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Development of berberine-based derivatives as novel antimicrobial agents

Siritron Samosorn
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DEVELOPMENT OF BERBERINE-BASED DERIVATIVES AS NOVEL ANTIMICROBIAL AGENTS

A thesis submitted in fulfillment of the requirements of the award of

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DOCTOR OF PHILOSOPHY

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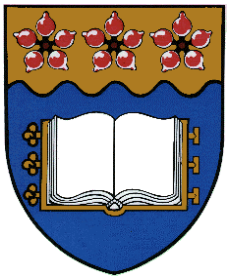

by

Siritron Samosorn, M.S. (Applied Chemistry)

Department of Chemistry

University of Wollongong

Wollongong, Australia

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AcOH	acetic acid
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ArH	aromatic proton
ATP	adenosine triphosphate
ax	axial
Boc	*tert*-butoxycarbonyl
$(Boc)_2O$	di-*tert*-butyl dicarbonate
br.d	broad doublet
br.m	broad multiplet
br.s	broad singlet
br.t	broad triplet
Bu	butyl
^{t}BuOH	*tert*-butanol
^{o}C	degree celcius
C	carbon
C. albicans	*Candida albicans*
CI	chemical ionization
13-CPTC	13-cyclopentylthio-5-hydroxy tetracycline
d	doublet
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
dd	doublet of doublets

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DMSO	dimethylsulfoxide
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E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
EI	electron impact
eq	(molar) equivalent/equatorial
ES	electrospray
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
FDA	food and drug administration
g	gram/s
gCOSY	gradient correlation spectroscopy
gHMBC	gradient heteronuclear multiple bond correlation
gHSQC	gradient heteronuclear single quantum correlation

H	hydrogen/proton
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HRP	horseradish peroxide
Hz	Hertz
IC_{50}	inhibitory concentration 50%
J	coupling constant
Lit.	literature
m	multiplet
M	molar (moles per litre)
m.p.	melting point
m/z	mass to charge ratio
MATE	multidrug and toxic compound extrusion
MDR	multidrug resistance
Me	methyl
MeOH	methanol
MFS	major facilitator superfamily
5'-MHC	5'-methoxyhydnocarpin
MHz	mekahertz
MIC	minimum inhibitory concentration
min	minute/s
mL	milliliters
μM	micromolar

mmol	millimoles
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectroscopy
NBS	*N*-bromosuccinimide
n-BuLi	*normal*-butyl lithium
NCS	*N*-chlorosuccinimide
NMR	nuclear magnetic resonance
OH	hydroxy
OMe	methoxy
P. aeruginosa	*Pseudomonus aeruginosa*
PBPs	Penicillin-binding proteins
PEG	polyethylene glycol
P. falciparum	*Plasmodium falciparum*
Ph	phenyl
$PhSO_2Cl$	benzenesulfonyl chloride
PLC	preparative thin layer liquid chromatography
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SARs	structure-activity relationships
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SMR	small multidrug resistance
TBDMS	*tert*-butyldimethylsilyl
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Abstract

Multidrug resistance (MDR) mediated by a drug efflux mechanism is one of the major drug resistance problems not only in bacteria but also in other microorganisms. NorA MDR efflux protein is a well characterized and major efflux pump in the pathogenic Gram-positive bacterium, *Staphylococcus aureus*. It contributes to the resistance to berberine and ciprofloxacin antibiotics by extrusion of these drugs from the cells of *S. aureus*. In order to overcome this type of drug resistance by dual action agents incorporating efflux pump inhibitor properties and antibacterial activity, a variety of new, aryl group-substituted 2-aryl-5-nitro-1*H*-indole efflux pump inhibitors were synthesized. In the synthesis of these 2-aryl-5-nitro-1*H*-indoles, a new procedure for the *N*-acylation of indoles was developed based on DCC/DMAP coupling with carboxylic acids. This method was particularly effective with 5-nitro-1*H*-indole. The activity of these indole derivatives as inhibitors of the NorA MDR pump in *S. aureus* was assessed. It was found that some of the 2-aryl-5-nitro-1*H*-indole derivatives potentiated the activity of the antibacterial agents berberine and ciprofloxacin against the resistant strain, K2361, of *S. aureus*. The new 2-aryl-5-nitro-1*H*-indole inhibitors were particularly effective in potentiating the antibacterial activity of berberine. The compound [4-benzyloxy-2-(5-nitro-1*H*-2-yl)-phenyl]-methanol (**43**) was the most potent NorA pump inhibitor found in this work.

A number of dual action antibacterial agents were designed and synthesized. These included dual action prodrugs, in which the MDR pump inhibitor and berberine were attached in the same molecule with enzymatically cleavable linkages (ester or amide groups), and dual action drugs with a non-cleavable linkage (methylene group). In the synthesis of the dual action agents, a direct new approach to 13-substituted berberine derivatives was found. This approach involved alkylation of 8-

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In the case of the dual action prodrugs, the amide prodrugs were more active than the ester prodrugs against the Gram-positive bacterium *S. aureus* and *vice versa* against the Gram-negative bacterium *S. enterica* Serovar Typhimurium. However, minimum inhibitory concentrations for all the dual action drugs and dual action prodrugs were near or at clinically useful concentrations (*ca.* 1μg/mL or less) as antibacterial agents against *S. enterica* Serovar Typhimurium SL1344R2, and they showed 400- to 1600-fold higher activity than the parent antibacterial agent berberine. The design principle of having in the one molecule an MDR inhibitor moiety and an antibacterial moiety was established as a viable one, which potentially could be extended to other types of antimicrobial agents.

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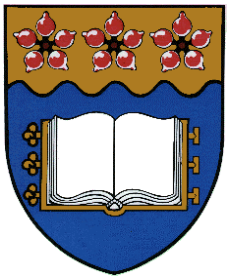

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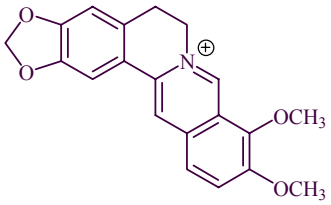
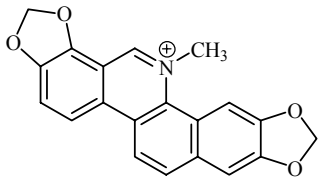
Chapter1: Introduction

1.1 Isoquinoline alkaloids as natural antimicrobial agents

Antimicrobials can be both natural products and synthetic chemicals, which are designed to inhibit or destroy pathogenic microorganisms, such as bacteria, fungi, protozoa, and viruses. Amongst the natural products, alkaloids play an important role as medicinal agents and as poisons, and they have been found in such varied sources as plants, animals, insects, marine invertebrates and microorganisms. A general definition of an alkaloid has been given by Pelletier[1] as "An alkaloid is a cyclic compound containing nitrogen in a negative oxidation state which is of limited distribution in living organisms." Alkaloids can be classified into several categories based on the chemical structure of their nucleus. Isoquinoline alkaloids are thus based on the isoquinoline nucleus. A few isoquinoline alkaloids, such as berberine and sanguinarine, are currently used clinically as antimicrobial agents,[2] but many others show antimicrobial activity. Berberine and sanguinarine occur in several genera of families including the Berberidaceae, Papaveraceae, and Rutaceae, and possess a variety of pharmacological properties including antimicrobial, antileukemic, antiulcerous, gastric antisecretory, and enzyme inhibitory activities.[2,3]



Berberine (**1**)



Sanguinarine

Berberine is a member of the protoberberine class of isoquinoline alkaloids, but sanguinarine is a member of the benzophenanthridine class of these alkaloids. The mechanism of antimicrobial activity of berberine and sanguinarine is related to their effect on DNA intercalation and inhibition reverse transcription and DNA synthesis in

microorganism cells.[4] The therapeutic uses of berberine are in the treatment of infected eyes and eye irritations (Murine^{TM}) while sanguinarine is used as an antiplaque in toothpaste and mouthwash.[5] Sanguinarine has been classified by the FDA as being unsafe for use in food and drugs.[6]

1.1.1 Antimicrobial activity and structure-activity relationships (SARs) of berberine and related alkaloids

Berberine was first isolated from the plant *Xanthoxylon cava* in 1926. Berberine extracts from plants have been used in the treatment of cholera and other bacterial diarrhoeas in Native American, Chinese, and Japanese traditional folk medicine for centuries. It has been reported that berberine and related alkaloids exhibit antibacterial activity against Gram-positive bacteria (e.g. *Staphylococcus aureus*, *Staphylococcus epidermidis, Enterococcus faecalis, Bacillus subtilis*) and Gram-negative bacteria (e.g. *Escherichia coli, Klebsiella pneumoniae*), antifungal activity against *Candida albicans* and *Aspergdlus fumigatus*, antiplasmodal activity against *Plasmodium falciparum*, and several other pharmacological activities. The extensive antimicrobial screening data (Amin *et al*.[7]) of berberine sulfate are shown in Table 1-1. Moreover, it has recently been reported that berberine possesses anti-tumour activity.[8] It is likely that berberine and its analogues show these activities because of the presence of the iminium ion moiety in the aromatic structure which could react with nucleophilic amino acid residues in enzymes and receptors of microorganisms. There have been some previous studies on the relationships between the structure and biological activity of these compounds in order to determine the structural regions important for antimicrobial activity. These studies may be summarized as follows:

Table 1-1 Antibacterial activity of berberinium sulfate[7]

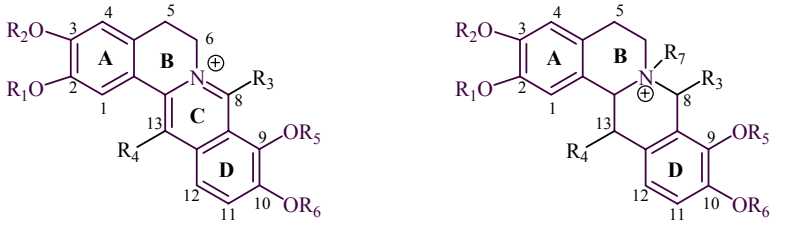
Organism	Minimum growth inhibitory concentration	
	μg/mL	μM*
Bacillus pumilus	25	57.7
Bacillus cerus	50	115.5
Bacillus sublilis	25	57.7
Corynebacterium diphtheriae	6.2	14.3
Escherichia coli	50 – greater than 100	115.5 – greater than 230.9
Klebsiella pneumoniae	25	57.7
Pseudomonas pyocyanea	>100	>230.9
Pseudomonas fluorescens	>100	>230.9
Salmonella paratyphi	>100	>230.9
Salmonella schottmuelleri	>100	>230.9
Salmonella typhimurium	>100	>230.9
Salmonella typhi	>100	>230.9
Shigella boydii	12.5	28.9
Staphylococcus aureus	6.2 - 50.0	14.3 - 115.5
Staphylococcus albus	50	115.5
Streptococcus pyogenes	12.5	28.9
Vibrio cholerae Inaba 569B	25	57.7
Vibrio cholerae E1 Tor Ogawa	50	115.5
Xanthomonas citri	3.1	7.2
Xanthomonas campestris	6.2	14.3
Xanthomonas malvacearum	12.5	28.9
Erwinia carotovora	100	230.9
Pseudomonas mangiferae	>100	>230.9
Pseudomonas solanacearum	>100	>230.9
Candida utilis	12.5	28.9
Candida albicans	12.5	28.9
Candida tropicalis	3.1	7.2
Sporotrichum schenkii	6.2	14.3

*The minimum inhibitory concentrations were converted from μg/mL into μM, using a calculated molecular weight of berberinium sulfate of 433 daltons.

Influence of the oxygen substituents in ring A

According to Iwasa *et al,*[9] the antimalarial activity of protoberberine alkaloids was influenced by the type of oxygen substituent on ring A (Figure 1-1a). Compounds having a methylenedioxy group on ring A (where $R_1 + R_2 = CH_2$) showed a higher activity against *P. falciparum* than compounds having a methoxy group at the same

positions, (R_1 = R_2 = OCH_3). In addition, replacement of a methoxy group at C-2 or C-3 by a hydroxy group resulted in an increase in activity.



a) Berberine derivative b) Tetrahydroberberine derivative

Figure 1-1 General structures and numbering of protoberberine alkaloid derivatives for SARs

Influence of substituents on ring C

There have been several investigations by Iwasa *et al*[9-12] on the effect of substituents at C-8, C-13, and of the quaternary ammonium ion of this ring on antibacterial and antimalarial activities. For example, the introduction of an alkyl group at C-8 (R_3 = CH_3, C_2H_5, *n*-C_3H_7, and *n*-C_4H_9) (Figure 1-1a) increased the antibacterial activity against *S. aureus* and *B. subtilis* as the alkyl side chain length increased. Adding a bromine atom at C-12 on ring D of these compounds also increased antibacterial activity (Figure 1-1a). Substitution of various alkyl groups at C-13 (R_4, Figure 1-1a) was evaluated and optimized. The alkyl side chain length was increased from methyl to *n*-hexyl group, with the 13-hexylberberine derivatives found to exhibit the highest antibacterial activity. The R_4 substituent at C-13 also was changed to OH, OMe, OEt, OCOOEt, and $OCON(Me)_2$. Their antimalarial activities were lower than berberine itself against *P. falciparum*. Reduction of protoberberinium salts (Figure 1-1a) to tetrahydroberberine derivatives (Figure 1-1b) significantly reduced the antibacterial activity against *S. aureus* and *B. subtilis*. Making the *N*-metho salts (R_7 = Me in Figure 1-1b), and also adding a methyl group at C-13 caused an increase in activity. Moreover, changing of the B/C junction of the *N*-metho salt derivatives in the

tetrahydroberberine series from *trans* to *cis* caused a decrease in the activity. The SARs for antibacterial and antimalarial activities were not compared by Iwasa.

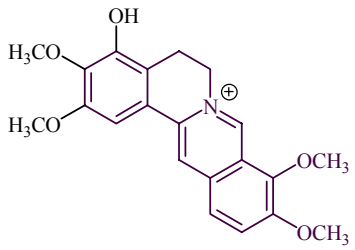
Influence of type and position of oxygen on ring D

The type of oxygen group at C-9 strongly affected the antimicrobial activity in the berberine series. It has been reported[13] that berberine and berberrubine, the latter differing in structure from berberine only at C-9, where R_5 = H (Figure 1-1a), both mediate DNA cleavage but in the DNA cleavage assay berberrubine was more active than berberine. Alkylation of the hydroxy group of berberrubine at C-9 with an alkyl halide, where the alkyl groups were hexyl, heptyl, octyl, nonyl, decyl, and undecyl groups, gave alkyl ether products with higher antibacterial activities against Gram-positive bacteria than those for berberrubine. The activities increased as the alkyl chain length increased up to dodecyl, but alkyl chains longer than the dodecyl had decreased activity. In the same way, acylation of the hydroxy group of berberrubine at C-9 was also investigated. The acyl groups were varied from octanoyl, decanoyl, lauroyl, myristoyl, to palmitoyl groups. The antibacterial activities increased as the length of the chain increased, but then decreased with longer chains than the lauroyl group.[14,15]

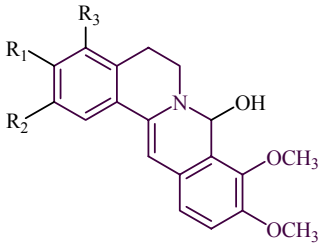
1.1.2 Cytotoxicity of berberine and related alkaloids

Berberine is known to possess cytotoxic activity. The IC_{50} values are 7.32 μM against KB cells (human carcinoma of the nasopharynx),[16] 0.03 μM against Hela (human uterus carcinoma), less than 0.03 μM against $SVKO_3$ (human ovary carcinoma), Fadu (human pharynx carcinoma), Hep-2 (human larynx carcinoma) and moderate toxicity against primary cultures from mouse embryos and human fibroblasts. The cytotoxic activity comparison of berberine with related alkaloids was evaluated using

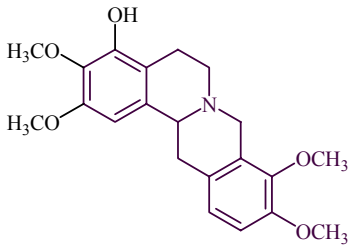
human cancer cell lines. Berberine showed higher cytotoxicity than lincangenine, 8-hydroxydihydroberberine and 8-hydroxylincangenine, and thaicanine which has a *trans*-quinolizidine conformation.[17]



Lincangenine



8-Hydroxylincangenine: $R_1 = R_2 = OCH_3$, $R_3 = OH$
8-Hydroxydihydroberberine: $R_1 = R_2 = OCH_2O$, $R_3 = H$



Thaicanine

In addition, Iwasa *et al*[18] investigated the cytotoxicity of several protoberberine alkaloids against human cancer cell lines (lung, colon, CNS, stomach, ovarian, breast, renal, melanoma), and they showed that the cytotoxic activity paralleled the antimicrobial activity. Compounds bearing a methylenedioxy group at C-2 and C-3 were more cytotoxic than those with a methoxy group at the same positions. Compounds having alkyl side chains at C-13 also showed cytotoxicity, with an increase in cytotoxicity occurring with an increasing chain length.

1.1.3 Structural features for antimicrobial and cytotoxic activities

In summary, it has been established that the following features are important for antimicrobial and cytotoxic activities in the berberine series:

1. a quaternary nitrogen atom
2. aromaticity of ring C
3. the type of *O*-alkyl substituent on rings A and D
4. the size of the substituent at C-13.

1.2 Antibacterial drug resistance problem

A number of antibiotics were developed in the 20th century to combat bacterial infections. Penicillin, one of the earliest antibiotics, is produced by the fungal species *Penicillium* sp. and was found to have the ability to kill *S. aureus*, a bacterium responsible for causing skin infections. Other antibiotics found in the same period, and shown in Table 1-2, include the sulfonamides and streptomycin which were used to combat a wide range of bacterial infections.[19]

Table 1-2 Evolution of resistance to antibiotics[20]

Antibiotic	Year deployed	Resistance observed
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracyclin	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	late1960s

The discovery of successful antibiotics encouraged scientists to research and develop new antibiotics. However, pathogenic microbes also have tried to develop intrinsic self-protection to combat these antibiotics resulting in the emergence of bacterial resistance (Table 1-2). Antibiotic resistant bacteria have now become a major worldwide health problem. During the past six to seven decades, over 100 antibiotics/antibacterial agents have been discovered and developed. The major classes of antibacterial agents are β-lactams (e.g. penicillin, methicillin, cephalosporins), aminoglycosides, tetracyclines, sulfonamides, macrolides (e.g. erythromycin),

quinolones and glycopeptides (e.g. vancomycin). These antibiotic classes are grouped according to their targets at the surface of the bacterial cell or inside the cell.[21] One of the most significant antibiotic resistance problems observed in clinical practice is the increase in the number of the isolates of methicillin-resistant *S. aureus* (MRSA) strains. MRSA is the most common antibiotic-resistant organism in hospitals, and ranks as the most frequently isolated pathogen associated with blood stream infections in North America between 1997-2001.[22] Several variations of multidrug resistance (MDR) have been found in MRSA isolates, with resistances to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics, fluoroquinolones and other antibiotics. Vancomycin was the last line of defence against serious infections caused by MRSA, but the emergence of vancomycin resistance of *S. aureus* was reported in Japan in 1996,[23] and subsequently in many other countries. Similarly, *Enterococcal* strains are also commonly isolated pathogens in blood steam infections in North America.[22] Many *Enterococcus faecium* isolates are now vancomycin-resistant and *E. faecalis* isolates also showed resistance to aminoglycosides and β-lactams. Unfortunately, the dual drug quinupristin-dalfopristin (Synercid™, a new drug that has activity against MDR *S. aureus* and has been introduced into hospital use) does not control *E. faecalis*.[24,25] Clearly, new drugs are urgently needed to overcome the serious multidrug resistance problem in pathogenic bacteria.

1.3 Mechanisms of antimicrobial resistance

Antibacterial agents can kill bacteria or stop their growth by attacking three main targets in bacterial systems: bacterial cell wall biosynthesis, bacterial protein synthesis, and bacterial DNA replication and repair.[26] In response, bacteria have developed a number of resistance mechanisms to protect themselves from antibacterial agents.

These bacterial survival strategies fall into three major types, as outlined in the following sub-sections.

1.3.1 Resistance by antibacterial alteration

Resistance to a wide range of antibacterial agents can be achieved by modification of the antibacterial agents. This mechanism results in destruction of the antibacterials by deactivating the drugs to inactive forms. For example, deactivation of penicillin and cephalosporin antibiotics can be caused by β-lactamase enzymes, which are produced by resistant bacteria (Figure 1-2). The strained four-membered β-lactam ring, the active structural moiety in the penicillin antibiotics, can be opened by β-lactamase to give penicilloic acid, which is inactive as an antibiotic.[27]

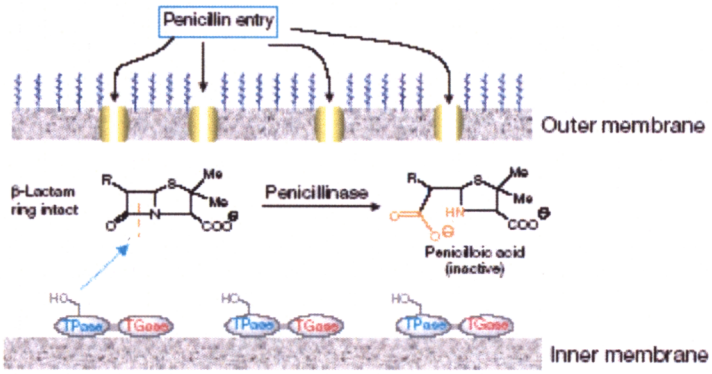


Figure 1-2 Antibiotic destruction by enzymes produced by resistant bacteria (Figure from reference 26)

1.3.2 Resistance by bacterial target modification

This mechanism involves modification of the drug target to such an extent that it is insensitive to the antibiotic, whilst still keeping its essential cellular function. An example of this occurs in vancomycin-resistant *Enterococci* (VRE). The mode of action of vancomycin involves the disrupting of bacterial cell wall synthesis.

Peptidoglycan is an important protein in bacterial cell wall synthesis. Cross-linking of peptidoglycans occurs at the D-Ala-D-Ala terminus of peptidoglycan and this is important for cell wall strength. Vancomycin acts by binding to the D-Ala-D-Ala terminus of the peptidoglycan and preventing this essential cross-linking by the transpeptidase enzyme. The *vanHA* genes of *E. faecium* encode a new pathway of cell biosynthesis which involves a change in the D-Ala terminus to an ester linkage with D-lactate (Figure 1-3) that results in a poor binding of vancomycin to the modified peptidoglycan terminus of *N*-acyl-D-Ala-D-lactate and allows the VRE to survive.[28,29]

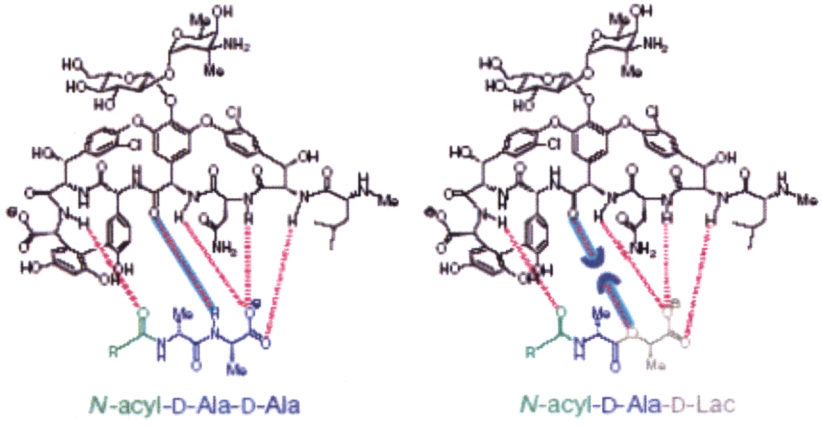


Figure 1-3 Binding of vancomycin to D-Ala-D-Ala as peptidoglycan models (Figure from reference 26)

1.3.3 Resistance by reducing antibacterial permeability

This resistance mechanism reduces the intracellular accumulation of antibacterial agents in bacterial cells through transmembrane proteins. The action of membrane-based efflux pumps has been shown to play an important role in the recent development of multidrug resistance to antibiotics.[30,31] Basically, the development of efflux-mediated resistance occurs through the up-regulation of genes encoding transporters that efficiently extrude drugs from the bacterial cell and result in a low ineffective

concentration of the drugs in the cell. For example, tetracycline and erythromycin antibiotics are pumped out from bacterial cells (e.g. *E. coli*) *via* efflux pump protein (Figure 1-4). The pump exports these drugs from the bacterial cell faster than the drug diffuses into the cell, so the intracellular drug concentration is not high enough to destroy the cell.

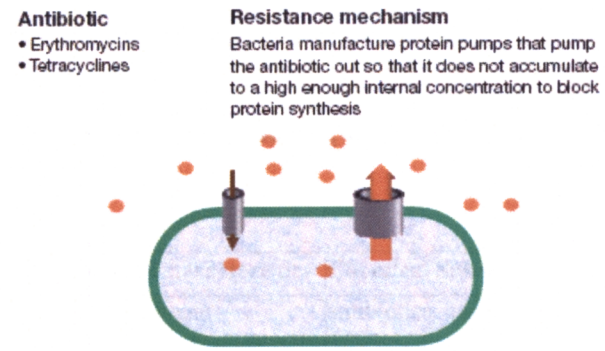


Figure 1-4 Drugs are pumped out of bacterial cells *via* efflux pump proteins (Figure from reference 26)

1.4 Bacterial efflux pump classification

To date, five families of bacterial drug efflux pumps have been identified based on the energy source used for extruding substrates and on sequence similarity (Table 1-3). Bacteria can possess efflux proteins from one or more families. The Major Facilitator Superfamily (MFS), Resistance-Nodulation Division Family (RND), Small Multidrug Resistance Family (SMR), and Multidrug and Toxic Compound Extrusion Family (MATE) use a proton motive force (PMF), pH gradient and electrochemical formation to efflux antibacterial agents in exchange for protons. The ATP-Binding Cassette Superfamily (ABC) of transporter proteins derive their energy from ATP hydrolysis.[30]

MFS transporters contain about 400 amino acids and have been classified into two subfamilies, which have 12 and 14 transmembrane helices (Figure 1-5). The pumps

with 14-helix transporters are QacA, EmrB from *E.coli*, TetK from *S. aureus*, TetL from *Bacillus stearothermophilus*, and TcmA from *Streptomyces glaucescens*. Others having 12-helix transporters are Blt and Bmr from *Bacillus subtilis*, EmeD from *E. coli*, and NorA from *S. aureus*. The substrates of this family are a variety of organic cations, including quaternary ammonium compounds such as benzalkonium chlorides, which are pumped out by QacA, plus uncharged drugs such as chloramphenicol which is extruded by NorA and Bmr.[32]

Table 1-3 Antibacterial Resistance Efflux Families, Biochemical Characteristics, Efflux Substrates and Bacteria Hosts[33]

Family	Biochemical Characteristics	Chemical Substrates	Bacterial Hosts (genera)
Major Facilitator Superfamily (MFS)	12 or 14-membrane spanning segments	antibiotics, quaternary ammonium compounds basic dyes, phosphonium ions	*Mycobacterium* *Lactobacillus* *Staphylococcus* *Bacillus* *Escherichia* *Streptococcus* *Vibrio*
Resistance Nodule Cell Division (RND)	Multi-component segments: membrane protein	basic dyes, detergents antibiotics, fatty acids	*Escherichia* *Pseudomonas* *Neisseria* *Haemophilus*
Small Multidrug Resistance Family (SMR)	Approximately 100-120 amino acids in primary structure, 4 helices	antibiotics, quaternary ammonium compounds antiseptics, tertraphenyl phosphonium, ethidium	*Bacillus* *Escherichia* *Mycobacterium* *Staphylococcus*
Multidrug and Toxic Compound Extrusion Family (ABC)	12-putative membrane spanning segments	dyes, fluoroquinolones aminoglycosides	*Haemophilus* *Vibrio* *Bacillus*

SMR transporters are the smallest known translocases with about 110 amino acids, and only 4 transmembrane domains; an example is the EmrE transporter from *E. coli*. The substrates are similar to the MFS, namely hydrophobic cations, but the range

is narrower than that of the MFS transorters.[34] Many multidrug transporters of the MFS and SMR families have acidic residues at a similar position in helix 1, suggesting that they are using a similar mechanism to bind to similar substrates.[35]

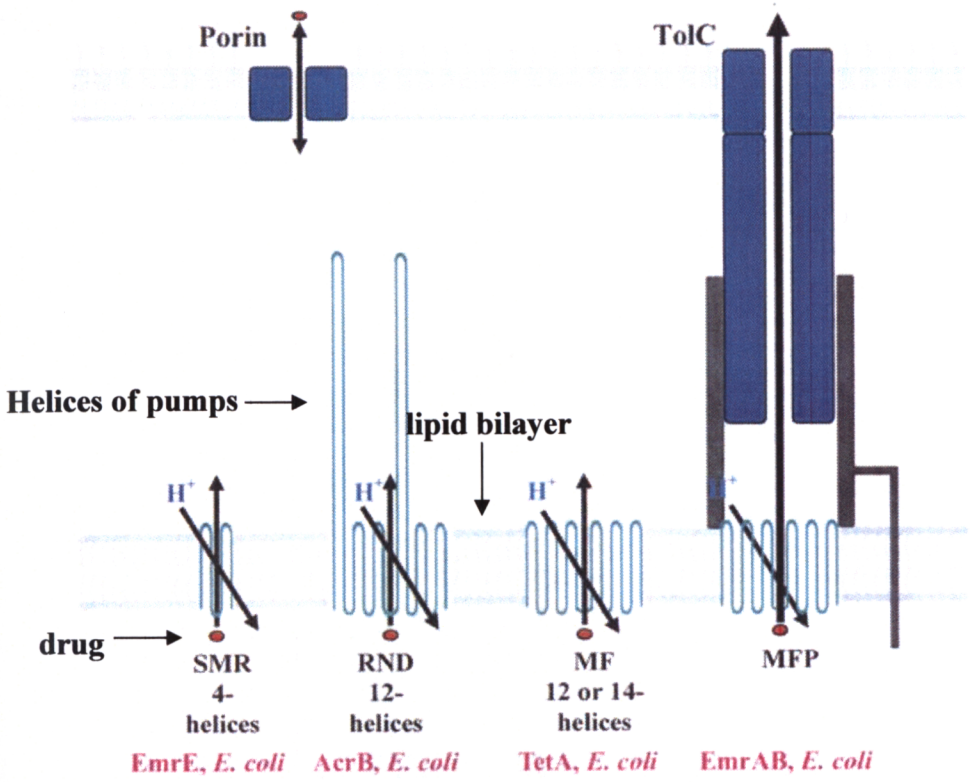


Figure 1-5 Diagram of the membrane topology of proton-driven drug pumps in Gram-negative bacteria[35]

RND transporters contain about 1000 amino acids, which make them much bigger than the MFS transporters, with a similar 12-helical structure except for extracytoplasmic domains between helices 1 and 2 and between helices 7 and 8 (Figure 1-5). A mutation of the pumps in this family leads to susceptibility to a wide range of substrates, which carry positive, negative or no charge, suggesting that the pumps can capture their substrates, which are only partially inserted into the membrane lipid bilayer.[36] Recently, the crystal structures of outer membrane TolC[37] and inner (cytoplasmic) membrane AcrB[38] transporter proteins in *E. coli* have been solved while

that of AcrA protein is still unknown (Figure 1-6). The structures of TolC and AcrB proteins were predicted to line up to from one continuous channel, which is connected together with the AcrA protein. This system exports a wide range of substrates from both cytoplasm and periplasm to the outside of the bacterial cells. This double protection system confers resistance to antibiotics and makes *E. coli* dangerous pathogenic bacterium.

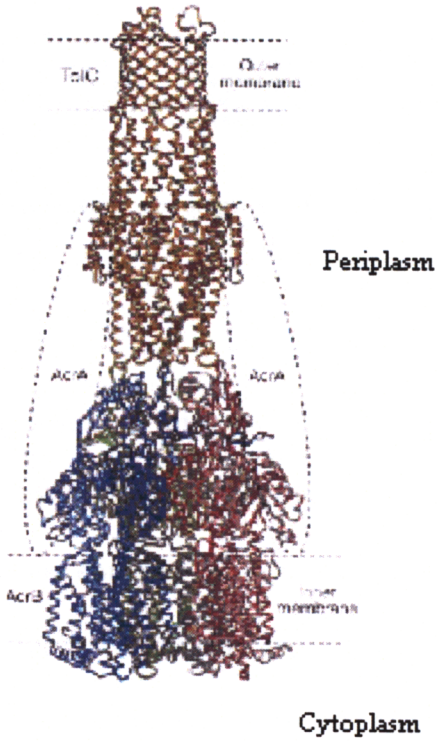


Figure 1-6 Proposed model of the AcrB-AcrA-TolC drug export complex in *E. coli*[38]

MATE transporters were the most recently identified family, with 12 transmembrane helices containing a total of 450 amino acids. The NorM transporter from *Vibrio parahaemolyticus* has been characterized as a multidrug Na^+- antiporter, which confers resistance to cationic compounds, dyes, fluoroquinolones, and aminoglycosides.[39] No proposed mechanism has been reported.

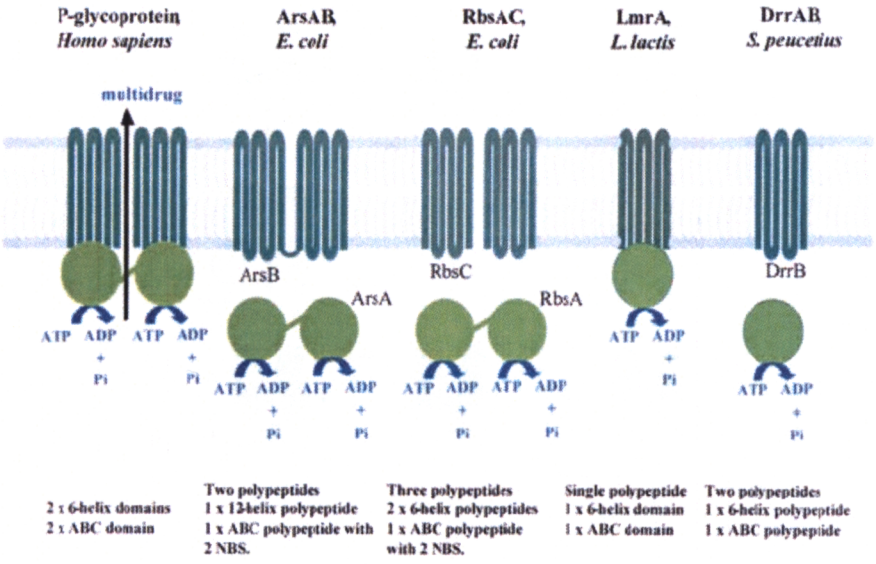


Figure 1-7 Diagram of the membrane topology of drug transporters belonging to the ABC superfamily[35]

ABC transporters represent a minority of efflux pumps. They derive their transport energy from ATP hydrolysis and have two similar halves, each containing two parts of twelve transmembrane domains arranged into six α-helices, and a nucleotide-binding domain (NBD) (Figure 1-7). The wide range of substrates of this family includes dyes, ionophoric peptides, lipids and steroids. Recently, the MacB transporter from *E. coli* has been identified as being involved in the extrusion of macrolide antibiotics.[35] The proposed general mechanism of the pumps in this family can be described by the process involved in the LmrA pump from *Lactococcus lactis*, which is a multidrug transporter with structural and functional identity with the P-glycoprotein (P-pg) pump in humans (Figure 1-8). The interconversion of two conformational forms of the protein is induced by ATP, which have a high-affinity transport-competency in one intracellular site, and a low-affinity drug-release site in an extracellular region.

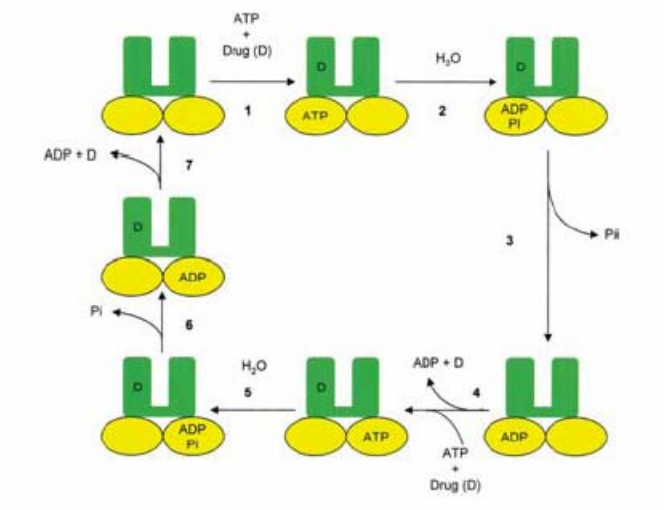


Figure 1-8 Simple model of P-gp transport

"The NBDs are represented in yellow and the membrane-spanning domains in green. Step 1: binding of ATP to the NBD of one half of the transporter triggers drug binding to a high-affinity intracellular drug-binding site. Step 2-3: ATP is hydrolysed, occluding the drug-binding site. Step 3-4: release of the generated Pi leads to the exposure of the drug at an extracellular-facing, low-affinity drug-binding site on the same domain. At this point the drug can be released. As a result of the remaining ADP and co-operative interaction between the two NBDs, ATP is likely to bind to the non-liganded NBD. Step 4-5: further conformational changes are likely to result in the exposure of a high-affinity drug-binding site on the intracellular side of the membrane domain that is associated with the liganded NBD. Step 5-6: drug binding at this site results in ATP hydrolysis and the drug-binding site becomes occluded. Step 7-1: release of Pi exposes a low-affinity drug-binding site at the extracellular side and the drug is released. Release of the drug and ADP allows the return of the transporter to the original conformation, and the cycle can begin again."[35]

1.5 Bacterial MDR pump inhibitors

There are a few proposed mechanisms of inhibitor action against MDR transporters: these include direct binding of the inhibitor to the binding sites of the pump protein causing no drug transport,[40] destruction of the pumps' energy by

inhibiting binding of ATP and modifying protein structure by an inhibitor interaction with the cell membrane.[29,41]

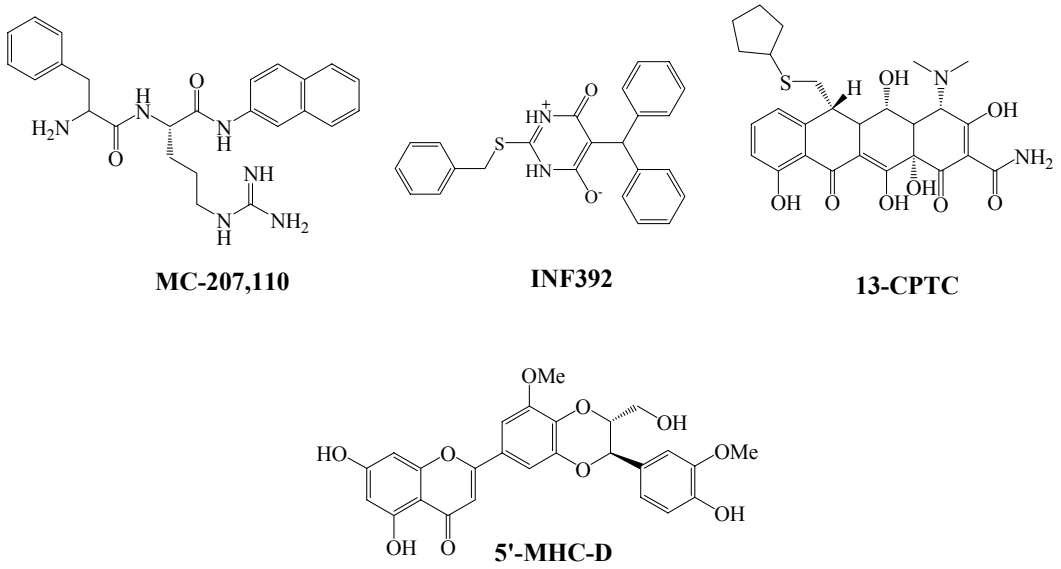


Figure 1-9 Inhibitors of MDR pumps

A number of potential inhibitors of bacterial efflux pumps have been discovered, such as MC-207,110, a broad-range inhibitor and the first inhibitor active against multiple RND transporters in Gram-negative bacteria, which was produced by Microcide Pharmaceuticals.[42] It is an aminonaphthalene derivative of a phenylalanine-arginine dipeptide (Figure 1-9), and it was found to possess superior activity to inhibit RND multidrug pumps of *P. aeruginosa, Enterobacteriacea, Haemophilus influenza* and *Stenotrophomonas maltophilia*, which potentiates the effects of fluoroquinolone antibiotics.[42-44] Several semisynthetic tetracycline analogues have been synthesised, of which the most potent analogue with ability to inhibit the TetB protein, is 13-cyclopentylthio-5-hydroxytetracycline (13-CPTC, Figure 1-9). This compound has been shown to inhibit tetracycline efflux in *E. coli*.[45] INF 392, the most potent synthetic NorA pump inhibitor of a series of INF analogues (Figure 1-9), has been shown to potentiate the bacteriocidal activity of ciprofloxacin or ethidium bromide in *S. aureus*; INF392 also reduced the number of spontaneous mutants in *S. aureus* to ciprofloxacin

by 50-fold or more.[33,46] 5′-Methoxyhydnocarpin-D (5′-MHC-D, which had originally been given the name 5′-methoxyhydnocarpin, 5′-MHC),[47-49] a natural potent inhibitor of the NorA pump, was found in a leaf extract of Barberry (*Berberis fremontii*) and was found to potentiate the activity of typical substrates of the NorA pump, such as: berberine, ethidium bromide, and triphenylphosphonium ion.[47] Some inhibitors of the NorA pump will be discussed further in Chapter 3 as part of this thesis.

1.6 Definitions of dual action prodrugs and dual action drugs

Dual action prodrugs, also known as mutual prodrugs, are pharmacological derivatives of two different but generally synergistic drug molecules, combined together with a covalent linkage, which requires spontaneous or enzymatic transformation within the body to release the two active compounds.[50,51] Over the past two decades, the prodrug concept has been optimized in an attempt to solve some problems found in a large number of existing drugs, and has also become an integral part of the new drug design process. The application of a prodrug approach has been successful in the enhancement of the parent drug activity, for example, with dual action antibiotic hybrids of cephalosporins and quinolones that attack bacteria with two completely different modes of action. Cephalosporins are active against *Streptococci* and belong to the β-lactam class of antibiotics, and quinolones are active against β-lactam-resistant strains.

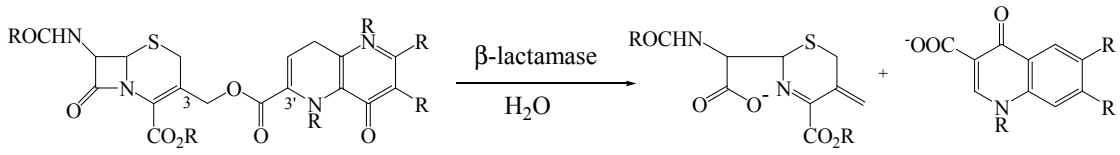


Figure 1-10 Release of quinolone on hydrolysis of a cephalosporin-quinolone ester prodrug

For a quinolone to be microbiologically active then its 3-carboxyl group must be free. The 3-carboxyl group of the quinolone is combined to a cephalosporin nucleus at

position 3 *via* an ester link (Figure 1-10). Quinolone activity is generated by one of three mechanisms. Firstly, when the prodrug is in the presence of an active β-lactamase, which catalyzes the hydrolysis of the amide bond in the β-lactam ring, resulting in ring opening and subsequent release of the quinolone carboxylic acid; secondly, when the prodrug undergoes spontaneous hydrolysis; and thirdly, when the penicillin-binding proteins (PBPs) in the cytoplasmic membrane of bacteria are acylated by the cephalosporin causing inhibition of cell-wall synthesis and cell death *via* the standard mode of action of β-lactam antibiotics. Therefore, the dual action prodrug cephalosporin-quinolone esters act as cephasporins and also as prodrugs for quinolones.[52-54]

Dual action drugs (or hybrid drugs) are pharmacological derivatives of two different drug molecules attached to each other and do not require biotransformations to release the two active compounds.[50] The dual action prodrug and dual action drug approaches of parent compounds A and B can be illustrated as shown in Figure 1-11.

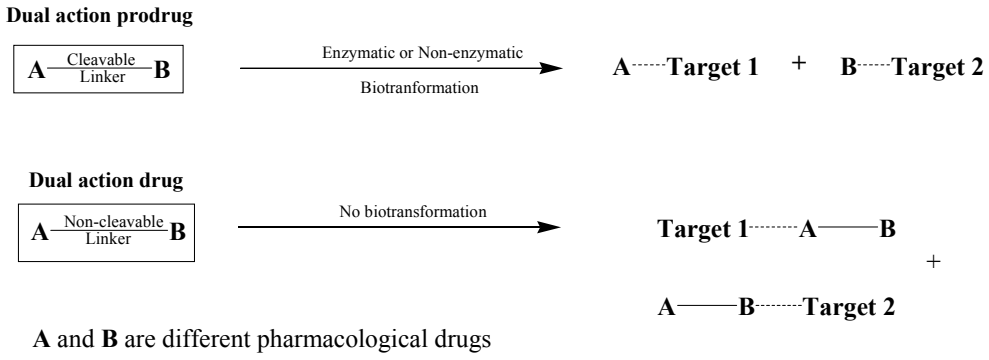


Figure 1-11 Diagram illustrating dual action prodrug and drug approaches

The main aim of the work in this thesis was to attempt to overcome the antibacterial resistance problem in certain bacteria using compounds based on both the dual action prodrug and dual action drug concepts. Both design concepts were to

incorporate two molecular components having different biochemical targets, and were to be delivered to the target sites in the bacterial cell in effective concentrations. Some of the advantages and disadvantages of dual action drugs and dual action prodrugs are summarized in Table 1-4, and a comparison made with the administration of two separate drugs (dual drug approach). This last approach has been used successfully in antibacterial therapy, for example with Augmentin (amoxicillin (antibacterial) and potassium clavulanate (β-lactamase inhibitor)).[55] A key potential advantage of the dual action drugs or prodrugs was the synchronous (or near synchronous) delivery in high concentration of active components to different bacterial target sites.

Table 1-4 Comparison of dual action drugs and dual action prodrugs with two separate drugs (dual drugs).

Type	Advantages	Disadvantages
Dual Drugs	• Known components administered	• Formulation; administration • Different pharmacokinetics of each drug • Drug may not synchronously accumulate at bacterial sites • More side effects
Dual Action Drugs	• Synchronous delivery to different bacterial target sites and in high concentrations • Slower development of resistance • Improved formulation • Improved chemical stability • Decreased toxicity	• Each activity may be reduced (e.g. steric reasons) • Molecular weight high
Dual Action Prodrugs	• Synchronous or near synchronous delivery of active agents • Slower development of resistance? • Improved formulation • Improved chemical stability • Decreased toxicity	• Synthetic difficulties • Molecular weight high

1.6.1 Design principles of dual action antimicrobials

One of the three resistance mechanisms of bacteria to antibiotics, as mentioned above**,** is mediated through reducing antibacterial permeability. Recently, there has been a report by Stermitz *et al*.[47] on the synergistic antibacterial activity in components of a medicinal plant, *Berberis fremontii*, involving the alkaloid berberine (**1**) and the flavonolignan 5´-MHC-D (Figure 1-9). Berberine possesses a planar aromatic cationic center that is thought to provide the antimicrobial activity and also to serve as a recognition site by efflux pump proteins in the microbial cells; berberine is pumped out from the cells thus reducing its potency. 5´-MHC-D flavonolignan is not a typical MDR substrate or antibacterial agent, but it can potentiate the activity of berberine by blocking the MDR (NorA) pump in *S. aureus* as shown in Figure 1-12.

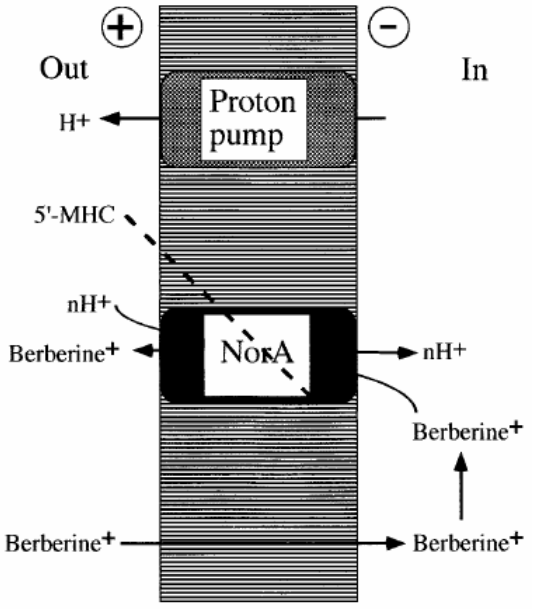
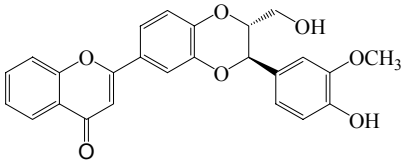
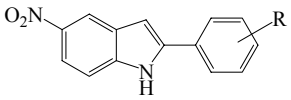


Figure 1-12 "A model of the synergistic action of berberine and an MDR inhibitor that are both produced by *B. fremontii*. Berberine accumulates in the cell driven by the membrane potential. The NorA pump extrudes berberine. The MDR inhibitor 5'-MHC, which has been renamed to 5'-MHC-D and confirmed its structure by HMBC NMR spectroscopic data,[49] blocks the NorA pump, potentiating the antibiotic action of berberine."[47]

This knowledge could be adapted to attack resistant strains of pathogenic bacteria by using an MDR pump inhibitor in combination with an antimicrobial agent which is normally effluxed by this pump. Thus, the design of dual action prodrugs and dual action drugs to combat antibacterial resistance by reducing this antibacterial permeability mechanism could be achieved potentially via a combination of antibacterial agent and MDR pump inhibitor in the same molecule. The linkage between the two moieties could be designed to cleave under bacterial enzymatic action (e.g. ester or amide hydrolysis) for dual action prodrugs, or to be a non-cleavable under enzymatic action for dual action drugs. The prodrugs should deliver synchronously (or near synchronously) the antibacterial agent and the MDR pump inhibitor in high concentration at or near the appropriate bacterial sites, and a dual mode of action would be exerted. Additionally, this approach could help to eliminate the undesirable properties associated with administering the two parent molecules separately (dual drugs) as summarized in Table 1-4. Similar considerations could apply to the development of novel antimalarial agents.



5,7-Deoxyhydnocarpin-D



2-Aryl-5-nitro-1*H*-indole derivatives

There are a variety of antimicrobial agents and many types of MDR pump inhibitors. In this project the antimicrobial agent of interest was the alkaloid berberine, which has a range of antimicrobial activity, and the MDR pump inhibitor was focussed on the flavonolignan 5,7-deoxyhydnocarpin-D,[56] the most potent synthetic NorA pump inhibitor, and on the simpler 2-aryl-5-nitro-1*H*-indole NorA pump inhibitor derivatives.

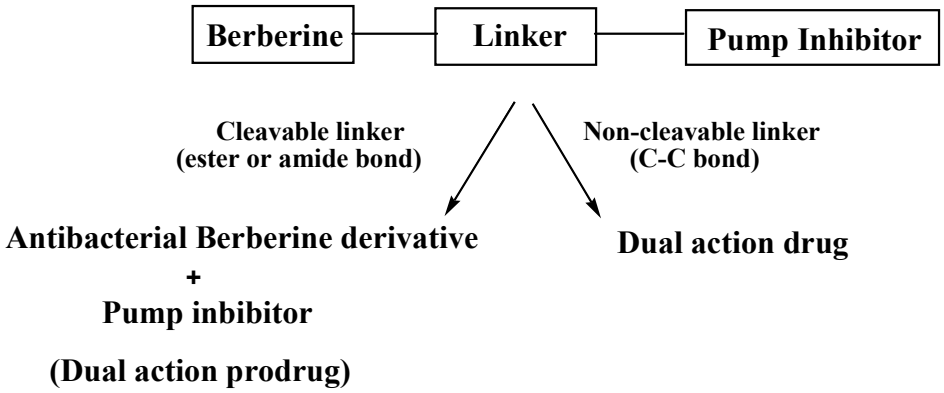


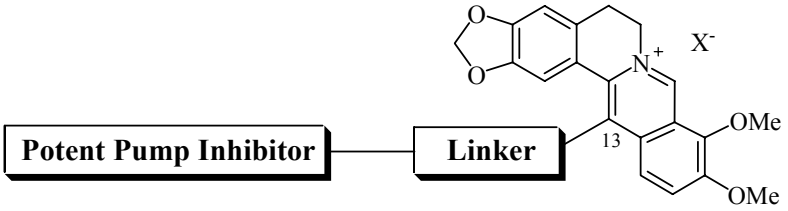
Figure 1-13 Diagram illustrating the dual action prodrug and dual action drug designs

As shown in Figure 1-13, the dual action prodrugs and dual action drugs of efflux pump inhibitor-berberine prodrugs are formed to enhance a drug's utility. Once inside the bacterial cell the dual action efflux pump inhibitor-berberine prodrugs with cleavable linkages should revert to the antibacterial berberine derivative and efflux pump inhibitor at the same time by an enzymatic or non-enzymatic process, and then show their activities when they reach the target sites in high concentration. The dual action efflux pump inhibitor-berberine hybrids with a non-cleavable linkage are similar to the ones with cleavable linkage, but would not require a post-barrier transformation process to release the two parent moieties.

1.7 Aims of project

The aims of this project were:

1. To design new antimicrobial agents of berberine-based derivatives based on the dual action prodrug and dual action drug concepts.



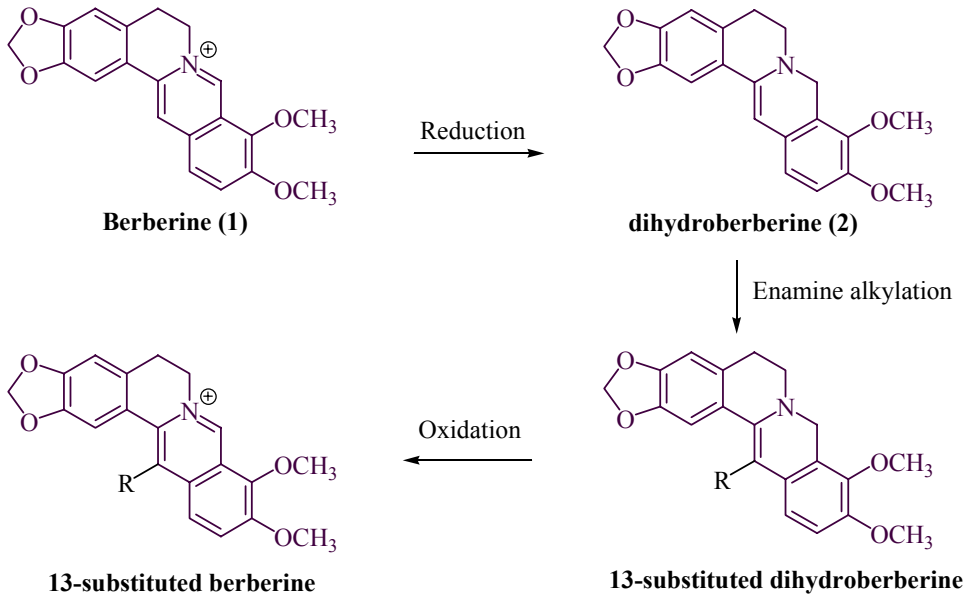
Model of dual action agents

2. To develop a synthetic route to the dual action agents.
3. To synthesize variants on the dual action agents with different enzyme-sensitive linking groups (substituted ester; amide), and enzyme-insensitive linking groups.
4. To assess the antibacterial potency (and spectrum of activity) and antimalarial activity of the new derivatives produced, and to define structure-activity relationships.
5. To identify leads for further drug development.
6. To measure MDR inhibition of the new efflux pump inhibitors in the NorA efflux pump.

Chapter2: Synthesis of berberine derivatives

On the basis of the SARs of berberine analogues discussed in Chapter 1, substitution at C-13 of berberine can increase the antibacterial activity. Thus, a combination of an efflux pump inhibitor *via* the 13-position of berberine, based upon the dual action prodrug and dual action drug concepts, formed the basis for the synthetic targets in this study. The inhibitor was expected to act synergistically by preventing the expulsion of the berberine-based pump substrate.

