2005

Development of berberine-based derivatives as novel antimicrobial agents

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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
the degree

DOCTOR OF PHILOSOPHY

from

UNIVERSITY OF WOLLONGONG

by

Siritron Samosorn, M.S. (Applied Chemistry)

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University of Wollongong
Wollongong, Australia
June 2005
Declaration

The work described in this Thesis does not contain any material that has been submitted for the award of any higher degree in this or any other University, and to the best of my knowledge and belief contains no material previously published by any other person, except where due reference has been acknowledged.

Siritron Samosorn

8th June 2005
Publications

Sections of the work described in this thesis have been reported in the following publications:


Acknowledgements

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## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>ArCH</td>
<td>CH in aromatic ring</td>
</tr>
<tr>
<td>ArH</td>
<td>aromatic proton</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ax</td>
<td>axial</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>(Boc)₂O</td>
<td>di-tert-butyl dicarbonate</td>
</tr>
<tr>
<td>br.d</td>
<td>broad doublet</td>
</tr>
<tr>
<td>br.m</td>
<td>broad multiplet</td>
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<td>tert-butanol</td>
</tr>
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<td>°C</td>
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<td>C</td>
<td>carbon</td>
</tr>
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<td>C. albicans</td>
<td>Candida albicans</td>
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<tr>
<td>CI</td>
<td>chemical ionization</td>
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<tr>
<td>13-CPTC</td>
<td>13-cyclopentylthio-5-hydroxy tetracycline</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
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<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets</td>
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</table>
ddd doublet of doublet of doublets
decomp. decomposition
DEPT distortionless enhancement by polarization transfer
DHU dicyclohexylurea
DMAP dimethylaminopyridine
DMF $N,N$-dimethylformamide
DMSO dimethylsulfoxide
DNA deoxyribose nucleic acid
dt doublet of triplet

$E. \text{coli}$ *Escherichia coli*

EDCI $1$-$[3$-(dimethylamino)propyl]-$3$-ethylcarbodiimide$

$E. \text{faecalis}$ *Enterococcus faecalis*

$E. \text{faecium}$ *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
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* *Pseudomonas aeruginosa*
PBP    Penicillin-binding proteins
PEG    polyethylene glycol
*P. falciparum* *Plasmodium falciparum*
Ph    phenyl
PhSO2Cl benzenesulfonyl chloride
PLC    preparative thin layer liquid chromatography
P-pg   P-glycoprotein
ppm    parts per million
PS    petroleum spirit
Rf    retention factor
RFU    relative fluorescence unit
RND   resistance nodulation division
rt    room temperature
s     singlet
SARs  structure-activity relationships

*S. aureus*  *Staphylococcus aureus*

*S. cereavaesiae*  *Saccharomyces cereavaesiae*

SMR  small multidrug resistance

TBDMS  *tert*-butyldimethylsilyl

td  triplet of doublet

TEA  triethylamine

THF  tetrahydrofuran

TLC  thin layer chromatography

TMS  tetramethylsilane

UV  ultraviolet

VLC  vacuum liquid chromatography

VRE  vancomycin-resistant *Enterococci*

$\delta$  chemical shift in parts per million downfield from TMS
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-1H-indole efflux pump inhibitors were synthesized. In the synthesis of these 2-aryl-5-nitro-1H-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-1H-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-1H-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-1H-indole inhibitors were particularly effective in potentiating the antibacterial activity of berberine. The compound [4-benzyloxy-2-(5-nitro-1H-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-
allyldihydroberberine followed by the elimination of propene. The antimicrobial activity of these indole-berberine compounds was assessed against a variety of pathogenic microorganisms. One of the dual action drugs, 9,10-dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)benzyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizininium bromide (64), was a potent antimicrobial agent at a clinically viable concentration against various bacteria in vitro, including *Staphylococcus aureus* K2361, *Enterococcus faecalis* V583, and *Salmonella enterica* Serovar Typhimurium SL1344R2. This compound also had good activity against the protozoan, *Plasmodium falciparum* K1 (in vitro).

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.
Table of Contents

Declaration ................................. i
Publications ............................... ii
Acknowledgements ......................... iii
List of Abbreviations ....................... v
Abstract .................................. xi

Chapter 1: Introduction .................... 1
  1.1 Isoquinoline alkaloids as natural antimicrobial agents 1
    1.1.1 Antimicrobial activity and structure-activity relationships (SARs) of berberine and related alkaloids 2
    1.1.2 Cytotoxicity of berberine and related alkaloids 5
    1.1.3 Structural features for antimicrobial and cytotoxic activities 6
  1.2 Antibacterial drug resistance problem 7
  1.3 Mechanisms of antimicrobial resistance 8
    1.3.1 Resistance by antibacterial alteration 9
    1.3.2 Resistance by bacterial target modification 9
    1.3.3 Resistance by reducing antibacterial permeability 10
  1.4 Bacterial efflux pump classification 11
  1.5 Bacterial MDR pump inhibitors 16
  1.6 Definitions of dual action prodrugs and dual action drugs 18
    1.6.1 Design principles of antimicrobials 21
  1.7 Aims of project .......................... 24

Chapter 2: Synthesis of berberine derivatives 25
2.1 Retrosynthesis of efflux pump inhibitor-berberine dual action agents 26

2.2 Synthetic strategies 28

2.2.1 Model Study 1 28

2.2.1.1 Reduction of berberine chloride 29

2.2.1.2 Enamine alkylation and reduction 31

2.2.1.3 Ester hydrolysis 32

2.2.1.4 Esterification 33

2.2.1.5 Oxidation 34

2.2.2 Model Study 2 35

2.2.2.1 Allylation 36

2.2.2.2 Enamine alkylation 37

Chapter 3: Synthesis of Efflux pump inhibitors 40

3.1 NorA efflux pump 40

3.2 Substrates of the NorA efflux pump 40

3.3 Inhibitors of the NorA efflux pump 42

3.3.1 Synthesis of 5,7-deoxyhydnocarpin-D (12) 48

3.3.1.1 Acetylation of the regioisomer 12 52

3.3.2 Synthesis of 2-aryl-5-nitro-1H-indoles 54

3.3.3 Attempted synthesis of 2-aryl-5-nitro-1H-indoles via Fischer indolization 56

3.3.4 Synthetic strategy of 2-aryl-5-nitro-1H-indole derivatives via Palladium cyclization 60

3.3.4.1 N-acylation of indoles 62

3.3.4.2 Cyclization of the N-acylated indoles 71
3.3.4.3 Amide hydrolysis of the cyclized products 76
3.3.4.4 Reduction of acids to alcohols 78
3.3.4.5 Attempted conversion of alcohols to amines 79
3.3.4.6 Reduction of azides to amines 82
3.3.4.7 Preparation of α-bromoacetamides 84
3.3.4.8 Preparation of α-bromoesters 85
3.3.4.9 Preparation of indole benzyl bromide derivatives from the alcohols 86
3.3.4.10 Attempted N-protection of indole 42 89

Chapter 4: Synthesis of the berberine-indole dual action agents 92

4.1 Synthesis of the berberine-indole prodrugs with a cleavable linkage 92
4.1.1 Synthesis of the ester prodrug (60) 93
4.1.2 Synthesis of the ester prodrug (61) 94
4.1.3 Synthesis of the amide prodrug (62) 95
4.1.4 Synthesis of the amide prodrug (63) 96
4.1.5 Synthesis of the dual action drug (64) 97
4.1.6 Synthesis of the dual action drug (65) 97

4.2 Attempted linking group expansion of berberine-indole hybrids 99
4.2.1 Attempted synthesis of a berberine-indole hybrid via a cross metathesis reaction 100
4.2.2 Synthesis of 2-(tert-butyldimethylsilyloxy)ethanol (68) 106
4.2.3 Attempted O-alkylation of 55a 106

4.3 Hydrolysis of the ester linked berberine-indole prodrug 107

Chapter 5: Biological test results 109

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

5.2 MDR pump inhibitory testing results

5.3 Antimicrobial testing results (Direct activity)
   5.3.1 5-Nitroindoles
   5.3.2 Berberine derivatives

5.4 Cytotoxicity results

Chapter 6: Conclusion and Future Directions

6.1 Conclusions

6.2 Future directions

Chapter 7: Experimental

7.1 General

7.2 Dihydroberberine route to 13-substituted berberines (Chapter 2)
   7.2.1 Preparation of 9,10-Dimethoxy-5,8-dihydro-6H-benzo[g]-1,3-benzodioxolo[5,6-\textit{a}]quinolizine (2)
   7.2.2 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6H-benzo[g]-1,3-benzodioxolo[5,6-\textit{a}]quinolizin-13-yl)-acetic acid ethyl ester (3)
   7.2.3 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6H-benzo[g]-1,3-benzodioxolo[5,6-\textit{a}]quinolizin-13-yl)-acetic acid (4)
   7.2.4 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6H-benzo[g]-1,3-benzodioxolo[5,6-\textit{a}]quinolizin-13-yl)-acetic acid benzyl ester (5)
   7.2.5 Preparation of 13-Ethoxycarbonylmethyl-9,10-dimethoxy-5,6-dihydro benzo[g]-1,3-benzodioxolo[5,6-\textit{a}]quinolizinium iodide (6\textit{a})
   7.2.6 Preparation of 13-Benzylxycarbonylmethyl-9,10-dimethoxy-5,6-dihydro
dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium iodide (7a)  
and bromide (7b)  

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

7.3 8-Allyldihydroberberine route (Chapter 2)  

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

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)  

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

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)  

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

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

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

7.4.2 Acetylation of 5,7-Deoxyhydnocarpin-D  

7.5 Synthesis of synthetic bacterial pump blocking agents (Chapter 3)  

7.5.1 Attempted Fischer indole synthesis  

7.5.2 N-Acylation reaction
7.5.2.1 Preparation of 1-Benzoyl-5-nitro-1\textit{H}-indole (18) 152
7.5.2.2 Preparation of 1-(4-methoxybenzoyl)-5-nitro-1\textit{H}-indole (19) 152
7.5.2.3 Preparation of 1-(4-benzyloxybenzoyl)-5-nitro-1\textit{H}-indole (22) 153
7.5.2.4 Preparation of 5-nitro-1-phenylethanoyl-1\textit{H}-indole (23) 155
7.5.2.5 Preparation of 1-benzoyl-1\textit{H}-indole (24) 156
7.5.2.6 Preparation of 1-(4-methoxybenzoyl)-1\textit{H}-indole (25) 156
7.5.2.7 Preparation of 1-(2-methoxybenzoyl)-1\textit{H}-indole (26) 157
7.5.2.8 Preparation of 1-benzoyl-5-methoxy-1\textit{H}-indole (27) 158
7.5.2.9 Preparation of 5-methoxy-1-(4-methoxybenzoyl)-1\textit{H}-indole (28) 158
7.5.2.10 Preparation of 1-benzoyl-5-fluoro-1\textit{H}-indole (29) 159
7.5.2.11 Preparation of 5-fluoro-1-(4-methoxybenzoyl)-1\textit{H}-indole (30) 160
7.5.2.12 Preparation of 5-fluoro-1-(2-methoxybenzoyl)-1\textit{H}-indole (31) 160
7.5.2.13 Preparation of 1-benzoyl-5-nitro-1\textit{H}-indole (18) 161
7.5.2.14 Preparation of 1-(4-methoxybenzoyl)-5-nitro-1\textit{H}-indole (19) 162
7.5.2.15 Preparation of 1-(2-methoxybenzoyl)-5-nitro-1\textit{H}-indole (32) 162
7.5.2.16 Preparation of 5-nitro-1-phenylethanoyl-1\textit{H}-indole (23) 163
7.5.2.17 Preparation of 1-(4-benzyloxybenzoyl)-5-nitro-1\textit{H}-indole (22) 163

7.5.3 Cyclization reactions 164
7.5.3.1 Preparation of 2-Nitro-isoindolo[2,1-\textit{a}]indol-6-one (33) 164
7.5.3.2 Preparation of 9-Methoxy-2-nitro-isoindolo[2,1-\textit{a}]indol-6-one (34) 165
7.5.3.3 Preparation of 9-Benzylxy-2-nitro-isoindolo[2,1-\textit{a}]indol-6-one (35) 165
7.5.3.4 Conversion of 36 to 35 167

7.5.4 Ring opening reactions 167
7.5.4.1 Preparation of 2-(5-Nitro-1\textit{H}-indol-2-yl)benzoic acid (38) 167
7.5.4.2 Preparation of 4-Methoxy-2-(5-nitro-1\textit{H}-indol-2-yl)benzoic acid (39) 168
7.5.4.3 Preparation of 4-Benzylloxy-2-(5-nitro-1\textit{H}-indol-2-yl)-benzoic acid (40) 169
7.5.5 Reduction reactions 169
7.5.5.1 Preparation of [2-(5-Nitro-1\textit{H}-indol-2-yl)-phenyl]-methanol (41) 169
7.5.5.2 Preparation of [4-Methoxy-2-(5-nitro-1\textit{H}-indol-2-yl)-phenyl]-methanol (42) 170
7.5.5.3 Preparation of [4-Benzylloxy-2-(5-nitro-1\textit{H}-indol-2-yl)-phenyl]-methanol (43) 171
7.5.6 Amination reactions 172
7.5.6.1 Preparation of 2-(2-Azidomethyl-phenyl)-5-nitro-1\textit{H}-indole (44) 172
7.5.6.2 Preparation of 2-(5-nitro-1\textit{H}-indol-2-yl)-benzylamine (45) 173
7.5.6.3 Preparation of 2-(2-Azidomethyl-5-methoxy-phenyl)-5-nitro-1\textit{H}-indole (47) 174
7.5.6.4 Preparation of 4-Methoxy-2-(5-nitro-1\textit{H}-indol-2-yl)-benzylamine (48) 175
7.5.7 \textit{N}-Alkylation reactions 176
7.5.7.1 Preparation of 2-Bromo-\textit{N}-[2-(5-nitro-1\textit{H}-indol-2-yl)benzyl]-acetamide (49) 176
7.5.7.2 Preparation of 2-Bromo-\textit{N}-[4-methoxy-2-(5-nitro-1\textit{H}-indol-2-yl)benzyl]-acetamide (50) 177
7.5.8 \textit{O}-Alkylation reactions 178
7.5.8.1 Preparation of Bromoacetic acid 2-(5-nitro-1H-indol-2-yl)-benzyl ester (51) 178

7.5.8.2 Preparation of Bromoacetic acid 4-methoxy-2-(5-nitro-1H-indol-2-yl)phenyl)-benzyl ester (52) 178

7.5.9 Bromination reactions 179

7.5.9.1 Preparation of 2-(2-Bromomethylphenyl)-5-nitro-1H-indole (53) 179

7.5.9.2 Attempted bromination of the benzyl alcohol 42 180

7.5.9.3 Preparation of 2-(5-Methoxy-2-vinyl-phenyl)-5-nitro-1H-indole (55) 181

7.5.9.4 Attempted N-protection of the benzyl alcohol 42 182

7.6 Alkylation reactions (Chapter 4) 184

7.6.1 Preparation of 9,10-Dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)-benzylloxycarbonyl-methyl]-5,6-dihydro-benzo[g]-1,3-benzodioxolo [5,6-a]quino-lizinium bromide (60) 184

7.6.2 Preparation of 9,10-Dimethoxy-13-[4-methoxy-2-(5-nitro-1H-indol-2-yl)-benzylloxycar-bonylmethyl]-5,6-dihydro-benzo[g]-1,3-benzodioxolo[5,6-a] quinolinizinium bromide (61) 185

7.6.3 Preparation of 9,10-Dimethoxy-13-{{2-(5-nitro-1H-indol-2-yl)-benzylcarbamoyl}-methyl}-5,6-dihydro-benzo[g]-1,3-benzodioxolo [5,6-a]quino-lizinium bromide (62) 186

7.6.4 Preparation of 9,10-Dimethoxy-13-{{4-methoxy-2-(5-nitro-1H-indol-2-yl)-benzylcarbamoyl}-methyl}-5,6-dihydro-benzo[g]-1,3-benzodioxolo[5,6-a]quinolinizinium bromide (63) 187

7.6.5 Preparation of 9,10-Dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)benzyl]-
5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-α]quinolizininium bromide (64) 188

7.6.6 Preparation of 9,10-Dimethoxy-13-[4-methoxy-2-(5-nitro-1H-indol-2-yl)benzyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-α]quinolizininium chloride (65) 189

7.7 Attempted synthesis to increase the bond length between berberine and pump blocker (Chapter 4) 190

7.7.1 Preparation of 13-Allyl-9,10-dimethoxy-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-α]quinolizininium bromide (66) 190

7.7.2 Attempted cross metathesis reaction of 66 191

7.7.3 Attempted O-alkylation of 42 191

7.8 Enzymatic hydrolysis of 9,10-Dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)benzyloxy carbonyl-methyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-α]quinolizininium bromide (60) 194

References 195

Appendices 203
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Department of Chemistry
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Wollongong, Australia
June 2005
Declaration

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Siritron Samosorn

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To Dr. Kirsten Benkendorff and Dr. Carey Rogers for providing me your microbiology skills and teaching me how to manipulate and undertake the antibacterial testing.

