and 1031 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.39 (d, *J* 8.8, 2H, H5), 7.27 (m, 1H, Ar–H), 7.04 (m, 2H, Ar–H), 6.93 (m, 1H, Ar–H), 6.80 (d, *J* 8.8, 2H, H6), 5.51 (s, 1H, H4), 5.26 (s, 1H, H3), 4.70 (s, 2H, H8), 3.74 (s, 3H, H7), 3.67 (m, 5H, H11 + H1), 3.28 (s, 2H, H9), 3.19 (td, *J* 5.6 and 2.8, 1H, H1), 2.99 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.85 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.45 (ddd, *J* 11.2, 6.0 and 3.2, 4H, H10); δc (100 MHz; $CDCl_3$) 168.1, 166.5, 158.1, 156.6, 135.9, 130.8, 130.2, 121.7, 120.9, 115.9, 114.8, 114.1, 82.6, 80.3, 66.8, 60.2, 65.8, 56.2, 55.5, 51.9, 47.3, 39.2, 36.3 and 15.2; *m/z* 463.5340 (M^+); (Found: M^+, 463.5340. $C_{26}H_{29}N_3O_5$ requires *M*, 463.5334).

***N*-(1,1,3,3-*Tetramethylbutyl*)-2-{3-[(4-*morpholin*-4-*ylbut*-2-*ynyl*)*oxy*]*phenyl*}-2-(2-*oxoazetidin*-1-*yl*)*acetamide*, BL21**



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.1:9.9) to yield the product as a white powder, (0.110 g, 62%); m.p. 86 – 89 °C; R_f (EtOAc) 0.38; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1737 (C=O), 1694 (C=O) and 1266 (C–N) and 1035 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.27 (m, 1H, Ar–H), 6.93 (m, 3H, Ar–H), 5.93 (s, 1H, H4), 5.19 (s, 1H, H3), 4.69 (s, 2H, H8), 3.69 (m, 4H, H11), 3.59 (td, *J* 5.6 and 6.0, 1H, H1), 3.30 (s, 2H, H9), 3.14 (td, *J* 6.0 and 2.8, 1H, H1), 2.95 (ddd, *J* 14.8, 6.0 and 2.8, 1H, H2), 2.84 (ddd, *J* 14.8, 6.0 and 2.8, 1H, H2), 2.49 (m, 4H, H10), 1.74 (d, *J* 14.8, 1H, H6), 1.56 (d, *J* 14.8, 1H, H6), 1.38 (s, 3H, H5), 1.34 (s, 3H, H5), 0.89 (s, 9H, H7); δc (100 MHz; $CDCl_3$) 168.1, 167.2, 155.6, 130.1, 121.1, 115.2, 115.0, 82.5, 80.1, 66.8, 65.8, 60.4, 56.2, 55.8, 52.1, 52.0, 47.4, 39.0, 36.3, 31.7, 31.6, 31.5, 31.4, 29.0 and 28.7; *m/z* 469.6256 (M^+); (Found: M^+, 469.6256. $C_{27}H_{39}N_3O_4$ requires *M*, 469.6244) (Found: C, 69.13; H, 8.54; N, 9.06. requires C, 69.05; H, 8.37; N, 8.95%)

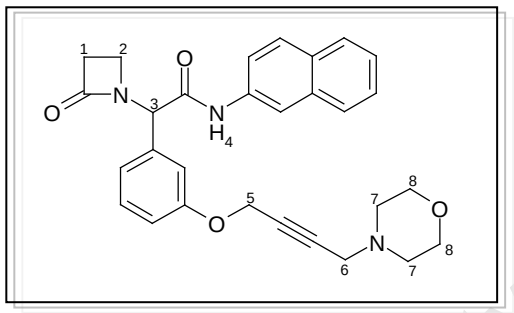
***N*-(1-*Methylbutyl*)-2-{3-[(4-*morpholin*-4-*ylbut*-2-*ynyl*)*oxy*]*phenyl*}-2-(2-*oxoazetidin*-1-*yl*)*acetamide*, BL22**



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.1:9.9) to yield the product as a colourless oil, (0.148 g, 91%); R_f (EtOAc) 0.33; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1735 (C=O), 1695 (C=O), 1266 (C–N) and 1024 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.24 (m, 1H, Ar–H), 6.95 (m, 3H, Ar–H),

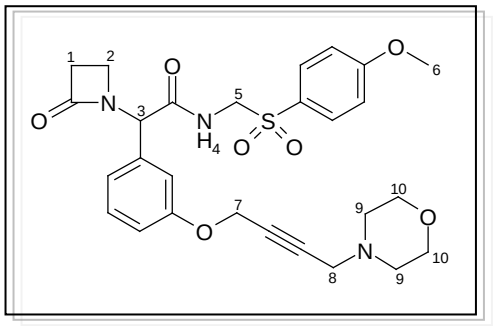
6.10 (m, 1H, H4), 5.29 (s, 1H, H3), 4.69 (s, 2H, H6), 3.95 (m, 1H, H5), 3.68 (m, 4H, H9), 3.60 (td, *J* 5.6 and 3.2, 1H, H1), 3.30 (s, 2H, H7), 3.12 (td, *J* 5.6 and 3.2, 1H, H1), 2.94 (ddd, *J* 14.8, 5.6 and 3.2, 1H, H2), 2.85 (ddd, *J* 14.8, 5.6 and 3.2, 1H, H2), 2.48 (m, 4H, H8), 1.4 – 0.7(m, 10H, alkyl–H); δc (100 MHz; $CDCl_3$) 167.8, 167.6, 158.1, 136.4, 130.1, 120.9, 115.4, 114.9, 82.5, 80.2, 66.8, 65.8, 59.7, 59.6, 56.2, 52.0, 47.4, 45.5, 45.4, 38.9, 38.8, 36.3, 27.3 and 27.2; *m/z* 427.5444 (M^+); (Found: M^+, 427.5444. $C_{24}H_{33}N_3O_4$ requires *M*, 427.5437).

***N*-2-*Naphthyl*-2-*{3-[(4-morpholin-4-ylbut-2-ynyl)oxy]phenyl}*-2-*(2-oxoazetidin-1-yl)aceta mide*, BL23**



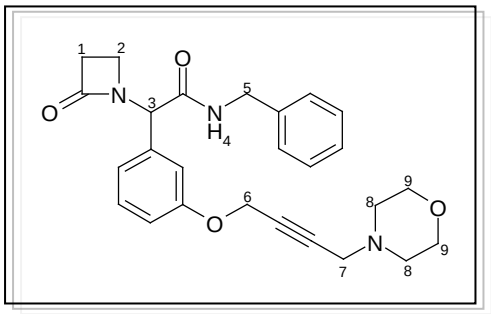
Crude residue was purified via column chromatography, eluting with Hex/EtOAc (3:7) to EtOAc, to yield the product as a light-brown oil, (0.152 g, 82%); R_f (EtOAc) 0.26; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1737 (C=O), 1694 (C=O), 1266 (C–N) and 1021 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.73 (m, 3H, Ar–H), 7.4 – 7.2 (m, 5H, Ar–H), 7.08 (m, 2H, Ar–H), 6.95 (m, 1H, Ar–H), 5.60 (s, 1H, H4), 5.26 (s, 1H, H3), 4.71 (s, 2H, H5), 3.71 (m, 5H, H8 + H1), 3.25 (m, 3H, H6 + H1), 3.03 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.92 (ddd, *J* 14.8, 5.6 and 2.8, 1H, H2), 2.45 (m, 4H, H7); δc (100 MHz; $CDCl_3$) 168.3, 167.0, 158.1, 135.7, 135.2, 133.8, 130.8, 130.2, 128.7, 127.7, 127.5, 126.5, 125.1, 120.9, 119.8, 116.9, 116.0, 114.8, 82.6, 80.2, 66.8, 65.8, 60.6, 56.2, 51.9, 47.3, 39.3 and 36.3; *m/z* 483.5674 (M^+); (Found: M^+, 483.5674. $C_{29}H_{29}N_3O_4$ requires *M*, 483.5670).