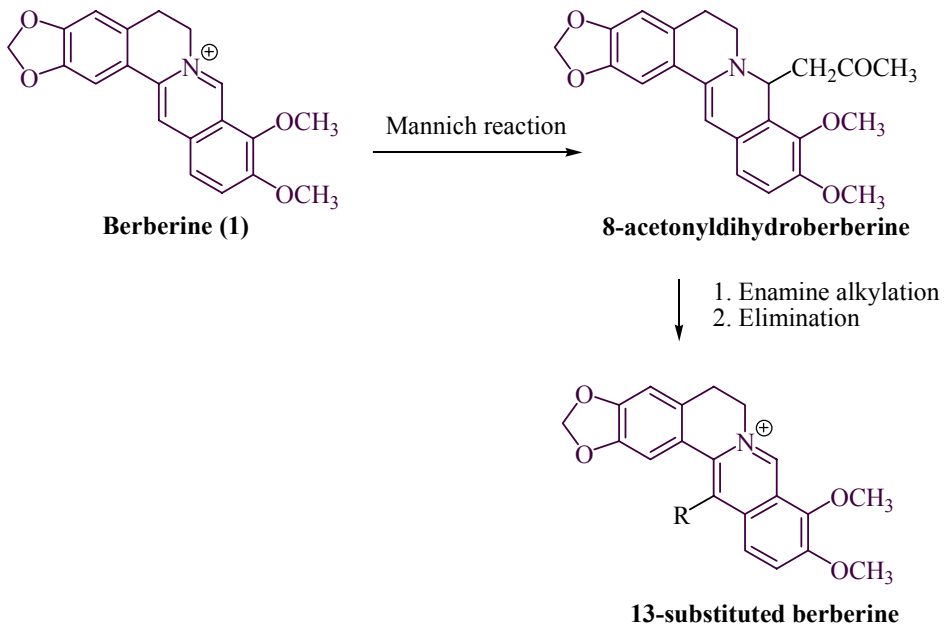
A number of 13-substitued berberine derivatives were thus prepared from berberine chloride in 3 steps *via* dihydroberberine (Scheme 2-1) or in 2 steps *via* 8-acetonyldihydroberberine derivatives (Scheme 2-2).[9,57,58]



Scheme 2-1 Preparation of 13-substituted berberine derivatives from berberine *via* dihydroberberine (Note: for simplicity the counterion for the salts is omitted in this Scheme and, on occasion, also in subsequent Schemes)

In the first synthetic approach, berberine (**1**) was reduced with sodium borohydride[59] and potassium carbonate in methanol[57] to afford dihydroberberine (**2**),

followed by enamine alkylation with electrophiles (R-X), and subsequent oxidation with *N*-chlorosuccinimide (NCS) or *N*-bromosuccinimide (NBS) to give the 13-substitued berberine salt derivatives. The target compounds were also prepared in 2 steps (Scheme 2-2) by treatment of **1** with acetone and aqueous sodium hydroxide solution to afford 8-acetonyldihydroberberine and subsequent enamine alkylation with electrophiles (R-X) followed by elimination of acetone to afford the salt derivatives. One problem associated with the 8-acetonyldihydroberberine route is that the berberine salt (**1**) can be regenerated quite readily by elimination.[60]

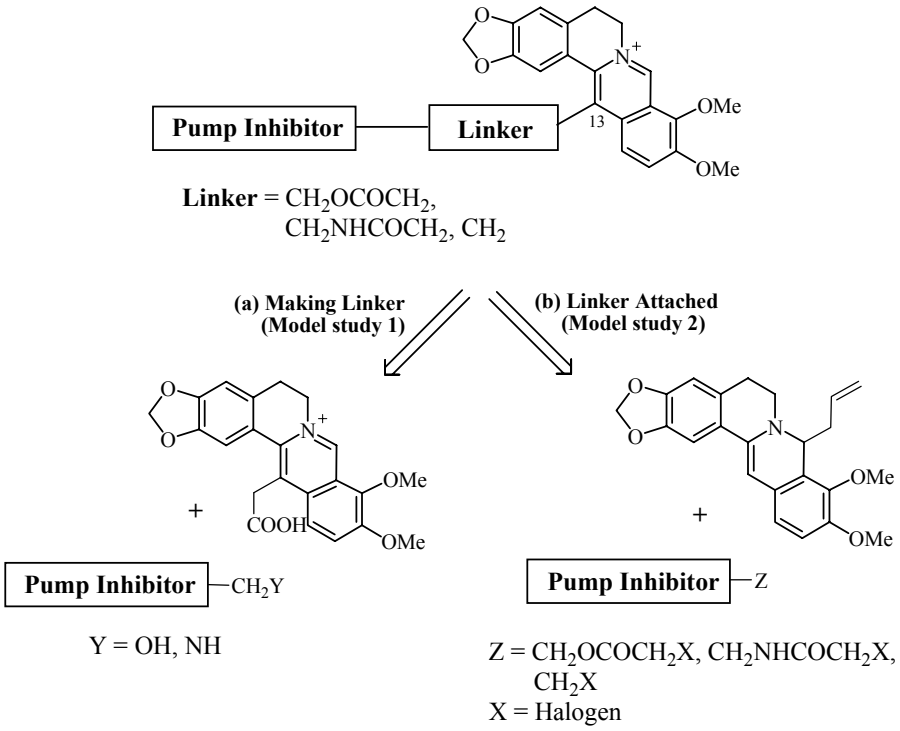


Scheme 2-2 Preparation of 13-substituted berberines in 2 steps *via* 8-actonyldihydroberberine

2.1 Retrosynthesis of efflux pump inhibitor-berberine dual action agents

The preparation of efflux pump inhibitor-berberine dual action agents with a cleavable linker can be achieved *via* the DCC coupling of berberine acid derivative with

a range of pump inhibitors. The alcohol derivative of the pump inhibitors can be attached to a berberine acid derivative *via* an ester linkage. Similarly, the amine derivatives of pump blockers can be attached to a berberine acid derivative to form an amide linkage (Model study 1). Alternatively, the dual action agents may be achieved *via* enamine alkylation of dihydroberberine derivatives with an inhibitor containing an alkyl halide group to give products both with cleavable and non-cleavable linkages (Model study 2). The general retrosynthetic analysis with respect to the two model studies is shown in Scheme 2-3.



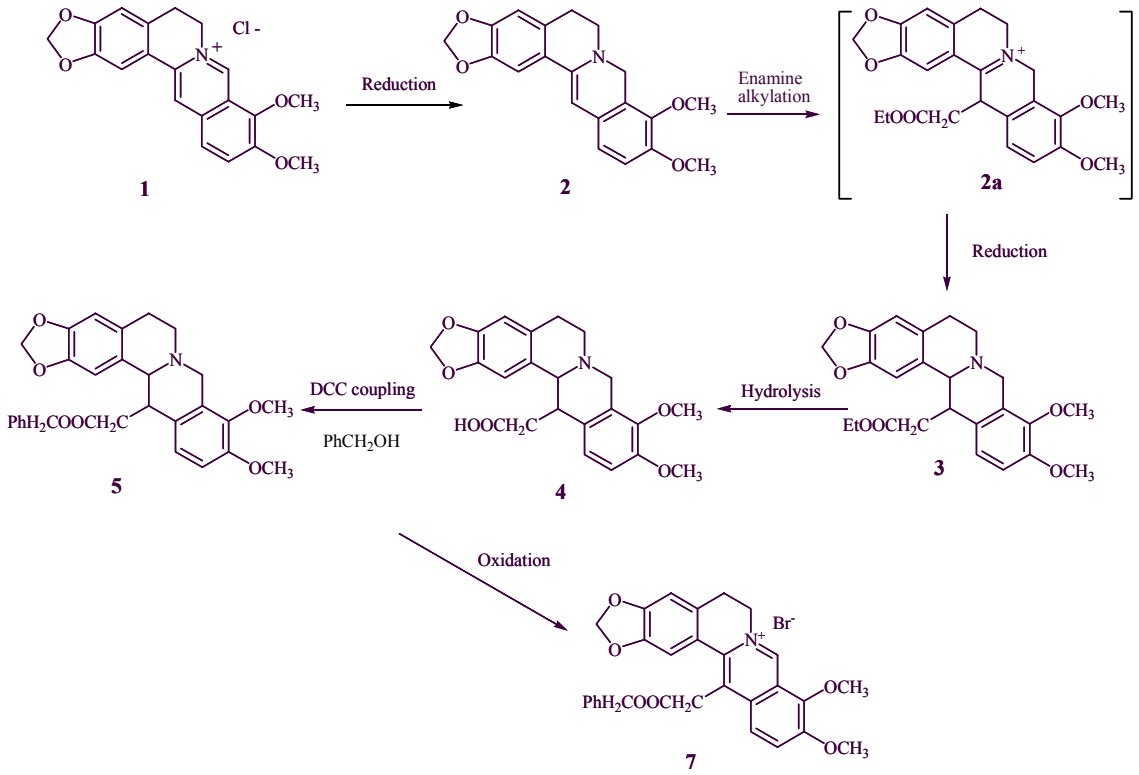
Scheme 2-3 General retrosynthetic analysis of efflux pump inhibitor-berberine dual action agents

2.2 Synthetic strategies

2.2.1 Model Study 1

In this model study, in summary, the tetrahydroberberine acid derivative **4** was prepared (Scheme 2-4), and then coupled with benzyl alcohol as an MDR inhibitor model. Subsequent oxidation then gave the berberine salt (**7**).

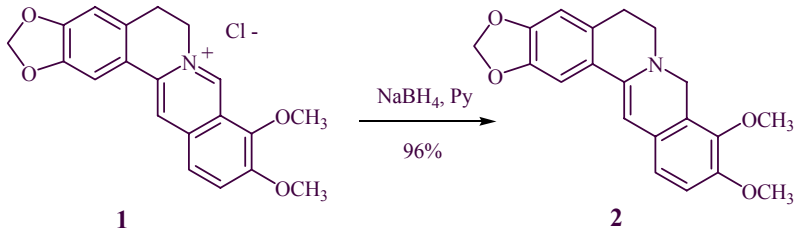
Preparation of the model target compound **7** began from commercially available berberine chloride (**1**), which was reduced with sodium borohydride to give dihydroberberine (**2**). Enamine alkylation of **2** with ethyl bromoactetate produced the 13-substituted iminium salt (**2a**) as an unstable product, which was reduced immediately with sodium borohydride in methanol to afford the 13-substituted tetrahydroberberine (**3**). Hydrolysis of **3** with an aqueous solution of lithium hydroxide in methanol gave the tetrahydroberberine acid (**4**). DCC coupling of **4** with benzyl alcohol then afforded the benzyl ester (**5**), followed by oxidation with NBS to yield the model target compound (**7**). A detailed discussion of these steps is given in the following sub-sections.



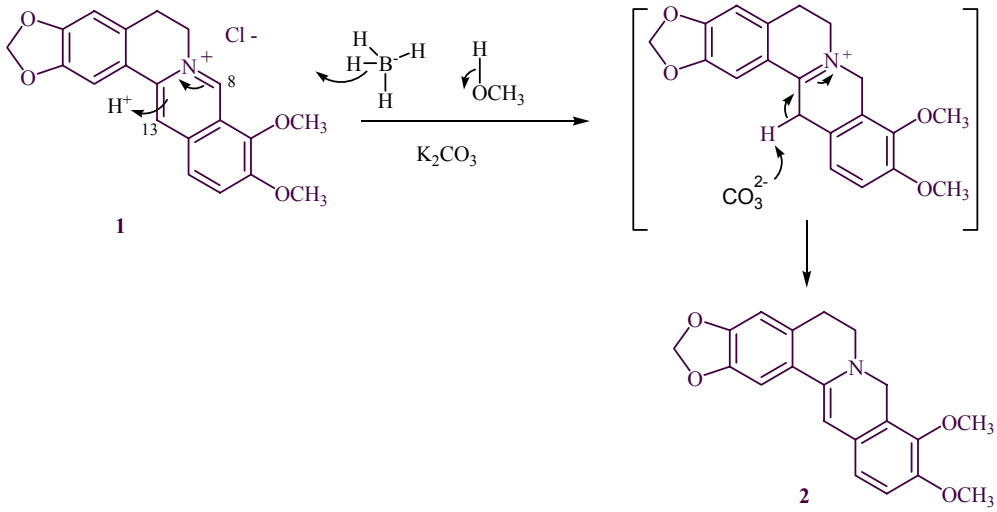
Scheme 2-4 Synthetic strategy for preparation of efflux pump inhibitor-berberine prodrug *via* DCC coupling.

2.2.1.1 Reduction of berberine chloride

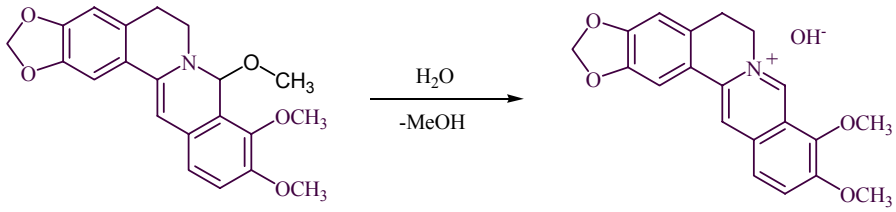
Berberine chloride (**1**) has been reported to be reduced to its enamine (**2**) in high yield using $NaBH_4$ in an aprotic solvent (e.g. pyridine) or in a protic solvent (e.g. MeOH) in the presence of K_2CO_3, the yield of **2** were 73%[59] and 98%[57], respectively. In the present work, compound **2** was prepared by both methods. Reaction of **1** with $NaBH_4$ in pyridine (Scheme 2-5) was found in the present study to afford a higher yield (96%) of **2** than from $NaBH_4$ and MeOH in the presence of K_2CO_3 (53%, Scheme 2-6). A possible mechanism for the latter reagent conditions is *via* a hydride transfer to C-8, and then abstraction of the proton at C-13 by carbonate (Scheme 2-6). This method was easier to handle than the $NaBH_4$/pyridine method which required anhydrous conditions, and the unpleasant solvent, pyridine.



Scheme 2-5 Preparation of dihydroberberine (2) using $NaBH_4$ in pyridine



Scheme 2-6 Preparation of dihydroberberine (2) using $NaBH_4$ in MeOH and K_2CO_3

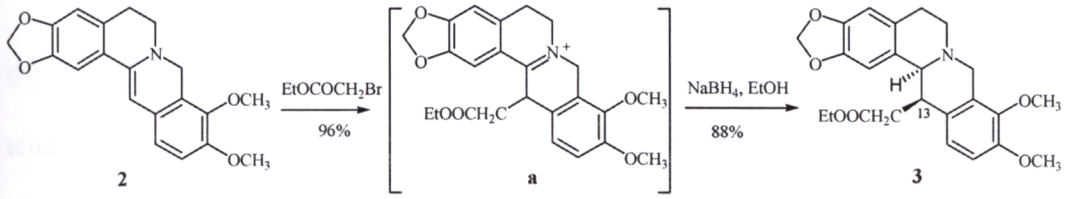


Scheme 2-7 Possible by-product berberine hydroxide generated in the presence of strong base

In the $NaBH_4$/MeOH reaction, a possible by-product could be formed from MeOH in the presence of strong base, which can generate a methoxide ion. The methoxide ion could attack at the C-8 atom of **1** to form an 8-methoxy enamine. Subsequently, the enamine formed might be protonated by water in the reaction and followed by washing successively the crude product with water (a stronger acid than methanol), leading to elimination of methanol and formation ultimately of the hydroxide (rather than methoxide) of **1** (Scheme 2-7).[61]

2.2.1.2 Enamine alkylation and reduction

The alkylation of enamine **2** with ethyl bromoacetate produced the non-purified iminium salt intermediate **a** in high yield (Scheme 2-8).



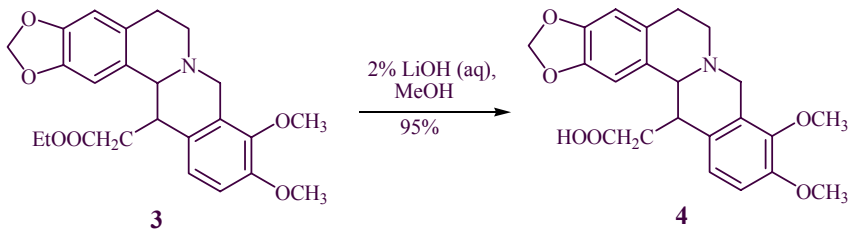
Scheme 2-8 Preparation of 13-ethylethanoate tetrahydroberberine (3)

Under various anhydrous reaction conditions, the highest yield of **a** obtained was 96% using neat ethyl bromoacetate and a reaction temperature of 100°C. Initially, the reaction was tried using a solvent, e.g. toluene, to dissolve the starting enamine **2** first, then an excess of dry ethyl bromoactate was added to the solution, and the reaction was then carried out at 80°C. The yields obtained were in the range of 60-70%, with many low polarity by-products being observed on TLC. Fortunately, the by-products were easily removed by trituration with toluene. As a result of the instability of the intermediate **a**, it was reduced immediately by $NaBH_4$ in EtOH at room temperature to yield the ester **3** in 88% yield. The structure of **3** was confirmed by the ^1H NMR spectrum, which showed the loss of the signal attributed to H-13, but a signal assigned to H-13a as a broad singlet at δ 3.72 was observed instead. In addition, the data showed a set of signals assigned to the methylene protons at δ 2.30 (1H), 2.44 (1H), and 3.98 (2H), which were attributed to the CH_2CO and OCH_2 groups respectively, together with a triplet signal ascribed to the methyl substituent of the ethyl ester at δ 1.15. MS (CI)

showed the molecular ion peak at *m/z* 426. All the spectroscopic data was consistent with the addition of an ethyl ethanoate substituent to the dihydroberberine at C-13.

2.2.1.3 Ester hydrolysis

The hydrolysis of ester **3** was attempted in both acidic and basic conditions. In the case of acidic hydrolysis, 2M HCl and 1M H_2SO_4 did not hydrolyze ester **3** at room temperature. Monitoring of the reaction by TLC before work-up of the reaction showed the disappearance of the starting ester **3** and the presence of a more polar component than ester **3**. After neutralization with $NaHCO_3$, and extraction with EtOAc, the product spot on TLC (silica gel) reverted back to the same R_f as the starting ester **3**. It is likely that **3** was protonated on the nitrogen atom by the acid without hydrolysis occurring, and then the free base of **3** was produced after neutralization. In contrast to the acidic hydrolysis, the hydrolysis of **3** under basic conditions proceeded smoothly. Thus, reaction of **3** with 2% LiOH in MeOH at 60°C[62] gave the carboxylic acid **4** in 95% yield (Scheme 2-9) after acidification. The ^{1}H NMR and ^{13}C NMR spectra confirmed the structure of acid **4** with the absence of ethyl group signals, and the presence of a carboxylic acid carbonyl signal at δ 173.6 in the ^{13}C NMR, together with the MS (EI) spectrum which showed a molecular ion peak at *m/z* 397 consistent with this product.

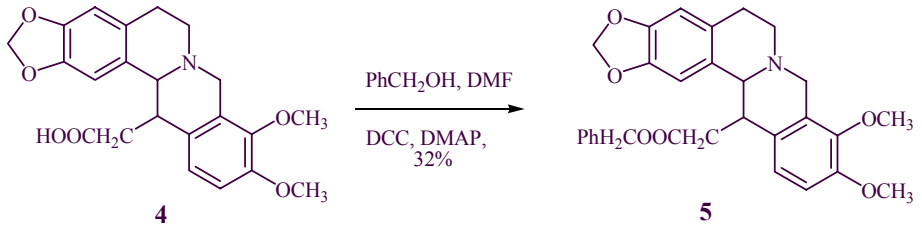


3 **4**

Scheme 2-9 Preparation of acid derivative 4

2.2.1.4 Esterification

The esterification of carboxylic acid **3** with benzyl alcohol was accomplished in the presence of DCC and DMAP under anhydrous conditions. Attempts to esterify the acid **4** with benzyl alcohol using EDCI with DMAP or DCC with HOBt in anhydrous DMF were not successful. The benzyl ester **5** was obtained in 32% yield (Scheme 2-10) with DCC and DMAP in DMF.

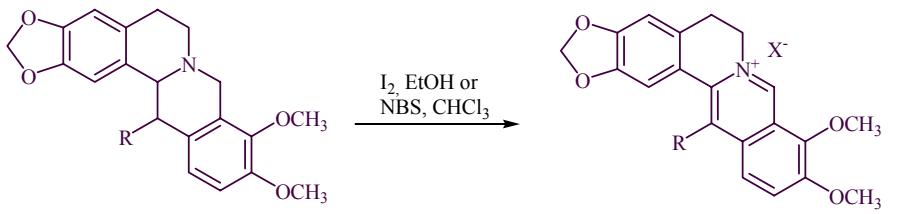


Scheme 2-10 Preparation of the benzyl ester berberine derivative 5

Initially the esterification was attempted using 1.5 eq of benzyl alcohol, 1.0 eq of acid **4** in DMF in the presence of DCC and DMAP and heating at 40^{o}C for 5 days. The reaction was monitored by TLC (silica gel) until no starting acid **4** could be detected, and then the reaction mixture was concentrated by distillation at 110^{o}C to remove the DMF. Removal of the excess benzyl alcohol required increasing the distillation temperature to 150^{o}C, which gave many unwanted by-products. However, using exactly 1 equivalent of benzyl alcohol overcame this difficulty. In the ^{1}H NMR spectrum of **5**, signals assigned to five aromatic protons at δ 7.19-7.35 were apparent, together with signals at δ 2.37 and 2.52 that were assigned to the CH_2CO protons, and at δ 4.97 which was ascribed to the OCH_2 protons.

2.2.1.5 Oxidation

The oxidation of tetrahydroberberines to their berberine salt derivatives was achieved using an oxidizing agent, such as I_2 in EtOH and NBS in $CHCl_3$. Following a literature procedure,[63] an initial trial oxidation reaction of ester **3** was performed using I_2 in EtOH. The iodide salt **6a** was produced in 97% yield (Scheme 2-11).



(**3**) R = CH_2COOCH_2Et
(**5**) R = CH_2COOCH_2Ph

(**6a**) X = I, R = CH_2COOCH_2Et, 97%
(**7a**) X = I, R = CH_2COOCH_2Ph, 16%
(**7b**) X = Br, R = CH_2COOCH_2Ph, 85%

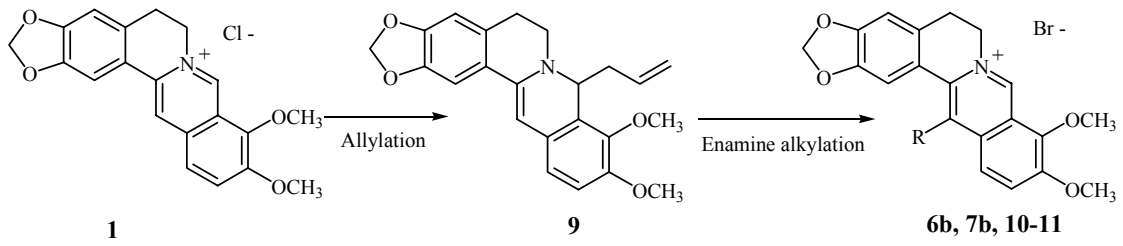
Scheme 2-11 Oxidation of tetrahydroberberines to their berberine salt derivatives

Under similar conditions to those used for the oxidation of **6a**, the ester **5** was oxidized to produce **7** in low yield. Unlike the oxidation of **3**, the oxidation of **5** did not give any insoluble product material after the addition of Na_2SO_3 to destroy the excess I_2. Thus, the reaction mixture was evaporated and chromatographed on silica gel, but due to the very strong interaction between the silica gel and product **7a,** purification was quite difficult. In a modified procedure, a large volume of EtOH was used in the oxidation reaction, and purification on alumina gave **7a** in 16% yield. Following a general literature procedure,[57] the oxidation of ester **5** was also carried out with NBS in chloroform to afford the bromide salt **7b** in high yield (85%). This method was milder and quicker than that of iodine in EtOH. Moreover, the purification of product **7b** did not require recourse to chromatography. The structure of **7b** was confirmed by the presence of a singlet signal in the ^1H NMR ascribed to H-8, which moved dramatically

downfield from δ 3.50 and 4.18 (2H, in **5**) to 10.56 (1H, in **7**). Additionally, two proton signals assigned to H-8 and H-13a in the starting material **5** were absent in the ^{1}H NMR spectrum of the product **7b**. The MS (ES) showed a positive ion peak at *m/z* 484, which corresponded to the molecular weight of compound **7**.

2.2.2 Model Study 2

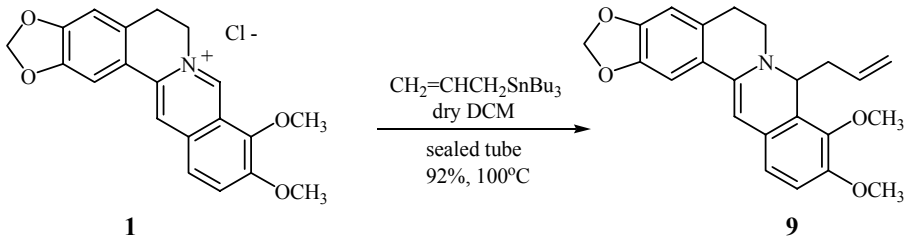
The basic synthetic strategy was to prepare the enamine (**9**) first, followed by alkylation to the 13-substituted berberinium salts. A brief discussion on the two-step preparation of the model target compounds **6b, 7b, 10-11** began from the commercially available berberine chloride (**1**). Compound **1** was reacted with allyltributyltin to give 8-allyldihydroberberine (**9**). Subsequent enamine alkylation of **9** with a range of alkylating agents produced the model targets (**6b, 7b, 10-11**) in moderate yield. The detailed discussion is given in the following sub-sections.



Scheme 2-12 Synthetic strategy for preparation of model efflux pump inhibitor-berberine prodrugs *via* enamine alkylation.

2.2.2.1 Allylation

α-Allylation of isoquinoline alkaloids can be accomplished by reaction with allyltin reagents, resulting in high yields of product. Commercially available allyltributyltin was used as the allylating agent in the preparation of the enamine **9**. Based on an adapted literature procedure,[64] the reaction was achieved by simply adding excess allyltributyltin to a suspension of berberine chloride (**1**) in DCM in a sealed tube and heating at 100°C for 8 hours. The product **9** was then obtained in 92% yield.



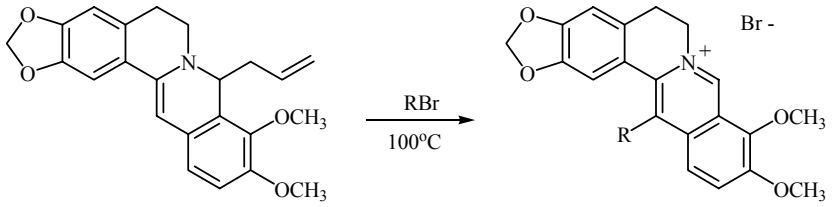
1 **9**

Scheme 2-13 Preparation of 8-allyldihydroberberine 9

The structure of **9** was confirmed by the presence of a characteristic signal pattern attributed to the allyl substituent in the ^{1}H NMR spectrum. It showed two groups of multiplet signals at δ 4.83-4.92 (2H) and 5.74-5.88 (1H), which were characteristic of methylene and methine protons attached to an sp^2 carbon (CH_2=CH-). A multiplet signal at δ 4.83-4.92 (2H) was assigned to the allylic methylene protons of the allyl substituent. In addition, the signal ascribed to H-8 moved dramatically upfield from δ 9.76 in the chloride salt of **1** to δ 4.83-4.92 in the enamine **9**, with an accompanying change in multiplicity from a singlet in **1** to a multiplet in **9** due to the coupling between H-8 and the allylic sp^3 methylene group. The MS CI) showed a molecular ion signal at *m/z* 378, which matched the molecular weight of compound **9**.

2.2.2.2 Enamine alkylation

13-Substituted berberine derivatives can be accessed by *C*-alkylation of 8-acetonyldihydroberberine as mentioned above in Scheme 2-2. The problem with the 8-acetonyldihydroberberine route is that the berberine salt (**1**) itself can be regenerated *via* a retro-Mannich reaction, which is competitive with the enamine alkylation to generate 13-substitued berberine derivatives.[60] To avoid this problem, the new 8-allyldihydroberberine **9** route was utilized. Heating of enamine **9** with a variety of bromide derivatives (ethyl bromoacetate, benzyl bromoacetate, phenacyl bromide and benzyl bromide) at 100°C afforded the 13-substituted salts (**6b, 7b, 10-11**) in moderate yield (scheme 2-14). It was found that using neat bromide alkylating agent derivatives gave better yields than with an added solvent except in the case of phenacyl bromide, for which a solution in acetonitrile was preferable.



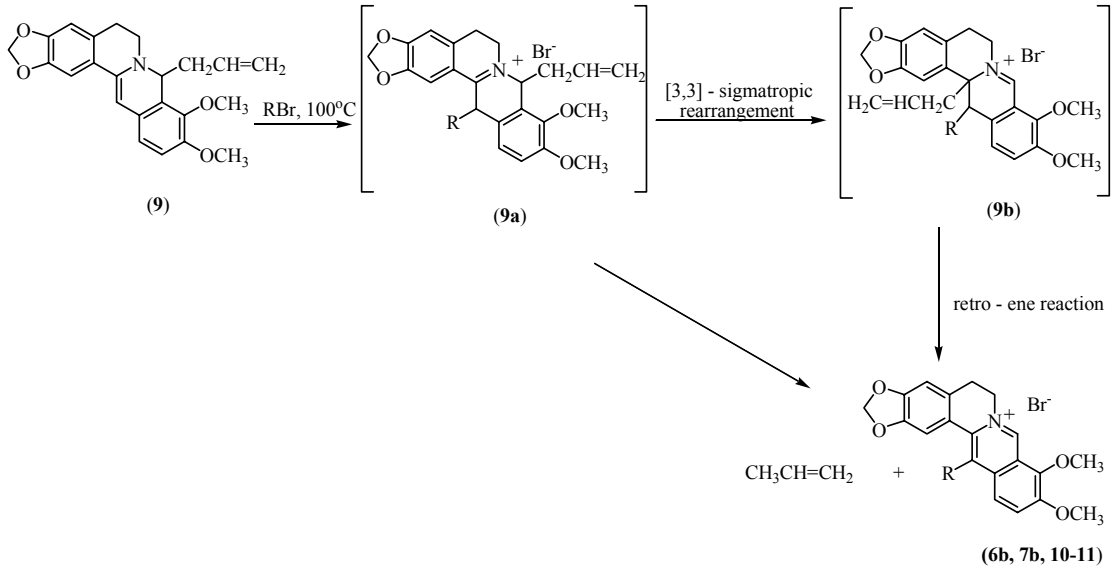
9

(**6b**) R = $CH_2CO_2CH_2CH_3$, 59%
(**7**) R = $CH_2CO_2CH_2Ph$, 57%
(**10**) R = CH_2COPh, 55%
(**11**) R = CH_2Ph, 62%

Scheme 2-14 Preparation of 13-substituted berberine salt derivatives

Mechanistically (Scheme 2-15), the formation of the salts may be rationalized in terms of initial enamine alkylation of **9** to give the intermidiate **9a**, followed by a [3,3]-sigmatropic rearrangement to **9b** and a subsequent retro-ene reaction[65] to afford the salts **6b**, **7b**, **10-11** and propene. The alternative step of direct elimination of propene from **9a** cannot be excluded however. While the acetonyldihydroberberine route to 13-substituted berberinium salts may also involve a [3,3]-sigmatropic rearrangement of a

tautomeric enol intermediate, this is considered less likely in view of the very low enol concentration expected; the driving force again would be the eventual formation of the aromatic ring in the berberine.



(**9**) (**9a**) (**9b**) (**6b, 7b, 10-11**)

Scheme 2-15 Proposed mechanism of formation of 13-substituted berberine salt derivatives

The structures of the 13-substituted berberine salts were confirmed by NMR spectroscopic analysis and mass spectrometry. The ^{1}H NMR spectrum of **6b, 7b, 10** and **11** revealed the loss of the allyl group with no signals present that could be ascribed to this substituent. The presence of a signal assigned to the H-8 proton appeared in the region of δ 10.46-10.61 as a singlet, while a triplet in the range of δ 2.9-3.2 was ascribed to the C5 protons. A broad signal at δ 4.6-5.0 was ascribed to the C-6 protons, which the methoxy group signals appeared at δ 3.8-4.2 and four aromatic methine proton signals were also apparent. In addition, a signal ascribed to the methine carbon of C-8 in the region of δ 145-148 was observed in the ^{13}C NMR of the products. Besides the characteristic signal pattern attributed to the berberinium salt nucleus, the expected ethyl ethanoate substituent signals of compound **6b** were seen in the ^{1}H NMR spectrum at δ 1.37 (3H) as a triplet, 4.36 (2H) as a quartet and 4.27 (2H) as a singlet. The MS (ES)

showed a positive ion signal at *m/z* 422 which was consistent with the molecular ion of **6b**. Compound **10** was also confirmed by a singlet signal attributed to the methylene protons attached to the carbonyl function at δ 5.07, and a signal integrating for five aromatic protons in the aromatic region. Moreover, the presence of a signal assigned to the CO in the ^{13}C NMR spectrum was observed at δ 196.7 and MS (ES) showed a positive ion peak at *m/z* 454 $[MH]^+$, which was consistent with the molecular ion of **10**. The ^{1}H and ^{13}C NMR spectra of compound **11** were broadly similar to those of **10** except for the absence of a carbonyl group signal in the ^{13}C NMR spectrum in the former compound.

In summary, the "model study 2" provided a concise new route to access 13-substituted berberine salts and it was chosen over "model study 1" for the later preparation of the berberine-pump inhibitor dual action agents. In order to use this route, it was necessary to prepare a variety of alkyl halide derivatives of the pump inhibitors. The efflux pump inhibitors and their synthesis are discussed in the following sub-sections.

Chapter3: Synthesis of Efflux pump inhibitors

3.1 NorA efflux pump

NorA protein is a major multidrug transport protein in the human pathogenic bacterium *S. aureus*, an organism which possesses several multidrug efflux pump proteins.[66] The NorA pump is a member of the Major Facilitator (MF) Superfamily having 12 transmembrane-spanning segments and is dependant on the transmembrane proton gradient to transport potentially harmful compounds out of the bacterial cell, and is driven by the proton motive force as a source of energy. The NorA protein is located in the cytoplasmic membrane and is encoded by the naturally occurring chromosomal *norA* gene, conferring an intrinsic resistance to a variety of structurally unrelated antibiotics.[67] The mechanism by which the NorA efflux protein recognizes multiple structurally dissimilar substrates is still unclear.

3.2 Substrates of the NorA efflux pump

As noted above, the NorA efflux pump actively exports a wide variety of compounds that are all structurally very different from each other, making it a powerful first line of defence for *S. aureus* against toxic molecules. Both naturally occurring and synthetic compounds can be pump substrates, including hydrophilic fluoroquinolones and monocationic organic compounds such as berberine, ethidium bromide, tetraphenylphosphonium, benzalkonium, chlorhexidine, pentamidine, norfloxacin and others (Figure 3-1).[66,68] The precise mechanism of multidrug recognition of the NorA pump is unclear due to the difficulty of performing high-resolution X-ray structural analysis on integral membrane proteins. Unfortunately none of the efflux pump proteins belonging to the MF superfamily have been crystallized with bound substrates,

which would provide a better understanding of how the efflux pump proteins can bind and transport a wide range of structurally dissimilar substrates. Recently, the structures of the first few homologous transporters to MFS-type multidrug efflux transporters, lactose permease LacY and glycerol-3-phosphate transporter GlpT, which are not multidrug efflux transporters *per se*, have been solved. These structures confirmed the proposal that their hydrophilic substrates were bound to the MF superfamily transporters within an intramembranous cavity.[69-71]

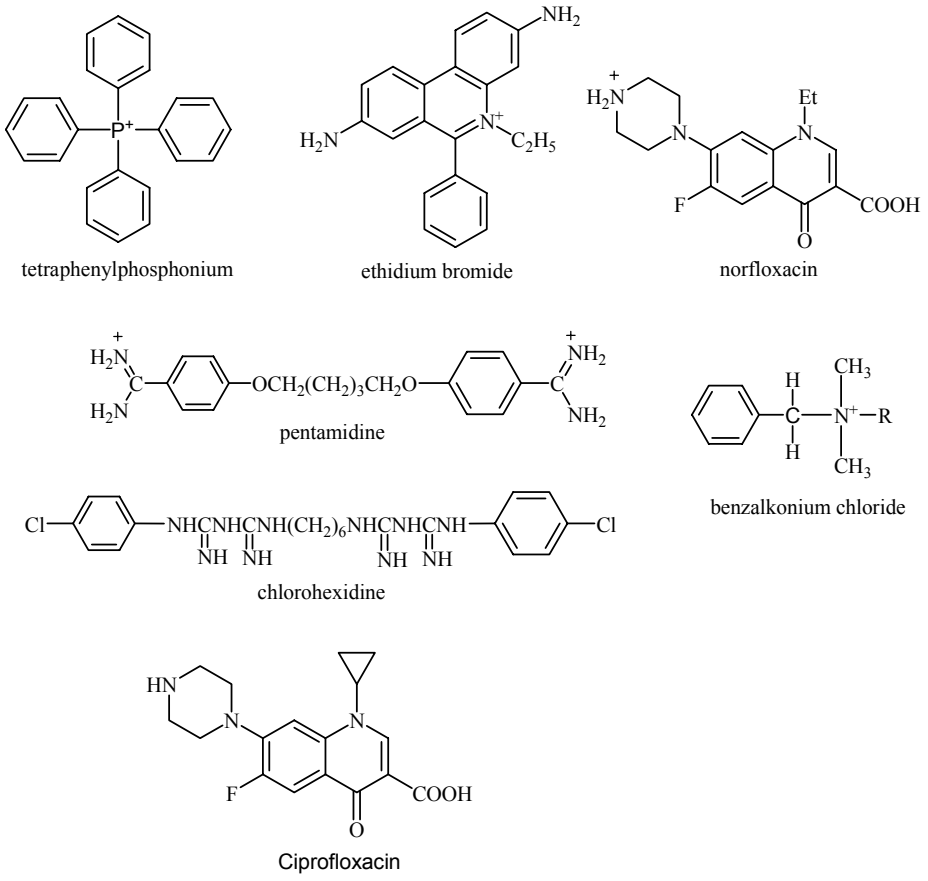


Figure 3-1 Chemical structures of some NorA substrates

3.3 Inhibitors of the NorA efflux pump

Recently, efflux pump inhibitors of the NorA efflux pump have been investigated in order to potentiate the activity of antibiotics which are substrates of the efflux pump. A variety of natural product and synthetic inhibitors of the NorA efflux pump have been reported.

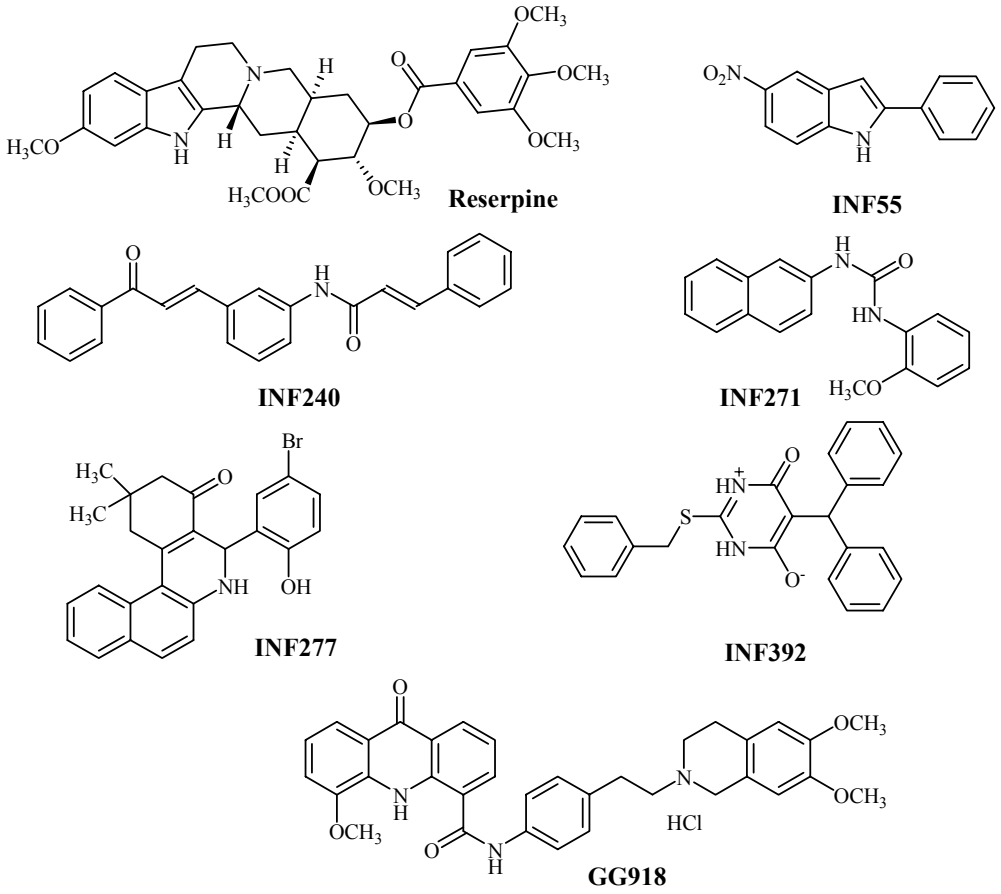


Figure 3-2 Chemical structures of NorA inhibitors

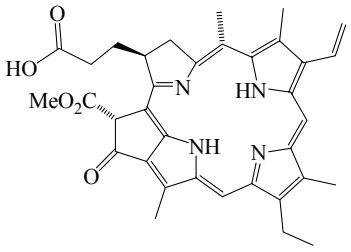
Reserpine (Figure 3-2), an indole alkaloid obtained from root extracts of *Rauwolfia serpentina*, was the first compound identified[68] as a potential inhibitor of NorA, which, when combined with a hydrophilic fluoroquinolone antibiotic, can potentiate the antibiotic potency. Reserpine enhanced the activity of ciprofloxacin (MIC = 8 μg/mL) against a fluoroquinolone-resistant strain of *S. aureus* SA-1199B, a strain

that overexpresses NorA, by eight-fold at a concentration of 20 μg/mL.[72] Reserpine also prevented emergence of fluoroquinolone resistance in *S. aureus*[73] and *S. pneumoniae*.[74] Since reserpine is neurotoxic to humans at the concentration required for NorA inhibition it cannot be used clinically as a NorA inhibitor.[46] This lead to a search for other compounds with more selective activity.

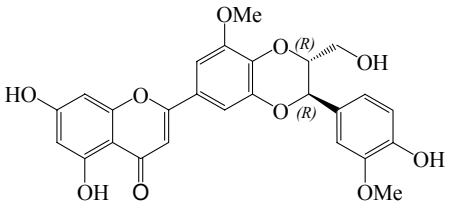
A number of structurally different inhibitors of NorA, such as the synthetic INF analogues, have been reported by Influx Inc. (Chicago, IL) (Figure 3-2). Screening of a chemical library containing 9600 compounds for NorA inhibitor activity resulted in 399 compounds (4%) which were structurally unrelated, and which had inhibitory activity which was at least equipotent to that of reserpine. The chemical inhibitor library was divided into several groups. The first group consisted of indole derivatives, of which 30 compounds were found to be active, and 7 of these were nitroindoles. The second active group included compounds containing trichloromethylaminal functionality. However, it was considered likely that compounds with this group would be toxic to humans and therefore no further characterization of this group was conducted. The third group contained biphenyl urea derivatives, with 11 compounds present in this library being found to be active. The last group contained structurally dissimilar compounds including INF392, INF277, and INF 240 (Figure 3-2). Five of the most potent INF analogue inhibitors were tested in combination with ciprofloxacin against *Staphylococcus aureus* strain SA-1199B and found to act synergistically and also reversed ciprofloxacin resistance four-fold (MIC of ciprofloxacin 2 versus 8 μg/mL) at a concentration of 0.2, 0.4, 0.8, 1.5 and 1.5 μg/mL for INF392, INF240, INF277, INF271, and INF55, respectively.[46] The most potent inhibitor of all the INF analogues was INF392, which was more potent than reserpine by fifty-fold. Also, INF392 reduced

the MIC values of ethidium bromide and ciprofloxacin by eight-fold against SA-1199B at a concentration of 0.4 μg/mL.[75]

A synthetic inhibitor of P-glycoprotein-mediated mammalian tumour multidrug resistance, GG918 (Figure 3-2), was discovered to be a NorA inhibitor and was equipotent to reserpine in potentiating the activity of norfloxacin and ciprofloxacin against some strains of *S. aureus*. In combination with ciprofloxacin, GG918 reduced the MIC of ciprofloxacin against SA-1199B eight-fold at a concentration of 10 μg/mL, but did not show synergistic activity at the same concentration against the RN4220 strain of S. aureus, which carries a gene encoding the MsrA macrolide efflux protein. Moreover, GG918 enhanced the activity of norfloxacin eight-fold against SA-1199B and by four-fold against RN4220 at a concentration of 10 μg/mL.[72] There was no significant inhibitory activity of GG918 in combination with moxifloxacin against all tested strains.



pheophorbide *a*

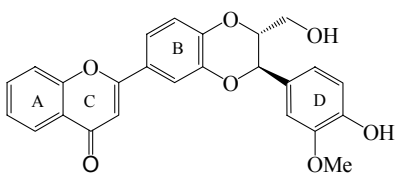


5'-methoxyhydnocarpin-D

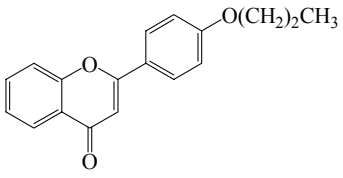
Figure 3-3 Naturally occurring NorA inhibitors from *Berberis* species

Berberine-producing plants from *Berberis* species have been found to produce potent NorA inhibitors, including porphyrin pheophorbide *a* and the flavonolignan 5′-MHC-D,[47-49] that act synergistically with the antibacterial berberine against *S. aureus*. Berberine contains a planar aromatic cationic center which is recognized by efflux

proteins in bacterial cells, resulting in its extrusion from the bacterial cells. Pheophorbide *a*, and 5′-MHC-D, have no antibacterial activity alone but have been shown to boost the activity of an ineffective antibiotic such as berberine against a NorA-producing strain of *S. aureus*. These results may help explain why *Berberis* plants are relatively free of bacterial plant infections. The MIC of pheophorbide *a* and 5′-MHC-D were 0.5 and 1.2 μg/mL, respectively, in the presence of 30 μg/mL of berberine a concentration (which is one-eighth of its MIC against wild-type *S. aureus* RN4222).[48] Many synthetic flavonolignans and simple flavones were synthesized in order to determine structure-activity relationships (SARs) for synergistic activity with berberine against *S. aureus* RN4222. Many of those compounds showed a synergistic action with a sub-inhibitory concentration of berberine (30 μg/mL, 1/8 of the MIC) in the MIC range of 0.08-163 μg/mL. The most potent flavonolignan-based NorA inhibitor was 5,7-deoxyhydnocarpin-D and the most potent flavone-based inhibitor was 4′-*n*-propoxyflavone (Figure 3-4) with MIC values of 0.08 and 0.4 μg/ml, respectively, in combination with a sub-inhibitory concentration of berberine in *S. aureus*. The results revealed that the free hydroxy groups in ring A were not necessary for NorA inhibitory activity.[56] The effect of stereochemistry on activity was not reported for the 5,7-deoxyhydnocarpin-D flavonolignan.



5,7-deoxyhydnocarpin-D



4'-*n*-propoxyflavone

Figure 3-4 The most potent synthetic flavonolignan (relative configuration shown) and flavone of NorA inhibitors

Recently, some naturally occurring flavonol and isoflavone NorA inhibitors (Figure 3-5) have been identified. A weak antibacterial, α-linolenic acid (MIC, 62.5 μg/mL against *S. aureus* wild-type 8325-4), was isolated from the leaf and stem extracts of *Lupinus argenteus*, along with the flavonoids chrysoplenetin and biochanin A which potentiated the antibacterial activity of that acid.

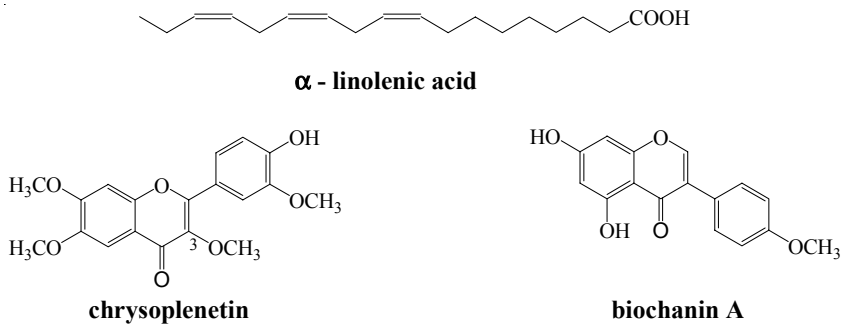


Figure 3-5 Natural flavonol and isoflavone NorA inhibitors, and the antibacterial, α-linolenic acid

Biochanin A was the most potent inhibitor in this plant, and it was shown to completely inhibit *S. aureus* and *B. megaterium* (11561, M. Cannon) growth at a concentration of 6.25 μg/mL in combination with a sub-inhibitory concentration of berberine (30 μg/mL) or a sub-inhibitory concentration of α-linolenic acid (30 μg/mL). Moreover, a SAR study of the flavone series found that the monomethoxy B-ring derivatives of isoflavones were more potent than disubstitued B-ring derivatives.[56,76]

Additionally, in either the flavonolignans or flavonols, it was found that compounds bearing a free hydroxy group at C-3 in ring C did not show efflux pump inhibitory activity. In contrast, compounds bearing a methoxy group at the same position showed inhibitory activity in *S. aureus*. For example, a combination of chrysoplenetin, a natural flavonol extracted from *Artemisa annua*, at a concentration of

6.25 μg/mL with a sub-inhibitory dose of berberine, completely inhibited the growth of *S. aureus* 8325-4.[77]

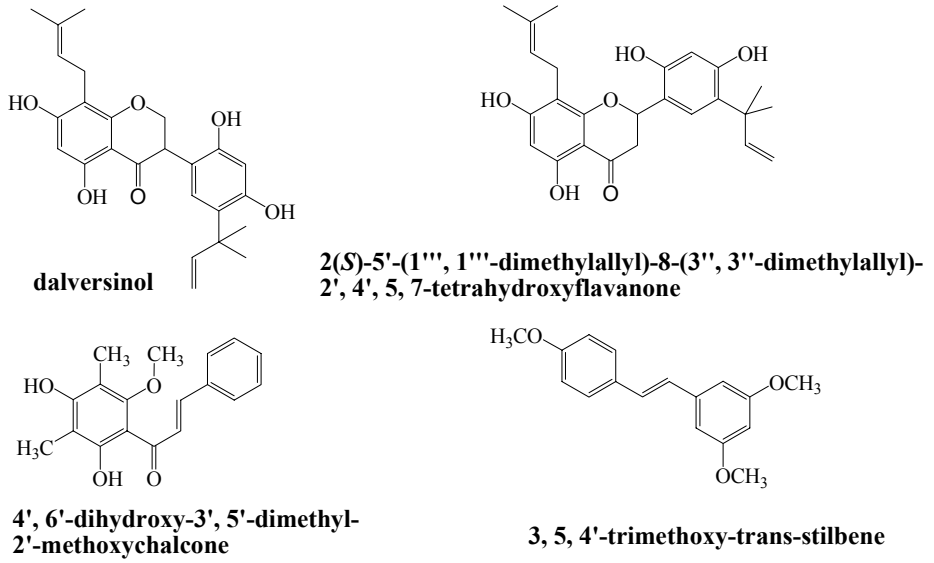
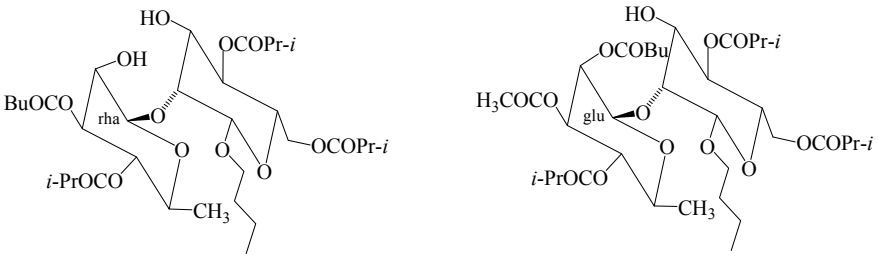


Figure 3-6 Natural phenolic compounds from *Dalea versicolor*

More recently, a new antibacterial flavonoid and six known phenolic compounds (Figure 3-6) were isolated[78] from the plant, *Dalea versicolor*, and they showed direct or synergistic activities against *S. aureus* and *B. cereus*. Both the methoxychalcone derivative and stilbene derivative were equally the most potent inhibitors in this plant at a concentration of 3.3 μg/mL in combination with a sub-inhibitory concentration of berberine against *S. aureus* 8325-4, whereas dalversinol and the tetrahydroxyflavanone derivative (Figure 3-6) had direct activity with MICs of 31.3 and 7.8 μg/mL, respectively. Typically, medicinal plants produce efflux pump inhibitors to potentiate their own weak antibiotics. However, *Dalea versicolor* was the first plant reported in the literature that produced both strong antibacterials and efflux pump inhibitors. It is likely that dalversinol and the tetrahydroxyflavanone derivative, which have a flavanone nucleus, might not be NorA pump substrates.



tetraester of neohesperidoside **pentaester of neohesperidoside**

Figure 3-7 Natural polyacylated neohesperidoside efflux inhibitors

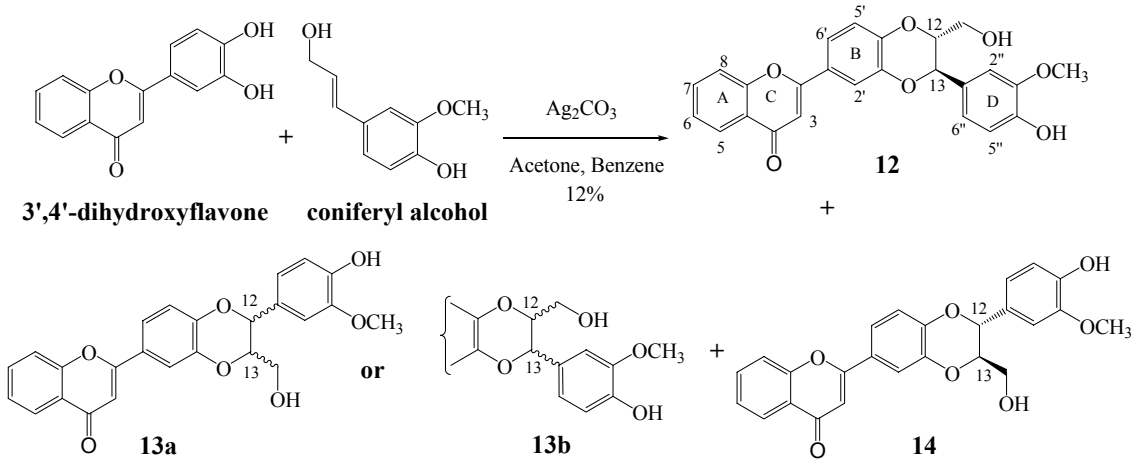
Polyacylated neohesperidosides (Figure 3-7) isolated from *Geranium caespitosum* were found to potentiate the antibacterial activity of berberine, rhein (an anthraquinone), ciprofloxacin and norfloxacin against *S. aureus* 8325-4. Neither the monoester nor diester derivatives showed potentiation activity with berberine, whereas the tetraester and pentaester derivatives did with MIC values of 3.12-6.25 μg/mL in the presence of a sub-inhibitory concentration of berberine.[79]

From all the literature data, the synthetic NorA inhibitor 5,7-deoxyhydnocarpin-D,[56] with an MIC value of 0.08 μg/mL, is the most potent inhibitor discovered so far. In comparison to reserpine having efflux pump inhibitory activity at a concentration of 20 μg/mL in combination with a sub-inhibitory dose of berberine,[77] it was found that 5,7-deoxyhydnocarpin-D was more active than reserpine by 250-fold against *S. aureus* 8325-4. Thus, in the present study this inhibitor was chosen initially for synthesis and ultimately for combination with berberine to make dual action prodrugs and dual action antibacterials.

3.3.1 Synthesis of 5,7-deoxyhydnocarpin-D (12)

Following a literature procedure,[56] commercially available coniferyl alcohol and 3′,4′-dihydroxyflavone were oxidatively coupled using Ag_2CO_3. Either Ag_2CO_3 or

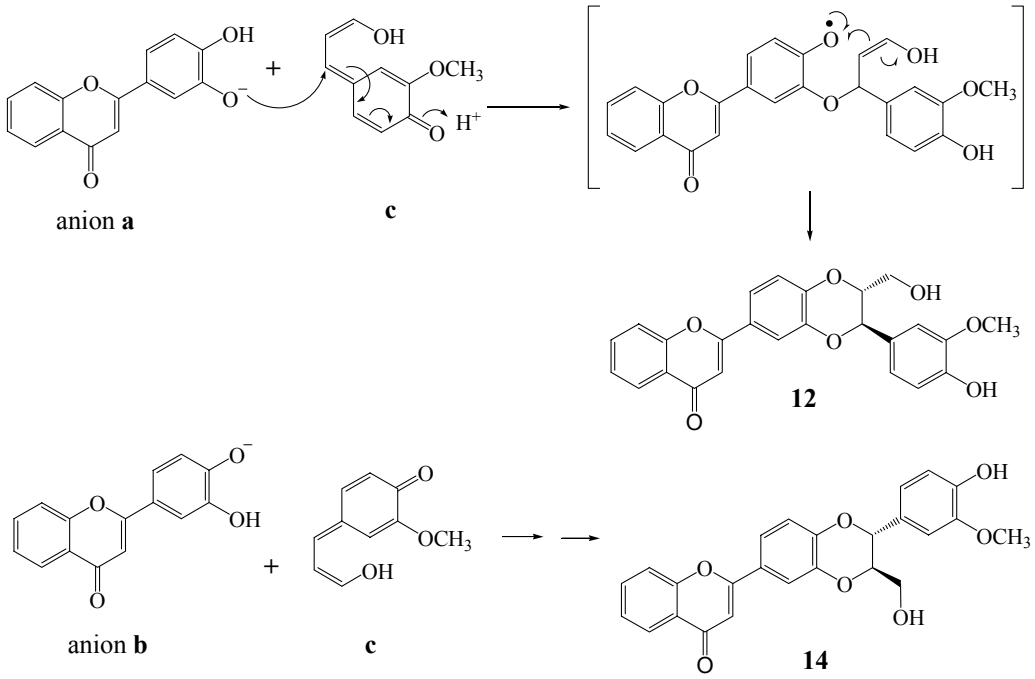
horseradish peroxide (HRP) was used previously[56] for the preparation of **12** and **14** yielding a mixture of different regioisomers. The silver reactions gave **12** as a major regioisomer, having ring D at C-13 with the beta position, whereas the HRP reactions gave **14** as the major regioisomer product having ring D at C-12 with the alpha position.



Scheme 3-1 Preparation of flavonolignan 12

The reaction with Ag_2CO_3 was performed in a solvent mixture of 2:1 benzene/acetone at 60°C for 10h. Purification of the crude product by column chromatography gave only one major fraction, which was shown to be a mixture of regio- and stereoisomers on the basis of the ^1H NMR spectroscopic data. Further purification of the fraction was performed by PLC with multiple development, resulting in 7 product bands with similar R_f values. Three of these bands were processed to afford the regiopure compound **12** as a major product, and two minor products, **13(a or b)** and **14** (Scheme 3-1), which were confirmed by NMR spectroscopic analysis. Guz *et al.*[49,56] were not able to separate a mixture of **12** and **14** using HPLC methods. As acetylation of the mixture **12**/**14** facilitated an easier separation of the mixed products,

the NMR spectroscopic data of the peracetate derivative of **12** were reported by these authors.



Scheme 3-2 Free radical mechanism for the formation of 12 and 14

Mechanistically, the coupling of coniferyl alcohol and 3′,4′-dihydroxyflavone is likely to proceed *via* the corresponding phenoxy radicals and phenoxide anions, which then react *via* an intermolecular Michael-type addition of the phenoxide anion **a** or **b** on the oxidation product **c**, followed by intramolecular free radical coupling[80] to give either **12** or **14** (Scheme 3-2). The formation of **12** and **14** depended on whether anions **a** or **b** were formed, with anion **a** being the precursor of product **12** and anion **b** being the precursor of product **14**.[81]

The structure of **12** was confirmed by NMR spectroscopic analysis and mass spectrometry. In the ^{1}H NMR spectrum, the four ring A aromatic protons gave signals in the aromatic region (δ 7.06-8.20), which appeared as two doublet of doublets (dd) attributed to H-5 and H-8, and two doublet of doublet of doublets (ddd) were ascribed to H-6 and H-7. Three aromatic proton signals ascribed to those of ring B were also in a

similar region to those of ring A and they appeared as a *meta*-coupled doublet assigned to H-2′, and an *ortho*-coupled doublet assigned to H-5′ and H-6′. A singlet signal attributed to H-3 of ring C appeared at δ 6.72. Three aromatic proton signals for ring D appeared as an *ortho*-coupled doublet assigned to H-5′′ and H-6′′ at δ 6.95 (2H), and as a singlet attributed to H-2′′, as well as methoxy and hydroxy signals at δ 3.92 and 5.83 as a singlet and broad singlet, respectively. The hydroxymethyl proton signals appeared at δ 3.58 and 3.85 as two doublet of doublets sets, and the presence of signals at δ 5.00 (d, J = 8.4 Hz) and δ 4.08-4.14 (m), for the methine protons H12/H13; these could correspond to either isomer **12a** or **12b** (Figure 3-8).