To Prof. Kim Lewis and Anthony Ball, Northeastern University, USA for your microbiological specialist aspect, and carrying out the bacterial multidrug resistance pump inhibitory testing together with antibacterial testing; Dr. Sumalee Kamchonwongpaisan, National Science and Technology Development Agency, Thailand for conducting antimalarial testing; Avexa, Ltd., Australia for performing the antibacterial testing; and Ms. Kara Vine for operating cytotoxicity testing for my samples.

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# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>ArCH</td>
<td>CH in aromatic ring</td>
</tr>
<tr>
<td>ArH</td>
<td>aromatic proton</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ax</td>
<td>axial</td>
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<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>(Boc)$_2$O</td>
<td>di-tert-butyl dicarbonate</td>
</tr>
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<td>br.d</td>
<td>broad doublet</td>
</tr>
<tr>
<td>br.m</td>
<td>broad multiplet</td>
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<td>br.s</td>
<td>broad singlet</td>
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<tr>
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<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Candida albicans</td>
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<tr>
<td>CI</td>
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<tr>
<td>13-CPTC</td>
<td>13-cyclopentylthio-5-hydroxy tetracycline</td>
</tr>
<tr>
<td>d</td>
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<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<tr>
<td>dd</td>
<td>doublet of doublets</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ddd</td>
<td>doublet of doublet of doublets</td>
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<tr>
<td>decomp.</td>
<td>decomposition</td>
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<td>DEPT</td>
<td>distortionless enhancement by polarization transfer</td>
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<td>dicyclohexylurea</td>
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<td>DMAP</td>
<td>dimethylaminopyridine</td>
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<td>DMF</td>
<td>(N,N)-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
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<td>dt</td>
<td>doublet of triplet</td>
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<tr>
<td>(E. coli)</td>
<td>(Escherichia coli)</td>
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<tr>
<td>EDCI</td>
<td>1-([3-(dimethylamino)propyl])-3-ethylcarbodiimide</td>
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<tr>
<td>(E. faecalis)</td>
<td>(Enterococcus faecalis)</td>
</tr>
<tr>
<td>(E. faecium)</td>
<td>(Enterococcus faecium)</td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
</tr>
<tr>
<td>eq</td>
<td>(molar) equivalent/equatorial</td>
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<td>ES</td>
<td>electrospray</td>
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<td>FDA</td>
<td>food and drug administration</td>
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<td>g</td>
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<td>gradient correlation spectroscopy</td>
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<tr>
<td>gHMBC</td>
<td>gradient heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>gHSQC</td>
<td>gradient heteronuclear single quantum correlation</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>----------</td>
<td>-------------------------------------------------</td>
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<tr>
<td>H</td>
<td>hydrogen/proton</td>
</tr>
<tr>
<td>HOBT</td>
<td>1-hydroxybenzotriazole</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
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<td>horseradish peroxide</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>inhibitory concentration 50%</td>
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<tr>
<td>J</td>
<td>coupling constant</td>
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<td>Lit.</td>
<td>literature</td>
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<tr>
<td>m</td>
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<tr>
<td>M</td>
<td>molar (moles per litre)</td>
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<tr>
<td>m.p.</td>
<td>melting point</td>
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<td>m/z</td>
<td>mass to charge ratio</td>
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<td>MATE</td>
<td>multidrug and toxic compound extrusion</td>
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<td>MDR</td>
<td>multidrug resistance</td>
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<tr>
<td>Me</td>
<td>methyl</td>
</tr>
<tr>
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<td>methanol</td>
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<tr>
<td>MFS</td>
<td>major facilitator superfamily</td>
</tr>
<tr>
<td>5'-MHC</td>
<td>5'-methoxyhydnocarpin</td>
</tr>
<tr>
<td>MHz</td>
<td>mekahertz</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>min</td>
<td>minute/s</td>
</tr>
<tr>
<td>mL</td>
<td>milliliters</td>
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<tr>
<td>µM</td>
<td>micromolar</td>
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mmol \quad \text{millimoles}

MRSA \quad \text{methicillin-resistant } Staphylococcus aureus

MS \quad \text{mass spectroscopy}

NBS \quad N\text{-bromosuccinimide}

n\text{-BuLi} \quad \text{normal-butyl lithium}

NCS \quad N\text{-chlorosuccinimide}

NMR \quad \text{nuclear magnetic resonance}

OH \quad \text{hydroxy}

OMe \quad \text{methoxy}

P. aeruginosa \quad \text{Pseudomonas aeruginosa}

PBPs \quad \text{Penicillin-binding proteins}

PEG \quad \text{polyethylene glycol}

P. falciparum \quad \text{Plasmodium falciparum}

Ph \quad \text{phenyl}

PhSO₂Cl \quad \text{benzenesulfonyl chloride}

PLC \quad \text{preparative thin layer liquid chromatography}

P-pg \quad \text{P-glycoprotein}

ppm \quad \text{parts per million}

PS \quad \text{petroleum spirit}

R_f \quad \text{retention factor}

RFU \quad \text{relative fluorescence unit}

RND \quad \text{resistance nodulation division}

rt \quad \text{room temperature}

s \quad \text{singlet}
<table>
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<td>structure-activity relationships</td>
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<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>S. cereavaesiae</td>
<td><em>Saccharomyces cereavaesiae</em></td>
</tr>
<tr>
<td>SMR</td>
<td>small multidrug resistance</td>
</tr>
<tr>
<td>TBDMS</td>
<td>tert-butyldimethylsilyl</td>
</tr>
<tr>
<td>td</td>
<td>triplet of doublet</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VLC</td>
<td>vacuum liquid chromatography</td>
</tr>
<tr>
<td>VRE</td>
<td>vancomycin-resistant <em>Enterococci</em></td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift in parts per million downfield from TMS</td>
</tr>
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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-1H-indole efflux pump inhibitors were synthesized. In the synthesis of these 2-aryl-5-nitro-1H-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-1H-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-1H-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-1H-indole inhibitors were particularly effective in potentiating the antibacterial activity of berberine. The compound [4-benzyloxy-2-(5-nitro-1H-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-
allyldihydroberberine followed by the elimination of propene. The antimicrobial activity of these indole-berberine compounds was assessed against a variety of pathogenic microorganisms. One of the dual action drugs, 9,10-dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)benzyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolinizinium bromide (64), was a potent antimicrobial agent at a clinically viable concentration against various bacteria in vitro, including Staphylococcus aureus K2361, Enterococcus faecalis V583, and Salmonella enterica Serovar Typhimurium SL1344R2. This compound also had good activity against the protozoan, Plasmodium falciparum K1 (in vitro).

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.
Table of Contents

Declaration i

Publications ii

Acknowledgements iii

List of Abbreviations v

Abstract xi

Chapter 1: Introduction 1

1.1 Isoquinoline alkaloids as natural antimicrobial agents 1

1.1.1 Antimicrobial activity and structure-activity relationships (SARs) of berberine and related alkaloids 2

1.1.2 Cytotoxicity of berberine and related alkaloids 5

1.1.3 Structural features for antimicrobial and cytotoxic activities 6

1.2 Antibacterial drug resistance problem 7

1.3 Mechanisms of antimicrobial resistance 8

1.3.1 Resistance by antibacterial alteration 9

1.3.2 Resistance by bacterial target modification 9

1.3.3 Resistance by reducing antibacterial permeability 10

1.4 Bacterial efflux pump classification 11

1.5 Bacterial MDR pump inhibitors 16

1.6 Definitions of dual action prodrugs and dual action drugs 18

1.6.1 Design principles of antimicrobials 21

1.7 Aims of project 24

Chapter 2: Synthesis of berberine derivatives 25
2.1 Retrosynthesis of efflux pump inhibitor-berberine dual action agents 26

2.2 Synthetic strategies 28

2.2.1 Model Study 1 28

2.2.1.1 Reduction of berberine chloride 29

2.2.1.2 Enamine alkylation and reduction 31

2.2.1.3 Ester hydrolysis 32

2.2.1.4 Esterification 33

2.2.1.5 Oxidation 34

2.2.2 Model Study 2 35

2.2.2.1 Allylation 36

2.2.2.2 Enamine alkylation 37

Chapter 3: Synthesis of Efflux pump inhibitors 40

3.1 NorA efflux pump 40

3.2 Substrates of the NorA efflux pump 40

3.3 Inhibitors of the NorA efflux pump 42

3.3.1 Synthesis of 5,7-deoxyhydnocarpin-D (12) 48

3.3.1.1 Acetylation of the regioisomer 12 52

3.3.2 Synthesis of 2-aryl-5-nitro-1H-indoles 54

3.3.3 Attempted synthesis of 2-aryl-5-nitro-1H-indoles via Fischer indolization 56

3.3.4 Synthetic strategy of 2-aryl-5-nitro-1H-indole derivatives via Palladium cyclization 60

3.3.4.1 N-acylation of indoles 62

3.3.4.2 Cyclization of the N-acylated indoles 71
3.3.4.3 Amide hydrolysis of the cyclized products 76
3.3.4.4 Reduction of acids to alcohols 78
3.3.4.5 Attempted conversion of alcohols to amines 79
3.3.4.6 Reduction of azides to amines 82
3.3.4.7 Preparation of α-bromoacetamides 84
3.3.4.8 Preparation of α-bromoesters 85
3.3.4.9 Preparation of indole benzyl bromide derivatives from the alcohols 86
3.3.4.10 Attempted N-protection of indole 42 89

Chapter 4: Synthesis of the berberine-indole dual action agents 92

4.1 Synthesis of the berberine-indole prodrugs with a cleavable linkage 92

4.1.1 Synthesis of the ester prodrug (60) 93
4.1.2 Synthesis of the ester prodrug (61) 94
4.1.3 Synthesis of the amide prodrug (62) 95
4.1.4 Synthesis of the amide prodrug (63) 96
4.1.5 Synthesis of the dual action drug (64) 97
4.1.6 Synthesis of the dual action drug (65) 97

4.2 Attempted linking group expansion of berberine-indole hybrids 99

4.2.1 Attempted synthesis of a berberine-indole hybrid via a cross metathesis reaction 100
4.2.2 Synthesis of 2-(tert-butyldimethylsilyloxy)ethanol (68) 106
4.2.3 Attempted O-alkylation of 55a 106

4.3 Hydrolysis of the ester linked berberine-indole prodrug 107

Chapter 5: Biological test results 109

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 110

5.2 MDR pump inhibitory testing results 113

5.3 Antimicrobial testing results (Direct activity) 119

5.3.1 5-Nitroindoles 119

5.3.2 Berberine derivatives 121

5.4 Cytotoxicity results 128

Chapter 6: Conclusion and Future Directions 129

6.1 Conclusions 129

6.2 Future directions 131

Chapter 7: Experimental 133

7.1 General 133

7.2 Dihydroberberine route to 13-substituted berberines (Chapter 2) 135

7.2.1 Preparation of 9,10-Dimethoxy-5,8-dihydro-6H-benzo[g]-1,3-benzodioxolo[5,6-\(\alpha\)]quinolizine (2) 135

7.2.2 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6H-benzo[g]-1,3-benzodioxolo[5,6-\(\alpha\)]quinolizin-13-yl)-acetic acid ethyl ester (3) 136

7.2.3 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6H-benzo[g]-1,3-benzodioxolo[5,6-\(\alpha\)]quinolizin-13-yl)-acetic acid (4) 137

7.2.4 Preparation of (9,10-Dimethoxy-5,8,13,13a-tetrahydro-6H-benzo[g]-1,3-benzodioxolo[5,6-\(\alpha\)]quinolizin-13-yl)-acetic acid benzyl ester (5) 137

7.2.5 Preparation of 13-Ethoxycarbonylmethyl-9,10-dimethoxy-5,6-dihydro benzo[g]-1,3-benzodioxolo[5,6-\(\alpha\)]quinolizinium iodide (6a) 140

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

7.2.7 Preparation of 13-Carboxymethyl-9,10-dimethoxy-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium iodide (8) 142

7.3 8-Allyldihydroberberine route (Chapter 2) 142

7.3.1 Preparation of 8-Allyl-9,10-dimethoxy-5,8-dihydro-6H-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizine (9) 142

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) 143

7.3.3 Preparation of 13-Benzylxycarbonylmethyl-9,10-dimethoxy-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (7b) from 8-Allyldihydroberberine (9) 144

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) 145

7.3.5 Preparation of 13-benzyl-9,10-dimethoxy-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (11) 146

7.4 Synthesis of natural bacterial pump blocking agents (Chapter 3) 147

7.4.1 Preparation of 5,7-Deoxyhydnocarpin-D (12) 147

7.4.2 Acetylation of 5,7-Deoxyhydnocarpin-D 149

7.5 Synthesis of synthetic bacterial pump blocking agents (Chapter 3) 149

7.5.1 Attempted Fischer indole synthesis 149

7.5.2 N-Acylation reaction 152
7.5.2.1 Preparation of 1-Benzoyl-5-nitro-1\(H\)-indole (18) 152
7.5.2.2 Preparation of 1-(4-methoxybenzoyl)-5-nitro-1\(H\)-indole (19) 152
7.5.2.3 Preparation of 1-(4-benzyloxybenzoyl)-5-nitro-1\(H\)-indole (22) 153
7.5.2.4 Preparation of 5-nitro-1-phenylethanoyl-1\(H\)-indole (23) 155
7.5.2.5 Preparation of 1-benzoyl-1\(H\)-indole (24) 156
7.5.2.6 Preparation of 1-(4-methoxybenzoyl)-1\(H\)-indole (25) 156
7.5.2.7 Preparation of 1-(2-methoxybenzoyl)-1\(H\)-indole (26) 157
7.5.2.8 Preparation of 1-benzoyl-5-methoxy-1\(H\)-indole (27) 158
7.5.2.9 Preparation of 5-methoxy-1-(4-methoxybenzoyl)-1\(H\)-indole (28) 158
7.5.2.10 Preparation of 1-benzoyl-5-fluoro-1\(H\)-indole (29) 159
7.5.2.11 Preparation of 5-fluoro-1-(4-methoxybenzoyl)-1\(H\)-indole (30) 160
7.5.2.12 Preparation of 5-fluoro-1-(2-methoxybenzoyl)-1\(H\)-indole (31) 160
7.5.2.13 Preparation of 1-benzoyl-5-nitro-1\(H\)-indole (18) 161
7.5.2.14 Preparation of 1-(4-methoxybenzoyl)-5-nitro-1\(H\)-indole (19) 162
7.5.2.15 Preparation of 1-(2-methoxybenzoyl)-5-nitro-1\(H\)-indole (32) 162
7.5.2.16 Preparation of 5-nitro-1-phenylethanoyl-1\(H\)-indole (23) 163
7.5.2.17 Preparation of 1-(4-benzyloxybenzoyl)-5-nitro-1\(H\)-indole (22) 163
7.5.3 Cyclization reactions 164
7.5.3.1 Preparation of 2-Nitro-isoiindolo[(2,1-a)indol-6-one (33) 164
7.5.3.2 Preparation of 9-Methoxy-2-nitro-isoiindolo[(2,1-a)indol-6-one (34) 165
7.5.3.3 Preparation of 9-Benzylxy-2-nitro-isoiindolo[(2,1-a)indol-6-one (35) 165
7.5.3.4 Conversion of 36 to 35 167
7.5.4 Ring opening reactions 167
7.5.4.1 Preparation of 2-(5-Nitro-1H-indol-2-yl)benzoic acid (38) 167
7.5.4.2 Preparation of 4-Methoxy-2-(5-nitro-1H-indol-2-yl)benzoic acid (39) 168
7.5.4.3 Preparation of 4-Benzylxoy-2-(5-nitro-1H-indol-2-yl)-benzoic acid (40) 169
7.5.5 Reduction reactions 169
7.5.5.1 Preparation of [2-(5-Nitro-1H-indol-2-yl)-phenyl]-methanol (41) 169
7.5.5.2 Preparation of [4-Methoxy-2-(5-nitro-1H-indol-2-yl)-phenyl]-methanol (42) 170
7.5.5.3 Preparation of [4-Benzylxoy-2-(5-nitro-1H-indol-2-yl)-phenyl]-methanol (43) 171
7.5.6 Amination reactions 172
7.5.6.1 Preparation of 2-(2-Azidomethyl-phenyl)-5-nitro-1H-indole (44) 172
7.5.6.2 Preparation of 2-(5-nitro-1H-indol-2-yl)-benzylamine (45) 173
7.5.6.3 Preparation of 2-(2-Azidomethyl-5-methoxy-phenyl)-5-nitro-1H-indole (47) 174
7.5.6.4 Preparation of 4-Methoxy-2-(5-nitro-1H-indol-2-yl)-benzylamine (48) 175
7.5.7 N-Alkylation reactions 176
7.5.7.1 Preparation of 2-Bromo-N-[2-(5-nitro-1H-indol-2-yl)benzyl]-acetamide (49) 176
7.5.7.2 Preparation of 2-Bromo-N-[4-methoxy-2-(5-nitro-1H-indol-2-yl)-benzyl]-acetamide (50) 177
7.5.8 O-Alkylation reactions 178
7.5.8.1 Preparation of Bromoacetic acid 2-(5-nitro-1H-indol-2-yl)-benzyl ester (51) 178

