COPYRIGHT AND USE OF THIS THESIS

This thesis must be used in accordance with the provisions of the Copyright Act 1968.

Reproduction of material protected by copyright may be an infringement of copyright and copyright owners may be entitled to take legal action against persons who infringe their copyright.

Section 51 (2) of the Copyright Act permits an authorized officer of a university library or archives to provide a copy (by communication or otherwise) of an unpublished thesis kept in the library or archives, to a person who satisfies the authorized officer that he or she requires the reproduction for the purposes of research or study.

The Copyright Act grants the creator of a work a number of moral rights, specifically the right of attribution, the right against false attribution and the right of integrity.

You may infringe the author’s moral rights if you:

- fail to acknowledge the author of this thesis if you quote sections from the work
- attribute this thesis to another author
- subject this thesis to derogatory treatment which may prejudice the author’s reputation

For further information contact the University’s Director of Copyright Services

sydney.edu.au/copyright
Targeting β-alanyl aminopeptidase in *Pseudomonas aeruginosa*

Mohamed Ali Sharkasi

A thesis submitted for the fulfillment of the degree of

Doctor of Philosophy

Faculty of Pharmacy

The University of Sydney
DECLARATION

The work described in this thesis was constructed under the supervision of professor Paul Groundwater, professor David Hibbs and Dr. Bret Church in the Faculty of Pharmacy, The University of Sydney.

It is the work of the candidate and is not currently being submitted for any other degree.

Mohamed Ali Sharkasi

May, 2014
ACKNOWLEDGMENTS

First and foremost thank you Allah for everything. I would like to express my deepest appreciation to my supervisor Professor Paul Groundwater for his continuous support and encouragement to finish this project. Paul has guided me in all the time of my degree especially during the Libyan War.

I am also grateful to my co-supervisors Professor David Hibbs and Dr. Bret Church for their support, invaluable advices, and comments.

I would also like to thank Emma and Munikumar for their help and training in homology modeling.

My special thanks and respect for Dr. Rajeshwar and Dr. Linda for their training and support in chemical synthesis.

My best regards go to Professor Stuart and Dr. Nathan in the School of Molecular Bioscience for their help with biological tests.

I am also very grateful to Vivian, who is a PhD student for her helping to complete the MTT assay.

Special thanks to all research students in Lab. N213. especially Kaiser, Abram, Zechariah and Ran.

Personally, I would like to express my deep gratitude to my lovely family: my wife Nada, who added a wonderful joy to my life by joining me here. My parents in law, for their usual
support, and during the war. My parents, Afaf and Ali, for their encouragements, support and prayers.
### TABLE OF CONTENTS

1. **Introduction** .................................................................................................................. 2

1.1 Antibacterial Resistance ............................................................................................... 2

1.2 The Impact of MDR Organisms on Public Health and Healthcare Economics ................. 4

1.3 Mechanisms of Antibiotic Resistance ........................................................................... 6

1.4 Novel antibiotics ............................................................................................................. 7

1.4.1 Lipoglycopeptides .................................................................................................. 8

1.4.2 Oxazolidinones ...................................................................................................... 9

1.4.3 Lipopeptides ......................................................................................................... 10

1.4.4 Signermycin B ..................................................................................................... 11

1.4.5 Actinonin ............................................................................................................. 12

1.4.6 β-Lactones ........................................................................................................... 13

1.4.7 Platensimycin ....................................................................................................... 13

1.5 *Pseudomonas aeruginosa* .......................................................................................... 15

1.5.1 Pathogenesis ......................................................................................................... 16

1.5.2 Detection of *Pseudomonas aeruginosa* .................................................................. 19

1.5.3 Treatment of *P. aeruginosa* infections .................................................................... 21

1.5.4 Multi-drug resistance in *P. aeruginosa* .................................................................. 22

1.5.5 The *Pseudomonas aeruginosa* genome ................................................................... 25

1.6 Aminopeptidase Enzymes ........................................................................................... 25

1.7 Targeting β-alanyl aminopeptidase in *P. aeruginosa* ..................................................... 26

1.8 Protein Structure Prediction ........................................................................................... 27

1.9 Homology Modeling ....................................................................................................... 28
1.9.1 Identification of template protein .......................................................... 29
1.9.2 Sequence Alignment .............................................................................. 29
1.9.3 Model Construction ............................................................................. 30
1.9.3.1 Modeling by rigid-body assembly ....................................................... 30
1.9.3.2 Modeling by segment matching .......................................................... 31
1.9.3.3 Modeling by satisfaction of spatial restraints ....................................... 31
1.9.4 Model validation .................................................................................... 31
1.9.4.1 Ramachandran Plot ........................................................................ 32
1.9.4.2 Root Mean Square Deviation (RMSD) .............................................. 32
1.9.4.3 Molprobity ....................................................................................... 32
1.10 Protein-Ligand Docking .......................................................................... 33
1.11 β-Alanyl aminopeptidase and homology modeling .................................. 34
1.12 Aims ......................................................................................................... 36
1.13 Significance ............................................................................................. 37
2. **Homology Modeling** .............................................................................. 39
2.1 Determining the target sequence ............................................................... 39
2.2 Identification of a template ..................................................................... 40
2.2.1 L-Aminopeptidase D-Ala-esterase / amidase (DmpA) from *Ochrobactrum anthropi* ......................................................................................... 42
2.3 Alignment of target and template proteins .............................................. 43
2.4 Modeling of the target enzyme ................................................................. 46
2.5 Model validation ....................................................................................... 48
2.5.1 Ramachandran plots .......................................................................... 48
2.5.2 Molprobity ........................................................................................... 51
2.5.3 Docking of a known substrate ......................................................... 52
  2.5.3.1 Protein preparation ........................................................................... 52
  2.5.3.2 Preparation of the ligand ................................................................. 53
  2.5.3.3 Receptor grid generation ................................................................. 55
  2.5.3.4 Induced fit docking .......................................................................... 55
2.5.4 Ligand Receptor interactions ............................................................... 55
2.6 *In Silico* screening .................................................................................. 58
2.7 Selection of hit compounds ...................................................................... 59
2.8 *In silico* screening round two ................................................................. 61
3. *Synthesis* .................................................................................................. 67
  3.1 Results and Discussion ........................................................................... 68
    3.1.1 Synthesis of 4-amino-N-(2-aminoethyl)phenylsulfonamide ............... 74
    3.1.2 Coupling of the most active sulfonamides with β-alanine .................... 77
    3.1.3 Coupling of Ciprofloxacin with β-alanine ........................................... 80
  3.2 Round two HTVS screening .................................................................... 83
  3.3 Experimental section ............................................................................. 84
    3.3.1 Spectroscopic instrumentation............................................................. 84
    3.3.2 Preparation of Sulfonamides ............................................................... 85
      3.3.2.1 Preparation of tert-butyl (2-phenylsulfonamido)ethylcarbamate 4a ........ 85
      3.3.2.2 Preparation of N-(2-aminoethyl)benzenesulfonamide trifluoroacetate salt 1a . 86
      3.3.2.3 Preparation of tert-butyl (2-(4-methylphenyl)-sulfonamido)ethylcarbamate 4b ................................................................. 87
      3.3.2.4 Preparation of N-(2-aminoethyl)-4-methylphenylsulfonamide trifluoroacetate salt 1b ................................................................. 88
3.3.2.5 Preparation of tert-butyl \(2-(4\text{-chlorophenylsulfonamido})\text{-ethyl}\)carbamate \(4c\) 89
3.3.2.6 Preparation of \(N-(2\text{-aminoethyl})-4\text{-chlorophenylsulfonamide}\) trifluoroacetate salt \(1c\) .................................................................................. 90
3.3.2.7 Preparation of tert-butyl \(2-(4\text{-nitrophenyl})\text{-sulfonamido}\text{-ethyl}\)carbamate \(4d\) .................. 91
3.3.2.8 Preparation of \(N-(2\text{-aminoethyl})-4\text{-nitrophenylsulfonamide}\) trifluoroacetate salt \(1d\) .................................................................................. 92
3.3.2.9 Preparation of tert-butyl \(2-(4\text{-chlorophenylsulfonamido})\text{-ethyl}\)carbamate \(4e\) ... 93
3.3.2.10 Preparation of \(N-(2\text{-aminoethyl})-2\text{-nitrophenylsulfonamide}\) trifluoroacetate salt \(1e\) .................................................................................. 94
3.3.2.11 Preparation of tert-butyl \(2-(4\text{-acetamidophenyl})\text{sulfonamide}\text{-ethyl}\)carbamate \(4f\) .... 95
3.3.2.12 Preparation of \(N\{4-(N-(2\text{-aminoethylsulfamoyl})\text{phenyl})\text{acetamide}\) trifluoroacetate \(1f\) .................................................................................. 96
3.3.2.13 Preparation of 4-amino-\(N-(2\text{-aminoethyl})\text{benzenesulfonamide}\) \(1g\) .................... 97
3.3.3 Coupling of sulfonamides with \(\beta\)-alanine ........................................................................ 98
3.3.3.1 Synthesis of tert-butyl \[3\text{-oxo-3}\text{-}2-(\text{phenylsulfonamido})\text{ethyl}\]\text{amino}\text{propylcarbamate} \(7a\) ......................... 98
3.3.3.2 Preparation of 3-amino-\(N\{2-(\text{phenylsulfonamido})\text{ethyl}\}\)propanamide trifluoroacetate \(8a\) .................................................................................. 99
3.3.3.3 Synthesis of tert-butyl \[3\{2-(4\text{-methylphenylsulfonamido})\text{ethyl}\}\text{amino}\text{-3-oxopropylcarbamate} \(7b\) .................................................................................. 100
3.3.3.4 Preparation of 3-amino-\(N\{2-(4\text{-methylphenylsulfonamido})\text{ethyl}\}\)propanamide trifluoroacetate \(8b\) .................................................................................. 101
3.3.3.5 Synthesis of tert-Butyl \[3\{2-(4\text{-chlorophenylsulfonamido})\text{ethyl}\}\text{amino}\text{-3-oxopropylcarbamate} \(7c\) .................................................................................. 102
3.3.3.6 Preparation of 3-amino-\(N\{2-(4\text{-chlorophenylsulfonamido})\text{ethyl}\}\)propanamide trifluoroacetate \(8c\) .................................................................................. 104
3.3.3.7 Synthesis of tert-butyl [3-{2-(4-nitrophenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7d ................................................................. 105
3.3.3.8 Preparation of 3-amino-N-{2-(4-nitrophenylsulfonamido)ethyl}propanamide 8d ................................................................. 106
3.3.3.9 Synthesis of tert-butyl [3-{2-(2-nitrophenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7e ................................................................. 107
3.3.3.10 Preparation of 3-amino-N-{2-(2-nitrophenylsulfonamido)ethyl}propanamide 8e ................................................................. 108
3.4.1 Coupling of Ciprofloxacin with 'Boc-β-alanine 10 ........................................ 109
3.4.2 Synthesis of N''-(β-alanyl)ciprofloxacin 11 ........................................ 111
3.5.1 Preparation of N^7-(3-phthalimidopropyl)theophylline 14 .................. 112
3.5.2 Preparation of N^7-(3-aminopropyl)theophylline 13 ......................... 113
4. **Biological activity tests** ........................................................................... 116
4.1 Antimicrobial susceptibility tests ............................................................ 116
4.1.1 Disc diffusion assay ........................................................................... 116
4.1.2 Experimental procedure ..................................................................... 117
4.1.3 Results ............................................................................................... 119
4.2.1 Minimum inhibitory concentration (MIC) or micro-dilution assay ...... 126
4.2.2 Experimental procedure ..................................................................... 126
4.2.3 Results ............................................................................................... 128
4.3 Cytotoxic effect of compounds on a human cell line .............................. 134
4.3.1 Preparation of MTT solution ............................................................... 135
4.3.2 Prostate cancer (PC3 cell line) assay .................................................. 135
4.3.3 Preparation of HeKa (primary human keratinocytes-adult) cells ........ 136
4.3.4 Results from cytotoxicity tests ......................................................... 136
5. Conclusions ........................................................................................................... 140
6. References .......................................................................................................... 145
LIST OF FIGURES