12 ≡ 12a: C-13 ring D **14 ≡ 12b: C-12 ring D**

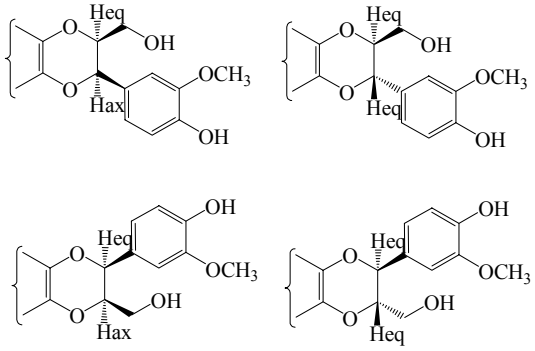
Figure 3-8 Possible structures of 12 and 14

Connectivities in structure **12** were established from the HMBC experiment, and the upfield chemical shift value of H-13 (δ 5.00, d) (relative to the value for H-12 at δ 5.03 (d) in **14**) confirmed[49] the regioisomer shown (**12** ≡ **12a**; Figure 3-8).

The structure of the regioisomer **14** was confirmed by ^{1}H NMR spectroscopic analysis, in which the ^{1}H NMR spectrum was almost identical to that of **12** including a characteristic doublet signal at δ 5.03 (d, J = 8.4 Hz) for H-12$_{ax}$-H-13$_{ax}$ coupling. The chemical shift of the signal for H-12 (in **14**) was slightly more downfield than that of H-13 (in **12**). Thus, the isomer **14** was assigned as **12b** (Figure 3-8).

The ^{1}H NMR spectrum of **13** was similar to that of **12** and **14** except for the presence of signals attributed to H-12 and H-13 at δ 5.27 (d, J = 3.0 Hz) and δ 4.57-4.63

(m) corresponding to either structure **13a** or **13b** (Scheme 3-1). Therefore, if **13** was a mixture of **12** and **14**, two sets of doublet signals in the region of δ 4.9-5.0 with a vicinal coupling constant for diaxial protons H-12, H-13 ($J_{ax,ax}$ ~ 8-13 Hz) would be expected in the ^{1}H NMR spectrum. On the contrary, the ^{1}H NMR spectrum of **13** revealed a single doublet signal at δ 5.27 (d, J = 3.0 Hz) indicating a vicinal coupling of axial and equatorial protons ($J_{ax,eq}$ ~ 2-6 Hz) or of diequatorial protons ($J_{eq,eq}$ ~ 2-5 Hz).[82] Therefore **13**, therefore, is tentatively assigned as one of the isomers as shown in Scheme 3-3.

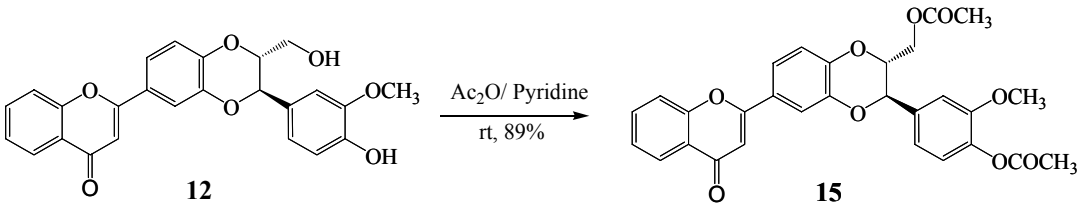


Scheme 3-3 Possible regioisomers of 13 (relative configurations shown)

Unfortunately no spectroscopic data for compounds **12**, **13**, and **14** were reported in the literature. The data for the peracetate derivative of **12** were reported however and thus acetylation of this compound was undertaken.

3.3.1.1 Acetylation of the regioisomer 12

Acetylation of **12** was preformed using standard acetic anhydride/pyridine conditions. The structure of the diacetate product **15** was confirmed by NMR spectroscopic analysis and mass spectrometry.



Scheme 3-4 Preparation of the peracetate derivative of 12

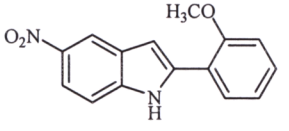
Table 3-1 A comparison of ^{1}H NMR spectroscopic data for 14 with those reported[56] for the peracetate of 5,7-deoxyhydnocarpin-D

^{1}H	15 (δ ppm, $CDCl_3$)	Report Data[56] (δ ppm, $CDCl_3$)
OAc	2.06 (s)	2.09 (s)
OAc	2.31 (s)	2.35 (s)
OCH_3	3.85 (s)	3.89 (s)
H-11	4.02 (dd, 12.3, 4.2)	4.04 (dd, 12.4, 4.4)
H-12	4.26-4.36 (m)	4.36 (m)
H-11	4.38 (dd, 12.2, 3.2)	4.42 (dd, 12.4, 3.2)
H-13	4.98 (d, 7.8)	5.01 (d, 8.0)
H-3	6.73 (s)	6.76 (s)
H-6"	6.97 (dd, 7.8, 1.5)	7.02 (m)
H-2"	6.99 (s)	7.00 (d, 2.0)
H-5"	7.09 (d, 8.4)	7.12 (d, 8.0)
H-8	7.09 (d, 8.4)	7.12 (d, 8.0)
H-7	7.39 (br.t, 8.1)	7.42 (ddd, 8.2, 6.8, 0.8)
H-5'	7.48-7.54 (m)	7.54 (dd, 8.4, 1.6)
H-6'	7.48-7.54 (m)	7.63 (dd, 8.0, 2.0)
H-2'	7.58 (d, 2.1)	7.61 (d, 2.0)
H-6	7.63-7.70 (m)	7.70 (ddd, 8.6, 7.0, 1.6)
H-5	8.20 (dd, 7.8, 1.5)	8.23 (dd, 8.4, 1.6)

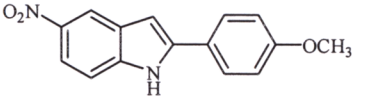
NMR spectroscopic data for **15** was almost the same as that for the peracetate of 5,7-deoxyhydnocarpin-D reported in the literature (Table 3-1).[56] Unfortunately, the key correlation of H-13 to C-3´ was not seen in the HMBC spectrum as expected. Thus, the identity of **15** with the peracetate of 5,7-deoxyhydnocarpin-D would not be confirmed. Due to the difficulties experienced, further work on compound **12** and its coupling to a berberine acid derivative, was not pursued.

3.3.2 Synthesis of 2-aryl-5-nitro-1*H*-indoles

Synthetically more accessible efflux pump inhibitors were then investigated. INF 55, one of the most active synthetic NorA efflux pump inhibitors amongst the indole analogues, was the inhibitor of choice (Figure 3-2). At a concentration of 1.5 μg/mL, INF55 potentiated the activity of ciprofloxacin four-fold against *S. aureus* SA1199-B. Thus, the next target pump inhibitors were designed to modify INF55 (5-nitro-2-phenyl-1*H*-indole) in order to provide a linking functionality in the new target molecule, which could be attached to the antibacterial berberine based on the dual action prodrug and dual action drug concepts.



2-(2-methoxyphenyl)-5-nitro-1*H*-indole

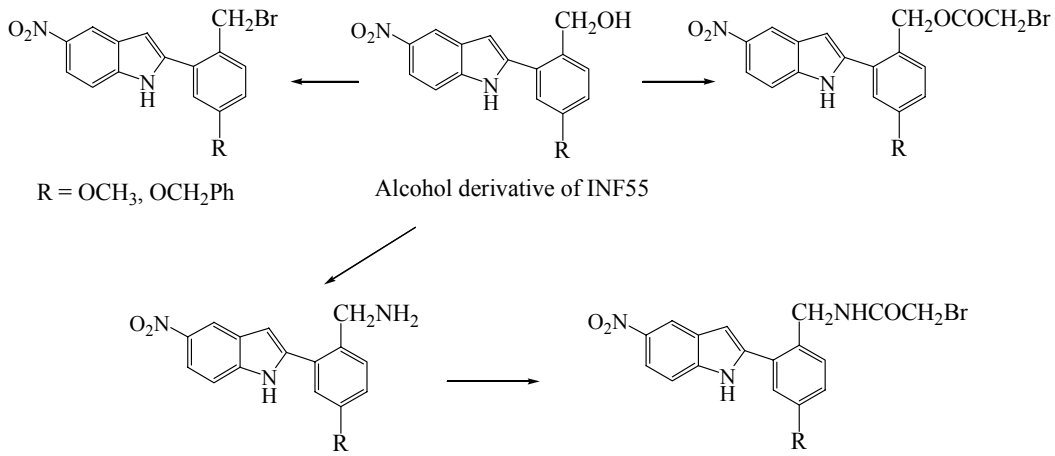


2-(4-methoxyphenyl)-5-nitro-1*H*-indole

Figure 3-9 Related INF55 derivatives were predicted to possess NorA inhibitory activity[83]

Some related 2-aryl-5-nitro-1*H*-indole derivatives (Figure 3-9) have been predicted[83] by CoMFA (3D-QSAR) analysis to have pump blocking activity. In particular, 2-(2-methoxyphenyl)-5-nitro-1*H*-indole was predicted to show comparable activity to INF55, with *ortho*-substitution on the 2-phenyl ring, while 2-(4-methoxyphenyl)-5-nitro-1*H*-indole, with *para*-substitution on the 2-phenyl ring was predicted to be less active than INF55. Therefore, the linking functionality for the 2-aryl-5-nitro-1*H*-indole derivatives in the present study was designed to be preferentially at the *ortho*-position on the 2-phenyl ring. Subsequently, simple lipophilic groups such as OCH_3 and OCH_2Ph, which could also act as H-bond acceptors, were added as substituents in the *meta*-position on the 2-aryl ring in order to investigate structure-

activity relationships. However, preparation of the linking functionality for these designed compounds at the *para*-position on the 2-phenyl ring was not ruled out and an attempt to access these *via* the Fischer indole synthesis was also undertaken (Section 3.3.3).

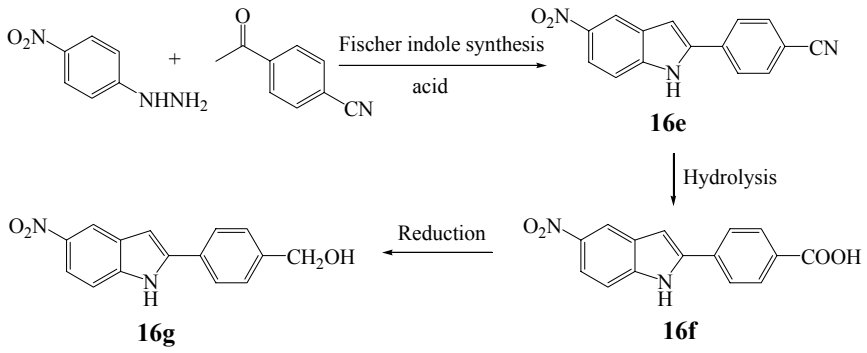


Scheme 3-5 Designed INF55 derivatives to access the dual action prodrugs and dual action drugs

Following the model study 1 (Section 2.2.1), an alcohol derivative of INF55 (Scheme 3-5) was eventually chosen as the first functionalised pump inhibitor target in order to access ultimately the dual action prodrug with a cleavable ester linkage. Subsequently, the alcohol derivative was converted to an amine for making the dual action prodrug with a potentially cleavable amide linkage. Alternatively, the alcohol could be converted to a variety of halide derivatives, such as; bromoacetate, bromoacetamide, and bromide derivatives, in order to access the dual action ester and amide prodrugs, and dual action drugs *via* the methodology established in model study 2 (Section 2.2.2).

3.3.3 Attempted synthesis of 2-aryl-5-nitro-1*H*-indoles *via* Fischer indolization

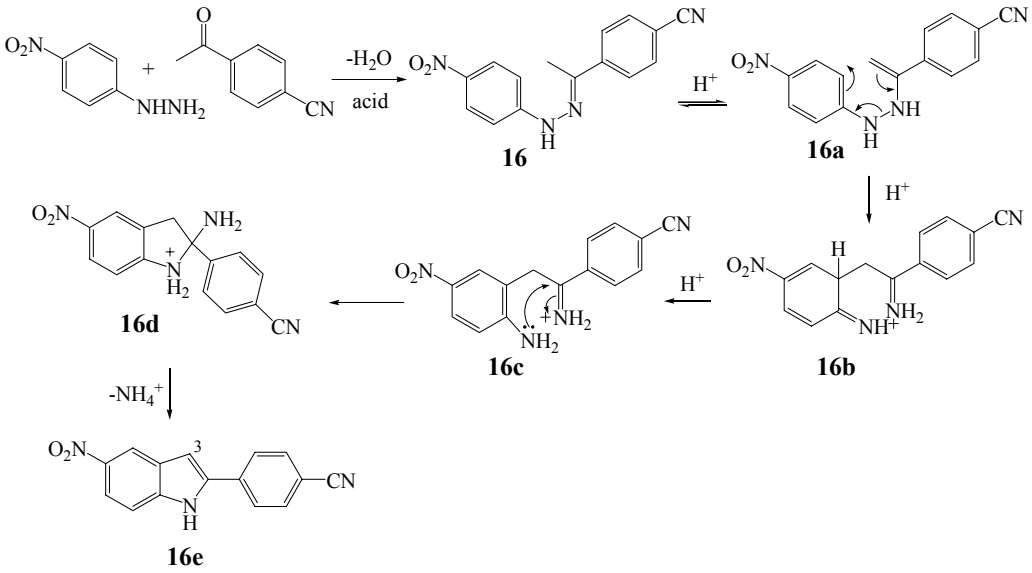
Prior to the synthesis of the *ortho*-substituted 2-aryl group analogues of INF55, some preliminary work was undertaken aimed at *para*-substituted analogues. Synthesis of the alcohol derivative **16g** of 5-nitro-2-phenyl-1*H*-indole (Scheme 3-6) was the first synthetic target in this early phase of the work. It was expected this derivative could be accessed by the well established Fischer indole route for synthesizing substituted indoles *via* arylhydrazones. It was proposed that using commercially available 4-nitrophenylhydrazine and 4-acetylbenzonitrile as reagents with an acid catalyst, the indole **16e** could be prepared. Subsequent hydrolysis of the benzonitrile **16e** would afford the benzoic acid **16f**, and then selective reduction of the acid would give the desired benzyl alcohol **16g**.



Scheme 3-6 Synthetic strategy to 4-(5-nitro-1*H*-indol-2-yl)benzyl alcohol (16g)

The general mechanism[84]for the Fisher indolisation reaction is shown in Scheme 3-7. Proton-tautomerization of hydrazone **16,** which is simply prepared by the treatment of 4-nitrophenylhydrazine and 4-acetylbenzonitrile with an acid catalyst, generates the enehydrazine **16a**. A subsequent [3,3]-sigmatropic rearrangement, the key step of the

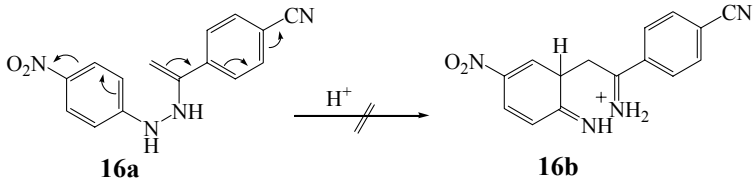
mechanism, gives **16b** and then elimination of ammonia affords the desired indole **16e**. An acid catalyst is necessary to accelerate the formation of the enehydrazine **16a** from the proton-tautomerization of hydrazone **16.** A variety of acid catalysts can be employed, for example metal halides (zinc chloride is the most frequently used), mineral acids (hydrochloric, sulfuric, and polyphosphoric acids), and organic acids (actetic acid).[85-88] Moreover, microwave irradiation has been used to facilitate this reaction.[89]



Scheme 3-7 Mechanism for the Fischer indole synthesis of 16e

The Fischer indole synthesis of **16e** from 4-nitrophenylhydrazine and 4-acetylbenzonitrile was attempted a number of times with various types of acid catalysts such as *p*-toluenesulfonic acid, glacial acetic acid, hydrochloric acid, phosphoric acid, polyphosphoric acid and BF_3-etherate. All reactions gave the same product, the hydrazone **16**, which was confirmed by ^{1}H NMR and MS spectroscopic data. The ^{1}H NMR spectrum of **16** in $CDCl_3$ showed four doublet signals which integrated for eight aromatic protons with *ortho*-coupling at δ 7.40 (2H) and 8.15 (2H) which were

attributed to H-2, H-6 and H-3, H-5 on the aromatic ring bearing the *para*-nitro substituent, and at δ 7.86 (2H) and 8.00 (2H) ascribed to H-2′, H-6′ and H-3′, H-5′ on the aromatic ring bearing the *para*-cyano substituent. A singlet signal at δ 3.35 (3H) was assigned to the methyl group adjacent to the imine functionality together with a singlet signal at δ 10.43 attributed to the hydrazone NH proton. The MS (EI) spectrum showed a signal at *m/z* 280, which represented the molecular ion of compound **16**. Both the ^{1}H NMR and MS spectroscopic data were consistent with the hydrazone **16** and not consistent with the indole **16e** for which a characteristic signal (δ 6.5–7.0) for the H-3 proton of the indole would be expected. The problem associated with this method was the inability to access the key [3,3]-sigmatropic rearrangement (**16a** to **16b**) (Scheme 3-8) possibly due to the presence of electron-withdrawing substituents (-NO_2 and –CN groups) on both aromatic rings, which may hinder the protonation step and rearrangement.



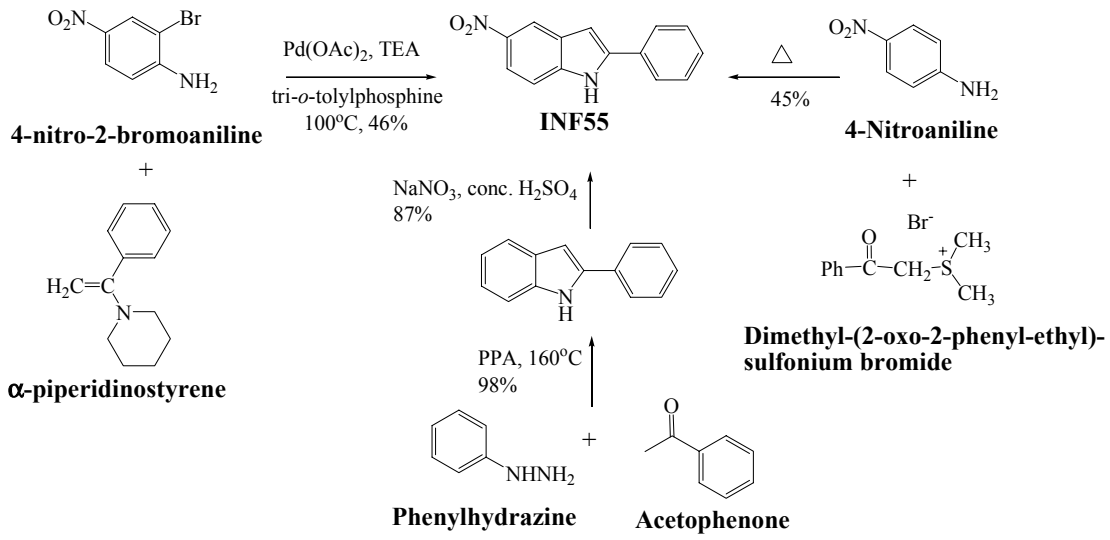
16a **16b**

Scheme 3-8 Effect of electron-withdrawing groups on the [3,3]-sigmatropic rearrangement of 16a

More severe conditions were then investigated involving microwave irradiation at high energy levels with short heating periods. The reactions were carried out under acid conditions using either formic acid or neat $ZnCl_2$.[90] Unfortunately, none of the desired product **16b** was observed, and only hydrazone **16** was detected in formic acid reaction. With neat $ZnCl_2$ the conditions were too severe and no products were isolated.

Therefore, alternative methods for the synthesis of 5-nitro-2-phenyl-1*H*-indole derivatives bearing alcohol functionality were considered and the Fischer indole synthesis strategy was abandoned.

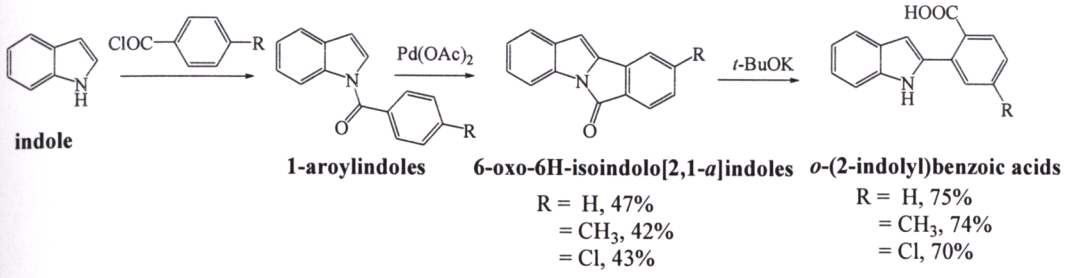
Several routes have been described for the synthesis of 5-nitro-2-phenyl-1*H*-indole (Scheme 3-9), including a palladium-assisted reaction of 4-nitro-2-bromoaniline and α-piperidinostyrene,[91] the Fischer indole synthesis of acetophenone with phenylhydrazine and subsequent nitration,[92] and reaction of 4-nitroaniline and dimethyl-(2-oxo-2-phenyl-ethyl)-sulfonium bromide followed by cyclisation.[93] However, there are limitations with respect to functional group tolerances and selective introduction of functionalities at desired positions in these approaches. With the commercial availability of 5-nitro-1*H*-indole in mind, particular attention in the alternative approaches focussed on preformed indoles and introduction of an *ortho*-substituted aryl group at the indolic C2 position.



Scheme 3-9 Preparation of 5-nitro-2-phenyl-1*H*-indole (INF55)

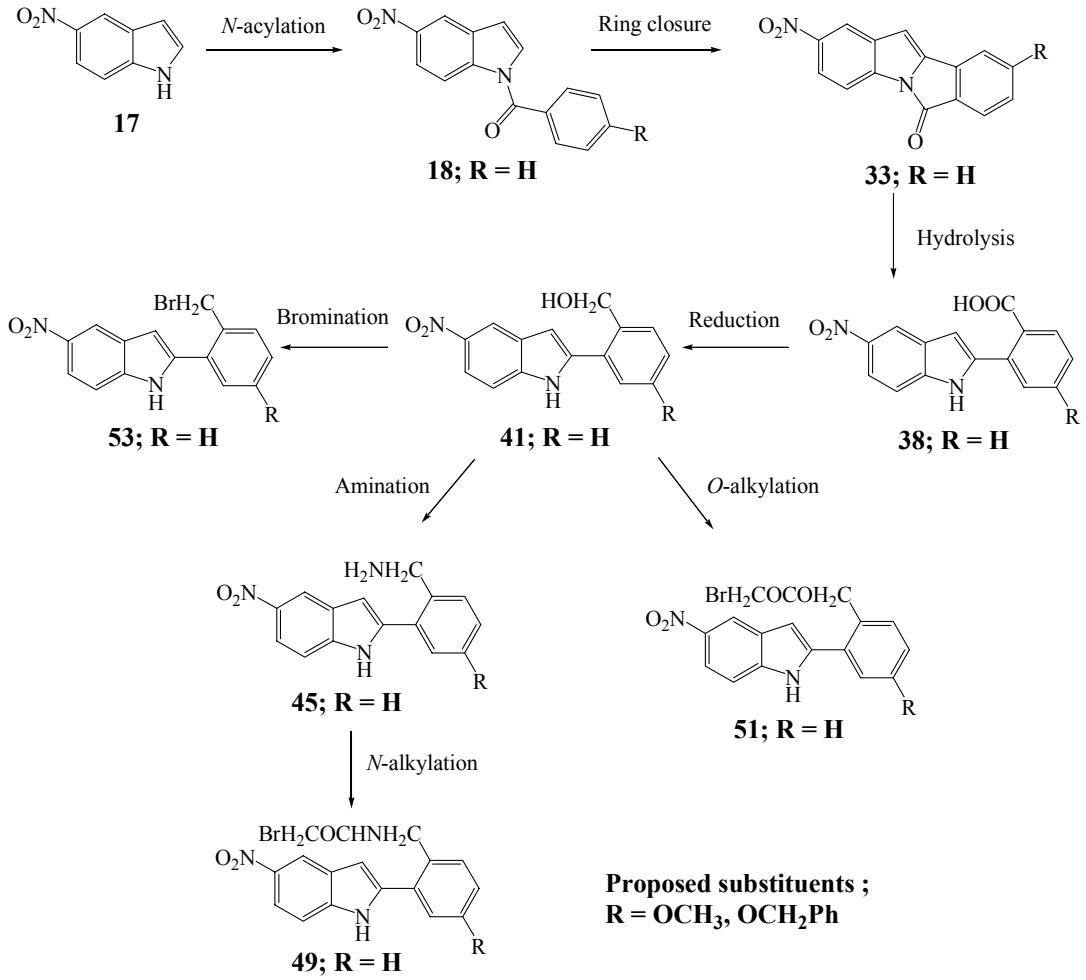
3.3.4 Synthetic strategy for 2-aryl-5-nitro-1*H*-indole derivatives *via* palladium cyclization

A regioselective synthesis of 2-arylindoles from indole was reported by Itahara.[94,95] His work involved the synthesis of 2-(o-indolyl)benzoic acids *via* ring closure of 1-aroylindoles using palladium acetate followed by hydrolysis (Scheme 3-10).



Scheme 3-10 Preparation of *o*-(2-indolyl)benzoic acids *via* intramolecular ring closure of 1-aroylindoles[94,95]

Itahara's approach was of interest as it was a regioselective and concise three-step method with potential for the introduction of a range of substituents. It was expected that the 2-(*o*-indolyl)benzoic acids produced could be easily reduced to the desired primary alcohols, and the latter then converted to other substituted derivatives. Thus, the general method of Itahara was adapted in the current work using commercially available 5-nitro-1*H*-indole (**17**) as a starting material for the synthesis of 2-(*o*-indolyl)benzyl alcohols as shown in Scheme 3-11.



Scheme 3-11 Synthetic strategy for 5-nitro-2-phenyl-1*H*-indole derivatives

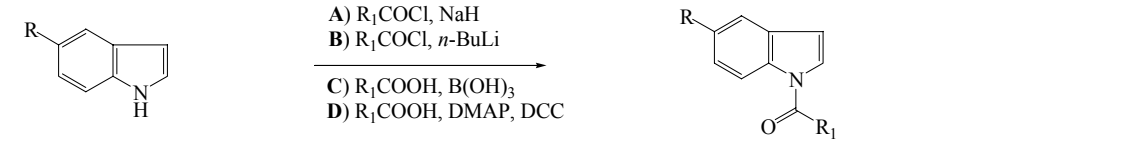
The basic synthetic strategy was to initially prepare *N*-benzoyl-5-nitroindole (**18**) by *N*-acylation of the commercially available 5-nitro-1*H*-indole (**17**), followed by an intramolecular oxidative ring-closure by palladium cyclisation, then ring-opening by amide hydrolysis and subsequent specific reduction of the benzoic acid **38** to afford the benzyl alcohol **41** which could then be converted into various alkylating agents **49**, **51** and **53**. The bromoacetate **51** was prepared from **41** in one-step by *O*-alkylation and the benzyl bromide **53** was also prepared from **41** in one-step by bromination, whereas the bromoacetamide **49** was prepared from the benzylamine **45** obtained in turn from amination of **41**. Similarly, the other methoxy and benzyloxy derivatives of alcohol **41**

were to be prepared and then the various alkylating agents also would be synthesized following the same synthetic strategy.

3.3.4.1 *N*-acylation of indoles

The *N*-acylation of indoles is a well established reaction that is used to protect the indolic nitrogen with an acyl group[96,97] which can then be easily removed by base hydrolysis[98], and also to provide precursors for intramolecular cyclization.[99] The most common *N*-acylation method requires the generation of an indole anion (by addition of a base, e.g. BuLi,[100] NaH,[101,102] KOH,[102] and K_2CO_3[103]) followed by nucleophilic attack on an acyl halide. *N*-acetylation of indoles can be achieved in high yields using acetic anhydride in combination with TEA and a catalytic amount of DMAP.[104] Also direct *N*-acylation of indole with carboxylic acids catalyzed by boric acid affords *N*-acylated indoles in moderate yield.[105]

In the present work, a variety of *N*-acylated indoles (Scheme 3-12) were prepared *via* the acid chloride method with strong bases, NaH (method **A**) or *n*-BuLi (method **B**), at low temperature, together with the direct coupling method with carboxylic acids and boric acid (method **C**). Additionally, a new method using carboxylic acids with DCC



R = H, OCH_3, NO_2, F

(18-32)

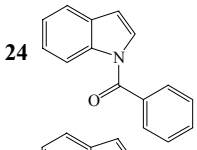
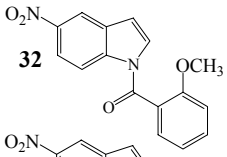
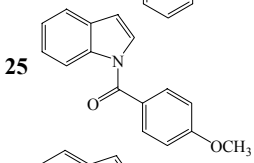
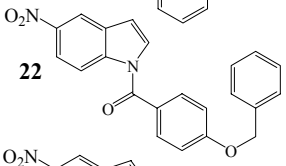
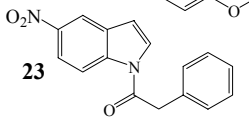
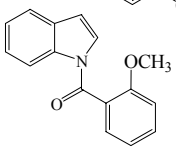
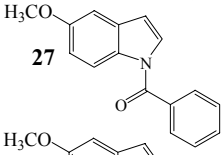
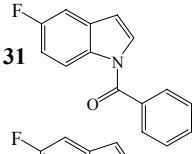
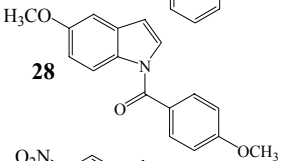
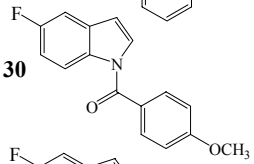
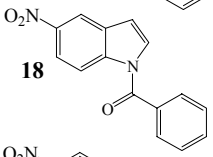
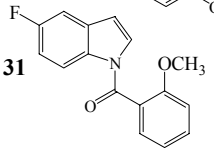
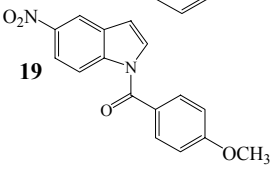
24 R = H, R_1 = Ph
25 R = H, R_1 = Ph-*p*-OCH_3
26 R = H, R_1 =Ph-*o*-OCH_3
27 R = OCH_3, R_1 = Ph
28 R = OCH_3, R_1 = Ph-*p*-OCH_3
18 R = NO_2, R_1 = Ph
19 R = NO_2, R_1 = Ph-*p*-OCH_3
32 R = NO_2, R_1 =Ph-*o*-OCH_3
22 R = NO_2, R_1 =Ph-*p*-OBn
23 R = NO_2, R_1 = CH_2Ph
29 R = F, R_1 = Ph
30 R = F, R_1 = Ph-*p*-OCH_3
31 R = F, R_1 = Ph-*o*-OCH_3

Scheme 3-12 Methods for *N*-acylation of indoles

and DMAP as carboxylate activators at room temperature (method **D**) was explored. It was found the NaH and *n*-BuLi methods under low temperature conditions worked well with commercially available benzoyl chlorides only, leading to the desired *N*-acylated indole products in high yields except for **22** with the 4-benzyloxybenzoyl chloride, which was very sensitive to moisture. To avoid the use of acid chlorides, direct coupling of carboxylic acids to the indoles using boric acid in mesitylene at reflux was trialled, but only low yields of *N*-acylated products were obtained. Thus, investigation of a simpler and milder new method for the direct acylation of indoles with carboxylic acids using DCC as the coupling agent was developed in this project. High yields were obtained when an electron-withdrawing group was present at C-5, however this method was less effective with a C-5 electron donating group (Table 3-2).

A variety of *N*-acylated products of indoles bearing electron-donating and electron-withdrawing groups were synthesized by methods **A**-**D** (Scheme 3-12) in order to compare yields and to find the optimal approaches. The results from Table 3-1 revealed the most efficient methods for the *N*-acylation of indole and its derivatives bearing an electron-donating group at C-5 were methods **A** and **B** using the highly flammable NaH and *n*-BuLi, respectively, and the appropriate acid chlorides. Method **B** (using *n*-BuLi) required a shorter reaction time and less reaction steps than method **A**. In the case of indoles with electron-withdrawing groups at C-5, the simple and efficient method for *N*-acylation was method **D** using carboxylic acids and DCC/DMAP at room temperature, which involved milder conditions and avoided the extra step of acid chloride preparation when they were not available commercially.

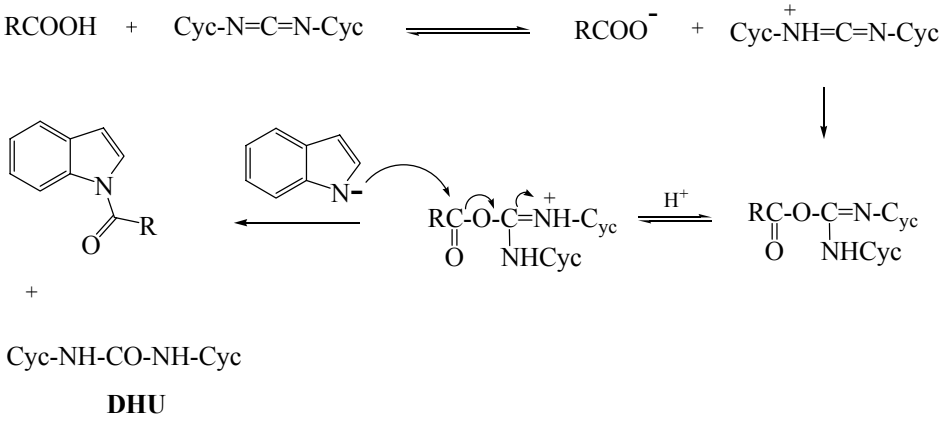
Table 3-2 Percentage yields of *N*-acylated products of indoles

Product	Method	Yield (%)	Product	Method	Yield (%)
24 	**A**	88	**32** 	**D**	80
	C	45[104]			
	D	32			
25 	**A**	75[95]	**22** 	**B**	30
				D	95
	D	46	**23** 	**A**	19
26 				**C**	4
	D	34			
27 	**A**	72[95]	**31** 	**D**	89
	D	15			
28 	**D**	9	**30** 	**D**	87
18 	**A**	89	**31** 	**D**	85
	D	91			
19 	**B**	44			
	D	95			

3.3.4.1.1 Discussion of the *N*-acylation of indole

The preparation of *N*-benzoyl indole **24** was carried out *via* methods **A** and **D** and yields were compared with the yield reported for method **C**.[105] Method **A** required reaction of indole with NaH for 2 h to ensure complete formation of the indole sodium salt. Subsequent alkylation of the deprotonated indole with benzoyl chloride at –60°C to 70°C then afforded the desired product **24,** which had almost the same R_f value as the starting indole, resulting in a difficult purification by chromatography since some starting material remained. Recrystallization of **24** from warm ethanol afforded **24** in 88% yield. In comparison, method **D** involved a direct acylation of indole with benzoic

acid using the dehydrating agent DCC in the presence of DMAP to afford **24** in low yield (32%) together with unreacted indole. In this case the low yield is probably a result of incomplete deprotonation of the indolic NH (*p*Ka ~ 17) by the DMAP, since the indolide anion is presumably involved in the key penultimate nucleophilic attack on the acid-DCC intermediate (Scheme 3-13). In general terms, the carboxylic acid forms an adduct with DCC which then acts as a good leaving group, on subsequent nucleophilic attack by the indolide anion (generated by DMAP deprotonation) to afford the desired amide and dicyclohexylurea. An additional preparation of **24** *via* direct acylation of indole with benzoic acid in the presence of boric acid has been reported previously by Terashima and afforded **24** in 45% yield.[105] The role of boric acid in this reaction is unclear.



Scheme 3-13 Mechanism of the DCC coupling of indole and carboxylic acid in the presence of DMAP

The preparation of **25** was also achieved by method **D** using indole and 4-methoxybenzoic acid with DCC/DMAP. The reaction was carried out overnight at room temperature to afford **25** in 46% yield. The amide **25** had also been made previously by Welstead in 75% yield[96] by method **A** using NaH and 4-methoxybenzoyl chloride. Thus in this case method **A** was again better than method **D**.

The preparation of **26** was accomplished by method **D** using indole with 2-methoxybenzoic acid and DCC/DMAP. The reaction was carried out for 3 days giving **26** in 34% yield. The structure of **26** was confirmed by NMR spectroscopy and mass spectrometry. The ^{1}H NMR spectrum revealed the absence of a broad singlet signal near δ 8.00 which could be assigned to the indolic NH. The presence of a 3-proton singlet at δ 3.79 was attributed to the aromatic methoxy group. A broad doublet signal at δ 8.44 was assigned to H-7. The broad signal for H-7 appeared to be associated with partial conformational restriction in the acylated region of the molecule. Signals attributed to H-3′, 4′, 5′ and 6′ in the *o*-methoxybenzoyl moiety appeared as multiplets in the region of δ 7.00-7.60. Additionally, in the ^{13}C NMR spectrum a signal at δ 167.2 was assigned to the carbonyl carbon, while the HRMS (EI) spectrum supported the supported the molecular formula of **26.**

3.3.4.1.2 Discussion of the *N*-acylation of 5-methoxyindole

The preparation of **27** (Table 3-2) was achieved by method **D** using 5-methoxyindole with benzoic acid and DCC/DMAP. The reaction mixture was stirred at room temperature overnight to afford **27** in 32% yield, whereas the preparation of **27** has been reported in 72% yield by method **A** using 5-methoxyindole with benzoyl chloride and a strong base (NaH).[96] With an electron-donating group at C-5 in the indole, product formation in method **D** proceeded generally in low yield, and was not successful in the attempted synthesis of acylated product from 5-methoxyindole and 2-methoxybenzoic acid. Since the methoxy substituent is an electron-donating group, it would increase electron density adjacent to the indole nitrogen in the *para* position reducing the acidity of the 5-methoxyindole and hence retarding indolide anion

formation *via* reaction with DMAP. The structure of **27** was confirmed by ^{1}H NMR spectroscopic data and mass spectrometry. The ^{1}H NMR spectrum showed the presence of a singlet signal ascribed to the methoxy group at δ 3.88 and the absence of a broad singlet signal which could be attributed to an indolic proton. Two sets of multiplets between δ 7.48-7.75, integrating for a total of 5 protons, were attributed to the aromatic protons of the benzoyl moiety. Additional confirmation of the structure was provided by the HRMS (EI) spectrum, which showed a molecular ion signal at *m/z* 251.0950, consistent with the molecular formula of **27**. In a similar result to that of **27**, application of method **D** to the reaction of 5-methoxyindole and 4-methoxybenzoic acid afforded the *N*-acyl product **28** in only 9% yield.

3.3.4.1.3 Discussion of the *N*-acylation of 5-nitroindole

In contrast to the results with 5-methoxyindole, the preparation of **18** (Table 3-2) was successful using both method **A** and **D** from 5-nitroindole and benzoic acid, resulting in high yields (89-91%) without chromatographic purification, whereas this compound previously has been prepared in only moderate yield by Cho *et al.* in a study of selective cyclooxygenase-2 inhibitors.[103] Cho's method involved reaction of 5-nitroindole and benzoyl chloride with potassium carbonate in DMF to afford **18** in 43% yield. In the current study, the structure of **18** was confirmed by NMR spectroscopic analysis and mass spectrometry. The ^{1}H NMR spectrum showed a multiplet signal (δ 7.54-7.80) integrating for five protons which was attributed to the aromatic protons of the benzoyl moiety. Additional confirmation of the structure was provided by the HRMS (EI) spectrum with a signal at *m/z* 266.0688, which was consistent with the molecular ion of compound **18**.

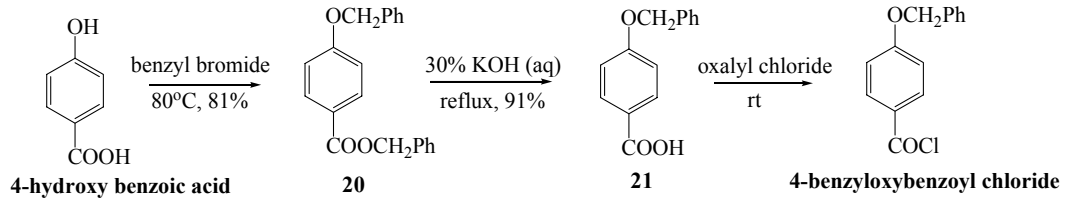
In the attempt to explore the effects of DMAP, DCC and carboxylic acid concentrations on the reaction outcome, 5-nitroindole and 4-methoxybenzoic acid were reacted using varying amounts of DMAP, DCC and the carboxylic acid. All reactions gave product **19** in high yields (91-97%) as shown in Table 3-3. Taking into account reaction time, the preferred conditions were those for entry 1.

Table 3-3 A variety of reagent amounts for the synthesis of 19 *via* method D

Entry	**5-nitroindole** (eq)	**acid** (eq)	**DCC** (eq)	**DMAP** (eq)	**reaction time** (h)	**yield** (%)
1	1	2	2	1	4	95
2	1	1.3	1.3	0.1	72	97
3	1	2	2	0.1	6	91

Moreover, the preparation of **19** was carried out from 5-nitroindole and 4-methoxybenzoyl chloride *via* *n*-BuLi (method **B**) to afforded **19** in moderate yield (44%). Confirmation of the structure of **19** was provided by NMR spectroscopic analysis and mass spectrometry. The ^{1}H NMR spectrum revealed a singlet at δ 3.92 which integrated for three protons and was attributed to the methoxy protons. The appearance of two equivalent signals integrating for two protons each at δ 7.05 and 7.77 were attributed to the aromatic protons in the *para*-substituted benzoyl moiety. Furthermore, a signal ascribed to the carbonyl carbon was seen in the ^{13}C NMR spectrum at δ 167.9, and in the HRMS (EI) a molecular ion peak was observed at *m/z* 296.0796 which corresponded to the molecular weight of **19**.

Following the optimal conditions established for the synthesis of **19** *via* method **D**, compound **32** was prepared from 5-nitroindole and 2-methoxybenzoic acid to afford **32** in 80% yield after recrystallization. The structure of **32** was confirmed by NMR spectroscopic analysis and mass spectrometry.



4-hydroxy benzoic acid **20** **21** **4-benzyloxybenzoyl chloride**

Scheme 3-14 Preparation of 4-benzyloxybenzoyl chloride

The first attempted preparation of the amide **22** was carried out by method **B** using 5-nitroindole and the moisture-sensitive 4-benzyloxybenzoyl chloride. The preparation of 4-benzyloxybenzoyl chloride (Scheme 3-14) was based on a literature procedure[106] using 4-hydroxybenzoic acid and benzyl bromide in K_2CO_3 to generate **20** in 81% yield. Subsequent hydrolysis of the ester **20** using KOH afforded the acid **21** in 91% yield, followed by reaction with oxalyl chloride under anhydrous conditions to afford the corresponding acid chloride. The acid chloride was found to be unstable due to moisture sensitivity and could not be chromatographed, and thus the yield of the subsequent acylated indolic product **22** was low (30%). Following the procedure used to prepare **19** by method **D**, **22** was prepared from 5-nitroindole and the acid **21**. The reaction was carried out at 40°C in order to accelerate the reaction. However, the result was different from what was expected. The reaction was monitored by TLC for 5 days, and a consistent ratio of 1 to 1 of starting indole and product **22** was observed after day 2 of the reaction. The reaction was stopped after 5 days and the mixture chromatographed to afford **22** in 52% yield. It is likely that the *N*-acylated product **22** was partially hydrolyzed under the prolonged basic conditions regenerating some of the 5-nitroindole. The structure of **22** was confirmed by NMR spectroscopy and mass spectrometry. The ^{1}H NMR spectra of **22** were similar to **19** with the same signal pattern above 6 ppm except for additional aromatic multiplet signals (δ 7.36-7.48) integrating for five protons attributed to the five aromatic protons of the benzyl

substituent. The ^{13}C NMR spectrum also showed an additional five aromatic signals in the range of δ 127.5-135.8 when compared to the spectrum for **19**. Moreover, the ^{1}H NMR spectrum showed a distinct signal at δ 5.19 integrating for two protons, which was ascribed to the methylene protons of the benzyloxy substituent and a methylene carbon signal was also seen in the ^{13}C NMR at δ 70.3. The molecular formula of **22** was established by HRMS (EI).

Following the procedure described by Terashima[105] (method **C**), the reaction of 5-nitroindole and phenylacetic acid and boric acid in mesitylene at 185^{o}C was investigated for the preparation of **23** (Table 3-2). However, the *N*-acylated product **23** was only obtained in very low yield (4%). Similarly, with method **A** using 5-nitroindole and phenyl acetyl chloride with NaH in DMF at –60 to 70^{o}C overnight, amide **23** was again obtained in only low yield (19%). The structure of **23** was confirmed by NMR spectroscopic analysis and mass spectrometry. The ^{1}H NMR spectrum showed a signal integrating for two protons at δ 4.29 which was attributed to the methylene protons of the phenyl acetyl moiety. An aromatic signal integrating for five protons was seen at δ 7.29-7.42 as a multiplet and was ascribed to the five aromatic protons of the phenyl acetyl moiety. Additionally, the ^{13}C NMR spectrum showed a signal attributed to the carbonyl carbon at δ 164.9 while the HRMS (CI) data was consistent with the molecular formula of **23**.

3.3.4.1.4 Discussion of the *N*-acylation of 5-fluoroindole

The preparation of a series of *N*-acylated products (**29**-**31**) (Table 3-2) of 5-fluoroindole with various carboxylic acids (benzoic acid, 4-methoxybenzoic acid and 2-methoxybenzoic acid) was carried out by method **D**. Compounds **29**-**31**were obtained

in high yield (85-89%) after chromatography. The structures of **29**-**31** were confirmed by NMR spectroscopic analysis and mass spectrometry. The ^{1}H NMR spectra of those compounds revealed the absence of a signal attributable to an indolic NH proton. The ^{1}H NMR spectrum of **29** showed the presence of aromatic signals (δ 7.40-7.65 as multiplets) integrating for five protons consistent with the presence of the benzoyl moiety. Further confirmation was provided in the ^{13}C NMR spectrum through a signal attributed to the carbonyl carbon at δ 168.5 as well as by the HRMS (EI) spectrum which gave a molecular ion peak at *m/z* 239.0738, consistent with the molecular formula of **29**.

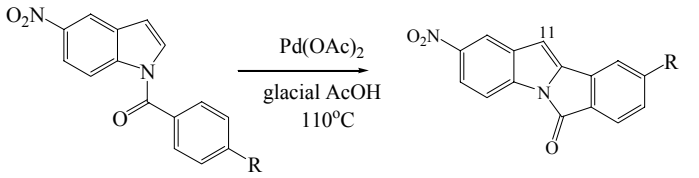
The ^{1}H NMR spectrum of **30** was very similar to that of **29** except for the loss of one aromatic signal around δ 6.90-7.80 and the presence of a singlet signal (δ 3.91) integrating for three protons assigned to the methoxy protons. The HRMS (EI) spectrum afforded a molecular ion peak at *m/z* 269.0854, consistent with the molecular formula of **29**.

All spectroscopic data for **31** were closely comparable to those of **30**, with a small difference in splitting pattern of the signals attributed to the aromatic protons of the benzoyl moiety in a range of δ 6.90-7.80, and the signal assigned to H-7 at δ 8.44 appeared as a broadened multiplet in **31**. The HRMS (EI) spectrum showed a signal at *m/z* 269.0854, representing the molecular ion of **31**.

3.3.4.2 Cyclization of the *N*-acylated indoles

The cyclisation of the *N*-acylated indoles **18**, **19** and **22** was accomplished *via* a palladium(II)-promoted oxidative intramolecular reaction. Accordingly, following a method for the intramolecular reaction of 1-aroylindoles by palladium acetate which has

been reported previously by Itahara[94] and shown in Scheme 3-10, the *N*-acylated indole **18** (Scheme 3-15) was reacted with one equivalent of palladium(II) acetate in glacial acetic acid. The reaction was carried out at 110°C to afford the expected ring-closed product **33.**



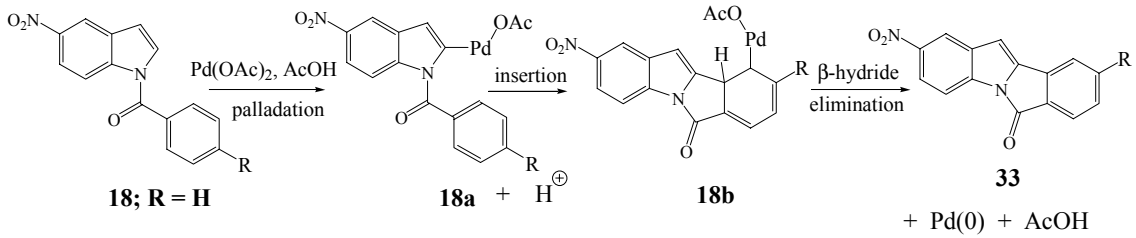
18; R =H
19; R = OCH_3
22; R = OCH_2Ph
37; R = OH, 35%

33; R =H, 50%
34; R = OCH_3, 74%
35; R = OCH_2Ph, 20%
36; R = OH, 22%

Scheme 3-15 Intramolecular ring-closure of the *N*-acylated indoles

The reaction mixture, which still contained some of the starting indole **18**, was separated by flash chromatography. The separation was very difficult however due to the very similar chromatographic properties of **33** and **18** and the tendency of **33** to elute very slowly from the column. Thus, the separation by flash chromatography was changed to vacuum liquid chromatography (VLC) which used reduced pressure and this then gave a more rapid separation. Additionally, **33** had unusual solubility properties, in that it was only partially soluble in various solvents such as DCM, DCM/MeOH, PS/EtOAc and DMSO. After chromatography, **33** was obtained in 50% yield. The ^1H NMR spectrum of **33** revealed the loss of the doublet signal at δ 7.50 which could be ascribed to H-2 in the starting material, together with the loss of an aromatic proton signal. The characteristic signal attributed to H-11 appeared at δ 6.79 as a singlet, which strongly supported the structure of **18.**

Mechanistically,[107,108] the oxidative cyclization of the *N*-acylated product **18** probably involves initial indole C-2 palladation to form an σ–indolylpalladium(II) complex **18a**, which would be promoted by protonation of the acetate ligand of $Pd(OAc)_2$ by acetic acid (Scheme 3-16). Subsequently, complex **18a** could undergo an intramolecular insertion into the benzoyl ring to give **18b**, followed by β-hydride elimination of **18b** to afford the ring-closed product **33**.



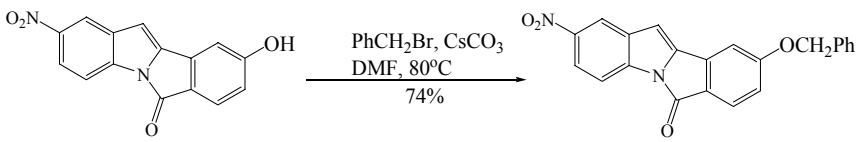
Scheme 3-16 Mechanism for the palladium(II)-promoted oxidative cyclization

As this reaction consumed one equivalent of $Pd(OAc)_2$, which is expensive and could not be recovered, the cyclization was then attempted using a catalytic amount of $Pd(OAc)_2$. Following a literature procedure[109,110] using cupric acetate to reoxidize Pd(0) to Pd(II), a solution of acetic acid solution containing **18**, 0.1 equivalents of $Pd(OAc)_2$ and 2.6 equivalents of $Cu(OAc)_2.H_2O$ was heated under an air atmosphere at 95°C for 5 days to give **33** in 29% yield, with over 50% of the starting material **18** still remaining. The attempted reoxidation of the Pd metal back to Pd(II) by $Cu(OAc)_2$ was thus unsuccessful and hence one equivalent of Pd(II) was still required for the preparation of derivatives of **18**.

In further work, the *N*-acylated product **19** was reacted with 1.0 equivalent of $Pd(OAc)_2$ in glacial acetic acid to afford the ring-closed product **34** in high yield (74%, Scheme 3-15). It is likely that the electron releasing *para*-substitutent of **18a** (R =

OCH_3) would facilitate the insertion step for ring closure (Scheme 3-16). Confirmation of the structure of **34** was again provided by NMR spectroscopic analysis and mass spectrometry. The ^{1}H NMR showed a characteristic signal for ring-closed product at δ 6.77 as a singlet attributed to H-11 and which had no correlation with any nearby proton in the gCOSY spectrum. The absence of the signal which could be ascribed to H-2 around δ 7.55 was also noted. Further support for the structure was provided by the HRMS (EI) with a signal at *m/z* 294.0648, indicating the molecular formula of compound **34**.

Another derivative of **22** (R = OCH_2Ph, Scheme 3-15) was prepared by reaction of the *N*-acylated product **22** and $Pd(OAc)_2$ in glacial acetic acid to afford the ring-closed product **35** in 20% yield and the unexpected products **36** and **37** in 20 and 35% yields, respectively (Scheme 3-15). Debenzylation can be readily accomplished under acidic conditions at reflux.[111] Therefore, the cleavage of the benzyloxy subsituent in **22** and **35** could both occur together with the cyclization of **22**. The mixture was purified by VLC and **35** was easily removed from the chromatographically inseparable mixture of **36** and **37**. Fortunately, the components of **36** and **37** had different solubility properties, as **37** was completely soluble in 2% MeOH in DCM but **36** was not. Thus, a suspension of the mixture of **36** and **37** in 2% MeOH in DCM was filtered to give the ring-closed product **36** as a solid and **37** was retained in the filtrate. The phenol **36** could be converted to **35** in good yield by benzylation of the phenoxide anion with benzyl bromide (Scheme 3-17).



36 **35**

Scheme 3-17 Benzylation of compound 35 with benzyl bromide

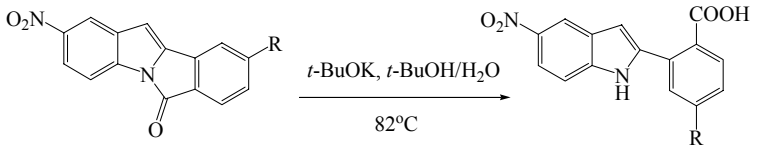
In the ^{1}H NMR of **35** a diagnostic singlet at δ 6.79 was assigned to H-11. Unfortunately, the ^{13}C NMR spectrum in $CDCl_3$ as solvent revealed only methine/methylene carbon signals and the quaternary carbon signals were not seen. Due to the partial solubility of compound **35** in $CDCl_3$, the concentration of **35** was too dilute for the quaternary carbon signals to be detected. The ^{13}C NMR spectrum was reattempted in DMSO-d_6, and in a solvent mix of $CDCl_3/CD_3OD$, in which the solubility of **35** was much higher. The ^{13}C NMR experiments were carried out at 25°C overnight, however the same problem arose as with the $CDCl_3$ spectrum with no quaternary carbon signals being seen and some of **35** precipitated in the NMR tube. The mass spectral data indicated cyclisation to **35** had occurred.

The structure of **36** was also confirmed by NMR spectroscopic analysis. The ^{1}H NMR revealed a characteristic singlet signal assigned to H-11 at δ 7.07. The absence of a signal which could be ascribed to the methylene protons of the benzyloxy substituent around δ 5.20 was also noted.

The structure of **37** was confirmed similarly by ^{1}H NMR spectroscopic analysis. The ^{1}H NMR showed the loss of signals which could be assigned to the methylene protons and the five aromatic protons of benzyloxy substituent. A doublet signal at δ 6.72 was attributed to H-3 and the gCOSY spectrum showed it was coupled to a doublet signal at δ 7.53, which was thus assigned to H-2. The HRMS (EI) spectrum showed a molecular ion peak at *m/z* 282.0638, indicating the molecular formula of **37** to be $C_{15}H_{10}N_2O_4$.

3.3.4.3 Amide hydrolysis of the cyclized products

N, *N*-Disubstituted amides can be hydrolysed under acidic or alkaline conditions. Based on the method of Itahara,[95] alkaline hydrolysis of the cyclized products **33-35** was achieved at 82°C using *t*-BuOK in *t*-BuOH containing a small amount of H_2O. The amide hydrolysis was unsuccessful under normal conditions with either HCl/MeOH in the presence of H_2O or NaOH/MeOH in the presence of H_2O.



33; R = H
34; R = OCH_3
35; R = OCH_2Ph

38; R = H, 95%
39; R = OCH_3, 94%
40; R = OCH_2Ph, 96%

Scheme 3-18 Amide hydrolysis of the cyclized products 33-35

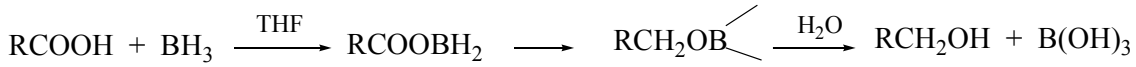
On TLC analysis of the first attempted hydrolysis of **33,** with *t*-BuOK in *t*-BuOH (82°C, N_2) for 12h only one clean product spot could be detected and none of the starting material **33**. The reaction was then stopped and the literature work-up procedure was followed. The reaction mixture was evaporated and diluted with a large amount of water. The mixture was then carefully neutralized with dilute HCl and extracted with diethyl ether. The ether extracts were dried with sodium sulfate and evaporated to give a brown semicrystalline residue which was triturated with ether/hexane to give the desired product **38** but, surprisingly, in very low yield (11%). It was suspected the work-up procedure might be the problem and thus it was reconsidered. The hydrolysis of **33** was reattempted with *t*-BuOK in *t*-BuOH containing a small amount of H_2O at 82°C, and after 12h, the reaction mixture was concentrated and then added to a large amount of ice water. The reaction mixture was then acidified

to pH1 using dilute HCl to give a yellow suspension. The suspension was saturated with solid NaCl and then vigorously stirred until the yellow suspension changed to a yellow solution. The reaction solution was then extracted with diethyl ether to afford the desired acid **38** in high yield (95%). Presumably the effect of the NaCl was important in reducing acid solubility in the aqueous phase and thus assisting extraction of the product into the diethyl ether. Structural confirmation of **38** was provided by NMR spectroscopic analysis and mass spectrometry. The ^{1}H NMR spectrum showed a similar signal pattern to the ^{1}H NMR spectrum of the starting cyclized product **33**. However, the ^{13}C NMR spectrum displayed a downfield signal attributed to the carbonyl carbon of a carboxylic acid at δ 171.7, while the HRMS (EI) spectrum supported the molecular formula of **38**.

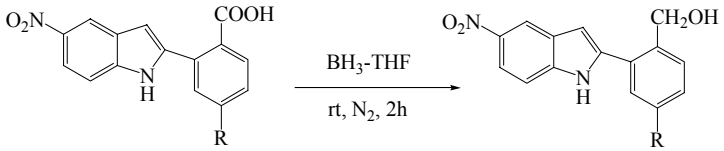
Following the modified work-up procedure, the other two acid derivatives **39** and **40** were prepared from **34** and **35** in the same manner, and were obtained in 94% and 96% yields, respectively. The structure confirmation of **39** and **40** was provided by NMR spectroscopic analysis and mass spectrometry. The ^{1}H NMR spectrum of **39** showed a similar spectrum to the starting material **34** but with additional signal which could be attributed to the indolic proton at δ 12.07 and this was confirmed by gHMBC analysis. The ^{13}C NMR spectrum revealed a signal ascribed to the carbonyl carbon of the acid at δ 167.9 and the HRMS (EI) spectrum had a molecular ion peak at *m/z* 312.0749, supportive of the molecular formula of **39**. The ^{1}H NMR spectra of **40** showed similar signals to those of **39,** together with extra signals which could be assigned to the benzylic ether group.