7.5.8.2 Preparation of Bromoacetic acid 4-methoxy-2-(5-nitro-1H-indol-2-yl)phenyl)-benzyl ester (52) 178

7.5.9 Bromination reactions 179

7.5.9.1 Preparation of 2-(2-Bromomethylphenyl)-5-nitro-1H-indole (53) 179

7.5.9.2 Attempted bromination of the benzyl alcohol 42 180

7.5.9.3 Preparation of 2-(5-Methoxy-2-vinyl-phenyl)-5-nitro-1H-indole (55) 181

7.5.9.4 Attempted N-protection of the benzyl alcohol 42 182

7.6 Alkylation reactions (Chapter 4) 184

7.6.1 Preparation of 9,10-Dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)-benzyloxy carbonyl-methyl]-5,6-dihydro-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (60) 184

7.6.2 Preparation of 9,10-Dimethoxy-13-[4-methoxy-2-(5-nitro-1H-indol-2-yl)-benzyloxy carbonylmethyl]-5,6-dihydro-benzo[g]-1,3-benzodioxolo[5,6-a] quinolizinium bromide (61) 185

7.6.3 Preparation of 9,10-Dimethoxy-13-\{[2-(5-nitro-1H-indol-2-yl)-benzylcarbamoyl]-methyl\}-5,6-dihydro-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (62) 186

7.6.4 Preparation of 9,10-Dimethoxy-13-\{[4-methoxy-2-(5-nitro-1H-indol-2-yl)-benzylcarbamoyl]-methyl\}-5,6-dihydro-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (63) 187

7.6.5 Preparation of 9,10-Dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)benzyl]-
5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizininium bromide (64) 188

7.6.6 Preparation of 9,10-Dimethoxy-13-[4-methoxy-2-(5-nitro-1H-indol-2-yl)benzyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizininium chloride (65) 189

7.7 Attempted synthesis to increase the bond length between berberine and pump blocker (Chapter 4) 190

7.7.1 Preparation of 13-Allyl-9,10-dimethoxy-5,6-dihydrobenzo[g]-1,3 benzodioxolo[5,6-a]quinolizininium bromide (66) 190

7.7.2 Attempted cross metathesis reaction of 66 191

7.7.3 Attempted O-alkylation of 42 191

7.8 Enzymatic hydrolysis of 9,10-Dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)benzyloxy carbonyl-methyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizininium bromide (60) 194

References 195

Appendices 203
Chapter 1: 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 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. The therapeutic uses of berberine are in the treatment of infected eyes and eye irritations (Murine™) while sanguinarine is used as an antiplaque in toothpaste and mouthwash. Sanguinarine has been classified by the FDA as being unsafe for use in food and drugs.

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 Aspergillus fumigatus, antiplasmodial activity against Plasmodium falciparum, and several other pharmacological activities. The extensive antimicrobial screening data (Amin et al.) of berberine sulfate are shown in Table 1-1. Moreover, it has recently been reported that berberine possesses anti-tumour activity. 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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Minimum growth inhibitory concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mL</td>
</tr>
<tr>
<td>Bacillus pumilus</td>
<td>25</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>50</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>25</td>
</tr>
<tr>
<td>Corynebacterium diphtheriae</td>
<td>6.2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>50 – greater than 100</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>25</td>
</tr>
<tr>
<td>Pseudomonas pyocyanea</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Salmonella paratyphi</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Salmonella schottmuelleri</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Shigella boydii</td>
<td>12.5</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6.2 - 50.0</td>
</tr>
<tr>
<td>Staphylococcus albus</td>
<td>50</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>12.5</td>
</tr>
<tr>
<td>Vibrio cholerae Inaba 569B</td>
<td>25</td>
</tr>
<tr>
<td>Vibrio cholerae El Tor Ogawa</td>
<td>50</td>
</tr>
<tr>
<td>Xanthomonas citri</td>
<td>3.1</td>
</tr>
<tr>
<td>Xanthomonas campestris</td>
<td>6.2</td>
</tr>
<tr>
<td>Xanthomonas malvacearum</td>
<td>12.5</td>
</tr>
<tr>
<td>Erwinia carotovora</td>
<td>100</td>
</tr>
<tr>
<td>Pseudomonas mangiferae</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Pseudomonas solanacearum</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Candida utilis</td>
<td>12.5</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>12.5</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>3.1</td>
</tr>
<tr>
<td>Sporothrichum schenckii</td>
<td>6.2</td>
</tr>
</tbody>
</table>

*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 R1 + R2 = CH2) showed a higher activity against P. falciparum than compounds having a methoxy group at the same
positions, \((R_1 = R_2 = \text{OCH}_3)\). In addition, replacement of a methoxy group at C-2 or C-3 by a hydroxy group resulted in an increase in activity.

![Figure 1-1 General structures and numbering of protoberberine alkaloid derivatives for SARs](image)

**Influence of substituents on ring C**

There have been several investigations by Iwasa et al\(^9\text{-}^{12}\) 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 \((R_3 = \text{CH}_3, \text{C}_2\text{H}_5, \text{n-}C_3\text{H}_7, \text{and n-}C_4\text{H}_9)\) (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 \((R_4, \text{Figure 1-1a})\) 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 \((R_7 = \text{Me in Figure 1-1b})\), 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\(^\text{13}\) 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.\(^\text{14,15}\)

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),\(^\text{16}\) 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.\textsuperscript{17}

In addition, Iwasa et al.\textsuperscript{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.\(^\text{19}\)

Table 1-2 Evolution of resistance to antibiotics\(^\text{20}\)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Year deployed</th>
<th>Resistance observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonamides</td>
<td>1930s</td>
<td>1940s</td>
</tr>
<tr>
<td>Penicillin</td>
<td>1943</td>
<td>1946</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1943</td>
<td>1959</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1947</td>
<td>1959</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1948</td>
<td>1953</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1952</td>
<td>1988</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1956</td>
<td>1988</td>
</tr>
<tr>
<td>Methicillin</td>
<td>1960</td>
<td>1961</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1961</td>
<td>1973</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>1960s</td>
<td>late1960s</td>
</tr>
</tbody>
</table>

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 \(\beta\)-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.\textsuperscript{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 \textit{S. aureus} (MRSA) strains. MRSA is the most common antibiotic-resistant organism in hospitals, and ranks as the most frequently isolated pathogen associated with bloodstream infections in North America between 1997-2001.\textsuperscript{22} Several variations of multidrug resistance (MDR) have been found in MRSA isolates, with resistances to macrolide-lincosamide-streptogramin B (MLS\textsubscript{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 \textit{S. aureus} was reported in Japan in 1996,\textsuperscript{23} and subsequently in many other countries. Similarly, \textit{Enterococcal} strains are also commonly isolated pathogens in bloodstream infections in North America.\textsuperscript{22} Many \textit{Enterococcus faecium} isolates are now vancomycin-resistant and \textit{E. faecalis} isolates also showed resistance to aminoglycosides and \(\beta\)-lactams. Unfortunately, the dual drug quinupristin-dalfopristin (Synercid\textsuperscript{TM}, a new drug that has activity against MDR \textit{S. aureus} and has been introduced into hospital use) does not control \textit{E. faecalis}.\textsuperscript{24,25} Clearly, new drugs are urgently needed to overcome the serious multidrug resistance problem in pathogenic bacteria.

\subsection*{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.\textsuperscript{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.

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

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)](image)

### 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.\

**Table 1-3 Antibacterial Resistance Efflux Families, Biochemical Characteristics, Efflux Substrates and Bacteria Hosts**

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

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 transporters. 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.

![Diagram of the membrane topology of proton-driven drug pumps in Gram-negative bacteria](image)

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. Recently, the crystal structures of outer membrane TolC and inner (cytoplasmic) membrane AcrB 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 form 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*](image)

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.
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. 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.
“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 cooperative interaction between the two NBDs, ATP is likely to bind to the non-ligated 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.”

### 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, destruction of the pumps’ energy by
inhibiting binding of ATP and modifying protein structure by an inhibitor interaction with the cell membrane.\textsuperscript{29,41}

![Chemical structures of MC-207,110, INF392, 13-CPTC, and 5'-MHC-D](image)

**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.\textsuperscript{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, Enterobacteriaceae, Haemophilus influenza* and *Stenotrophomonas maltophilia*, which potentiates the effects of fluoroquinolone antibiotics.\textsuperscript{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*.\textsuperscript{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.
18

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 (\textit{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 \textit{Streptococci} and belong to the \(\beta\) -lactam class of antibiotics, and quinolones are active against \(\beta\)-lactam-resistant strains.

\[
\begin{align*}
\text{ROCHN} \ & \ \text{S} \ & \ \text{O} \ & \ \text{CO}_2\text{R} \\
\ & \ \text{O} \ & \ \text{O} \ & \ \text{NR} \\
\ & \ \text{RN} \ & \ \text{R} \\
\ & \ \text{R} \ & \ \text{3} \ & \ \text{3′}
\end{align*}
\]

\[
\begin{align*}
\text{N} \ & \ \text{S} \ & \ \text{O} \ & \ \text{CO}_2\text{R} \\
\ & \ \text{ROCHN} \ & \ \text{S} \ & \ \text{O} \\
\ & \ \text{O} \ & \ \text{NR} \\
\ & \ \text{H}_2\text{O} \ & \ \text{OOC} \ & \ \text{O}
\end{align*}
\]

\textbf{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.

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)).\textsuperscript{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).

<table>
<thead>
<tr>
<th>Type</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dual Drugs</td>
<td>• Known components administered</td>
<td>• Formulation; administration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Different pharmacokinetics of each drug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Drug may not synchronously accumulate at bacterial sites</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• More side effects</td>
</tr>
<tr>
<td>Dual Action Drugs</td>
<td>• Synchronous delivery to different bacterial target sites and in high concentrations</td>
<td>• Each activity may be reduced (e.g. steric reasons)</td>
</tr>
<tr>
<td></td>
<td>• Slower development of resistance</td>
<td>• Molecular weight high</td>
</tr>
<tr>
<td></td>
<td>• Improved formulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Improved chemical stability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Decreased toxicity</td>
<td></td>
</tr>
<tr>
<td>Dual Action</td>
<td>• Synchronous or near synchronous delivery of active agents</td>
<td>• Synthetic difficulties</td>
</tr>
<tr>
<td>Prodrugs</td>
<td>• Slower development of resistance?</td>
<td>• Molecular weight high</td>
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<tr>
<td></td>
<td>• Improved formulation</td>
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<tr>
<td></td>
<td>• Improved chemical stability</td>
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<tr>
<td></td>
<td>• Decreased toxicity</td>
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</tr>
</tbody>
</table>
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.\textsuperscript{47} on the synergistic antibacterial activity in components of a medicinal plant, \textit{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 \textit{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 \textit{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,\textsuperscript{49} blocks the NorA pump, potentiating the antibiotic action of berberine."\textsuperscript{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.

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, the most potent synthetic NorA pump inhibitor, and on the simpler 2-aryl-5-nitro-1\(H\)-indole NorA pump inhibitor derivatives.
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.

Figure 1-13 Diagram illustrating the dual action prodrug and dual action drug designs
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](image)

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.
Chapter 2: 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-substituted 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 borohydride59 and potassium carbonate in methanol57 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-substituted
berberine salt derivatives. The target compounds were also prepared in 2 steps (Scheme
2-2) by treatment of I with acetone and aqueous sodium hydroxide solution to afford 8-
acetyloidydroberberine and subsequent enamine alkylation with electrophiles (R-X)
followed by elimination of acetone to afford the salt derivatives. One problem
associated with the 8-acetyloidydroberberine route is that the berberine salt (I) can be
regenerated quite readily by elimination.60

![Scheme 2-2 Preparation of 13-substituted berberines in 2 steps via 8-acetyloidydroberberine](image)

**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 bromoacetate 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 subsections.
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 NaBH₄ in an aprotic solvent (e.g. pyridine) or in a protic solvent (e.g. MeOH) in the presence of K₂CO₃, 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 NaBH₄ in pyridine (Scheme 2-5) was found in the present study to afford a higher yield (96%) of 2 than from NaBH₄ and MeOH in the presence of K₂CO₃ (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 NaBH₄/pyridine method which required anhydrous conditions, and the unpleasant solvent, pyridine.
Scheme 2-5 Preparation of dihydroberberine (2) using NaBH₄ in pyridine

Scheme 2-6 Preparation of dihydroberberine (2) using NaBH₄ in MeOH and K₂CO₃

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

In the NaBH₄/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 bromoacetate 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 NaBH₄ 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 CH₂CO and OCH₂ groups respectively, together with a triplet signal ascribed to the methyl substituent of the ethyl ester at δ 1.15. MS (Cl)
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$_2$SO$_4$ 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°-$^\circ$C to 62°-$^\circ$C 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.

![Scheme 2-9 Preparation of acid derivative 4](image-url)
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°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°C to remove the DMF. Removal of the excess benzyl alcohol required increasing the distillation temperature to 150°C, which gave many unwanted by-products. However, using exactly 1 equivalent of benzyl alcohol overcame this difficulty. In the 1H 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 CH2CO protons, and at δ 4.97 which was ascribed to the OCH2 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).

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$_2$SO$_3$ 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 $^1$H 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 1H 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 allylttributyltin to give 8-allyldihydroberberine (9). Subsequent enamine alkylation of 9 with a range of alkylation 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

\( \alpha \)-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,\(^6\) 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.

![Scheme 2-13 Preparation of 8-allyldihydroberberine 9](image)

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 \( \delta \) 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 \( \delta \) 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 \( \delta \) 9.76 in the chloride salt of 1 to \( \delta \) 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 Cl 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-substituted berberine derivatives. 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.

![Scheme 2-14 Preparation of 13-substituted berberine salt derivatives](image)

Mechanistically (Scheme 2-15), the formation of the salts may be rationalized in terms of initial enamine alkylation of 9 to give the intermediate 9a, followed by a [3,3]-sigmatropic rearrangement to 9b and a subsequent retro-ene reaction 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 acetonylldihydroberberine 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.

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 $\delta$ 10.46-10.61 as a singlet, while a triplet in the range of $\delta$ 2.9-3.2 was ascribed to the C5 protons. A broad signal at $\delta$ 4.6-5.0 was ascribed to the C-6 protons, which the methoxy group signals appeared at $\delta$ 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 $\delta$ 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 $\delta$ 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 \( \delta \) 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 \( \delta \) 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.
Chapter 3: 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. 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. 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). 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

![Chemical structures of some NorA substrates](image)

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.

![Chemical structures of NorA inhibitors](image)

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 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.\textsuperscript{72} Reserpine also prevented emergence of fluoroquinolone resistance in \textit{S. aureus}\textsuperscript{73} and \textit{S. pneumoniae}\textsuperscript{74}. Since reserpine is neurotoxic to humans at the concentration required for NorA inhibition it cannot be used clinically as a NorA inhibitor.\textsuperscript{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 \textit{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.\textsuperscript{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.\textsuperscript{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 \textit{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 \textit{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.\textsuperscript{72} There was no significant inhibitory activity of GG918 in combination with moxifloxacin against all tested strains.