***N*-{[(4-Methoxyphenyl)sulfonyl]methyl}-2-{3-[(4-morpholin-4-ylbut-2-ynyl)oxy]phenyl}-2-*(*2-oxoazetidin-1-yl)acetamide*, BL24**



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.1:9.9 to 0.3:9.7), to yield the product as a light-brown oil, (0.143 g, 69%); R_f (EtOAc) 0.16; ν_{max} (DCM)/cm^{-1} 2305 (C≡C), 1745 (C=O), 1700 (C=O), 1324 (O=S=O), 1266 (C–N), 1145 (O=S=O) and 1031 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.64 (m, 3H, Ar–H), 7.25 (m, 2H, Ar–H), 6.94 (m, 1H, Ar–H), 6.89 (m, 1H, Ar–H), 6.77 (m, 1H, Ar–H), 5.36 (s, 1H, H3), 4.74 (dd, *J* 14.0 and 6.8, 1H, H5), 4.72 (s, 2H, H7), 4.51 (dd, *J* 14.0 and 6.8, 1H, H5), 3.70 (m, 4H, H10), 3.41 (td, *J* 5.6 and 2.8, 1H, H1), 3.34 (s, 2H, H8), 3.02 (td, *J* 5.6 and 2.8, 1H, H1), 2.90 (ddd, *J* 14.4, 5.6 and 2.8, 1H, H2), 2.81 (ddd, *J* 14.4, 5.6 and 2.8, 1H, H2), 2.51 (m, 4H, H9), 2.41 (s, 3H, -OCH_3); δc (100 MHz; $CDCl_3$) 168.3, 167.7, 158.0, 145.3, 134.9, 134.0, 130.1, 130.0, 128.8, 121.0, 116.1, 114.9, 82.3, 80.5, 66.7, 66.6, 65.8, 60.2, 59.0, 56.1, 52.3, 51.9, 47.3, 38.8, 36.3, 21.7 and 15.3; *m/z* 541.6257 (M^+); (Found: M^+, 541.6257. $C_{27}H_{31}N_3O_7S$ requires *M*, 541.6250).

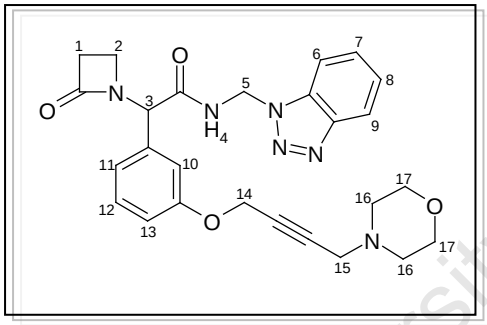
***N*-Benzyl-2-{3-[(4-morpholin-4-ylbut-2-ynyl)oxy]phenyl}-2-(2-oxoazetidin-1-yl)acetamide, BL25**



Crude residue was purified via column chromatography, eluting with MeOH/EtOAc (0.3:9.7), to yield the product as a light-brown oil, (0.168 g, 99%); R_f (MeOH/EtOAc, 0.3:9.7) 0.26; ν_{max} (DCM)/cm^{-1} 2306 (C≡C), 1745 (C=O), 1683 (C=O), 1264 (C–N) and 1024 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 7.62 (m, 3H, Ar–H),

7.12 (m, 3H, Ar–H), 6.95 (m, 3H, Ar–H), 6.76 (br s, 1H, H4), 5.37 (s, 1H, H3), 4.67 (m, 2H, H6), 4.43 (dd, *J* 14.8 and 6.0, 1H, H5), 4.38 (dd, *J* 14.8 and 6.0, 1H, H5), 3.60 (m, 5H, H9 + H1), 3.28 (s, 2H, H7), 3.12 (td, *J* 5.6 and 2.8, 1H, H1), 2.93 (ddd, *J* 14.8, 5.6 and 2.4, 1H, H2), 2.82 (ddd, *J* 14.8, 5.6 and 2.4, 1H, H2), 2.45 (m, 4H, H8); δc (100 MHz; $CDCl_3$) 168.4, 167.9, 158.1, 137.8, 136.0,130.1, 128.7, 127.7, 127.5, 121.0, 115.6, 115.0, 82.5, 80.2, 66.7, 59.6, 56.2, 52.0, 47.3, 43.7, 39.0, 36.3 and 29.7; *m/z* 447.5351 (M^+); (Found: M^+, 437.5351. $C_{26}H_{29}N_3O_4$ requires *M*, 447.5340).

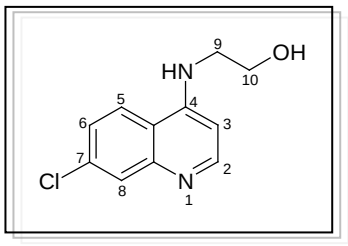
***N*-(1*H*-1,2,3-*Benzotriazol*-1-*ylmethyl*)-2-{3-[(4-*morpholin*-4-*ylbut*-2-*ynyl*)*oxy*]*phenyl*}-2-(2-*oxoazetidin*-1-*yl*)*acetamide*, BL26**



Crude residue was purified via column chromatography, eluting with EtOAc to yield the product as a brown powder, (0.113 g, 60%); m.p. 131 – 134 °C; R_f (MeOH/DCM, 0.5:9.5) 0.21; ν_{max} (DCM)/cm^{-1} 2304 (C≡C), 2163 (N=N/N–N), 1743 (C=O), 1705 (C=O), 1265 (C–N) and 1035 (C–O (ether)); δ_H (400 MHz; $CDCl_3$) 8.15 (t, *J* 6.4, 1H, H4) 7.98 (dt, *J* 8.4 and 2.0, 1H, H11), 7.87 (dt, *J* 8.4 and 2.0, 1H, H13), 7.48 (td, *J* 7.6 and 1.6, 1H, H7/H8), 7.35 (td, *J* 7.6 and 1.6, 1H, H7/H8), 7.11 (t, *J* 8.4, 1H, H12), 6.84 (dd, *J* 7.6 and 1.6, 1H, H6/H9), 6.80 (t, *J* 2.0, 1H, H10), 6.70 (dd, *J* 7.6 and 1.6, 1H, H6/H9), 6.11 (dd, *J* 14.0 and 6.4, 1H, H5), 5.99 (dd, *J* 14.0 and 6.4, 1H, H5), 5.38 (s, 1H, H3), 4.55 (t, *J* 1.6, 2H, H14), 3.66 (m, 4H, H17), 3.51 (td, *J* 5.6 and 2.8, 1H, H1), 3.28 (t, *J* 1.6, 2H, H15), 3.04 (td, *J* 5.6 and 2.8, 1H, H1), 2.91 (ddd, *J* 14.4, 5.6 and 2.8, 1H, H2), 2.80 (ddd, *J* 14.4, 5.6 and 2.8, 1H, H2), 2.43 (m, 4H, H16); δc (100 MHz; $CDCl_3$) 169.5, 169.4, 167.9, 158.0, 146.0, 135.0, 130.1, 128.0, 124.0, 120.8, 119.7, 116.1, 114.9, 110.7, 82.5, 80.2, 66.8, 59.2, 56.0, 51.8, 51.0, 47.3, 38.8 and 36.4; *m/z* 488.5468 (M^+); (Found: M^+, 488.5468.