3.3.4.4 Reduction of acids to alcohols

Carboxylic acids are easily reduced to primary alcohol by $LiAlH_4$ which is a powerful but non-selective reagent. Alternative reagents were thus considered and selective reduction of benzoic acid derivatives **38-40** in the presence of the nitro substituent to the corresponding alcohols was accomplished with borane in tetrahydrofuran without disturbing the reducible nitro substituent. Moreover, the reaction was remarkably facile and proceeded in high yield. A possible mechanism involves the formation of an acyloxyborane in the first step (Scheme 3-19). Subsequently, this moiety can undergo further reaction with borane and eventually hydrolysis to the corresponding alcohol and boric acid.[112,113]



Scheme 3-19 Possible mechanism for reduction of carboxylic acids with borane in tetrahydrofuran



38; R = H
39; R = OCH_3
40; R = OCH_2Ph

41; R = H, 99%
42; R = OCH_3, 95%
43; R = OCH_2Ph, 85%

Scheme 3-20 Selective reduction of the benzoic acid derivatives in the presence of a nitro substituent

The benzoic acid **38** was reacted[112] with excess borane-tetrahydrofuran complex to afford the corresponding alcohol **41** in high yield (99%) (Scheme 3-20). The aromatic nitro group is inert toward borane. In the ^1H NMR spectrum of **41** the

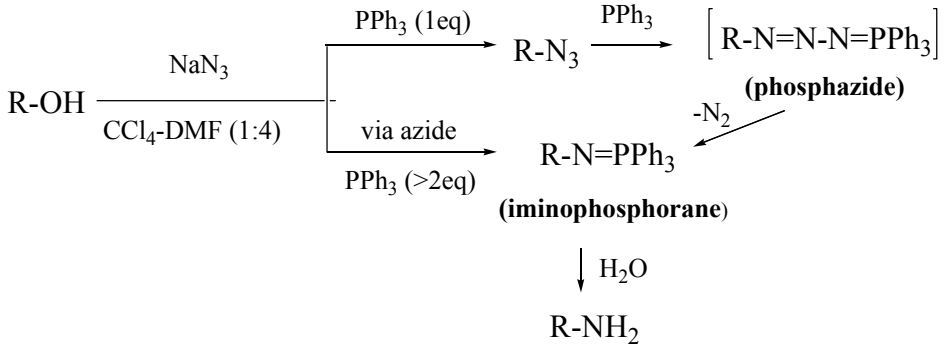
presence of the new hydroxymethyl group was confirmed by a signal integrating for two protons at δ 4.77. A signal for this methylene carbon was also apparent at δ 65.1 in the ^{13}C NMR spectrum, and this assignment was confirmed by a DEPT experiment..

The other benzyl alcohol derivatives **42** and **43** were prepared similarly from their benzoic acid derivatives **39** and **40** in 95% and 85% yield, respectively (Scheme 3-20). The ^{1}H NMR analysis of **42** confirmed the formation of the benzyl alcohol derivative with the presence of a signal ascribed to the methylene protons at δ 4.70. The presence of a signal attributed to a methylene carbon at δ 64.6 and the absence of a carbonyl group signal was noted in the ^{13}C NMR spectrum. Correspondingly, the structure of **43** was confirmed by the disappearance of the signal assigned to the carbonyl carbon at δ 167.9 in the starting material **40** and the appearance of a signal attributed to the methylene carbon at δ 70.2 in the ^{13}C NMR spectrum. Further confirmation of the structure of the benzyl alcohol derivative **43** was provided by the presence of a two proton singlet signal attributed to a methylene proton at δ 5.08 in the ^{1}H NMR spectrum; an M^{+} signal at *m/z* 374.1256 in the HRMS (EI) spectrum was consistent with the molecular formula $C_{22}H_{18}N_2O_4$.

3.3.4.5 Attempted conversion of alcohols to amines

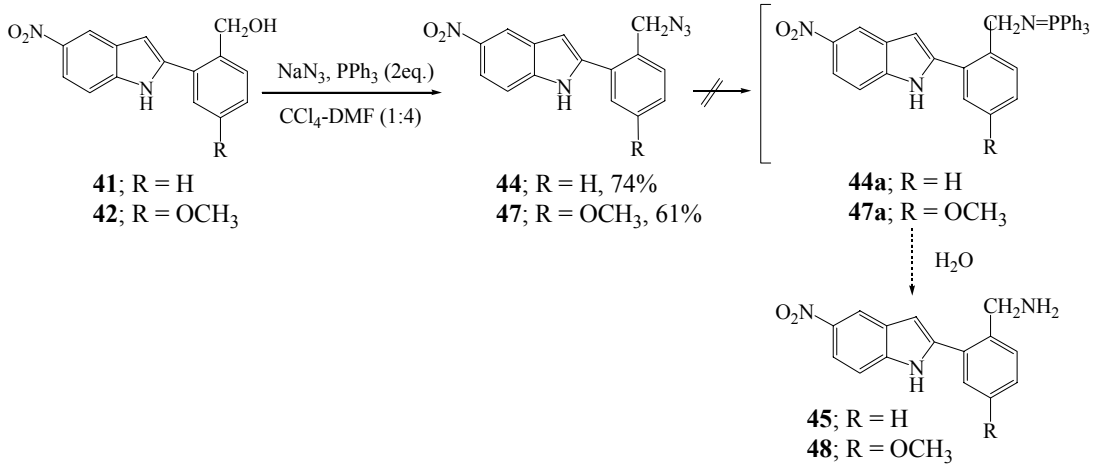
Typical methods to convert primary alcohols to primary amines involve a three-step procedure *via* the transformation of the alcohol to the corresponding halide or sulfonate, which then undergoes nucleophilic substitution by the azide anion to afford an alkyl azide and subsequent reduction of the azide to the desired primary amine.[114-116] Alternatively the conversion of an alcohol to such an amine can be achieved in a facile

one-pot process by a combination of reactions. An alcohol may be converted to the corresponding azide/amine by using NaN_3 and PPh_3 in 20% CCl_4 in DMF.[117]



Scheme 3-21 A facile one pot methodology for the conversion of alcohols to azides or amines

Treatment of alcohols with NaN_3 and two equivalents of PPh_3 in CCl_4-DMF (1:4) at 90°C afforded their corresponding amines[117] (Staudinger reaction) in excellent yield (85-95%). Using one equivalent of PPh_3 in the same manner[117] afforded the corresponding azides. Azide formation occurs with the first equivalent of PPh_3, and the azide can then react with the second equivalent of PPh_3 producing the iminophosphorane intermediate (*via* the phosphazide), which can then be converted to the amine by hydrolysis with water (Scheme 3-21). Thus treatment of alcohol **41** with NaN_3 and PPh_3 (2 equivalents) in 25% CCl_4 in DMF at 90°C, followed by the addition of excess water to hydrolyze the expected iminophosphorane intermediate **44a,** afforded the undesired azide **44** without any of the desired amine **45** (or intermediate **44a)**. The azide **44** was obtained in 74% yield.



41; R = H
42; R = OCH_3

44; R = H, 74%
47; R = OCH_3, 61%

44a; R = H
47a; R = OCH_3

45; R = H
48; R = OCH_3

Scheme 3-22 Attempted preparation of the benzylamines 45 and 48

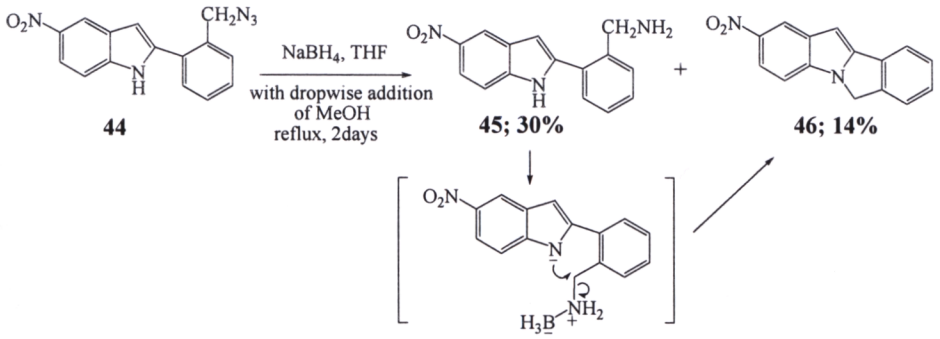
The reaction was reattempted in the same manner as previously except that the reaction time was extended to ensure that the azide **44** had sufficient time to react with the second equivalent of PPh_3. Water was then added to the reaction with warming at 50°C overnight as suggested in the literature,[117,118] however neither intermediate **44a** nor amine **45** were observed. After chromatography, only the azide **44** was obtained in 74% yield. The ^1H NMR spectrum of **44** showed a distinct 2-proton singlet at δ 4.40 which was attributed to the methylene protons, the signal position being upfield relative to the corresponding signal in the starting alcohol **41**. The ^{13}C NMR spectrum also displayed a signal ascribed to the methylene carbon at δ 54.0, which was consistent with a CH_2 which is shielded by a neighbouring azide group. Additionally, the HRMS (EI) spectrum revealed a molecular ion signal at *m/z* 294.0987, consistent with the molecular formula $C_{15}H_{12}N_5O_2$.

The azide derivative **47** was also prepared in the same manner as for **44**, from treatment of the alcohol **42** with NaN_3 and 2 equivalents of PPh_3 at 90°C. It is possible that with both **44** and **47**, the temperature was not sufficiently high for the phosphazide

intermediate formation and hence formation of **44a** and **47a**. Alternative azide reduction methods were then investigated.

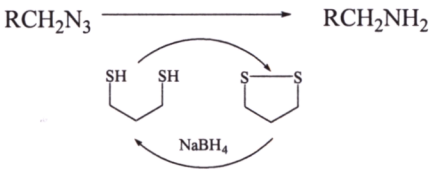
3.3.4.6 Reduction of azides to amines

Azides can be reduced easily to primary amines by a number of reducing agents, including $NaBH_4$, which usually gives poor yields but is selective. However, the reducing power of $NaBH_4$ is augmented by using either THF or *t*-BuOH as a solvent with dropwise addition of MeOH. Following a general literature procedure,[119] the azide **44** was reduced with $NaBH_4$ in THF-MeOH at reflux with stirring for 2 days. TLC analysis of the reaction mixture indicated some of the starting material **44** still remained together with the formation of two new products, one at a lower R_f than the azide **44** and another as a streak from the baseline. After separation of the former product, NMR analysis and mass spectrometry suggested that it was the cyclized product **46.** The ^1H NMR spectrum revealed the loss of the signal previously ascribed to the indolic NH proton and the appearance of a downfield singlet integrating for two protons at δ 5.12, which was attributed to the methylene protons. The HRMS (EI) spectrum, which showed a molecular ion signal at *m/z* 250.0732, was also consistent with the molecular formula of the cyclized product **46**. It is possible that **46** forms from the amine **45** *via* its borane complex, followed by nucleophilic attack on a methylene carbon by an indolide anion which may be generated in the basic conditions (Scheme 3-23). The other product, which was observed as a streak from the baseline on TLC, was predicted to be the desired amine **45**, which would bind strongly to the acidic silica gel. The amine **45** was separated by column chromatography with an eluting solvent mix of DCM in MeOH plus triethylamine.



Scheme 3-23 Reduction of the azide 44 to amine 45 with $NaBH_4$ in THF with dropwise addition of MeOH

The ^1H NMR spectrum of **45** showed an upfield singlet signal integrating for two protons at δ 3.99 which was assigned to the methylene protons adjacent to the amino group, and a signal ascribed to the indolic NH was apparent at δ 13.63. The HRMS (EI) data was consistent with the molecular formula of **45**.

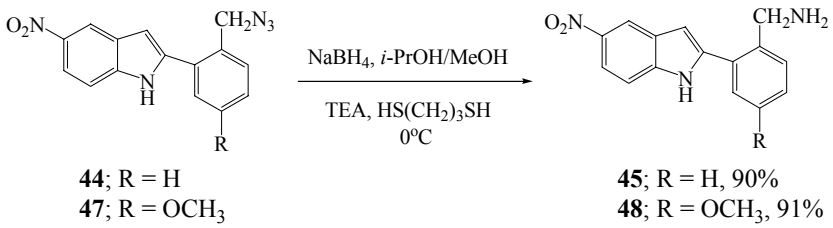


Scheme 3-24 General scheme for the conversion of azides to amines using $NaBH_4$ and 1,3-propanedithiol as a catalyst[120]

The azide reduction was reattempted following a method described by Pei[120] using $NaBH_4$ and 1,3-propanedithiol as a catalyst in *i*-PrOH and TEA at room temperature. Azides may be selectively reduced to amines by 1,3-propanedithiol which is oxidized to the cyclic disulfide (Scheme 3-24). Sodium borohydride can then be used to reduce the cyclic disulfide back to the 1,3-propanedithiol thus reducing the amount of 1,3-propanedithiol required for complete reduction. Unfortunately, the reduction of the azide **44** still gave a 3:1 mixture of the amine **45** and the unwanted cyclized product **46**.

To avoid this unwanted cyclization, the reaction was undertaken at a lower temperature for a shorter time. The azide **44** was then successfully reduced by 1,3-propanedithiol (3 eq.) and $NaBH_4$ (15 eq.) in 35% MeOH in *i*-PrOH and TEA at 0°C for 130 min to afford the amine **45** in 90% yield.

The other benzylamine derivative **48** was obtained from the benzyl azide **47** in 91% yield in the same manner as for **45** (Scheme 3-25). In the ^{1}H NMR spectrum of **48**, the methylene protons were assigned to the signal at δ 3.91, further upfield from the corresponding signal (δ 4.26) in the starting azide **47**.



44; R = H
47; R = OCH_3

45; R = H, 90%
48; R = OCH_3, 91%

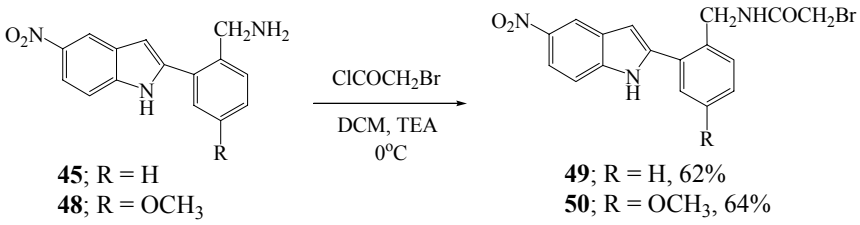
Scheme 3-25 Reduction of the azides 44 and 47 to the amines 45 and 48, respectively

Since the palladium-induced oxidative cyclization of **22** gave **35** in low yield and two by-products **36** and **37**, the synthesis of benzyloxy analogues of azide and amine derivatives was thus omitted. Although the by-product **36** can be converted to **35**, there is an extra step involved.

3.3.4.7 Preparation of α-bromoacetamides

The final step in the preparation of the required alkylating agents was adapted from a literature precedent.[121] The amine **45** was reacted with bromoacetyl chloride in DCM and TEA to afford the bromoacetamide **49** in 62% yield (Scheme 3-26). The ^{1}H NMR spectrum of **49** revealed the presence of two singlet signals integrating for two

protons each at δ 3.85 and 4.45 which were attributed to two groups of methylene protons in the molecule. Further confirmation of the structure was obtained by the appearance of a signal ascribed to a carbonyl carbon at δ 167.2 in the ^{13}C NMR spectrum, together with the HRMS (EI) spectrum which showed a molecular ion peak at *m/z* 389.0197, consistent with the molecular formula of **49**.



45; R = H
48; R = OCH_3

49; R = H, 62%
50; R = OCH_3, 64%

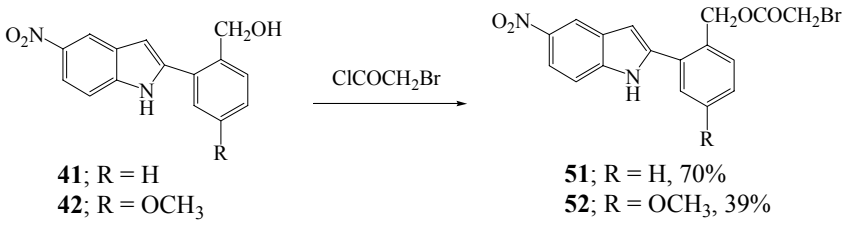
Scheme 3-26 Preparation of bromoacetamides 49 and 50

The bromoactamide derivative **50** was prepared in 64% yield from the amine **48** in the same manner as for **49**. Analysis of the ^{1}H NMR spectrum of **50** revealed two methylene proton signals as two singlets at δ 3.91 and 4.44, and the ^{13}C NMR spectrum confirmed the presence of a carbonyl carbon signal at δ 167.1. HRMS (EI) spectral analysis indicated a molecular ion at *m/z* 417.0332, supporting the molecular formula of **50**.

3.3.4.8 Preparation of α-bromoesters

The α-bromoester derivatives **51** and **52** (Scheme 3-27) were prepared from the corresponding alcohol derivatives **41** and **42**, respectively. Following a literature procedure,[122] the alcohol **41** was reacted with bromoacetyl chloride in TEA and THF at 0°C for 2h. As the formation of the desired bromoester **51** was not observed by TLC

analysis, the reaction was heated at 50°C for 5h to then afford **51** in 70% yield. The ^{1}H NMR spectrum of **51** revealed two singlet signals attributed to two groups of methylene protons at δ 3.97 and 5.31, together with a distinct signal ascribed to the carbonyl carbon in the ^{13}C NMR at δ 167.0. The mass spectral analysis HRMS (EI) showed a molecular ion at *m/z* 338.0056, consistent with the structure of **51**.



41; R = H
42; R = OCH_3

51; R = H, 70%
52; R = OCH_3, 39%

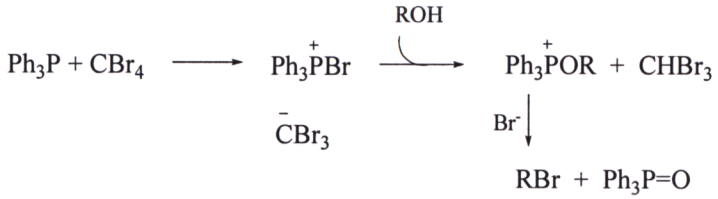
Scheme 3-27 Preparation of bromoesters 51 and 52

The α-bromoester **52** was prepared in the same manner as the α-bromoacetamides **49** and **50**. The desired product **52** was obtained in 39% yield with some starting material **42** still being present. The ^{1}H NMR spectrum of **52** showed a downfield singlet signal integrating for two protons at δ 5.17, which was attributed to the benzylic methylene protons, and a new signal integrating for two protons at δ 3.88 which was assigned to the bromomethyl group. The ^{13}C NMR spectrum of **52** had a signal at δ 166.8 which was consistent with an ester carbonyl group.

3.3.4.9 Preparation of indole benzyl bromide derivatives from the alcohols

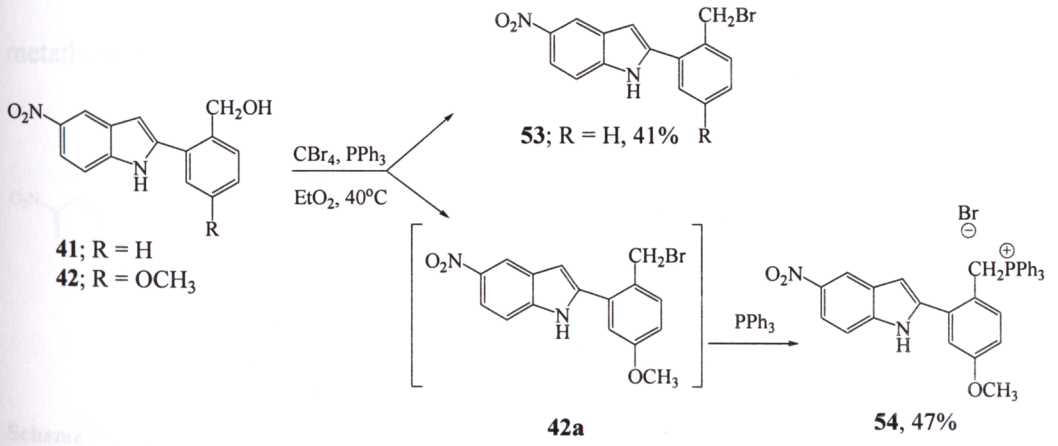
Various methods for the conversion of alcohols into their corresponding alkyl bromides are known. The most commonly used preparative route is the reaction of an alcohol with triphenylphosphine and carbon tetrabromide (Scheme 3-28).[123,124] Thus the

preparation of benzyl bromide **53** was conducted[125] in anhydrous diethyl ether at room temperature for 2 days, but no reaction occurred. The reaction was therefore repeated at a higher temperature (40°C, 2 days) to push the bromination to completion. The desired benzyl bromide **53** was obtained in 41% yield together with triphenylphosphine oxide as a by-product (Scheme 3-29).



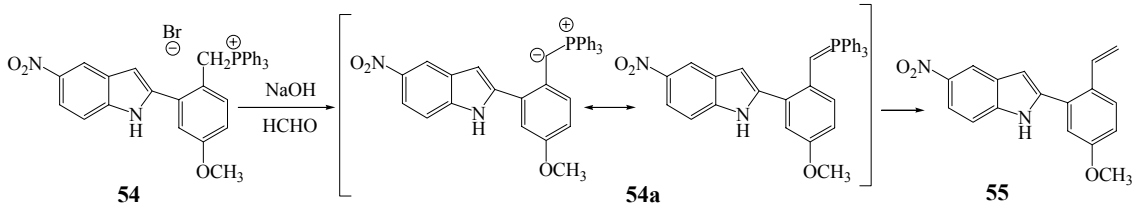
Scheme 3-28 General scheme for the bromination of alcohols using carbon tetrabromide and triphenylphosphine[126]

The structure of **53** was confirmed by NMR spectroscopy, with the spectra being similar to the starting alcohol **41**, while the formula was established from the HRMS (EI) data.



Scheme 3-29 Bromination of the alcohol 41 into the benzyl bromide 53

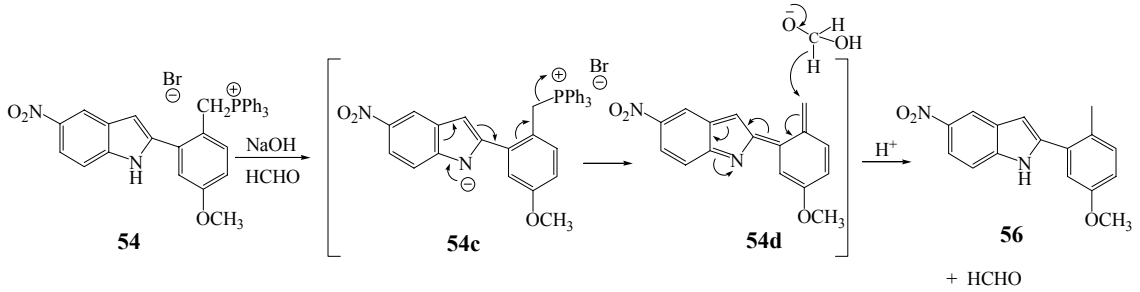
In contrast, attempted bromination of alcohol **42** in the same manner as for the reaction with **41** (Scheme 3-29) failed to furnish the desired benzyl bromide **42a**. Instead, the benzylphosphonium salt **54** was only obtained. In the ^{1}H NMR of **54**, 15 aromatic protons at δ 7.18-7.97 were present, plus two signal ascribed to methylene protons at δ 5.17. The presence of a very weak singlet signal at δ 29.2 in the ^{13}C NMR spectrum was attributed to the methylene carbon, although a doublet would be expected[127] for this signal from coupling with the adjacent phosphorus atom. This signal assignment was confirmed, by gHSQC and DEPT spectral analysis. High resolution mass spectral analysis (ES) revealed a positive ion at *m/z* 543.1838, corresponding to the formula of the cation in the salt **54**. It is likely that the desired product **42a** (Scheme 3-29) was formed in the reaction, but this then proceeded readily to give **54** on reaction with an excess of triphenylphosphine. The bromide **42a** would be further activated towards such a reaction by the *para*-methoxy group in the benzylic bromide. Although undesired in this reaction, these conditions are common for making a phosphonium salt from an alkyl halide and phosphine. Thus it was decided to make the undesired phosphonium salt **54** into a potentially useful alkene **55** (for cross metathesis reactions) *via* the Wittig reaction.



Scheme 3-30 Preparation of the alkene 55 *via* the Wittig reaction

The phosphonium salt **54** was treated with NaOH to generate ylid **54a,** which was then reacted with formaldehyde to give the alkene **55** (Scheme 3-30). Unfortunately,

the desired alkene **55** was obtained in only 3% yield together with an undesired alkyl derivative **56** as a major product in 49% yield.



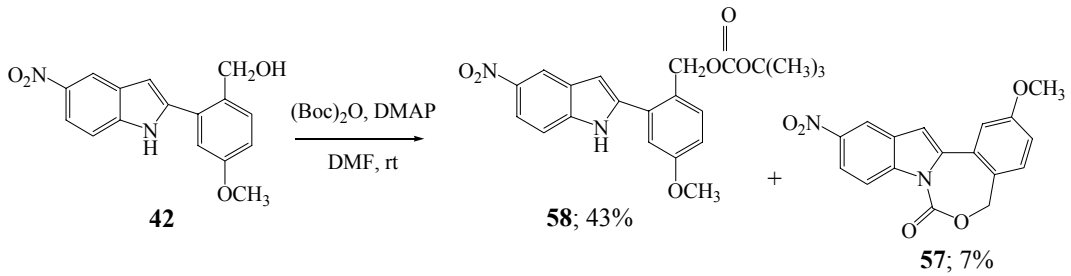
Scheme 3-31 Possible mechanism for the formation of 56

The normal Wittig reaction (Scheme 3-30) would proceed *via* the phosphorus ylide **54a,** being generated in turn by reaction of the phosphonium salt **54** with sodium hydroxide. Subsequent reaction with formaldehyde would give **55**. It is proposed that an alternative pathway from **54** and sodium hydroxide could also occur *via* the indolyl anion **54c**, which is stabilized by the electron-withdrawing nitro group. Elimination of triphenylphosphine from **54c** would afford **54d**, which could be the re-aromatized to give product **56** *via* hydride transfer (from $H_2C(OH)O^-$ formed in turn from HCHO and OH^-). A non-nucleophilic base could obviate this last step but this reaction was not examined further.

3.3.4.10 Attempted *N*-protection of indole 42

In order to attempt to prevent the formation of undesired products during the formation of the benzylic bromide from **42**, protection of the indolic nitrogen of **42** was investigated. It was hoped to Boc protect the indolic nitrogen and thus deactivate the ring before the bromination. Accordingly, direct *N*-boc protection was attempted using

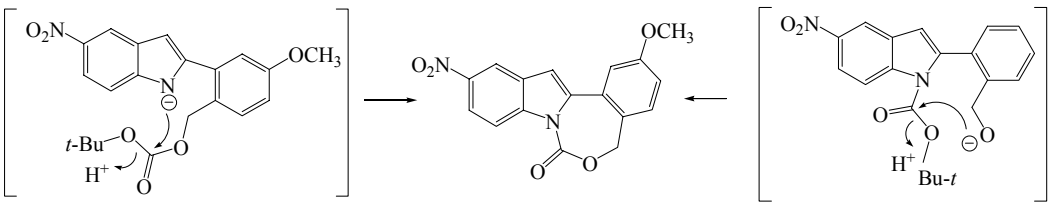
the alcohol **42** with $(Boc)_2O$ and DMAP.[128] NMR spectroscopic analysis and mass spectrometry revealed that the *O*-acylated compound **58** (Scheme 3-32) was the major product, together with a small amount of **57**, with no *N*-boc product.



42 **58**; 43% **57**; 7%

Scheme 3-32 Attempted *N*-boc protection of 42

The ^1H NMR spectrum of **58** showed the presence of a singlet signal at δ 1.52 integrating for nine protons which was attributed to the *t*-butyl group. A distinct singlet signal assigned to the NH proton at δ 9.99 confirmed that **58** was the *O*-boc and not the *N*-boc product. In addition, the correlation of a methylene proton signal at δ 5.11 to a carbonyl carbon signal at δ 153.5 was seen in the gHMBC spectrum and mass spectral (CI) analysis confirmed the molecular ion peak at *m/z* 399 ($C_{21}H_{23}N_2O_6$, $[MH]^+$), consistent with the molecular formula of **58**.

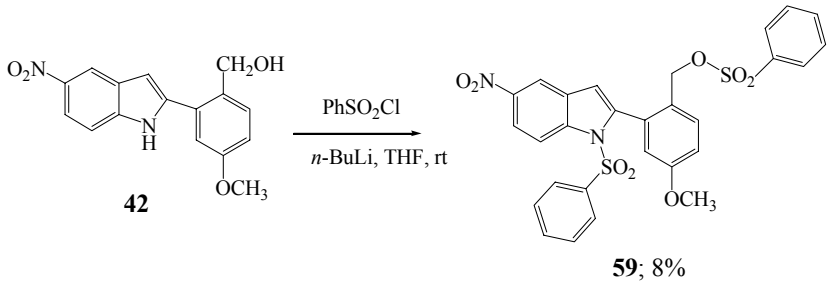


58a **57**; 7% **58b**

Scheme 3-33 Possible formation of the cyclized product 57

As the alcohol **42** contains two acidic protons (NH and OH) with similar *p*Ka values, which would be competitively deprotonated, the minor product **57** might be formed from an intermediate **58a** and/or **58b** (Scheme 3-33). The ^{1}H NMR spectrum of the minor product **57** showed a signal integrating for two protons at δ 4.91 as a broad doublet and this was attributed to the ring methylene group. Mass spectral (CI) analysis confirmed the molecular formula of **57** *via* a base peak at *m/z* 325 ($C_{17}H_{13}N_2O_5$, $[MH]^+$).

The *N*-protection of **42** was reattempted with a sulfonamide derivative which is less susceptible to nucleophilic attack than the carbamate type protecting groups.[129] However, reaction of the alcohol **42** with benzenesulfonyl chloride and *n*-BuLi at room temperature afforded only **59** in 8% yield (Scheme 3-34), without any of the desired *N*-sulfonyl product.



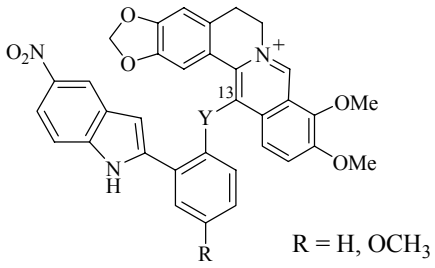
42 **59**; 8%

Scheme 3-34 Attempted *N*-sulfonyl group protection of 42

Due to the unsuccessful selective *N*-protection of **42**, the subsequent bromination step to achieve the alkyl bromide derivative **42a** (Scheme 3-29) was not pursued further. It was decided to make a dual action drug **65** (Chapter 4, Scheme 4-5) in the next step using the alcohol **42** instead of **42a**, as discussed in Chapter 4.

Chapter4:

Synthesis of the berberine-indole dual action agents



Dual action prodrugs; Y = CH_2OCOCH_2-(C13), $CH_2NHCOCH_2$-(C13)
Dual action drugs; Y = CH_2

Figure 4-1 The structures of the desired dual action agents

The aim of the synthetic work in this chapter was to synthesize berberine-indole dual action agents (Figure 4-1) based on the dual action prodrug and dual action drug concepts. The hybrids consisted of berberine linked at its C-13 position by an ester, amide or alkyl group to a 2-aryl-5-nitroindole analogue. Before the berberine and the indole derivatives were combined, a series of model compounds were produced, of which the first was 13-benzylberberine (Chapter 2, Section 2.2.2.2), with the benzyl group representing the 2-aryl-5-nitroindole moiety. Eventually, synthesis of the desired berberine-indole molecules was achieved based on the two-step strategy of "model study 2" (Chapter 2, Section 2.2.2) as described in the following sections.

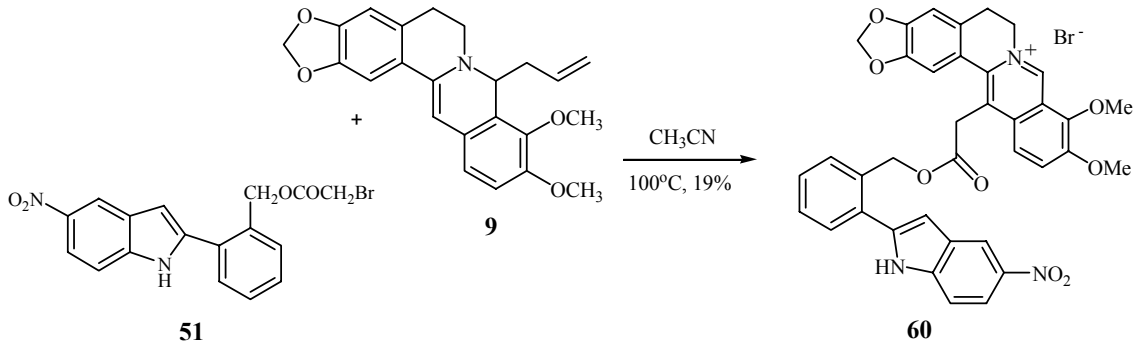
4.1 Synthesis of the berberine-indole prodrugs with a cleavable linkage

The dual action prodrugs containing berberine and an indole were designed to link the berberine and indole moieties together *via* an ester or amide group. These linkers

were expected to cleave under enzymatic action (by esterases or amidases) in the bacterial cells resulting in synchronous release in high concentration near the bacterial target sites. The synthesis of the ester and amide-linked prodrugs is discussed in the following sections.

4.1.1 Synthesis of the ester prodrug (60)

The ester bromide derivative of indole **51** was combined with the enamine **9** following the "model study 2" coupling procedure (Chapter 2, Section 2.2.2). The enamine **9,** which was discussed in Chapter 2, was reacted with the bromoester **51** in dry CH_3CN at 100°C for 2 days (Scheme 4-2). Analysis of the crude reaction mixture by TLC (silica gel, 8% MeOH in DCM) showed product was formed which streaked up from the baseline. The pure ester prodrug **60** was isolated in 19% yield after multiple preparative layer chromatography and recrystallization from 1% MeOH in DCM. It is likely that some product was lost in the purification process due to it strong adsorption on silica gel.



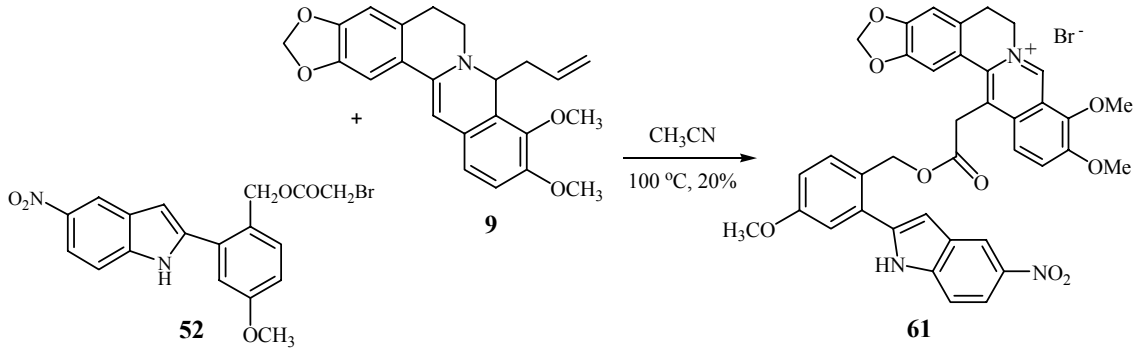
Scheme 4-2 Preparation of a dual action prodrug 60 with an ester linkage

The structure of the ester prodrug **60** was confirmed by NMR spectroscopic analysis and mass spectrometry. The ^1H NMR spectrum in $CDCl_3$ revealed the loss of

the signals attributed to the allyl substituent protons and the C-13 proton in the starting enamine **9,** and the presence of a singlet signal ascribed to the C-8 proton appeared at δ 9.91. The ^{1}H NMR spectrum also verified the presence of the indole moiety, with a singlet signal attributed to the indolic NH proton at δ 12.19, the expected set of signals integrating for seven protons ascribed to the aromatic protons of the indole, and a characteristic singlet signal assigned to the C-3′ proton at δ 6.74. In addition, two singlet signals attributed to the methylene protons of the ester linking group at δ 4.48 (CH_2CO) and 5.39 (CH_2O) were observed. The carbonyl carbon of the ester group was confirmed by a signal in the ^{13}C NMR spectrum at δ 170.3, and a downfield signal at δ 145.8 was ascribed to the C-8 methine carbon in the berberine moiety. Further support for the structural assignment was provided by HRMS (ES) with a signal at *m/z* 664.2034, consistent with the formula of the quaternary ammonium ion component of **60**.

4.1.2 Synthesis of the ester prodrug (61)

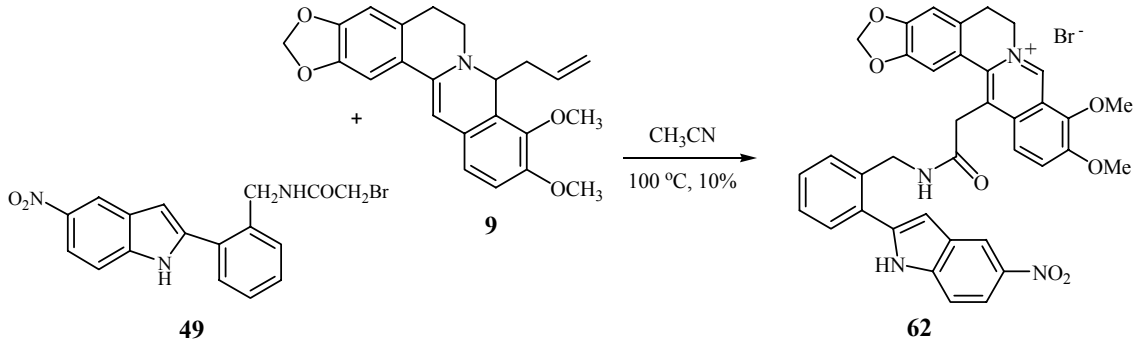
Under the same reaction conditions as for **60**, the ester prodrug **61** was produced from enamine **9** and the bromoester **52** in CH_3CN at 100^{o}C for 2 days. The ester **61** was obtained in 20% yield after extensive chromatographic treatment of the reaction mixture. The ^{1}H and ^{13}C NMR spectra of **61** were similar to those of the ester **60** except for the absence of an aromatic proton signal attributed to the C-4′′ proton in the latter. Moreover, the presence of a signal at δ 3.84 integrating for three protons and attributed to the methoxy groups was observed in the ^{1}H NMR spectrum, and for the methoxy carbon at δ 55.6 in the ^{13}C NMR spectrum. The HRMS (ES) spectrum showed a signal at *m/z* 674.2134, which was consistent with the positive ion component of compound **61.**



Scheme 4-3 Preparation of the dual action prodrug 61 with an ester linkage

4.1.3 Synthesis of the amide prodrug (62)

The reaction conditions for the formation of the amide prodrug **62** were the same as those used to synthesize the ester prodrugs (**60**, **61**) and the same purification problems, including the loss of some of the product **62,** occurred due to the polarity of the molecule. The enamine **9** was reacted with the bromoacetamide indole derivative **49** in dry CH_3CN at 100°C for 2 days. The amide **62** was obtained in only 10% yield after recrystallization.



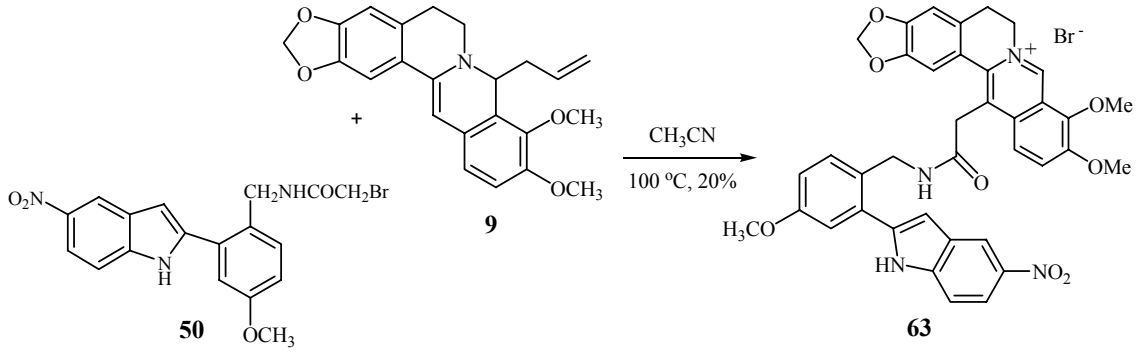
Scheme 4-4 Preparation of dual-action prodrug 62 with an amide linkage

The ^1H and ^{13}C NMR spectra in CD_3OD of amide **62** were almost the same as those for the ester **60,** except for an upfield singlet signal attributed to the methylene protons

adjacent to the amide at δ 4.69 (CH_2NH). In the ^{13}C NMR spectrum, an upfield signal at δ 42.0 was ascribed to the methylene carbon adjacent to the amide nitrogen atom. The HRMS (ES) showed a signal at *m/z* 643.2184, representing the positive ion component of compound **62.**

4.1.4 Synthesis of the amide prodrug (63)

A mixture of the enamine **9** and the bromoacetamide indole derivative **50** in dry CH_3CN was heated at 100°C for 2 days. After multiple chromatographic purification and recrystallization, **63** was obtained in 20% yield.

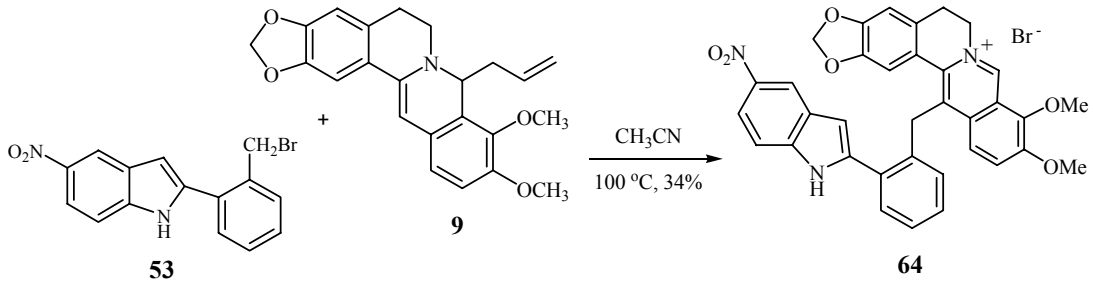


Scheme 4-5 Preparation of the dual-action prodrug 63 with an amide linkage

Characteristic peaks in the ^1H and ^{13}C NMR spectra of **63** included an extra signal integrating for three protons at δ 3.87 ascribed to the methoxy group in the indole portion, as well as a further signal at δ 56.0 in the ^{13}C NMR attributed to this group. The HRMS (ES) showed a peak at *m/z* 673.2305, representing the positive ion of compound **63.**

4.1.5 Synthesis of the dual action drug (64)

The preparation of the dual action drug **64** used the same reaction conditions as those used in the synthesis of the other ester and amide prodrugs. The enamine **9** and the bromide **53** in dry CH_3CN were heated at 100°C for 1 day, to afford **64** in 34% yield. The product was separated chromatographically but multiple development preparative layer chromatography was not required. The structure of compound **64** was elucidated by NMR spectroscopy and mass spectrometry. The ^1H and ^{13}C NMR spectra in CD_3OD had signals representative of the berberine and 2-aryl-5-nitroindole moieties. In addition, signals attributed to the methylene linking group for those two moieties were observed at δ 4.84 in the ^1H NMR spectrum and at δ 36.4 in the ^{13}C spectrum. The HRMS (ES) spectrum provided further evidence with a signal at *m/z* 586.1984, which was consistent with the positive ion component of compound **64**.

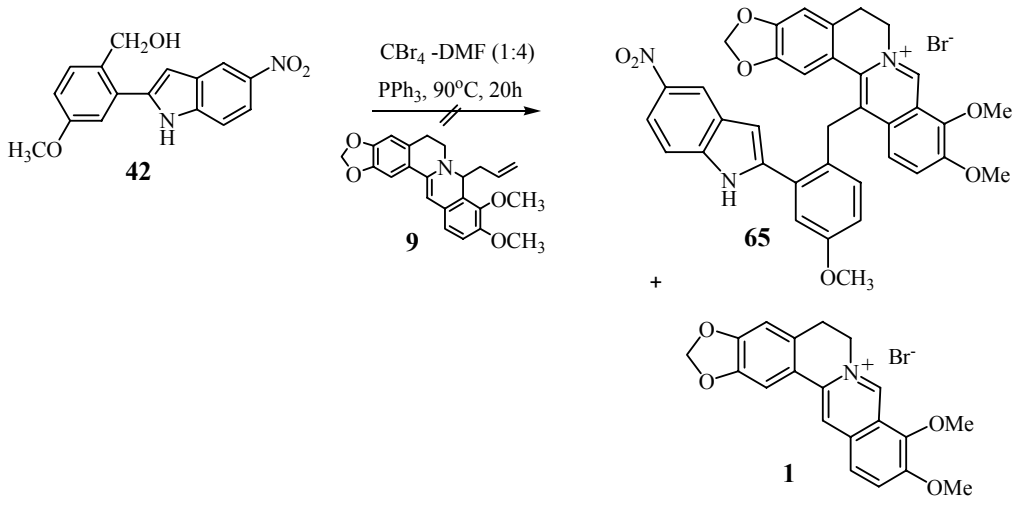


Scheme 4-6 Preparation of the dual action drug 64

4.1.6 Synthesis of the dual action drug (65)

Due to problems in the preparation of the *para*-methoxybenzyl bromide 5-nitroindole derivative from **42** (Chapter 3), one pot procedures for the formation of **65** from the alcohol **42** were investigated. The first attempted synthesis of **65** (Scheme 4-7) was based on triphenylphosphine-carbon tetrabromide as the reagent combination to

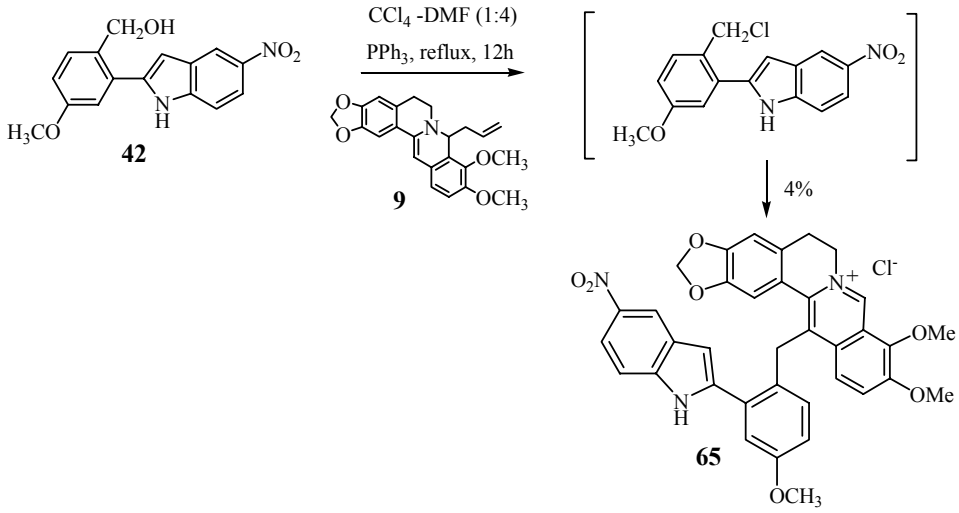
generate the alkyl bromide *in situ* from the alcohol **42** in the first step, and then attack by the enamine **9** to ultimately afford the bromide salt of **65**. This reaction was carried out at 90°C for 20 hours and produced many products as indicated by TLC analysis. The only product isolated was the unwanted berberinium bromide (**1**) in 12% yield. Unfortunately there was no evidence for the expected bromide salt **65**. It seemed likely that the alkyl bromide derivative had been generated from the benzyl alcohol **42** and immediately cyclized to **46** (Scheme3-23; Section 3.3.4.6) instead of reacting with the enamine **9**. Hydrogen bromide, which may be generated in this reaction, could protonate the enamine **9** and then propene elimination would give the bromide salt **1**.



Scheme 4-7 Attempted synthesis of the dual action drug 65

A further reaction was then attempted with the less reactive carbon tetrachloride in place of carbon tetrabromide. A mixture of alcohol **42**, enamine **9**, and triphenylphosphine in CCl_4 and DMF was heated at 90°C for 12 hours (Scheme 4-8). The crude reaction mixture was purified by multiple preparative layer chromatography and the product recrystallized to give the chloride salt **65** in 4% yield. The ^{1}H and ^{13}C NMR spectra in CD_3OD of **65** were similar to those of **64** except for one less aromatic

proton signal and the presence of signals which could be ascribed to the aromatic methoxy group (δ 3.83 in the ^{1}H NMR; δ 56.0 in the ^{13}C NMR). The HRMS (ES) spectrum showed a signal at *m/z* 616.2090, which is consistent with the positive ion component of compound **65.**



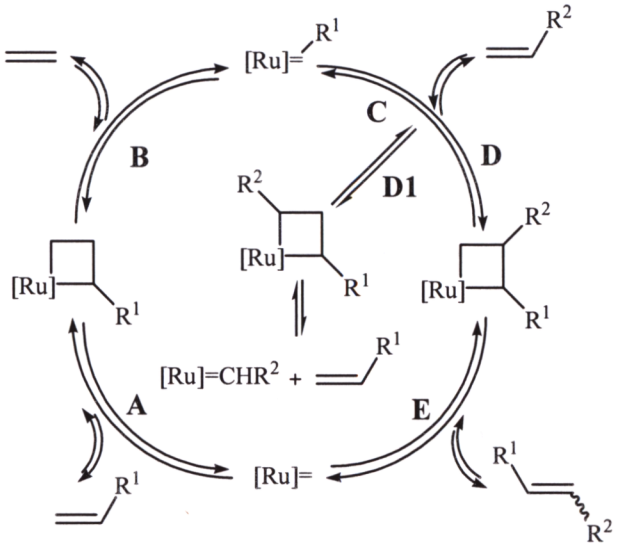
Scheme 4-8 Preparation of the dual action drug 65

4.2 Attempted linking group expansion of berberine-indole hybrids

The purpose of expanding the length of the linking group of the berberine-indole hybrid was to create increased flexibility between the two moieties in the dual action drugs. This would hopefully allow more successful attack on the bacterial target sites and hence increased antimicrobial activity.

4.2.1 Attempted synthesis of a berberine-indole hybrid *via* a cross metathesis reaction

Cross metathesis is an important reaction in organic synthesis, in which two independent alkenes are combined into one molecule using appropriate catalysts (e.g. molybdenum, or ruthenium complexes).[130,131] When two terminal alkenes are treated with a transition metal catalyst with the ability to catalyze the exchange of alkylidene groups of two independent alkenes, they produce a new compound linked *via* an alkene with the loss of ethylene.

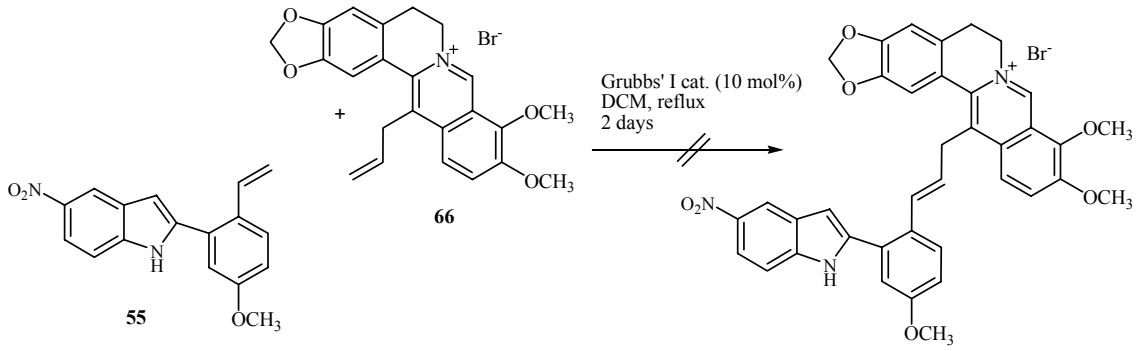


Scheme 4-9 Schematic representation of the Ru-based cross metathesis mechanism[132]

The catalytic mechanism cycle proceeds *via* initial [2+2] cycloaddition and consequent cycloreversion to give the desired product (Scheme 4-9). Initially, the cycle is initiated by coordination of an alkene to the ruthenium metal to form a ruthenacycle (step A) which rapidly releases ethylene gas and gives a newly substituted alkylidene (step B). Then a cycloaddition reaction of the second alkene (step C) with the alkylidine from step B gives a metallacyclobutane in step D1, or D which is more

preferred. Cycloreversion of the metallacyclobutane results in the desired cross-metathesis product and the active catalytic species is regenerated (step E).

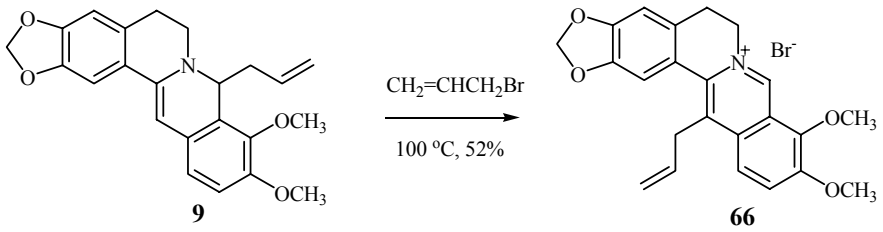
Cross metathesis of alkenes with a ruthenium complex catalyst was chosen to carry out the linking group expansion in the berberine-indole hybrids. Thus, the synthetic plan proposed the coupling of 13-allylberberine bromide **66** to the vinyl substituted 2-aryl-5-nitroindole analogue **55** *via* a cross metathesis reaction induced by polymer bound benzylidene-*bis* (tricyclohexylphosphine)-dichlororuthenium (Grubbs' I catalyst) (Scheme 4-10). The Grubbs' II catalyst was not investigated due to time constraints.



Scheme 4-10 Attempted linking group expansion of the berberine-indole hybrid

The starting allyl berberine **66** was prepared by reaction of 8-allyldihydroberberine **9** and excess neat allyl bromide at 100^{o}C for 2 hours (Scheme 4-11). After chromatography, the allyl berberine **66** was obtained in 52% yield. This compound had been made previously by Ikekawa *et al*., but they did not give any spectroscopic data in their patent.[133] Confirmation of the structure of **66** was provided by NMR spectroscopic analysis and mass spectrometry. The ^{1}H and ^{13}C NMR spectra showed the characteristic signals of 13-substituted berberinium salts as observed in compounds **60**-**65**. Additionally, the ^{1}H NMR spectrum showed a set of distinctive

signals attributed to the allyl group at δ 3.90-4.01 (CH_2**H**$_2$CH=CH_2) as a multiplet, 4.91 and 5.43 (CH_2CH=C**H**$_2$) as broad doublets, and 6.31-6.42 (CH_2C**H**=CH_2) as a multiplet. The ^{13}C NMR spectrum had signals that confirmed the structure of **66** including a set of signals attributed to an allyl group at δ 34.7 (**C**H_2CH=CH_2), 120.8 (CH_2CH=**C**H_2), and 135.1 (CH_2**C**H=CH_2). The HRMS (ES) spectrum provided additional evidence with a signal consistent with the positive ion of **66** at *m/z* 376.1548.



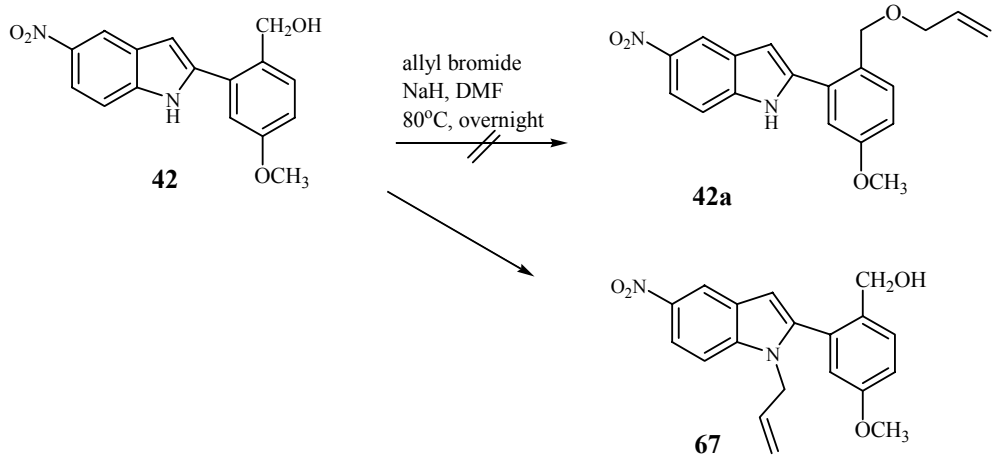
9 **66**

Scheme 4-11 Preparation of the allyl berberine salt 66

The other alkene component, **55** was prepared as discussed in Chapter 3 (Section 3.3.4.9). The cross metathesis reaction of **66** and **55** was catalyzed by 10 mol% of Grubbs' I ruthenium catalyst (polymer bound) in dry DCM with heating at reflux for 2 days under a nitrogen atmosphere. After chromatography, two unidentified products were isolated and some of the starting materials **66** and **55** were also recovered. Unfortunately, none of the expected indole-berberine cross metathesis product was obtained and only trace amounts of the two isolated products were obtained which did not permit full spectroscopic identification. The MS (CI) spectrum of the unknown products showed molecular ion peaks at *m/z* 208 in the first case and at *m/z* 313 in the second case. These peaks did not correspond to possible homodimeric products.

The cross metathesis reaction shown in Scheme 4-10 was tested by changing the alkene **55** to another alkene derivative. It was proposed that the vinyl group in **55** may

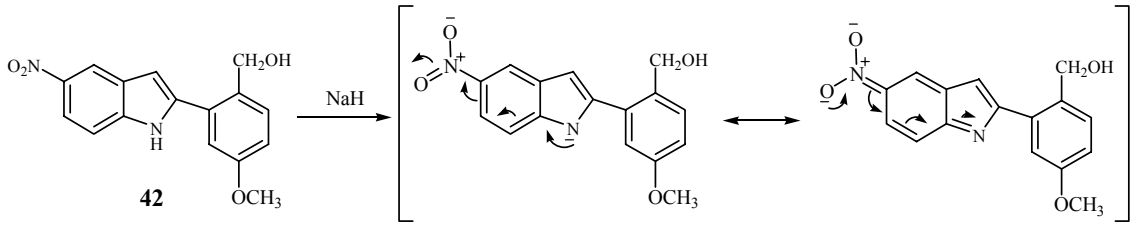
be too short, thus causing difficulty in coupling to **66** on steric grounds. Thus, the allyl derivative **42a** was designed to replace **55** for use in a cross metathesis reaction with **66**.