Figure 1 Worldwide emergence of multidrug resistant bacteria ............................. 3
Figure 2 Gram stain of Pseudomonas. ........................................................................ 16
Figure 3 Chromogenic effect in *P. aeruginosa* in the left two dishes (with no colour generation) with other strains of Pseudomonas in the right hand dishes ........... 20
Figure 4 Generation of a purple colour by the action of BAAP on a yellow β-alanylaminophenoxazinone substrate .......................................................... 21
Figure 5 Steps involved in the homology modeling of β-alanyl aminopeptidase........... 35
Figure 6 β-Alanine specific aminopeptidase [*Pseudomonas sp. MCI3434*] sequence in FASTA format ...................................................................................................... 40
Figure 7 Blast search for template using Prime (Maestro version 9.3)......................... 41
Figure 8 The catalytic mechanism of DmpA ............................................................... 43
Figure 9 Sequence alignment window, the red H is represents a residue in a helical region, and Blue E represents amino acids with extended regions (beta sheets). (Prime, Maestro version 9.3) ......................................................................................... 44
Figure 10 Alignment of the amino acid sequences of four β-peptidyl aminopeptidases: BapA from strain 3-2W4, BapA from strainY2, BapA from *Pseudomonas sp. MCI3434 BAE02664* (the target), and DmpA from *O. anthropi* (the template). Red represents the residue identical in all four enzymes and yellow represents residues identical in three of the four enzymes (ALINE program). .45
Figure 11 Solid ribbon representation of the homology model generated for β-alanyl aminopeptidase in Discovery Studio Visualizer Version 3 ................................. 47
Figure 12 Ramachandran plot for β-alanine aminopeptidase from Maestro 9.3. Amino acid representations are; Glycine (triangle), Proline (square), other amino acids (circle). Residues outside the yellow and red areas are in unfavourable regions, as the Red and yellow regions represent the favored and allowed regions. ...... 49
Figure 13 Distribution of residues in unacceptable regions of the BAAP model, and the distance of closest amino acid (Gly233) in Å, as shown in pink. Maestro V 9.3 ........................................................................................................................................ 50
Figure 14  Protein Preparation Wizard window in Maestro V.9.3 .................................53
Figure 15  The 3D structure of β-alanylaminophenoxazinone, 1 Maestro 9.3 .................54
Figure 16  LigPrep window in Maestro 9.3 ....................................................................54
Figure 17  A 2D Ligand interaction diagram. B 3D Diagram of ligand 1 in binding pocket of β-alanyl aminopeptidase (Maestro version 9.3) ......................................................56
Figure 18  β-Alanyl aminopeptidase model with substrate 1 bound to the proposed active site in Maestro V 9.3 ....................................................................................................57
Figure 19  Compounds obtained from HTVS round 2, for testing against β-alanyl aminopeptidase (P. aeruginosa growth inhibitory) activity .................................65
Figure 20  Single crystal X-ray structure of sulfonamide 1a...........................................70
Figure 21  Single crystal X-ray structure of sulfonamide 1d...........................................72
Figure 22  The zone of inhibition........................................................................................116
Figure 23  Disc diffusion test for N-(2-aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d, showing dose dependent response against PAO1 starting from lower row and from left to right 0, 10, and 20, and upper row from left 50, and 100 mg/ml.................................................................118
Figure 24  Disc diffusion test for compounds 4a, 4b, 4c, 4d, (first row), 1a, 1c, 1b, 4e, (second row), 4f, 1d, 1e (third row) against PAO1 at 0, 10, 50, and 100 mg/ml. .........................................................................................................................120
Figure 25  Disc diffusion test for compounds 1c (first row), and 1b (second row) against B. subtilis (first column), P. vulgaris (second column), and PAO1 (third column) at 100 mg/ml concentration.................................................................125
Figure 26  Micro-dilution assay of N-(2-aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d against PAO1 ..........................................................................................127
Figure 27  Effect on bacterial growth of PAO1 of 1d (A) and acetone solvent (B) ............129
Figure 28  Micro-dilution assay in PAO1 ..............................................................................130
Figure 29  Micro-dilution assay in B. subtilis .....................................................................131
Figure 30  Micro-dilution assay in P. vulgaris .....................................................................132
Figure 31  Micro-dilution for ciprofloxacin and its β-alanyl analog 11 in PAO1, *B. subtilis*, and *P. vulgaris* .......................................................... 133

Figure 32  Cell viability (MTT assay) for the active compounds in the PC3 cell line ..... 137

Figure 33  Cell viability (MTT assay) for the active compounds in HeKa cells ............ 138
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Multidrug resistant (MDR) bacteria</td>
<td>4</td>
</tr>
<tr>
<td>Table 2</td>
<td>Novel antibacterial agents</td>
<td>14</td>
</tr>
<tr>
<td>Table 3</td>
<td>Molprobity and clash score results from assessment of the modeled enzyme using Molprobity</td>
<td>51</td>
</tr>
<tr>
<td>Table 4</td>
<td>Comparison of the active sites of the target β-alanyl aminopeptidase and template 1B65_A</td>
<td>58</td>
</tr>
<tr>
<td>Table 5</td>
<td>In silico screening results Glide (Maestro version 9.3)</td>
<td>59</td>
</tr>
<tr>
<td>Table 6</td>
<td>In silico screening results, showing compounds with highest docking scores</td>
<td>60</td>
</tr>
<tr>
<td>Table 7</td>
<td>In silico screening results, showing compounds with highest docking scores from Chembridge database subsets 0 and 0.1</td>
<td>62</td>
</tr>
<tr>
<td>Table 8</td>
<td>% Yields for synthesis of N-protected 4a-g and deprotected sulfonamides 1a-g.</td>
<td>69</td>
</tr>
<tr>
<td>Table 9</td>
<td>Bond lengths for Compound 1a</td>
<td>71</td>
</tr>
<tr>
<td>Table 10</td>
<td>Bond lengths for Compound 1d</td>
<td>73</td>
</tr>
<tr>
<td>Table 11</td>
<td>Disc diffusion assay results for the active compounds at 6 mg/ml on disc</td>
<td>121</td>
</tr>
<tr>
<td>Table 12</td>
<td>Disc diffusion results for active compounds against PAO1, B. subtilis, and P. vulgaris</td>
<td>123</td>
</tr>
<tr>
<td>Table 13</td>
<td>Disc diffusion assay for the active compounds against PAO1 (at 100 mg/ml concentration)</td>
<td>141</td>
</tr>
<tr>
<td>Table 14</td>
<td>Disc diffusion assay for the active compounds at 100 mg/ml concentration against PAO1, B. subtilis, and P. vulgaris</td>
<td>142</td>
</tr>
</tbody>
</table>
CONFERENCE

Conference Presentations


SUMMARY

The aim of this study was to perform homology modeling of the β-alanyl aminopeptidase sequence of *Pseudomonas aeruginosa*, and to use the model obtained in the design, synthesis and testing of inhibitors in order to evaluate the cellular role of this enzyme.

A 3D model of β-alanyl aminopeptidase was derived and evaluated using Maestro (version 9.3, Schrödinger). Virtual database screening was then conducted in order to discover inhibitors which would be predicted to bind to the active site of the enzyme. Twenty-seven hit compounds with the highest docking scores were synthesized, purified and the *in vitro* antimicrobial activities for these synthesized agents, against both Gram-negative and Gram-positive strains were evaluated using both disc diffusion and micro-dilution assays.


Based on disc diffusion assay, against Gram positive and Gram negative strains, 3-Amino-*N*-{2-(2-nitrophenylsulfonamido)ethyl}propanamide trifluoroacetate 8e had the greatest selectivity, followed by *N*-(2-aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate 1c and *N*-(2-aminoethyl)-4-methylbenzenesulfonamide trifluoroacetate salt 1b.
Additionally, compound \( N\)-(2-aminoethyl)-4-nitrophenylsulfonamide trifluoroacetate 1d showed the greatest anti-pseudomonal effect, with an MIC of 78.125 \( \mu \)g/ml. This effect was not specific as it also had the greatest effect against \( B. subtilis \) and \( P. vulgaris \), with an MIC of 156.25 \( \mu \)g/ml for both species.

These sulfonamides (and their \( \beta \)-alanyl derivatives) represent new leads in the search for antimicrobial agents for the treatment of \( P. aeruginosa \). Although compounds 8e, 1c, and 1b have selective effects upon PA01, their anti-pseudomonal activities are not sufficient for the development of potent anti-pseudomonal agents, suggesting that \( \beta \)-alanyl aminopeptidase has no physiological role in the growth of \( Pseudomonas aeruginosa \). The results obtained in this work do, however, support further work to enhance the activity of these non-classical sulphonamides, as well as proteomics and protein purification in order to isolate and study \( \beta \)-alanyl aminopeptidase crystallographically.
CHAPTER ONE

INTRODUCTION
1. Introduction

1.1 Antibacterial Resistance

Since their initial discovery by Fleming in 1928, and their introduction to the clinic in the late 1940s, penicillins have been used for treatment of bacterial infections in millions of patients, resulting in significant reductions in the mortality and morbidity rates associated with these infections,(1,2) *e.g.* meningitis and pneumonia. In the late 1950s, some Staphylococci were found to be resistant to penicillin,(3) and in the following years resistance also developed to later classes of antibacterial agents such as the cephalosporins and fluoroquinolones.(4,5) It soon became clear that microorganisms have developed a number of different mechanisms for resistance to antibiotics and multi-drug resistant (MDR) bacteria or ‘superbugs’ have now spread worldwide, posing a serious threat to global public health, **Figure 1**.
The extent of antimicrobial resistance varies around the world. Many bacterial species exhibit multi-drug resistance, including, MDR Gram positive and negative organisms, example of which are given in Table 1.
Table 1  Multidrug resistant (MDR) bacteria

<table>
<thead>
<tr>
<th>Gram positive</th>
<th>Gram negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>Acinetobacter baumannii</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>Streptococcus pneumonia</td>
<td>Salmonella</td>
</tr>
</tbody>
</table>

1.2 The Impact of MDR Organisms on Public Health and Healthcare Economics

Drug resistant pathogenic bacteria may result in more frequent periods of illness, longer hospital stays and increased health care costs. Multi-drug resistance is exceedingly common and there is a high prevalence rate. For example, according to a report from the European Molecular Biology Organization (EMBO), infections caused by MRSA and VRSA result in 5000 deaths annually worldwide,(8) while in the US 800,000 to 4 million patients are infected with Salmonella each year, of which 500 cases are fatal.(9) Almost all strains responsible for Staphylococcal infections in the US have some resistance to penicillin, and many are also resistant to newer classes of antibacterial agents.(10) In the US, the annual hospital expenditure associated with treating infections caused by MRSA is $3-4 billion.(11) In Australia and New Zealand, Staphylococcus aureus bacteraemia has a
20% mortality rate, which is significantly higher than for infections caused by methicillin-sensitive *S. aureus*. (12) The prevalence of antimicrobial resistance is increasing worldwide; for example, a study conducted in the US showed that *Pseudomonas aeruginosa* resistance to fluoroquinolones, imipenem, and ceftazidime increased by 37, 32 and 22%, respectively, between 1997 and 2001. (13) Antimicrobial resistance has been recognized as a global crisis and this situation may partly be due to the improper use (or overuse) of antibiotics, which can lead to the emergence and persistence of MDR organisms. Closer control of antibiotic prescriptions is thus required to overcome the problems associated with antimicrobial resistance, provide patients with suitable antibiotics, and to ensure cost-effective treatment. (14) Antimicrobial Stewardship (AMS) is a healthcare intervention program run in many countries which aims to improve antibiotic use through the use of a range of resources and expertise. AMS approaches may produce different strategies and guidelines for antibiotics uses by using resources from infectious diseases physicians, pharmacists, and microbiologists, as well as governance procedures to help in the implementation of these guidelines. (15)
1.3 Mechanisms of Antibiotic Resistance

Antimicrobial resistance in bacteria results from one of two main mechanisms:

- Acquired resistance — bacteria can acquire resistance by acquisition of DNA from other bacteria by horizontal gene transfer, which is mediated by conjugation, transduction or transformation.