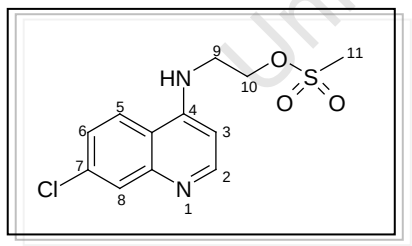
$C_{26}H_{28}N_6O_4$ requires *M*, 488.5461) (Found: C, 64.28.; H, 6.05; N, 17.29. requires C, 63.92; H, 5.78; N, 17.20%)

2-*(7-Chloroquinolin-4-ylamino)-ethanol*, 4a



Triethylamine (0.2 ml, 1.52 mmol, 0.3 eq) was added drop-wise to a solution of 4,7-dichloroquinoline (1.00 g, 5.04 mmol, 1.0 eq) and K_2CO_3 (0.34 g, 2.52 mmol, 0.5 eq) in ethanolamine (6.4 ml, 20.0 eq), and refluxed at 140 °C for 4 hours. The product **4a** precipitated on cooling, and was filtered and washed with brine and H_2O. Pure product was recrystallised from MeOH as white powder (1.071 g, 95%). m.p. 213 – 215 °C (lit. 214 °C)[8]. R_f (MeOH/DCM, 1:9) 0.17. δ_H (400 MHz; DMSO-d_6) 8.36 (d, *J* 5.6, 1H, H2), 8.23 (d, *J* 8.8, 1H, H5), 7.76 (d, *J* 2.0, 1H, H8), 7.41 (dd, *J* 8.8 and 2.4, 1H, H6), 7.21 (t, *J* 5.6, 1H, NH), 6.48 (d, *J* 5.2, 1H, H3), 4.80 (t, *J* 5.2, 1H, OH), 3.64 (t, *J* 5.6, 2H, H9), 3.35 (q, *J* 5.6, 2H, H10).

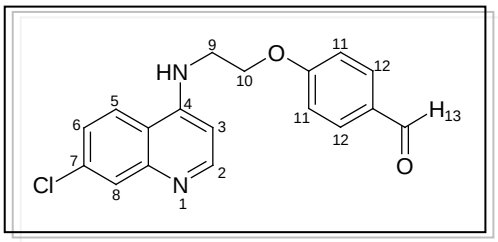
***Methanesulfonic acid* 2-*(7-chloroquinolin-4-ylamino)-ethyl ester*, 4b**



A solution of **4a** (0.90 g, 4.041 mmol, 1.0 eq) in pyridine (3.7 ml) was stirred at 0 °C for 30 min, after which a solution of methanesulfonyl chloride (0.78 ml, 2.5 eq) was added drop-wise. After stirring further for 5 hours at 0 °C, the mixture was diluted with 25% aqueous ammonium hydroxide solution until pH 8, extracted with EtOAc (3 x 20 ml), dried (Na_2SO_4) and solvent evaporated. The residue was recrystallised from MeOH/H_2O to yield white needle-like crystals (1.095 g, 90 %). m.p. 138 – 140 °C (lit. 138 – 140 °C)[9]. Rf (MeOH/DCM, 1:9) 0.41. δ_H (400 MHz; DMSO-d_6) 8.41 (d, *J* 5.2, 1H, H2), 8.22 (d, *J* 8.8, 1H, H5), 7.79 (d, *J* 2.0, 1H, H8), 7.45 (dd, *J* 8.8 and *J*

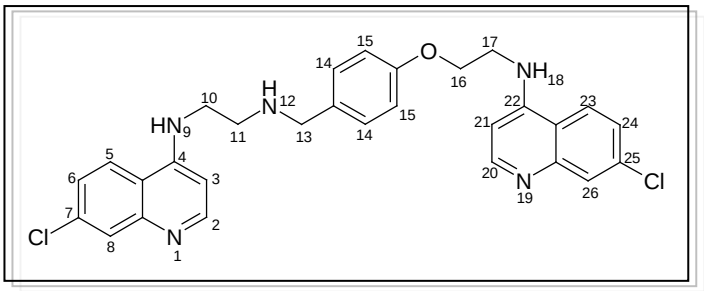
2.4, 1H, H6), 7.45 (t, *J* 5.6, 1H, NH), 6.57 (d, *J* 5.6, 1H, H3), 4.41 (t, *J* 5.6, 2H, H10), 3.65 (q, *J* 5.6, 2H, H9), 3.14 (s, 3H, H11).

***4-[2-(7-Chloroquinolin-4-ylamino)-ethoxy]-benzaldehyde*, 4c**



4-Hydroxybenzaldehyde (2.284 g, 18.70 mmol, 1.5 eq), **4b** (3.750 g, 12.47 mmol, 1.0 eq) and K_2CO_3 (2.757 g, 19.95 mmol, 1.6 eq) were stirred in DMF (100 ml) and refluxed between 140 – 150 °C for 5 hours. Upon cooling, the mixture was diluted with H_2O (100 ml), extracted with EtOAc (3 x 200 ml), washed with brine (200 ml), dried (Na_2SO_4) and solvent evaporated to yield the crude product as brown powder. As the product is slightly soluble in EtOAc, the crude product was washed with EtOAc (10 ml) to remove excess benzaldehyde, filtered and recrystallised from MeOH/H_2O to yield cream-coloured powder (3.10 g, 76%). m.p. 179 – 181 °C (lit. 175 – 176 °C)[9]. R_f (MeOH/DCM, 0.5:9.5) 0.18. δ_H (300 MHz; DMSO-d_6) 9.86 (s, 1H, H13), 8.42 (d, *J* 5.1, 1H, H2), 8.26 (d, *J* 9.0, 1H, H5), 7.84 (d, *J* 8.4, 2H, H12), 7.79 (d, *J* 2.1, 1H, H8), 7.47 (t, *J* 5.1, 1H, NH), 7.43 (dd, *J* 8.7 and 1.8, 1H, H6), 7.13 (d, *J* 8.7, 2H, H11), 6.60 (d, *J* 5.4, 1H, H3), 4.37 (t, *J* 5.1, 2H, H10), 3.71 (q, *J* 5.1, 2H, H9).