Scheme 4-12 Unsuccessful *O*-alkylation of alcohol 42

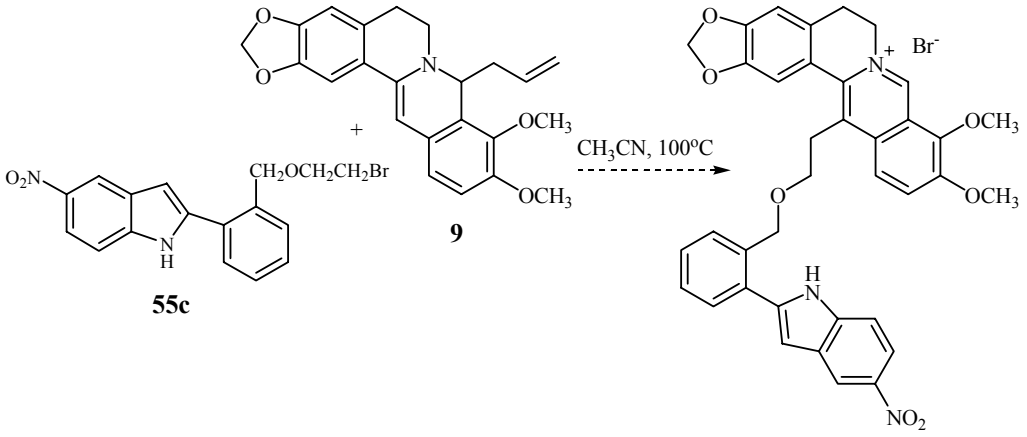
In the attempted preparation of **42a**, allyl bromide was used as the alkylating reagent, dry THF as a solvent, and TEA as a base to abstract the hydroxyl group proton in **42**. The reaction mixture was heated at 50°C for 30 hours under a nitrogen atmosphere, but none of the product **42a** was obtained. After chromatogryphy, only the starting alcohol **42** was recovered. It seems likely that the electron-donating *para*-methoxy substituent on the benzyl alcohol moiety decreased the acidity of the benzylic hydroxy proton. Thus, a stronger base was indicated and NaH was used instead of TEA in the next trial. Alcohol **42** was heated for 12 hours at 80°C in DMF with 1.3 mole equivalents of allyl bromide and 1.0 mole equivalent of NaH[101] (Scheme 4-12). After chromatography, the *N*-allyl product **67** was obtained in 21% yield together with two unidentified products which were lower in polarity than **67** (from TLC analysis). It is likely that the indolic NH proton is more acidic than the benzylic hydroxy proton due to the presence of the 5-nitro group (electron withdrawing group). The nitro group would

stabilize the indolyl anion through delocalization of the negative charge (Scheme 4-13), and hence *N*-alkylation would be preferred over *O*-alkylation.



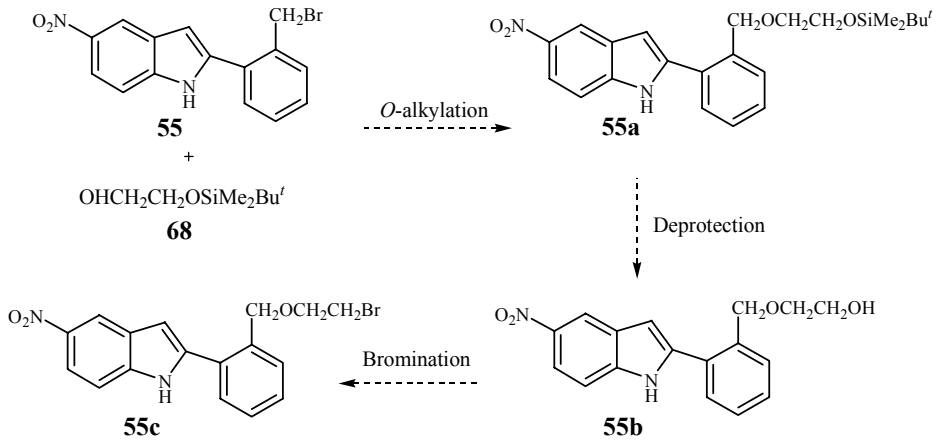
Scheme 4-13 Resonance stabilization of 5-nitroindolyl anion

The ^{1}H NMR spectrum of **67** consisted of six aromatic proton signals with the expected splitting patterns, a singlet at δ 6.71 attributed to the C-3 proton of the indole nucleus, a singlet at δ 3.83 integrating for three protons ascribed to the methoxy group, and a singlet at δ 4.48 integrating for two protons and assigned to the methylene group. Moreover, the presence of an allyl group was indicated by characteristic signals at δ 4.61 (CH_2**H**$_2$CH=CH_2) as a doublet, 4.85 and 5.16 (CH_2CH=C**H**$_2$) as two doublets, and 5.78-5.91 (CH_2C**H**=CH_2) as a multiplet; no signal which could be ascribed to the indolic NH proton was observed. The ^{13}C NMR spectrum and 2D analysis provided additional confirmation of the structure of **67**. The HMBC experimental data indicated that the methylene proton signal observed at δ 3.60 (allyl group) correlated with the signal at δ 139.6 (C-7a) and it also correlated with the signal at δ 131.3 (C-2). This clearly indicated that the allyl group was attached to the nitrogen of the indole nucleus. The HRMS (CI) showed a signal at *m/z* 339.1349, consistent with the molecular ion of compound **67.**



Scheme 4-14 Proposed alternative synthesis of a berberine-indole hybrid with an expanded linking group

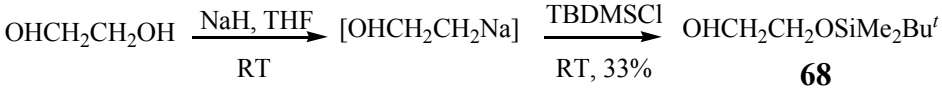
At this point an alternative route to the more flexible hybrid molecules was devised based on an enamine alkylation reaction of **9** with 2-aryl-5-nitroindole derivatives containing longer side chains and a bromo substituent to act as an alkylating agent (Scheme 4-14). The initial target was the bromide **55c**. The synthetic plan for **55c** (Scheme 4-15) involved a selective monoprotection of ethylene glycol (as its TBDMS ether), subsequent *O*-alkylation with the bromide **55** to produce **55a**, then deprotection to give the free alcohol **55b**, and bromination to eventually afford the bromide **55c**.



Scheme 4-15 Proposed synthesis of 55c

4.2.2 Synthesis of 2-(*tert*-butyldimethylsilanyloxy)ethanol (68)

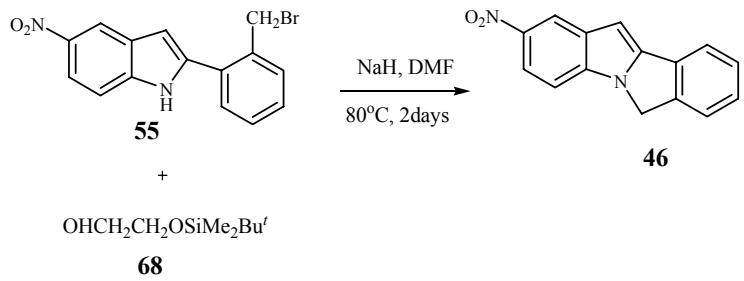
The selective monosilylation of ethylene glycol was achieved following a literature procedure which described the synthesis of this compound.[134] Ethylene glycol was treated with 1 equiv. of NaH to form a monosodium salt as an opaque white precipitate, and then silylating with TBDMSCl. After chromatography, the silyl alcohol **68** was obtained in 33% yield.



Scheme 4-16 Monosilylation of ethylene glycol

4.2.3 Attempted *O*-alkylation of 55a

The alkylation of 2-(*tert*-butyldimethylsilanyloxy)ethanol (**68**) with the previously prepared alkylating agent **55** was carried out using similar reaction conditions to those used for the synthesis of **67** (Scheme 4-12). The monosilylated ethanol in DMF was treated with NaH to form a sodium alkoxide salt, and then the bromide **55** was added and the mixture heated at 80°C for 2 days. The major product obtained (69% yield) was the cyclised compound **46** (see Chapter 3, Scheme 3-23); none of the required compound **55a** was observed. Due to time constraints, no further work on the extended linking chains was undertaken.



Scheme 4-17 Attempted *O*-alkylation of 2-(*tert*-butyldimethylsilanyloxy)ethanol (68)

4.3 Hydrolysis of the ester linked berberine-indole prodrug

An enzymatic hydrolysis experiment was undertaken only on the berberine-indole prodrug with an ester linkage, which was a model dual-action prodrug compound for enzymatic hydrolysis. The enzyme used was the commercially available porcine liver carboxyl esterase (EC 3.1.1.1). This experiment investigated the prodrug concept that two parent drugs would be released after enzymatic ester cleavage of the prodrug.

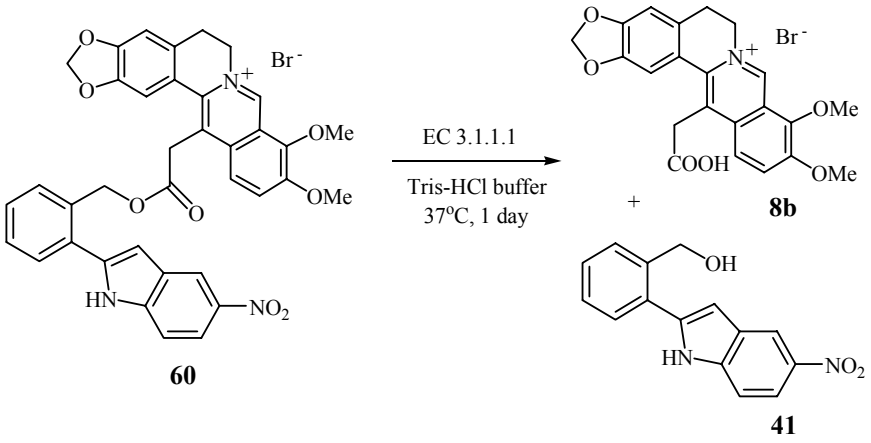


Figure 4-18 Enzymatic hydrolysis of ester prodrug 60

Following a general literature procedure,[135] the ester prodrug **60** in DMSO was reacted with the porcine liver carboxyl esterase (EC 3.1.1.1) in the presence of Tris-HCl buffer solution (pH 7.2) at 37°C for 1 day. After this time, organic products were extracted with DCM. TLC analysis of this concentrated extract indicated the presence of trace amounts of the alcohol **41**. It is likely that the solubility of prodrug **60** in DMSO-Tris-HCl buffer solution may have been a problem, since formation of a suspension was noted after addition of the buffer solution to a solution of the ester **60** in DMSO. When acetone was used instead of DMSO in the enzymatic reaction, a small amount of **41** was again detected. These results provided some indication that the ester

prodrug **60** does hydrolyse in the presence of esterase or undergoes spontaneous hydrolysis slowly with time. Further studies are needed to confirm these using bacterial esterases. Also, the hydrolysis of the amide prodrug with hydrolases[136] still needs to be investigated.

Chapter5: Biological test results

The biological activities of the test compounds were determined by antimicrobial assays against various microorganisms including bacteria, yeasts and a protozoan, but mainly bacteria. Subsequently, the most active compounds were assessed for cytotoxicity against human cancer cells. The antibacterial testing was divided into two phases. The first phase of testing was performed in the University of Wollongong to assess the initial antibacterial activity. The second phase was performed in the Department of Biology, Northeastern University, Boston, USA, in collaboration with Prof. Kim Lewis and Mr. Anthony Ball, for more detailed systematic studies of both antibacterial and MDR pump inhibitory activities. Additional antibacterial testing of a few active compounds was also done by Avexa Ltd., Melbourne, and antimalarial testing (protozoan; *Plasmodium falciparum*) of the most active antibacterial compound was done by Dr. Sumalee Kamchonwongpaisan, Protein-Ligand Engineering and Antimalarial Screening Laboratories, National Centre for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (BIOTEC), Bangkok, Thailand. Limited testing against the yeast, *Saccharomyces cereavaesiae* and the yeast-like fungus *Candida albicans* was also undertaken at Northeastern University. Details of the testing methodologies are given in Appendices I-V.

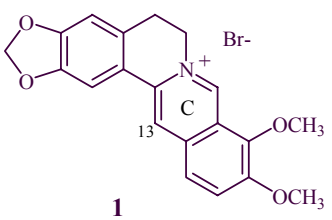
5.1 Preliminary antibacterial testing results against *Staphylococcus aureus* ACM844 and *Escherichia coli* ACM845 using a combination of FDA and antimicrobial (cell lysis/cell stasis) assays

The initial results of antibacterial testing of 7 berberine derivatives (**3, 5-8, 10-11**), 2 indoles (**38**, **41**), a potential dual action prodrug **60** and a dual action drug **64,** together with MDR inhibitory testing of a mixture of **11** and **38**, and **11** and **41**, provided valuable initial information about the antibacterial activity against Gram-positive (*S. aureus* ACM844) and Gram-negative (*E. coli* ACM845) human pathogenic bacteria using a combination of fluorescein diacetate (FDA) and antimicrobial (cell lysis/cell stasis) assays[137] (Table 5-1). With the FDA assay, it is not possible to distinguish whether antimicrobial agents cause cell death or cell stasis, and thus the antimicrobial (cell lysis/cell stasis) assay was used after completion of the FDA assay to assess the ability of the cells to recover. Therefore, the combination of both assays produced an accurate assessment of antimicrobial activity.[137] The antimicrobial activity in the cell lysis/cell stasis assay was determined as the lowest concentration that caused over 90% cells unable to recover and is defined as the minimum inhibitory concentration (MIC). This value is given in μg/mL and in μM concentrations in Table 5-1.

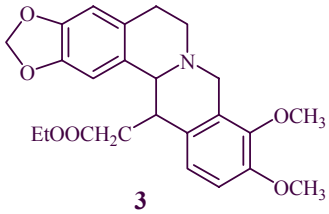
Table 5-1 Minimum Inhibitory Concentration (MIC) values of berberine bromide, berberine derivatives, indole derivatives and a mixture of indole and berberine derivatives against *S. aureus* ACM844 and *E.coli* ACM845 using the combination of FDA and antimicrobial (cell lysis/cell stasis) assays. NT indicates the sample was not tested. * indicates result of antibacterial screening.

Entry	Compound	MIC, μg/mL (μM)	
		S. aureus ACM844	***E. coli*** ACM845
1	**1, Br**	75.00(180.33)	NT
2	**3**	100.00(235.03)	NT
3	**5**	>100.00(>205.11)	100.00(205.11)
4	**6a**	100.00(182.03)	NT
5	**7**	100.00(177.28)	100.00(177.28)
6	**8**	100.00(191.82)	100.00(191.82)
7	**10**	50.00(93.62)	NT
8	**11**	50.00(98.80)	NT
9	**38**	100.00(354.28)	100.00(354.28)
10	**41**	50.00(186.38)	<10.00(<37.28)
11	**11+38** (1:1 weight ratio)	50.00	NT
12*	**11+41** (1:1 weight ratio)	10.00-25.00	NT
13	**41** in the presence of **11** at 0.5 MIC	10.00	NT
14*	**60**	10.00-100.00(13.80-137.97)	NT
15	**64**	10.00(15.00)	NT

The antibacterial activity results (Table 5-1) showed that compounds **3** and **5** had low antibacterial activity, and both were less active than **1**. Since it has been established[11] that aromaticity in ring C is essential for protoberberine alkaloids to exhibit high antimicrobial activity (as discussed in Chapter 1, section 1.1), the low activity of tetrahydroberberines **3** and **5** was not unexpected although other variables could be involved. Moreover, it has been reported that introduction of an ester group at the C-13 position (ring C) of **1** resulted in a decrease in the activity.[9] Therefore, it was not surprising that the activities of **3** and **5** were lower than that of **1**. Similarly, compound **6a** and **7** were slightly less active than **1** probably as the effect of the lipophilic ester group was introduced at the C-13 position.



1



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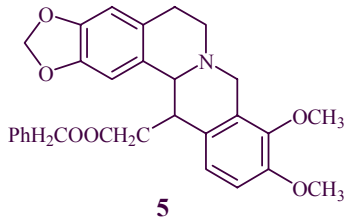
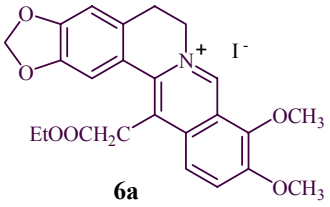
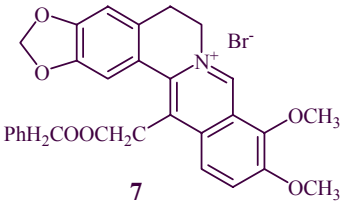


5



6a



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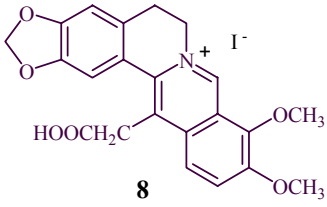
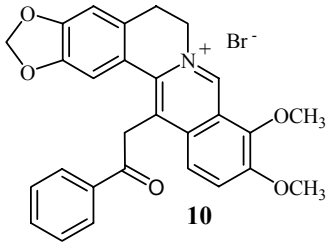


8



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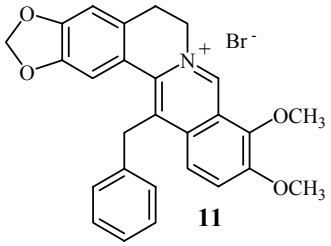
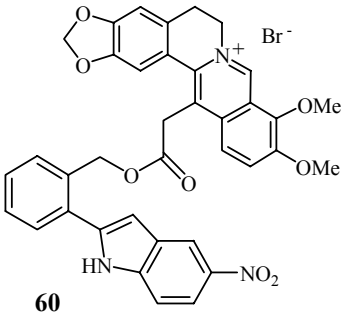
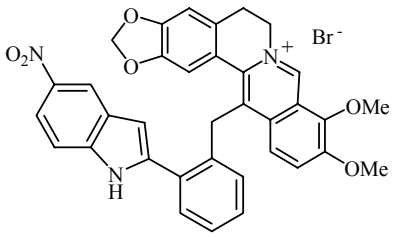


11

Introduction of a carboxylic acid group at the C-13 position of **1** caused a decrease in antibacterial activity (comparing **8** with **1**). Substitution of hydrogen at the C-13 position of **1** by a lipophilic phenacyl group (**10**) or benzyl group (**11**) resulted in an increase in the activity.

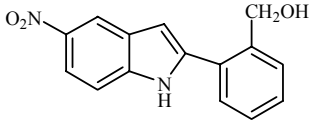


60

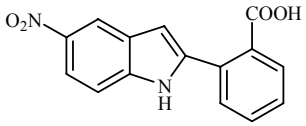


64

The related 13-substituted berberine derivatives **60** and **64**, which were expected to have dual antimicrobial and MDR pump inhibitory activities, were both more active than **1**, although the MIC for **60** was not accurately determined in these preliminary studies. Compound **64**, at least, was also more active than the benzyl analogue **11**. This evidence indicated that there was a significant advantage associated with a 2-phenyl-5-nitro-1*H*-indole substituent at the C-13 position.



41



38

The 2-phenyl-5-nitro-1*H*-indole analogues **38** and **41** had inherent antibacterial activity, with **38** being more active than **41**. Interestingly, **41** was more active against Gram-negative bacteria (*E. coli* ACM845) than Gram-positive bacteria (*S. aureus* ACM844). In addition, **38** and **41** were tested in *S. aureus* in the presence of the antibacterial agent **11** (entry **11**-**13**), and it was found that **41** was able to potentiate the antibacterial activity of **11** in line with **41** having MDR pump inhibitory activity.

The FDA and antimicrobial (cell lysis/cell stasis) assays provided preliminary results for the antibacterial agents (**60** and **64**) and the potential MDR pump blocking agents (**38** and **41**). Therefore, systematic antimicrobial activity testing and MDR pump inhibitory activity testing of all target compounds were indicated and these tests were undertaken by microbiologists at Northeastern University, Boston. The detailed results of the antimicrobial activity and MDR pump inhibitory activity are given in the next section.

5.2 MDR pump inhibitory testing results

The initial antibacterial activity results in the previous section showed that 2-phenyl-5-nitro-1*H*-indole derivatives may be NorA MDR pump inhibitors and potentiators of the antibacterial activity of berberine or berberine derivatives in *S. aureus*. To confirm and quantify the MDR pump inhibitory activity of the 2-phenyl-5-nitro-1*H*-indole derivatives, a set of these indoles was tested in the presence of a sub-inhibitory concentration (no effect on bacteria at this concentration) of a NorA MDR pump-substrate and antibacterial agent which was either berberine (**1**) chloride or

ciprofloxacin. The potentiating activity of the 2-phenyl-5-nitro-1*H*-indole derivatives on berberine **1** chloride in a wild type *S. aureus* strain 8325-4 was described by the fractional inhibitory concentration (FIC) index (Table 5-2), and the MDR pump inhibitor property was examined using this index as an indication of the activity. In the case of the potentiating activity of these indoles on ciprofloxacin, the FIC index was not able to be calculated because the exact MICs for direct activity of the indoles against *S. aureus* K2361 (Table 5-4) were not available. The MDR pump inhibitory activity results for the 2-phenyl-5-nitro-1*H*-indole derivatives are shown in Tables 5-3 and 5-4, and the direct activity (antibacterial activity) results for all test compounds (Figure 5-1; fold out sheet page 128) are shown in Tables 5-5 and 5-6 against strains expressing the MDR efflux pump in Gram-positive bacteria, Gram-negative bacteria and yeasts. In all tests berberine **1** was used as its chloride salt.

Table 5-2 Indicator of potentiating activity for 2-aryl-5-nitro-1*H*-indole derivatives with the antibacterial agent berberine (1) chloride using wild-type *S. aureus* 8325-4 which expresses the NorA MDR pump.

	FIC index[a]		FIC index[a]
Compound	**Berberine (1) chloride**	**Compound**	**Berberine (1) chloride**
38	1.12	**44**	0.24
39	<0.37	**45**	0.13
41	0.37	**47**	<0.14
42	<0.37	**48**	<0.13
43	<0.13	**56**	<0.15

[a] The fractional inhibitory concentration (FIC) was calculated for each inhibitor and berberine (**1**) chloride in combination by using the following formulas: FIC of antibacterial agent = MIC of antibacterial agent in combination/MIC of antibacterial agent alone, FIC of inhibitor = MIC of inhibitor in combination/MIC of inhibitor alone, and FIC index = FIC of antibacterial agent + FIC of inhibitor. Potentiating activity was defined as an FIC index of <0.5.[46]

Most of the test compounds were synergistic with a sub-inhibitory concentration of **1.** The FIC indices (Table 5-2) for compounds **39**, **41**-**45**, **47**, **48**, and **56** had values

less than 0.5, which indicated that these compounds were synergistic in potentiating the antibacterial activity of **1** while compound **38** was not. It appeared that the use of **1** in combination with an inhibitor of the MDR pump (**41** - **45**, **47**, **48**, and **56**) significantly improved the efficacy of the antibacterial **1** by inhibiting its efflux.

Table 5-3 Minimum Inhibitory Concentration (MIC) value for 2-phenyl-5-nitro-1*H*-indole derivatives (MDR pump inhibitory activity) against Gram-positive bacteria: *Staphylococcus aureus* K1758 (lacking NorA MDR pump), *S. aureus* 8325-4 (expressing NorA MDR pump), *S. aureus* K2361 (overexpressing the NorA MDR pump), *Enterococcus faecalis* V583, and *E. faecium* DO in the presence of sub-inhibitory concentration of berberine (1) chloride. NT indicates the sample was not tested. "-" indicates sample showed no activity when tested at a concentration of 50μg/mL.

Compound	MIC, μg/mL (μM)				
	S. aureus			*E. faecalis*	*E. faecium*
	K1758 +**1**, 3μg/mL	8325-4 +**1**, 30μg/mL	K2361 +**1**, 30μg/mL	V583 +**1**, 30μg/mL	DO +**1**, 30μg/mL
38	-	-	-	-	NT
39	12.50 (40.03)	12.50 (40.03)	12.50 (40.03)	-	NT
41	6.25 (23.30)	12.50 (46.59)	12.50 (46.59)	-	NT
42	3.13 (10.49)	12.50 (41.90)	6.25 (20.95)	-	NT
43	**0.39 (1.04)**	**0.39 (1.04)**	**0.78 (2.08)**	-	NT
44	0.78 (2.66)	6.25 (21.31)	3.13 (10.67)	-	NT
45	<0.24 (<0.90)	0.39 (1.46)	12.50 (46.77)	12.50 (46.77)	25.00 (93.53)
47	0.39 (1.21)	1.56 (4.83)	1.56 (4.83)	-	NT
48	0.78 (2.62)	0.39 (1.31)	12.50 (42.04)	25.00 (84.09)	12.50 (84.09)
56	0.39 (1.38)	1.56 (5.53)	-	-	NT
INF55	0.78 (3.28)	0.24 (1.01)	3.13 (13.15)	NT	NT

Note: inhibitors **41** and **44** were chosen for MDR inhibitory activity testing against *Salmonella enterica* Serovar Typhimurium SL1344R2 in the presence of either a fixed sub-inhibitory concentration of 30μg/mL berberine (**1**) or 50ng/mL ciprofloxacin. The results showed that the MIC for **41** was 3.13μg/mL (11.67μM) in the presence of **1,** but no activity in the presence of ciprofloxacin. The MICs for **44** were 0.24μg/mL (0.82μM) in the presence of **1**, and 3.13μg/mL (10.67μM) in the presence of ciprofloxacin.

As shown in Table 5-3, alcohol analogue **43** (with benzyloxy substitution on the 2-phenyl ring) showed the highest MDR inhibitory activity with an MIC of 0.78μg/mL (over 4-fold more potent than that of the parent INF55) in the presence of **1** against a mutant strain of *S. aureus* K2361 which overexpresses the NorA pump, but lower activity in the presence of ciprofloxacin with an MIC of 50.00μg/mL (Table 5-4). The lipophilicity of the benzyloxy substituent and the weak acidity of the benzyl alcohol substituent (*p*Ka ~ 12-13) might be of importance in mediating this synergistic activity. A similar pattern of synergy was shown with the other inhibitors **41-42**, **44-45**, **47-48** and **56**, which preferably potentiated the activity of **1** over ciprofloxacin (Tables 5-3 and 5-4). This would suggest that, compared to ciprofloxacin, **1** was a more preferred substrate for the NorA MDR pump, and that the less significant potentiating activity of those inhibitors with ciprofloxacin might relate to molecular hydrophilicity and structural characteristics of ciprofloxacin that may reduce recognition and efflux.

The azide analogues **44** and **47** bearing a methoxy substituent in the 2-aryl ring exhibited a 4- and 2-fold less blocking activity than **43**, respectively, against *S. aureus* K2361. In addition, the azide **44** was able to completely inhibit the Gram-negative bacterium *Salmonalla enterica* Serovar Typhimurium SL1344 R2 at MICs of 0.24 and 3.13μg/mL (see Note, Table 5-3) in the presence of a sub-inhibitory concentration of **1** and ciprofloxacin, respectively. It might be noted that the dipolar azide functionality, $N=N^{+}=N^{-}$, seems to be associated with the ability of the molecule to inhibit the bacterial efflux pumps in both Gram-positive and Gram-negative bacteria. However, one can not disregard the lipophilicity of the methoxy substituent in the azide **47,** since this compound showed higher activity than **44** (which lacked the methoxy group) against *S. aureus* K 2361. This is consistent with the effect of alkoxy substituents in the 2-aryl group on the activity of the alcohol analogues **41-43**. Without the alkoxy substituents

(**41, 44**), there was a 2- to 16-fold increase in MICs against *S. aureus* K 2361. Therefore, the substitution of a benzyloxy group in the 2-phenyl ring of the azide analogue might increase its MDR inhibitory activity in both *S. aureus* and *S. enterica*, and this would be of interest for future work.

Interestingly, two amine analogues, **45** and **48,** had moderate activity in the presence of **1** against *E. faecalis* V583 and *E. faecium* DO, while the other analogues had no activity against these bacteria. In addition, **45** and **48** also moderately potentiated the activity of either **1** (Table 5-3) or ciprofloxacin (Table 5-4) against *S. aureus* K2361.

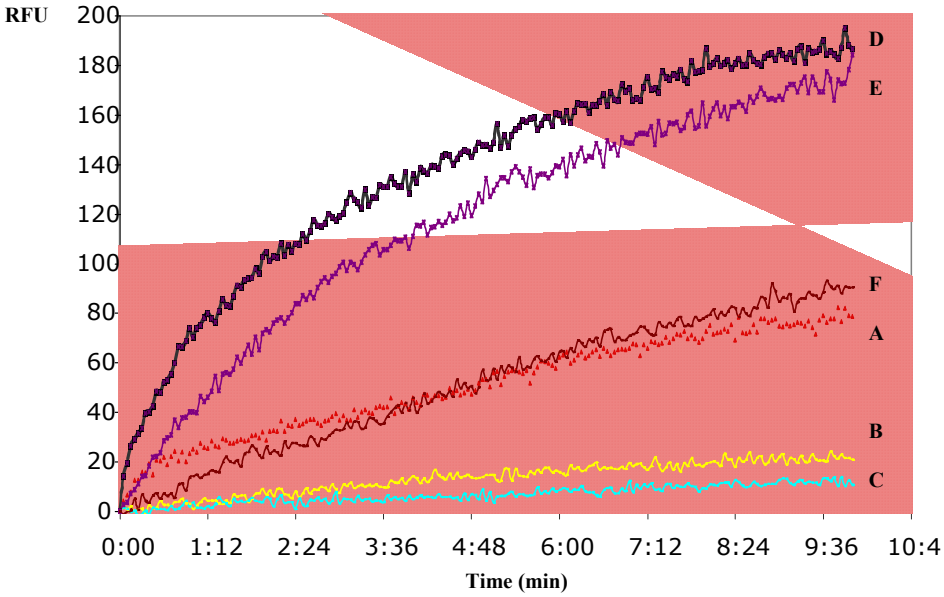
Table 5-4 Minimum Inhibitory Concentration (MIC) value for 2-phenyl-5-nitro-1*H*-indole derivatives (MDR pump inhibitory activity) against *S. aureus* K1758 (lacking NorA MDR pump), *S. aureus* 8325-4 (expressing NorA MDR pump), *S. aureus* K2361 (overexpressing NorA MDR pump) in the presence of sub-inhibitory concentration of ciprofloxacin (Cip.). NT indicates the sample was not tested. "-" indicates the sample showed no activity when tested at a concentration of 50μg/mL.

Compound	MIC, μg/mL (μM)		
	S. aureus		
	K1758	8325-4 +**Cip.**, 40ng/mL	K2361 +**Cip.**, 500ng/mL
38	NT	-	-
39	NT	-	-
41	NT	-	50.00 (186.38)
42	NT	-	-
43	NT	-	50.00 (133.55)
44	NT	-	12.50 (42.62)
45	NT	-	25.00 (93.53)
47	NT	-	-
48	NT	-	12.50 (42.04)
56	NT	-	-

Acid analogues **38** and **39** (with a methoxy substituent in the 2-aryl ring), were either inactive or were only slightly active against all test strains. As shown in Table 5-

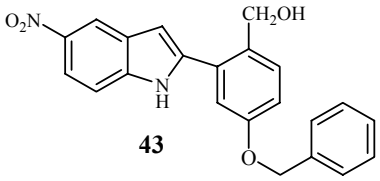
2, **38** had no synergistic activity in potentiating the antibacterial activity of either **1** or ciprofloxacin; it is possible that **38** and **39** are competitive MDR substrates.

Since **43** was the most active NorA MDR inhibitor, a berberine (**1**) uptake assay in the presence of inhibitor **43** (5µg/mL) was performed. As berberine is a planar cationic molecule, it fluoresces when located inside the cell and bound to DNA. The uptake assay evaluated NorA pump inhibition by **43** through suppressing the berberine efflux. The rate of berberine uptake (Figure 5-2) as measured by fluorescence emission showed a sharp increase in berberine accumulation within the cells over a period of 10 minutes when in the presence of **43** compared to the parent NorA MDR inhibitor INF55. **43** is over 20-fold more active than INF55 over this period, and it potentiated the uptake of berberine in three strains of *S. aureus*: the wild-type 8325-4, the NorA knock out K1754, and the overexpressing NorA K2361.

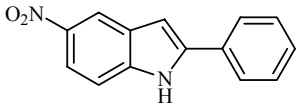


A = INF55 (8325-4), **B** = INF55 (K1754), **C** = INF55 (K2361), **D** = **43** (8325-4), **E** = **43** (K1754), **F** = **43** (K2361)

Figure 5-2 Uptake of berberine as potentiated by MDR inhibitors, 43 or INF55. Fluorescence is given in relative fluorescence units (RFU). The inhibitor concentration was 5µg/mL (for INF55 and 43). The background fluorescence of berberine was blanked to zero. Cells were in HEPES buffer pH7 at OD 0.15. The tests were done with *S. aureus* 8325-4, *S. aureus* K1754, and *S. aureus* K2361.



43



INF55

5.3 Antimicrobial testing results (Direct activity)

The set of 2-phenyl-5-nitro-1*H*-indole derivatives, the berberine carboxylic acid derivative (**8**), and dual-action prodrugs and dual-action drugs (**60**-**65**) (Figure 5-1; page 128) were tested for direct antimicrobial activity against human pathogenic Gram-positive and Gram-negative bacteria, and yeasts. The data are shown in Tables 5-5 and 5-6.

5.3.1 Nitroindoles

In general, the 2-phenyl-5-nitroindole derivatives had weak or no intrinsic antimicrobial activity against *S. aureus* (Table 5-5). Surprisingly, the amine **48** had moderate intrinsic antibacterial activity (12.5μg/mL) against the Gram-negative bacterium *E. coli,* which has an effective permeability barrier (outer membrane and MDR pumps) to hinder drug accumulation in its cells and also to transport unwanted molecules out of the cells. Gram-positive bacteria lack this outer membrane which would restrict the access of amphipathic compounds into their cells. Gram-negative bacteria are thus normally harder to kill than the Gram-positive ones.[138,139] A similar result with the other Gram-negative bacterium *S. enterica* Serovar Typhimurium was seen with the alcohol derivative **43** (MIC, 12.5μg/mL). This suggested that compounds **43** and **48** might have another mode of antibacterial action in the Gram-negative bacteria, apart from the MDR pump inhibition and synergistic action with antibiotics.

All compounds in the indole series were inactive against the Gram-positive bacterium *E. faecalis*, the Gram-negative bacterium *P. aeruginosa*, and the yeast, *Saccharomyces cereavaesiae* and the yeast-like fungus *Candida albicans*, (Table 5-5).

Table 5-5 Minimum Inhibitory Concentration (MIC) value for indole derivatives (direct activity) against bacteria Gram-positive (*Staphylococcus aureus*; 3 strains) and Gram-negative (*Escherichia coli*, *Salmonella enterica*), and yeast (*Saccharomyces cereavaesiae*). NT indicates the sample was not tested. "-" indicates sample showed no activity tested at concentration 50μg/mL.

	MIC, μg/mL (μM)					
Compd	***S. aureus***			***E. coli***	***S. enterica*** Serovar Typhimurium	***S. cerevisiae***
	K1758	8325-4	K 2361	K12	SL1344 R2	BY4742
38	-	-	-	-	-	
39	NT	-	-	-	-	-
41	50.00 (186.38)	50.00 (186.38)	-	-	25.00 (93.19)	50.00 (186.38)
42	-	-	-	-	-	-
43	NT	-	-	-	**12.50 (33.39)**	-
44	3.13 (10.67)	50.00 (170.48)	-	-	-	-
45	50.00 (187.07)	50.00 (187.07)	-	-	25.00 (93.53)	-
47	-	-	-	-	-	-
48	25.00 (84.09)	-	50.00 (168.17)	**12.50 (42.04)**	25.00 (84.09)	-
56	-	-	-	-	-	-
INF55	250.00 (1050.42)	250.00 (1050.42)	250.00 (1050.42)	NT	NT	NT

Note: all compounds in Table 5-5 were additionally tested direct activity against *Enterococcus faecalis* V583, *Pseudomonas aeruginosa* PA1, *Candida albicans* F5, and *Candida albicans* F5 M432, which showed no direct activity at concentration of 50μg/mL.

5.3.2 Berberine derivatives

The ester prodrugs **60**-**61**, the amide prodrugs **62**-**63** and the dual action drugs (or hybrid drugs) **64**-**65** were synthesized with the aim of increasing antibacterial potency of berberine or analogues **64** and **65** were obtained from a combination of berberine (**1**) and the 2-phenyl-5-nitro-1*H*-indole derivatives with a methylene linkage. Similarly, the combination of **1** and the 2-phenyl-5-nitro-1*H*-indole derivatives with an ester linkage afforded **60** and **61,** and those with an amide linking group also gave **62** and **63.** Esterification of the berberine carboxylic acid derivative **8**, which had 5- and 50-fold greater antibacterial activity than **1** (Table 5-6) against wild-type *S. aureus* 8325-4 and *S. enterica* Serovar Typhimurium, respectively, with the indole alcohol derivative **41** (or **42**) led to the corresponding ester prodrugs **60** (or **61**). Similarly, amidation of **8** with the indole amine derivative **45** (or **48**) led to the corresponding amide prodrugs **62** (or **63**). The prodrugs **60**-**63** required bacterial enzymatic hydrolysis (esterase and amidase) to release the MDR pump inhibitor component and berberine derivative **8** at or near target sites in bacterial cells. These compounds were tested against human pathogenic bacteria (both Gram-positive and Gram-negative) and yeasts. The data are shown in Table 5-6.

The ester prodrugs **60** and **61** exhibited moderate antibacterial activity against the NorA overexpressing strain of *S. aureus* K2361, with MICs of 25.00μg/mL for **60** and 12.50μg/mL for **61**, respectively. The two amide prodrugs **62** and **63** were comparable in activity and more active than the esters **60** and **61**; the amides **62** and **63** showed 2- to 4-fold greater antibacterial activity at the same MIC values of 6.25μg/mL against the NorA overexpressing *S. aureus* strain.

Table 5-6 Minimum Inhibitory Concentration (MIC) value for berberine derivatives, dual action prodrugs and dual action drugs (direct activity) against Gram-positive (*Staphylococcus aureus*; 3 strains, and *Escherichia coli*) and Gram-negative (*Salmonella enterica*). NT indicates the sample was not tested. "-" indicates sample showed no activity tested at a concentration of 50μg/mL.

Compound	MIC, μg/mL (μM)				
	S. aureus			*E. faecalis*	*S. enterica* Serovar
	K1758	8325-4	K2361	V583	Typhimurium SL1344 R2
Ciprofloxacin	0.24 (0.72)	1.00 (3.00)	4.00 (12.00)	NT	NT
1, Cl	15.60 (41.96)	250.00 (672.39)	500.00 (1344.77)	500.00 (1344.77)	1250.00 (3361.93)
8	50.00 (95.92)	50.00 (95.92)	NT	-	25.00 (47.96)
60	3.13 (4.32)	16.00 (22.07)	25.00 (34.49)	50.00 (68.98)	1.56 (2.15)
61	3.13 (4.15)	6.25 (8.28)	12.50 (16.57)	12.50 (16.57)	**0.78 (1.08)**
62	3.13 (4.33)	3.13 (4.33)	6.25 (8.64)	-	3.13 (4.33)
63	6.25 (8.29)	12.50 (16.59)	6.25 (8.29)	-	3.13 (4.15)
64	**1.56 (2.34)**	**0.97 (1.46)**	**1.98 (2.97)**	**1.56 (2.34)**	**0.78 (1.17)**
65	3.13 (4.80)	3.13 (4.80)	6.25 (9.58)	12.50 (19.17)	3.13 (4.80)

Note: all compounds in Table 5-6 were additionally tested direct activity against Gram-negative bacteria (*E. coli* K12, *Pseudomonas aeruginosa* PA1) and yeasts (*Saccharomyces cereavaesiae* BY4742, *Candida albicans* F5, and *Candida albicans* F5 M432), which showed no direct activity at a concentration of 50μg/mL.

The most active compound against *S. aureus* was the dual action drug **64** with an MIC against the mutant strain overexpressing the NorA pump at clinically useful concentrations (1.98μg/mL, over 250-fold more potent than the parent antibiotic **1**). With this compound, it appeared that it had both strong NorA pump blocking activity as well as separate direct antibacterial potency. A similar result was observed with the dual action drug **65,** although it was 3-fold less active than **64** against the *S. aureus* strain K2361. It seemed that the presence of a methoxy substituent in the aryl ring of the inhibitor moiety of **65,** resulted in a decrease in the activity against *S. aureus*. The MICs for **60-65** against *S. aureus* K2361 (the strain overexpressing the NorA pump)

compared with K1754 (the strain lacking the NorA pump) indicated that compounds **62-65** completely inhibited the efflux activity of NorA pump in this bacterium as the MICs for these compounds in both strains were the same or similar (note that there was only a 2-fold difference in the MICs with both strains, which might not be significant). The MICs for compounds **60-61** in these two strains were quite different however, indicating that the NorA pump was partially inhibited by these ester prodrugs.

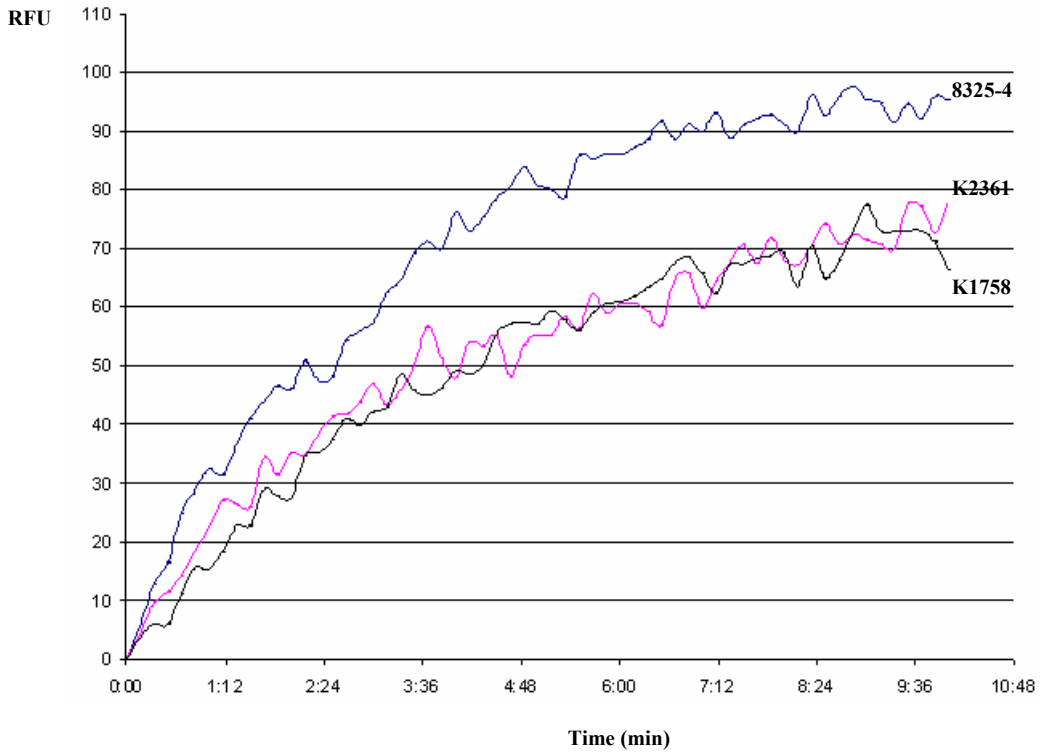
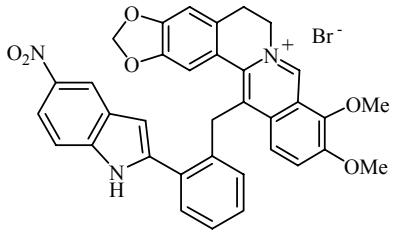
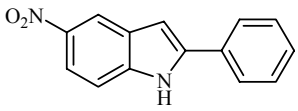


Figure 5-3 Graphs showing the accumulation of compound 64 (5µg/mL) inside *Staphylococcus aureus* cells over a period of 10 minutes. Fluorescence is given in relative fluorescence units (RFU). INF55 at 5µg/mL plus berberine 5µg/mL is not shown but would be represented by a horizontal line at –30 RFU's. The background fluorescence of berberine was blanked to zero. Cells were in HEPES buffer pH7 at OD 0.15. The tests were done with *S. aureus* 8325-4, *S. aureus* K1754, and *S. aureus* K2361.

Verification of the dual action of the most active compound **64** was subsequently achieved by a direct uptake assay in 3 strains of *S. aureus*.[47] The uptake assay measured the fluorescence generated by **64**, a planar cationic species, when it interacted with DNA in the bacterial cells. Due to the disabling of the MDR pumps, it led to an accumulation of **64** in the cells. The fluorescence from the complex of **64**-DNA (Figure 5-3) was found to be increased compared with that from berberine plus INF55 (known NorA inhibitor, Figure 5-1 and Table 5-3). This uptake study confirmed that **64** had a double action of MDR pump inhibition and antibacterial activity.



64



INF55

Interestingly, with the Gram-positive bacterium *E. faecalis*, only the hybrid drug **64** was able to completely inhibit the bacterial growth at a clinically useful concentration of 1.56μg/mL while the others were moderately active (**61** and **65** with MICs of 12.5μg/mL) or inactive (**60**, **62**, and **63**).

Unexpectedly, the ester prodrug **61** and the hybrid drug **64** were extremely active against the Gram-negative bacterium *S. enterica* Serovar Typhimurium at the same MIC of 0.78μg/mL (over 1600-fold more potent than berberine **1**). The amine prodrugs **62** and **63**, and hybrid drug **65** were strongly active at the same concentration of 3.13μg/mL against this bacterium while the ester prodrug **60** was more active at the concentration of 1.56μg/mL. This data indicated that the ester prodrugs **60-61** had more specific efficacy for the Gram-negative bacterium *S. enterica* Serovar Typhimurium than for *S. aureus*. The ester prodrug **60** and the hybrid **64** were also tested against the

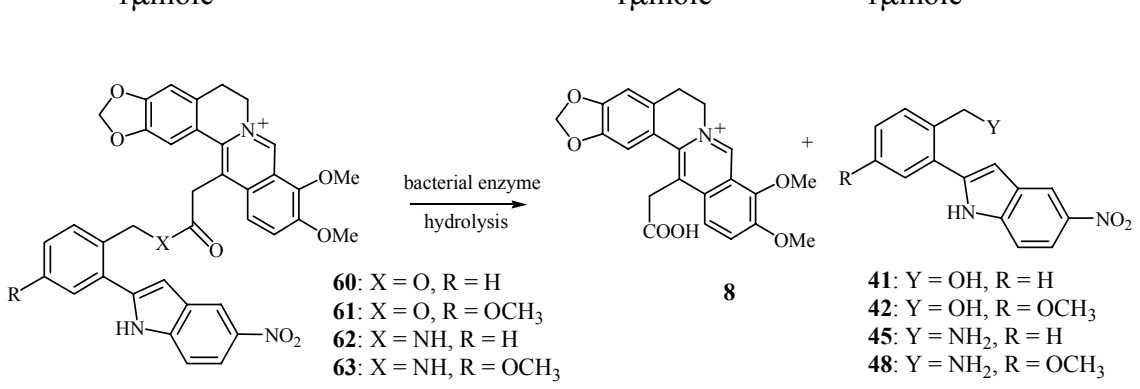
other wild-type strain of *S. aureus*, ATCC 6538P, and 4 strains of *Enterococcus faecium*. The hybrid drug **64** was strongly active against *S. aureus*, whereas the ester prodrug **60** was only moderately active. As expected, both **60** and **64** were inactive against all strains of *E. faecium*, vancomycin resistant *Enterococcus* (VRE) strains due to unrelated resistance mechanisms to the MDR pump. The resistance mechanism of these strains involves modification of the peptidoglycan drug target as discussed in Chapter1, section 1.3.2. Moreover, **64** was tested for antimalarial activity *in vitro* against an anti-folate sensitive strain (TM4) and an anti-folate resistant strain (K1) of *Plasmodium falciparum*. The result showed good activity with an IC_{50} of 1.66μg/mL against the anti-folate resistant strain, which possesses an efflux-related resistance phenotype.[140]

Table 5-7 Additional Minimum Inhibitory Concentration (MIC) values for 60 and 64 (direct activity) against *Staphylococcus aureus* ATCC 6538P, 4 strains of *Enterococcus faecium* (VRE243 and VRE987 are sensitive to vancomycin, and VRE449 and VRE820 are resistant to vancomycin), and 2 strains of *Plasmodium falciparum* (TM4 is an anti-folate sensitive strain and K1 is an anti-folate resistant strain).

Compd	MIC, μg/mL (μM)					IC_{50}, μg/mL (μM)	
	S. aureus	*E. faecium*				*P. falciparum*	
	ATCC 6538P	VRE243	VRE449	VRE820	VRE987	TM4	K1
60	31.25 (43.11)	>125.00 (>172.46)	>125.00 (>172.46)	>125.00 (>172.46)	>125.00 (>172.46)	NT	NT
64	**0.98 (1.47)**	>125.00 (187.54)	125.00 (187.54)	125.00 (187.54)	>125.00 (>187.54)	**7.93 (11.90)**	**1.66 (2.49)**

In summary, all compounds **60-65** showed moderate to strong direct antimicrobial activity and were much more potent than the parent berberine (**1**). The MICs of **64** were near or below 1μg/mL against both *S. aureus* and *S. enterica* Serovar Typhimurium, which are clinically useful concentrations. The hybrid drug **64** was the most potent

antimicrobial compound developed in this research, substantiating the principle of the hybrid molecule approach. With the dual action prodrugs **60-63**, there is evidence that antimicrobial berberine acid derivative (**8**) fragment and the MDR pump inhibitor fragment were possibly released by bacterial hydrolase enzymes. Also this partial hydrolytic cleavage had been noted using commercially available pig liver esterase at pH 7.2 for 24h (Chapter 4, Section 4.3). If it is assumed that the prodrugs **60-63** were hydrolysed completely by bacterial enzymes then they could release the two fragments expected in a 1:1 equimolar ratio as shown in Scheme 5-1.



Scheme 5-1 Potential release of 2 active components from the dual action prodrugs 60-63 by bacterial esterase or amidase as follows: 8 and 41 released from 60, 8 and 42 released from 61, 8 and 45 released from 62, and 8 and 48 released from 63.

In order to prove the assumption of full hydrolysis, separate mixtures of the hydrolysis products, the berberine acid **8** and each of the indoles (**41, 42, 45** or **47**) in a 1:1 ratio, were tested against both wild-type 8325-4 and mutant K1758 strains of *S. aureus*. The results are shown in Table 5-8. It should be noted that a 1:1 weight ratio was used in the testing instead of the more accurate 1:1 molar ratio.

The MIC value for a separate mixture of **8** and **41** in a 1:1 weight ratio in the antibacterial testing was compared with calculated MICs for the fragments **41** and **8**

which would be generated from the prodrug **60** (MIC, 16.00μg/mL = 22.07μM) assuming complete bacterial enzymatic hydrolysis (Table 5-8). The calculated MICs showed that the concentration of **41** and **8** required for wild-type (8325-4) strain growth inhibition was only 5.92μg/mL (22.07μM) and 11.51μg/mL (22.07μM), respectively. The 1:1 mixture of **41** and **8** gave an experimental MIC of 6.25μg/mL.

Table 5-8 Comparison of direct activity of co-administration of the MDR pump inhibitor and the berberine acid derivative 8 in a 1:1 weight ratio, to the dual action prodrugs (60-63).

	MIC, μg/mL (μM)			MIC, μg/mL (μM)	
Compd	***S. aureus***		**Compd**	***S. aureus***	
	8325-4	**K1758**		**8325-4**	**K1758**
60	16.00 (22.07)	3.13 (4.32)	**61**	6.25 (8.28)	3.13 (4.15)
8 + 41 (calcd)	**8** = 11.51 (22.07) **41** = 5.92 (22.07)	**8** = 2.25 (4.32) **41** = 1.16 (4.32)	**8 + 42 (calcd)**	**8** = 4.32 (8.28) **42** = 2.47 (8.28)	**8** = 2.16 (4.15) **42** = 1.24 (4.15)
8 + 41 (exp.)	**8** = 6.25 (11.99) **41** = 6.25 (23.30)	**8** = 3.13 (6.00) **41** = 3.13 (11.67)	**8 + 42 (exp.)**	**8** = 6.25 (11.99) **42** = 6.25 (20.95)	**8** = 6.25 (11.99) **42** = 6.25 (20.95)
62	3.13 (4.33)	3.13 (4.33)	**63**	12.50 (16.59)	6.25 (8.29)
8 + 45 (calcd)	**8** = 2.26 (4.33) **45** = 1.16 (4.33)	**8** = 2.26 (4.33) **45** = 1.16 (4.33)	**8 + 48 (calcd)**	**8** = 8.65 (16.59) **48** = 4.93 (16.59)	**8** = 4.32 (8.29) **48** = 2.46 (8.29)
8 + 45 (exp.)	**8** = 12.50 (23.98) **45** = 12.50 (46.77)	**8** = 12.50 (23.98) **45** = 12.50 (46.77)	**8 + 48 (exp.)**	**8** = 12.50 (23.98) **48** = 12.50 (42.04)	**8** = 3.13 (6.00) **48** = 3.13 (10.53)

These data indicated that the concentration of a separate mixture of **41** and **8** required for bacterial growth inhibition corresponded approximately to the calculated maximum concentrations of fragments **41** and **8** which could be released from prodrug **60**. Similar results were obtained with the other ester prodrug **61** and the amide prodrug **63**. Therefore, the ester and amide prodrugs **60-61** and **63** seemed to be cleaved by bacterial hydrolytic enzymes as anticipated. The calculated intracellular concentrations of the two fragments **8** and **45** released from the amide prodrug **62** were lower than the actual concentrations of a 1:1 separate mixture of **8** and **45** from antibacterial testing.

The amide prodrug **62** seemed to be stable in bacterial cells, and might have strong intrinsic antibacterial activity.

In order to fully establish bacterial esterase or amide hydrolysis in the prodrugs, it would be necessary to examine the effect of bacterial lysates on these compounds. Time constraints did not allow this to be undertaken.

5.4 Cytotoxicity results

Only the inhibitor **41**, the corresponding ester prodrug **60**, and the hybrid drug **64** were tested for cytotoxicity against human histiocytic lymphoma cells (cell line U937) by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2*H* tetrazolium, inner salt (MTS) assay, and the results are shown in Table 5-9.

Table 5-9 Preliminary minimum inhibitory concentration (MIC) of 40, 60, and 64 against human hystiocytic lymphoma cells (cell line U937).

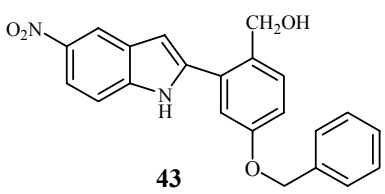
	Compound		
MIC	**41**	**60**	**64**
μg/mL	>100.00	>100.00	>100.00
(μM)	(327.76)	(137.97)	(>150.04)

The MIC value of each compound tested was greater than 100μg/mL, indicating they were only weakly or non-cytotoxic. There was over 50-fold difference in the MIC of **60** and **64** for antimicrobial activity versus cytotoxicity.

Chapter6: Conclusions and Future Directions

6.1 Conclusions

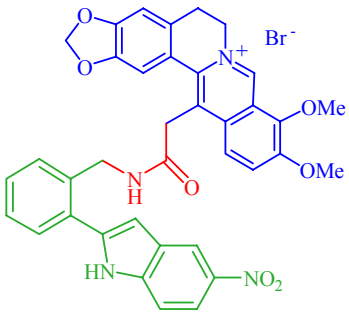
The synthesis of a range of new 2-aryl-5-nitroindole derivatives as potent multidrug resistance pump (MDR) inhibitors was achieved using a palladium-mediated cyclisation to establish the crucial indole C2-aryl C bond formation. Biological testing of these compounds showed that all compounds (**39, 41-45, 47-48,** and **56**) apart from the acid derivative **38** potentiated the action of the antibacterial agent berberine (**1**) by blocking the NorA MDR pump in *S. aureus*. The alcohol **43** was the most effective inhibitor in the 2-aryl-5-nitroindole series against all strains of *Staphylococcus aureus*, together with showing a moderate intrinsic activity against *Salmonella enterica* Serovar Typhimurium SL1344R2. The novel alcohol **43** was thus a new dual action antibacterial agent.



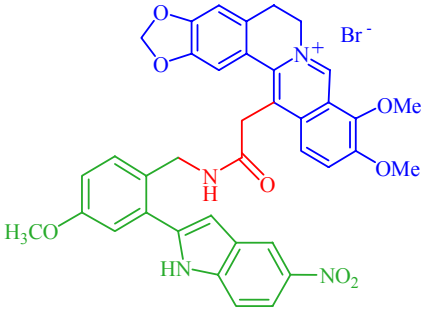
43

Other novel dual action antibacterial agents were designed and synthesized. The lead compounds (**60, 62,** and **64**) were based on a combination of berberine (**1**) and 2-phenyl-5-nitroindole derivatives with enzymatically cleavable (ester and amide) and non-cleavable (methylene) linking groups. The coupling of the two active components was achieved by a one-step synthesis. This involved the reaction of 8-allyldihydroberberine (**9**) with the appropriate alkylating agent from the 2-phenyl-5-nitro-1*H*-indole derivative *via* an enamine alkylation/propene elimination strategy to afford the potential dual action prodrugs (**60, 62**) and the dual action drug (**64**) in low to moderate yields. Similarly, methoxyaryl derivatives (**61, 63,** and **65**) of these leads were synthesized and investigated for antibacterial activity. Biological testing of the dual action prodrugs and dual action drugs (**60-65**) showed that all compounds were active as antibacterial agents. The dual action drug **64** was the most active antibacterial

agent against both Gram-positive and Gram-negative bacteria. Compound **64** exhibited a 250-fold greater antibacterial activity than the parent antibacterial berberine (**1**) against a resistant strain of *S. aureus*, K2361, which overexpresses the NorA MDR pump. Compound **64** also showed a 320-fold greater activity than **1** against *Enterococcus faecalis* V583, and a 1600-fold greater activity than **1** against *Salmonella enterica* Serovar Typhimurium SL1344R2. The methoxy substituted derivative of **64**, the compound **65**, was slightly less active than **64** against all strains tested. Compound **64** also showed good antimalarial activity (*Plasmodium falciparum*) *in vitro*. An additional uptake assay against *S. aureus* for compound it was confirmed that **64** had dual activity as a NorA inhibitor and as an antibacterial.

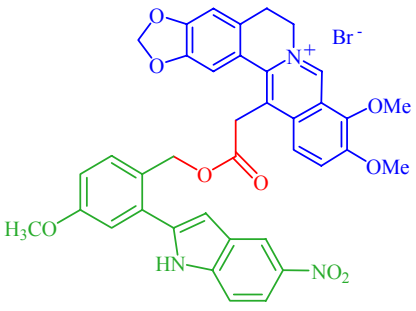


62

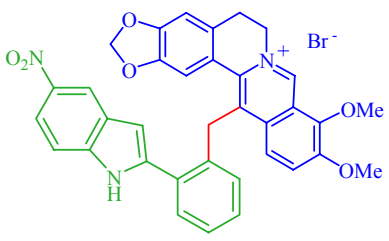


63

In the series of ester and amide dual action prodrugs **60**-**63**, the amides **62** and **63** had higher activities than the esters **60** and **61** against *S. aureus* strain K2361. However, the esters **60** and **61** were more potent than the amides **62** and **63** against *S. enterica* Serovar Typhimurium SL1344 R2.