- Inherent resistance — in which the bacteria have innate resistance to a class of antibacterial agents. For example, Pseudomonas aeruginosa has low susceptibility to hydrophobic antibiotics as they have difficulty in penetrating its outer membrane.(5, 16)

‘Superbugs’ exhibit resistance to antibacterial agents by a variety of mechanisms including:

- Drug inactivation or modification; for example bacteria can produce β-lactamase enzymes, which cleave the essential β-lactam ring of these antibiotics by catalyzing its hydrolysis.(17)

- Mutation of the antibacterial target site, for example, alteration of the drug binding site can lead to the decreased binding of the antibacterial agent to its target. For example, Walsh (2003) listed 19 clinically used agents which can be inactivated by this mechanism. This is the mechanism found in most resistance in Gram-positive bacteria to vancomycin.(18) Another example of this mechanism is the alteration of
biochemical pathways; this resistance mechanism is utilized by sulfonamide-resistant bacteria, which can mutate the dihydropteroate synthase (DHPS) gene in the folic acid pathway. DHPS is the target of the sulfonamides and the biosynthesis of folic acid, a process unique to prokaryotes, is inhibited by sulfonamides.(19)

- Active efflux of the antibiotic; for example, *Pseudomonas aeruginosa* resistance to the fluoroquinolones is due to reduced intracellular antibiotic concentrations. Walsh has described the multidrug resistance inducing efflux pumps, which contribute to resistance to multiple classes of antibacterial agents.(18)

1.4 Novel antibiotics

The persistent spread of antibiotic resistant bacteria and the decrease in the number of newly approved antibacterial agents means that the discovery of novel antibiotics to counteract the bacterial resistance of currently used antibacterial agents is urgent.(20) Pharmaceutical companies are losing interest in the antibiotics market because these drugs simply are not as profitable as drugs that treat chronic diseases (long-course treatment). The golden age of discovery new antibiotics is past and the period between 1983 and 2001 just 47 novel antibiotics were approved in North America, since 1998 nine new antibiotics were approved, only two of them had a new mode of action: linezolid (Pharmacia and Pfizer) and daptomycin (Cubist).(21) Newly developed antibiotics and novel antibiotics with new targets will be described below.
1.4.1 Lipoglycopeptides

Telavancin a semi-synthetic vancomycin derivative is a recently introduced (2009) lipoglycopeptide antibiotic (Vabativ®). It acts by both inhibiting bacterial peptidoglycan production and decreasing membrane depolarization.(22) Dalbavancin is another lipoglycopeptide under clinical trial with a longer half-life, up to 250 h.(23)
1.4.2 Oxazolidinones

Linezolid is a new oxazolidinone, which has a structure which is different from previously known oxazolidinones. Linezolid’s mode of action involves the inhibition of bacterial
protein synthesis. Other oxazolidinones are Radezolid, which is in phase 2 clinical trials, and Tedizolid, which is in phase 3 clinical trials. (24)

![Linezolid](image)

**1.4.3 Lipopeptides**

One of the more important classes of antibiotics are the lipopeptides. Mx-2401 is a lipopeptide that inhibits peptidoglycan synthesis by targeting C55-P, a substrate which plays an important role in peptidoglycan biosynthetic pathways, and targeting this substrate thus leads to inhibition of the cell wall precursor lipid production. (25)
1.4.4 Signermycin B

Signermycin B is a new antibiotic which targets histidine kinase (WalK). Histidine kinase (WalK) is one of the two essential components — histidine Kinase (WalK) and WalR (response regulator) — which are important for Gram positive bacterial growth and cell wall metabolism. (26)
Bacterial cell division is considered to be a promising target for new antibiotics, and Actinonin is an antibacterial agent that can inhibit peptide deformylase (PDF), an enzyme which has a key role in bacterial cell division. \(^{(27)}\)
1.4.6 \( \beta \)-Lactones

The \( \beta \)-lactones are natural compounds with promise as antibiotics due to their ability to inhibit homoserine transacetylase (HTA), an enzyme which is required for the biosynthesis of methionine and so is important for bacteria growth, e.g Obafluorin.(28)

![Obafluorin](image)

1.4.7 Platensimycin

Platensimycin is a recently discovered antibiotic which targets the enzymes involved in fatty acid biosynthesis and so acts on the formation of the cell membrane of Gram-positive bacteria. Platensimycin inhibits \( \beta \)-ketoacyl synthases I/II which are essential for the production of bacterial cell membrane fatty acids.(29)
As described before there is an urgent need for the discovery of new antibiotics to compete with the significant increase in bacterial resistance and Table 2 lists some of the newly developed antibacterial agents and their investigational status. The aim of many researchers who are working in this area is to find novel compounds that have antibacterial properties via new mechanism and target.(20,30)

Table 2  Novel antibacterial agents (30)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFN-1252</td>
<td>Fatty acid production</td>
<td>In Clinical trials</td>
</tr>
<tr>
<td>MX-2401</td>
<td>Bacteria cell wall</td>
<td>In Clinical trials</td>
</tr>
<tr>
<td>Radezolid</td>
<td>Protein synthesis</td>
<td>In Clinical trials</td>
</tr>
<tr>
<td>Surotomycin</td>
<td>Membrane depolarization</td>
<td>In Clinical trials</td>
</tr>
<tr>
<td>BAL30072</td>
<td>Bacterial cell wall</td>
<td>In Clinical trials</td>
</tr>
<tr>
<td>TR-701</td>
<td>Protein synthesis</td>
<td>In Clinical trials</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Target</td>
<td>Date</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Fidaxomicin</td>
<td>RNA polymerase</td>
<td>Launched in 2011</td>
</tr>
<tr>
<td>Biapenem</td>
<td>Bacterial cell wall</td>
<td>Launched in 2002</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>Protein synthesis</td>
<td>Approved in 2004</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>Bacterial cell membrane</td>
<td>Launched in 2003</td>
</tr>
<tr>
<td>Linezolid</td>
<td>Protein synthesis</td>
<td>Launched in 2000</td>
</tr>
</tbody>
</table>

1.5 **Pseudomonas aeruginosa**

*Pseudomonas aeruginosa* is a Gram-negative aerobic bacterium rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm, which is non-spore forming and non-fermentative, and belongs to the Pseudomonadaceae family, **Figure 2**. *P. aeruginosa* secretes a variety of pigments, including the blue-green pyocyanin, fluorescent pyoverdine and red-brown pyorubin. Most *P. aeruginosa* strains are motile, it is widely distributed in the environment, and normally found in soil, water, humans and animal samples. This pathogen has simple nutritional requirements and can grow in the absence of oxygen and over a wide temperature range.\(^{(31,32)}\) All these characteristics help contribute to the classification of *P. aeruginosa* as an opportunistic pathogen — it infects damaged tissues and can produce fatal infections in immuno-compromised individuals. *P. aeruginosa* is a nosocomial pathogen that is extremely well adapted to hospital conditions and can be found growing on medical equipment, such as catheters, often resulting in cross infection in clinics and hospitals.\(^{(31)}\)
1.5.1 Pathogenesis

*P. aeruginosa* is one of the most effective opportunistic nosocomial pathogens and is ranked as the second most common cause of hospital acquired infections in the US according to National Nosocomial Infection Surveillance System.(33) *P. aeruginosa* is responsible for around 20% of hospital acquired diseases and can lead to infections in both immunocompromised and immunocompetent patients. It can infect the respiratory tract, urinary tract, burns, wounds, and also the blood stream.(34)

**Heart.** *P. aeruginosa* can cause endocarditis, which is associated with a high mortality rate, by establishing itself on the endocardium through direct invasion from the blood-stream. Interestingly, there is a relationship between an increase in *Pseudomonas aeruginosa* induced endocarditis and narcotic addiction.(31,35)

Figure 2    Gram stain of Pseudomonas.(31)
**Urinary tract.** Urinary tract infections (UTI) which are caused by *P. aeruginosa* are usually nosocomial infections associated with urinary tract instruments or surgery during hospitalization. The cause of 40% of Pseudomonas bacteraemia is the invasion of *P. aeruginosa* from the urinary system to the blood-stream.(31,36)

**Blood.** Approximately one quarter of all nosocomial Gram negative *P. aeruginosa* infections are due to bacteraemia in immunocompromised individuals.(31) According to Vostrugina et al., *Staphylococcus aureus* and *P. aeruginosa* are the most common causes of bacteraemia in burn patients.(37)

**Respiratory tract.** Respiratory systems are the most likely to be infected by *P. aeruginosa*. Ventilator associated pneumonia (VAP) is a common disease in intensive care units, and has high mortality and morbidity rates.(38) It has been found that 60% of ventilator associated pneumonia cases are due to Gram negative bacilli and *P. aeruginosa*. VAP leads to increased healthcare costs due to prolonged hospitalization periods.(39)

**Cystic fibrosis (CF).** CF is one of the most prevalent genetically acquired diseases affecting young Australians; mostly affecting the lungs and the digestive system. *P. aeruginosa* is one of the most common pathogens which cause lung infections and is the main cause of morbidity and mortality in CF patients.(40)

**Brain.** *Pseudomonas aeruginosa* can cause meningitis either as a primary infection (with a 39% fatality rate), or a secondary infection (with a 66% fatality rate). The pathogen can invade the central nervous system (CNS) from either the urinary tract via the blood or the paranasal sinus.(31,36)
**Ear.** As *P. aeruginosa* flourishes in moist conditions, swimmers are especially prone to Pseudomonas otitis externa, which is the most common cause of chronic otitis media (approximately 70% of cases) and can spread through vascular channels to the leptomeninges, and so lead to meningitis.(41) The most serious Pseudomonal infection of the ear is Malignant Otitis Externa (MOE) in diabetics and elderly patients(4) and *P. aeruginosa* is the causative agent in most cases.(31,42)

**Eye.** *P. aeruginosa* is the most common Gram-negative bacterium causing bacterial keratitis. Patients can lose the entire eye if infected with this pathogen due to the bacterium secreting enzymes such as elastase and alkaline protease.(31)

**Bone.** Osteoarthritis and osteomyelitis can be caused by *P. aeruginosa* and these infections are associated with surgery, compound fractures, drug addiction and extension burns. The most frequently targeted sites for these infections are the vertebral column, pelvis, and sternoclavicular joint.(31,36)

**Gastrointestinal tract.** Many types of *Pseudomonas* infections can occur in the gastrointestinal tract of immunosuppressed patients, such as pediatric diarrhea, gastroenteritis, and typhlitis.(31) Typhlitis is the major GIT infection caused by *P. aeruginosa* and it may involve the whole colon.(43) It has also been shown that *P. aeruginosa* can cause diarrhea, especially in immunocompromised patients.(44)

**Skin.** Although normal dry skin does not support the growth of *P. aeruginosa* it has been found that infections can occur in moist skin. Approximately 25% of individuals with burns are more exposed to this bacterium, which can release protease and toxins and may cause
serious problems.(31,36) Dermatological infections that can be caused by *P. aeruginosa* include; green nail syndrome, toeweb infection, ecthyma gangrenosum, *Pseudomonas* septicemia, and *Pseudomonas pyoderma.*(45)

1.5.2 Detection of *Pseudomonas aeruginosa*

The Gram stain procedure is initially used to identify *P. aeruginosa* but there are many other methods to detect this pathogen, such as testing its growth in blood agar or eosin–methylthionine chloride blue agar. It can also be identified by its fruity odour, or fluorescent green colonies when the infected area is exposed to UV light.(31) A new technique for identifying *P. aeruginosa* targets a specific enzyme and indicates the presence of *P. aeruginosa* with either chromogenic or fluorogenic substrates allowing for the rapid and accurate detection of the pathogen, Figure 3.(46) Groundwater *et al.* reported that phenoxazinones can be used as chromogenic substrates in targeting β-alanyl aminopeptidase (BAAP).(47) The β-alanyl aminopeptidase sequence has been found in the genome of *Pseudomonas* Sp. MCI3434, and has unique substrate specificity for β-alanyl dipeptide compared to D-alanine.(48) β-Alanyl aminopeptidase is present in the periplasm of *P. aeruginosa* in significant quantities.(49) These chromogenic substrates, *e.g.* 1, are cleaved only by *Pseudomonas* strains expressing this enzyme, with the generation of a purple colour indicating the release of the core chromogen 2, Figure 4. When 7-N-(β-alanylamino)-1-pentylphenoxazinone substrate 1 was employed, it gave a low degree of
diffusion in nutrient media and the colonies of *P. aeruginosa* were easily distinguishable,(47) as shown in **Figure 3**.