![Chemical structures](image)

\textbf{Figure 3-3 Naturally occurring NorA inhibitors from \textit{Berberis} species}

Berberine-producing plants from \textit{Berberis} species have been found to produce potent NorA inhibitors, including porphyrin pheophorbide \textit{a} and the flavonolignan 5’-MHC-D,\textsuperscript{47-49} that act synergistically with the antibacterial berberine against \textit{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 \textit{S. aureus}. These results may help explain why \textit{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 \textit{S. aureus} RN4222).\textsuperscript{48} Many synthetic flavonolignans and simple flavones were synthesized in order to determine structure-activity relationships (SARs) for synergistic activity with berberine against \textit{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´-\textit{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 \textit{S. aureus}. The results revealed that the free hydroxy groups in ring A were not necessary for NorA inhibitory activity.\textsuperscript{56} The effect of stereochemistry on activity was not reported for the 5,7-deoxyhydnocarpin-D flavonolignan.

![Chemical structures](image)

\textbf{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, \(\alpha\)-linolenic acid (MIC, 62.5 \(\mu\)g/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.

![Chemical structures](image)

**Figure 3-5 Natural flavonol and isoflavone NorA inhibitors, and the antibacterial, \(\alpha\)-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 \(\mu\)g/mL in combination with a sub-inhibitory concentration of berberine (30 \(\mu\)g/mL) or a sub-inhibitory concentration of \(\alpha\)-linolenic acid (30 \(\mu\)g/mL). Moreover, a SAR study of the flavone series found that the monomethoxy B-ring derivatives of isoflavones were more potent than disubstituted 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

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46
6.25 µg/mL with a sub-inhibitory dose of berberine, completely inhibited the growth of *S. aureus* 8325-4.77

![Dalversinol](image1.png) ![2(S)-5''-((1''',1''''-dimethylallyl)-8-(3''',3''''-dimethylallyl)-2',4',5,7-tetrahydroxyflavanone](image2.png)

![4',6'-dihydroxy-3',5'-dimethyl-2'-methoxychalcone](image3.png) ![3,5,4'-trimethoxy-trans-stilbene](image4.png)

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

More recently, a new antibacterial flavonoid and six known phenolic compounds (Figure 3-6) were isolated78 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.
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.  

From all the literature data, the synthetic NorA inhibitor 5,7-deoxyhydnocarpin-D, 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, 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, commercially available coniferyl alcohol and 3’,4’-dihydroxyflavone were oxidatively coupled using Ag₂CO₃. Either Ag₂CO₃ or
horseradish peroxide (HRP) was used previously\textsuperscript{56} for the preparation of \textbf{12} and \textbf{14} yielding a mixture of different regioisomers. The silver reactions gave \textbf{12} as a major regioisomer, having ring D at C-13 with the beta position, whereas the HRP reactions gave \textbf{14} as the major regioisomer product having ring D at C-12 with the alpha position.

The reaction with $\text{Ag}_2\text{CO}_3$ was performed in a solvent mixture of 2:1 benzene/acetone at 60$^\circ$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 $^1$H 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 \textbf{12} as a major product, and two minor products, \textbf{13(a or b)} and \textbf{14} (Scheme 3-1), which were confirmed by NMR spectroscopic analysis. Guz et al.$\textsuperscript{49,56}$ were not able to separate a mixture of \textbf{12} and \textbf{14} using HPLC methods. As acetylation of the mixture \textbf{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.

![Chemical structures](image)

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

Mechanistically, the coupling of coniferol 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\(^8\)\(^0\) 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.\(^8\)\(^1\)

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 (\(\delta\) 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).

![Structures](image)

**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) confirmed49 the regioisomer shown (12 = 12a; Figure 3-8).

The structure of the regioisomer 14 was confirmed by 1H NMR spectroscopic analysis, in which the 1H 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-12ax-H-13ax 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 1H 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 $\delta$ 4.9-5.0 with a vicinal coupling constant for diaxial protons H-12, H-13 ($J_{ax,ax} \approx 8-13$ 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 $\delta$ 5.27 (d, $J = 3.0$ Hz) indicating a vicinal coupling of axial and equatorial protons ($J_{ax,eq} \approx 2-6$ Hz) or of diequatorial protons ($J_{eq,eq} \approx 2-5$ Hz). 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

<table>
<thead>
<tr>
<th>(^1)H</th>
<th>15 ((\delta) ppm, CDCl(_3))</th>
<th>Report Data(^{56}) ((\delta) ppm, CDCl(_3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAc</td>
<td>2.06 (s)</td>
<td>2.09 (s)</td>
</tr>
<tr>
<td>OAc</td>
<td>2.31 (s)</td>
<td>2.35 (s)</td>
</tr>
<tr>
<td>OCH(_3)</td>
<td>3.85 (s)</td>
<td>3.89 (s)</td>
</tr>
<tr>
<td>H-11</td>
<td>4.02 (dd, 12.3, 4.2)</td>
<td>4.04 (dd, 12.4, 4.4)</td>
</tr>
<tr>
<td>H-12</td>
<td>4.26-4.36 (m)</td>
<td>4.36 (m)</td>
</tr>
<tr>
<td>H-11</td>
<td>4.38 (dd, 12.2, 3.2)</td>
<td>4.42 (dd, 12.4, 3.2)</td>
</tr>
<tr>
<td>H-13</td>
<td>4.98 (d, 7.8)</td>
<td>5.01 (d, 8.0)</td>
</tr>
<tr>
<td>H-3</td>
<td>6.73 (s)</td>
<td>6.76 (s)</td>
</tr>
<tr>
<td>H-6'</td>
<td>6.97 (dd, 7.8, 1.5)</td>
<td>7.02 (m)</td>
</tr>
<tr>
<td>H-2''</td>
<td>6.99 (s)</td>
<td>7.00 (d, 2.0)</td>
</tr>
<tr>
<td>H-5''</td>
<td>7.09 (d, 8.4)</td>
<td>7.12 (d, 8.0)</td>
</tr>
<tr>
<td>H-8</td>
<td>7.09 (d, 8.4)</td>
<td>7.12 (d, 8.0)</td>
</tr>
<tr>
<td>H-7</td>
<td>7.39 (br.t, 8.1)</td>
<td>7.42 (ddd, 8.2, 6.8, 0.8)</td>
</tr>
<tr>
<td>H-5'</td>
<td>7.48-7.54 (m)</td>
<td>7.54 (dd, 8.4, 1.6)</td>
</tr>
<tr>
<td>H-6'</td>
<td>7.48-7.54 (m)</td>
<td>7.63 (ddd, 8.0, 2.0)</td>
</tr>
<tr>
<td>H-2'</td>
<td>7.58 (d, 2.1)</td>
<td>7.61 (d, 2.0)</td>
</tr>
<tr>
<td>H-6</td>
<td>7.63-7.70 (m)</td>
<td>7.70 (ddd, 8.6, 7.0, 1.6)</td>
</tr>
<tr>
<td>H-5</td>
<td>8.20 (dd, 7.8, 1.5)</td>
<td>8.23 (dd, 8.4, 1.6)</td>
</tr>
</tbody>
</table>

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-1H-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-1H-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.

![Figure 3-9 Related INF55 derivatives were predicted to possess NorA inhibitory activity](image)

Some related 2-aryl-5-nitro-1H-indole derivatives (Figure 3-9) have been predicted by CoMFA (3D-QSAR) analysis to have pump blocking activity. In particular, 2-(2-methoxyphenyl)-5-nitro-1H-indole was predicted to show comparable activity to INF55, with *ortho*-substitution on the 2-phenyl ring, while 2-(4-methoxyphenyl)-5-nitro-1H-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-1H-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 OCH₃ and OCH₂Ph, 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](image)

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-1H-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-1H-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 benzyol alcohol 16g.

The general mechanism for the Fischer 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 enehydrazone 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 (acetic acid). Moreover, microwave irradiation has been used to facilitate this reaction.

![Scheme 3-7 Mechanism for the Fischer indole synthesis of 16e](image)

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 $\delta$ 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 \textit{para}-nitro substituent, and at $\delta$ 7.86 (2H) and 8.00 (2H) ascribed to H-2’, H-6’ and H-3’, H-5’ on the aromatic ring bearing the \textit{para}-cyano substituent. A singlet signal at $\delta$ 3.35 (3H) was assigned to the methyl group adjacent to the imine functionality together with a singlet signal at $\delta$ 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 ($\delta$ 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.

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

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$_2$. 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\textit{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\textit{H}-indole (Scheme 3-9), including a palladium-assisted reaction of 4-nitro-2-bromoaniline and \( \alpha \)-piperidinostyrene,\textsuperscript{91} the Fischer indole synthesis of acetophenone with phenylhydrazine and subsequent nitration,\textsuperscript{92} and reaction of 4-nitroaniline and dimethyl-(2-oxo-2-phenyl-ethyl)-sulfonium bromide followed by cyclisation.\textsuperscript{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\textit{H}-indole in mind, particular attention in the alternative approaches focussed on preformed indoles and introduction of an \textit{ortho}-substituted aryl group at the indolic C2 position.
3.3.4 Synthetic strategy for 2-aryl-5-nitro-1\(H\)-indole derivatives \textit{via} palladium cyclization

A regioselective synthesis of 2-arylinoles from indole was reported by \cite{10,11} His work involved the synthesis of 2-(o-indolyl)benzoic acids \textit{via} ring closure of 1-aroylindoles using palladium acetate followed by hydrolysis (Scheme 3-10).

\begin{center}
\includegraphics[width=0.8\textwidth]{scheme310.png}
\end{center}

Scheme 3-10 Preparation of o-(2-indolyl)benzoic acids \textit{via} intramolecular ring closure of 1-aroylindoles\cite{10,11}

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-1H-indole derivatives

The basic synthetic strategy was to initially prepare N-benzoyl-5-nitroindole (18) by N-acylation of the commercially available 5-nitro-1H-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 alkylation 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\textsuperscript{96,97} which can then be easily removed by base hydrolysis\textsuperscript{98}, and also to provide precursors for intramolecular cyclization.\textsuperscript{99} The most common N-acylation method requires the generation of an indole anion (by addition of a base, e.g. BuLi,\textsuperscript{100} NaH,\textsuperscript{101,102} KOH,\textsuperscript{102} and K\textsubscript{2}CO\textsubscript{3}\textsuperscript{103}) followed by nucleophilic attack on an acyl halide. N-acylation of indoles can be achieved in high yields using acetic anhydride in combination with TEA and a catalytic amount of DMAP.\textsuperscript{104} Also direct N-acylation of indole with carboxylic acids catalyzed by boric acid affords N-acylated indoles in moderate yield.\textsuperscript{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 \textit{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\textsubscript{3}, NO\textsubscript{2}, F