***N-(7-Chloroquinolin-4-yl)-N'-{4-[2-(7-chloroquinolin-4-ylamino)-ethoxy]-benzyl}-ethane-1,2-diamine*, 4d**



The amine **4d** (0.5 g, 2.255 mmol, 1.0 eq) and aldehyde **QN01** (0.737 g, 2.255 mmol, 1.0 eq) were stirred in anhydrous MeOH (75 ml) under

N_2 at room temperature for 5 hours Sodium borohydride (0.171 g, 2.510 mmol, 2.0 eq) was added, and the reaction mixture was stirred further overnight. The sodium borohydride was destroyed by the slow addition of H_2O (50 ml), extracted with DCM (3 x 100 ml), dried (Na_2SO_4) and solvent evaporated to yield ***4e*** as light brown crystals (1.115 g, 92.8 %). m.p. 79 – 81 °C. R_f (MeOH/DCM/NH_4OH, 0.5:9.5:0.1) 0.44. ν_{max} ($CHCl_3$)/cm^{-1} 3290 (N-H), 3017 (Ar-H), 2942 (CH_2's), 1582 (Ar-C), 1215 (C-N) 753 (C-Cl); δ_H (400 MHz; DMSO-d_6) 8.40 (d, *J* 5.2, 1H, H20/H2), 8.35 (d, *J* 5.6, 1H, H2/H20), 8.26 (d, *J* 9.2, 1H, H23/H5), 8.20 (d, *J* 9.2, 1H, H5/H23), 7.78 (d, *J* 2.4, 1H, H26/H8), 7.76 (d, *J* 2.4, 1H, H8/H26), 7.45 (s, 1H, NH), 7.42 (dd, *J* 9.2 and 2.4, 1H, H24/H6), 7.40 (dd, *J* 9.2 and 2.0, 1H, H6/H24), 7.21 (d, *J* 8.4, 2H, H15), 7.17 (s, 1H, NH), 6.85 (d, *J* 8.4, 2H, H14), 6.56 (d, *J* 5.2, 1H, H21/H3), 6.44 (d, *J* 5.2, 1H, H3/H21), 5.73 (s, 2H, H13), 4.20 (d, *J* 5.6, 2H, H16), 3.66 (m, 4H, H11 + H17), 2.76 (t, *J* 6.4, 2H, H10); δc (100 MHz; DMSO-d_6) 157.1, 151,9, 151.8, 150.1, 150.0, 149.1, 149.0, 133.4, 133.3, 133.0, 129.0, 127.8, 127.5, 127.4, 124.1, 124.0, 123.9, 117.4, 114.1, 114.0, 103.2, 98.8, 98.7, 65.5, 65.5, 52.0, 46.5, 42.5, 41.9; *m/z* 532.48 (M^+); (Found: M^+, 532.48. $C_{29}H_{27}Cl_2N_5O$ requires *M*, 532.48) (Found: C, 65.69, H, 5.01, N, 13.37 %; Requires C, 65.42, H, 5.11, N, 13.15 %).

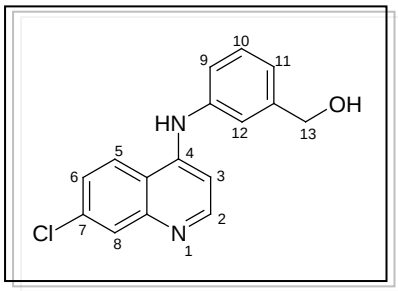
General procedure for the synthesis of **6a** – **6e**

The amine (0.902 mmol, 1.2 eq) and the aldehyde (0.752 mmol, 1.0 eq) were stirred in MeOH (12 ml) at 25 °C for 30 min, followed by the addition of the carboxylic acid (0.752 mmol, 1.0 eq) and the isocyanide (0.752 mmol, 1.0 eq) drop-wise. After stirring for 3 – 5 days, the solvent was removed under pressure, and the residue purified via column chromatography.

General procedure for the *benzyl alcohols* **9a** and **9b** and benzoic acid **9c**.

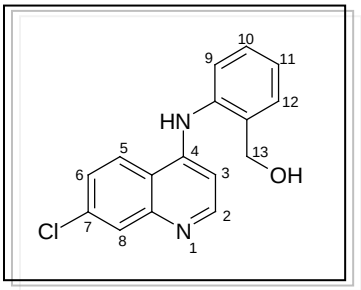
4,7-Dichloroquinoline (1.0 eq) and the amine (1.0 eq) were refluxed in EtOH at 80 – 85 °C for 3 h. The volume was reduced *in vacuo*, followed by drop-wise addition of ice-cold ammonium hydroxide solution (5%) until pH 8, and the resulting precipitate collected. In the case of **10b**, sticky oil was formed instead of precipitation. The oil was extracted into DCM, washed with H_2O, dried over Na_2SO_4, filtered and solvent reduced.

***{3-[(7-Chloroquinolin-4-yl)amino]phenyl}methanol*, 9a**



Pure product (0.68 g, 98%) was yielded as cream-coloured powder, m.p. 247 – 250 °C; R_f (MeOH/DCM, 0.5:9.5) 0.23; ν_{max} (DCM)/cm^{-1} 3260 (O–H), 2880 (C–O) and 1358 (O–H bend); δ_H (400 MHz; DMSO-d_6) 9.07 (s, 1H, NH), 8.44 (d, *J* 5.3, 1H, H2), 8.42 (d, *J* 8.8, 1H, H5), 7.87 (d, *J* 2.2, 1H, H8), 7.54 (dd, *J* 8.8 and 2.2, 1H, H6), 7.35 (t, *J* 7.9, 1H, H10), 7.33 (s, 1H, H12), 7.21 (d, *J* 7.9, 1H, H9), 7.09 (d, *J* 7.9, 1H, H11), 6.91 (d, *J* 5.3, 1H, H3), 5.22 (s, 1H, OH) and 4.52 (s, 2H, H13); δc (75.5 MHz; DMSO-d_6) 151.8, 149.5, 148.0, 144.0, 139.9, 133.8, 129.0, 127.5, 124.8, 124.4, 122.0, 120.8, 120.5, 118.2, 101.7 and 62.6; *m/z* 284.7441 (M^+); (Found: M^+, 284.7441. $C_{16}H_{13}ClN_2O$ requires *M*, 284.7450) (Found: C, 67.66; H, 4.41; N, 9.50. requires C, 67.69; H, 4.60; N, 9.84%).

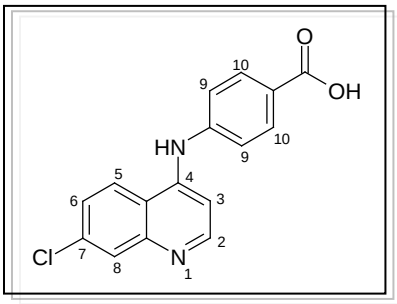
***{2-[(7-Chloroquinolin-4-yl)amino]phenyl}methanol*, 9b**



The crude residue was washed with DCM (30ml) to yield pure **9b** (3.0 g, 45%) as white needles, m.p. 194 – 197 °C; R_f (MeOH/DCM, 0.5:9.5) 0.26; ν_{max} (DCM)/cm^{-1} 3260 (O–H),

2881 (C–O) and 1358 (O–H bend); δ_H (300 MHz; DMSO-d_6) 8.80 (br s, 1H, NH), 8.38 (d, *J* 8.7, 1H, H5), 8.36 (d, *J* 5.4, 1H, H2), 7.89 (d, *J* 2.4, 1H, H8), 7.61 (d, *J* 7.2, 1H, H9 or 12), 7.56 (dd, *J* 8.7 and 2.4, 1H, H6), 7.33 (m, 3H, Ar-H), 6.29 (d, *J* 5.4, 1H, H3) and 4.50 (s, 2H, H13); δc (75.5 MHz; DMSO-d_6) 151.8, 149.3, 149.0, 138.1, 136.6, 133.7, 128.0, 127.8, 127.6, 126.0, 125.9, 123.9, 117.6, 101.0 and 59.4; *m/z* 284.7445 (M^+); (Found: M^+, 284.7445. $C_{16}H_{13}ClN_2O$ requires *M*, 284.7450) (Found: C, 67.89; H, 4.26; N, 9.96. $C_{16}H_{13}ClN_2O$ requires C, 67.69; H, 4.60; N, 9.84%).