**Figure 3** Chromogenic effect in *P. aeruginosa* in the left two dishes (with no colour generation) with other strains of Pseudomonas in the right hand dishes (47)
Figure 4  Generation of a purple colour by the action of BAAP on a yellow β-alanylaminophenoxazinone substrate (47)

1.5.3 Treatment of *P. aeruginosa* infections

An early start to a suitable antimicrobial treatment improves patient outcomes while, in contrast, inadequate therapy is a cause of *P. aeruginosa* associated mortality in hospitals.(50) Antibiotics which have activity against *P. aeruginosa* are classified below according to their mechanism of action;

1. Antibiotics which interfere with cell wall synthesis; cephalosporins (such as ceftazidime, cefepime, cefoperazone, and cefpirome), penicillins (such as ureidopenicillins and carboxypenicillins) and carbapenems (such as meropenem, imipenem and doripenem).

2. Antibiotics which inhibit protein synthesis; aminoglycosides (such as gentamicin, amikacin, and tobramycin).
3. Antibiotics which interfere with nucleic acid synthesis; quinolones (such as ciprofloxacin, levofloxacin, and moxifloxacin).

4. Antibiotics which disrupt bacterial membrane structures; polymyxins (such as polymyxin B and colistin).(51)

*P. aeruginosa* is resistant to a number of antimicrobial agents, and amikacin, ceftazidime, imipenem, and ciprofloxacin are the commonly prescribed antibiotics for Pseudomonas infections.

### 1.5.4 Multi-drug resistance in *P. aeruginosa*

*P. aeruginosa* is considered to be a superbug, and can develop resistance to antibiotics through all of the mechanisms described in section 1.3. It has both intrinsic and acquired resistance to a wide range of antimicrobial agents — this superbug can use one or more enzymatic or mutational resistance mechanisms against many classes of antibiotic agents, making it an economic and public health concern.(52) The economic concerns arise due to the fact that patients who are infected with *P. aeruginosa* require extended hospital stays to control their infection. Public health problems also arise as *P. aeruginosa* infections may be responsible for increases in both the mortality and morbidity rates associated with nosocomial-acquired diseases. In Iran, a survey carried out between January and April 2003 in Ghotbeddin Burn hospital found that most *P. aeruginosa* strains isolated from burns patients had resistance to all anti-Pseudomonal antibiotics, except meropenem and imipenem.(53) In a recent study, conducted in Bathinda, in India, the susceptibility of 193 *P. aeruginosa* samples to anti-Pseudomonal agents was assessed over the period March
2009 to March 2010 — 60% of the Pseudomonas isolates were found to be resistant to cephalosporins, 75% were resistant to cefotaxime, 63% were resistant to ceftriaxone, 79% were resistant to gentamicin, and 73% of the isolates were resistant to ciprofloxacin.(54)

The specific antibiotic resistance mechanisms exhibited by *P. aeruginosa* include:

**The production of β-lactamases**

As mentioned previously, β-lactamases catalyse the cleavage of the amide bond in the β-lactam ring, leading to the deactivation of these agents. There are over 300 different β-lactamases, which are present in the bacterial periplasmic space. These enzymes represent one of the major causes of *P. aeruginosa* resistance to β-lactam antibiotics. The chromosomal β-lactamase, AmpC hydrolyses a wide range of β-lactam antimicrobial agents, such as aztreonam and third generation cephalosporins.(55,56) Another example are the extended spectrum β-lactamases (ESBL), which hydrolyse even third generation cephalosporins, but not carbapenems.(57)

**The production of aminoglycoside modifying enzymes (AMEs)**

Aminoglycoside antibiotics act by inhibiting protein synthesis and can disturb the bacterial cell membrane. *P. aeruginosa* produces AMEs in response to treatment with aminoglycoside antibiotics. AMEs are divided into three major classes; aminoglycoside phosphoryltransferases, aminoglycoside acetyltransferases, and aminoglycoside adenyllyltransferases; all of which inhibit the binding ability of aminoglycoside agents to the ribosomal subunit.(52) An example of an AME which is secreted by *P. aeruginosa* is
aminoglycoside acetyltransferase, AAC (6')-li, which is linked with resistance to gentamicin, and tobramycin. Another example is adenine nucleotide translocator, ANT (2'')-I, which is associated with *P. aeruginosa* resistance to gentamicin and tobramycin.(52)

**The expression of efflux pumps**

Efflux pumps play a crucial role in *P. aeruginosa* resistance to many antimicrobials, and this is a non-enzymatic route of β–lactam resistance in *P. aeruginosa*. This resistance mechanism involves the over-expression of efflux systems which can be the result of mutations in regulatory genes. Four over-expressed efflux pumps have been characterized in *P. aeruginosa*; MexA–MexB–OprM, MexC–MexD–OprJ, MexE–MexF–OprN, and MexX–MexY–OprM. These efflux systems play a very important role in the multi-drug resistance of *P. aeruginosa*, especially to aminoglycosides, fluoroquinolones, polymyxin B and β–lactam antibiotics.(55,56) An example of resistance *via* this mechanism is the resistance of *P. aeruginosa* to tigecycline.(52,58)

**Outer-membrane modification**

Antimicrobial agents have to pass through the porin channels in the outer membrane of Gram negative bacteria in order to enter the cell and exert their activity. Livermore has demonstrated that the low outer membrane permeability of *P. aeruginosa* to hydrophilic compounds is associated with high resistance to β–lactams agents such as imipenem.(59) According to Pechere and Kohler, many imipenem resistant *P. aeruginosa* clinical isolates
are differentiated by a deficiency in the OprD porins, which promote the uptake of carbapenems and basic amino acids.(55)

1.5.5 The *Pseudomonas aeruginosa* genome

It is clear that *P. aeruginosa* has developed many different ways to resist the effect of antibiotics and can survive in water, animals, plants, and soil, and recent research has attempted to identify the source of its adaptability. Stover *et al.* proposed that the secret lies within the genome of *P. aeruginosa* which, at 6.3 million base pairs, is one of the largest and most complicated bacterial genomes. For comparison, the genome of *Bacillus subtilis*, contains 4.2 Mbp, while those of *Escherichia coli*, (4.6 Mbp) and *Mycobacterium tuberculosis*, (4.4 Mbp) are also considerably smaller. An understanding of the genome should hopefully allow for the development of new antibacterial agents which target specific proteins, and thus represent new strategies for the treatment of infections caused by *P. aeruginosa*.(60)

1.6 Aminopeptidase Enzymes

Aminopeptidases are widely distributed in both prokaryotic and eukaryotic organisms and, catalyze the cleavage of amino acid residues at the N-terminal position of proteins. Bacterial aminopeptidases are localized in the cytoplasm, on the membrane, and in periplasm and, to date, more than a hundred bacterial aminopeptidases have been detected. Aminopeptidases are classified according to their substrate specificity, into broad-
narrow-acting and according to their catalytic mechanism into metallo-, cysteine and serine aminopeptidases. Cahan et al. have reported that P. aeruginosa secretes an aminopeptidase which belongs to the metalloprotease family. Komeda and Asano detected β-alanyl aminopeptidase in Pseudomonas bacteria in 2005, while Fricke and Aurch found that at least four different aminopeptidases occur in the periplasm, leucyl aminopeptidase, glutamyl aminopeptidase, alanyl aminopeptidase, and prolyl aminopeptidase. Fuchs et al. suggested that β-alanyl aminopeptidase from P. aeruginosa is a novel class of enzyme, which could be targeted for the preparation of novel antibacterials. As the physiological function of β-alanyl aminopeptidase in P. aeruginosa is currently unknown this is an area which requires further research.

### 1.7 Targeting β-alanyl aminopeptidase in P. aeruginosa

Targeting of any species-specific enzymatic activity of this microorganism could help rapid detection of the pathogen and enable an early start of the appropriate treatment. An example of a technique for bacterial detection is the distinction of Gram negative from Gram positive bacteria by a method which also employs an aminopeptidase: only Gram negative bacteria gave yellow stains within 5 minutes when L-alanine-4-nitroanilide-impregnated filter paper was placed on bacterial colonies.

Another example of targeting aminopeptidase activity in P. aeruginosa for detection purposes was mentioned previously, in which the β-alanylaminopeptidase activity of P.
*aeruginosa* was used to generate a purple colour from a light yellow coloured chromogenic enzyme substrate.\(^{(47)}\)

In addition to its use in the detection of *P. aeruginosa*, if β-alanyl aminopeptidase is essential for the growth of this organism, inhibition could provide a new class of anti-pseudomonal agents.

### 1.8 Protein Structure Prediction

Enzymes are large biological molecules responsible for cellular process and play a crucial role in many diseases. An understanding of the protein structure which is linked to their function is very important for the discovery of new effective treatments for disease.\(^{(65)}\) As there are often difficulties in obtaining sufficient amounts of pure protein for high quality crystal structure, homology modeling and protein threading approaches help to predict the three dimensional structure of enzymes from their primary amino acid sequence and so help in analyzing the structure and its properties. A subsequent structure based drug design approach can significantly save both the cost and time involved in discovering effective therapeutic agents, and the three dimensional structure of the target is required for this process, in order to understand the function and the interactions with the ligands.\(^{(66)}\)
1.9 Homology Modeling

Homology modeling, or comparative modeling, is the process by which computational algorithms are used to predict three dimensional structure of a protein starting from its primary amino acid sequence. This method is currently the most reliable approach for the prediction of protein 3D structure, the principle being based on protein sequence similarity, as more similar enzyme sequences will have more similar structures and functions.\(^{(67,68)}\)

In this approach, the enzyme sequence with an unknown 3D structure (Target) is aligned with one or more protein sequences with a known 3D structure (Template). The accuracy of the homology modeling approach is dependent upon the similarity between the target and template sequences being greater than 30\% in order to obtain good alignment.\(^{(65)}\) The limitations of this technique can arise from the selection of the wrong template, and the incorrect alignment of the target and template sequences.\(^{(69)}\)

The four main steps in homology modeling are;

- Identification of one or more known templates with similar primary sequence to the target.
- Alignment of the target and template sequences.
- Generation of the model(s), and
- Evaluation of the model(s) generated.
1.9.1 Identification of template protein

The first step in homology modeling is to identify one or more proteins with a known three-dimensional structure to serve as the template. These proteins should have amino acid sequence similarity to the target sequence. The sequence selection of the best templates is considered to be a crucial factor in obtaining an accurate model, and this is performed by a sensitive sequence-based search for the closest template. BLAST (Basic Local Alignment Search Tool) is an algorithm for the comparison of the amino acid sequences of different proteins. There are different software packages for BLAST, which can be used to identify the best template such as:

- Protein-protein BLAST (BLASTP), which compares the sequence of proteins and
- Nucleotide-nucleotide BLAST (BLASTN), which compares DNA sequences.