61



64

The ester **61** was the most active compound against the *S. enterica* strain SL1344 R2 in the prodrug series, and was comparable in activity to the dual action drug **64** (MIC; 0.78 μg/mL). It is probable that the prodrugs **60-63** may have intrinsic antibacterial activity without enzymatic hydrolysis being involved. As an amide bond has greater stability than an ester bond, the rate of bacterial enzymatic hydrolysis in **62** or **63** might be slower than hydrolysis of the ester **60** or **61**, resulting in slightly higher activities than the ester prodrugs against *S. aureus* K2361. Thus, the dual action of the amide prodrugs may not require bacterial enzymatic action to generate the fragment **8** and the corresponding MDR pump inhibitor (**45** or **48**). On the other hand, the microbiological evidence pointed to hydrolysis of the esters **60** and **61** by bacterial esterase, with the fragments being the antibacterial berberine acid **8** and a corresponding MDR pump inhibitor (**41** or **42**) produced synchronously in high concentration. Partial hydrolysis of **60** was observed in the presence of pig liver esterase.

In summary, a potent dual action drug, **64,** was found. The dual action mechanism is thus a promising way to combat the problem of antibiotic resistance by drug efflux. Also, compound **43** is the most potent NorA MDR pump inhibitor against *S. aureus* in the 2-aryl-5-nitro-1*H*-indole series. Therefore, the variety of novel MDR pump inhibitors, dual action prodrugs and dual action drugs discovered in this project should provide a useful basis for future antimicrobial drug developments.

6.2 Future directions

To verify ester or amide hydrolysis within bacterial cells, bacterial enzymatic hydrolysis experiments on the ester and amide prodrugs (**60-63**) is required in future work.

Also, prior to *in vivo* antibacterial studies, plasma stability studies on the prodrugs need to be undertaken and modifications to the ester or amide groups made, if necessary, in order to avoid hydrolysis before delivery to the bacteria.

A combination of the most potent inhibitor **43** with berberine **1** should also be investigated. Such a compound may be a novel triple action drug against the Gram-negative pathogen *Salmonella enterica*.

Chapter7: Experimental

7.1 General

All melting points were determined using a Reichert hot-stage melting point apparatus and are uncorrected.

The ^{1}H and ^{13}C NMR were determined at 299.92 and 75.42 MHz with a Varian Unity-300 spectrometer, and at 499.91 and 125.71 MHz with Varian Inova-500 spectrometer. Unless otherwise stated, the spectra were obtained from solutions in $CDCl_3$ and referenced to TMS (proton) and the chloroform mid-line (77) (carbon). Chemical shifts of the outer peaks are given for specified multiplet patterns in the ^{1}H-NMR spectra. The assignments were made by standard gradient correlation spectroscopy (gCOSY), gradient heteronuclear single quantum correlation (gHSQC) and gradient heteronuclear multiple bond correlation (gHMBC) spectroscopy. The same superscript assignments may be reversed for the signals designated in the same compound. *J* values for mutually coupled systems were calculated from chemical shift differences and were the same or very close to being the same; coupling was confirmed from gCOSY spectra.

MS (CI) and (EI) were obtained using a Shimadzu QP-5000 spectrometer with isobutane as the ionising gas in the CI mode, and with a source temperature of 250^{o}C. High resolution (CI) MS (for MH^{+}) and (EI) MS (for M^{+}) were run using a VG Autospec spectrometer operating at 70 eV and a source temperature of 250^{o}C with PFK reference and methane as ionising gas in CI mode, and high resolution (ES) MS (for MH^{+}) with a Micromass Qtof 2 mass spectrometer using a cone voltage of 30V and polyethylene glycol (PEG) as an internal reference.

Elemental microanalyses were determined by Mr. G. Blazak at the University of Queensland.

Berberine chloride was obtained from the Sigma Chemical Company and dried over phosphorus pentoxide at 80^{o}C under reduced pressure for 8 h before use.[59] NBS was recrystallized from water and dried over phosphorus pentoxide before use. Solvents were purified and dried by standard techniques.[141]

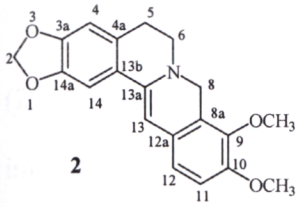
Chromatography using Merck Kieselgel 60 silica gel (230-400 mesh) was performed under medium pressure or by vacuum liquid chromatography (VLC). Preparative TLC was done on Merck Silica gel 60 F_{254} and Aluminium oxide F_{254} with a thickness of 0.2 mm on aluminium sheet. All chromatographic solvent proportions are volume for volume. Reactions were monitored by thin-layer chromatography (TLC) on Merck silica gel 60 F_{254} and Aluminium oxide F_{254} on aluminium sheets, and the compounds were detected by examination under ultraviolet light and by exposure to iodine vapour.

Organic solvents were dried with anhydrous sodium sulfate and removed under reduced pressure (*in vacuo*) by a Büchi rotary evaporator.

Microwave reactions were performed in a Milestone Ethos Sel Microwave Solvent Extractor, employing Easywave software, and using internal reaction temperature control. Sealed Teflon reaction vessels were used and washed with concentrated nitric acid between uses. Magnetic stirring was used to stir reaction mixtures.

7.2 Dihydroberberine route to 13-substituted berberines (Chapter 2)

7.2.1 Preparation of 9,10-Dimethoxy-5,8-dihydro-6*H*-benzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizine (2)[57,59]

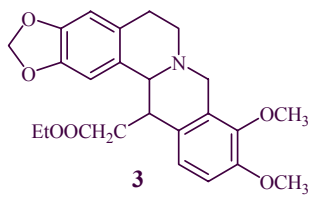


2

Method 1: To a solution of dry berberine hydrochloride (**1**) (3.0g, 8.1mmol) in pyridine (18mL) was added sodium borohydride (360mg, 9.5mmol) and the mixture stirred at room temperature for 20min. More sodium borohydride (300mg, 7.9mmol) was added and stirring was continued for 30min. The reaction mixture was then poured into ice water (200mL). The precipitated solid was filtered and dried to give the enamine **2** (2.6g, 96%) as a yellow solid, m.p. 123-125°C (Lit.[142] 157-158°C; recrystallised from DCM-MeOH). ^{1}H-NMR (300MHz, $CDCl_3$): δ 2.88 (t, *J* = 5.9 Hz, 2H, H-5), 3.13 (t, *J* = 5.9 Hz, 2H, H-6), 3.85 (s, 6H, OCH_3), 4.32 (br.s, 2H, H-8), 5.94 (s, 2H, OCH_2O), 5.95 (s, 1H, H-13), 6.58 (br.s, 1H, H-11), 6.74 (s, 2H, H-12, H-4), 7.17 (s, 1H, H-14). CIMS: *m/z*, $[MH]^+$: 338 (50 %).

Method 2: To a solution of berberine hydrochloride.2.5 H_2O (**1**) (0.7g, 1.7mmol) in methanol (25mL) was added K_2CO_3 (0.8g, 60mmol), and then sodium borohydride (24.0mg, 0.64mmol) was slowly added to the suspension which was then stirred at 0°C for 8 h. The mixture was concentrated and then added to ice water (200mL). The precipitated solid was filtered and washed thoroughly with water until the washing were neutral. The solid was then washed with MeOH (50mL) to remove starting material **1**, and dried to give the enamine **2** (0.3g, 53%) as a yellow solid.

7.2.2 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6*H*-benzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizin-13-yl)-acetic acid ethyl ester (3)

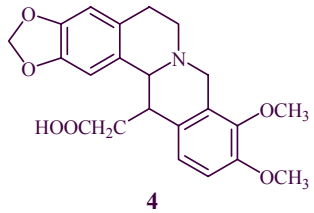


3

Dry ethyl bromoacetate (30mL, 270.5mmol) was added dropwise with stirring to dihydroberberine **2** (2.62g, 0.78mmol) at 0°C under a nitrogen atmosphere. The solution was heated to 100°C for 1h to give a suspension. Dry toluene (25mL) was added to the suspension, the precipitate filtered and then dried to give the iminium salt intermediate **2a** (3.78g, 96%). The unstable intermediate **2a** (3.78g, 8.9mmol) was dissolved in absolute ethanol (50mL) and stirred at 0°C. Sodium borohydride (400mg, 10.5mmol) was added to the suspension which was then stirred at room temperature for 20min. More sodium borohydride (400mg, 10.5mmol) was added and further stirred for 1h. The mixture was then concentrated by solvent evaporation *in vacuo*. Water (200mL) was added to the crude product and the mixture then extracted with diethyl diethyl ether (3 x 150mL). The combined diethyl ether extract was washed with water, dried and evaporated. The crude product was chromotographed on silica gel (DCM) to afford the *ester* **3** (2.8g, 88%) as a yellow solid, m.p. 103-104°C. ^{1}H-NMR (300MHz, $CDCl_3$): δ 1.15 (t, *J* = 7.2 Hz, 3H, CH_3), 2.30 (dd, *J* = 15.6, 8.4 Hz, 1H, CH_2CO), 2.44 (dd, *J* = 15.6, 8.4 Hz, 1H, CH_2CO), 2.50-2.61 (m, 2H, H-5, H-6), 2.99-3.13 (m, 2H, H-5, H-6), 3.52 (d, *J* = 16 Hz, 1H, H-8), 3.61-3.68 (m, 1H, H-13), 3.72 (br.s, 1H, C-13a), 3.85 (s, 6H, OCH_3), 3.98 (q, *J* =7 Hz, 2H, C**H**$_2$CH$_3$), 4.10 (d, *J* = 16 Hz, 1H, H-8), 5.91 (d, *J* = 1.3 Hz, 1H, OCH_2O), 5.92 (d, *J* = 1.3 Hz, 1H, OCH_2O), 6.58 (s, 1H, H-4), 6.74 (s, 1H, H-14), 5.76 (d, *J* = 8.4 Hz, 1H, H-11), 7.00 (d, *J* = 8.4 Hz, 1H, H-12). ^{13}C NMR (75MHz, $CDCl_3$): δ 14.11 (CH_3), 29.8 (C5), 38.1 (**C**H_2CO), 40.5 (C13), 51.0 (C6), 54.2 (C8), 55.8 (OCH_3), 60.0 (OCH_3), 60.1 (**C**H_2CH_3), 63.0 (C13a),

100.8 (OCH_2O), 105.9 (C4)[a], 108.3 (C14)[a], 110.6 (C12)[b], 124.1 (C11)[c], 128.4 (C13b)[d], 128.5 (C8a)[e], 129.8 (C4a)[d], 132.4 (C12a)[e], 144.9 (C14a)[f], 146.0 (C9), 146.4 (C3a)[f], 150.6 (C10), 173.6 (CO). HMRS (EI); *m/z* calcd for $C_{24}H_{27}NO_6$ $[M]^+$: 425.1838; found: 425.1837.

7.2.3 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6*H*-benzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizin-13-yl)-acetic acid(4)



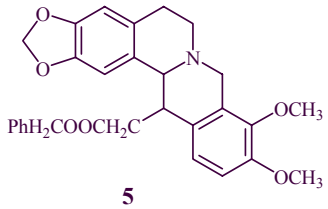
4

Method 1: A solution of **3** (18.0mg, 0.04mmol) in 2M HCl (1.5mL) was stirred for 30h at room temperature with shielding from light, and was then evaporated and added to ice water (20mL). The mixture was neutralized by saturated $NaHCO_3$ solution and then extracted with EtOAc (3 x 20mL). The combined EtOAc extracts were washed with water, dried and evaporated to give only the starting material **3** (18.0mg). The reaction conditions were, therefore, changed to use 1M H_2SO_4 in THF under the same procedure but no hydrolysis was observed.

Method 2: To a solution of **3** (521.0mg, 1.19mmol) in MeOH (30mL) was added a 2% aqueous solution of lithium hydroxide (40mL) and the mixture heated at reflux for 1 h. The cooled reaction mixture was then evaporated. The crude product was added to ice water (200mL) and acidified to pH 1 with 1M HCl. The precipitate was filtered, washed with water and dried to afford the *acid* **4** (460.1mg, 95%) as an opaque white solid, m.p. 202-205°C. ^{1}H-NMR (300MHz, $CDCl_3$): δ 2.59-2.85 (m, 4H, H-5, H-6, CH_2CO), 3.18-3.34 (m, 2H, H-5, H-6), 3.42-3.50 (m, 1H, H-13), 3.73 (d, *J* = 15.6 Hz, 1H, H-8), 3.87 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.06 (d, *J* = 3.3 Hz, 1H, H-13a), 4.32 (d, *J* = 15.6 Hz, 1H, H-8), 5.98 (s, 2H, OCH_2O), 6.60 (s, 1H, H-4)[a], 6.64 (s, 1H, H-14)[a], 6.86 (d, *J* = 8.7 Hz, 1H, H-12)[b], 7.00 (d, *J* = 8.7 Hz, 1H, H-11)[b]. ^{13}C NMR (75MHz,

DMSO-d_6): δ 28.2 (C5), 37.8 (**C**H_2CO), 43.3 (C13), 50.1 (C6), 53.0 (C8), 55.7 (OCH_3), 59.6 (OCH_3), 62.2 (C13a), 100.8 (OCH_2O), 105.6 (C4)[c], 108.2 (C14)[c], 111.6 (C12), 123.9 (C11), 126.3 (C8a)[d], 127.1 (C4a)[e], 128.7 (C13b)[e], 131.0 (C8a)[d], 144.3 (C9)[f], 145.9 (C3a)[g], 146.3 (C14a)[g], 150.3 (C10)[f], 173.6 (CO). HRMS (EI); *m/z* calcd for $C_{22}H_{23}NO_6$ $[M]^+$: 397.1525; found: 397.1516.

7.2.4 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6*H*-benzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizin-13-yl)-acetic acid benzyl ester (5)



5

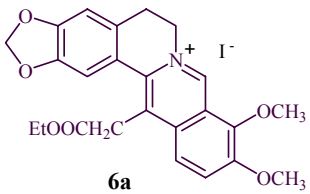
Method 1: To a mixture of the acid **4** (23.0mg, 0.06mmol), HOBT (2mg, 0.01mmol) and DCC (15.0mg, 0.07mmol) was added anhydrous DMF (1mL) at 0°C under a nitrogen atmosphere. The mixture was then stirred at room temperature for 5 min. A solution of 20% dry benzyl alcohol in anhydrous DMF (0.15mL, 0.29mmol) was then added and the mixture stirred at room temperature for 2 days. The reaction mixture was monitored by TLC (silica gel, 2%DCM in MeOH), but no reaction of the starting material **4** was observed.

Method 2: To a mixture of the acid **4** (23.0mg, 0.06mmol), DMAP (7.0mg, 0.06mmol) and EDCI (9.2mg, 0.06mmol) was added anhydrous DMF (1mL) at 0°C under a nitrogen atmosphere. The mixture was then stirred at room temperature for 5 min. A solution of 5% dry benzyl alcohol in anhydrous DMF (0.15mL, 0.08mmol) was then added and the mixture stirred at 40°C for 2 days. The mixture was monitored by TLC (silica gel, 2%DCM in MeOH), but no reaction of the starting material **4** was observed.

Method 3: To a mixture of the acid **4** (115.0mg, 0.29mmol), DMAP (5mg, 0.04mmol) and DCC (76.0mg, 0.37mmol) was added anhydrous DMF (1mL) at 0°C under a nitrogen atmosphere. The mixture was then stirred at room temperature for 5 min. A solution of 20% dry benzyl alcohol in anhydrous DMF (0.15mL, 0.29mmol) was then added and the mixture stirred at 80°C for 30h. The reaction mixture was evaporated *in vacuo*. The residue was chromatographed on silica gel (0.5% MeOH in DCM) to afford the *ester* **5** (45.0mg, 32%) as a yellow solid, m.p.104-105°C. ^{1}H-NMR (300MHz, $CDCl_3$): δ 2.37 (dd, *J* = 15.5, 4.1 Hz, 1H, CH_2CO), 2.52 (dd, *J* = 15.5, 8.9 Hz, 1H, CH_2CO), 2.48-2.60 (m, 2H, H-5, H-6), 2.94-3.32 (m, 2H, H-5, H-6), 3.50 (d, *J* = 15.9 Hz, 1H, H-8), 3.63-3.69 (m, 1H, H-13), 3.71 (br.s, 1H, H-13a), 3.83 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 4.18 (d, *J* = 16.2 Hz, 1H, H-8), 4.97 (d, *J* = 2.1, 2H, OCH_2), 5.88 (d, *J* = 1.2 Hz, 1H, OCH_2O), 5.89 (d, *J* = 1.5 Hz, 1H, OCH_2O), 6.55 (s, 1H, H-4)[a], 6.68 (d, J = 8.7 Hz, 1H, H-11)[b], 6.74 (s, 1H, H-14)[a], 6.90 (d, *J* = 8.4 Hz, 1H, H-12)[b], 7.19-7.35 (m, 5H, ArH). ^{13}C NMR (75MHz, $CDCl_3$): δ 29.8 (C5), 38.0 (**C**H_2CO), 40.7 (C13), 51.0 (C6), 54.2 (CH_2O), 55.7 (OCH_3), 60.0 (OCH_3), 63.0 (C13a), 65.9 (CH_2O), 100.7 (OCH_2O), 105.8 (C4)[c], 108.2 (C14)[c], 110.5 (C11)[d], 124.0 (C12)[d], 127.4 (C8a)[e], 127.8 (ArCH), 127.9 (2C, ArCH), 128.2 (C4a)[f], 128.2 (2C, ArCH), 128.3 (C13b)[f], 132.0 (C12a)[e], 135.9 (ArC), 144.8 (C9)[g], 145.8 (C14a)[h], 146.3 (C3a)[h], 150.5 (C10)[g], 173.3 (CO). HRMS (EI): *m/z* calcd for $C_{29}H_{29}NO_6$ $[M]^+$: 487.1995; found: 487.1988.

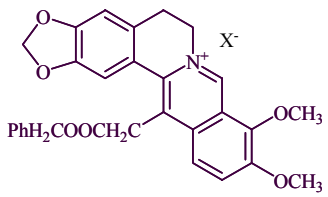
7.2.5 Preparation of 13-(Ethoxycarbonylmethyl)-9,10-dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium iodide (6a)[58]

To a solution of the ester **3** (100.0mg, 0.24mmol) in absolute EtOH (10mL), was added iodine (179.2mg, 0.71mmol) and the mixture stirred at 60°C for 12 h. More iodine (100.0mg, 0.39mmol) was then added and the mixture stirred for a further 12 h. The excess iodine was decomposed by the addition of sodium thiosulfate until the brown solution was changed to a yellow solution and a white precipitate was formed. The insoluble substance was filtered and the filtrate was then evaporated. The residue was crystallized from warm EtOH to afford the iodide salt **6a** (125.5mg, 97%) as a yellow solid, m.p. 155-157°C (Lit.[58] 165°C). ^{1}H-NMR (300MHz, $CDCl_3$): δ 1.35 (t, *J* = 7.2 Hz, 3H, CH_3), 3.26 (br.s, 2H, H-5), 4.04 (s, 3H, OCH_3), 4.25 (s, 2H, CH_2CO), 4.33 (q, *J* = 7.2 Hz, 2H, C**H**$_2$CH$_3$), 4.38 (s, 3H, OCH_3), 5.09 (br.s, 2H, H-6), 6.08 (s, 2H, OCH_2O), 6.87 (s, 1H, H-4), 7.21 (s, 1H, H-14), 7.70 (d, *J* = 9.0 Hz, 1H, H-11), 7.84 (d, *J* = 9.3 Hz, 1H, H-12), 10.34 (s, 1H, H-8).



6a

7.2.6 Preparation of 13-Benzyloxycarbonylmethyl-9,10-dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium iodide (7a) and bromide (7b)



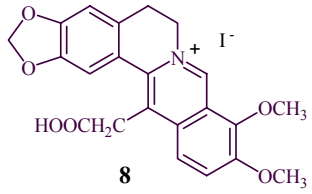
7a : X = I; 7b: X = Br

Method 1, Iodide salt: To a solution of ester **5** (79.0mg, 0.16mmol) in ethanol (150mL) was added iodine (80.0mg, 0.32mmol). The solution was heated at reflux for 1h. The excess iodine was decomposed by addition of

sodium thiosulfate until the brown solution was changed to a yellow solution and a white precipitate was formed. The insoluble substance was filtered and the solvent was then evaporated. The crude product was chromatographed on alumina by PLC (2% MeOH in DCM) to afford the *quinolizinium iodide* **7a** (15.7mg, 16%) as a yellow solid.

Method 2, Bromide salt: To a mixture of the ester **5** (30mg, 0.06mmol) and NBS (21.8mg, 0.12mmol) was added dry $CHCl_3$ (2mL) and the solution stirred at room temperature for 1h. The mixture was then washed with water (50mL). The chloroform layer was dried and concentrated. The residue was triturated with diethyl ether (10mL) and then filtered to afford the *quinolizinium bromide* **7b** (38.2mg, 85%) as a yellow solid, m.p. 124-126^{o}C. ^{1}H-NMR (300MHz, $CDCl_3$): δ 3.25 (t, J = 5.7 Hz, 2H, H-5), 4.05 (s, 3H, OCH_3), 4.31 (s, 2H, CH_2CO), 4.39 (s, 3H, OCH_3), 5.14-5.30 (br.m, 2H, H-6), 5.31 (s, 2H, OCH_2), 6.10 (s, 2H, OCH_2O), 6.89 (s, 1H, H-4)[a], 7.22 (s, 1H, H-14)[a], 7.39 (s, 5H, ArH), 7.57 (d, J = 9.3 Hz, 1H, H-11), 7.68 (d, J = 9.3 Hz, 1H, H-12), 10.56 (s, 1H, H-8). ^{13}C NMR (75MHz, $CDCl_3$): δ 28.6 (C5), 37.2 (CH_2CO), 57.0 (OCH_3), 57.4 (C6), 63.1 (OCH_3), 68.0 (OCH_2), 102.1 (OCH_2O), 108.6 (C4)[b], 109.1 (C14)[b], 119.1 (C13b)[c], 119.6 (C11), 121.6 (C8a), 125.4 (C12), 125.7 (C12a)[d], 128.7 (5C, ArCH), 133.2 (C13)[d], 134.1 (C4a)[c], 134.7 (ArC), 137.5 (C13a), 146.5 (C8), 147.2 (C9)[e], 147.3 (C14a), 150.1 (C3a), 150.5 (C10)[e], 170.3 (CO). HRMS (ES): *m/z* calcd for $C_{29}H_{26}NO_6$ $[M]^+$: 484.1760; found: 484.1735. *Anal*.Calcd. for $C_{29}H_{26}NO_6Br.1.5H_2O$: C, 58.89 ; H, 4.94; N, 2.37%. Found: C, 58.70; H, 5.06; N, 2.26%.

7.2.7 Preparation of 13-Carboxymethyl-9,10-dimethoxy-5,6-dihydro-benzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium iodide (8)



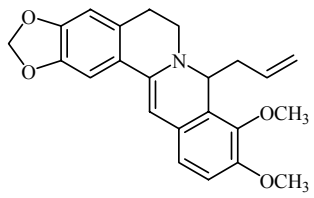
8

To a solution of the ester **6a** (90.0mg, 0.16mmol) in MeOH (5mL), was added a 2% aqueous solution of LiOH (6mL) and the mixture was then heated at reflux for 30min. The cooled reaction mixture was then evaporated. The crude product was added to ice water (100mL) and acidified to pH1 with 1M HCl. The precipitate was filtered and washed with water. The solid was dried and then recrystallized from 3% EtOH in DCM to afford the *quinolizinium iodide* **8** (47.4mg, 53%) as a white solid, m.p. >250°C. ^{1}H-NMR (300MHz, CD_3OD): δ 3.12 (t, *J* = 5.7 Hz, 2H, H-5), 4.09 (s, 3H, OCH_3), 4.18 (s, 2H, CH_2CO), 4.19 (s, 3H, OCH_3), 4.80 (br.s, 2H, H-6), 4.89 (s, 2H, OCH_2O), 7.00 (s, 1H, H-4)[a], 7.70 (s, 1H, H-14)[a], 8.07 (d, *J* = 9.9 Hz, 1H, H-11)[b], 8.12 (d, *J* = 9.9 Hz, 1H, H-12)[b], 9.78 (s, 1H, H-8). ^{13}C NMR (125MHz, CD_3OD): δ 29.1 (C5), 40.2 (**C**H_2CO), 49.2 (OCH_3), 57.5 (C6), 62.6 (OCH_3), 103.6 (OCH_2O), 109.1 (C4)[c], 111.1 (C14)[c], 122.0 (C8a), 122.4 (C11)[d], 123.0 (C12a), 127.2 (C12)[d], 132.1 (C4a), 134.8 (C13), 135.6 (C13a)[e], 138.6 (C13b)[e], 145.0 (C8), 146.1 (C9)[f], 148.8 (C3a)[g], 151.4 (C14a)[g], 151.7 (C10)[f], 177.1 (CO). HRMS (ES): *m/z* calcd for $C_{22}H_{20}NO_6$ $[M]^+$: 394.1291; found: 394.1282.

7.3 8-Allyldihydroberberine route

7.3.1 Preparation of 8-Allyl-9,10-dimethoxy-5,8-dihydro-6*H*-benzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizine (9)[143]

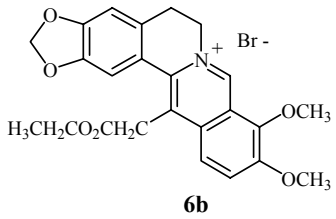
To a suspension of dry berberine chloride **(1)** (1.30g, 3.50mmol) in dry DCM (30mL) was added allyl tri-*n*-butyltin (3mL, 9.39mmol) in a sealed tube and the mixture



9

then heated to 100°C for 8h. The resulting brown-yellow solution was cooled to room temperature and then concentrated. The residue was crystallized from MeOH by placing the mixture in a freezer overnight. The crystals were filtered, washed with cold MeOH and dried to give the allyl enamine **9** (1.12g) as a yellow crystalline solid. The filtrate was concentrated and the residue recrystallized from MeOH to yield further **9** (0.10g) for a total yield of pure **9** of 1.22g (92%); m.p. 109-110°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 2.38-2.50 (m, 2H, C**H**$_2$CH=CH$_2$), 2.78-2.90 (m, 2H, H-5), 3.28-3.36 (m, 1H, H-6), 3.44-3.58 (m, 1H, H-6), 3.84 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 4.83-4.92 (m, 1H, H-8), 4.83-4.92 (m, 2H, CH_2CH=C**H**$_2$), 5.74-5.88 (m, 1H, CH_2C**H**=CH_2), 5.80 (s, 1H, H-13), 5.94 (d, *J* = 3.3 Hz, 2H, OCH_2O), 6.58 (s, 1H, H-4)[a], 6.72 (d, *J* = 8.4 Hz, 1H, H-12), 6.76 (d, *J* = 8.4 Hz, 1H, H-11), 7.14 (s, 1H, H-14)[a]. ^{13}C NMR (75MHz, $CDCl_3$): δ 30.6 (C5), 36.5 (**C**H_2CH=CH_2), 47.3 (C6), 56.0 (OCH_3), 58.3 (C8), 60.7 (OCH_3), 94.6 (C13), 100.9 (OCH_2O), 104.2 (C14)[b], 107.7 (C4)[b], 116.6 (CH_2CH=**C**H_2), 111.7 (C12), 118.2 (C11), 123.0 (C12a), 125.8 (C4a)[c], 128.0 (C8a), 128.7 (C13b)[c], 135.5 (CH_2**C**H=CH_2), 138.9 (C13a), 144.0 (C9)[d], 146.4 (C14a), 149.7 (C10)[d], 147.0 (C3a). HRMS (CI): *m/z* calcd for $C_{23}H_{24}NO_4$ $[MH]^+$: 378.1705; found: 378.1703. *Anal*.Calcd. for $C_{23}H_{23}NO_4$: C, 73.19; H, 6.14; N, 3.71. Found: C, 73.33; H, 6.17; N, 3.60.

7.3.2 Preparation of 13-Ethoxycarbonylmethyl-9,10-dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (6b) from 8-Allyldihydroberberine (9)



6b

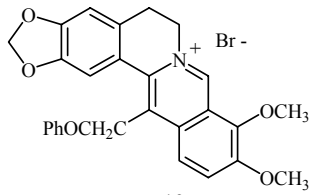
Dry ethyl bromoacetate (2mL, 18.03mmol) was added dropwise with stirring to 8-allyldihydroberberine (**9**) (50.5mg, 0.13mmol) at 0°C under a nitrogen atmosphere. The solution was then heated to 100°C for 2.5h to give a suspension. Dry toluene (5mL) was added to the suspension, the mixture filtered and the filtrate was then evaporated to give **6b** (57.3mg) as an orange-yellow amorphous solid. The solid was recrystallized from 1% MeOH in DCM, and diethyl diethyl ether to give the *quinolizinium bromide* **6b** (39.3mg, 59%) as yellow needles; m.p. 202-204°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 1.37 (t, *J* = 7.2 Hz, 3H, $OCH_2C\mathbf{H}_3$), 3.20-3.35 (br.t, 2H, H-5), 4.07 (s, 3H, OCH_3), 4.27 (s, 2H, CH_2CO), 4.36 (q, *J* = 7.2 Hz, 2H, OCH_2), 4.41 (s, 3H, OCH_3), 5.30-5.70 (br.m, 2H, H-6), 6.10 (s, 2H, OCH_2O), 6.90 (s, 1H, H-4), 7.24 (s, 1H, H-14), 7.71 (d, *J* = 9.3 Hz, 1H, H-11), 7.84 (d, *J* = 9.0 Hz, 1H, H-12), 10.60 (s, 1H, H-8). ^{13}C NMR (75MHz, $CDCl_3$): δ 14.3 ($CH_2\mathbf{C}H_3$), 28.5 (C5), 37.2 ($\mathbf{C}H_2CO$), 57.0 (OCH_3), 57.4 (C6), 62.3 (OCH_2), 63.1 (OCH_3), 102.1 (OCH_2O), 108.6 (C4), 109.0 (C14), 119.3 (C13b)[a], 119.6 (C11), 121.6 (C8a), 125.6 (C4a)[a], 125.8 (C12), 133.2 (C13), 133.9 (C12a), 137.4 (C13a), 146.2 (C9)[b], 146.9 (C14a), 147.3 (C8), 150.0 (C3a), 150.5 (C10)[b], 170.4, (CO). HRMS (ES): *m/z* calcd for $C_{24}H_{24}NO_6$ $[M]^+$: 422.1604; found: 422.1596. *Anal.*Calcd. for $C_{24}H_{24}NO_6Br.0.2CH_2Cl_2$: C, 55.97 ; H, 4.74; N, 2.70%. Found: C, 55.83; H, 4.85; N, 2.54%.

7.3.3 Preparation of 13-Benzyloxycarbonylmethyl-9,10-dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (7b) from 8-Allyldihydroberberine (9)

Dry benzyl bromoacetate (1.5mL, 9.47mmol) was added dropwise with stirring to 8- allyldihydroberberine **(9)** (36.4mg, 0.10mmol) at 0°C under a nitrogen atmosphere.

The solution was then heated to 100°C for 2.5h. The reaction mixture was cooled, then adsorbed on celite and chromatographed on silica gel (4%MeOH in DCM) to give the *quinolizinium bromide* **7b** (31.2mg, 57%) as a yellow solid. All spectroscopic data for **7b** was the same as that noted for this compound previously (Section 6.2.6).

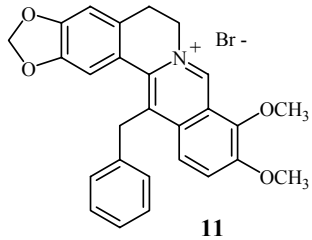
7.3.4 Preparation of 9,10-Dimethoxy-13-(2-oxo-2-phenyl-ethyl)-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (10)



10

To a solution of 8-allyldihydroberberine **(9)** (46mg, 0.12mmol) in dry CH_3CN (2mL) was added phenacyl bromide (46mg, 0.23mmol) under a nitrogen atmosphere. The mixture was refluxed for 4h, then concentrated and the residue chromatographed on silica gel (4% MeOH in DCM) to give the *quinolizinium bromide* **10** (35.6mg, 55%) as a yellow solid; m.p.181-183°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.20-3.35 (br.m, 2H, H-5), 4.02 (s, 3H, OCH_3), 4.33 (s, 3H, OCH_3), 5.07 (s, 2H, CH_2CO), 5.10-5.30 (br.m, 2H, H-6), 5.99 (s, 2H, OCH_2O), 6.90 (s, 2H, H-14 and H-4), 7.47 (d, *J* = 9.3 Hz, 1H, H-11), 7.62 (t, *J* = 7.8 Hz, 2H, ArH), 7.70-7.78 (m, 1H, ArH), 7.77 (d, *J* = 9.3 Hz, 1H, H-12), 8.19 (br.d, *J* = 7.5Hz, 2H, ArH), 10.46 (s, 1H, H-8). ^{13}C NMR (75MHz, $CDCl_3$): δ 28.6 (C5), 42.03 (**C**H_2CO), 57.0 (OCH_3), 57.3 (C6), 63.0 (OCH_3), 102.0 (OCH_2O), 108.4 (C4)[a], 108.6 (C14)[a], 119.8 (C4a), 119.9 (C11), 121.7 (C8a), 125.7 (C12), 127.2 (C13b), 128.5 (2C, ArCH), 129.2 (2C, ArCH), 133.4 (C13)[b], 133.9 (C12a)[b], 134.5 (ArCH), 135.2 (ArC), 137.6 (C13a), 146.0 (C9)[c], 146.5 (C8), 147.3 (C14a), 150.0 (C3a), 155.3 (C10)[c], 196.7 (CO). HRMS (ES): *m/z* calcd for $C_{28}H_{24}NO_5$ $[M]^+$: 454.1654; found: 454.1649. *Anal*.Calcd. for $C_{28}H_{24}NO_5Br.1.4CH_3OH$: C, 60.96; H, 5.15; N, 2.42. Found: C, 60.69; H, 5.17; N, 2.39.

7.3.5 Preparation of 13-Benzyl-9,10-dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (11)



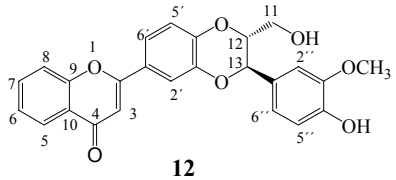
11

Dry benzyl bromide (3.0mL, 25.26mmol) was added dropwise with stirring to 8-allyldihydroberberine (**9**) (205.0mg, 0.54mmol) at 0°C under a nitrogen atmosphere. The mixture was then heated to 100°C for 11h. The resulting suspension was cooled to room temperature and dry toluene (*ca* 10mL) added. The slurry was suction filtered to give yellow solids (270.0mg). The solids were recrystallized from 1% MeOH in DCM, and diethyl ether, to give the *quinolizinium bromide* **11** (170.6mg, 62%) as yellow crystals; m.p. 223-225°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.27 (t, *J* = 5.4 Hz, 2H, H-5), 4.02 (s, 3H, OCH_3), 4.38 (s, 3H, OCH_3), 4.68 (s, 2H, CH_2Ph), 5.20-5.35 (m, 2H, H-6), 6.00 (s, 2H, OCH_2O), 6.95 (s, 1H, H-14), 6.88 (s, 1H, H-4), 7.11 (br.d, *J* = 7.2 Hz, 2H, ArH), 7.26-7.42 (m, 3H, ArH), 7.62 (d, *J* = 9.6 Hz, 1H, H-12)[a], 7.71 (d, *J* = 9.6 Hz, 1H, H-11)[a], 10.61 (s, 1H, H8). ^{13}C NMR (75MHz, $CDCl_3$ + CD_3OD): δ 28.3 (C5), 36.4 (**C**H_2Ph), 56.7 (OCH_3), 56.8 (C6), 62.6 (OCH_3), 101.9 (OCH_2O), 108.4 (C4)[b], 108.6 (C14)[b], 119.7 (C4a), 120.9 (C12)[c], 121.5 (C8a), 125.6 (C11)[c], 127.0 (ArCH), 127.6 (2C, ArCH), 129.2 (2C, ArCH), 130.3 (C13b), 133.3 (C13)[d], 133.5 (C12a)[d], 137.4 (C13a), 137.9 (ArC), 145.5 (C9)[e], 145.6 (C8), 147.0 (C14a), 149.8 (C3a), 150.1 (C10)[e]. HRMS(ES): *m/z* calcd for $C_{27}H_{24}NO_4$ $[M]^+$: 426.1705; found: 426.1715. *Anal*.Calcd. for $C_{27}H_{24}NO_4Br$: C, 64.04; H, 4.78; N, 2.77. Found: C, 63.65; H, 5.02; N, 2.55

7.4 Synthesis of natural bacterial pump blocking agents (Chapter 3)

7.4.1 Preparation of 5,7-Deoxyhydnocarpin-D (12)[144]

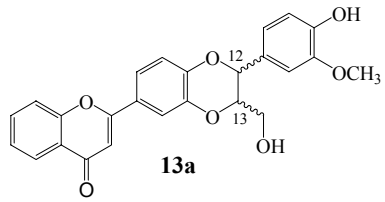
To a mixture of 3′,4′-dihydroflavone (160.6mg, 0.63mmol) and coniferyl alcohol (98.5mg, 0.55mmol) were added dry benzene (12.5mL) and dry acetone (6.25mL) under a nitrogen atmosphere. The mixture was warmed up to 60°C with stirring for 10min, and then silver carbonate (70.0mg, 0.25mmol) was added to the reaction mixture and further stirred vigorously for 10h. The mixture was then allowed to cool to room temperature, filtered through celite, and washed with 5% MeOH in DCM. The filtrate was evaporated to give an orange-yellow oil. The oil was then chromatographed on silica gel (DCM with a gradient elution to MeOH) to give a major fraction (57.2mg, eluent: 2% MeOH in DCM). Further purification of this major fraction was achieved by multiple development PLC on silica gel (1% MeOH in DCM) to give **12** (29.0mg, 12%) as an opaque white solid, **13** (2.0mg, 1%) as an opaque white solid, and **14** (3.2mg, 1%) as an opaque white solid.



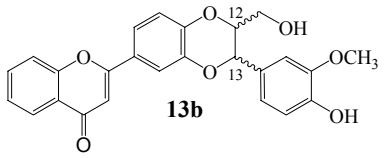
12

12: ^{1}H NMR (300MHz, $CDCl_3$): δ 3.58 (dd, *J* = 12.8, 3.5 Hz, 1H, H-11), 3.85 (dd, *J* = 12.6, 2.4 Hz, 1H, H-11), 3.92 (s, 3H, OCH_3), 4.08-4.14 (m, 1H, H-12), 5.00 (d, *J* = 8.4 Hz, 1H, H-13), 5.83 (br.s, 1H, OH), 6.72 (s, 1H, H-3), 6.95 (d, *J* = 7.8 Hz, 2H, H-5′′, H-6′′), 6.96 (s, 1H, H-2′′), 7.06 (d, *J* = 8.7 Hz, 1H, H-5′), 7.39 (ddd, *J* = 8.1, 7.2, 0.9 Hz, 1H, H-7), 7.46 (dd, *J* = 8.4, 2.1 Hz, 1H, H-8), 7.49 (d, *J* = 9.0 Hz, 1H, H-6′), 7.58 (d, *J* = 2.1 Hz, 1H, H-2′), 7.66 (ddd, *J* = 8.4, 7.2, 1.5 Hz, 1H, H-6), 8.20 (dd, *J* = 8.1, 1.2 Hz, 1H, H-5). ^{13}C NMR (75MHz, $CDCl_3$): δ 55.9 (OCH_3), 60.9 (C11), 76.3 (C13), 78.8 (C12), 105.9 (C3), 109.9 (C2′′), 114.9 (C2′), 115.3 (C5′′), 117.5 (C5′),

117.8 (C5), 120.0 (C6′), 120.5 (C6′′), 123.4 (C1′), 124.4 (C10), 125.2 (C8), 125.3 (C7), 127.1 (C1′′), 133.8 (C6), 144.0 (C3′), 146.5 (C4′′), 146.0 (C3′′), 147.2 (C4′), 155.9 (C9), 163.5 (C2), 178.8 (C4). HRMS (CI); *m/z* calcd for $C_{25}H_{21}O_7$ $[MH]^+$: 433.1287; found: 433.1278.

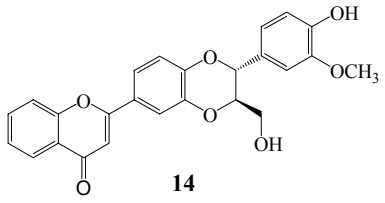


13a



13b

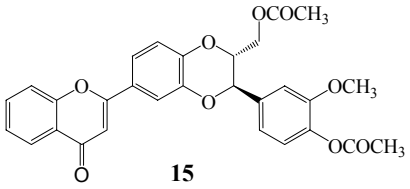
13: ^{1}H NMR (300MHz, $CDCl_3$): δ 3.55-3.63 (m, 1H, H-11), 3.71-3.79 (m, 1H, H-11), 3.90 (s, 3H, OCH_3), 4.57-4.63 (m, 1H, H-13 of **13a** or H-12 of **13b**), 5.27 (d, *J* = 3.0 Hz, 1H, H-12 of **13a** or H-13 of **13b**), 5.67 (s, 1H, OH), 6.74 (s, 1H, H-3), 6.89 (d, *J* = 6.9 Hz, 1H, H-5′′), 6.91 (s, 1H, H-2′′), 6.95 (d, *J* = 8.7 Hz, 1H, H-6′′), 7.12 (d, *J* = 8.4 Hz, 1H, H-5′), 7.40 (br.t, *J* = 8.1 Hz, 1H, H-7), 7.51 (dd, *J* = 8.4, 2.1 Hz, 1H, H-8), 7.53 (dd, *J* = 7.8 Hz, 1H, H-6′), 7.59 (d, *J* = 2.1 Hz, 1H, H-2′), 7.68 (ddd, *J* = 8.4, 7.2, 1.8 Hz, 1H, H-6), 8.21 (dd, *J* = 8.1, 1.5 Hz, 1H, H-5). HRMS (CI); *m/z* calcd for $C_{25}H_{21}O_7$ $[MH]^+$: 433.1287; found: 433.1281.



14

14: ^{1}H NMR (300MHz, $CDCl_3$): δ 3.52-3.62 (m, 1H, H-11), 3.85 (br.d, *J* = 13.2 Hz, 1H, H-11), 3.92 (s, 3H, OCH_3), 4.07-4.10 (m, 1H, H-13), 5.03 (d, *J* = 8.4 Hz, 1H, H-12), 5.72 (s, 1H, OH), 6.74 (s, 1H, H-3), 6.94 (d, *J* = 12.3 Hz, 2H, H-5′′, H-6′′), 6.97 (s, 1H, H-2′′), 7.08 (d, *J* = 8.7 Hz, 1H, H-5′), 7.40 (br.t, *J* = 7.7 Hz, 1H, H-7), 7.49 (dd, *J* = 8.3, 2.6 Hz, 1H, H-8), 7.52 (d, *J* = 8.1 Hz, 1H, H-6′), 7.58 (d, *J* = 2.1 Hz, 1H, H-2), 7.68 (td, *J* = 7.8, 1.8 Hz, 1H, H-6), 8.22 (br.d, *J* = 7.5 Hz, 1H, H-5). HRMS (CI); *m/z* calcd for $C_{25}H_{21}O_7$ $[MH]^+$: 433.1287; found: 433.1284.

7.4.2 Acetylation of 5,7-Deoxyhydnocarpin-D[144]

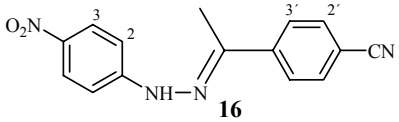


15

To a solution of 5,7-deoxyhydnocarpin-D (**12**) (9.4mg, 0.02mmol) in dry pyridine (0.1mL) was added excess acetic anhydride (0.9mL) and the mixture stirred at room temperature for 12h. The mixture was evaporated and then chromatographed on silica gel by PLC (DCM) to give the diacetate product **15** (10.0mg, 89%) as an opaque white solid, m.p. 166-168^{o}C (Lit.[144] 172-174^{o}C). ^{1}H NMR (300MHz, $CDCl_3$): δ 2.06 (s, 3H, $COCH_3$), 2.31 (s, 3H, $COCH_3$), 3.85 (s, 3H, OCH_3), 4.02 (dd, *J* = 12.3, 4.2 Hz, 1H, H-11), 4.26-4.36 (m, 1H, H-12), 4.38 (dd, *J* = 12.2, 3.2 Hz, 1H, H-11), 4.98 (d, *J* = 7.8 Hz, 1H, H-13), 6.73 (s, 1H, H-3), 6.97 (dd, *J* = 7.8, 1.5 Hz, 1H, H-6′′), 6.99 (s, 1H, H-2′′), 7.09 (d, *J* = 8.4 Hz, 2H, H-8, H-5′′), 7.39 (br.t, *J* = 8.1 Hz, 1H, H-7), 7.48-7.54 (m, 2H, H-5′, H-6′), 7.58 (d, *J* = 2.1 Hz, 1H, H-2′), 7.63-7.70 (m, 1H, H-6), 8.20 (dd, *J* = 7.8, 1.5 Hz, 1H, H-5). ^{13}C NMR (75MHz, $CDCl_3$): δ 20.7 (2C, CO**C**H_3), 55.0 (OCH_3), 62.5 (C11), 75.9 (C12), 76.4 (C13), 106.6 (C3), 110.5 (C2′′), 115.4 (C2′), 117.7 (C5′′), 117.9 (C5′)[a], 119.7 (C6′′), 120.3 (C6′)[a], 123.3 (C1′), 123.8 (C8), 125.1 (C7), 125.3 (C1′′), 125.6 (C5), 133.6 (C6), 133.9 (C10), 140.6 (C3′)[b], 143.6 (C4′′), 145.8 (C4′)[b], 151.6 (C3′′), 156.0 (C9), 162.7 (C2), 168.5 (CO), 170.2 (CO), 178.2 (C4). HMRS (EI); *m/z* calcd for $C_{29}H_{24}O_9$ $[M]^+$: 516.1420; found; 516.1419.

7.5 Synthesis of synthetic bacterial pump blocking agents

7.5.1 Attempted Fischer indole synthesis



16

Method 1: A mixture of 4-acetylbenzonitrile (145.0mg, 1.0mmol) and 4-nitrophenylhydrazine (153.0mg, 1.0mmol) in glacial acetic acid (1mL) and 1M HCl in diethyl ether (1mL)

was refluxed at 115°C under a nitrogen atmosphere for 1 h. The solution was poured into ice (50g), then adjusted to pH 8 with saturated aqueous Na_2CO_3, and extracted with DCM (3 x 30mL). The combined DCM extracts were dried, evaporated, and chromatographed on silica gel (DCM) to give the hydrazone **16** (181.0mg, 65%) as an orange solid. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.35 (s, 3H, CH_3), 7.40 (d, *J* = 9.3 Hz, 2H, H-2), 7.86 (d, *J* = 8.4 Hz, 2H, H-3′), 8.00 (d, *J* = 8.7 Hz, 2H, H-2′), 8.15 (d, *J* = 9.0 Hz, 2H, H-3), 10.43 (s, 1H, NH). EIMS: *m/z*, $[M]^+$: 280 (50%).

Method 2: To a solution of 4-acetylbenzonitrile (145.0mg, 1.0mmol) in isopropanol (10mL) and 1M HCl in diethyl ether (3mL), was added 4-nitrophenylhydrazine (153.0mg, 1.0mmol) and refluxed at 80°C for 25min. The suspension was cooled to room temperature. The precipitate was filtered, washed thoroughly with water, then hexane, and dried to give the hydrazone **16** (180.1mg, 64%).

Method 3: The hydrazone **16** (100.0mg, 0.36mmol) and 98-100% formic acid (1mL) were mixed in a microwave vessel which was irradiated at 110°C for 10min. The mixture was allowed to cool down to room temperature, then poured into water (5mL) and boiled for 2min to hydrolyze any *N*-formyl derivative, which might have formed. The suspension was cooled to room temperature. The precipitate was filtered, washed thoroughly with water, and dried to give only the hydrazone starting material **16.**

Method 4: The hydrazone **16** (1.12g, 4.0mmol) and zinc chloride powder (1.61g, 16mmol) were mixed in a microwave vessel without solvent. The solid mixture was irradiated at180°C for 1min using CH_3CN for temperature probe vessel. The mixture gave a black solid as carbonaceous residue.

Method 5: A mixture of the hydrazone **16** (41.6mg, 0.15mmol) and *p*-toluenesulfonic acid monohydrate (56.5mg, 0.30mmol) in xylene (3mL) was refluxed at

140^{o}C for 5h. The mixture was washed with water (5 x 3mL). The xylene layer was dried and evaporated to give the hydrazone starting material **16**.

Method 6: To a solution of polyphosphoric acid (72.4mg) in xylene (3mL) at 80^{o}C, was added the hydrazone **16** (35mg, 0.13mmol) and heated at 110^{o}C for 1 h. The reaction was worked up in the same manner as in *method 5* to give the hydrazone starting material **16**.

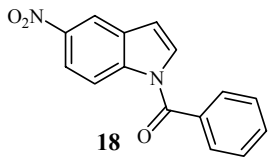
Method 7: A mixture of the hydrazone **16** (100.0mg, 0.36mmol), 85% phosphoric acid (0.6mL) and toluene (2mL) was stirred vigorously at 100^{o}C for 2h. The toluene layer was decanted and fresh toluene (5mL) was added and further stirred for 4h. The toluene was decanted again, then combined with the first toluene layer, dried, and evaporated to give the hydrazone starting material **16**.

Method 8: To a suspension of the hydrazone **16** (100.0mg, 0.36mmol) in glacial acetic acid (3mL), was added a solution of BF_3-diethyl etherate and the mixture heated at reflux 110^{o}C under a nitrogen atmosphere for 1h. The reaction was cooled to room temperature and poured into iced water (100mL). The suspension was filtered and dried to give a dark green solid. The solid was dissolved in DCM and filtered. The DCM filtrate was evaporated to give the hydrazone **16** and two other unidentified components in small amounts (these components on TLC analysis an silica gel did not give a brown colouration with iodine vapour).

7.5.2 *N*-Acylation reaction

Acid chloride route

7.5.2.1 Preparation of 1-Benzoyl-5-nitro-*1H*-indole (18)[103]



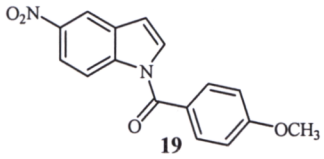
18

To a suspension of sodium hydride (1.55g of 50% dispersion in mineral oil, 31.3mmol) in anhydrous DMF (24mL) at 0°C, was added, with stirring, a solution of 5-nitroindole (**17**) (2.90g, 17.9mmol) in DMF (50mL). The mixture was stirred for 30min at 0°C. The mixture was then warmed to room temperature and stirring continued for 2h. Excess DMF (180mL) was added and then the mixture was cooled to –60°C in a dry ice/acetone bath. A solution of benzoyl chloride (3mL, 25.9mmol) in DMF (12mL) was added dropwise to the cooled mixture, which was allowed to stir overnight with warming to 70°C. The mixture was evaporated, added to ice water (700mL), and stirred vigorously for 4h. The precipitate was filtered, washed with cold water, air dried, and washed with EtOAc (to remove traces of the starting material), yielding **18** (4.22g, 89%) as an opaque white solid, m.p. 158-159°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 6.77 (dd, *J* = 3.8, 0.5 Hz, 1H, H-3), 7.50 (d, *J* = 3.9 Hz, 1H, H-2), 7.54-7.62 (m, 2H, H-3′), 7.63-7.70 (m, 1H, H-4′), 7.72-7.80 (m, 2H, H-2′), 8.26 (dd, *J* = 9.2, 2.3 Hz, 1H, H-6), 8.47 (dd, *J* = 9.0, 0.6 Hz, 1H, H-7), 8.52 (d, *J* = 2.1 Hz, 1H, H-4). HRMS (EI); *m/z* calcd for $C_{15}H_{10}N_2O_3$ $[M]^+$: 266.0691; found: 266.0688.

7.5.2.2 Preparation of 1-(4-Methoxybenzoyl)-5-nitro-1*H*-indole (19)

To a solution of **17** (2.01g, 12.4mmol) in dry THF (250mL) at –60°C in a dry ice/acetone bath was added dropwise of 2M *n*-BuLi solution in cyclohexane (6.20mL, 14.8mmol) and stirred for 30 min. 4-Methoxybenzoyl chloride (2.0mL, 14.8mmol) was

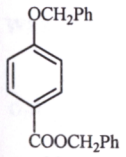
added to the reaction mixture which was further stirred for 3h. The mixture was then quenched with saturated $NaHCO_3$ solution (2mL), dried and evaporated to dryness. The residue was chromatographed on silica gel (40% DCM in PS) to afford **19** (1.62g, 44.2%) as a pale yellow solid, m.p.199-200°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.92 (s, 3H, OCH_3), 6.76 (d, J = 3.8 Hz, 1H, H-3), 7.05 (d, J = 8.8 Hz, 2H, H-3′), 7.55 (d, J = 3.8 Hz, 1H, H-2), 7.77 (dd, J = 7.0, 1.8 Hz, 2H, H-2′), 8.25 (dd, J = 9.1, 2.1 Hz, 1H, H-6), 8.41 (d, J = 9.1 Hz, 1H, H-7), 8.54 (d, J = 2.1 Hz, 1H, H-4). ^{13}C NMR (75MHz, $CDCl_3$): δ 55.6 (OCH_3), 108.3 (C3), 114.2 (2C, C3′), 116.2 (C7), 117.2 (C4), 119.9 (C6), 125.1 (C1′), 130.4 (C3a), 130.6 (C2), 132.0 (2C, C2′), 129.1 (C7a), 114.1 (C5), 163.4 (C4′), 167.9 (CO). HRMS (EI); *m/z* calcd for $C_{16}H_{12}N_2O_4$ $[M]^+$: 296.0797; found: 296.0796.



19

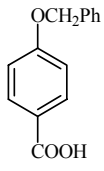
7.5.2.3 Preparation of 1-(4-Benzyloxybenzoyl)-5-nitro-1*H*-indole (22)

Preparation of acid chloride: To a suspension of *p*-hydroxybenzoic acid (3.0g, 22.8mmol) and dry K_2CO_3 (7.0g, 50.0mmol) in dry DMF (60mL), was added dropwise of benzyl bromide (5.4mL, 45.8mmol) under a nitrogen atmosphere. The reaction mixture was heated at reflux at 80°C for 6h. The mixture was evaporated, added to ice water (500mL), and acidified with 5M HCl to pH1. The suspension was filtered, washed several times with H_2O, then dried and chromatographed on silica gel by VLC (60% DCM in PS) to give benzyl 4-(benzyloxy) benzoate (**20**, 5.62g, 81%) as an off-white solid, m.p. 93-95°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 5.09 (s, 2H, OCH_2), 5.33 (s, 2H, $COOCH_2$), 6.97 (d, J = 8.7 Hz, 2H, H-3), 7.32-7.44 (m, 10H, ArH), 8.03 (d, J = 9.0 Hz, 2H, H-2).



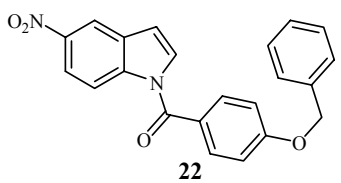
20

The suspension of **20** (5.0g, 15.7mmol) in MeOH (50mL) was added 30% aqueous solution of KOH (10mL) and heated at reflux for 6h. The solvent was evaporated, added to ice water (300mL), and acidified to pH1. The white precipitate was filtered and washed thoroughly with water and 20% DCM in PS (100mL) to remove unreacted dibenzylated starting material. The precipitate was crystallized from diethyl ether to give 4-benzyloxybenzoic acid (**21,**[106] 3.25g, 91%) as white needles, m.p. 185-187°C (Lit.[145] 187-190°C). ^{1}H NMR (300MHz, $CDCl_3$ + CD_3OD): δ 5.13 (s, 2H, OCH_2), 7.00 (d, *J* = 9.0 Hz, 2H, H-3), 7.34-7.46 (m, 5H, ArH), 8.01 (d, *J* = 9.0 Hz, 2H, H-2). ^{13}C NMR (75MHz, $CDCl_3$): δ 69.7 (OCH_2), 114.1 (2C, ArCH), 122.6 (ArC), 127.1 (2C, ArCH), 127.8 (ArCH), 128.3 (2C, C-3), 131.5 (2C, C-2), 135.9 (C-1), 162.3 (C-4), 168.5 (CO).



21

The suspension of **21** (1.0g, 4.7mmol) in a 1:1 mixed solvent of THF/Benzene (10mL) was added dropwise of oxalyl chloride (1.28mL, 6.9mmol) at room temperature and stirred for 30h. The reaction mixture was monitored by alumina (2% MeOH in DCM). The mixture was then concentrated to give crude of 4-(benzyloxy)benzoyl chloride, which was used in the next step immediately without purification because it was air sensitive.

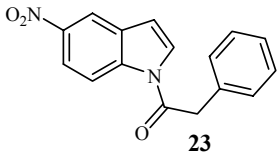


22

Preparation of acylated product: To a solution of 5-nitroindole (0.3g, 2.0mmol) in dry THF (40mL) at –60°C in a dry ice/acetone bath was added dropwise 2M *n*-BuLi solution in cyclohexane (1.0mL, 2.0mmol) and the mixture then stirred for 30min. The crude 4-(benzyloxy)benzoyl chloride (0.5g, 2.2mmol) was added to the reaction mixture and further stirred for 4.5h. The mixture was then quenched with saturated $NaHCO_3$ solution (2mL), dried and evaporated to dryness. The residue was chromatographed on silica gel by VLC (60% DCM in PS) to afford **22** (0.2g, 30%) as off-white needles, m.p.

177-179°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 5.19 (s, 2H, OCH_2), 6.77 (dd, *J* = 3.8, 0.8 Hz, 1H, H-3), 7.12 (d, *J* = 9.0Hz, 2H, H-3′), 7.36-7.48 (m, 5H, ArH), 7.55 (d, *J* = 3.6 Hz, 1H, H-2), 7.77 (d, *J* = 8.7 Hz, 2H, H-2′), 8.25 (dd, *J* = 9.3, 2.1 Hz, 1H, H-6), 8.41 (d, *J* = 9.0 Hz, 1H, H-7), 8.54 (d, *J* = 2.1 Hz, 1H, H-4). ^{13}C NMR (75MHz, $CDCl_3$): δ 70.3 (OCH_2), 108.3 (C3), 115.0 (2C, C3′), 116.2 (C7), 117.2 (C4), 119.6 (C6), 125.4 (C1′), 127.5 (2C, C3′′), 128.4 (2C, C4′′), 128.8 (C2′′), 130.4 (C3a), 130.5 (C2), 132.1 (2C, C2′), 135.8 (C1′′), 139.1 (C7a), 144.2 (C5), 162.5 (C4′), 167.9 (CO). HMRS (EI); *m/z* calcd for $C_{22}H_{16}N_2O_4$ $[M]^+$: 372.1110; found; 372.1105.