***4-[(7-Chloroquinolin-4-yl)amino]benzoic acid*, 9c**

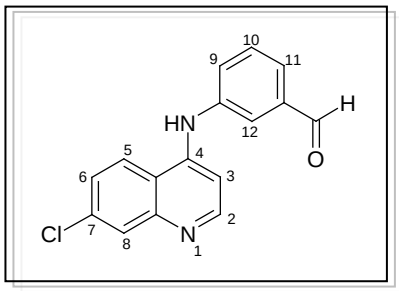


Pure product (0.68 g, 98%) was collected as light yellow powder, m.p. >326 °C; R_f (MeOH/DCM, 0.8:9.2) 0.24; ν_{max} (DCM)/cm^{-1} 3038 (O–H), 1569 (C=O) and 1384 (C–O); δ_H (400 MHz; DMSO-d_6) 8.492 (d, *J* 5.6, 1H, H2), 8.429 (d, *J* 8.8, 1H, H5), 7.910 (d, *J* 8.0, 1H, H10), 7.893 (d, *J* 2.4, 1H, H8), 7.544 (dd, *J* 8.8 and 2.4, 1H, H6), 7.317 (d, *J* 8.0, 2H, H9) and 7.054 (d, *J* 5.6, 1H, H3); δc (100 MHz; DMSO-d_6) 168.2, 152.1, 149.8, 147.5, 142.3, 134.0, 131.8, 130.4 (x2), 127.8, 125.2, 124.4 (x2), 120.3, 118.5 and 103.1; *m/z* 298.7298 (M^+); (Found: M^+, 298.7298. $C_{16}H_{11}ClN_2O_2$ requires *M*, 298.7285) (Found: C, 64.07; H, 4.17; N, 9.55. requires C, 64.33; H, 3.71; N, 9.38%).

General procedure for *aldehydes* **11a** and **11b**

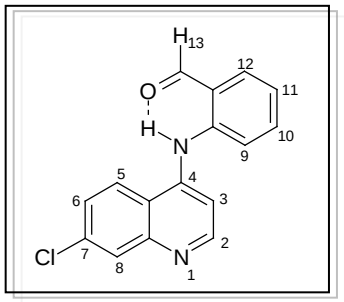
SO_3·Pyr (2.0 eq) was added to a solution of the benzyl alcohol **10a** (**10b)** (1.0 eq) and Et_3N (4.0 eq) in DMSO and stirred for 2 days at 26 °C. The reaction mixture was diluted with EtOAc, washed with H_2O (2 times) and brine, dried over Na_2SO_4, filtered and solvent removed *in vacuo*.

3-*[(7-Chloroquinolin-4-yl)amino]benzaldehyde,* 11a



Crude product isolated as cream-coloured powder (1.44 g, 58%). ^{1}H NMR shows that the crude material contained only 58% of the desired product. Attempts of purification were unsuccessful, as product was too polar and could only be dissolved in DMSO, which was difficult to be chromatographed. Hence the crude product was used for the subsequent reactions.

2-*[(7-Chloroquinolin-4-yl)amino]benzaldehyde,* 11b



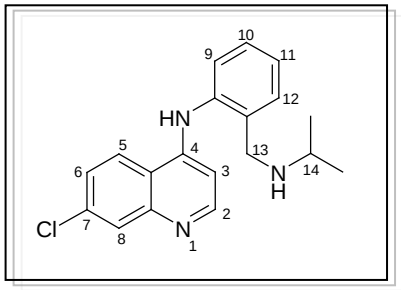
The crude residue was washed with DCM (10 ml) and the *aldehyde* **11b** (0.97 g, 94%) was collected as light yellow powder, m.p. 164 – 166 °C; R_f (EtOAc/Hex, 7:3) 0.74; ν_{max} (DCM)/cm^{-1} 2829, 2749 (aldehydic C–H stretch), 1668 (C=O) and 1378 (aldehydic C–H bend); δ_H (300 MHz; DMSO-d_6) 10.25 (s, 1H, NH), 10.07 (s, 1H, H13), 8.61 (d, *J* 5.1, 1H, H2), 8.27 (d, *J* 9.0, 1H, H5), 7.98 (d, *J* 1.8, 1H, H8), 7.93 (dd, *J* 7.5 and 1.8, 1H, H12), 7.68 (m, 2H, H9 and H10), 7.28 (t, *J* 7.5, 1H, H11), 7.62 (d, *J* 8.4, 1H, H6) and 7.20 (d, *J* 5.4, 1H, H3); δc (75.5 MHz; DMSO-d_6) 193.9, 152.1, 149.4, 145.8, 142.9, 135.7, 134.2, 133.8, 128.0, 126.2, 125.2, 123.5, 122.8, 120.0 and 105.4; *m/z* 282.7298 (M^+); (Found: M^+, 282.7298. $C_{16}H_{11}ClN_2O$ requires *M*, 282.7291) (Found: C, 68.23; H, 3.67; N, 10.04. Requires C, 67.97; H, 3.92; N, 9.91%).

General method for the *amines* **12a**, **12b** and **13a**

The aldehyde **11a** (**11b)** (1.0 eq) was dissolved in MeOH/DMF (5:2), followed by the drop-wise addition of the amine (1.0 eq) and stirred at 26 °C for 5 h. $NaBH_4$ (2.0 eq) was then added and stirred further overnight (For the reaction where diethylamine was used,

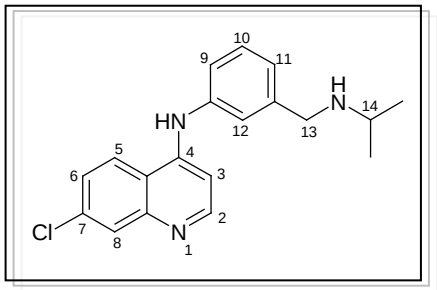
$NaCNBH_3$ was used as the reducing agent). The borohydride was destroyed by the slow addition of H_2O, and the mixture extracted with DCM. The combined organic layer was washed with H_2O (3 times), dried over Na_2SO_4, filtered and solvent removed *in vacuo*. The residue was purified by column chromatography slowly increasing the polarity from 0.2 : 9.8 to 1 : 9 : 0.1 MeOH/DCM/Et_3N to yield the pure product.