In this work BLASTP matching methods were used, in which a pair-wise comparison of the target sequence and a protein with known 3D structure is conducted. The most suitable template is the one which has high sequence similarity with the target, and this protein is preferably from the same family as the target.(68,69)

1.9.2 Sequence Alignment

As the template selection is based on the initial alignment, this alignment is not sufficient to be used for construction of a model and a more reliable alignment is needed to align structurally equivalent amino acids and to correlate common structural features such as the
secondary structure of the catalytic sites. As the sequence alignment is the arrangement of the amino acid sequences of the target and template, this alignment is generally described by sequence similarity and sequence identity which are represented as a percentage. The template can give an accurate model for the target enzyme if the sequence identity is greater than 60%, while if the sequence identity is between 20-35%, the template is considered to have low homology and this described as the twilight zone. There are many programs for sequence alignment, such as CLUSTAL, FASTA, and BLAST alignment.\(^{(70,71)}\) and the BLAST alignment was used in this work.

1.9.3 Model Construction

Starting from the target template alignment, a 3D model can be generated using different methods, modeling by rigid-body assembly, modeling by segment matching and modeling by satisfaction of spatial restraints.

1.9.3.1 Modeling by rigid-body assembly

Modeling by rigid-body assembly is a widely used technique which is based upon finding the similarity between the secondary structures of the template and the target, then enforcing the template’s secondary structure features on the correlated parts of the target.\(^{(72)}\)
1.9.3.2  Modeling by segment matching

In segment matching modeling, the models are generated by using a number of atomic positions from the template structures as guiding points, this approach can help to construct both side chain and main chain of the protein model by using a database of short regions of protein structure, geometry, and energy rules.(73)

1.9.3.3  Modeling by satisfaction of spatial restraints

In modeling by satisfaction of spatial restraints, the spatial restraints are obtained from the target-template sequence alignment, and this technique can use many different types of information about the target, which can be collected from fluorescence spectroscopy, NMR spectra, and other experimental information.(74) Restraint-based modeling is probably the most promising technique of all homology modeling techniques.

1.9.4  Model validation

Validation is a crucial step that can be conducted at different levels of structure, and it is important to evaluate the predicted model to check if there are any potential errors, or if there are inaccuracies in generating the protein structure.(68) A model with high sequence homology alignment will require less effort in model validation than a modeling with low homology.(70) There are a number of evaluation approaches, such as PROCHECK and MolProbity, these programs are used in the assessment of stereochemical quality, bond
lengths, the conformation of the side chains, angle planes, and the Ramachandran plot of the predicted model.(65,75)

1.9.4.1 Ramachandran Plot

The Ramachandran plot (or Ramachandran diagram) is a technique which is used to validate the generated model by measuring the distribution of the amino acids within the Ramachandran area. It is a means of visualizing the backbone dihedral angles, $\psi$ against $\phi$, of amino acids in the protein.(76) This tool allows us to check the overall quality of the generated protein structure by measuring the distribution of the amino acids within the favorable and forbidden regions of the plot.(77)

1.9.4.2 Root Mean Square Deviation (RMSD)

The generated model can also be assessed by calculating the backbone root mean square deviation RMSD (Å), which measures the distance between two sets of points. RMSD shows the accuracy of the generated model compared to well-known structures and for identical 3D structures the RMSD is 0.

1.9.4.3 Molprobity

Molprobity is a web service for the assessment of a 3D model of a protein and the evaluation of the number of serious clashes (clashscore; $\geq 0.4$Å) per 1000 atoms.(78)
1.10 Protein-Ligand Docking

Docking is a method which predicts the suitable conformations of the ligand when it interacts with the target protein. In addition, docking allows prediction of possible ligand binding sites and the discovery of novel ligands based on virtual screening. (79) The docking steps include:

- Protein preparation
- Ligand preparation
- Grid generation
- Glide docking
- Induced fitting

A detailed description of these steps and the evaluation of docked models will be discussed in Chapter Two.

The main aims of Protein Ligand docking are; (80)

- The correct prediction of binding affinity of related molecules from a known active series,
- Accurate structure modeling, and,
- The discovery of new ligands through virtual screening.

Virtual screening can be conducted using virtual libraries of either compounds that have yet to be synthesized, or compounds available for purchase from chemical databases. This screening can be performed using the Structure Based Virtual Screening (SBVS) or, the
High Throughput Screening (HTS) approach. SBVS is considered to be most successful if the target structure has been determined at high resolution.(81)

### 1.11 β-Alanyl aminopeptidase and homology modeling

A knowledge of the three-dimensional structure of β-alanyl aminopeptidase is important for an understanding the mechanism of this enzyme, as well as for the structure-based design of small molecules inhibitors which target it. The process of new drug design is greatly benefitted by the 3D structural analysis of target enzymes and their interaction with potential ligands, but there is currently no 3D structure for β–alanyl aminopeptidase enzyme.

In this case homology modeling, as outlined in Figure 5, was required for the generation of the 3D structure, based upon the amino acid sequences of β-alanyl aminopeptidase, as will be described in the next chapter.
Figure 5  Steps involved in the homology modeling and virtual screening of β- alanyl aminopeptidase
1.12 Aims

As mentioned previously, an aminopeptidase is an enzyme which catalyzes the cleavage of amino acid residues at the N-terminus of a peptide. A number of research groups have suggested the presence of β-alanyl aminopeptidase in Pseudomonas species. Groundwater and co-workers targeted the β-alanyl aminopeptidase of *Pseudomonas aeruginosa* through the synthesis of chromogenic substrates (β-alanylresorufamines) which are selectively hydrolysed by this enzyme. Lyczaka *et al.* described how *P. aeruginosa* enzymes contribute to infections caused by this organism but the precise role of β-alanyl aminopeptidase in *P. aeruginosa* is still unknown. With no published data on the 3D structure of this enzyme, the aims of this research are to perform homology modeling of the β-alanyl aminopeptidase sequence of *P. aeruginosa* and to use the model generated to design and synthesize inhibitors in order to evaluate its cellular role, and perhaps discover new antimicrobial agents with the potential to inhibit the growth of *P. aeruginosa*. 
1.13 Significance

3D enzyme structures help to provide a fuller understanding of protein function and aid in the discovery of an inhibitor through the use of structure databases. To date, the 3D structure of β–alanyl aminopeptidase is not known. As *P. aeruginosa* exhibits multi-drug resistance, with few effective marketed antibiotics available and, as there has been a steady decrease in the number of approved antimicrobials, there is increased interest in the search for new agents for the treatment of *P. aeruginosa* infections. The generation of a 3D model of β–alanyl aminopeptidase should allow the design and synthesis of inhibitors which have potential as novel specific agents for the inhibition of the growth of MDR *P. aeruginosa*. In addition, by testing the effect of inhibitors on *P. aeruginosa* growth, we should gain a greater understanding of the role of β–alanyl aminopeptidase in *P. aeruginosa*. 
CHAPTER TWO

HOMOLOGY MODELING
2. Homology Modeling

In order to construct a 3D model for β-alanyl aminopeptidase, the following steps were conducted:

- The primary amino acid sequence of the target was determined.
- The best template was selected.
- The template was aligned with the target enzyme sequence.
- The target protein structure was modeled, and
- The model generated was evaluated through the docking of a known ligand.

Schrodinger Maestro 9.3 software was used in the comparative modeling “Prime” (86) and docking studies “Glide” (87) in this project.

2.1 Determining the target sequence

β-Alanyl aminopeptidase is an enzyme from Pseudomonas spp. which is specific for the cleavage of β-alanyl dipeptides.(47,48,62) The sequence of β-alanyl aminopeptidase was determined from the National Center for Biotechnology Information (NCBI) database, and is available from GenBank: BAE02664.1 under the title ‘beta-alanine specific aminopeptidase [Pseudomonas sp. MCI3434]’ (NCBI). The 366 amino acid sequence of the target protein is shown in Figure 6.
After input of the MCI3434 sequence in FASTA format the next step was to find sequence homologs.

2.2 Identification of a template

After downloading the primary amino acid sequence for the target, the second and most crucial step in homology modeling is the selection of accurate homologs. In order to build the most appropriate model, the template with the greatest similarity to the target sequence (in terms of both structure and function) must be selected. The target sequence was imported into the Prime program workspace (Maestro version 9.3) then the search for a template was performed using a BLAST homology search (the default is to use BLAST to search the non-redundant PDB database). A list of potential templates was obtained and displayed in the homologs table and the highest-scoring template was selected by default, as shown in Figure 7. 1B65_A was selected (with 43% sequence identity) as the template:
1B65_A corresponds to Chain A of L-aminopeptidase D-Ala-esterase amidase (DmpA) from *Ochrobactrum anthropi*.

**Figure 7**  Blast search for template using Prime (Maestro version 9.3)
2.2.1 L-Aminopeptidase D-Ala-esterase / amidase (DmpA) from *Ochrobactrum anthropi*

DmpA has a sequence identity of 43% with the target sequence, it is a serine aminopeptidase enzyme which has a distinctive substrate specificity — it is the only known enzyme which can catalyze the removal of either an L- or a D-amino acid from the amino terminus of a peptide, and is, therefore, called L-aminopeptidase D-ala-esterase / amidase. The physiological role of DmpA has not yet been deduced. The DmpA crystal structure (PDB 1B65) was carried out by Bompard-Gilles *et al.*, and shows a homotetrameric protein of 375 amino acids; each monomer contains two polypeptides (α chain 1-249; β chain 250-375) which can be generated by autocatalytic cleavage of the Gly249-Ser250 bond. The catalytic residues for 1B65 are; Y146, N218, S250, S288 and G289 Figure 8. Glu144A is close to the active site and it is the only negatively charged amino acid in the substrate binding pocket, it is important for the stabilization of the positive charge on the substrate.(88)
Figure 8  The catalytic mechanism of DmpA (adapted from ref. 88)

2.3  Alignment of target and template proteins

After the selection of a template with a known 3D structure (in this case 1B65 chain A [1B65_A]), an automated alignment in Prime was carried out in order to align the target and template sequences. There are several ways to produce an alignment for use in building a structure:

- Accept the BLAST / PSI-BLAST alignment generated in the find homologs step.
- Import an alignment (Prime can also use an alignment produced in another alignment program).
Generate or import secondary structure predictions, then use the Align program to create a new alignment.

Edit any of these alignments manually.

In this project the generated alignment from the template selection step was used, as the conserved amino acids for 1B65_A are well aligned with the target sequence, as shown in Figure 9.

Figure 9  Sequence alignment window, the red H represents a residue in a helical region, and Blue E represents amino acids with extended regions (beta sheets). (Prime, Maestro version 9.3)
A survey of the literature showed that there are only four known β-peptidyl aminopeptidases from *Proteobacteria*, and Geueke *et al.* have reported their alignment, Figure 10.(82,89)

**Figure 10** Alignment of the amino acid sequences of four β-peptidyl aminopeptidases: BapA from strain 3-2W4, BapA from strain Y2, BapA from *Pseudomonas sp.* MCI3434 BAE02664 (the target), and DmpA from *O. anthropi* (the template) Red represents to the residue identical in all four enzymes and yellow represents residues identical in three of the four enzymes (ALINE program).(89)
2.4 Modeling of the target enzyme

Starting from the appropriate sequence alignment, a 3D structure for the target had to be constructed and this building process included;

1. Coordination of the copying of backbone atoms for aligned area and side chains of conserved amino acid.

2. Building insertion and closing the deletions in the alignment.

3. Minimization of all atoms not derived directly from the template, and

4. Prediction of the side chain orientations for non-conserved residues.

By default, Prime software builds insertions, predicts side chain conformations of non-conserved amino acids, and closes gaps to generate a model with no physical clashes. Once the model building process was finished, the model was displayed in the workspace superimposed on the template. The 3D structure for the target was obtained using Prime (Maestro version 9.3) shown in Figure 11.
Figure 11  Solid ribbon representation of the homology model generated for β-alanyl aminopeptidase in Discovery Studio Visualizer Version 3.(90)
2.5 Model validation

Once the 3D model of β-alanyl aminopeptidase had been constructed, it was necessary to assess the accuracy of the model generated, and this was done via a Ramachandran plot, Molprobity, and the docking of a known target substrate 1 (a chromogenic β-alanylaminophenoxazinone).(47)

![Chemical structure of substrate 1]

2.5.1 Ramachandran plots

A Ramachandran plot was used to evaluate the model generated, in which each amino acid residue is represented by a point, and the plot indicates the distribution of the amino acids in the favourable and unfavourable regions of the model. The Ramachandran plot tool in Maestro version 9.3 was used to validate the 3D model of β-alanine aminopeptidase, as shown in Figure 12. The number of amino acids residues in unacceptable areas represents 6.8 % of the total residues, and these are located in areas which are distant from the
receptor binding site, while 93.2% of the amino acid residues were found within acceptable regions of the model.