24 R = H, R\textsubscript{1} = Ph
25 R = H, R\textsubscript{1} = Ph-\textit{p}-OCH\textsubscript{3}
26 R = H, R\textsubscript{1} = Ph-\textit{o}-OCH\textsubscript{3}
27 R = OCH\textsubscript{3}, R\textsubscript{1} = Ph
28 R = OCH\textsubscript{3}, R\textsubscript{1} = Ph-\textit{p}-OCH\textsubscript{3}
29 R = OCH\textsubscript{3}, R\textsubscript{1} = Ph-\textit{o}-OCH\textsubscript{3}
30 R = NO\textsubscript{2}, R\textsubscript{1} = \textit{Ph-\textit{p}-OCH\textsubscript{3}}
31 R = NO\textsubscript{2}, R\textsubscript{1} = Ph-\textit{o}-OCH\textsubscript{3}
32 R = NO\textsubscript{2}, R\textsubscript{1} = Ph-\textit{o}-OCH\textsubscript{3}
```

Scheme 3-12 Methods for N-acylation of indoles

62
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

<table>
<thead>
<tr>
<th>Product</th>
<th>Method</th>
<th>Yield (%)</th>
<th>Product</th>
<th>Method</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Indole 24" /></td>
<td>A</td>
<td>88</td>
<td><img src="image" alt="Indole 32" /></td>
<td>D</td>
<td>80</td>
</tr>
<tr>
<td><img src="image" alt="Indole 25" /></td>
<td>C</td>
<td>45</td>
<td><img src="image" alt="Indole 32" /></td>
<td>B</td>
<td>30</td>
</tr>
<tr>
<td><img src="image" alt="Indole 25" /></td>
<td>D</td>
<td>32</td>
<td><img src="image" alt="Indole 22" /></td>
<td>B</td>
<td>95</td>
</tr>
<tr>
<td><img src="image" alt="Indole 26" /></td>
<td>A</td>
<td>75</td>
<td><img src="image" alt="Indole 23" /></td>
<td>D</td>
<td>95</td>
</tr>
<tr>
<td><img src="image" alt="Indole 26" /></td>
<td>D</td>
<td>46</td>
<td><img src="image" alt="Indole 32" /></td>
<td>A</td>
<td>19</td>
</tr>
<tr>
<td><img src="image" alt="Indole 27" /></td>
<td>A</td>
<td>72</td>
<td><img src="image" alt="Indole 27" /></td>
<td>C</td>
<td>4</td>
</tr>
<tr>
<td><img src="image" alt="Indole 28" /></td>
<td>D</td>
<td>15</td>
<td><img src="image" alt="Indole 31" /></td>
<td>D</td>
<td>89</td>
</tr>
<tr>
<td><img src="image" alt="Indole 28" /></td>
<td>D</td>
<td>9</td>
<td><img src="image" alt="Indole 30" /></td>
<td>D</td>
<td>87</td>
</tr>
<tr>
<td><img src="image" alt="Indole 18" /></td>
<td>A</td>
<td>89</td>
<td><img src="image" alt="Indole 19" /></td>
<td>B</td>
<td>44</td>
</tr>
<tr>
<td><img src="image" alt="Indole 18" /></td>
<td>D</td>
<td>91</td>
<td><img src="image" alt="Indole 31" /></td>
<td>D</td>
<td>85</td>
</tr>
<tr>
<td><img src="image" alt="Indole 19" /></td>
<td>B</td>
<td>95</td>
<td><img src="image" alt="Indole 31" /></td>
<td>D</td>
<td>85</td>
</tr>
</tbody>
</table>

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. 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 Rf 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 \((pK_a \sim 17)\) 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.

\[
\text{RCOOH} + \text{Cyc-N}=\text{C}=\text{N-Cyc} \rightarrow \text{RCOO}^- + \text{Cyc-NH}=\text{C}=\text{N-Cyc}
\]

\[
\text{R} + \text{RC}_{\text{O}}=\text{C}=\text{NH-Cyc} \xrightarrow{\text{H}^+} \text{R-OC}=\text{N-Cyc}
\]

Cyc-NH-CO-NH-Cyc

DHU

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 $\delta$ 8.00 which could be assigned to the indolic NH. The presence of a 3-proton singlet at $\delta$ 3.79 was attributed to the aromatic methoxy group. A broad doublet signal at $\delta$ 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 $\delta$ 7.00-7.60. Additionally, in the $^{13}$C NMR spectrum a signal at $\delta$ 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). 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 $\delta$ 3.88 and the absence of a broad singlet signal which could be attributed to an indolic proton. Two sets of multiplets between $\delta$ 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. 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 ($\delta$ 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

<table>
<thead>
<tr>
<th>Entry</th>
<th>5-nitroindole (eq)</th>
<th>acid (eq)</th>
<th>DCC (eq)</th>
<th>DMAP (eq)</th>
<th>reaction time (h)</th>
<th>yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.3</td>
<td>1.3</td>
<td>0.1</td>
<td>72</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0.1</td>
<td>6</td>
<td>91</td>
</tr>
</tbody>
</table>

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 1H 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 13C 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.
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 using 4-hydroxybenzoic acid and benzyl bromide in K₂CO₃ 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 ¹H 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 $\delta$ 127.5-135.8 when compared to the spectrum for 19. Moreover, the $^1$H NMR spectrum showed a distinct signal at $\delta$ 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 $\delta$ 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°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°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 $\delta$ 4.29 which was attributed to the methylene protons of the phenyl acetyl moiety. An aromatic signal integrating for five protons was seen at $\delta$ 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 $\delta$ 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 ($\delta$ 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 $\delta$ 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 $\delta$ 6.90-7.80 and the presence of a singlet signal ($\delta$ 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 $\delta$ 6.90-7.80, and the signal assigned to H-7 at $\delta$ 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-aryloindoles by palladium acetate which has
been reported previously by Itahara\textsuperscript{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.

\begin{center}
\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme_3-15}
\caption{Scheme 3-15 Intramolecular ring-closure of the N-acylated indoles}
\end{figure}
\end{center}

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 \textsuperscript{1}H NMR spectrum of 33 revealed the loss of the doublet signal at \( \delta 7.50 \) 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 \( \delta 6.79 \) as a singlet, which strongly supported the structure of 18.
Mechanistically, the oxidative cyclization of the N-acylated product probably involves initial indole C-2 palladation to form an σ-indolylpalladium(II) complex \( \text{18a} \), which would be promoted by protonation of the acetate ligand of \( \text{Pd(OAc)}_2 \) by acetic acid (Scheme 3-16). Subsequently, complex \( \text{18a} \) could undergo an intramolecular insertion into the benzoyl ring to give \( \text{18b} \), followed by β-hydride elimination of \( \text{18b} \) to afford the ring-closed product \( \text{33} \).

![Scheme 3-16 Mechanism for the palladium(II)-promoted oxidative cyclization](image)

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

In further work, the N-acylated product \( \text{19} \) was reacted with 1.0 equivalent of \( \text{Pd(OAc)}_2 \) in glacial acetic acid to afford the ring-closed product \( \text{34} \) in high yield (74%, Scheme 3-15). It is likely that the electron releasing para-substituent of \( \text{18a} \) (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 ^1H NMR showed a characteristic signal for ring-closed product at \( \delta \) 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 \( \delta \) 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 substituent 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).

\[
\begin{align*}
\text{36} & \quad \xrightarrow{\text{PhCH}_2\text{Br, CsCO}_3, \text{DMF, 80°C}} \quad 74\% \\
\text{OCH}_3\text{Ph} & \quad \text{35}
\end{align*}
\]

Scheme 3-17 Benzylation of compound 35 with benzyl bromide
In the $^1$H NMR of 35 a diagnostic singlet at $\delta$ 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$_3$OD, 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 $\delta$ 7.07. The absence of a signal which could be ascribed to the methylene protons of the benzyloxy substituent around $\delta$ 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 $\delta$ 6.72 was attributed to H-3 and the gCOSY spectrum showed it was coupled to a doublet signal at $\delta$ 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$_2$O$_4$. 

75
3.3.4.3 Amide hydrolysis of the cyclized products

$\text{N}, \text{N-Disubstituted amides can be hydrolysed under acidic or alkaline conditions.}$

Based on the method of Itahara, 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$_2$O. The amide hydrolysis was unsuccessful under normal conditions with either HCl/MeOH in the presence of H$_2$O or NaOH/MeOH in the presence of H$_2$O.

\begin{equation*}
\text{O}_2\text{N} \backslash \text{N} \backslash \text{COOH} \\
\text{R} \\
\text{NH} \backslash \text{O}_2\text{N} \\
\text{R} \\
\text{R} \\
\end{equation*}

33; R = H
34; R = OCH$_3$
35; R = OCH$_2$Ph

\begin{equation*}
\text{O}_2\text{N} \backslash \text{N} \backslash \text{COOH} \\
\text{R} \\
\text{NH} \backslash \text{O}_2\text{N} \\
\text{R} \\
\text{R} \\
\end{equation*}

38; R = H, 95%
39; R = OCH$_3$, 94%
40; R = OCH$_2$Ph, 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$_2$O 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 pH 1 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 $\delta$ 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 $\delta$ 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 $\delta$ 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 LiAlH₄ 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

\[
\text{RCOOH} + \text{BH}_3 \xrightarrow{\text{THF}} \text{RCOOBH}_2 \xrightarrow{\text{H}_2\text{O}} \text{RCH}_2\text{OBH}_2 \xrightarrow{\text{H}_2\text{O}} \text{RCH}_2\text{OH} + \text{B(OH)}_3
\]

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

The benzoic acid 38 was reacted¹¹² 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 \(^1\text{H}\) 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 13C 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 1H 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 13C 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 13C 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 1H NMR spectrum; an M+ signal at m/z 374.1256 in the HRMS (EI) spectrum was consistent with the molecular formula C22H18N2O4.

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 NaN₃ and PPh₃ in 20% CCl₄ in DMF.¹¹⁷

\[
\begin{align*}
R-OH + NaN_3 &\rightarrow R-N_3 \\
&\text{via azide} \\
PPh₃ (>2eq) &\rightarrow R-N=PPh₃ \\
&\text{iminophosphorane} \\
H_2O &\rightarrow R-NH₂
\end{align*}
\]

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

Treatment of alcohols with NaN₃ and two equivalents of PPh₃ in CCl₄-DMF (1:4) at 90°C afforded their corresponding amines¹¹⁷ (Staudinger reaction) in excellent yield (85-95%). Using one equivalent of PPh₃ in the same manner¹¹⁷ afforded the corresponding azides. Azide formation occurs with the first equivalent of PPh₃, and the azide can then react with the second equivalent of PPh₃ 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 NaN₃ and PPh₃ (2 equivalents) in 25% CCl₄ 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.
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 PPh₃. 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 ¹³C NMR spectrum also displayed a signal ascribed to the methylene carbon at δ 54.0, which was consistent with a CH₂ 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 C₁₅H₁₂N₅O₂.

The azide derivative 47 was also prepared in the same manner as for 44, from treatment of the alcohol 42 with NaN₃ and 2 equivalents of PPh₃ 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 NaBH₄, which usually gives poor yields but is selective. However, the reducing power of NaBH₄ is augmented by using either THF or t-BuOH as a solvent with dropwise addition of MeOH. Following a general literature procedure, the azide 44 was reduced with NaBH₄ 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 Rf 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 NaBH₄ in THF with dropwise addition of MeOH

The $^1$H NMR spectrum of 45 showed an upfield singlet signal integrating for two protons at $\delta$ 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 $\delta$ 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 NaBH₄ and 1,3-propanedithiol as a catalyst$^{120}$

The azide reduction was reattempted following a method described by Pei$^{120}$ using NaBH₄ 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 NaBH₄ (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 ¹H 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.

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 ¹H 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.

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.

![Scheme 3-27 Preparation of bromoesters 51 and 52](image)

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). Thus the
preparation of benzyl bromide 53 was conducted\textsuperscript{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^\circ 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).

\[
\text{Ph}_3\text{P} + \text{CBr}_4 \rightarrow \text{Ph}_3\text{PBr} \rightarrow \text{Ph}_3\text{POR} + \text{CHBr}_3
\]

Scheme 3-28 General scheme for the bromination of alcohols using carbon tetrabromide and triphenylphosphine\textsuperscript{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.

\[
\text{O}_2\text{N} \quad \text{CH}_2\text{Br} \\
\text{41; } R = \text{H} \quad \text{53; } R = \text{H}, 41\% \\
\text{42; } R = \text{OCH}_3 \\
\text{O}_2\text{N} \quad \text{CH}_2\text{Br} \\
\text{42a} \quad \text{54, 47\%}
\]

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 1H 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 13C NMR spectrum was attributed to the methylene carbon, although a doublet would be expected127 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](image)

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 H₂C(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 benzyl 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. 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.

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 (C₂₁H₂₃N₂O₆, [MH]⁺), consistent with the molecular formula of 58.

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 $\delta$ 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. 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.

![Scheme 3-34 Attempted N-sulfonyl group protection of 42](image-url)

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.
Chapter 4:

Synthesis of the berberine-indole 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$_3$CN 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](image)

The structure of the ester prodrug 60 was confirmed by NMR spectroscopic analysis and mass spectrometry. The $^1$H 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 \( \delta \) 
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 \( \delta \) 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\(^{\prime}\) proton at \( \delta \) 6.74. In addition, two 
singlet signals attributed to the methylene protons of the ester linking group at \( \delta \) 4.48 
(CH\(_2\)CO) and 5.39 (CH\(_2\)O) were observed. The carbonyl carbon of the ester group was 
confirmed by a signal in the \(^13\)C NMR spectrum at \( \delta \) 170.3, and a downfield signal at \( \delta \) 
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\(_3\)CN at 100°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\(^{\prime}\prime\) proton in the latter. 
Moreover, the presence of a signal at \( \delta \) 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 \( \delta \) 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.
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$_3$CN at 100°C for 2 days. The amide 62 was obtained in only 10% yield after recrystallization.

The $^1$H and $^{13}$C NMR spectra in CD$_3$OD 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$_2$NH). 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$_3$CN 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 $^1$H 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 CH₃CN 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 ¹³C NMR spectra in CD₃OD 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 ¹³C 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](image)

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 (Scheme 3-23; 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.

![Reaction Scheme](image)

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 CCl₄ 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 ¹H and ¹³C NMR spectra in CD₃OD of 65 were similar to those of 64 except for one less aromatic

![Chemical Structures](image)
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](image)

### 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 \textit{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).\textsuperscript{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 \textit{via} an alkene with the loss of ethylene.

The catalytic mechanism cycle proceeds \textit{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 D, 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](image)

The starting allyl berberine 66 was prepared by reaction of 8-allyldihydroberberine 9 and excess neat allyl bromide at 100°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. 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 $\delta$ 3.90-4.01 (CH$_2$CH=CH$_2$) as a multiplet, 4.91 and 5.43 (CH$_2$CH=CH$_2$) as broad doublets, and 6.31-6.42 (CH$_2$CH=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 $\delta$ 34.7 (CH$_2$CH=CH$_2$), 120.8 (CH$_2$CH=CH$_2$), and 135.1 (CH$_2$CH=CH$_2$). The HRMS (ES) spectrum provided additional evidence with a signal consistent with the positive ion of 66 at $m/z$ 376.1548.

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](image)