***7-Chloro-N-{3-[(isopropylamino)methyl]phenyl}quinolin-4-amine,* 12a**



The product was yielded as beige-coloured powder (0.07 g, 77%), m.p. 124 – 127 °C; R_f (MeOH/DCM, 0.5:9.5) 0.14; ν_{max} (DCM)/cm^{-1} 3458 (N-H), 2963 (CH_3) and 1164 (C–N); δ_H (400 MHz; $CDCl_3$) 8.54 (d, *J* 5.3, 1H, H2), 8.01 (d, *J* 2.0, 1H, H8), 7.88 (d, *J* 9.0, 1H, H5), 7.43 (dd, *J* 9.0 and 2.0, 1H, H6), 7.35 (t, *J* 7.7, 1H, H10), 7.29 (br s, 1H, H12), 7.19 (br d, *J* 7.7, 1H, H9), 7.15 (br d, *J* 7.9, 1H, H11), 6.96 (d, *J* 5.3, 1H, H3), 3.81 (s, 1H, H13), 2.90 (septet, *J* 6.2, 1H, H14) and 1.13 (d, *J* 6.2, 6H, 2 x CH_3); δc (100 MHz; DMSO-d_6) 151.9, 149.5, 148.0, 142.1, 140.0, 133.8, 129.0, 127.6, 124.8, 124.4, 123.8, 122.2, 120.9, 118.3, 91.4, 50.0, 47.4 and 22.4; *m/z* 325.8415 (M^+); (Found: M^+, 325.8415. $C_{19}H_{20}ClN_4$ requires *M*, 325.8409) (Found: C, 69.28; H, 6.14; N, 12.97. requires C, 70.04; H, 6.19; N, 12.90%).

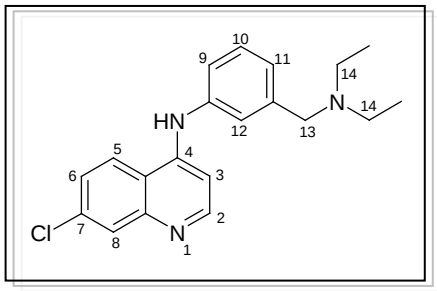
***7-Chloro-N-{2-[(isopropylamino)methyl]phenyl}quinolin-4-amine,* 12b**



Product yielded as beige-coloured powder (0.22 g, 65%), m.p. 118 – 121 °C; R_f (MeOH/DCM, 0.5:9.5) 0.26; ν_{max} (DCM)/cm^{-1} 3458 (N–H), 2960 (CH_3) and 1162 (C–N); δ_H (300 MHz; $CDCl_3$) 8.524 (d, *J* 5.4, 1H, H2), 8.081 (d,

J 9.0, 1H, H5), 8.006 (d, *J* 2.1, 1H, H8), 7.562 (dd, *J* 8.4 and 0.9, 1H, H9), 7.423 (dd, *J* 9.0 and 2.1, 1H, H6), 7.332 (m, 2H, H10 and H11), 7.079 (dd, *J* 8.7 and 0.9, 1H, H12), 7.042 (d, *J* 5.4, 1H, H3), 3.881 (s, 2H, H13), 2.945 (septet, *J* 6.3, 1H, H14) and 1.210 (d, *J* 6.3, 6H, 2 x CH_3); δc (75.5 MHz; $CDCl_3$) 155.6, 151.9, 149.8, 140.7, 135.2, 130.8, 129.7, 128.6, 125.7 (x2), 123.4, 122.7, 118.85, 107.0, 101.6, 49.0, 45.7, 22.5 and 9.1; *m/z* 325.8417 (M^+); (Found: M^+, 325.8417. $C_{19}H_{20}ClN_4$ requires *M*, 325.8409) (Found: C, 69.29; H, 6.12; N, 12.60. requires C, 70.04; H, 6.19; N, 12.90%).

***7-Chloro-N-{3-[(diethylamino)methyl]phenyl}quinolin-4-amine*, 13a**



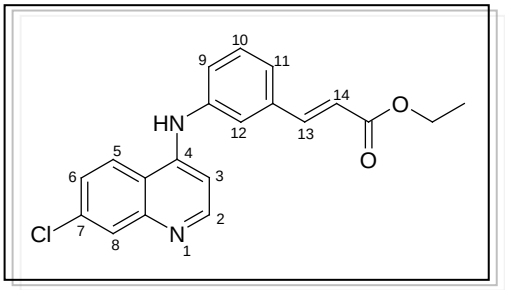
Product yielded as beige-coloured powder (0.16 g, 68%), m.p. 142 – 145 °C; R_f (MeOH/DCM, 0.5:9.5) 0.14; ν_{max} (DCM)/cm^{-1} 2968 (CH_3) and 1158 (C–N); δ_H (400 MHz; $CDCl_3$) 8.600 (d, *J* 5.2, 1H, H2), 8.05 (d, *J* 2.4, 1H, H8), 7.93 (d, *J* 8.8, 1H, H5), 7.79 (s, 1H, H12), 7.47 (dd, *J* 8.8 and 2.4, 1H, H6), 7.34 (t, *J* 7.6, 1H, H10), 7.23 (d, *J* 7.6, 1H, H9), 7.15 (d, *J* 7.6, 1H, H11), 7.01 (d, *J* 5.2, 1H, H3), 3.67 (s, 2H, H13), 2.64 (q, *J* 7.2, 4H, 2 x H14) and 1.11 (t, *J* 7.2, 6H, 2 x CH_3); δc (100 MHz; DMSO-d_6) 151.8, 149.5, 148.0, 140.0, 133.8, 129.0, 127.6, 124.8, 124.4, 124.2, 122.6, 122.0, 120.9, 120.5, 101.7, 62.6, 56.6, 46.2 and 11.5 (x2); *m/z* 339.8681 (M^+); (Found: M^+, 339.8681. $C_{20}H_{22}ClN_3$ requires *M*, 339.8676) (Found: C, 70.88; H, 6.22; N, 12.42. requires C, 70.68; H, 6.57; N, 12.36%).

General procedure for the Wittig products **14a** and **14b**

Ethyl bromoacetate (1.0 eq) was added drop-wise to a solution of triphenylphosphine (1.2 eq) in tolune under N_2, and stirred for 8 h. The mixture was left standing overnight at rt for precipitation. The crystals were filtered, washed with cold toluene and dried to yield pure

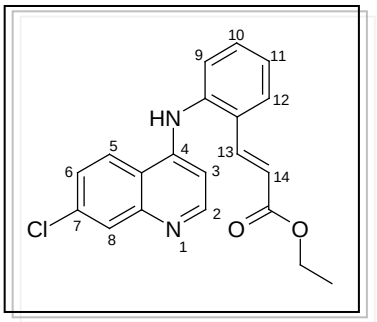
phosphonium salt. The salt was dissolved in anhydrous MeOH under N_2, followed by the drop-wise addition of NaOMe (1.2 eq, dissolved in MeOH) at 0 °C and stirred for 20 min. The aldehyde **11a** (**11b**) (0.9 eq) was then added to the mixture, and stirred further at 26 °C under N_2. Equal amounts of H_2O and aqueous $NaHCO_3$ were added to the reaction mixture, and the resulting mixture extracted with EtOAC (3 times), washed with brine, dried over Na_2SO_4, filtered and solvent removed *in vacuo*.