**Figure 12**  Ramachandran plot for β-alanine aminopeptidase from Maestro 9.3. Amino acid representations are; Glycine (triangle), Proline (square), other amino acids (circle). Residues outside the yellow and red areas are in unfavourable regions, as the Red and yellow regions represent the favored and allowed regions
The amino acids that distributed in unfavorable regions in the model are: Gly 203, Gly 233, Gly 85, Gly 26, Gly 212, Gly 341, Gly 343, Gly 161, Gly 177, Gly 46, Gly 39, Gly 156, Gly 13, Gln 189, Gln 116, Val 41, Thr 340, Ser 89, and Leu 291, and, of these, the closest residue to the active site is Gly 233, with a distance of 6.93 Å as shown in Figure 13.

**Figure 13** Distribution of residues in unacceptable regions of the BAAP model, and the distance of closest amino acid (Gly233) in Å, as shown in pink. Maestro V 9.3
2.5.2 Molprobity

The generated model was also assessed using the Molprobity website after uploading the 3D structure of the target. The Molprobity summary, representing the clash score and the structure’s percentile value are shown in Table 3.(91)

**Table 3** Molprobity and clash score results from assessment of the modeled enzyme using Molprobity

<table>
<thead>
<tr>
<th>Homology Model of β-alanyl aminopeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clashscore, all atoms</td>
</tr>
<tr>
<td>Clashscore is the number of serious steric overlaps (&gt; 0.4 Å) per 1000 atoms.</td>
</tr>
<tr>
<td>Poor rotamers</td>
</tr>
<tr>
<td>MolProbity score</td>
</tr>
<tr>
<td>Cβ deviations &gt; 0.25 Å</td>
</tr>
<tr>
<td>Bad backbone bonds:</td>
</tr>
</tbody>
</table>

In the two results columns, the left column gives the raw count, the right column gives the percentage.

*100\(^{th}\) percentile is the best among structures of comparable resolution, 0\(^{th}\) percentile is the worst.

The MolProbity score combines the clashscore and poor rotamer score into a single score.
2.5.3 Docking of a known substrate

The modeled structure of β-alanyl aminopeptidase was prepared for docking with a β-alanylaminophenoxazinone substrate 1, a known chromogenic compound which is hydrolyzed (with removal of the β-alanyl residue) only by β-alanyl aminopeptidase from *Pseudomonas aeruginosa*. Docking was conducted using the Glide program in Maestro version 9.3.

2.5.3.1 Protein preparation

The overall quality of the docking is dependent upon the quality of the enzyme structure, so the first step was to prepare the β-alanyl aminopeptidase 3D structure through; fixing the structure, deleting unwanted chains and water molecules, and finally performing the optimization of the fixed structure. All of these steps were performed using the Protein Preparation wizard in Maestro version 9.3, using the default settings shown in Figure 14. The automatic protein preparation tasks allow the conversion of a protein from its raw state (that is, missing hydrogen atoms or having a charge state) to a state which is acceptable for the Maestro software.
Figure 14  Protein Preparation Wizard window in Maestro V.9.3

2.5.3.2  Preparation of the ligand

The β-alanylamino phenoxazinone substrate 3D structure was drawn, and then refined in LigPrep (Maestro version 9.3) as shown in Figure 15 and Figure 16. (93)
Figure 15  The 3D structure of β-alanylaminophenoxazinone, 1

Figure 16  LigPrep window in Maestro 9.3
2.5.3.3 Receptor grid generation

The main role of receptor grid generation is to produce a grid containing the location of the potential binding pocket, which can be generated either automatically or by specifying the amino acids enclosing a binding site. In this work, the grid was generated by selection of the conserved amino acids in the template, as Bompard-Gilles et al. have reported, the following catalytic residues from the target β-alanyl aminopeptidase were selected; Glutamic acid (Glu) 127, Tyrosine (Tyr) 129, Asparagine (Asn) 201, Serine (Ser) 239, Serine (Ser) 277, and Glycine (Gly) 278 from.(88)

2.5.3.4 Induced fit docking

Induced fit docking provides a highly accurate docking result via the following steps: initial docking (Glide), induced fit (Prime), and redocking (Glide). In this project the β-alanylaminophenoxazinone 1 was docked into the β-alanyl aminopeptidase model using induced fit docking methodology in Maestro version 9.3, giving a docking score of -9.6.

2.5.4 Ligand Receptor interactions

From Figures 17 and 18, it can be clearly seen that the β-alanylaminophenoxazinone 1 fits nicely into the active site forming four hydrogen bond interactions with the amino acid residues Glu 127, Tyr 129, Gly 278, and Asn 72 of the model.
Figure 17  
A 2D Ligand interaction diagram. B 3D Diagram of ligand 1 in binding pocket of β-alanyl aminopeptidase (Maestro version 9.3)
Figure 18  β-Alanyl aminopeptidase model with substrate 1 bound to the proposed active site in Maestro V 9.3
If we look more closely at the substrate binding pocket, it is clear that all amino acids involved in the ligand binding site interaction are conserved in the Template 1B65_A, Table 4, especially GLU 127 which is the only acidic amino acid close to the active site and which may be responsible for stabilization of the \( \alpha \)-amino group of the substrate. (88)

Table 4  Comparison of the active sites of the target \( \beta \)-alanyl aminopeptidase and template 1B65_A

<table>
<thead>
<tr>
<th>Proposed catalytic function</th>
<th>1B65_A</th>
<th>( \beta )-Alanyl aminopeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxyanion Hole 1</td>
<td>Tyr 146</td>
<td>Tyr 129</td>
</tr>
<tr>
<td>Oxyanion Hole 2</td>
<td>Asn 218</td>
<td>Asn 201</td>
</tr>
<tr>
<td>Stabilisation</td>
<td>Glu 144</td>
<td>Glu 127</td>
</tr>
<tr>
<td>Indirect involvement in</td>
<td>Ser 250, Ser 288, and Gly 289</td>
<td>Ser 239, Ser 277, and Gly 278</td>
</tr>
<tr>
<td>catalytic mechanism</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6  \textit{In Silico screening}

After the homology model of \( \beta \)-alanyl aminopeptidase was constructed and evaluated, \textit{in silico} structure based high throughput virtual screening (HTVS), in Maestro version 9.3, was used to predict \( \beta \)-alanyl aminopeptidase inhibitors using over 100,000 molecules obtained from the Chembridge database.
2.7 Selection of hit compounds

Virtual screening was performed to obtain the best substrate fit with the target, and the results were ranked by docking score, as shown in Table 5. The lead compounds were identified according to their docking score and their synthesis will be described in the next chapter.

Table 5  In silico screening results Glide (Maestro version 9.3)

<table>
<thead>
<tr>
<th>ID</th>
<th>Title</th>
<th>Docking score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ZINC09426195</td>
<td>-7.891</td>
</tr>
<tr>
<td>2</td>
<td>ZINC11757529</td>
<td>-7.865</td>
</tr>
<tr>
<td>3</td>
<td>ZINC09095060</td>
<td>-7.735</td>
</tr>
<tr>
<td>4</td>
<td>ZINC02884757</td>
<td>-7.650</td>
</tr>
<tr>
<td>5</td>
<td>ZINC04344119</td>
<td>-7.513</td>
</tr>
<tr>
<td>6</td>
<td>ZINC09426194</td>
<td>-7.368</td>
</tr>
<tr>
<td>7</td>
<td>ZINC19169028</td>
<td>-7.302</td>
</tr>
<tr>
<td>8</td>
<td>ZINC19093808</td>
<td>-7.194</td>
</tr>
<tr>
<td>9</td>
<td>ZINC19765764</td>
<td>-7.093</td>
</tr>
<tr>
<td>10</td>
<td>ZINC02571732</td>
<td>-6.960</td>
</tr>
<tr>
<td>11</td>
<td>ZINC04227458</td>
<td>-6.953</td>
</tr>
<tr>
<td>12</td>
<td>ZINC12304536</td>
<td>-6.922</td>
</tr>
<tr>
<td>13</td>
<td>ZINC19765499</td>
<td>-6.922</td>
</tr>
<tr>
<td>14</td>
<td>ZINC20436111</td>
<td>-6.911</td>
</tr>
</tbody>
</table>
As can be seen from Table 6, sulfonamide containing hits have some of the highest docking scores and so represent possible leads in the search for β-alanyl aminopeptidase inhibitors. If these leads were specific inhibitors of an enzyme expressed only by this bacterium, these compounds would represent probes of the function of this enzyme in this bacterium and, possibly, potential new, highly specific antibacterial agents for the treatment of *P. aeruginosa* based infections. The next chapters detail the synthesis of these compounds and their biological testing.

**Table 6**  *In silico* screening results, showing compounds with highest docking scores

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Compound Structure</th>
<th>Docking score</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZINC09426195</td>
<td><img src="image1" alt="Compound Structure" /></td>
<td>-7.891</td>
</tr>
<tr>
<td>ZINC11757529</td>
<td><img src="image2" alt="Compound Structure" /></td>
<td>-7.865</td>
</tr>
<tr>
<td>ZINC09095060</td>
<td><img src="image3" alt="Compound Structure" /></td>
<td>-7.735</td>
</tr>
</tbody>
</table>
2.8  *In silico* screening round two

After the first virtual screening results, the HTVS was conducted to find other classes of compounds which could act as leads for the design of specific inhibitors of β-alanyl aminopeptidase. This process was performed using the same procedure (Protein preparation and Grid receptor generation panel), but with 35,000 compounds from different Chembridge database, subsets (0, 0.1). From the best scoring 10 hit compounds identified through HTVS result, two compounds **Figure 19** were selected for β-alanyl aminopeptidase inhibition testing on the basis of visual inspection, cost, ease of synthesis and / or commercial availability, *Table 7.*
Table 7  *In silico* screening results, showing compounds with highest docking scores from Chembridge database subsets 0 and 0.1

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Compound Structure</th>
<th>Docking score</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZINC12445668</td>
<td><img src="image" alt="Compound Structure" /></td>
<td>-8.006</td>
</tr>
<tr>
<td>ZINC32580868</td>
<td><img src="image" alt="Compound Structure" /></td>
<td>-7.684</td>
</tr>
<tr>
<td>ZINC02485430</td>
<td><img src="image" alt="Compound Structure" /></td>
<td>-7.613</td>
</tr>
<tr>
<td>Compound ID</td>
<td>Molecular Structure</td>
<td>Value</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>ZINC00978443</td>
<td><img src="image1.png" alt="Molecular Structure" /></td>
<td>-7.583</td>
</tr>
<tr>
<td>ZINC19838419</td>
<td><img src="image2.png" alt="Molecular Structure" /></td>
<td>-7.494</td>
</tr>
<tr>
<td>ZINC14967779</td>
<td><img src="image3.png" alt="Molecular Structure" /></td>
<td>-7.357</td>
</tr>
<tr>
<td>ZINC14967784</td>
<td><img src="image4.png" alt="Molecular Structure" /></td>
<td>-7.311</td>
</tr>
<tr>
<td>ID</td>
<td>Structure</td>
<td>Score</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
<td>-------</td>
</tr>
<tr>
<td>ZINC19113241</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>-7.303</td>
</tr>
<tr>
<td>ZINC23478413</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>-7.277</td>
</tr>
<tr>
<td>ZINC02075451</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>-6.495</td>
</tr>
</tbody>
</table>
Figure 19  Compounds obtained from HTVS round 2, for testing against β-alanyl aminopeptidase (P. aeruginosa growth inhibitory) activity
CHAPTER THREE

SYNTHESIS
3. Synthesis

As can be seen from the results of the high-throughput virtual screening (HTVS) (Scheme 1), some of the compounds with the best docking scores are sulfonamides. Therefore, a number of new sulfonamides, and their β-alanyl derivatives were synthesized according to their docking scores and the availability of suitable starting materials.