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 chromatography, 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.

\[ \text{Scheme 4-13 Resonance stabilization of 5-nitroindolyl anion} \]

The \(^1\)H NMR spectrum of 67 consisted of six aromatic proton signals with the expected splitting patterns, a singlet at \( \delta \) 6.71 attributed to the C-3 proton of the indole nucleus, a singlet at \( \delta \) 3.83 integrating for three protons ascribed to the methoxy group, and a singlet at \( \delta \) 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 \( \delta \) 4.61 (\( \text{CH}_2\text{CH}^=\text{CH}_2 \)) as a doublet, 4.85 and 5.16 (\( \text{CH}_2\text{CH}^=\text{CH}_2 \)) as two doublets, and 5.78-5.91 (\( \text{CH}_2\text{CH}^=\text{CH}_2 \)) 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 \( \delta \) 3.60 (allyl group) correlated with the signal at \( \delta \) 139.6 (C-7a) and it also correlated with the signal at \( \delta \) 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. 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.

\[
\text{OHCH}_2\text{CH}_2\text{OH} \xrightarrow{\text{NaH, THF}} \text{[OHCH}_2\text{CH}_2\text{Na]} \xrightarrow{TBDMSCl} \text{OHCH}_2\text{CH}_2\text{OSiMe}_2\text{Bu}^t, \text{RT, 33\% yield}
\]

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 alkylation 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.

\[
\text{O}_2\text{N} \quad \text{CH}_2\text{Br} \quad \text{NaH, DMF} \quad 80^\circ\text{C, 2days}
\]

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, 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\textsuperscript{136} still needs to be investigated.
Chapter 5: 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\(^\text{137}\) (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.\(^\text{137}\) 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.

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

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.
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.

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-1H-indole substituent at the C-13 position.
The 2-phenyl-5-nitro-1H-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-1H-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-1H-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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>FIC index(^a) Berberine (1) chloride</th>
<th>FIC index(^a) Berberine (1) chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>1.12</td>
<td>44</td>
</tr>
<tr>
<td>39</td>
<td>&lt;0.37</td>
<td>45</td>
</tr>
<tr>
<td>41</td>
<td>0.37</td>
<td>47</td>
</tr>
<tr>
<td>42</td>
<td>&lt;0.37</td>
<td>48</td>
</tr>
<tr>
<td>43</td>
<td>&lt;0.13</td>
<td>56</td>
</tr>
</tbody>
</table>

\(^a\) 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-1H-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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC, µg/mL (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td></td>
<td>K1758</td>
</tr>
<tr>
<td>+1, 3µg/mL</td>
<td>+1, 30µg/mL</td>
</tr>
<tr>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>39</td>
<td>12.50 (40.03)</td>
</tr>
<tr>
<td>41</td>
<td>6.25 (23.30)</td>
</tr>
<tr>
<td>42</td>
<td>3.13 (10.49)</td>
</tr>
<tr>
<td>43</td>
<td>0.39 (1.04)</td>
</tr>
<tr>
<td>44</td>
<td>0.78 (2.66)</td>
</tr>
<tr>
<td>45</td>
<td>&lt;0.24 (&lt;0.90)</td>
</tr>
<tr>
<td>47</td>
<td>0.39 (1.21)</td>
</tr>
<tr>
<td>48</td>
<td>0.78 (2.62)</td>
</tr>
<tr>
<td>56</td>
<td>0.39 (1.38)</td>
</tr>
<tr>
<td>INF55</td>
<td>0.78 (3.28)</td>
</tr>
</tbody>
</table>

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 0.78 µg/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 50.00 µg/mL (Table 5-4). The lipophilicity of the benzyloxy substituent and the weak acidity of the benzyl alcohol substituent (pKa ~ 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 *Salmonella enterica* Serovar Typhimurium SL1344 R2 at MICs of 0.24 and 3.13 µg/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 \textit{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 \textit{S. aureus} and \textit{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 \textit{E. faecalis} V583 and \textit{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 \textit{S. aureus} K2361.

Table 5-4 Minimum Inhibitory Concentration (MIC) value for 2-phenyl-5-nitro-1H-indole derivatives (MDR pump inhibitory activity) against \textit{S. aureus} K1758 (lacking NorA MDR pump), \textit{S. aureus} 8325-4 (expressing NorA MDR pump), \textit{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 50\(\mu\text{g/mL}\).

<table>
<thead>
<tr>
<th>Compound</th>
<th>(S. aureus)</th>
<th>MIC, (\mu\text{g/mL} (\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K1758</td>
<td>8325-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+Cip., 40ng/mL</td>
</tr>
<tr>
<td>38</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>39</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>47</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>NT</td>
<td>-</td>
</tr>
</tbody>
</table>

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.

![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.](image-url)
5.3 Antimicrobial testing results (Direct activity)

The set of 2-phenyl-5-nitro-1H-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 (12.5 μg/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, 12.5 μg/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 cereavisiae* 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 cereavisiae*). NT indicates the sample was not tested. “-” indicates sample showed no activity tested at concentration 50µg/mL.

<table>
<thead>
<tr>
<th>Compd</th>
<th>S. aureus</th>
<th>E. coli</th>
<th>S. enterica Serovar Typhimurium</th>
<th>S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K1758</td>
<td>8325-4</td>
<td>K 2361</td>
<td>K12</td>
</tr>
<tr>
<td>38</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>39</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>50.00 (186.38)</td>
<td>50.00 (186.38)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>3.13 (10.67)</td>
<td>50.00 (170.48)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>50.00 (187.07)</td>
<td>50.00 (187.07)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>47</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>25.00 (84.09)</td>
<td>-</td>
<td>50.00 (168.17)</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>INF55</td>
<td>250.00 (1050.42)</td>
<td>250.00 (1050.42)</td>
<td>250.00 (1050.42)</td>
<td>NT</td>
</tr>
</tbody>
</table>

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-1H-indole derivatives with a methylene linkage. Similarly, the combination of 1 and the 2-phenyl-5-nitro-1H-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.

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

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 cerevaesiae* 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*. 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.

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 \textit{S. aureus}, ATCC 6538P, and 4 strains of \textit{Enterococcus faecium}. The hybrid drug 64 was strongly active against \textit{S. aureus}, whereas the ester prodrug 60 was only moderately active. As expected, both 60 and 64 were inactive against all strains of \textit{E. faecium}, vancomycin resistant \textit{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 Chapter 1, section 1.3.2. Moreover, 64 was tested for antimalarial activity \textit{in vitro} against an anti-folate sensitive strain (TM4) and an anti-folate resistant strain (K1) of \textit{Plasmodium falciparum}. The result showed good activity with an IC\textsubscript{50} of 1.66\mu g/mL against the anti-folate resistant strain, which possesses an efflux-related resistance phenotype.\textsuperscript{140}

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

<table>
<thead>
<tr>
<th>Compd</th>
<th>\textit{S. aureus} ATCC 6538P</th>
<th>\textit{E. faecium}</th>
<th>\textit{P. falciparum}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC, \mu g/mL (\mu M)</td>
<td>IC\textsubscript{50}, \mu g/mL (\mu M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
<td>E. faecium</td>
<td>VRE243</td>
</tr>
<tr>
<td>60</td>
<td>31.25 (43.11)</td>
<td>&gt;125.00 (&gt;172.46)</td>
<td>&gt;125.00 (&gt;172.46)</td>
</tr>
<tr>
<td>64</td>
<td>0.98 (1.47)</td>
<td>&gt;125.00 (187.54)</td>
<td>125.00 (187.54)</td>
</tr>
</tbody>
</table>

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 1\mu g/mL against both \textit{S. aureus} and \textit{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.

\[
\begin{align*}
1 \mu\text{mole} & \quad 1 \mu\text{mole} & \quad 1 \mu\text{mole} \\
\text{bacterial enzyme} & & \\
\text{hydrolysis} & & \\
\end{align*}
\]

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).

<table>
<thead>
<tr>
<th>Compd</th>
<th>MIC, µg/mL (µM)</th>
<th>S. aureus</th>
<th>Compd</th>
<th>MIC, µg/mL (µM)</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8325-4</td>
<td>K1758</td>
<td>8325-4</td>
<td>K1758</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>16.00 (22.07)</td>
<td>3.13 (4.32)</td>
<td>61</td>
<td>6.25 (8.28)</td>
<td>3.13 (4.15)</td>
</tr>
<tr>
<td>8 + 41 (calcd)</td>
<td>8 = 11.51 (22.07)</td>
<td>8 = 2.25 (4.32)</td>
<td>41 = 5.92 (22.07)</td>
<td>8 = 4.32 (8.28)</td>
<td>41 = 1.16 (4.32)</td>
</tr>
<tr>
<td>8 + 42 (calcd)</td>
<td>8 = 6.25 (11.99)</td>
<td>8 = 3.13 (6.00)</td>
<td>41 = 6.25 (23.00)</td>
<td>8 = 2.47 (8.28)</td>
<td>42 = 1.24 (4.15)</td>
</tr>
<tr>
<td>8 + 41 (exp.)</td>
<td>8 = 6.25 (11.99)</td>
<td>8 = 3.13 (6.00)</td>
<td>41 = 6.25 (23.00)</td>
<td>8 = 6.25 (11.99)</td>
<td>42 = 1.24 (4.15)</td>
</tr>
<tr>
<td>62</td>
<td>3.13 (4.33)</td>
<td>3.13 (4.33)</td>
<td>63</td>
<td>12.50 (16.59)</td>
<td>6.25 (8.29)</td>
</tr>
<tr>
<td>8 + 45 (calcd)</td>
<td>8 = 2.26 (4.33)</td>
<td>8 = 2.26 (4.33)</td>
<td>45 = 1.16 (4.33)</td>
<td>8 = 4.32 (8.29)</td>
<td>48 = 2.46 (8.29)</td>
</tr>
<tr>
<td>8 + 48 (calcd)</td>
<td>8 = 12.50 (23.98)</td>
<td>8 = 12.50 (23.98)</td>
<td>45 = 12.50 (46.77)</td>
<td>8 = 3.13 (6.00)</td>
<td>48 = 3.13 (10.53)</td>
</tr>
</tbody>
</table>

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-y1)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H 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 histiocytic lymphoma cells (cell line U937).**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC</th>
<th>41</th>
<th>60</th>
<th>64</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/mL</td>
<td>&gt;100.00</td>
<td>&gt;100.00</td>
<td>&gt;100.00</td>
<td></td>
</tr>
<tr>
<td>(µM)</td>
<td>(327.76)</td>
<td>(137.97)</td>
<td>(&gt;150.04)</td>
<td></td>
</tr>
</tbody>
</table>

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.
Chapter 6: 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.

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-1H-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.

![Chemical structures](image)

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.
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-1H-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*. 
Chapter 7: 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 250ºC. 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 250ºC 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°C under reduced pressure for 8 h before use. NBS was recrystallized from water and dried over phosphorus pentoxide before use. Solvents were purified and dried by standard techniques.

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-6H-benzo[g]-1,3-benzodioxol[5,6-a]quinolizine (2)\(^{57,59}\)

*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\(_3\)): \(\delta\) 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\(_2\)O), 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\(_2\)O (1) (0.7g, 1.7mmol) in methanol (25mL) was added K\(_2\)CO\(_3\) (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 \((9,10\text{-Dimethoxy-5,8,13,13a-tetrahydro-6H-benzo}[g]\text{-1,3-benzodioxolo}[5,6-a]quinolizin-13-yl)\text{-acetic acid ethyl ester (3)}\)

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 chromatographed 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$_2$CO), 2.44 (dd, $J = 15.6$, 8.4 Hz, 1H, CH$_2$CO), 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, CH$_2$CH$_3$), 4.10 (d, $J = 16$ Hz, 1H, H-8), 5.91 (d, $J = 1.3$ Hz, 1H, OCH$_2$O), 5.92 (d, $J = 1.3$ Hz, 1H, OCH$_2$O), 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 (CH$_2$CO), 40.5 (C13), 51.0 (C6), 54.2 (C8), 55.8 (OCH$_3$), 60.0 (OCH$_3$), 60.1 (CH$_2$CH$_3$), 63.0 (C13a),
100.8 (OCH$_2$O), 105.9 (C4)$^a$, 108.3 (C14)$^a$, 110.6 (C12)$^b$, 124.1 (C11)$^c$, 128.4 (C13)$^d$, 128.5 (C8)$^e$, 129.8 (C4)$^d$, 132.4 (C12)$^e$, 144.9 (C14)$^f$, 146.0 (C9), 146.4 (C3)$^f$, 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)

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$_2$SO$_4$ 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$_3$): $\delta$ 2.59-2.85 (m, 4H, H-5, H-6, CH$_2$CO), 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$_2$O), 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-$d_6$: $\delta$ 28.2 (C5), 37.8 (CH$_2$CO), 43.3 (C13), 50.1 (C6), 53.0 (C8), 55.7 (OCH$_3$), 59.6 (OCH$_3$), 62.2 (C13a), 100.8 (OCH$_2$O), 105.6 (C4)$^c$, 108.2 (C14)$^c$, 111.6 (C12), 123.9 (C11), 126.3 (C8a)$^d$, 127.1 (C4a)$^c$, 128.7 (C13b)$^c$, 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-$a$]quinolinizin-13-yl)-acetic acid benzyl ester (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. ¹H-NMR (300MHz, CDCl₃): δ 2.37 (dd, J = 15.5, 4.1 Hz, 1H, CH₂CO), 2.52 (dd, J = 15.5, 8.9 Hz, 1H, CH₂CO), 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, OCH₃), 3.84 (s, 3H, OCH₃), 4.18 (d, J = 16.2 Hz, 1H, H-8), 4.97 (d, J = 2.1, 2H, OCH₂), 5.88 (d, J = 1.2 Hz, 1H, OCH₂O), 5.89 (d, J = 1.5 Hz, 1H, OCH₂O), 6.55 (s, 1H, H-4)⁹, 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). ¹³C NMR (75MHz, CDCl₃): δ 29.8 (C5), 38.0 (CH₂CO), 40.7 (C13), 51.0 (C6), 54.2 (CH₂O), 55.7 (OCH₃), 60.0 (OCH₂), 63.0 (C13a), 65.9 (CH₂O), 100.7 (OCH₂O), 105.8 (C4)⁹, 108.2 (C14)⁹, 110.5 (C11)b, 124.0 (C12)d, 127.4 (C8a)⁶, 127.8 (ArCH), 127.9 (2C, ArCH), 128.2 (C4a)⁷, 128.2 (2C, ArCH), 128.3 (C13b)⁷, 132.0 (C12a)⁸, 135.9 (ArC), 144.8 (C9)⁸, 145.8 (C14a)b, 146.3 (C3a)b, 150.5 (C10)b, 173.3 (CO). HRMS (EI): m/z calcd for C₂₉H₂₉NO₆ [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)\(^\text{58}\)

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.\(^\text{58}\) 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\(_2\)CO), 4.33 (q, \(J = 7.2\) Hz, 2H, CH\(_2\)CH\(_3\)), 4.38 (s, 3H, OCH\(_3\)), 5.09 (br.s, 2H, H-6), 6.08 (s, 2H, OCH\(_2\)O), 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).

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

**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 quinolizininium 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 CHCl₃ (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 quinolizininium bromide 7b (38.2mg, 85%) as a yellow
solid, m.p. 124-126°C. ¹H-NMR (300MHz, CDCl₃): δ 3.25 (t, J = 5.7 Hz, 2H, H-5),
4.05 (s, 3H, OCH₃), 4.31 (s, 2H, CH₂CO), 4.39 (s, 3H, OCH₃), 5.14-5.30 (br.m, 2H, H-
6), 5.31 (s, 2H, OCH₂), 6.10 (s, 2H, OCH₂O), 6.89 (s, 1H, H-4), 7.22 (s, 1H, H-14),
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). ¹³C NMR (75MHz, CDCl₃): δ 28.6 (C5), 37.2 (CH₂CO), 57.0 (OCH₃),
57.4 (C6), 63.1 (OCH₃), 68.0 (OCH₂O), 102.1 (OCH₂O), 108.6 (C4), 109.1 (C14),
119.1 (C13b), 119.6 (C11), 121.6 (C8a), 125.4 (C12), 125.7 (C12a), 128.7 (5C,
ArCH), 133.2 (C13), 134.1 (C4a), 134.7 (ArC), 137.5 (C13a), 146.5 (C8), 147.2 (C9),
147.3 (C14a), 150.1 (C3a), 150.5 (C10), 170.3 (CO). HRMS (ES): m/z calcd for
C₂₉H₂₆NO₆ [M⁺]: 484.1760; found: 484.1735. Anal. Calcd. for C₂₉H₂₆NO₆Br.1.5H₂O: 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)

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 quinolizinum iodide 8 (47.4mg,
53%) as a white solid, m.p. >250°C. 1H-NMR (300MHz, CD3OD): δ 3.12 (t, J = 5.7
Hz, 2H, H-5), 4.09 (s, 3H, OCH3), 4.18 (s, 2H, CH2CO), 4.19 (s, 3H, OCH3), 4.80 (br.s,
2H, H-6), 4.89 (s, 2H, OCH2O), 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). 13C NMR (125MHz,
CD3OD): δ 29.1 (C5), 40.2 (CH2CO), 49.2 (OCH3), 57.5 (C6), 62.6 (OCH3), 103.6
(OCH2O), 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)c, 138.6 (C13b)c, 145.0 (C8), 146.1
(C9)f, 148.8 (C3a)d, 151.4 (C14a)d, 151.7 (C10)f, 177.1 (CO). HRMS (ES): m/z calcd
for C22H20NO6 [M]+: 394.1291; found: 394.1282.

7.3 8-Allyldihydroberberine route

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

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
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. 1H NMR (300MHz, CDCl3): δ 2.38-2.50 (m, 2H, CH2CH=CH2), 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, OCH3), 3.89 (s, 3H, OCH3), 4.83-4.92 (m, 2H, CH2CH=CH2), 5.74-5.88 (m, 1H, CH2CH=CH2), 5.80 (s, 1H, H-13), 5.94 (d, J = 3.3 Hz, 2H, OCH2O), 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). 13C NMR (75MHz, CDCl3): δ 30.6 (C5), 36.5 (CH2CH=CH2), 47.3 (C6), 56.0 (OCH3), 58.3 (C8), 60.7 (OCH3), 94.6 (C13), 100.9 (OCH2O), 104.2 (C14)b, 107.7 (C4)b, 111.7 (C12), 118.2 (C11), 123.0 (C12a), 125.8 (C4a)c, 128.0 (C8a), 128.7 (C13b)c, 135.5 (CH2CH=CH2), 138.9 (C13a), 144.0 (C9)d, 146.4 (C14a), 149.7 (C10)d, 147.0 (C3a). HRMS (CI): m/z calc for C23H24NO4 [MH]+: 378.1705; found: 378.1703. Anal.Calcd. for C23H23NO4: 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)