***Ethyl (2E)*-3-*{3-[(7-chloroquinolin-4-yl)amino]phenyl}acrylate*, 14a**



Crude residue was purified via column chromatography, eluting with Hex/EtOAc (3:7) to yield the product **14a** (0.09 g, 76%) as beige-coloured powder, m.p. 75 – 78 °C; R_f (EtOAc/Hex, 7:3) 0.49; ν_{max} (DCM)/cm^{-1} 2925 (alkyl), 1710 (C=O), 1638 (C=C (alkene)), 1179 (C–C–O) and 1036 (O–C–C); δ_H (400 MHz; DMSO-d_6) 9.11 (s, 1H, H12), 8.48 (d, *J* 5.2, 1H, H2), 8.41 (d, *J* 9.2, 1H, H5), 7.89 (d, *J* 2.0, 1H, H8), 7.65 (d, *J* 15.6, 1H, H14), 7.56 (dd, *J* 9.2 and 2.0, 1H, H6), 7.50 (d, *J* 7.6, 1H, H9), 7.45 (t, *J* 7.6, 1H, H10), 7.38 (d, *J* 7.6, 1H, H11), 6.95 (d, *J* 5.2, 1H, H3), 6.60 (d, *J* 15.6, 1H, H13), 4.18 (q, *J* 7.2, 2H, CH_2) and 1.24 (t, *J* 7.2, 3H, CH_3); δc (100 MHz; DMSO-d_6) 166.0, 152.0, 149.5, 147.6, 144.0, 140.8, 135.3, 133.9, 130.0, 127.7, 125.0, 124.3, 123.6, 122.0, 118.7, 118.4, 102.2, 60.0 and 14.1; *m/z* 352.8209 (M^+); (Found: M^+, 352.8209. $C_{20}H_{17}ClN_2O_2$ requires *M*, 352.8202) (Found: C, 67.92; H, 4.77; N, 7.90. requires C, 68.09; H, 4.86; N, 7.94%).

***Ethyl (2E)*-3-*{2-[(7-chloroquinolin-4-yl)amino]phenyl}acrylate*, 14b**



Crude residue was purified via column chromatography, eluting with MeOH/DCM (0.01:0.99) to yield the product **14b** (0.1 g, 79%) as cream-coloured powder, m.p. 182 – 185 °C; R_f (DCM) 0.14; ν_{max} (DCM)/cm^{-1} 2919 (alkyl), 1714 (C=O), 1634 (C=C (alkene)), 1178 (C–C–O) and 1038 (O–C–C); δ_H (400 MHz; $CDCl_3$) 8.52 (d, *J* 5.2, 1H, H2), 8.03 (d, *J* 2.0, 1H, H8), 7.89 (d, *J* 9.0, 1H, H5), 7.80 (d, *J* 15.6, 1H, H14), 7.45 (dd, *J* 9.0 and 2.0, 1H, H6), 7.40 (m, 2H, Ar-H), 7.26 (m, 2H, Ar-H), 6.93 (d, *J* 5.2, 1H, H3), 6.44 (d, *J* 15.6, 1H, H13), 3.60 (q, *J* 7.2, 2H, CH_2) and 1.28 (t, *J* 7.2, 3H, CH_3); δc (100 MHz; DMSO-d_6) 166.1, 151.9, 151.8, 150.5, 139.7, 139.6, 134.0, 131.7, 127.8, 126.8, 125.0, 124.9, 124.3, 124.2, 119.1, 119.0, 101.8, 60.0 and 14.0; *m/z* 352.8210 (M^+); (Found: M^+, 352.8210. $C_{20}H_{17}ClN_2O_2$ requires *M*, 352.8202) (Found: C, 67.84; H, 4.72; N, 7.71. requires C, 68.09; H, 4.86; N, 7.94%).

8.2 Biological Testing Protocols

Preparation of stock solution of test compounds

All compounds were dissolved in DMSO or methanol to the concentration of 4 mg/ml, and stored at -20 °C until use. All samples were further diluted in complete medium to their desired concentrations on the day of the experiment, whilst ensuring the maximum concentration of DMSO or methanol to be under 0.5 % after dilution. Chloroquine was used as the positive control in all experiments. The 50 % inhibitory concentration (IC_{50}) 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 D10, K1 and Dd2 strains of *P. falciparum*

All samples were tested in duplicate on at least three occasions against the various strains of *Plasmodium falciparum*. Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen.[10] Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase (pLDH) assay using a modified method described by Makler.[11] The pLDH assay compares well with other available assays.[12] The 50% inhibitory concentration values of the compounds were determined at ten different concentrations.

in vitro Activities of compounds against 3D7 strain of *P. falciparum* using the Luciferase assay

Parasites expressing cytoplasmic luciferase were synchronized at ring stage using 5 % sorbitol solution (Sigma) for at least two cycles prior to the assay. Compounds were added to ring stage parasites and allowed to grow for 60 hours. To assay the plates, Bright-Glo® Luciferase Substrate (Promega) was dispensed. Immediately following, luminescence read by an EnVision multilable reader 2102 (Perkin Elmer). Growth in the presence of each compound is compared to DMSO controls.

Drug treatments of parasites for Western blotting and Immunofluorescence assays

Various drugs were added to 1 ml early trophozoite-stage parasite cultures at 1 % haematocrit, approximately 10 % parasitaemia, at final concentrations of approximately 5 times the IC_{50} values of respective drug. Experiments were performed in 24-well plates in triplication, and each plate was incubated at 37 °C for 5 h for Western blotting and 8 h for Immunofluorescence assays.

Incubations were terminated before the parasites enter the schizont stage. This was assessed by Giemsa-staining or fluorescence microscopy of 4',6'-diamidino-2-phenylindole (DAPI) stained parasites to ensure there were no more than two nuclei per parasite.

Parasite isolation by saponin lysis

Incubated parasite samples were collected in microcentrifuge tubes and centrifuged at 750 G for 3 min. The supernatant was removed and the infected RBC pellet resuspended in 0.5 ml PBS containing 0.1 % (w/v) saponin (Sigma-Aldrich, Steinheim, Germany) to lyse the RBCs. Parasites were pelleted at 1500 G for 3 min and washed extensively (5 times) by centrifugation in cold PBS to remove RBC membranes and excess haemoglobin.

SDS-PAGE and Western blotting

SDS-PAGE was performed according to the procedure described by Laemmli.[13] Parasite pellets of pre-incubated cultures were resuspended in 3 volumes of dH_2O and 1 volume of 4 x reducing SDS-PAGE sample buffer and boiled for 5 min to ensure protein denaturation. Samples (20 µl) were electrophoresed on a 10 % sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel at a constant current of 20 mA until properly resolved. Resolved proteins were transblotted onto Hybond-ECL nitrocellulose membranes (GE Healthcare) at 100 V for approximately 1 h. Membranes were subsequently incubated in blocking solution (2 % (w/v) fat-free milk powder, 0.1 % (v/v) Tween in PBS) for 45 min before being incubated in blocking solution containing rabbit anti-haemoglobin antiserum (1:1000) (Sigma-Aldrich, Steinheim, Germany) for 1 h. The membranes were washed in blocking solution and further incubated in blocking solution containing peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Sigma-Aldrich, Steinheim, Germany) for another hour. Membranes were washed thoroughly in PBS containing 0.1 % Tween and soaked in LumiGlo (an enhanced

chemilluminescence western blotting detection reagent) (KPL, Gaithersburg, MD). Membranes were exposed to Kodak Biomax Light autoradiography film for between 10 and 30 seconds and the film developed to visualize bound secondary antibodies. Photos of the developed films were taken with a Kodak DC290 digital camera with Kodak EDAS 290 gel documentation system. The net intensities of the individual haemoglobin bands were determined using Kodak 1D image analysis software version 3.5.

Immunofluorescence assay

Immunofluorescence assays were performed on untreated (control) and drug-pressured parasites to assess the effects of drugs on endocytic vesicle trafficking, and to visualize the location of haemoglobin inside the parasites.