Scheme 1  Compounds with high docking score from HTVS
3.1 Results and Discussion

The sulfonamides 1a-g were synthesized from commercially available benzenesulfonyl chlorides 2, by a process involving the initial nucleophilic substitution with a mono-protected ethylenediamine 3, followed by deprotection of the resulting aminoethylsulfonamide 4 using trifluoroacetic acid (TFA) ([Scheme 2, Table 8]).

\[ \text{Scheme 2} \quad (i) \, \text{H}_2\text{NCH}_2\text{CH}_2\text{NHBOc 3, DCM, } \text{iPr}_2\text{NEt, 24 h, r.t.}; \,(ii) \, \text{TFA, DCM, 2 h, 0}^\circ\text{C} \]
Coupling of sulfonyl chlorides 2 with the mono-protected primary amine 3 (Scheme 2) can be considered to be a general method for the preparation of the sulfonamides 1a-g, via the Boc-protected aminoethylsulfonamides 4a-g. The protected intermediates were characterized by \(^1\)H and \(^{13}\)C NMR spectroscopy, with the 9 aliphatic protons of the Boc-protecting group observed at \(\delta 1.35-1.45\) and the protons of the aromatic group at \(\delta 7.2-8.4\). The subsequent disappearance of the methyl and quaternary carbons of the Boc group upon
treatment with TFA confirmed the removal of the protecting group, as did the loss of the carbamate carbonyl stretch, and the sulfonamides 1a-g were characterized by IR, $^1$H and $^{13}$C NMR spectroscopy, and high resolution mass spectrometry (HRMS). X-Ray diffraction structures were obtained for two of the sulfonamides, 1a and 1d (Figures 20 and 21). Table 9 and Table 10 show bond lengths for the crystal structures of 1a and 1d.

**Figure 20**  Single crystal X-ray structure of sulfonamide 1a
Table 9  Bond lengths for Compound 1a

<table>
<thead>
<tr>
<th>Atom ID</th>
<th>Bond length</th>
</tr>
</thead>
<tbody>
<tr>
<td>O(2)-S(1)</td>
<td>1.440</td>
</tr>
<tr>
<td>O(1)-S(1)</td>
<td>1.425</td>
</tr>
<tr>
<td>N(1)-C(7)</td>
<td>1.474</td>
</tr>
<tr>
<td>N(1)-S(1)</td>
<td>1.610</td>
</tr>
<tr>
<td>C(8)-N(2)</td>
<td>1.491</td>
</tr>
<tr>
<td>C(8)-C(7)</td>
<td>1.524</td>
</tr>
<tr>
<td>O(3)-C(10)</td>
<td>1.229</td>
</tr>
<tr>
<td>O(4)-C(10)</td>
<td>1.255</td>
</tr>
<tr>
<td>F(1)-C(9)</td>
<td>1.289</td>
</tr>
<tr>
<td>F(2)-C(9)</td>
<td>1.309</td>
</tr>
<tr>
<td>F(3)-C(9)</td>
<td>1.274</td>
</tr>
<tr>
<td>S(1)-C(4)</td>
<td>1.771</td>
</tr>
<tr>
<td>C(4)-C(3)</td>
<td>1.368</td>
</tr>
<tr>
<td>Bond</td>
<td>Length (Å)</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------</td>
</tr>
<tr>
<td>C(4)-C(5)</td>
<td>1.381</td>
</tr>
<tr>
<td>C(5)-C(6)</td>
<td>1.387</td>
</tr>
<tr>
<td>C(1)-C(2)</td>
<td>1.369</td>
</tr>
<tr>
<td>C(1)-C(6)</td>
<td>1.371</td>
</tr>
<tr>
<td>C(3)-C(2)</td>
<td>1.399</td>
</tr>
<tr>
<td>C(10)-C(9)</td>
<td>1.527</td>
</tr>
</tbody>
</table>

**Figure 21**  Single crystal X-ray structure of sulfonamide 1d
### Table 10  Bond lengths for Compound 1d

<table>
<thead>
<tr>
<th>Atom ID</th>
<th>Bond length</th>
</tr>
</thead>
<tbody>
<tr>
<td>O(6)-C(10)</td>
<td>1.241</td>
</tr>
<tr>
<td>O(5)-C(10)</td>
<td>1.241</td>
</tr>
<tr>
<td>F(2)-C(9)</td>
<td>1.319</td>
</tr>
<tr>
<td>F(1)-C(9)</td>
<td>1.304</td>
</tr>
<tr>
<td>F(3)-C(9)</td>
<td>1.311</td>
</tr>
<tr>
<td>S(1)-O(3)</td>
<td>1.431</td>
</tr>
<tr>
<td>S(1)-O(4)</td>
<td>1.436</td>
</tr>
<tr>
<td>S(1)-N(2)</td>
<td>1.608</td>
</tr>
<tr>
<td>S(1)-C(4)</td>
<td>1.777</td>
</tr>
<tr>
<td>N(2)-C(7)</td>
<td>1.472</td>
</tr>
<tr>
<td>O(2)-N(1)</td>
<td>1.222</td>
</tr>
<tr>
<td>O(1)-N(1)</td>
<td>1.225</td>
</tr>
<tr>
<td>N(3)-C(8)</td>
<td>1.488</td>
</tr>
<tr>
<td>N(1)-C(1)</td>
<td>1.474</td>
</tr>
</tbody>
</table>
3.1.1 Synthesis of 4-amino-N-(2-aminoethyl)phenylsulfonamide

The reduction of the 4-nitro protected sulfonamide 4d was attempted with stannous chloride in ethanol, refluxing for 24 hours at 80 °C, as shown in Scheme 3.
Unfortunately, the reduction of compound 4d, was not successful, therefore the acetamido analogue 5 was prepared according to Scheme 4, then deprotected to give acetamido analogue 6, which had an acetyl methyl signal at $\delta_H 2.10$ and $\delta_C 23.3$, and which was then hydrolysed to produce the desired amino-substituted aminoethylphenylsulfonamide 1g, with the expected loss of the acetyl signals.
Scheme 4  
(i) H₂NCH₂CH₂NHBoc 3, DCM, iPr₂NEt, 24 h, r.t.; (ii) TFA, DCM, 2 h, 0°C; (iii) 2M HCl, reflux, 24 h
The structure of compound 6 was confirmed by NMR spectroscopy; the $^1$H NMR spectrum showed a singlet peak at $\delta$ 2.10 for the acetyl methyl, while the $^{13}$C NMR spectrum showed peaks at $\delta$ 23.3 for the CH$_3$ and at $\delta$ 168 for CONH. The IR spectrum showed a CONH stretch at 1716 cm$^{-1}$. The anilinosulfonamide 1g was characterized by IR, $^1$H and $^{13}$C NMR spectroscopy, and HRMS.

3.1.2 Coupling of the most active sulfonamides with β-alanine

Derivatives 8a-g with β-alanine linked to these sulfonamides 1a-g were then prepared, in order to increase binding to the enzyme, increase selectivity (as β-alanyl aminopeptidase is specific to P. aeruginosa) and to enhance prokaryotic cellular uptake of β-alanine coupled sulfonamides. The coupling reaction, Scheme 4, involved the use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as the coupling agent as its by-product has high aqueous solubility, thus making work-up easier. Once again, deprotection gave the TFA salt of the free amine, Scheme 5. The role of EDC can be described as in Scheme 6.
Scheme 5  
Coupling of sulfonamides with β-alanine
Scheme 6  EDC coupling mechanism.

The structures of all the β-alanyl substituted aminopropylsulfonamides 8a-g were confirmed by NMR, IR, and HRMS. The $^1$H NMR spectra for intermediates 7a-g showed a singlet peak at approximately $\delta_H 1.30$ for the 'butyl protons, and peaks at $\delta 2.6-3$ for the four methylene protons (CH$_2$CH$_2$) of the β-alanyl group. The $^{13}$C NMR spectra for the protected compounds 7a-g showed a singlet at $\sim \delta 28$ for the 'butyl methyls, and at $\delta 155$ and $\delta 170$ for the amide and carbamate C=O respectively. The IR spectra for these compounds showed NH, CO and S=O stretches. The $^1$H NMR spectra for the deprotected β-alanyl substituted sulfonamides 8a-g did not show the singlet peak at $\delta 1.3$ for the 'butyl
protons, but did show signals for the four protons of CH₂CH₂ of the β-alanyl group. The $^{13}$C NMR spectra for the deprotected analogues 8a-g did not show the singlet peak at $\delta$ 28 for the $t$-butyl methyls and only showed one C=O peak (at $\delta$ 170).

### 3.1.3 Coupling of Ciprofloxacin with β-alanine

In order to investigate the potential for preparing specific antibacterial prodrugs for *P. aeruginosa* through the synthesis of β-alanylaminopeptidase substrates, β-alanylciprofloxacin was also synthesized, as shown in **Scheme 7**. Ciprofloxacin 9 was coupled with N-Boc-β-alanine in DMF, in the presence of EDC and HOBT, and the usual workup gave the product 10 in 91% yield. The $^1$H NMR spectrum for the protected analogue 10 once again showed a signal at $\delta$ 1.30 for the $t$-butyl protons, and the $^{13}$C NMR spectrum showed 6 methylene signals, including one at $\delta$ 8.6 for the cyclopropyl methylenes, and a signal at $\delta$ 28.6 for the $t$-butyl methyls. The electrospray ionization mass spectrum showed the MH$^+$ ion at $m/z$ 503, corresponding to the molecular formula of C$_{25}$H$_{31}$O$_6$N$_4$F. Treatment of N-Boc-β-alanylciprofloxacin 10 with trifluoroacetic acid, in DCM at 0 °C for 2 h, gave β-alanylciprofloxacin 11 in 95% yield. The $^1$H NMR spectrum for β-alanylciprofloxacin trifluoroacetate salt 11 showed two signals, at $\delta$ 1.15 and 1.28, for the cyclopropyl methylene groups, two signals, at $\delta$ 2.69 and 2.99, for the β-alanyl methylenes, and another 2 signals for the piperdinyl methylenes, at $\delta$ 3.33 and 3.61. The deprotection was confirmed by the absence of signals for the $N$-$t$-butyloxycarbonyl group (Boc) in both the $^1$H and $^{13}$C NMR spectra, while the IR spectrum showed C=O
absorbances at 1782 cm\(^{-1}\). The electrospray ionization mass spectrometry showed the MH\(^+\) ion at \(m/z\) 403 and HRMS confirmed the molecular formula of C\(_{20}\)H\(_{23}\)O\(_4\)N\(_4\)F.
Scheme 7  Synthesis of β-alanyl prodrug of Ciprofloxacin 11
3.2 Round two HTVS screening

Scheme 8 shows the new hit compounds which were obtained from the Chembridge screening. Compound 12 was ordered from SPECS and compound 13 was synthesized by the Gabriel synthesis, involving the initial reaction of theophylline with $N$-(3-bromopropyl)phthalimide, followed by deprotection of the resulting 14 with hydrazine, as shown in Scheme 9.