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. 1H NMR (300MHz, CDCl3): δ 1.37 (t, J = 7.2 Hz, 3H, OCH2CH3), 3.20-3.35 (br.t, 2H, H-5), 4.07 (s, 3H, OCH3), 4.27 (s, 2H, CH2CO), 4.36 (q, J = 7.2 Hz, 2H, OCH2), 4.41 (s, 3H, OCH3), 5.30-5.70 (br.m, 2H, H-6), 6.10 (s, 2H, OCH2O), 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). 13C NMR (75MHz, CDCl3): δ 14.3 (CH2CH3), 28.5 (C5), 37.2 (CH2CO), 57.0 (OCH3), 57.4 (C6), 62.3 (OCH2), 63.1 (OCH3), 102.1 (OCH2O), 108.6 (C4), 109.0 (C14), 119.3 (C13b)a, 119.6 (C11), 121.6 (C8a), 125.6 (C4a)b, 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 C24H24NO6 [M]+: 422.1604; found: 422.1596. Anal.Calcd. for C24H24NO6Br.0.2CH2Cl2: 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-Benzylxycarbonylmethyl-9,10-dimethoxy-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinum 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)

To a solution of 8-allyldihydroberberine (9) (46mg, 0.12mmol) in dry CH$_3$CN (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$_3$): $\delta$ 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$_2$CO), 5.10-5.30 (br.m, 2H, H-6), 5.99 (s, 2H, OCH$_2$O), 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, $J = 7.5$Hz, 2H, ArH), 10.46 (s, 1H, H-8). $^{13}$C NMR (75MHz, CDCl$_3$): $\delta$ 28.6 (C5), 42.03 (CH$_2$CO), 57.0 (OCH$_3$), 57.3 (C6), 63.0 (OCH$_3$), 102.0 (OCH$_2$O), 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$_5$ $\left[\text{M}\right]^+$: 454.1654; found: 454.1649. Anal.Calcd. for C$_{28}$H$_{24}$NO$_5$Br.1.4CH$_3$OH: 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)

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$_3$): $\delta$ 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$_2$Ph), 5.20-5.35 (m, 2H, H-6), 6.00 (s, 2H, OCH$_2$O), 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$_3$OD): $\delta$ 28.3 (C5), 36.4 (CH$_2$Ph), 56.7 (OCH$_3$), 56.8 (C6), 62.6 (OCH$_3$), 101.9 (OCH$_2$O), 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)$^c$, 145.6 (C8), 147.0 (C14a), 149.8 (C3a), 150.1 (C10)$^d$. 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$_4$Br: 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)\textsuperscript{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.

\textbf{12}: \textsuperscript{1}H NMR (300MHz, CDCl\textsubscript{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\textsubscript{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). \textsuperscript{13}C NMR (75MHz, CDCl\textsubscript{3}): δ 55.9 (OCH\textsubscript{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.

13: \(^1\)H NMR (300MHz, CDCl\(_3\)): \(\delta\) 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 (dd, 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: \(^1\)H NMR (300MHz, CDCl\(_3\)): \(\delta\) 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’’), 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\textsuperscript{144}

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°C (Lit.\textsuperscript{144} 172-174°C). \textsuperscript{1}H NMR (300MHz, CDCl\textsubscript{3}): \(\delta\) 2.06 (s, 3H, COCH\textsubscript{3}), 2.31 (s, 3H, COCH\textsubscript{3}), 3.85 (s, 3H, OCH\textsubscript{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\textsuperscript{‘}'), 6.99 (s, 1H, H-2\textsuperscript{‘}'), 7.09 (d, \(J = 8.4\) Hz, 2H, H-8, H-5\textsuperscript{‘}'), 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). \textsuperscript{13}C NMR (75MHz, CDCl\textsubscript{3}): \(\delta\) 20.7 (2C, COCH\textsubscript{3}), 55.0 (OCH\textsubscript{3}), 62.5 (C11), 75.9 (C12), 76.4 (C13), 106.6 (C3), 110.5 (C2\textsuperscript{‘}'), 115.4 (C2'), 117.7 (C5'), 117.9 (C5'), 119.7 (C6'), 120.3 (C6')\textsuperscript{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')\textsuperscript{b}, 143.6 (C4'), 145.8 (C4')\textsuperscript{b}, 151.6 (C3''), 156.0 (C9), 162.7 (C2), 168.5 (CO), 170.2 (CO), 178.2 (C4). HMRS (EI); \textit{m/z} calcd for C\textsubscript{20}H\textsubscript{24}O\textsubscript{9} [M\textsuperscript{+}]: 516.1420; found; 516.1419.

7.5 Synthesis of synthetic bacterial pump blocking agents

7.5.1 Attempted Fischer indole synthesis

\textit{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 Na₂CO₃, 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. ¹H NMR (300MHz, CDCl₃): δ 3.35 (s, 3H, CH₃), 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 CH₃CN 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°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°C, was added the hydrazone 16 (35mg, 0.13mmol) and heated at 110°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°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 BF₃-diethyl etherate and the mixture heated at reflux 110°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*-Acylolation reaction

Acid chloride route

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

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. ¹H NMR (300MHz, CDCl3): 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 C₁₅H₁₀N₂O₃ [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 NaHCO₃ 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. ¹H NMR (300MHz, CDCl₃): δ 3.92 (s, 3H, OCH₃), 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). ¹³C NMR (75MHz, CDCl₃): δ 55.6 (OCH₃), 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 (El); m/z calcd for C₁₆H₁₂N₂O₄ [M⁺]: 296.0797; found: 296.0796.

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

Preparation of acid chloride: To a suspension of p-hydroxybenzoic acid (3.0g, 22.8mmol) and dry K₂CO₃ (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 H₂O, 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. ¹H NMR (300MHz, CDCl₃): δ 5.09 (s, 2H, OCH₂), 5.33 (s, 2H, COOCH₂), 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).
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**, 3.25g, 91%) as white needles, m.p. 185-187°C (Lit. 187-190°C). $^1$H NMR (300MHz, CDCl$_3$ + CD$_3$OD): 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).

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.

**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, $J = 9.0$Hz, 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$_2$O$_4$ [M]$^+$: 372.1110; found; 372.1105.

### 7.5.2.4 Preparation of 5-Nitro-1-phenylethanoyl-1H-indole (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$_2$CO), 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, $J = 9.3$, 3.6 Hz, 1H, H-7).
0.6 Hz, 1H, H-7). $^1$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$_2$O$_3$ [M]$^+$: 280.0848; found: 280.0835.

**Preparation of N-Acyl Indoles**

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

![Schema of 1-Benzoyl-1H-indole (24)](image)

To a solution of 1H-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). $^1$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)-1H-indole (25)

![Schema of 1-(4-Methoxybenzoyl)-1H-indole (25)](image)

To a solution of 1H-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. 137-139°C). \(^1\)H NMR (300MHz, CDCl\(_3\)): \(\delta\) 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\(_2\) \([\text{M}]^+\): 251.0946; found: 251.0947.

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

To a solution of 1H-indole (23.4mg, 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, 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\(_3\)): \(\delta\) 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\(_3\)): \(\delta\) 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\(_2\) \([\text{M}]^+\): 251.0946; found: 251.0955.
7.5.2.8 Preparation of 1-Benzoyl-5-methoxy-1H-indole (27)

To a solution of 5-methoxy-1H-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)-1H-indole (28)

To a solution of 5-methoxy-1H-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$_3$): $\delta$ 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-1H-indole (29)

To a solution of 5-fluoro-1H-indole (27.0mg, 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 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$_3$): $\delta$ 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$_3$): $\delta$ 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$_{18}$H$_{10}$NOF [M]$^+$: 239.0746; found: 239.0738.
7.5.2.11 Preparation of 5-Fluoro-1-(4-methoxybenzoyl)-1H-indole (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. ¹H NMR (300MHz, CDCl₃): δ 3.91 (s, 3H, OCH₃), 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). ¹³C NMR (75MHz, CDCl₃): δ 55.5 (OCH₃), 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 C₁₆H₁₂NO₂F [M⁺]: 269.0852; found: 269.0854

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

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 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$_3$): $\delta$ 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 (bddd, $J = 8.8$, 4.7 Hz, 1H, H-7). $^{13}$C NMR (75MHz, CDCl$_3$): $\delta$ 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$_2$F [M]$^+$: 269.0852; found: 269.0854.

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

To a solution of 5-nitro-1H-indole (17) (32.4mg, 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 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-1H-indole (19)

To a solution of 17 (32.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and \textit{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-1H-indole (32)

To a solution of 17 (32.4mg, 0.2mmol), DMAP (24.4mg, 0.2mmol) and \textit{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 \textit{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. 

\[^1\text{H}	ext{ NMR (300MHz, CDCl}_3\text{)}: \delta 3.71 (s, 3H, OCH}_3\text{), 6.61 (d, J = 3.5 Hz, 1H, H-3), 6.98 (d, J = 8.5 Hz, 1H, H-3'), 7.05 (t, J = 7.5 Hz, 1H, H-5'), 7.17 (d, J = 3.5 Hz, 1H, H-2), \]
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). ¹³C NMR (75MHz, CDCl₃): δ 55.7 (OCH₃), 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 C₁₆H₁₂N₂O₄ [M]+: 296.0797; found: 296.0804.

7.5.2.16 Preparation of 5-nitro-1-phenylethanoyl-₁H-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, trituated 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-₁H-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°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°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-isoindo[2,1-\(a\)]indol-6-one (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\(_3\)): \(\delta\) 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-\(d_6\)): \(\delta\) 104.2 (C11), 112.6 (C4), 118.8 (C1), 121.8 (C3), 122.8 (C7), 125.6 (C10), 130.2 (C9), 132.5 (C10a), 133.8 (C11a), 134.6 (C6a), 135.1 (C8), 136.1 (C4a), 141.1 (C10b), 143.9 (C2), 162.0 (C6). HRMS (EI); \(m/z\) calcd for C\(_{15}\)H\(_8\)N\(_2\)O\(_3\) [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)

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. ¹H NMR (300MHz, CDCl₃ + CD₃OD): δ 3.95 (s, 3H, OCH₃), 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). ¹³C NMR (75MHz, CDCl₃+CD₃OD): δ 55.8 (OCH₃), 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 C₁₆H₁₀N₂O₄ [M]+: 294.0641; found: 294.0648.

7.5.3.3 Preparation of 9-Benzylxyloxy-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: m.p. >250°C, $^1$H NMR (300MHz, CDCl$_3$ + CD$_3$OD): $\delta$ 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$_3$OD or DMSO-d$_6$) 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$_2$O$_4$ [M$^+$]: 370.0954; found: 370.0944.

36: m.p. 199-201°C, $^1$H NMR (300MHz, DMSO-d$_6$): $\delta$ 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-d$_6$): $\delta$ 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$_8$N$_2$O$_4$ [M$^+$]: 280.0484; found: 280.0480.

37: m.p. 201-203°C, $^1$H NMR (300MHz, DMSO-d$_6$): $\delta$ 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-d$_6$): 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$_2$O$_4$ [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-1H-indol-2-yl)benzoic acid (38)

A solution of t-BuOH (192mL) and H$_2$O (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 =
7.5.4.2 Preparation of 4-Methoxy-2-(5-nitro-1H-indol-2-yl)benzoic acid (39)

A solution of t-BuOH (88.5mL) and H₂O (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. 

1H NMR (300MHz, DMSO-d₆): δ 3.86 (s, 3H, OCH₃), 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). 13C NMR (75MHz, DMSO-d₆): δ 55.7 (OCH₃), 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´), 139.6 (C7a), 140.7 (C5), 141.5 (C2´), 161.3 (C4´), 167.9 (CO). HRMS (EI); m/z caled for C₁₅H₁₀N₂O₄ [M]+: 282.0641; found: 282.0643.
7.5.4.3 Preparation of 4-Benzylxoy-2-(5-nitro-1H-indol-2-yl)-benzoic acid (40)

A solution of t-BuOH (22.5mL) and H2O (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. 1H NMR (300MHz, DMSO-d6): δ 5.24 (s, 2H, OCH2), 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). 13C NMR (75MHz, DMSO-d6): δ 69.6 (OCH2), 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 C22H16N2O5 [M]+: 388.1059; found: 388.1069.

7.5.5 Reduction reactions

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

To a solution of 38 (997.0mg, 3.54mmol) in dry THF (90mL) was slowly added 1M BH3-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 H₂O (20mL) until the gas bubbling ceased. The aqueous layer was saturated with anhydrous K₂CO₃. The THF layer was separated and the aqueous 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°C. ¹H NMR (300MHz, CDCl₃): δ 4.77 (s, 2H, CH₂O), 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). ¹³C NMR (75MHz, CDCl₃): δ 65.1 (CH₂), 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 C₁₅H₁₂N₂O₃ [M]⁺: 268.0848; found: 268.0838.

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

To a solution of 39 (520.0mg, 1.67mmol) in dry THF (50mL) was slowly added 1M BH₃-THF complex solution (3.3mL, 3.3mmol) 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 H₂O (10mL) until the gas bubbling ceased. The aqueous layer was saturated with anhydrous K₂CO₃. The THF layer was separated and the aqueous 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. 1H NMR (300MHz, CDCl3): δ 3.89 (s, 3H, OCH₃), 4.70 (s, 2H, CH₂), 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). 13C NMR (75MHz, CDCl3): δ 55.5 (OCH₃), 64.6 (CH₂O), 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 C₁₆H₁₄N₂O₄ [M]+: 298.0954; found: 298.0941.

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

To a solution of 40 (200.0mg, 0.52mmol) in dry THF (15mL) was slowly added 1M BH₃-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 H₂O (2mL) until the gas bubbling ceased. The aqueous layer was saturated with anhydrous K₂CO₃. The THF layer was separated and the aqueous 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. 1H NMR (300MHz, CDCl3): δ 4.63 (s, 2H, CH₂OH), 5.08 (s, 2H, OCH₂), 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$_2$OH), 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 calcld for C$_{22}$H$_{18}$N$_2$O$_4$ [M]$^+$: 374.1267; found: 374.1256.

7.5.6 Amination reactions

7.5.6.1 Preparation of 2-(2-Azidomethyl-phenyl)-5-nitro-1H-indole (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$_2$O (10mL), and stirred for 10min. The mixture was diluted with diethyl ether (40mL) and washed thoroughly with H$_2$O. 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$_2$N$_3$), 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$_2$N$_3$), 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 calcld for C$_{15}$H$_{12}$N$_5$O$_2$ [MH]$^+$: 294.0991; found: 294.0987.
7.5.6.2 Preparation of 2-(5-Nitro-1H-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-isoirindolo[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: m.p. 127-129°C, ¹H NMR (300MHz, CDCl₃): δ 3.99 (s, 2H, CH₂), 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). ¹³C NMR (75MHz, CDCl₃): δ 46.0 (CH₂), 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 C₁₅H₁₃N₃O₂ [M]⁺: 267.1008; found: 267.0995.

46: m.p. 234-236°C, ¹H NMR (300MHz, CDCl₃): 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). ¹³C NMR (75MHz, CDCl₃): 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 calc for C_{15}H_{10}N_{2}O_{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
H2O (50mL) and extracted with 40% Et2O 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-1H-indole (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% CCl4 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 H2O (10mL), and stirred for 10min. The mixture was
diluted with diethyl ether (40mL) and washed thoroughly with H2O. 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. \(^1H\) NMR (300MHz, CDCl\(_3\)): \(\delta\) 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\(_3\)): \(\delta\) 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\(_5\)O\(_3\) [M]+: 323.1018; found: 323.1024.

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

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\(_2\)O (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. \(^1H\) NMR
(300MHz, CDCl₃): δ 3.86 (s, 3H, OCH₃), 3.91 (s, 2H, CH₂), 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). ¹³C NMR (75MHz, DMSO-d₆): δ 42.5 (CH₂), 55.3 (OCH₃), 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´), 134.9 (C2´), 140.7 (C5), 141.4 (C7a), 158.6 (C4´). HRMS (EI); m/z calcd for C₁₆H₁₅N₃O₃ [M]+: 297.1113; found: 297.1101.

7.5.7 N-Alkylation reactions

7.5.7.1 Preparation of 2-Bromo-N-[2-(5-nitro-1H-indol-2-yl)benzyl]acetamide (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 H₂O (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. ¹H NMR (300MHz, CDCl₃): δ 3.85 (s, 2H, CH₂Br), 4.45 (d, J = 6.6 Hz, 2H, CH₂N), 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). ¹³C NMR (75MHz, CDCl₃): δ 28.7 (CH₂Br), 42.3 (CH₂N), 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´), 176
139.7 (C7a), 140.9 (C1´), 141.7 (C5), 167.2 (CO). HRMS (EI); m/z calcd for C17H14N3O3Br [M]+: 389.0198; found: 389.0197.

7.5.7.2 Preparation of 2-Bromo-N-[4-methoxy-2-(5-nitro-1H-indol-2-yl)-benzyl]-acetamide (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 H2O (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. 1H NMR (300MHz, CDCl3): δ 3.84 (s, 3H, OCH3), 3.91 (s, 2H, CH2Br), 4.44 (d, J = 6 Hz, 2H, CH2), 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). 13C NMR (75MHz, CDCl3): δ 28.8 (CH2Br), 41.9 (CH2N), 55.5 (OCH3), 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 C18H16N3O4Br [M]+: 417.0324; found: 417.0332.