Glass coverslips were treated with 1 mg/ml poly *L*-lysine in PBS for 15 min, placed into 24-well plates and washed 4 times with PBS. Parasite pellets of drug-treated and untreated cultures were resuspended in 30 µl PBS and added to wells containing coverslips in 300 µl PBS. The plates were centrifuged at 100 G for 2 min to pellet the parasites onto glass coverslips. Glass coverslips were washed with PBS (4 times) to remove unbound parasites. Bound parasites were fixed onto coverslips with PBS containing 4 % PFA, 0.25 % GLA (w/v) for 15 min, and coverslips washed for a further 4 times. Unreacted aldehydes were quenched by immersing coverslips in 0.15 M glycine for 20 min, and washed (4 times) in PBS. Coverslips were incubated in blocking solution (PBS containing 2 % (w/v) BSA, 50 % (v/v) fetal calf serum (FCS), 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1 % (v/v) Tween 20) for 30 min, followed by 1 h incubation with rabbit anti-haemoglobin antiserum (Sigma-Aldrich, Steinheim, Germany) diluted 1:200 in blocking solution. The coverslips were then washed in PBS containing 0.1 % (w/v) BSA and 0.1 % (v/v) Tween 20 and further incubated in

blocking solution containing the secondary antibody (tetramethylrhodamine (TRITC)-conjugated goat anti-rabbit antiserum, 1:250)(Invitrogen, Carlsbar, CA) for 1 h in the dark. Coverslips were washed 4 times, and incubated in PBS containing 30 μg/ml 4',6'-diamidino-2-phenylindole (DAPI) for 1 min to stain the parasite nuclei. The coverslips were rinsed by dipping into water (3 times), dried by gently dabbing on soft tissue and mounted onto glass microscope slides in Permafluor polyvinyl alcohol mounting medium (Immunotech) and allowed to dry overnight in a dry dark container. Slides were examined by fluorescence microscopy (Nikon Eclipse E600 fluorescence microscope) under a 100 times oil immersion objective lens. Images of the parasites were capture by media Cybernetics CoolSNAP-Pro monochrome cooled charge-couple device camera. Only parasites at mid to late trophozoite stage were included (≦ 2 DAPI-stained nuclei) in subsequent transport vesicle enumeration.

Cytotoxicity Assays

HeLa (Human cervical carcinoma) (ATCC no CCL 2)

HeLa cells are adherent epithelial cells, maintained in Eagles minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 0.1mM non-essential amino acids, 1.0 mM sodium pyruvate and 10 % bovine fetal calf serum (FCS).

Preparation of cells for proliferation experiments

Cell cultures were prepared in the prescribed ATCC tissue culture medium (specified for each cell line) supplemented with 10% FCS in 75 cm^3 culture flasks and sub-cultured three times a week. Tissue culture medium was discarded from the tissue culture flask containing the cells that were to be used and ± 5 ml trypsin/versene was added. Care was taken to coat all interior surfaces with the solution, before it was discarded. A small amount

of trypsin/versene (2-5 ml) was added, enough to cover the adherent cells on the bottom of the flask. The culture flask with trypsin/versene cell mixture was incubated at 37 ºC for a few minutes until the cells detached from the flask. The trypsin/versene cell mixture was transferred to a 15 ml centrifuge tube. Tissue culture media supplemented with 10% bovine FCS was used to rinse the culture flask and this was added to the mixture in the centrifuge tube, thus neutralizing the action of the trypsin/versene. Cell cultures which grow in suspension were decanted directly into 15 ml centrifuge tubes. The cell suspension was centrifuged for 6 minutes at 200 G. The supernatant was discarded and the cell pellet was re-suspended in 1 ml of the tissue culture medium specified for that specific cell line, supplemented with 10% bovine FCS. The cell suspension was mixed repeatedly by pippetting repeatedly with an automatic pipette to make sure that the cells were separated from each other and that a uniform suspension was formed.

Counting of cells

50 µl of the cell suspension which was prepared as described above was added to 450 µl of white cell counting fluid and mixed well. A small volume of this suspension was put onto a Haemocytometer and the cells were counted using a Reichert Jung Micro Star microscope. Dilutions were then made with the tissue culture medium specified for the specific cell line used, supplemented with 10% bovine FCS to achieve the required cell concentration for the cell proliferation assay.

Cell proliferation assay

Tissue culture plates were divided into different sections for controls (untreated cells) and experimental compounds (treated cells). 80 µl of the tissue culture medium (specified for the specific cell line used) supplemented with 10% bovine FCS (60 µl in the case of the

lymphocytes earmarked to be stimulated) was dispensed into each well of a 96 well tissue culture plate. 100 µl cell suspension was added to each well. Cultures were incubated at 37 ºC in an atmosphere of 5 % CO_2 for an hour. 20 µl of dilutions of the drugs to be tested (different concentrations) was added to the wells. Untreated control wells received 20 µl of culture medium. A control with DMSO was also done to make sure that there was no DMSO effect. Lymphocytes earmarked to be stimulated received 20 µl PHA 5 minutes after the addition of the drug. Cultures were incubated at 37 ºC in an atmosphere of 5 % CO_2 for seven days with the exception of the lymphocytes, which were incubated for three days before the MTT was added. All the cell cultures were incubated before the MTT was added.

MTT assay

After the incubation period (five days) of the tissue culture plates, 20 µl MTT (5 mg/ml) was added to each well. Cultures were incubated for 4 h at 37 ºC in an atmosphere of 5 % CO_2 and then centrifuged for 10 minutes at 800 G. Supernatant was removed from each well without disturbing the pellet with a Pasteur pipette and cells were washed by addition of 150 µl PBS and centrifuged 10 minutes at 800 G. Supernatant was removed from each well without disturbing the pellet with a Pasteur pipette and plates were left an hour to dry off at 37 ºC. 100 µl DMSO was added to each well to solubilize the formazan crystals. Culture plates containing the DMSO/formazan solution were put on a shaker for about 1-2 hours and absorbance was measured on a Universal Microplate Reader (ELx800 UV, Bio-tek Instruments) using a wavelength of 570 nm and a reference wavelength of 630 nm.

Percentage of control of cell growth in drug treated wells was calculated by dividing the mean absorbance of the treated cells by the mean absorbance of the untreated controls x 100 and these values were used to determine the IC_{50} value.

β-Haematin inhibition assays

Serial dilutions of the drug solutions were carried out in triplicate in a 96-well plate. Concentrations of drug solution corresponded to 0 – 10 equivalents relative to haematin in the final mixture. Each well contained 10.12 µl of drug solution. 101.2 µl of haematin stock solution (1.68 mM in 0.1 M NaOH) was added to each well. After mixing the solutions, 58.7 µl of acetate solution (12.9 M, pH 5.0) pre-incubated at 60 ºC was added and the plate was incubated for 60 min at 60 ºC. Thereafter, 80 µl 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. 38 µl of supernatant was transferred to another plate and diluted to 250 µl with 30 % (v/v) pyridine solution (pH 7.5, 20 mM Hepes). Data were collected using a microplate reader and analyzed by absorbance measurement at 405 nm.[14]

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 µl) was mixed with 2.02 µl of each drug dissolved in 1.0 M HCl in methanol. Acetate solution (11.74 µl, 12.9 M, pH 5.0) pre-warmed at 60 ºC was added. After 60 minutes of incubation, 250 µl 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.[14]

8.3 References

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