7.5.2.4 Preparation of 5-Nitro-1-phenylethanoyl-1*H*-indole (23)



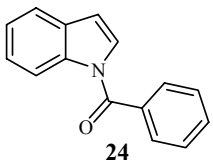
23

To a suspension of sodium hydride (63.0mg of a 50% dispersion in mineral oil, 1.3mmol) in anhydrous DMF (1mL) at 0°C, was added, with stirring, a solution of **17** (93.0mg, 0.57mmol) in DMF (3mL). The mixture was stirred for 30min at 0°C. The mixture was then warmed to room temperature and further stirred for 2h. Excess DMF (7mL) was added and then the mixture was cooled to –60°C in a dry ice/acetone bath. A solution of phenyl acetyl chloride (0.1mL, 0.75mmol) in DMF (0.4mL) was added dropwise to the cooled mixture and then allowed to stir overnight with warming to 70°C. The mixture was evaporated, then added to ice water (50mL), and stirred vigorously for 4h. The precipitate was filtered, washed with cold water and air-dried. The crude product was chromatographed on silica gel by VLC (4% EtOAc in PS) to give **23** (31.3mg, 19%) as an off white solid, m.p. 96-98°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 4.29 (s, 2H, CH_2CO), 6.76 (dd, *J* = 3.8, 0.8 Hz, 1H, H-3), 7.29-7.42 (m, 5H, ArH), 7.68 (d, *J* = 3.9 Hz, 1H, H-2), 8.24 (dd, *J* = 9.2, 2.3 Hz, 1H, H-6), 8.46 (d, *J* = 2.4 Hz, 1H, H-4), 8.60 (dt, *J* = 9.3,

0.6 Hz, 1H, H-7). ^{13}C NMR (75MHz, $CDCl_3$): δ 43.0 (CH_2), 109.8 (C3), 116.9 (C7), 117.0 (C4), 120.5 (C6), 127.6 (C2), 127.8 (ArCH), 129.1 (4C, ArCH), 130.2 (C3a), 132.5 (ArC), 138.8 (C7a), 144.4 (C5), 169.4 (CO). HMRS (EI); *m/z* calcd for $C_{16}H_{12}N_2O_3$ $[M]^+$: 280.0848; found: 280.0835.

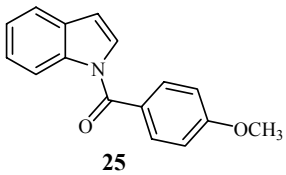
Preparation of *N*-Acyl Indoles

7.5.2.5 Preparation of 1-Benzoyl-1*H*-indole (24)


24

To a solution of 1*H*-indole (23.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and benzoic acid (48.8mg, 0.4mmol) in dry DCM (2mL) at 0°C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 15h. The resulting suspension was evaporated and chromatographed on silica gel by PLC (5% EtOAc in PS) (multiple development) to give **24** (14.0mg, 32%) as an off-white solid, m.p. 63-64°C (Lit.[96] 59-60°C and 67-68°C[94]). ^{1}H NMR (300MHz, $CDCl_3$): δ 6.57 (dd, *J* = 3.9, 0.6 Hz, 1H, H-3), 7.25 (d, *J* = 3.9 Hz, 1H, H-2), 7.27-7.40 (m, 2H, H-5, H-6), 7.44-7.50 (m, 2H, H-3′), 7.52-7.60 (m, 2H, H-4′, H-4), 7.68-7.73 (m, 2H, H-2′), 8.40 (dd, *J* = 8.4, 0.9 Hz, 1H, H-7). HRMS (EI): *m/z* calcd for $C_{15}H_{11}NO$ $[M]^+$: 221.0841; found: 221.0843.

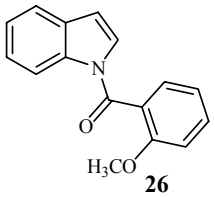
7.5.2.6 Preparation of 1-(4-Methoxybenzoyl)-1*H*-indole (25)


25

To a solution of 1*H*-indole (23.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and *p*-methoxybenzoic acid (60.9mg, 0.4mmol) in dry DCM (2mL) at 0°C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The

solution was then warmed to room temperature and stirred for 15 h. The resulting suspension was evaporated and chromatographed on silica gel (5% EtOAc in PS) to give the acylated product **25** (23.0mg, 46%) as an off-white solid, m.p. 138-139°C (Lit.[96] 137-139°C). ^{1}H NMR (300MHz, $CDCl_3$): δ 3.82 (s, 3H, OCH_3), 6.53 (dd, *J* = 3.8, 0.8 Hz, 1H, H-3), 6.91-6.99 (m, 2H, H-3′), 7.18-7.32 (m, 3H, H-2, H-4, H-6), 7.51-7.54 (m, 1H, H-5), 7.64-7.69 (m, 2H, H-2′), 8.26 (dd, *J* = 8.3, 1.1 Hz, 1H, H-7). HRMS (EI): *m/z* calcd for $C_{16}H_{13}NO_2$ $[M]^+$: 251.0946; found: 251.0947.

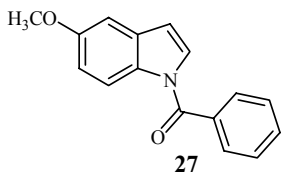
7.5.2.7 Preparation of 1-(2-Methoxybenzoyl)-1*H*-indole (26)



26

To a solution of 1*H*-indole (23.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and *o*-methoxybenzoic acid (60.9mg, 0.4mmol) in dry DCM (2mL) at 0^{o}C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 72h. The resulting suspension was evaporated and chromatographed on silica gel (5% EtOAc in PS) to give the acylated product **26** (17.1mg, 34%) as an off-white solid, m.p. 137-139°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.79 (s, 3H, OCH_3), 6.55 (dd, *J* = 3.8, 0.9 Hz, 1H, H-3), 7.03 (d, *J* = 8.5 Hz, 1H, H-3′), 7.07 (d, *J* = 3.3 Hz, 1H, H-2), 7.09 (td, *J* = 7.3, 0.9 Hz, 1H, H-5′), 7.33 (td, *J* = 7.6, 1.5 Hz, 1H, H-5), 7.37 (td, *J* = 7.3, 1.5 Hz, 1H, H-6), 7.45 (dd, *J* = 7.6, 1.8 Hz, 1H, H-6′), 7.51 (ddd, *J* = 8.5, 7.3, 1.8 Hz, 1H, H-4′), 7.57 (ddd, *J* = 7.6, 1.5, 0.6 Hz, 1H, H-4), 8.44 (bd, *J* = 8.2 Hz, 1H, H-7). ^{13}C NMR (75MHz, $CDCl_3$): δ 55.7 (OCH_3), 108.6 (C3), 111.4 (C3′), 116.5 (C7), 120.7 (C5′)[a], 120.8 (C4)[a], 123.9 (C5), 124.8 (C1′), 124.9 (C6), 127.4 (C2), 129.0 (C6′), 131.0 (C3a), 132.1 (C4′), 135.6 (C7a), 156.3 (C2′), 167.2 (CO). HRMS (EI): *m/z* calcd for $C_{16}H_{13}NO_2$ $[M]^+$: 251.0946; found: 251.0955.

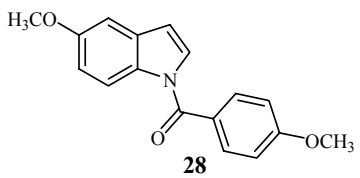
7.5.2.8 Preparation of 1-Benzoyl-5-methoxy-1*H*-indole (27)



27

To a solution of 5-methoxy-1*H*-indole (29.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and benzoic acid (48.8mg, 0.4mmol) in dry DCM (2mL) at 0°C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 15h. The resulting suspension was evaporated and chromatographed on silica gel (5% EtOAc in PS) to give the acylated product **27** (14.0mg, 32%) as an off-white solid, m.p. 106-107°C (Lit.[96] 109-111°C). ^{1}H NMR (300MHz, $CDCl_3$): δ 3.88 (s, 3H, OCH_3), 6.54 (dd, *J* = 3.8, 0.8 Hz, 1H, H-3), 7.00 (dd, *J* = 9.2, 2.6 Hz, 1H, H-6), 7.06 (d, *J* = 2.4 Hz, 1H, H-4), 7.26 (d, *J* = 2.1 Hz, 1H, H-2), 7.48-7.62 (m, 3H, H-3′, H-4′), 7.70-7.75 (m, 2H, H-2′), 8.31 (d, *J* = 8.7 Hz, 1H, H-7). HRMS (EI): *m/z* calcd for $C_{16}H_{13}NO_2$ $[M]^+$: 251.0946; found: 251.0950.

7.5.2.9 Preparation of 5-Methoxy-1-(4-methoxybenzoyl)-1*H*-indole (28)

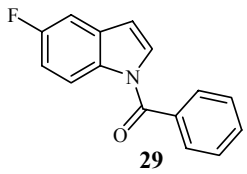


28

To a solution of 5-methoxy-1*H*-indole (29.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and *p*-methoxybenzoic acid (60.9mg, 0.4mmol) in dry DCM (2mL) at 0°C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 15h. The resulting suspension was evaporated and chromatographed on silica gel (5% EtOAc in PS) to give **28** (7.7mg, 15%) as an off-white solid, m.p. 105-106°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.81 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 6.47 (dd, *J* = 3.8, 0.6 Hz, 1H, H-3), 6.89 – 7.00 (m, 4H, H-4, H-6, H-3′), 7.26 (d, *J* = 3.8, 1H, H-2),

7.66 (tt, *J* = 9.7, 2.7 Hz, 2H, H-2′), 8.18 (d, *J* = 9.1 Hz, 1H, H-7). ^{13}C-NMR (75MHz, $CDCl_3$): δ 55.5 (OCH_3), 55.7 (OCH_3), 103.4 (C6), 108.0 (C3), 113.3 (C4), 113.8 (2C, C3′), 117.0 (C7), 126.6 (C1′), 128.4 (C2), 130.8 (C3a), 131.6 (2C, C2′), 131.6 (C7a), 156.5 (C5), 162.6 (C4′), 167.9 (CO). HRMS (EI): *m/z* calcd for $C_{17}H_{15}NO_3$ $[M]^+$: 281.1052; found: 281.1053.

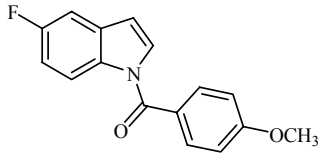
7.5.2.10 Preparation of 1-Benzoyl-5-fluoro-1*H*-indole (29)



29

To a solution of 5-fluoro-1*H*-indole (27.0mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and benzoic acid (48.8mg, 0.4mmol) in dry DCM (2mL) at 0^{o}C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 24h. The resulting suspension was evaporated and chromatographed on silica gel (5% EtOAc in PS) to give **29** (42.7mg, 89%) as an opaque white solid, m.p. 65-66°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 6.50 (d, *J* = 3.8 Hz, 1H, H-3), 7.03 (td, *J* = 9.1, 2.3 Hz, 1H, H-6), 7.18 (dd, *J* = 8.8, 2.6 Hz, 1H, H-4), 7.25 (d, *J* = 3.8 Hz, 1H, H-2), 7.46 (tt, *J* = 8.5, 1.5 Hz, 2H, H-3′), 7.54 (tt, 1H, *J* = 6.2, 1.2 Hz, 1H, H-4′), 7.65 (dd, *J* = 8.8, 1.8 Hz, 2H, H-2′), 8.31 (dd, *J* = 9.1, 5.3 Hz, 1H, H-7). ^{13}C NMR (75MHz, $CDCl_3$): δ 106.4 (d, *J* = 23.9 Hz, C4), 108.2 (d, *J* = 4.0 Hz, C3), 112.7 (d, *J* = 24.8 Hz, C6), 117.4 (d, *J* = 9.2 Hz, C7), 128.6 (2C, C3′), 129.1 (C2), 129.1 (2C, C2′), 131.7 (d, *J* = 10.1 Hz, C3a), 132.0 (C4′), 132.4 (C1′), 134.2 (C7a), 159.8 (d, *J* = 240.4 Hz, C5), 168.5 (CO). HRMS (EI): *m/z* calcd for $C_{15}H_{10}NOF$ $[M]^+$: 239.0746; found: 239.0738.

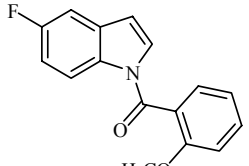
7.5.2.11 Preparation of 5-Fluoro-1-(4-methoxybenzoyl)- 1*H*-indole (30)



30

To a solution of 5-fluoro-*1H*-indole (27.0mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and *p*-methoxybenzoic acid (60.9mg, 0.4mmol) in dry DCM (2mL) at 0°C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 24h. The resulting suspension was evaporated and chromatographed on silica gel (5% EtOAc in PS) to give **30** (47.0mg, 87%) as an opaque white solid, m.p. 133-135°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.91 (s, 3H, OCH_3), 6.57 (d, *J* = 3.8, 0.9 Hz, 1H, H-3), 7.02 (dt, *J* = 9.4, 2.6 Hz, 2H, H-3′), 7.09 (td, *J* = 9.1, 2.6 Hz, 1H, H-6), 7.25 (dd, *J* = 8.5, 2.3 Hz, 1H, H-4), 7.40 (d, *J* = 3.8 Hz, 1H, H-2), 7.74 (dt, *J* = 9.7, 2.9 Hz, 2H, H-2′), 8.32 (ddd, *J* = 9.3, 4.8, 0.6 Hz, 1H, H-7). ^{13}C NMR (75MHz, $CDCl_3$): δ 55.5 (OCH_3), 106.3 (d, *J* = 23.9 Hz, C4), 107.7 (d, *J* = 3.7 Hz, C3), 112.5 (d, *J* = 25.0 Hz, C6), 113.9 (2C, C3′), 117.2 (d, *J* = 9.2 Hz, C7), 126.2 (C1′), 129.2 (C2), 131.5 (C3a), 131.7 (2C, C2′), 132.5 (C7a), 159.7 (d, *J* = 239.9 Hz, C5), 162.8 (C4′), 168.0 (CO). HRMS (EI): *m/z* calcd for $C_{16}H_{12}NO_2F$ $[M]^+$: 269.0852; found: 269.0854

7.5.2.12 Preparation of 5-Fluoro-1-(2-methoxybenzoyl)- 1*H*-indole (31)



31

To a solution of 5-fluoro-1*H*-indole (27.0mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and *p*-methoxybenzoic acid (60.9mg, 0.4mmol) in dry DCM (2mL) at 0°C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 3h. The resulting

suspension was evaporated and chromatographed on silica gel (5% EtOAc in PS) to give **31** (45.7mg, 85%) as a pale yellow solid, m.p. 100-101°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.79 (s, 3H, OCH_3), 6.50 (d, *J* = 3.8 Hz, 1H, H-3), 7.01 – 7.12 (m, 4H, H-3′, H-5′, H-2, H-6), 7.22 (dd, *J* = 8.2, 2.1 Hz, 1H, H-4) 7.44 (dd, *J* = 7.3, 2.3 Hz, 1H, H-6′), 7.51 (td, *J* = 8.5, 1.8 Hz, 1H, H-4′), 8.44 (bdd, *J* = 8.8, 4.7 Hz, 1H, H-7). ^{13}C NMR (75MHz, $CDCl_3$): δ 55.6 (OCH_3), 106.3 (d, *J* = 23.9 Hz, C4), 108.2 (d, *J* = 4.0 Hz, C3), 111.4 (C3′), 112.4 (d, *J* = 24.8 Hz, C6), 117.5 (d, *J* = 9.2 Hz, C7), 120.8 (C5′), 124.4 (C1′), 128.9 (C6′)[a], 129.1 (C2)[a], 131.9 (C3a)[b], 132.0 (C7a)[b], 132.3 (C4′), 156.3 (C2′), 159.8 (d, *J* = 240.1 Hz, C5), 167.0 (CO). HRMS (EI): *m/z* calcd for $C_{16}H_{12}NO_2F$ $[M]^+$: 269.0852; found: 269.0854.

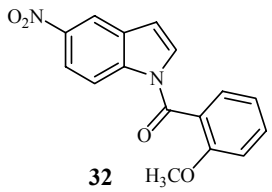
7.5.2.13 Preparation of 1-Benzoyl-5-nitro-1*H*-indole (18)

To a solution of 5-nitro-1*H*-indole (**17**) (32.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and benzoic acid (48.8mg, 0.4mmol) in dry DCM (2mL) at 0^{o}C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 6h. The resulting suspension was dried and to the crude residue was added MeOH (20mL). The suspension was filtered and the precipitate then washed with cold MeOH and dried to give **18** (40.4mg) as an opaque white solid. The filtrate was concentrated and recrystallized from MeOH to yield further **18** (8.0mg) for a total yield of pure product of 48.4 mg (92%). All spectroscopic data for **18** was the same as that noted for this compound previously (Section 6.5.2.1).

7.5.2.14 Preparation of 1-(4-Methoxybenzoyl)-5-nitro-1*H*-indole (19)

To a solution of **17** (32.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and *p*-methoxy benzoic acid (60.9mg, 0.4mmol) in dry DCM (2mL) at 0°C under a nitrogen atmosphere, was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 4h. The resulting suspension was dried and to the crude residue was added MeOH (20mL). The suspension was filtered and the precipitate then washed with cold MeOH and dried to give **19** (45.7mg) as a pale yellow solid. The filtrate was concentrated and recrystallized from MeOH to yield further **19** (10.4mg) for a total yield of pure product of 56.1mg (95%), m.p. 199-200°C. All spectroscopic data for **19** was the same as that noted for this compound previously (Section 6.5.2.2).

7.5.2.15 Preparation of 1-(2-Methoxybenzoyl)-5-nitro-1*H*-indole (32)



32

To a solution of **17** (32.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and *o*-methoxybenzoic acid (60.9mg, 0.4mmol) in dry DCM (2mL) at 0°C under a nitrogen atmosphere, which was added a solution of DCC (82.5mg, 0.4mmol) in DCM (1mL). The solution was then warmed to room temperature and stirred for 3h. The resulting suspension was dried *in vacuo* and to the crude residue was added MeOH (20mL). The suspension was filtered and the precipitate then washed with MeOH and dried to give **32** (38.1mg) as a slight yellow solid. The filtrate was concentrated and recrystallized from MeOH to yield further **32** (9.2mg) for a total yield of pure product of 47.3mg (80%), m.p. 144-145°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.71 (s, 3H, OCH_3), 6.61 (d, J = 3.5 Hz, 1H, H-3), 6.98 (d, J = 8.5 Hz, 1H, H-3′), 7.05 (t, J = 7.5 Hz, 1H, H-5′), 7.17 (d, J = 3.5 Hz, 1H, H-2),

7.41 (d, J = 7.3 Hz, 1H, H-6′), 7.49 (t, J = 7.6 Hz, 1H, H-4′), 8.17 (t, J = 9.1 Hz, 1H, H-6), 8.41-8.46 (m, 2H, H-4, H-7). ^{13}C NMR (75MHz, $CDCl_3$): δ 55.7 (OCH_3), 108.7 (C3), 111.5 (C3′), 116.5 (C7), 116.9 (C4), 120.1 (C6), 121.0 (C5′), 123.5 (C1′), 129.4 (C6′), 130.3 (C2), 130.9 (C3a), 133.0 (C4′), 138.5 (C7a), 144.3 (C5), 156.4 (C2′), 167.2 (CO). HRMS (EI): *m/z* calcd for $C_{16}H_{12}N_2O_4$ $[M]^+$: 296.0797; found: 296.0804.

7.5.2.16 Preparation of 5-nitro-1-phenylethanoyl-1*H*-indole (23)

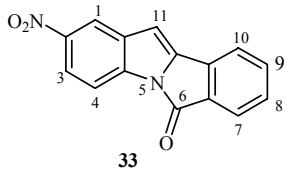
A mixture of **17** (320.6mg, 1.98mmol), phenyl acetic acid (299.0mg, 2.20mmol), and boric acid (37.3mg, 0.60mmol) in mesitylene (30mL) was heated at reflux for 2 days using a Dean-Stark water separator. The reaction mixture was evaporated, triturated with PS, filtered, and washed thoroughly with PS to remove the mesitylene. The crude solid was chromatographed on silica gel by VLC (10% EtOAc in PS) to give the starting indole (200.0mg, 1.23mmol), and **23** (9.0mg, 4%).

7.5.2.17 Preparation of 1-(4-benzyloxybenzoyl)-5-nitro-1*H*-indole (22)

To a solution of **17** (2.35g, 14.5mmol), DMAP (0.23g, 1.89mmol) and *p*-benzyloxybenzoic acid (**21**, 4.00g, 18.6mmol) in dry DCM (150mL) at 0^{o}C under a nitrogen atmosphere, was added a solution of DCC (3.89g, 18.6mmol) in DCM (50mL). The solution was then refluxed at 40^{o}C for 2 days. The resulting suspension was filtered and washed with DCM. The filtrate was evaporated and then chromatographed on silica gel by VLC (40% DCM in hexane) to give the starting indole (510.0mg, 3.15mmol) and **22** (2.80g, 52%).

7.5.3 Palladium Cyclization reactions

7.5.3.1 Preparation of 2-Nitro-isoindolo[2,1-*a*]indol-6-one (33)

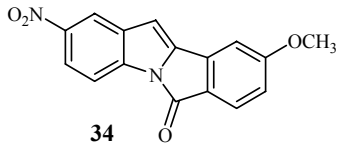


33

Method 1: A solution of **18** (200mg, 0.75mmol) and palladium (II) acetate (167.8mg, 0.75mmol) in glacial acetic acid (20mL) was heated at 110°C under a nitrogen atmosphere for 11h. The black suspension was filtered through celite and washed with acetone. The filtrate was then evaporated and added to ice water (200mL). The precipitate was filtered, dried and chromatographed on silica gel by VLC (40-60% DCM in PS) to give **33** (100.1mg, 50%) as yellow needles, m.p. 268.8°C (decomp.). ^{1}H NMR (300MHz, $CDCl_3$): δ 6.79 (d, *J* = 0.6 Hz, 1H, H-11), 7.43-7.49 (ddd, *J* = 8.4, 5.9, 2.6 Hz, 1H, H-9) 7.62-7.65 (m, 2H, H-10, H-8), 7.84 (dt, *J* = 7.5, 0.9 Hz, 1H, H-7), 7.98 (d, *J* = 8.7 Hz, 1H, H-4), 8.23 (dd, *J* = 8.7, 2.1 Hz, 1H, H-3), 8.41 (d, *J* = 2.1 Hz, 1H, H-1). ^{13}C NMR (125MHz, DMSO-d_6): δ 104.2 (C11), 112.6 (C4), 118.8 (C1), 121.8 (C3), 122.8 (C7), 125.6 (C10), 130.2 (C9)[a], 132.5 (C10a), 133.8 (C11a), 134.6 (C6a), 135.1 (C8)[a], 136.1 (C4a), 141.1 (C10b), 143.9 (C2), 162.0 (C6). HRMS (EI); *m/z* calcd for $C_{15}H_8N_2O_3$ $[M]^+$: 264.0535; found: 264.0533.

Method 2: A solution of **18** (103.7mg, 0.38mmol), palladium (II) acetate (8.5mg, 0.038mmol) and copper (II) acetate monohydrate (180mg, 0.99mmol) in glacial acetic acid (6mL) was heated at 95°C for 5 days. The reaction was worked up as in *method 1* to give **33** (30.2mg, 29%). All spectroscopic data were the same as those reported above.

7.5.3.2 Preparation of 9-Methoxy-2-nitro-isoindolo[2,1-*a*]indol-6-one (34)



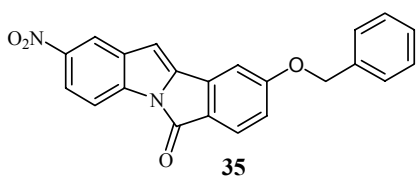
34

A solution of **19** (379mg, 1.28mmol) and palladium (II) acetate (287.3mg, 1.28mmol) in glacial acetic acid (80mL) was heated at 110°C under a nitrogen atmosphere for 16h. The black suspension was filtered through celite and washed with acetone. The filtrate was then evaporated and added to ice water (200mL). The precipitate was filtered, dried and chromatographed on silica gel by VLC (40–60% DCM in PS) to give **34** (279.4mg, 74%) as yellow needles, m.p. 238-240°C. ^{1}H NMR (300MHz, $CDCl_3$ + CD_3OD): δ 3.95 (s, 3H, OCH_3), 6.77 (s, 1H, H-11), 6.91 (dd, *J* = 8.7, 2.1 Hz, 1H, H-8), 7.11 (d, *J* = 2.7 Hz, 1H, H-10), 7.74 (d, *J* = 8.4 Hz, 1H, H-7), 7.95 (d, *J* = 8.7 Hz, 1H, H-4), 8.20 (dd, *J* = 8.7, 2.1 Hz, 1H, H-3), 8.40 (d, *J* = 2.1 Hz, 1H, H-1). ^{13}C NMR (75MHz, $CDCl_3$+CD_3OD): δ 55.8 (OCH_3), 103.0 (C11), 107.7 (C10), 112.6 (C4), 115.1 (C8), 118.3 (C1), 121.7 (C3), 125.0 (C10a), 127.5 (C7), 134.1 (C11a), 136.3 (C4a), 136.5 (C6a), 140.8 (C10b), 144.0 (C2), 162.3 (CO), 165.1 (C9). HRMS (EI); *m/z* calcd for $C_{16}H_{10}N_2O_4$ $[M]^+$: 294.0641; found: 294.0648.

7.5.3.3 Preparation of 9-Benzyloxy-2-nitro-isoindolo[2,1-*a*]indol-6-one (35)

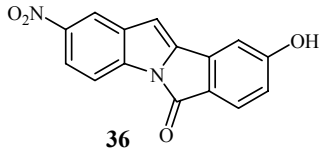
A solution of **22** (189.7mg, 0.51mmol) and palladium (II) acetate (120.7mg, 0.54mmol) in glacial acetic acid (34mL) was heated at 110°C under a nitrogen atmosphere for 20h. The black suspension was filtered through celite and then washed with acetone. The filtrate was then evaporated and added to ice water (100mL). The precipitate was filtered, dried and then chromatographed on silica gel by VLC (PS with gradient elution to DCM and then MeOH) to give **35** (35.0mg, 20%, eluent: 40-60% DCM in PS) as pale yellow needles, and the more polar fraction (eluent: 2% MeOH in

DCM) which gave two colors (gray and brown) in the same spot on TLC after exposure to iodine vapor. This fraction was added to 2% MeOH in DCM and then filtered to afford *9-hydroxy-2-nitro-isoindolo[2,1-a]indol-6-one* (**36**) (32.3mg, 22%) as a pale yellow solid, and the filtrate was evaporated to give *1-(4-hydroxybenzoyl)-5-nitro-1H-indole* (**37**) (50.1mg, 35%) as a pale yellow solid.



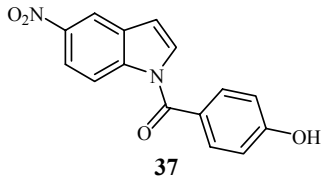
35

35: m.p. >250°C, ^{1}H NMR (300MHz, $CDCl_3$ + CD_3OD): δ 5.21 (s, 2H, OCH_2), 6.79 (s, 1H, H-11), 7.01 (dd, *J* = 8.7, 2.1 Hz, 1H, H-8), 7.22 (d, *J* = 2.1 Hz, 1H, H-10), 7.43-7.50 (m, 5H, ArH), 7.76 (d, *J* = 8.7 Hz, 1H, H-7), 7.97 (d, *J* = 8.7 Hz, 1H, H-6), 8.22 (dd, *J* = 8.7, 2.4 Hz, 1H, H-3), 8.42 (d, *J* = 2.1 Hz, 1H, H-1). ^{13}C NMR (75MHz, $CDCl_3$ + CD_3OD or DMSO-*d6*) spectrum showed only methine and methylene carbon signals without quaternary carbon signals due to precipitating whilst the experiment was underway. HRMS (EI); *m/z* calcd for $C_{22}H_{14}N_2O_4$ $[M]^+$: 370.0954; found: 370.0944.



36

36: m.p. 199-201°C, ^{1}H NMR (300MHz, DMSO-*d6*): δ 6.82 (dd, *J* = 8.6, 2.3 Hz, 1H, H-8), 7.07 (s, 1H, H-11), 7.16 (d, *J* = 2.1 Hz, 1H, H-10), 7.60 (d, *J* = 8.7 Hz, 1H, H-7), 7.81 (d, *J* = 9.0 Hz, 1H, H-4), 8.13 (dd, *J* = 8.7, 2.4 Hz, 1H, H-3), 8.45 (d, *J* = 1.8 Hz, 1H, H-1). ^{13}C NMR (75MHz, DMSO-*d6*): δ 103.6 (C11), 109.6 (C10), 112.1 (C4), 116.7 (C8), 118.6 (C1), 121.5 (C3), 122.7 (C10), 127.6 (C7), 134.1 (C11a), 135.9 (C4a), 136.2 (C6a)[a], 140.6 (C10b)[a], 143.4 (C2), 161.6 (CO), 163.9 (C9). HRMS (EI); *m/z* calcd for $C_{15}H_8N_2O_4$ $[M]^+$: 280.0484; found: 280.0480.



37

37: m.p. 201-203°C, ^{1}H NMR (300MHz, DMSO-*d6*): δ 6.92 (d, *J* = 3.3 Hz, 1H, H-3), 6.91-6.93 (m, 2H, aromatic), 7.64-7.67 (m, 2H, ArH), 7.73 (d, *J* = 3.3 Hz, 1H, H-2), 8.17 (dd, *J*

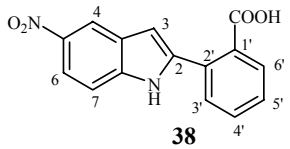
= 9.3, 2.4 Hz, 1H, H-6), 8.27 (d, *J* = 9.0 Hz, 1H, H-7), 8.60 (d, *J* = 1.8 Hz, 1H, H-4), 10.53 (br s, 1H, OH). ^{13}C NMR (75 MHz, DMSO-*d6*): δ 108.1 (C3), 115.6 (2C, ArCH), 115.8 (C7), 117.3 (C1′), 119.3 (C6), 123.0 (C4), 130.3 (C2), 131.7 (C3a), 132.5 (2C, ArCH), 138.6 (C7a), 143.4 (C5), 162.0 (C4′), 167.6 (CO). HRMS (EI); *m/z* calcd for $C_{15}H_{10}N_2O_4$ $[M]^+$: 282.0641; found: 282.0638.

7.5.3.4 Conversion of 36 to 35:

To a suspension of **36** (200.0mg, 0.71mmol) and cesium carbonate (230.0mg, 0.70mmol) in dry DMF (20mL), was added dropwise with stirring of benzyl bromide (0.1mL, 0.84mmol). The reaction was heated at 80°C under a nitrogen atmosphere for 7h. The reaction was allowed to cool to room temperature and then filtered. The precipitate was washed thoroughly with water and dried to give **35** as a pale yellow solid (196.1mg, 74%).

7.5.4 Ring opening reactions

7.5.4.1 Preparation of 2-(5-Nitro-1*H*-indol-2-yl)benzoic acid (38)

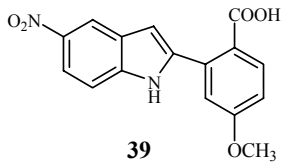


38

A solution of *t*-BuOH (192mL) and H_2O (19.2mL) containing *t*-BuOK (4.25 g, 37.9mmol) was added to **33** (1g, 3.79mmol) and heated at 82°C for 12h. The mixture was evaporated and added to ice water (400mL). The solution was acidified to pH1 with 5M HCl. The solution was stirred vigorously with solid NaCl for 2h, and then extracted with diethyl ether (3 x 400mL). The combined diethyl ether extracts were dried and evaporated to give **38** (1.016g, 95%) as a pale yellow solid, m.p. 250-252°C. ^{1}H NMR (300MHz, DMSO-d_6): δ 6.98 (s, 1H, H-3), 7.33-7.45 (m, 2H, H-4′, H-5′), 7.52 (d, *J* =

9.0 Hz, 1H, H-7), 7.67 (br.d, *J* = 7.2 Hz, 1H, H-3′), 7.75 (br.d, *J* = 7.5 Hz, 1H, H-6′), 7.94 (dd, *J* = 9.03, 2.4 Hz, 1H, H-6), 8.52 (d, *J* = 2.4, 1H, H-4). ^{13}C NMR (75MHz, DMSO-d_6): δ 102.2 (C3), 117.8 (C7), 116.3 (C6), 116.8 (C4), 127.5 (C3a), 127.8 (C5′), 128.5 (C2), 128.7 (C4′), 128.9 (C6′), 129.9 (C3′), 138.5 (C2′), 139.3 (C7a), 140.5 (C5), 142.2 (C1′), 171.7 (CO). HRMS (EI); *m/z* calcd for $C_{15}H_{10}N_2O_4$ $[M]^+$: 282.0641; found: 282.0643.

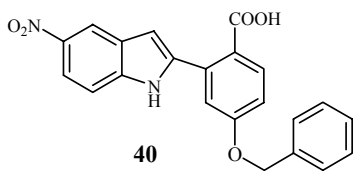
7.5.4.2 Preparation of 4-Methoxy-2-(5-nitro-1*H*-indol-2-yl)benzoic acid (39)



39

A solution of *t*-BuOH (88.5mL) and H_2O (8.9mL) containing *t*-BuOK (1.98g, 17.7mmol) was added to **34** (520.0mg, 1.77mmol) and heated at 82°C for 12h. The reaction mixture was evaporated and added to ice water (200mL). The solution was acidified to pH1 with 5M HCl. The solution was stirred vigorously with solid NaCl for 2h, and then extracted with diethyl ether (3 x 200mL). The combined diethyl ether extracts were dried and evaporated to give **39** (520.0g, 94%) as a pale yellow solid, m.p. 206-208°C. ^{1}H NMR (300MHz, DMSO-d_6): δ 3.86 (s, 3H, OCH_3), 6.78 (dd, *J* = 2.1, 0.6 Hz, 1H, H-3), 7.08 (dd, *J* = 7.8, 2.7 Hz, 1H, H-5′), 7.11 (s, 1H, H-3′), 7.50 (d, *J* = 8.7 Hz, 1H, H-7), 7.89 (d, *J* = 7.8 Hz, 1H, H-6′), 8.00 (dd, *J* = 9.0, 2.4 Hz, 1H, H-6), 8.55 (d, *J* = 2.4 Hz, 1H, H-4), 12.07 (s, 1H, NH). ^{13}C NMR (75MHz, DMSO-d_6): δ 55.7 (OCH_3), 103.3 (C3), 111.5 (C7), 114.0 (C5′), 116.4 (C3′), 116.7 (C6), 117.1 (C4), 123.8 (C2), 127.4 (C3a), 132.3 (C6′), 134.1 (C1′)[a], 139.6 (C7a), 140.7 (C5), 141.5 (C2′)[a], 161.3 (C4′), 167.9 (CO). HRMS (EI); *m/z* calcd for $C_{16}H_{12}N_2O_5$ $[M]^+$: 312.0746; found: 312.0749.

7.5.4.3 Preparation of 4-Benzyloxy-2-(5-nitro-1*H*-indol-2-yl)-benzoic acid (40)

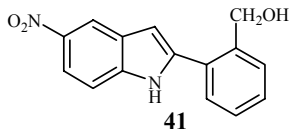


40

A solution of *t*-BuOH (22.5mL) and H_2O (2.3mL) containing *t*-BuOK (503.5mg, 4.49mmol) was added to **35** (166.0mg, 0.45mmol) and heated at 82°C for 12h. The reaction mixture was evaporated and added to ice water (70mL). The solution was acidified to pH1 with 5M HCl. The solution was stirred vigorously with solid NaCl for 2h, and then extracted with diethyl ether (3 x 50mL). The combined diethyl ether extracts were dried and evaporated to give **40** (167.0mg, 96%) as a pale yellow solid, m.p. 233-235°C. ^{1}H NMR (300MHz, DMSO-d_6): δ 5.24 (s, 2H, OCH_2), 6.78 (s, 1H, H-3), 7.16 (dd, *J* = 8.6, 2.6 Hz, 1H, H-5′), 7.24 (d, *J* = 2.7 Hz, 1H, H-3′), 7.30-7.50 (m, 5H, ArH), 7.52 (d, *J* = 9.0 Hz, 1H, H-7), 7.85 (d, *J* = 8.7, 1H, H-6′), 8.00 (dd, *J* = 8.9, 2.3 Hz, 1H, H-6), 8.55 (d, *J* = 2.4 Hz, 1H, H-4), 12.20 (s, 1H, NH), 12.57 (s, 1H, COOH). ^{13}C NMR (75MHz, DMSO-d_6): δ 69.6 (OCH_2), 103.2 (C3), 111.5 (C7), 114.6 (C5′), 116.6 (C6), 117.0 (C3′), 117.1 (C4), 124.1 (C2), 127.3 (C3a), 127.7 (2C, ArCH), 128.0 (ArCH), 128.4 (2C, ArCH), 132.1 (C6′), 133.8 (C2′), 136.4 (ArC), 139.6 (C7a), 140.6 (C5), 141.1 (C1′), 160.2 (C5′), 167.9 (CO). HRMS (EI); *m/z* calcd for $C_{22}H_{16}N_2O_5$ $[M]^+$: 388.1059; found: 388.1069.

7.5.5 Reduction reactions

7.5.5.1 Preparation of [2-(5-Nitro-1*H*-indol-2-yl)-phenyl]-methanol (41)

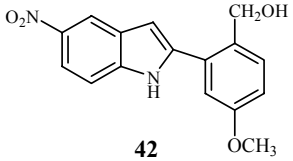


41

To a solution of **38** (997.0mg, 3.54mmol) in dry THF (90mL) was slowly added 1M BH_3-THF complex solution (7.1mL, 7.1mmol) at 0°C under a nitrogen atmosphere. After

vigorous stirring at room temperature for 2h, the excess hydride was carefully destroyed by adding a solution of 50% THF in H_2O (20mL) until the gas bubbling ceased. The aqueous layer was saturated with anhydrous K_2CO_3. The THF layer was separated and the aquoeus layer was extracted with diethyl ether. The combined THF and diethyl ether extracts were dried, then evaporated, and chromatographed on silica gel by VLC (2% MeOH in DCM) to give **41** (940.5mg, 99%) as bright yellow needles, m.p.132-134^{o}C. ^{1}H NMR (300MHz, $CDCl_3$): δ 4.77 (s, 2H, CH_2O), 6.91 (d, *J* = 1.2 Hz, 1H, H-3), 7.36-7.47 (m, 4H, H-7, ArH), 7.79 (br.d, *J* = 7.8 Hz, 1H, ArH), 8.12 (dd, *J* = 8.9, 2.4 Hz, 1H, H-6), 8.61 (d, *J* = 2.4 Hz, 1H, H-4), 10.92 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 65.1 (CH_2), 103.5 (C3), 111.2 (C7), 117.5 (C6), 117.6 (C4), 127.8 (C3a), 128.8 (ArCH), 129.4 (ArCH), 130.3 (ArCH), 131.1 (ArCH), 132.8 (C2), 135.8 (C2′), 139.6 (C7a), 141.4 (C1′), 141.8 (C5). HRMS (EI); *m/z* calcd for $C_{15}H_{12}N_2O_3$ $[M]^+$: 268.0848; found: 268.0838.

7.5.5.2 Preparation of [4-Methoxy-2-(5-nitro-1*H*-indol-2-yl)-phenyl]-methanol (42)

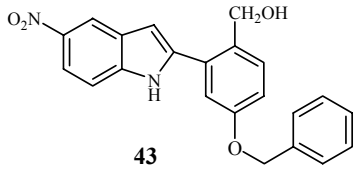


42

To a solution of **39** (520.0mg, 1.67mmol) in dry THF (50mL) was slowly added 1M BH_3-THF complex solution (3.3mL, 3.3mmol) at 0^{o}C under a nitrogen atmosphere. After vigorous stirring at room temperature for 2h, the excess hydride was carefully destroyed by adding a solution of 50% THF in H_2O (10mL) until the gas bubbling ceased. The aqueous layer was saturated with anhydrous K_2CO_3. The THF layer was separated and the aquoeus layer was extracted with diethyl ether. The combined THF and diethyl ether extracts were dried, then evaporated, and

chromatographed on silica gel by VLC (2% MeOH in DCM) to give **42** (470.7mg, 95%) as bright yellow needles, m.p. 206-208°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.89 (s, 3H, OCH_3), 4.70 (s, 2H, CH_2), 6.91 (d, *J* = 1.8 Hz, 1H, H-3), 6.92 (dd, *J* = 8.4, 2.7 Hz, 1H, H-5′), 7.30 (d, *J* = 2.7 Hz, 1H, H-3′), 7.34 (d, *J* = 8.7 Hz, 1H, H-6′), 7.46 (d, *J* = 8.7 Hz, 1H, H-7), 8.10 (dd, *J* = 8.7, 2.1 Hz, 1H, H-6), 8.62 (d, *J* = 2.1 Hz, 1H, H-4), 11.02 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 55.5 (OCH_3), 64.6 (CH_2O), 103.5 (C3), 111.3 (C7), 113.9 (C5′), 115.7 (C3′), 117.6 (C6), 117.7 (C4), 127.7 (C3a), 128.3 (C2), 132.6 (C6′), 134.2 (C1′)[a], 139.6 (C2′)[a], 141.4 (C7a), 141.8 (C5), 160.1 (C4′). HRMS (EI); *m/z* calcd for $C_{16}H_{14}N_2O_4$ $[M]^+$: 298.0954; found: 298.0941.

7.5.5.3 Preparation of [4-Benzyloxy-2-(5-nitro-1*H*-2-yl)-phenyl]-methanol (43)



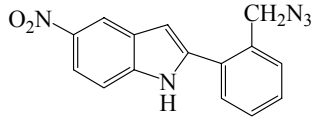
43

To a solution of **40** (200.0mg, 0.52mmol) in dry THF (15mL) was slowly added 1M BH_3-THF complex solution (1.0mL, 1.0mmol) at 0°C under a nitrogen atmosphere. After vigorous stirring at room temperature for 2h, the excess hydride was carefully destroyed by adding a solution of 50% THF in H_2O (2mL) until the gas bubbling ceased. The aqueous layer was saturated with anhydrous K_2CO_3. The THF layer was separated and the aquoeus layer was extracted with diethyl ether. The combined THF and diethyl ether extracts were dried, then evaporated, and chromatographed on silica gel by VLC (2% MeOH in DCM) to give **43** (163.5mg, 85%) as bright yellow needles, m.p. 70-72°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 4.63 (s, 2H, C**H**$_2$OH), 5.08 (s, 2H, OCH_2), 6.80 (dd, *J* = 2.0, 0.8 Hz, 1H, H-3), 6.90 (dd, *J* = 8.4, 2.7 Hz, 1H, H-5′), 7.23-7.42 (m, 8H, H-7, H-3′, H-6′, ArH), 8.03 (dd, *J* = 9.0, 2.1 Hz,

1H, H-6), 8.54 (d, *J* = 2.4 Hz, 1H, H-4), 10.92 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 64.6 (CH_2OH), 70.2 (OCH_2), 103.5 (C3), 111.3 (C7), 114.7 (C5′), 116.7 (C6′), 117.6 (C6), 117.7 (C4), 112.4 (2C, ArCH), 127.7 (C2), 128.2 (ArCH), 128.5 (C3a), 128.7 (2C, ArCH), 132.6 (C3′), 134.2 (ArC), 136.4 (C2′), 139.6 (C7a), 141.3 (C5), 141.9 (C1′), 159.3 (C4′). HRMS (EI); *m/z* calcd for $C_{22}H_{18}N_2O_4$ $[M]^+$: 374.1267; found: 374.1256.

7.5.6 Amination reactions

7.5.6.1 Preparation of 2-(2-Azidomethyl-phenyl)-5-nitro-1*H*-indole (44)

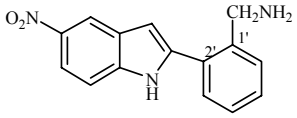


44

A mixture of **41** (285.0mg, 1.06mmol), sodium azide (75.5mg, 1.16mmol) and triphenyl phosphine (546.0mg, 2.08mmol) in a solution of 25% CCl_4 in DMF (10mL) was heated at 90°C under a nitrogen atmosphere for 5h. The reaction mixture was then cooled to room temperature, quenched by adding H_2O (10mL), and stirred for 10min. The mixture was diluted with diethyl ether (40mL) and washed thoroughly with H_2O. The organic layer was dried, then concentrated, and chromatographed on silica gel by VLC (30% DCM in PS) to give **44** (231.0mg, 74%) as bright yellow needles, m.p. 146-148°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 4.40 (s, 2H, CH_2N_3), 6,86 (dd, *J* = 2.1 Hz, 0.6 Hz, 1H, H-3), 7.44-7.54 (m, 4H, H-7, ArH), 7.69 (dd, *J* = 6.2, 1.7 Hz, 1H, ArH), 8.13 (dd, *J* = 9, 2.1 Hz, 1H, H-6), 8.61 (d, *J* = 2.1 Hz, 1H, H-4), 9.53 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$,): δ 54.0 (CH_2N_3), 104.7 (C3), 111.1 (C7), 117.8 (C6), 117.9 (C4), 127.8 (C3a), 129.2 (ArCH), 129.5 (ArCH), 130.7 (ArCH), 131.2 (ArCH), 132.1 (C2), 132.3 (C1′), 139.4 (C7a), 140.0 (C2′), 142.1 (C5). HRMS (CI); *m/z* calcd for $C_{15}H_{12}N_5O_2$ $[MH]^+$: 294.0991; found: 294.0987.

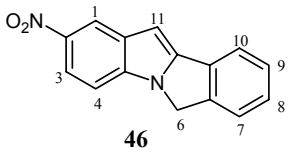
7.5.6.2 Preparation of 2-(5-Nitro-1*H*-indol-2-yl)-benzylamine (45)

Method 1: To a mixture of **44** (260.0mg, 0.89mmol) and sodium borohydride (25.0mg, 0.66mmol) in dry THF (10mL) heated at reflux, was slowly added MeOH (1.0mL) and the mixture stirred for 2h. Further sodium borohydride (25.0mg, 0.66mmol) was then added and stirring continued for 3 days. The mixture was cooled to room temperature and then 1M HCl (3mL) was added until the gas bubbling ceased. The mixture was basified to pH11 with a saturated NaOH solution and then extracted with DCM (3 x 20mL). The combined DCM extracts were dried, concentrated and then chromatographed on silica gel by VLC (PS by gradient elution to DCM, then MeOH and TEA) to give starting material **44** (70.2mg, 0.24mmol; eluent: 30% DCM in PS), *2-nitro-6H-isoindolo[2,1-a]indole* **46** (30.1mg, 14%; eluent: 40% DCM in PS) as a yellow solid, and the *benzylamine* **45** (70.6 mg, 30%; eluent: 30:1:2 DCM/MeOH/TEA) as a brown solid.



45

45: m.p. 127-129°C, ^{1}H NMR (300MHz, $CDCl_3$): δ 3.99 (s, 2H, CH_2), 6.87 (br.s, 1H, H-3), 7.26-7.46 (m, 4H, H-7, ArH), 7.78 (d, *J* = 7.5 Hz, 1H, ArH), 8.08 (dd, *J* = 8.7, 2.4 Hz, 1H, H-6), 8.61 (d, *J* = 2.1 Hz, 1H, H-4), 13.63 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 46.0 (CH_2), 102.5 (C3), 111.2 (C7), 117.1 (C6), 117.6 (C4), 127.9 (C3a), 128.5 (ArCH), 128.7 (ArCH), 130.3 (ArCH), 131.4 (ArCH), 133.1 (C2), 136.4 (C2′), 139.6 (C7a), 141.4 (C5), 142.7 (C1′). HRMS (EI); *m/z* calcd for $C_{15}H_{13}N_3O_2$ $[M]^+$: 267.1008; found: 267.0995.



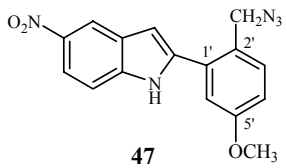
46

46: m.p. 234-236°C, ^{1}H NMR (300MHz, $CDCl_3$): δ 5.12 (s, 2H, H-6), 6.75 (s, 1H, H-11), 7.33 (d, *J* = 9.0 Hz, 1H, H-4), 7.38-7.51 (m, 3H, ArH), 7.74 (d, *J* = 7.2 Hz, 1H, ArH), 8.09 (dd, *J* = 9.0, 2.1 Hz, 1H, H-3), 8.58 (d, *J* = 2.1 Hz, 1H, H-1). ^{13}C NMR (75MHz, $CDCl_3$): δ 48.8

(C6), 93.6 (C11), 108.8 (C4), 117.3 (C3), 118.7 (C1), 121.6 (ArCH) 123.7 (ArCH), 128.3 (ArCH), 128.6 (ArCH), 132.0 (C10b), 136.6 (C11a), 141.3 (2C, C6a, C10a), 141.6 (C2), 147.1 (C4a). HRMS (EI); *m/z* calcd for $C_{15}H_{10}N_2O_2$ $[M]^+$: 250.0742; found: 250.0732.

Method 2: To a solution of **44** (99.6mg, 0.34mmol), TEA (0.09mL, 0.68mmol), and 2 drops of 1,3-propanedithiol (*ca.* 0.1mL, 1.0mmol) in 35% MeOH in *i*-PrOH (12mL), was added sodium borohydride (128.6mg, 3.4mmol) at 0°C. After 2h, more sodium borohydride (64mg, 1.7mmol) was added and the mixture stirred for a further 10min to complete the reaction. The reaction mixture was evaporated, then added to H_2O (50mL) and extracted with 40% Et_2O in PS (2 x 40mL). The aqueous layer was basified to pH11 with a saturated NaOH solution and extracted with DCM (3 x 50mL). The combined DCM extract was dried, concentrated and then chromatographed on silica gel by VLC (4% MeOH in DCM) to give the amine **45** (81.6mg, 90%) as a brown yellow solid after washing with 40% diethyl ether in PS to remove traces of 1,3-propanedithiol.

7.5.6.3 Preparation of 2-(2-Azidomethyl-5-methoxyphenyl)-5-nitro-1*H*-indole (47)

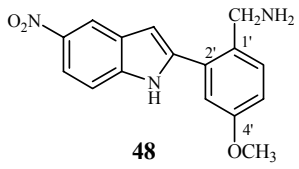


47

A mixture of **42** (298.0mg, 1.0mmol), sodium azide (65.0mg, 1.0mmol) and triphenylphosphine (524.6mg, 2.0mmol) in a solution of 25% CCl_4 in DMF (9mL) was heated to 90°C under a nitrogen atmosphere for 5h. The reaction mixture was cooled to room temperature, quenched by adding H_2O (10mL), and stirred for 10min. The mixture was diluted with diethyl ether (40mL) and washed thoroughly with H_2O. The organic layer

was dried, concentrated, and chromatographed on silica gel by VLC (40% DCM in PS) to give **47** (198.0mg, 61%) as bright yellow needles, m.p. 138-140°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.79 (s, 3H, OCH_3), 4.26 (s, 2H, CH_2), 6.78 (dd, *J* = 2.1, 0.9 Hz, 1H, H-3), 6.89 (dd, *J* = 8.7, 2.7 Hz, 1H, H-4′), 7.12 (d, *J* = 2.7 Hz, 1H, H-6′), 7.29 (d, *J* = 8.7 Hz, 1H, H-3′), 7.37 (d, *J* = 8.4 Hz, 1H, H-7), 8.03 (dd *J* = 9.0, 2.1 Hz, 1H, H-6), 8.52 (d, *J* = 2.1 Hz, 1H, H-4), 9.77 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 53.6 (CH_2), 55.5 (OCH_3), 104.6 (C3), 111.2 (C7), 114.2 (C5′), 116.1 (C6′), 117.7 (C4), 117.9 (C6), 124.6 (C2), 127.7 (C3a), 132.6 (C3′), 133.5 (C1′)[a], 139.5 (C7a), 140.0 (C2′)[a], 142.3 (C5), 160.1 (C5′). HRMS (EI); *m/z* calcd for $C_{16}H_{13}N_5O_3$ $[M]^+$: 323.1018; found: 323.1024.

7.5.6.4 Preparation of 4-Methoxy-2-(5-nitro-1*H*-indol-2-yl)-benzylamine (48)



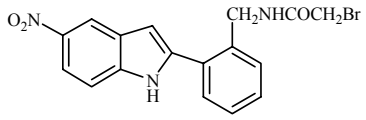
48

To a solution of **47** (109.0mg, 0.34mmol), TEA (0.09mL, 0.68mmol), and 2 drops of 1,3-propanedithiol (*ca.* 0.1mL, 1.0mmol) in *i*-PrOH (9mL) and MeOH (3mL), was added sodium borohydride (128.6mg, 3.4mmol) at 0°C. After 2h, more sodium borohydride (64mg, 1.7mmol) was added and stirring continued for 10min. The reaction mixture was evaporated, then added to H_2O (50mL) and extracted with 40% diethyl ether in PS (2 x 40mL). The aqueous layer was basified to pH11 with a saturated NaOH solution and extracted with DCM (3 x 50mL). The combined DCM extracts were dried, concentrated and then chromatographed on silica gel by VLC (4% MeOH in DCM) to give **48** (91.0mg, 91%) as a yellow solid after washing with 40% diethyl ether in PS to remove traces of 1,3-propanedithiol, m.p. 170-178°C. ^{1}H NMR

(300MHz, $CDCl_3$): δ 3.86 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2), 6.84 (br.s, 1H, H-3), 6.86 (dd, *J* = 7.9, 2.6 Hz, 1H, H-5′), 7.23 (d, *J* = 8.1 Hz, 1H, H-6′), 7.28 (s, 1H, H-6′), 7.40 (d, *J* = 9.3 Hz, 1H, H-7), 8.06 (dd, *J* = 9.3, 2.4 Hz, 1H, H-6), 8.59 (d, *J* = 2.1 Hz, 1H, H-4), 13.66 (s, 1H, NH). ^{13}C NMR (75MHz, DMSO-d_6): δ 42.5 (CH_2), 55.3 (OCH_3), 103.4 (C3), 111.9 (C7), 114.1 (C5′), 114.6 (C3′), 116.7 (C6), 117.0 (C4), 127.6 (C3a), 129.0 (C2), 131.8 (C6′), 132.9 (C1′)[a], 139.6 (C2′)[a], 140.7 (C5), 141.4 (C7a), 158.6 (C4′). HRMS (EI); *m/z* calcd for $C_{16}H_{15}N_3O_3$ $[M]^+$: 297.1113; found: 297.1101.

7.5.7 *N*-Alkylation reactions

7.5.7.1 Preparation of 2-Bromo-*N*-[2-(5-nitro-1*H*-indol-2-yl)benzyl]-acetamide (49)

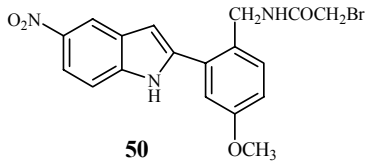


49

To a solution of **45** (70.6mg, 0.26mmol) and TEA (0.17mL, 1.2mmol) in dry DCM (4mL), was added bromoacetyl chloride (0.1mL, 1.2mmol) at 0°C under a nitrogen atmosphere, and stirred for 15min. The reaction mixture was then added to H_2O (20mL) and extracted with DCM (3 x 20mL). The combined DCM extracts were dried, concentrated and then chromatographed on silica gel (60% DCM in PS) to give **49** (63.8mg, 62%) as a yellow solid, m.p.181-183°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.85 (s, 2H, CH_2Br), 4.45 (d, *J* = 6.6 Hz, 2H, CH_2N), 6.65 (d, *J* = 1.5 Hz, 1H, H-3), 7.28-7.50 (m, 6H, H-7, NHCO, ArH), 8.02 (dd, *J* = 8.9, 2.3 Hz, 1H, H-6), 8.51 (d, *J* = 2.4 Hz, 1H, H-4), 11.36 (s, 1H, NH-indole). ^{13}C NMR (75MHz, $CDCl_3$): δ 28.7 (CH_2Br), 42.3 (CH_2N), 104.4 (C3), 111.3 (C7), 117.5 (2C, C-4, C-6), 127.9 (C3a), 128.3 (ArCH), 129.3 (ArCH), 129.5 (ArCH), 131.1 (ArCH), 131.6 (C2), 134.7 (C2′),

139.7 (C7a), 140.9 (C1′), 141.7 (C5), 167.2 (CO). HRMS (EI); *m/z* calcd for $C_{17}H_{14}N_3O_3Br$ $[M]^+$: 389.0198; found: 389.0197.

7.5.7.2 Preparation of 2-Bromo-*N*-[4-methoxy-2-(5-nitro-1*H*-indol-2-yl)-benzyl)-acetamide (50)

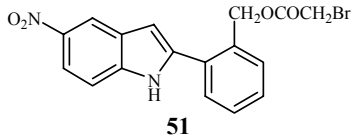


50

To a solution of **48** (80mg, 0.27mmol) and TEA (0.1mL, 0.27mmol) in dry DCM (8mL), was added bromoacetyl chloride (0.05mL, 0.60mmol) at 0°C under a nitrogen atmosphere for 15min. The reaction mixture was then added to H_2O (10mL) and extracted with DCM (3 x 10mL). The DCM extracts were dried, concentrated and then chromatographed on silica gel (50% DCM in PS) to give **50** (72.0mg 64%) as a yellow solid, m.p.176-179°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.84 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2Br), 4.44 (d, *J* = 6 Hz, 2H, CH_2), 6.72 (dd, *J* = 2.1, 0.8 Hz, 1H, H-3), 6.96 (dd, *J* = 8.4, 3.0 Hz, 1H, C-4′), 7.04 (d, *J* = 2.7 Hz, 1H, C-6′), 7.30 (d, *J* = 8.4 Hz, 1H, H-3′), 7.33 (br s, 1H, NHCO), 7.47 (d, *J* = 9.0 Hz, 1H, H-7), 8.09 (dd, *J* = 9.0, 2.4 Hz, 1H, H-6), 8.58 (d, *J* = 2.4 Hz, H-4), 11.52 (s, 1H, NH-indole). ^{13}C NMR (75MHz, $CDCl_3$): δ 28.8 (CH_2Br), 41.9 (CH_2N), 55.5 (OCH_3), 104.4 (C3), 111.3 (C7), 115.5 (C6′), 115.6 (C4′), 117.6 (2C, C4, C6), 126.8 (C2), 127.8 (C3a), 131.0 (C3′), 132.9 (C1′)[a], 139.7 (C7a), 141.0 (C2′)[a], 141.9 (C5), 159.2 (C5′), 167.1 (CO). HMRS (EI); *m/z* calcd for $C_{18}H_{16}N_3O_4Br$ $[M^+]$: 417.0324; found; 417.0332.

7.5.8 *O*-Alkylation reactions

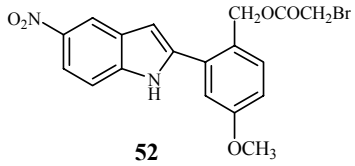
7.5.8.1 Preparation of Bromoacetic acid 2-(5-nitro-1*H*-indol-2-yl)-benzyl ester (51)



51

To a solution of **41** (200mg, 0.75mmol), anhydrous TEA (0.21mL, 1.5mmol) and dry THF (25mL), was slowly added a solution of bromoacetyl chloride (0.13mL, 1.5mmol) in THF (5mL) at 0°C under a nitrogen atmosphere and the mixture then heated at 50°C for 5h. The reaction mixture was then filtered and concentrated. The resulting residue was added to water (50mL), extracted with EtOAc (3 x 30mL) and washed with H_2O. The combined EtOAc extracts were dried and chromatographed on silica gel by VLC (20% EtOAc in PS) to give **51** (160.0mg, 70%) as yellow needles, m.p. 139-141°C. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.97 (s, 2H, CH_2Br), 5.31 (s, 2H, CH_2O), 6.83 (d, *J* = 1.8 Hz, 1H, H-3), 7.44-7.64 (m, 5H, H-7, ArH), 8.13 (dd, *J* = 9.0, 1.8 Hz, 1H, H-6), 8.62 (d, *J* = 1.8 Hz, 1H, H-4), 9.63 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 40.9 (CH_2Br), 66.4 (CH_2O), 104.9 (C3), 111.1 (C7), 117.7 (C6), 117.8 (C4), 127.8 (C3a), 129.1 (ArCH), 129.5 (ArCH), 130.3 (ArCH), 131.2 (ArCH), 132.2 (C2), 132.5 (C2´), 139.6 (C7a), 139.7 (C1´), 142.0 (C5), 167.0 (CO). HRMS (EI); *m/z* calcd for $C_{17}H_{13}N_2O_4Br$ $[M]^+$: 338.0059; found: 338.0056.