7.5.8  O-Alkylation reactions

7.5.8.1 Preparation of Bromoacetic acid 2-(5-nitro-1H-indol-2-yl)-benzyl ester (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 H₂O. 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. ¹H NMR (300MHz, CDCl₃): δ 3.97 (s, 2H, CH₂Br), 5.31 (s, 2H, CH₂O), 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). ¹³C NMR (75MHz, CDCl₃): δ 40.9 (CH₂Br), 66.4 (CH₂O), 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 C₁₇H₁₃N₂O₄Br [M]⁺: 338.0059; found: 338.0056.

7.5.8.2 Preparation of Bromoacetic acid 4-methoxy-2-(5-nitro-1H-indol-2-yl)phenyl-benzyl ester (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 H2O. 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. 1H NMR (300MHz, CDCl3): δ 3.81 (s, 3H, OCH3), 3.88 (s, 2H, CH2CO), 5.17(s, 2H, CH2O), 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). 13C NMR (75MHz, CDCl3): δ 25.9 (CH2Br), 55.5 (OCH3), 66.6 (CH2O), 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 (El); m/z caled for C18H15N2O5Br [M]⁺: 420.0144; found: 420.0151.

7.5.9 Bromination reactions

7.5.9.1 Preparation of 2-(2-Bromomethylphenyl)-5-nitro-1H-indole (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$_3$): \( \delta \) 4.64 (s, 2H, CH$_2$Br), 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$_3$): \( \delta \) 33.0 (CH$_2$Br), 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 (El); \( m/z \) calcd for C$_{15}$H$_{11}$N$_2$O$_2$Br $[M]^+ $: 330.0003; found: 329.9982.

7.5.9.2 Attempted bromination of the benzyl alcohol 42

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$_3$): \( \delta \) 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$_3$): \( \delta \) 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), 180
114.4 (C5), 159.7 (C5'). HRMS (ES): m/z calcd for C$_{34}$H$_{28}$N$_{2}$O$_{3}$P [M]$^+$: 543.1838; found: 543.1838.

7.5.9.3 Preparation of 2-(5-Methoxy-2-vinylphenyl)-5-nitro-1H-indole (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-methylphenyl)-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, CH=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=CH$_2$), 117.7 (C4), 117.9 (C6), 127.9 (C3a), 128.4 (C3''), 129.4 (C2''), 131.2 (C2), 134.8 (CH=CH$_2$), 139.1 (C7a), 140.1 (C1''), 142.1 (C5), 159.3 (C5'). HRMS (EI); m/z calcd for C$_{17}$H$_{14}$N$_{2}$O$_{3}$ [M]$^+$: 294.1004; found: 294.0988.

56: m.p. 194-196°C, $^1$H NMR (300MHz, CDCl$_3$ + CD$_3$OD): δ
2.43 (s, 3H, CH₃), 3.90 (s, 3H, OCH₃), 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'). ¹³C NMR (75MHz, CDCl₃ + CH₃OD): δ 20.0 (CH₃), 55.4 (OCH₃), 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')₉, 139.2 (C1')₉, 140.7 (C7a), 141.6 (C5), 157.6 (C5'). HRMS (Cl); m/z calcd for C₁₆H₁₅N₂O₃ [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: ¹H NMR (300MHz, CDCl₃): δ 3.83 (s, 3H, OCH₃), 4.91 (br.d, J = 9.6 Hz, 2H, OCH₂), 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 (Cl): m/z 325 (M+1, 100 %).

58: ¹H NMR (300MHz, CDCl₃): δ 1.52 (s, 9H, CH₃), 3.87 (s, 3H, OCH₃), 5.11 (s, 2H, OCH₂), 6.81 (d, J = 1.5 Hz, 3H, H-3).
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$_3$): $\delta$ 27.8 (3C, CH$_3$), 55.5 (OCH$_3$), 66.9 (OCH$_2$), 83.2 (OCMe$_3$), 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 chromatographed on silica gel by PLC (50% DCM in hexane) to give the dibenzenesulfonyl indole 59 (4.5mg, 8%) as an opaque white solid.

$^1$H NMR (300MHz, CDCl$_3$): $\delta$ 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-1H-indol-2-yl)-benzyloxycarbonyl-methyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (60)

A solution of the dihydroberberine 9 (100.0mg, 0.26mmol) and the bromoester 51 (108.8mg, 0.32mmol) in dry CH$_3$CN (8mL), was heated at reflux for 48h under a nitrogen atmosphere. The reaction mixture was then concentrated by evaporation of the CH$_3$CN. 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°C. $^1$H NMR (300MHz, DMSO-$d_6$): $\delta$ 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$_2$CO), 4.78 (br.s, 2H, H-6), 5.39 (s, 2H, CH$_2$O), 6.14 (s, 2H, OCH$_2$O), 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-$d_6$): $\delta$ 27.4 (C5), 36.5 (CH$_2$CO), 56.9 (C6), 57.1 (OCH$_3$), 61.2 (OCH$_3$), 65.5 (CH$_2$O), 102.3 (OCH$_2$O), 104.3 (C3´), 108.7 (C4)$_e$, 108.8 (C14)$_e$, 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´),
7.6.2 Preparation of 9,10-Dimethoxy-13-[4-methoxy-2-(5-nitro-1H-indol-2-yl)-benzyloxy carbonylmethyl]-5,6-dihydrobenzo[\textit{g}]-1,3-benzodioxolo[5,6-\textit{a}] quinolizinium bromide (61)

A solution of the dihydroberberine 9 (91.0mg, 0.24mmol) and the bromoester 52 (100.0mg, 0.24mmol) in dry CH$_3$CN (14mL) was heated at reflux for 48h under a nitrogen atmosphere. The reaction mixture was then concentrated by evaporation of the CH$_3$CN. 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-$d_6$): $\delta$ 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$_2$CO), 4.78 (s, 2H, H-6), 5.31 (s, 2H, CH$_2$O), 6.14 (s, 2H, OCH$_2$O), 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-$d_6$): $\delta$ 27.3 (C5), 36.5 (CH$_2$CO), 55.6 (OCH$_3$), 56.9 (C6), 57.0 (OCH$_3$), 62.1 (OCH$_3$), 65.4 (CH$_2$O), 102.3 (OCH$_2$O), 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)\textsuperscript{d}, 127.4 (C3a′), 132.7 (2C, C13, C6′′), 133.2 (C1′′)\textsuperscript{e}, 134.2 (C13b), 137.2 (C13a), 139.4 (C2′′)\textsuperscript{e}, 139.8 (C7a′), 140.9 (C5′), 144.3 (C9)\textsuperscript{f}, 145.7 (C8), 146.8 (C3a)\textsuperscript{g}, 149.5 (C14a)\textsuperscript{g}, 150.3 (C10)\textsuperscript{f}, 159.5 (C4′′), 170.3 (CO). HRMS (ES): \textit{m}/\textit{z} calcd for C\textsubscript{38}H\textsubscript{32}N\textsubscript{3}O\textsubscript{9} [M]+: 674.2139; found: 674.2134.

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

A solution of the dihydroberberine 9 (22.5mg, 0.06mmol) and the bromoamide 49 (25.0mg, 0.07mmol) in dry CH\textsubscript{3}CN (3mL) was heated at reflux for 48h under a nitrogen atmosphere. The reaction mixture was then concentrated by evaporation of the CH\textsubscript{3}CN. 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\degree\textsuperscript{C}. \textsuperscript{1}H NMR (500MHz, CD\textsubscript{3}OD): \textdelta 3.11 (br,t, \textit{J} = 4.8 Hz, 2H, H-5), 4.03 (s, 3H, OCH\textsubscript{3}), 4.19 (s, 3H, OCH\textsubscript{3}), 4.33 (s, 2H, CH\textsubscript{2}CO), 4.69 (s, 2H, CH\textsubscript{2}N), 4.88 (br.s, 2H, H-6), 6.12 (s, 2H, OCH\textsubscript{2}O), 6.83 (s, 1H, H-3′), 7.02 (s, 1H, H-4)\textsuperscript{a}, 7.40 (s, 1H, H-14)\textsuperscript{a}, 7.46-7.63 (m, 5H, H-7′, ArH), 7.82 (d, \textit{J} = 9.6 Hz, 1H, H-11)\textsuperscript{b}, 7.98 (d, \textit{J} = 9.6 Hz, 1H, H-12)\textsuperscript{b}, 8.06 (dd, \textit{J} = 9, 2.1 Hz, 1H, H-6′), 8.52 (d, \textit{J} = 2.1 Hz, 1H, H-4′), 9.84 (s, 1H, H-8). \textsuperscript{13}C NMR (125MHz, CD\textsubscript{3}OD): \textdelta 27.8 (C5), 37.3 (CH\textsubscript{2}CO), 42.0 (CH\textsubscript{2}N), 56.2 (OCH\textsubscript{3}), 57.3 (C6), 61.2 (OCH\textsubscript{3}), 102.5 (C2), 104.1 (C3′), 108.3 (C4)\textsuperscript{c}, 109.3 (C14)\textsuperscript{c}, 111.0 (C7′), 116.9 (C7′), 117.1 (C4′), 120.0 (C4a), 120.4 (C11)\textsuperscript{d}, 121.7 (C8a)\textsuperscript{e}, 126.2 (C12)\textsuperscript{d}, 127.8
(C12a)², 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´´)², 138.2 (C13a), 139.2 (C2´´)², 140.7 (C5´), 141.3 (7a´), 144.9 (C8), 145.1 (C9)², 147.9 (C3a)², 150.2 (C14a)², 150.7 (C10)², 170.8 (CO). HRMS (ES): m/z calcd for C37H31N4O7 [M]+: 643.2193; found: 643.2184.

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

A solution of the dihydroberberine 9 (58.0mg, 0.15mmol) and the bromoamide 50 (70.0mg, 0.16mmol) in dry CH3CN (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. ¹H NMR (300MHz, CD3OD): δ 3.12 (br t, J = 5.7 Hz, 2H, H-5), 3.87 (s, 3H, OCH3), 4.06 (s, 3H, OCH3), 4.19 (s, 3H, OCH3), 4.30 (s, 2H, CH2CO), 4.62 (s, 2H, CH2N), 4.79 (br.t, J = 5.7 Hz, 2H, H-6), 6.12 (s, 2H, OCH2O), 6.83 (d, J = 0.9 Hz, 1H, H-3´), 7.02 (s, 1H, H-4)², 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)², 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)², 7.94 (d, J = 9.3 Hz, 1H, H-11)², 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). ¹³C NMR (75MHz, CD3OD): δ 29.1 (C5), 38.5 (CH2CO), 42.9 (CH2N), 56.0 (OCH3), 57.5 (OCH3), 58.7 (C6), 62.7 (OCH3), 103.8 (C2), 105.2 (C3´), 187
109.4 (C4), 110.5 (C14), 112.3 (C7'), 115.7 (C5''), 116.0 (C3''), 118.2 (C4'), 118.3 (C6'), 121.4 (C12), 121.5 (C11), 122.8 (C8a), 127.4 (C4a), 128.9 (C12a), 129.0 (C2'), 129.3 (C3a'), 132.6 (C6'), 134.2 (C1'), 135.1 (C13), 135.2 (C13b), 139.4 (C13a), 141.2 (C2''), 141.5 (C7a'), 142.9 (C5'), 146.3 (C9), 146.4 (C8), 148.9 (C3a), 151.7 (C14a), 151.8 (C10), 160.7 (C4'), 171.9 (CO). HRMS (ES): m/z calcd for C_{38}H_{33}N_{4}O_{8} [M]^+: 673.2298; found: 673.2305.

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

A solution of the dihydroberberine 9 (91.0mg, 0.24mmol) and the benzyl bromide 53 (102.3mg, 0.30mmol) in dry CH_{3}CN (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_{3}OD): \(\delta\) 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_{2}Ph), 5.96 (s, 2H, OCH_{2}O), 6.72 (s, 1H, H3'), 6.86 (s, 1H, H-4), 6.90 (s, 1H, H-14), 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), 7.88 (dd, \(J = 9.0, 2.4\) Hz, 1H, H-6''), 7.94 (d, \(J = 9.3\) Hz, 1H, H-12), 8.34 (d, \(J = 2.1\) Hz, 1H, H-4''), 9.8 (s, 1H, H-8). \(^13\)C NMR (75MHz, CD_{3}OD): \(\delta\) 29.1 (C5), 36.4 (CH_{2}Ar), 57.5 (OCH_{3}), 58.8 (C6), 62.7 (OCH_{3}), 103.6 (OCH_{2}O), 105.6 (C3'), 109.3 (C4'), 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_{3}O_{6} [M]^+: 586.1978; found:

7.6.6 Preparation of 9,10-Dimethoxy-13-[4-methoxy-2-(5-nitro-1H-indol-2-yl)benzyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium chloride (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 CHCl₃ 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% CCl₄ 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, CD₃OD): δ 3.04 (t, 5.9 Hz, 2H, H-5), 3.83
(s, 3H, OCH₃), 4.06 (s, 3H, OCH₃), 4.19 (s, 3H, OCH₃), 4.78 (s, 2H, H-6), 4.80 (s, 2H, CH₂Ph), 6.00 (s, 2H, OCH₂O), 6.78 (s, 1H, H-3'), 6.84-6.91 (m, 2H, H-6′′, H-5′′), 6.92 (s, 1H, H-4)d, 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). ¹³C NMR (125MHz, CD₂OD): δ 29.0 (C5), 35.7 (CH₂Ph), 56.0 (OCH₃), 57.5 (OCH₃), 58.8 (OCH₃), 62.7 (C6), 103.6 (OCH₂O), 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)c, 127.3 (C11)d, 129.2 (C3a′), 130.3 (C2′), 131.6 (C3′′′), 133.1 (C13b), 134.3 (C1′′′)d, 135.0 (C12a)c, 135.2 (C13), 138.9 (C13a), 141.1 (2C, C7a′, C2′′′), 142.9 (C5′), 146.0 (C9)c, 146.2 (C8), 148.6 (C3a)b, 151.4 (C14a)b, 151.7 (C10)g, 160.2 (C4′′). HRMS (ES): m/z calcd for C₃₆H₃₀N₃O₇ [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

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, CDCl₃ + CD₃OD): δ 3.23 (t, J = 5.9 Hz, 2H, H-5), 3.90-4.01 (m, 2H, CH₂CH=CH₂), 4.06 (s, 3H, OCH₃),
4.36 (s, 3H, OCH₃), 4.91 (br.d, J = 17.4 Hz, 1H, CH₂CH=CH₂), 5.24 (br.s, 2H, H-6), 5.43 (br.d, J = 10.5 Hz, 1H, CH₂CH=CH₂), 6.07 (s, 2H, OCH₂O), 6.31-6.42 (m, 1H, CH₂CH=CH₂), 6.87 (s, 1H, H-4)⁵, 7.34 (s, 1H, H-14)⁵, 7.82 (s, 2H, H-11, H-12), 10.53 (s, 1H, H-8). ¹³C NMR (75MHz, CDCl₃ + CD₃OD): δ 28.5 (C5), 34.7 (CH₂CH=CH₂), 57.0 (OCH₃), 57.6 (C6), 63.2 (OCH₃), 102.0 (OCH₂O), 108.4 (C4)⁵, 108.8 (C14)⁵, 119.3 (C12)⁶, 120.0 (C13b)⁷, 120.8 (CH₂CH=CH₂), 121.7 (C8a)⁸, 125.4 (C11)⁸, 129.7 (C4a)⁷, 133.3 (C13), 133.5 (C12a)⁹, 135.1 (CH₂CH=CH₂), 136.9 (C13a), 145.9 (C9)⁹, 146.4 (C14a)⁹, 147.0 (C8), 149.7 (C3a)⁸, 150.3 (C10)⁹. HRMS (ES): m/z calcd for C₂₃H₂₂NO₄ [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).
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$_3$): $\delta$ 3.83 (s, 3H, OCH$_3$), 4.48 (s, 2H, CH$_2$), 4.61 (d, $J$ = 3.6 Hz, 2H, CH$_2$CH=CH$_2$), 4.85 (d, $J$ = 17.1 Hz, 1H, CH$_2$CH=CH$_2$), 5.16 (d, $J$ = 10.5 Hz, 1H, CH$_2$CH=CH$_2$), 5.78-5.91 (m, 1H, CH$_2$CH=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$_3$): $\delta$ 46.7 (CH$_2$CH=CH$_2$), 55.4 (OCH$_3$), 62.4 (CH$_2$OH), 104.9 (C3), 110.2 (C7), 115.3 (C4’), 116.2 (C6’), 117.2 (CH$_2$CH=CH$_2$), 117.4 (C6), 117.7 (C4), 127.1 (C3a), 130.4 (C3’), 131.3 (C2), 132.6 (C1’)a, 132.7 (CH$_2$CH=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$_2$O$_4$ [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 45 min. The mixture was poured into diethyl ether (200 mL), and then washed with 10% aqueous K₂CO₃ solution (60 mL) and brine (60 mL). 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 (1.13 g, 33%) as a pale yellow oil. ¹H NMR (300 MHz, CDCl₃): δ 0.01 (s, 6 H, Si(CH₃)₂), 0.82 (s, 9 H, C(CH₃)₃), 2.71 (br s, 1 H, OH), 3.51-3.64 (m, 4 H, CH₂). CIMS: m/z 177 ([MH]⁺, 100%).

To a suspension of sodium hydride (11.0 mg of a 50% dispersion in mineral oil, 0.23 mmol) in anhydrous DMF (1 mL) at 0 °C, was added a solution of 2-(tert-butyldimethylsilanyloxy)ethanol (49.0 mg, 0.28 mmol) in DMF (1 mL) and the mixture stirred for 30 min. The mixture was then warmed to room temperature and stirred for a further 2 h. Excess DMF (3 mL) was added and then the mixture was cooled to −60 °C in a dry ice/acetone bath. A solution of benzyl bromide 53 (75.9 mg, 0.23 mmol) in DMF (4 mL) was added dropwise to the cooled mixture and then the mixture was allowed to stir for 2 days at 80 °C. The mixture was evaporated, then added to ice water (30 mL), and stirred vigorously for 4 h. 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 (39.6 mg, 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-1H-indol-2-yl)-benzyloxy carbonyl-methyl]-5,6-dihydro benzo[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, 46 units: 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 tris-hydroxymethyl-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°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

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°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°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°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°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°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\textsuperscript{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: \textit{S. aureus} 8325-4 (wild-type strain which expresses NorA MDR pump), \textit{S. aureus} K1758 (NorA mutant strain lacking the NorA MDR pump), \textit{S. aureus} K2361 (resistance strain which overexpresses the NorA MDR pump), \textit{E. faecalis} V583, and \textit{E. faecium} DO; Gram-negative bacteria: \textit{E. coli} K12, \textit{S. enterica} Serovar Typhimurium SL1344R2, and \textit{P. aueruginosa} PA1; and Yeast: \textit{S. cerevisiae} BY4742, \textit{C. albicans} F5, and \textit{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°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°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\textsuperscript{146} (performed by Avexa Ltd., Australia)

Materials and Methods

The bacterial strains were used \textit{S. aureus} ATCC 6538P, and 4 strains of \textit{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 \(\mu\)g/mL MgCl\(_2\) and 2 \(\mu\)g/mL CaCl\(_2\) and was pre-warmed for 2-3 h at 37\(^\circ\)C before use. Mueller-Hinton Agar (MHA) Medium culture media was prepared with final concentrations of 1.5% Agar (Merck Agar 1.01614). \textit{S. aureus} was streaked onto MHA and the plate was incubated overnight at 37\(^\circ\)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 \(-140\)\(^\circ\)C. A cryovial was removed from \(-140\)\(^\circ\)C storage and thawed at room temperature. The MHA plate was streaked with a loopful of bacterial suspension and incubated overnight at 37\(^\circ\)C to create a parent plate (P1). The parent plate was stored at 4\(^\circ\)C. A daughter plate (D1) was incubated overnight at 37\(^\circ\)C and its loop of colony was used to inoculate a 125 mL flask containing 20 mL of MHB containing 25 \(\mu\)g/mL CaCl\(_2\).2H\(_2\)O) and 12.5 \(\mu\)g/mL MgCl\(_2\).6H\(_2\)O. The flask was shaken at 260 rpm for 18 h at 37\(^\circ\)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°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°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°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\textsuperscript{147} (performed by Kamchonwongpaisan, S., BIOTEC, Thailand)

Materials and Methods

Samples were made up in DMSO solution. Using the Microdilution Radioisotope Technique, the \textit{in vitro} antimalarial activity of the alkaloids was tested against \textit{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 \textit{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 $1 \times 10^{-5}$ to $1 \times 10^{-8}$ g/ml with 0.1% of the organic solvent. The plates were cultured under standard conditions for 24 hours and the $^3$H-hypoxanthine (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\textsuperscript{148} was used to culture the \textit{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°C in a CO₂ 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)-2H tetrazolium, inner salt (MTS) assay, the in vitro cytotoxicity of these compounds was tested against human histiocytic 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 (37ºC, 5% CO₂ 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™ Thermo Trace).

Cytotoxicity testing

Test compounds were tested for cytotoxicity against human histiocytic 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 N₂ or Ar gas until the compound was completely dry. Cells (2x10⁴ 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 N₂ 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% CO₂ 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.