7.5.8.2 Preparation of Bromoacetic acid 4-methoxy-2-(5-nitro-1*H*-indol-2-yl)phenyl)-benzyl ester (52)



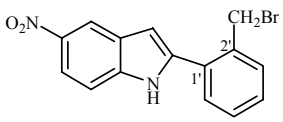
52

To a solution of **42** (220mg, 0.74mmol), anhydrous TEA (0.1mL, 0.74mmol) and dry DCM (20mL), was

slowly added a solution of bromoacetyl chloride (0.13mL, 1.5mmol) at 0°C under a nitrogen atmosphere, and the mixture then stirred for 15min. The reaction mixture was added to water (30mL), then extracted with DCM (3 x 30mL) and washed with H_2O. The combined DCM extracts were dried and chromatographed on silica gel by VLC (PS by gradient elution to DCM and then MeOH) to give the starting material **42** (31.1mg, 0.1mmol; eluent: 2% MeOH in DCM) and **52** (120.0mg, 39%; eluent: 60% DCM in PS) as an amorphous solid. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.81 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2CO), 5.17(s, 2H, CH_2O), 6.76 (dd, *J* = 2.1, 0.9 Hz, 1H, H-3), 6.91 (dd, *J* = 8.4, 2.7 Hz, 1H, H-5′), 7.06 (d, *J* = 2.7 Hz, 1H, H-3′), 7.40 (d, *J* = 9.3 Hz, 1H, H-7), 7.44 (d, *J* = 8.4 Hz, 1H, H-6′), 8.06 (dd, *J* = 9.0, 2.4 Hz, 1H, H-6), 8.54 (d, *J* = 2.4 Hz, 1H, H-4), 9.59 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 25.9 (CH_2Br), 55.5 (OCH_3), 66.6 (CH_2O), 104.9 (C3), 111.1 (C7), 114.6 (C5′), 115.8 (C6′), 117.8 (C4), 118.0 (C6), 124.2 (C2), 127.8 (C3a), 133.5 (C6′), 134.2 (C1′)[a], 139.5 (C7a), 140.0 (C2′)[a], 142.2 (C5), 160.3 (C4′), 166.8 (CO). HRMS (EI); *m/z* calcd for $C_{18}H_{15}N_2O_5Br$ $[M]^+$: 420.0144; found: 420.0151.

7.5.9 Bromination reactions

7.5.9.1 Preparation of 2-(2-Bromomethylphenyl)-5-nitro-1*H*-indole (53)

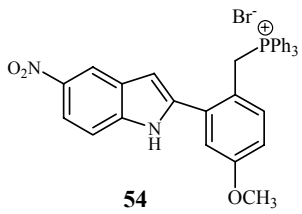


53

A yellow suspension of **41** (200mg, 0.75mmol), triphenylphosphine (390mg, 1.5mmol) and carbon tetrabromide (490mg, 1.5mmol) in dry diethyl ether (60mL) was stirred with warming at 40°C under a nitrogen atmosphere for 2 days. The reaction mixture was then filtered and the filtrate concentrated. The residual yellow oil was chromatographed on silica gel by VLC (20% EtOAc in PS) to give **53** (102.3mg, 41%) as a yellow solid,

m.p. 164-166°C. ^{1}H NMR (300MHz, $CDCl_3$,): δ 4.64 (s, 2H, CH_2Br), 6.93 (d, *J* = 1.2 Hz, 1H, H-3), 7.43-7.58 (m, 5H, H-7, ArH), 8.16 (dd, *J* = 9.0, 2.1 Hz, 1H, H-6), 8.64 (d, *J* = 2.1 Hz, 1H, H-4), 9.14 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 33.0 (CH_2Br), 105.1 (C3), 111.1 (C7), 117.8 (C4), 118.6 (C6), 127.9 (C3a), 129.4 (ArCH), 129.6 (ArCH), 130.5 (ArCH), 131.7 (ArCH), 131.8 (C2), 135.6 (C1′), 139.3 (C7a), 139.6 (C2′), 142.3 (C5). HRMS (EI); *m/z* calcd for $C_{15}H_{11}N_2O_2Br$ $[M]^+$: 330.0003; found: 329.9982.

7.5.9.2 Attempted bromination of the benzyl alcohol 42

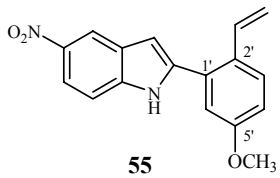


54

Method 1: A yellow suspension of **42** (510mg, 1.7mmol), triphenylphosphine (472mg, 1.8mmol) and carbon tetrabromide (597.0mg, 1.8mmol) in dry diethyl ether (80mL) was stirred at room temperature under a nitrogen atmosphere for 3 days. The reaction mixture was filtered and then concentrated. The residue was chromatographed on silica gel by VLC (1% MeOH in DCM) to give *[4-methoxy-2-(5-nitro-1H-indol-2-yl)-triphenyl-phosphonium bromide* (**54**) (500.0mg, 47%) as an orange solid. ^{1}H NMR (300MHz, $CDCl_3$,): δ 3.71 (s, 3H, OCH_3), 5.17 (d, *J* = 11.7 Hz, 2H, CH_2), 5.95 (s, 1H, H-3), 6.57 (d, *J* = 7.8 Hz, 1H, H-5′), 6.78 (d, *J* = 6.9 Hz, 1H, H-6′), 6.91 (br.s, 1H, H-3′), 7.18-7.97 (m, 15H, ArH), 7.80 (br.d, *J* = 9.3 Hz, 1H, H-7), 7.93 (br.d, *J* = 9.0 Hz, 1H, H-6), 8.30 (d, *J* = 1.5 Hz, 1H, H-4), 12.03 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$,): δ 29.2 (CH_2, very weak signal), 55.4 (OCH_3), 103.6 (C3), 112.5 (C7), 114.8 (C4′), 116.2 (d, *J* = 35.4 Hz, 3C, ArC), 116.4 (C2)[a], 116.8 (C6′), 116.9 (C4), 117.2 (C6), 117.5 (C1′)[a], 127.1 (C3a), 130.0 (d, *J* = 49.2 Hz, 6C, ArCH), 133.2 (C3′), 133.5 (d, *J* = 38.7 Hz, 6C, ArCH), 134.9 (3C, ArCH), 139.3 (C2′)[a], 140.0 (C7a),

141.4 (C5), 159.7 (C5′). HRMS (ES): *m/z* calcd for $C_{34}H_{28}N_2O_3P$ $[M]^+$: 543.1838; found: 543.1838.

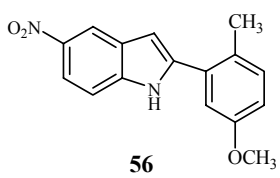
7.5.9.3 Preparation of 2-(5-Methoxy-2-vinylphenyl)-5-nitro-1*H*-indole (55)



55

To a solution of **54** (355.0mg, 0.57mmol) in DCM (20mL) was added 40mg/mL aqueous solution of formaldehyde (2mL, 2.66mmol). The reaction mixture was stirred vigorously, 0.1% aqueous NaOH (23mL, 0.57mmol) was added slowly over 10min at room temperature. Stirring of the reaction mixture was continued for 40min. The mixture was then washed sequentially with water (30mL), 1M HCl (30mL), water (30mL), and brine (30mL). The DCM layer was dried, evaporated and then chromatographed on silica gel (50% DCM in hexane) to give **55** (5.0mg, 3%) as a yellow solid and *2-(5-methoxy-2-methyl-phenyl)-5-nitro-1H-indole* (**56**) (82.7mg, 49%) as bright yellow needles.

55: m.p. 151-153°C, ^{1}H NMR (300MHz, $CDCl_3$,): δ 3.87 (s, 3H, OCH_3), 5.29 (dd, *J* = 11.0, 1.4 Hz, 1H, C=CH_2), 5.68 (dd, *J* = 17.3, 1.4 Hz, 1H, C=CH_2), 6.69 (dd, *J* = 2.1, 0.9 Hz, 1H, H-3), 6.88 (dd, *J* = 17.6, 11.0 Hz, 1H, C**H**=CH_2), 6.98 (dd, *J* = 8.4, 2.7 Hz, 1H, H-4′), 7.03 (d, *J* = 2.4 Hz, 1H, H-6′), 7.44 (d, *J* = 8.7 Hz, 1H, H-7), 7.58 (d, *J* = 8.4 Hz, 1H, H-3′), 8.13 (dd, *J* = 9.2, 2.3 Hz, 1H, H-6), 8.61 (d, *J* = 1.8 Hz, 2H, H-4, NH). ^{13}C NMR (75MHz, $CDCl_3$,): δ 55.5 (OCH_3), 105.2 (C3), 110.8 (C7), 114.2 (C6′), 115.0 (C4′), 115.3 (CH=**C**H_2), 117.7 (C4), 117.9 (C6), 127.9 (C3a), 128.4 (C3′), 129.4 (C2′)[a], 131.2 (C2), 134.8 (**C**H=CH_2), 139.1 (C7a), 140.1 (C1′)[a], 142.1 (C5), 159.3 (C5′). HRMS (EI); *m/z* calcd for $C_{17}H_{14}N_2O_3$ $[M]^+$: 294.1004; found: 294.0988.



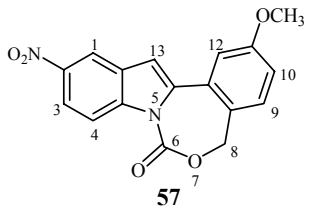
56

56: m.p. 194-196°C, ^{1}H NMR (300MHz, $CDCl_3$ + CD_3OD): δ

2.43 (s, 3H, CH_3), 3.90 (s, 3H, OCH_3), 6.75 (d, *J* = 0.9 Hz, 1H, H-3), 6.89 (dd, *J* = 8.5, 2.6 Hz, 1H, H-4′), 7.05 (d, *J* = 2.7 Hz, 1H, H-6′), 7.25 (d, *J* = 8.4 Hz, 1H, H-3′), 7.44 (d, *J* = 8.1 Hz, 1H, H-7), 8.10 (dd, *J* = 9.2, 2.3 Hz, 1H, H-6), 8.59 (d, *J* = 2.4 Hz, 1H, H-4). ^{13}C NMR (75MHz, $CDCl_3$ + CH_3OD): δ 20.0 (CH_3), 55.4 (OCH_3), 104.3 (C3), 110.7 (C7), 113.8 (C4′), 114.4 (C6′), 117.4 (C6), 117.5 (C4), 127.9 (C3a), 128.3 (C2), 132.1 (C3′), 132.3 (C2′)[a], 139.2 (C1′)[a], 140.7 (C7a), 141.6 (C5), 157.6 (C5′). HRMS (CI); *m/z* calcd for $C_{16}H_{15}N_2O_3$ $[MH]^+$: 283.1083; found: 283.1092.

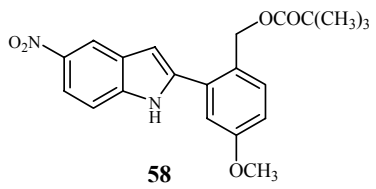
7.5.9.4 Attempted *N*-protection of the benzyl alcohol 42

Method 1: A mixture of **42** (20.0mg, 0.067mmol), $(Boc)_2O$ (14.65mg, 0.067mmol) and DMAP (8.2mg, 0.067mmol) in dry DMF (2mL), was stirred under a nitrogen atmosphere at room temperature for 24h. The mixture was evaporated and then diethyl ether (10mL) was added. The ethereal solution was washed with 10% citric acid, dried and then evaporated. The residue was chromatographed on silica gel by PLC (70% DCM in PS) to give the cyclized product **57** (1.5mg, 7%) as an opaque white solid and the *O*-Boc product **58** (11.5mg, 43%) as a yellow solid.



57

57: ^{1}H NMR (300MHz, $CDCl_3$): δ 3.83 (s, 3H, OCH_3), 4.91 (br.d, *J* = 9.6 Hz, 2H, OCH_2), 6.67 (s, 1H, H-13), 6.86 (d, *J* = 2.7 Hz, 1H, H-12), 6.97 (dd, *J* = 8.6, 2.6 Hz, 1H, H-10), 7.43 (d, *J* = 9.0 Hz, 1H, H-9), 8.23 (dd, *J* = 9.5, 2.3 Hz, 1H, H-3), 8.40 (d, *J* = 9.3 Hz, 1H, H-4), 4.48 (d, *J* = 2.4 Hz, 1H, H-1). MS (CI): *m/z* 325 (M+1, 100 %).

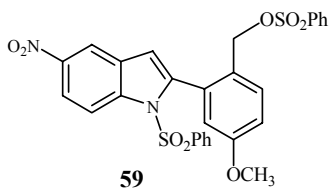


58

58: ^{1}H NMR (300MHz, $CDCl_3$): δ 1.52 (s, 9H, CH_3), 3.87 (s, 3H, OCH_3), 5.11 (s, 2H, OCH_2), 6.81 (d, *J* = 1.5

Hz, 1H, H-3), 6.97 (dd, *J* = 8.4, 2.7 Hz, 1H, H-5′), 7.11 (d, *J* = 2.7 Hz, 1H, H-3′), 7.45 (d, *J* = 9.0 Hz, 1H, H-7), 7.53 (d, *J* = 8.7 Hz, 1H, H-6′), 8.13 (dd, *J* = 8.9, 2.3 Hz, 1H, H-6), 8.61 (d, *J* = 2.4 Hz, 1H, H-4), 9.99 (s, 1H, NH). ^{13}C NMR (75MHz, $CDCl_3$): δ 27.8 (3C, CH_3), 55.5 (OCH_3), 66.9 (OCH_2), 83.2 (O**C**Me_3), 104.6 (C3), 111.1 (C7), 114.6 (C5′), 115.5 (C3′), 117.7 (2C, C4, C6), 124.9 (C1′)[a], 127.8 (C3a), 133.3 (C6′), 133.7 (C2), 139.4 (C7a), 140.4 (C2′)[a], 142.0 (C5), 153.5 (CO), 160.0 (C4′). MS (CI): *m/z* 399 (M+1, 50 %), 299 (50 %), 281 (100 %).

Method 2: To a solution of **42** (30.0mg, 0.1mmol) in dry THF (1mL) at –60°C in a dry ice/acetone bath under a nitrogen atmosphere, was added dropwise 2M *n*-BuLi solution in cyclohexane (0.05mL, 0.1mmol). The cooling bath was removed and the reaction mixture stirred for 45min while warming to 0°C. The reaction mixture was then re-cooled to –60°C, and a solution of benzenesulfonyl chloride (20.8mg, 0.15mmol) in dry THF was added (1mL). The mixture was allowed to warm slowly to room temperature and stirring continued overnight. A 5% $NaHCO_3$ solution (1mL) was added to the mixture which was then extracted with DCM (3 x 5mL). The combined DCM extracts were washed with 5% $NaHCO_3$, water and then brine. The solution was dried, evaporated, and then chromatrographed on silica gel by PLC (50% DCM in hexane) to give the dibenzenesulfonyl indole **59** (4.5mg, 8%) as an opaque white solid.

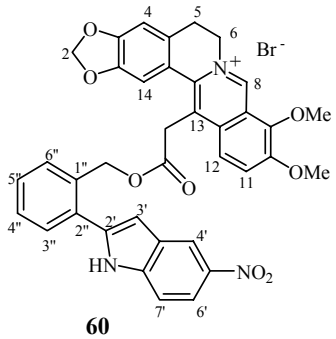


59

^{1}H NMR (300MHz, $CDCl_3$): δ 3.89 (s, 3H, OCH_3), 5.33 (s, 2H, OCH_2), 6.76 (d, *J* = 0.6 Hz, 1H, H-3), 6.91 (dd, *J* = 8.4, 2.1 Hz, 1H, H-6), 6.94-7.01 (m, 1H, ArH), 7.00 (d, *J* = 8.4 Hz, 1H, H-4′), 7.11 (d, *J* = 1.8 Hz, 1H, H-6′), 7.12-7.22 (m, 2H, ArH), 7.32-7.42 (m, 3H, H-3′, ArH), 7.52-7.59 (m, 2H, ArH), 7.76 (d, *J* = 8.7 Hz, 1H, H-7), 7.98 (d, *J* = 8.7 Hz, 1H, ArH), 8.17-8.25 (m, 2H, ArH), 8.42 (d, *J* = 1.8 Hz, 1H, H-4). MS (CI): *m/z* 579 (M+H, 1%), 295 (100%).

7.6 Alkylation reactions (Chapter 4)

7.6.1 Preparation of 9,10-Dimethoxy-13-[2-(5-nitro-1*H*-indol-2-yl)-benzyloxycarbonyl-methyl]-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (60)

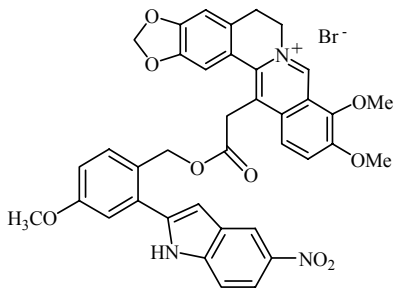


60

A solution of the dihydroberberine **9** (100.0mg, 0.26mmol) and the bromoester **51** (108.8mg, 0.32mmol) in dry CH_3CN (8mL), was heated at reflux for 48h under a nitrogen atmosphere. The reaction mixture was then concentrated by evaporation of the CH_3CN. The residue was chromatographed on silica gel (4% MeOH in DCM), followed by PLC (multiple development, silica gel, 4% MeOH in DCM) of the main fraction from the column. Subsequently, the polar fraction was crystallized from 1% MeOH in DCM to give **60** (66.6mg, 35%) as a yellow solid; m.p. 212-214^{o}C. ^{1}H NMR (300MHz, DMSO-d_6): δ 3.03 (br.t, J = 5.1 Hz, 2H, H-5), 4.04 (s, 3H, OCH_3), 4.07 (s, 3H, OCH_3), 4.48 (s, 2H, CH_2CO), 4.78 (br.s, 2H, H-6), 5.39 (s, 2H, CH_2O), 6.14 (s, 2H, OCH_2O), 6.74 (s, 1H, H-3′), 7.07 (s, 1H, H-4)[a], 7.13 (s, 1H, H-14)[a], 7.42-7.62 (m, 5H, H-7′, ArH), 7.87 (d, J = 9.3 Hz, 1H, H-11)[b], 8.00 (d, J = 8.1 Hz, 1H, H-6′), 8.03 (d, J = 9.3 Hz, 1H, H-12)[b], 8.48 (s, 1H, H-4′), 9.91 (s, 1H, H-8), 12.19 (s, 1H, NH). ^{13}C NMR (75MHz, DMSO-d_6): δ 27.4 (C5), 36.5 (CH_2CO), 56.9 (C6), 57.1 (OCH_3), 61.2 (OCH_3), 65.5 (CH_2O), 102.3 (OCH_2O), 104.3 (C3′), 108.7 (C4)[c], 108.8 (C14)[c], 111.9 (C7′), 117.2 (2C, C4′, C6′), 119.8 (C4a), 121.0 (C8a)[d], 121.1 (C11)[e], 126.0 (C12a)[d], 126.2 (C12)[e], 127.6 (C3a′), 129.0 (2C, C4′′, C5′′), 129.8 (C3′′)[f], 130.2 (C6′′)[f], 131.6 (C2′), 132.8 (C13), 133.2 (C2′′), 134.3 (C13b), 137.2 (C13a), 139.6 (C1′′), 139.9 (C7a′), 141.0 (C5′),

144.4 (C9)[g], 145.8 (C8), 146.9 (C3a)[h], 149.6 (C14a)[h], 150.4 (C10)[g], 170.3 (CO). HRMS (ES): *m/z* calcd for $C_{37}H_{30}N_3O_8$ $[M]^+$: 644.2033; found: 644.2034.

7.6.2 Preparation of 9,10-Dimethoxy-13-[4-methoxy-2-(5-nitro-1*H*-indol-2-yl)-benzyloxycarbonylmethyl]-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*] quinolizinium bromide (61)

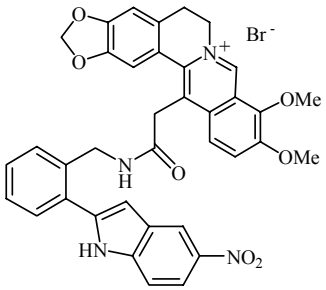


61

A solution of the dihydroberberine **9** (91.0mg, 0.24mmol) and the bromoester **52** (100.0mg, 0.24mmol) in dry CH_3CN (14mL) was heated at reflux for 48h under a nitrogen atmosphere. The reaction mixture was then concentrated by evaporation of the CH_3CN. The residue was chromatographed on silica gel (4% MeOH in DCM), followed by PLC (multiple development, silica gel, 4% MeOH in DCM) of the main fraction from the column. Subsequently, the polar fraction was crystallized from 1% MeOH in DCM to give **61** (40.7mg, 22%) as a yellow solid; m.p. 230°C (decomp.). ^{1}H NMR (500MHz, DMSO-d_6): δ 3.03 (t, J = 5.5 Hz, 2H, H-5), 3.84 (s, 3H, OCH_3), 4.04 (s, 3H, OCH_3), 4.06 (s, 3H, OCH_3), 4.47 (s, 2H, CH_2CO), 4.78 (s, 2H, H-6), 5.31 (s, 2H, CH_2O), 6.14 (s, 2H, OCH_2O), 6.72 (s, 1H, H-3′), 7.05 (dd, J = 5.1, 1.5 Hz, 1H, H-5′′), 7.08 (s, 1H, H-4)[a], 7.13 (s, 1H, H-14)[a], 7.16 (d, J = 1.5 Hz, 1H, H-3′′), 7.51 (d, J = 9.3 Hz, 1H, H-6′′), 7.54 (d, J = 9.6 Hz, 1H, H-7′), 7.85 (d, J = 5.7 Hz, 1H, H-11)[b], 8.01 (dd, J = 9.3, 1.2 Hz, 1H, H-6′), 8.03 (d, J = 5.7 Hz, 1H, H-12)[b], 8.46 (d, J = 1.2 Hz, 1H, H-4′), 9.90 (s,1H, H-8), 12.24 (s, 1H, NH). ^{13}C NMR (125MHz, DMSO-d_6): δ 27.3 (C5), 36.5 (CH_2CO), 55.6 (OCH_3), 56.9 (C6), 57.0 (OCH_3), 62.1 (OCH_3), 65.4 (CH_2O), 102.3 (OCH_2O), 104.2 (C3′), 108 6 (C4)[c], 108.7 (C14)[c], 111.9 (C7′), 114.4 (C5′′), 114.6 (C3′′), 117.1 (2C, C4′, C6′), 119.8 (C4a), 120.9 (2C, C12, C8a)[d], 125.0 (C2′), 126.1

(2C, C11, C12a)[d], 127.4 (C3a′), 132.7 (2C, C13, C6′′), 133.2 (C1′′)[e], 134.2 (C13b), 137.2 (C13a), 139.4 (C2′′)[e], 139.8 (C7a′), 140.9 (C5′), 144.3 (C9)[f], 145.7 (C8), 146.8 (C3a)[g], 149.5 (C14a)[g], 150.3 (C10)[f], 159.5 (C4′′), 170.3 (CO). HRMS (ES): *m/z* calcd for $C_{38}H_{32}N_3O_9$ $[M]^+$: 674.2139; found: 674.2134.

7.6.3 Preparation of 9,10-Dimethoxy-13-{[2-(5-nitro-1*H*-indol-2-yl)-benzylcarbamoyl]-methyl}-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (62)

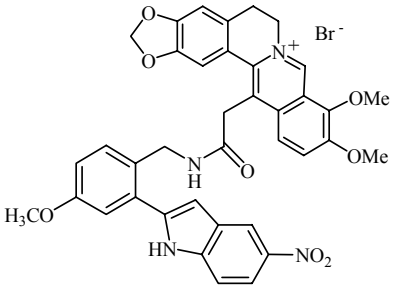


62

A solution of the dihydroberberine **9** (22.5mg, 0.06mmol) and the bromoamide **49** (25.0mg, 0.07mmol) in dry CH_3CN (3mL) was heated at reflux for 48h under a nitrogen atmosphere. The reaction mixture was then concentrated by evaporation of the CH_3CN. The residue was chromatographed on silica gel (4% MeOH in DCM), followed by PLC (multiple development, silica gel, 4% MeOH in DCM) of the polar fraction from the column. Subsequently, the polar fraction was crystallized from 1% MeOH in DCM to give **62** (5.0mg, 12%) as a yellow solid; m.p. 209-211^{o}C. ^{1}H NMR (500MHz, CD_3OD): δ 3.11 (br.t, *J* = 4.8 Hz, 2H, H-5), 4.03 (s, 3H, OCH_3), 4.19 (s, 3H, OCH_3), 4.33 (s, 2H, CH_2CO), 4.69 (s, 2H, CH_2N), 4.88 (br.s, 2H, H-6), 6.12 (s, 2H, OCH_2O), 6.83 (s, 1H, H-3′), 7.02 (s, 1H, H-4)[a], 7.40 (s, 1H, H-14)[a], 7.46-7.63 (m, 5H, H-7′, ArH), 7.82 (d, *J* = 9.6 Hz, 1H, H-11)[b], 7.98 (d, *J* = 9.6 Hz, 1H, H-12)[b], 8.06 (dd, *J* = 9, 2.1 Hz, 1H, H-6′), 8.52 (d, *J* = 2.1 Hz, 1H, H-4′), 9.84 (s, 1H, H-8). ^{13}C NMR (125MHz, CD_3OD): δ 27.8 (C5), 37.3 (**C**H_2CO), 42.0 (CH_2N), 56.2 (OCH_3), 57.3 (C6), 61.2 (OCH_3), 102.5 (C2), 104.1 (C3′), 108.3 (C4)[c], 109.3 (C14)[c], 111.0 (C7′), 116.9 (C7′), 117.1 (C4′), 120.0 (C4a), 120.4 (C11)[d], 121.7 (C8a)[e], 126.2 (C12)[d], 127.8

(C12a)[e], 127.9 (ArCH), 128.1 (C3a′), 128.8 (ArCH), 129.4 (ArCH), 129.8 (ArCH), 131.8 (C2′), 134.1 (2C, C13b, C13), 136.0 (C1′′)[f], 138.2 (C13a), 139.2 (C2′′)[f], 140.7 (C5′), 141.3 (7a′), 144.9 (C8), 145.1 (C9)[g], 147.9 (C3a)[h], 150.2 (C14a)[h], 150.7 (C10)[g], 170.8 (CO). HRMS (ES): *m/z* calcd for $C_{37}H_{31}N_4O_7$ $[M]^+$: 643.2193; found: 643.2184.

7.6.4 Preparation of 9,10-Dimethoxy-13-{[4-methoxy-2-(5-nitro-1*H*-indol-2-yl)-benzylcarbamoyl]-methyl}-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (63)

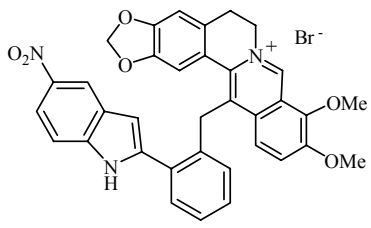


63

A solution of the dihydroberberine **9** (58.0mg, 0.15mmol) and the bromoamide **50** (70.0mg, 0.16mmol) in dry CH_3CN (3mL) was heated at reflux for 48h under a nitrogen atmosphere. The mixture was then concentrated and the residue triturated with diethyl ether. The precipitate was filtered and washed with diethyl ether. The solid was chromatographed on silica gel (4% MeOH in DCM), and the product was crystallized from 1% MeOH in DCM to give **63** (25.4mg, 22%) as a yellow solid; m.p. >250°C. ^{1}H NMR (300MHz, CD_3OD): δ 3.12 (br t, *J* = 5.7 Hz, 2H, H-5), 3.87 (s, 3H, OCH_3), 4.06 (s, 3H, OCH_3), 4.19 (s, 3H, OCH_3), 4.30 (s, 2H, CH_2CO), 4.62 (s, 2H, CH_2N), 4.79 (br.t, *J* = 5.7 Hz, 2H, H-6), 6.12 (s, 2H, OCH_2O), 6.83 (d, *J* = 0.9 Hz, 1H, H-3′), 7.02 (s, 1H, H-4)[a], 7.06 (dd, *J* = 8.6, 2.9 Hz, 1H, H-5′′), 7.14 (d, *J* = 2.4 Hz, 1H, H-3′′), 7.4 (s, 1H, H-14)[a], 7.48 (d, *J* = 9.0 Hz, 1H, H-7′), 7.52 (d, *J* = 8.4 Hz, 1H, H-6′′), 7.80 (d, *J* = 9.3 Hz, 1H, H-12)[b], 7.94 (d, *J* = 9.3 Hz, 1H, H-11)[b], 8.04 (dd, *J* = 9.2, 2.3 Hz, 1H, H-6′), 8.49 (d, *J* = 2.1 Hz, 1H, H-4′), 9.82 (s, 1H, H-8). ^{13}C NMR (75MHz, CD_3OD): δ 29.1 (C5), 38.5 (CH_2CO), 42.9 (CH_2N), 56.0 (OCH_3), 57.5 (OCH_3), 58.7 (C6), 62.7 (OCH_3), 103.8 (C2), 105.2 (C3′),

109.4 (C4)[c], 110.5 (C14)[c], 112.3 (C7′), 115.7 (C5′′), 116.0 (C3′′), 118.2 (C4′), 118.3 (C6′), 121.4 (C12)[d], 121.5 (C11)[d], 122.8 (C8a)[e], 127.4 (C4a), 128.9 (C12a)[e], 129.0 (C2′), 129.3 (C3a′), 132.6 (C6′′), 134.2 (C1′′)[f], 135.1 (C13), 135.2 (C13b), 139.4 (C13a), 141.2 (C2′′)[f], 141.5 (C7a′), 142.9 (C5′), 146.3 (C9)[g], 146.4 (C8), 148.9 (C3a)[h], 151.7 (C14a)[h], 151.8 (C10)[g], 160.7 (C4′′), 171.9 (CO). HRMS (ES): *m/z* calcd for $C_{38}H_{33}N_4O_8$ $[M]^+$: 673.2298; found: 673.2305.

7.6.5 Preparation of 9,10-Dimethoxy-13-[2-(5-nitro-1*H*-indol-2-yl)benzyl]-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (64)

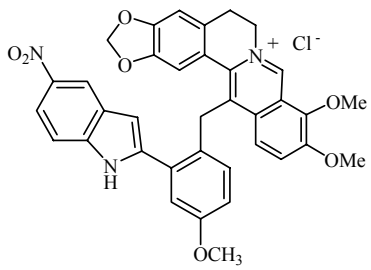


64

A solution of the dihydroberberine **9** (91.0mg, 0.24mmol) and the benzyl bromide **53** (102.3mg, 0.30mmol) in dry CH_3CN (7mL) was heated at reflux for 24h under a nitrogen atmosphere. The mixture was then concentrated and the residue triturated with diethyl ether. The precipitate was filtered and washed with diethyl ether. The solid was chromatographed on silica gel (6% MeOH in DCM) to give **64** (55.0mg, 35%) as a yellow solid; m.p. 206°C (decomp.). ^{1}H NMR (300MHz, CD_3OD): δ 3.03 (t, *J* = 5.5 Hz, 2H, H-5), 4.01 (s, 3H, OCH_3), 4.17 (s, 3H, OCH_3), 4.80 (br.s, 2H, H-6), 4.84 (s, 2H, CH_2Ph), 5.96 (s, 2H, OCH_2O), 6.72 (s, 1H, H3′), 6.86 (s, 1H, H-4)[a], 6.90 (s, 1H, H-14)[a], 6.96 (d, *J* = 7.8 Hz, 1H, H-6′′), 7.27 (td, *J* = 7.7, 1.5 Hz, 1H, H-5′′), 7.37 (br.t, *J* = 7.5 Hz, 1H, H-4′′), 7.42 (d, *J* = 9.0 Hz, 1H, H-7′), 7.58 (dd, *J* = 7.7, 1.1 Hz, 1H, H-3′′), 7.78 (d, *J* = 9.3 Hz, 1H, H-11)[b], 7.88 (dd, *J* = 9.0, 2.4 Hz, 1H, H-6′), 7.94 (d, *J* = 9.3 Hz, 1H, H-12)[b], 8.34 (d, *J* = 2.1 Hz, 1H, H-4′), 9.8 (s, 1H, H-8). ^{13}C NMR (75MHz, CD_3OD): δ 29.1 (C5), 36.4 (CH_2Ar), 57.5 (OCH_3), 58.8 (C6), 62.7 (OCH_3), 103.6 (OCH_2O), 105.6 (C3′), 109.3 (C4)[c], 109.8

(C14)[c], 112.3 (C7′), 118.2 (C6′), 118.3 (C4′), 121.4 (C4a), 122.5 (C11)[d], 122.9 (C8a)[e], 127.3 (C12)[d], 128.6 (C4′′), 129.2 (C3a′), 130.4 (2C, C6′′, C5′′), 131.8 (C3′′), 132.8 (C12a)[e], 133.4 (C2′), 135.0 (C13b), 135.1 (C13), 138.6 (C1′′)[f], 139.0 (C13a), 141.2 (C7a′), 141.5 (C2′′)[f], 142.9 (C5′), 146.1 (C8), 146.2 (C9)[g], 148.6 (C3a)[h], 151.4 (C14a)[h], 151.7 (C10)[g]. HRMS (ES): *m/z* calcd for $C_{35}H_{28}N_3O_6$ $[M]^+$: 586.1978; found: 586.1984.

7.6.6 Preparation of 9,10-Dimethoxy-13-[4-methoxy-2-(5-nitro-1*H*-indol-2-yl)benzyl]-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium chloride (65)



65

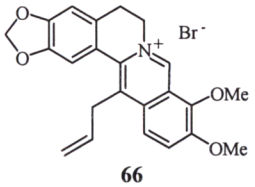
Method 1: A suspension of **42** (30.0mg, 0.10mmol), dihydroberberine **9** (37.9mg, 0.1mmol), triphenylphosphine (52.5mg, 0.20mmol), and carbon tetrabromide (331.6mg, 1.0mmol) in DMF (1mL) was heated at 90°C for 20h under a nitrogen atmosphere. The mixture was then evaporated and the residue chromatographed on silica gel (6% MeOH in DCM) to give a yellow solid (9.4mg). The solid was crystallized from 2% MeOH in $CHCl_3$ to give berberine bromide (5.1mg, 12.2%) as a yellow solid.

Method 2: A solution of **42** (20.0mg, 0.07mmol), dihydroberberine **9** (25.0mg, 0.07mmol), and triphenylphosphine (35.1mg, 0.13mmol) in 25% CCl_4 in DMF (1mL) was heated at 90°C for 12h under a nitrogen atmosphere. The mixture was then evaporated and the residue chromatographed on silica gel (4% MeOH in DCM), followed by multiple development PLC on silica gel (4% MeOH in DCM). An almost pure fraction was crystallized from hot MeOH to give **65** (1.8mg, 4%) as a yellow solid; m.p. 230°C (decomp.). ^{1}H NMR (500MHz, CD_3OD): δ 3.04 (t, 5.9 Hz, 2H, H-5), 3.83

(s, 3H, OCH_3), 4.06 (s, 3H, OCH_3), 4.19 (s, 3H, OCH_3), 4.78 (s, 2H, H-6), 4.80 (s, 2H, CH_2Ph), 6.00 (s, 2H, OCH_2O), 6.78 (s, 1H, H-3′), 6.84-6.91 (m, 2H, H-6′′, H-5′′), 6.92 (s, 1H, H-4)[a], 7.00 (s, 1H, H-14)[a], 7.22 (d, J = 2.4 Hz, 1H, H-3′′), 7.48 (d, J = 8.7 Hz, 1H, H-7′), 7.81 (d, J = 9.6 Hz, 1H, H-12)[b], 8.00 (d, J = 9.5 Hz, 1H, H-11)[b], 8.02 (dd, J = 8.9, 2.2 Hz, 1H, H-6′), 8.47 (d, J = 2.1 Hz, 1H, H-4′), 9.81 (s, 1H, H-8). ^{13}C NMR (125MHz, CD_3OD): δ 29.0 (C5), 35.7 (CH_2Ph), 56.0 (OCH_3), 57.5 (OCH_3), 58.8 (OCH_3), 62.7 (C6), 103.6 (OCH_2O), 105.7 (C3′), 109.3 (C4)[c], 109.8 (C14)[c], 112.3 (C7′), 115.5 (C5′′), 117.3 (C3′′), 118.3 (2C, C4′, C6′), 121.4 (C4a), 122.6 (C12)[d], 122.9 (C8a)[e], 127.3 (C11)[d], 129.2 (C3a′), 130.3 (C2′), 131.6 (C3′′), 133.1 (C13b), 134.3 (C1′′)[f], 135.0 (C12a)[e], 135.2 (C13), 138.9 (C13a), 141.1 (2C, C7a′,C2′′[f]), 142.9 (C5′), 146.0 (C9)[g], 146.2 (C8), 148.6 (C3a)[h], 151.4 (C14a)[h], 151.7 (C10)[g], 160.2 (C4′′). HRMS (ES): *m/z* calcd for $C_{36}H_{30}N_3O_7$ $[M]^+$: 616.2084; found: 616.2090.

7.7 Attempted synthesis to increase the bond length between berberine and pump blocker (Chapter 4)

7.7.1 Preparation of 13-Allyl-9,10-dimethoxy-5,6-dihydrobenzo[*g*]-1,3 benzodioxolo[5,6-*a*]quinolizinium bromide (66)[133]



66

A suspension of the dihydroberberine **9** (380.0mg, 1.01mmol) and allyl bromide (2mL) was refluxed at 100°C under a nitrogen atmosphere for 2h. Diethyl ether (10mL) was added to the reaction until no further precipitate formed. The precipitate was filtered and then chromatographed on silica gel (3% MeOH in DCM) to give **66** (241.0mg, 52%) as a yellow solid. ^1H NMR (300MHz, $CDCl_3$ + CD_3OD): δ 3.23 (t, J = 5.9 Hz, 2H, H-5), 3.90-4.01 (m, 2H, $CH_2CH{=}CH_2$), 4.06 (s, 3H, OCH_3),

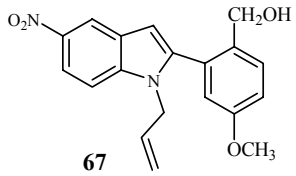
4.36 (s, 3H, OCH_3), 4.91 (br.d, *J* = 17.4 Hz, 1H, $CH_2CH=C\mathbf{H}_2$), 5.24 (br.s, 2H, H-6), 5.43 (br.d, *J* = 10.5 Hz, 1H, $CH_2CH=C\mathbf{H}_2$), 6.07 (s, 2H, OCH_2O), 6.31-6.42 (m, 1H, $CH_2C\mathbf{H}=CH_2$), 6.87 (s, 1H, H-4)[a], 7.34 (s, 1H, H-14)[a], 7.82 (s, 2H, H-11, H-12), 10.53 (s, 1H, H-8). ^{13}C NMR (75MHz, $CDCl_3$ + CD_3OD): δ 28.5 (C5), 34.7 ($\mathbf{C}H_2CH=CH_2$), 57.0 (OCH_3), 57.6 (C6), 63.2 (OCH_3), 102.0 (OCH_2O), 108.4 (C4)[b], 108.8 (C14)[b], 119.3 (C12)[c], 120.0 (C13b)[d], 120.8 ($CH_2CH=\mathbf{C}H_2$), 121.7 (C8a)[e], 125.4 (C11)[c], 129.7 (C4a)[d], 133.3 (C13), 133.5 (C12a)[e], 135.1 ($CH_2\mathbf{C}H=CH_2$), 136.9 (C13a), 145.9 (C9)[f], 146.4 (C14a)[g], 147.0 (C8), 149.7 (C3a)[g], 150.3 (C10)[f]. HRMS (ES): *m/z* calcd for $C_{23}H_{22}NO_4$ $[M]^+$: 376.1549; found: 376.1548.

7.7.2 Attempted cross metathesis reaction of 66

A mixture of **66** (5.1mg, 0.01mmol), indole **55** (4.0mg, 0.01mmol) and polymer bound benzylidine-bis(triscyclohexylphosphine)-dichlororuthenium 0.1mmol/g (1.4mg, 0.001mmol), in dry DCM (5mL) was stirred and heated at refluxed for 2 days under a nitrogen atmosphere. The catalyst was then removed by filtration of the mixture. The filtrate was then evaporated. The residue was chromatographed on silica gel (3% MeOH in DCM) to give a two component mixture of product (5.2mg), which could not be identified. MS (CI) of the mixture: *m/z*, $[MH]^+$ showed 208 (100%) and 313 (100%).

7.7.3 Attempted *O*-alkylation of 42

Method 1: To a solution of the benzyl alcohol **42** (13.0mg, 0.05mmol) in 10% TEA in THF (2mL) was added 10% allyl bromide in THF (0.1mL, 0.01mmol) at 0°C under a nitrogen atmosphere. The solution was then heated at 50°C for 30h. The reaction mixture was then evaporated and the residue chromatographed on silica gel by VLC (1% MeOH in DCM) to give only starting material **42** (10.0mg).



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Method 2: To a suspension of sodium hydride (5.0mg of a 50% dispersion in mineral oil, 0.1mmol) in anhydrous DMF (1mL) at 0°C, was added a solution of **42** (30.0mg, 0.1mmol) in DMF (2mL) and the mixture then stirred for 30min. The mixture was warmed to room temperature and stirred for a further 2h. Excess DMF (2mL) was added and then the mixture was cooled to –60°C in a dry ice/acetone bath. A solution of 10% allyl bromide in DMF (0.1mL, 0.13mmol) was added dropwise to the cooled mixture and then allowed to stir overnight at 80°C. The mixture was evaporated and the residue added to ice water (20mL), and the mixture stirred vigorously for 4h. The precipitate was filtered, washed with cold water, and dried. The residue was chromatographed on silica gel by PLC (50% DCM in hexane) to give **67** (7.0mg, 21%) as an amorphous solid. ^{1}H NMR (300MHz, $CDCl_3$): δ 3.83 (s, 3H, OCH_3), 4.48 (s, 2H, CH_2), 4.61 (d, *J* = 3.6 Hz, 2H, C**H**$_2$CH=CH_2), 4.85 (d, *J* = 17.1 Hz, 1H, CH_2CH=C**H**$_2$), 5.16 (d, *J* = 10.5 Hz, 1H, CH_2CH=C**H**$_2$), 5.78-5.91 (m, 1H, CH_2C**H**=CH_2), 6.71 (s, 1H, H-3), 6.87 (d, *J* = 2.4 Hz, 1H, H-6′), 7.04 (dd, *J* = 8.7, 2.4 Hz, 1H, H-4′), 7.35 (d, *J* = 9.3 Hz, 1H, H-7), 7.52 (d, *J* = 8.4 Hz, 1H, H-3′), 8.13 (dd, *J* = 8.9, 2.0 Hz, 1H, H-6), 8.59 (d, *J* = 1.8 Hz, 1H, H-4). ^{13}C NMR (75MHz, $CDCl_3$): δ 46.7 (**C**H_2CH=CH_2), 55.4 (OCH_3), 62.4 (CH_2OH), 104.9 (C3), 110.2 (C7), 115.3 (C4′), 116.2 (C6′), 117.2 (CH_2CH=**C**H_2), 117.4 (C6), 117.7 (C4), 127.1 (C3a), 130.4 (C3′), 131.3 (C2), 132.6 (C1′)[a], 132.7 (CH_2**C**H=CH_2), 139.6 (C7a), 141.6 (C2′)[a], 141.9 (C5), 158.7 (C5′). HRMS (CI): *m/z* calcd for $C_{19}H_{19}N_2O_4$ $[MH]^+$: 339.1345; found: 339.1349.

Method 3: To a suspension of sodium hydride (50% dispersion in mineral oil, 1.6g, 36.0mmol, after being washed with dry diethyl ether) in dry THF (30mL) was added ethylene glycol (2mL, 36.0mmol) at room temperature and the mixture stirred for 1h. After this time, *tert*-butyldimethylsilyl chloride (5.4g, 36.0mmol) was slowly added

to the mixture and vigorous stirring was continued for 45min. The mixture was poured into diethyl ether (200mL), and then washed with 10% aqueous K_2CO_3 solution (60mL) and brine (60mL). The diethyl ether layer was dried, concentrated and then chromatographed on silica gel by VLC (20% EtOAc in hexane) to give 2-(*tert*-butyldimethylsilanyloxy)ethanol (1.13g, 33%) as a pale yellow oil. ^{1}H NMR[134] (300MHz, $CDCl_3$): δ 0.01 (s, 6H, $Si(CH_3)_2$), 0.82 (s, 9H, $C(CH_3)_3$), 2.71 (br s, 1H, OH), 3.51-3.64 (m, 4H, CH_2). CIMS: *m/z* 177 ($[MH]^+$, 100%).

To a suspension of sodium hydride (11.0mg of a 50% dispersion in mineral oil, 0.23mmol) in anhydrous DMF (1mL) at 0^{o}C, was added a solution of 2-(*tert*-butyldimethylsilanyloxy)ethanol (49.0mg, 0.28mmol) in DMF (1mL) and the mixture stirred for 30min. The mixture was then warmed to room temperature and stirred for a further 2h. Excess DMF (3mL) was added and then the mixture was cooled to –60^{o}C in a dry ice/acetone bath. A solution of benzyl bromide **53** (75.9mg, 0.23mmol) in DMF (4mL) was added dropwise to the cooled mixture and then the mixture was allowed to stir for 2 days at 80^{o}C. The mixture was evaporated, then added to ice water (30mL), and stirred vigorously for 4h. The precipitate was filtered, then washed with cold water, and dried. The residue was chromatographed on silica gel by PLC (30% DCM in hexane) to give the cyclised product **46** (39.6mg, 69%). All spectroscopic data for **46** was the same as that noted for this compound previously (Section 6.5.6.2).

7.8 Enzymatic hydrolysis of 9,10-Dimethoxy-13-[2-(5-nitro-1*H*-indol-2-yl)-benzyloxycarbonyl-methyl]-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (60)

To a solution of **60** (4.7mg, 0.006mmol) in DMSO (30μL), was added a suspension of porcine liver carboxyl esterase (EC 3.1.1.1, 1mg, 46units: one unit represents 1μmol product produced per min) in Tris-HCl buffer solution at pH7.2 (1mL). The buffer solution was prepared as follows: 0.2M trishydroxymethyl-aminomethane (25mL) was mixed with 0.1M HCl (45mL), then adjusted to pH7.2, and the volume made up to 100mL with water. The reaction mixture was warmed to 37^{o}C, and stirred at this temperature for 24 h. The suspension was then extracted with DCM (3 x 3mL) and the DCM extract washed with water (10mL). The DCM extract was dried and evaporated to give a residue in which the benzyl alcohol **41** was identified by TLC analysis (silica gel, DCM) and comparison with the authentic alcohol.

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APPENDIX I

Antibacterial activity testing using Fluorescein Diacetate (FDA) and Antimicrobial (cell lysis/ cell stasis) assays[137]

Materials and Methods

Maintenance and preparation of microbial cultures

Stock cultures of *S. aureus* ACM844 and *E. coli*ACM845 were obtained from the Culture Collection at the University of Queensland and maintained at –78^{o}C in 15% glycerol. The cultures were prepared by streaking onto Nutrient Agar (NA) (Oxoid CM3; pH 7.4). After an overnight incubation, single colonies were used to inoculate sterile liquid media. The broth consisted of yeast extract (5g; ICN 103303-17), peptone (10g; Oxoid L37), and NaCl (5g, ICN 102892) in distilled water (1L). Inoculated broths were placed on an orbital shaker (150rpm) and incubated overnight at 37^{o}C.

FDA assay

The overnight cultures of the microorganisms were diluted to an absorbance of 0.12 (600nm) and grown to 0.18 (*ca* 30min; 37^{o}C; 150rpm). The wells of a 96 well tissue culture plate (3072 Microtest III, Becton Dickinson) were filled with 175µL of the culture. To each well 20µL of the test compound or appropriate control was added. Three replicates were made at each test concentration. The microtiter plate was incubated for 30min at 37^{o}C before 5µL FDA (0.2% solution in acetone) was added. Incubation was continued for a further 2h or until the production of fluorescein was easily visible under an ultraviolet lamp (λ 254nm). The results were simply recorded as positive or negative according to the detection of fluorescence.

Test compounds used in the assay were dissolved in acetone (100%, AR Grade) and tested at a maximum concentration of 10mg/mL. Two procedural controls, consisting of 20µL of acetone and 20µL of Milli Q water with FDA, were added to each test plate (three replicates) to determine any effects of the acetone on the viability of the cells. Additional procedural controls included 20µL of test substance in 175µL of broth with FDA, to ascertain whether the test compound hydrolysed FDA, and 195µL of broth with FDA to check for contamination in the broth.

Antimicrobial (cell lysis/ cell stasis) assay

After the FDA assay was completed, 20µL of culture (four replicates) from all the wells that did not show fluorescence, were spread on to agar to determine if the cells could recover. The plates were incubated overnight at 37^{o}C. Counts of visibly growing colonies were performed and compared to a dilution series of a control culture from the FDA plate containing acetone.

APPENDIX II

Antibacterial activity and MDR inhibitory activity testing using turbidometric assay[56] (performed by Lewis, K. and Ball, A., Northeastern University, USA)

Materials and Methods

Microbial strains used for antimicrobial activity testing were Gram-positive bacteria: *S. aureus* 8325-4 (wild-type strain which expresses NorA MDR pump), *S. aureus* K1758 (NorA mutant strain lacking the NorA MDR pump), *S. aureus* K2361 (resistance strain which overexpresses the NorA MDR pump), *E. faecalis* V583, and *E. faecium* DO; Gram-negative bacteria: *E. coli* K12, *S. enterica* Serovar Typhimurium SL1344R2, and *P. auerginosa* PA1; and Yeast: *S. cerevisiae* BY4742, *C. albicans* F5, and *C. albicans* F5 M432.

Cell Culturing and Susceptibility Testing

Growth of microbe and susceptibility measurements were performed according to the National Center for Clinical Laboratory Standards Recommendations. All strains were cultured in Mueller-Hinton (MH) broth overnight with aeration at 37^{o}C. Cells were then inoculated into fresh MH medium at a 1:10 dilution and were allowed to grow for 1h. This suspension was dilute 1:2000 into MH broth and 0.05 mL was dispensed per well of microtiter plates. For measurements of direct antimicrobial activity, test compounds were dissolved in DMSO at 10mg/mL and then serially diluted with water. The final volume of a well was 0.2 mL, and the cell concentration was 10^5 cells/mL. All tests were done in triplicate. Minimum inhibitory concentrations (MICs)

were determined by serial 2-fold dilution of test compounds. Two procedural controls were used. One contained cells and MH broth to observe the normal growth rate of microorganisms within the wells. Another one contained only MH broth to observe any contamination. MIC was defined as a concentration (μg/mL) of an antimicrobial that completely prevented cell growth during an 18h incubation at 37^{o}C. Growth was assayed with a microtiter plate reader (Bio-Rad) by absorption at 600nm.

Test for MDR inhibitory activity were done in similarly, but with antibiotic (Berberine chloride or Ciprofloxacin) present at a sub-inhibitory concentration throughout. The test substance was then serially diluted 2-fold, and MIC for test substances was then defined as their minimal concentration that completely inhibited cell growth in the presence of sub-inhibitory concentration of antibiotic.

Effect of NorA inhibitors on antibacterial accumulation in bacterial cells using uptake assay (performed by Lewis, K. and Ball, A., Northeastern University, USA*)*

Materials and Methods

Microbial strains used for antimicrobial activity testing were Gram-positive bacteria: *S. aureus* 8325-4 (wild-type strain which expresses NorA MDR pump), *S. aureus* K1758 (NorA mutant strain lacking the NorA MDR pump), *S. aureus* K2361 (resistance strain which overexpresses the NorA MDR pump).

Cells were grown to an OD of 1.5 (600nm) in 1 milliliter of MH broth. Cells were centrifuged at 12,000 Rpm for two minutes and washed in 20mM HEPES/KOH buffer pH 7 twice. Cells were resuspended to an OD of 0.6 in 20mM HEPES/KOH buffer

containing 10mM sucrose and incubated for 1h. Cells were centrifuged and washed with 20mM HEPES buffer and resuspended to an OD 0.3. Cells were then added to 96 well microtiter plates at an OD of 0.15. Uptake of berberine can be measured due to its ability to intercalate with DNA. Accumulation of berberine 30μg/mL was measured by fluorescence (λ 355nm excitation, λ 517nm emission) in the presence or absence of inhibitor 10μg/mL.

APPENDIX III

Antibacterial activity testing[146] (performed by Avexa Ltd., Australia)

Materials and Methods

The bacterial strains were used *S. aureus* ATCC 6538P, and 4 strains of *E. faecium* VRE243, VRE449, VRE820 and VRE987. It should be noted that, VRE243 and 987 were sensitive to vancomycin and VRE449 and VRE820 were resistant to vancomycin.

Antimicrobial assay

The Mueller-Hinton Broth (MHB) Medium culture media was prepared with final concentrations of 1 μg/mL $MgCl_2$ and 2 μg/mL $CaCl_2$ and was pre-warmed for 2-3 h at 37^{o}C before use**.** Mueller-Hinton Agar (MHA) Medium culture media was prepared with final concentrations of 1.5% Agar (Merck Agar 1.01614). *S. aureus* was streaked onto MHA and the plate was incubated overnight at 37^{o}C. From this plate, 10 cryovial were prepared by looping several colonies into 0.5 mL of 20% glycerol solution and were immediately stored at −140^{o}C. A cryovial was removed from −140^{o}C storage and thawed at room temperature. The MHA plate was streaked with a loopful of bacterial suspension and incubated overnight at 37^{o}C to create a parent plate (P1). The parent plate was stored at 4^{o}C. A daughter plate (D1) was incubated overnight at 37^{o}C and its loop of colony was used to inoculate a 125 mL flask containing 20 mL of MHB containing 25 μg/mL $CaCl_2.2H_2O$) and 12.5 μg/mL $MgCl_2.6H_2O$. The flask was shaken at 260 rpm for 18 h at 37^{o}C on an orbital incubator shaker. The parent plates 1 and 2

were each used twice to generate two daughter plates (D1 and D2) before being discarded.

The standardised inocula for assays was prepared as 1/10 dilution of seed cultures by adding 250 µL of the cultures to 2,250 µL of MHB in a disposable cuvette and the required dilution factor was calculated by dividing the observed OD_{650}. Sufficient volumes of the final inoculum cultures were prepared in pre-warmed MHB (37^{o}C) by diluting the standardised cultures to the required final concentration (10^{8} dilution).

Assay Procedure for 96-well Microtitre Plates

To each well of the 96-well microtitre plate was added 50 µL of liquid medium and 50 µL of test compound solution which was prepared by dissolving in 2.5 % DMSO was added in triplicate to the top of the microtitre plate. A vancomycin control set (triplicate) and a compound negative control set (triplicate) were also set up on each plate. The inoculated culture medium was incubated at 37^{o}C for 30 min shaking it at 130 rpm and using the multichannel pipette and multistepper pipette the adding, transferring and mixing of the inoculum were performed on the wells of the plates. The plates were incubated at 37^{o}C for 18 h shaking at 100 rpm in an environment with 90% relative humidity and the results were recorded as the highest dilution of test compound that prevented bacterial growth (MIC). The MIC was also determined for DMSO (2.5%) as a control measure.

APPENDIX IV

Antimalarial activity testing[147] (performed by Kamchonwongpaisan, S., BIOTEC, Thailand)

Materials and Methods

Samples were made up in DMSO solution. Using the Microdilution Radioisotope Technique, the *in vitro* antimalarial activity of the alkaloids was tested against *P. falciparum*, TM4 and K1 strains. The first strain is an anti-folate sensitive one while the second is an antifolate resistant strain.

Antimalarial assay

The sample (25 µl, in the culture medium) was placed in triplicate in a 96-well plate. Red blood cells (200 µl) infected with *P. falciparum* with a cell suspension (1.5%) of parasitemia (0.5-1%) were added to the wells. The range of the final concentrations of the samples varied from 1 x 10^{-5} to 1 x 10^{-8} g/ml with 0.1% of the organic solvent. The plates were cultured under standard conditions for 24 hours and the ^{3}H-hyphoxanthine (25 µl, 0.5 mCi) was added. The culture was incubated for 18-20 hours. The parasites' DNA was then harvested from the culture onto glass fibre filters. A radiation counter determined the amount of ^{3}H-hypoxanthine. The inhibitory concentration of the sample was determined from its dose-response curves or by calculation.

The Trager and Jensen method[148] was used to culture the *P. falciparum* K1 strain. The parasites were maintained in human red blood cells in a culture medium. RPMI

1640 was supplemented with 25 mM HEPES, 0.2% sodium bicarbonate, and 8% human serum, at 37^{o}C in a CO_2 incubator.

APPENDIX V

Cytotoxicity testing (performed by Vine, K., University of Wollongong)

Materials and Methods

Test compounds **40**, **60** and **64** were made up in 10% MeOH in DCM solution. Using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2*H* tetrazolium, inner salt (MTS) assay, the *in vitro* cytotoxicity of these compounds was tested against human hystiocytic lymphoma cells (cell line U937).

The U937 cancer cells were supplied by the Garvan Institute of Medical Research, Sydney, Australia. Cells were routinely maintained in vitro (37^{o}C, 5% CO_2 in humidified atmosphere) in a culture medium of RPMI-1640 media supplemented with 2mM L-glutamine, 5.6% (2g/L) sodium bicarbonate (Univar Analytical Reagents, Ajax Chemicals) and 5% Foetal Calf Serum (FCS) (MultiSer^{TM} Thermo Trace).

Cytotoxicity testing

Test compounds were tested for cytotoxicity against human hystiocytic lymphoma cells (cell line U937). Test compound dissolved in 10% MeOH in DCM solution was added to the wells of a sterile, 96 well microtitre plate from 100µg/mL to 1µg/mL. The 10% MeOH/DCM in the test solution was removed by either N_2 or Ar gas until the compound was completely dry. Cells ($2x10^4$ cells/well) in RPMI/5% FBS were added to the test compounds to give a final volume of 100µL. Positive control cultures were incubated with RPMI/5% FCS only, or in the case of solvent controls, 100µL EtOH dried with N_2 or Ar gas. Negative control cultures were incubated with RPMI/5% FCS

and 50% EtOH, all in triplicate. The cells were incubated for 24h at 37°C (5% CO_2 atmosphere), and then MTS (20□L) was added in the dark. The cells were incubated for a further 3h to allow colour development. Plates were read in the SpectroMax® 250 plate reader using Softmax Pro software V. 4.0 (Molecular devices, USA) at 490 nm.