Scheme 8 Chembridge screening hits
Scheme 9  Gabriel synthesis of compound 13

3.3  Experimental section

3.3.1  Spectroscopic instrumentation

$^1$H NMR and $^{13}$C NMR spectra were obtained on a Varian 400MR spectrometer, with an SMS autosampler, which has pulsed field gradients and a PFG 5mm ATB broadband probe $^1$H/$^{19}$F/$^1$X. Mass spectra were determined on Thermo Scientific TSQ Quantum Access Max LCMS/MS. HRMS were determined on a Bruker 7T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR) in the Mass Spectrometry Facility at the School of Chemistry, University of Sydney. IR spectra were recorded on a SHIMADZU FTIR-8400S spectrometer. Melting points were measured on a Stuart melting point apparatus.
3.3.2 Preparation of Sulfonamides

3.3.2.1 Preparation of tert-butyl (2-phenylsulfonamido)ethylcarbamate 4a

Benzenesulfonyl chloride (0.48 ml, 3.75 mmol) was added to a mixture of N-Boc-ethylenediamine (0.49 ml, 3.1 mmol) and diisopropylethylamine (0.65 ml, 4.7 mmol) in DCM (5 ml) and the mixture was stirred for 24 hours at room temperature, under nitrogen. When the reaction was complete (as indicated by TLC), the reaction mixture was diluted with DCM (10 ml) and washed with water (2 × 20 ml), then brine (2 × 25 ml). The combined organic layers were dried with anhydrous Na₂SO₄, filtered, and the solvent evaporated under reduced pressure to give tert-butyl (2-phenylsulfonamido)ethylcarbamate 4a, as a white solid (565 mg, 85 %); mp 110-115 °C; IR (nujol) νmax /cm⁻¹ 3363 (NH), 3286 (NH), 1725 (C=O), 1687 (CONH), 1456 (Ar-C), 1340 (S=O), 1161(C-N), 750 (Ar-H); ¹H NMR (400MHz, CDCl₃): δ (ppm) 1.42 (9H, s, C(CH₃)₃), 3.05 (2H, q, J = 5.6 Hz, NHCH₂CH₂), 3.23 (2H, q, J = 5.6 Hz, CH₂CH₂NH), 4.87 (1H, s, NH), 5.32 (1H, s, NH), 7.49-7.60 (3H, m, ArH), 7.86 (2H, m, ArH); ¹³C NMR (CDCl₃): δ (ppm) 28.3 (3 × CH₃),
40.2 (CH₂), 43.7 (CH₂), 76.8 (quat., CMe₃), 126.9 (2 × CH), 129.1 (2 × CH), 132.6 (CH), 139.7 (C-SO₂); ESIMS m/z 324 (21 %), 323 (MNa⁺, 100), 267 (14), 201 (9).

3.3.2.2 Preparation of N-(2-aminoethyl)benzenesulfonamide trifluoroacetate salt 1a

tert-Butyl (2-phenylsulfonamido)ethylcarbamate 4a (195 mg, 0.65 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution. The reaction mixture was stirred in an ice bath for 2 hours, until all the starting material was consumed, and the excess TFA was evaporated to yield N-(2-aminoethyl)benzenesulfonamide trifluoroacetate salt 1a, as white crystals (160 mg, 82 %); mp 152-156 °C (Found: MH⁺, 201.0693. Calc. for C₈H₁₃N₂O₂S: MH, 201.0692); IR (nujol) νmax/cm⁻¹ 3163 (NH), 1377 (CH₂), 1348 (S=O), 1089 (C-N), 1456 (Ar-C), 721 (Ar-H); ¹H NMR (400MHz, d₆-DMSO): δ (ppm) 2.83 (2H, t, J = 6.4 Hz, NHC₂H₂NH), 2.90 (2H, t, J = 6.4 Hz, NHCH₂CH₂NH), 7.64 (3H, m, ArH), 7.79 (2H, m, ArH); ¹³C NMR (d₆-DMSO): δ (ppm) 31.1 (CH₂), 39.8 (CH₂), 117.9 (CF₃), 127.6 (2 × CH), 129.8 (2 × CH), 133.2 (CH), 139.8 (C-SO₂), 161.8 (C=O); ESIMS m/z 223 (MNa⁺, 13 %), 202 (16), 201 (MH⁺, 100), 184, (16).
3.3.2.3 Preparation of tert-butyl \{2-(4-methylphenyl)-sulfonamido\}ethylcarbamate 4b

\[
\begin{align*}
\text{O} & \quad \text{S} \\
\text{N} & \quad \text{N} \\
\text{CMe}_3 & \quad \text{O}
\end{align*}
\]

\(N\)-Boc-ethylenediamine (0.49 ml, 3.12 mmol) was added to a mixture of 4-methylbenzenesulfonyl chloride (714 mg, 3.75 mmol) and diisopropylethylamine (0.65 ml, 4.7 mmol) in DCM (5 ml) and the mixture was stirred for 24 hours at room temperature, under nitrogen. When the reaction was complete (as indicated by TLC), the reaction mixture was diluted with DCM (10 ml) and washed with water (2 × 20 ml), then brine (2 × 25 ml). The combined organic layers were dried over anhydrous \(\text{Na}_2\text{SO}_4\), filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica, eluting with ethyl acetate-hexane (0:100 to 50:50), to yield tert-butyl \{2-(4-methylphenyl)sulfonamido\}ethylcarbamate 4b, as a white solid (505 mg, 71 %); mp 137-139 °C; IR (nujol) \(\nu_{\text{max}} /\text{cm}^{-1}\) 3371 (NH), 3286 (NH), 1695 (CO), 1458 (Ar-C), 1377 (CH\(_2\)), 1335 (S=O), 1157 (C-O); \(^1\text{H NMR (400MHz, CDCl}_3\)): \(\delta \) (ppm) 1.42 (9H, s, C(CH\(_3\))^3), 2.42 (3H, s, CH\(_3\)), 3.05 (2H, q, \(J = 5.8\) Hz, NHCH\(_2\)CH\(_2\)NH), 3.20 (2H, q, \(J = 5.8\) Hz, NHCH\(_2\)CH\(_2\)NH), 4.86 (1H, s, NH), 5.10 (1H, s, NH), 7.29 (2H, d, \(J = 8.4\) Hz, ArH), 7.73 (2H, d, \(J = 8.4\) Hz, ArH); \(^{13}\text{C NMR (CDCl}_3\)): \(\delta \) (ppm) 21.5 (CH\(_3\)), 28.3 (3 × CH\(_3\)), 40.2
3.3.2.4 Preparation of N-(2-aminoethyl)-4-methylphenylsulfonamide trifluoroacetate salt 1b

\[
\text{SO} \quad \text{NH} \quad \text{NH}_{3} \\
\text{CF}_{3}\text{COO} \\
\text{Me}
\]

*tert*-Butyl {2-(4-methylphenyl)sulfonamido}ethylcarbamate 4b (200 mg, 0.64 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution. The reaction mixture was stirred in an ice bath for 2 hours, until the reaction was complete, and the excess TFA was allowed to evaporate to yield white crystals of N-(2-aminoethyl)-4-methylbenzenesulfonamide trifluoroacetate salt 1b (190 mg, 95 %); mp 150-154 °C (Found: MH\(^+\), 215.0849. Calc. for C\(_9\)H\(_{14}\)N\(_2\)O\(_2\)S: MH, 215.0848); IR (nujol) \(\nu_{\max}/\text{cm}^{-1}\) 3198 (NH), 3177 (NH), 1460 (C=C), 1377 (CH\(_2\)), 1333 (S=O), 748 (Ar-H); \(^1\)H NMR (400MHz, \(d_6\)-DMSO): \(\delta\) (ppm) 2.37 (3H, s, CH\(_3\)), 2.83 (2H, q, \(J = 5.2\) Hz, NHCH\(_2\)CH\(_2\)NH), 2.89 (2H, q, \(J = 5.2\) Hz, NHCH\(_2\)CH\(_2\)NH), 7.41 (2H, d, \(J = 8.8\) Hz, ArH), 7.68 (2H, d, \(J = 8.8\) Hz, ArH); \(^{13}\)C NMR (\(d_6\)-DMSO): \(\delta\) (ppm) 21.4 (CH\(_3\)), 39.2 (CH\(_2\)), 39.8 (CH\(_2\)), 118.1 (CF\(_3\)), 127.8 (2 \(\times\) CH), 130.1 (2 \(\times\) CH), 137.8 (quat.), 143.5 (quat.), 161.8 (C=O); ESIMS \(m/z\) 237 (MNa\(^+\), 19 %), 215 (MH\(^+\), 100), 198 (14).
3.3.2.5  Preparation of tert-butyl \{2-(4-chlorophenylsulfonamido)-ethyl\}carbamate 4c

\[
\begin{align*}
\text{Cl} & \quad \text{SO} & \quad \text{NH} & \quad \text{O} & \quad \text{CH}_3 \\
\text{NH} & \quad \text{O} & \quad \text{S} \end{align*}
\]

4-Chlorobenzenesulfonyl chloride (553 mg, 2.62 mmol) was added to a mixture of N-Boc-ethylenediamine (0.35 ml, 2.18 mmol) and diisopropylethylamine (0.55 ml, 3.27 mmol) in DCM (5 ml), and the mixture was stirred for 24 hours at room temperature, under nitrogen. When the reaction was complete (as indicated by TLC), the reaction mixture was diluted with DCM (10 ml) and washed with water (2 × 20 ml), then brine (2 × 25 ml). The combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and the solvent evaporated under pressure to give tert-butyl \{2-(4-chlorophenylsulfonamido)ethyl\}carbamate 4c as a white solid (450 mg, 81 %); mp 129-132 °C; IR (nujol) \(v_{\text{max}}/\text{cm}^{-1}\) 3396 (NH), 3276 (NH), 1683 (CO), 1458 (Ar-C), 1377 (CH$_2$), 1345 (S=O), 1153 (C-O); $^1$H NMR (400MHz, CDCl$_3$): $\delta$ (ppm) 1.42 (9H, s, C(CH$_3$)$_3$), 3.05 (2H, q, \(J = 5.6\) Hz, NHCH$_2$CH$_2$NH), 3.22 (2H, q, \(J = 5.6\) Hz, NHCH$_2$CH$_2$NH), 4.88 (1H, s, NH), 5.45 (1H, s, NH), 7.29 (2H, d, \(J = 9.0\) Hz, ArH), 7.74 (2H, d, \(J = 9.0\) Hz, ArH); $^{13}$C NMR (CDCl$_3$): $\delta$ (ppm) 28.3 (CH$_3$)$_3$, 40.2 (CH$_2$), 43.9 (CH$_2$), 80.1 (quat.,CMe$_3$), 128.4 (2 × CH), 129.4 (2 × CH), 138.3 (quat.), 139.1 (quat.), 156.6 (C=O); ESIMS \(m/z\) 375 (18), 359 (39 %), 357 (MNa$^+$, 100), 300 (10).
3.3.2.6 Preparation of N-(2-aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate salt 1c

![Chemical Structure]

tert-Butyl (2-(4-chlorophenylsulfamido)ethyl)carbamate 4c (155 mg, 0.46 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution. The reaction mixture was stirred in ice for 2 hours until the reaction was complete (as indicated by TLC), and the excess TFA was evaporated to give white crystals of N-(2-aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate 1c (140 mg, 90 %); mp 151-156 °C (Found: MH⁺, 235.0303. Calc. for C₈H₁₁ClN₂O₂S: MH, 235.0302); IR (nujol) νmax/cm⁻¹ 3087 (NH), 1460 (Ar-C), 1341 (S=O); ¹H NMR (400MHz, d₆-DMSO): δ (ppm) 2.82 (2H, t, J = 6.0 Hz, NHCH₂CH₂NH), 2.92 (2H, t, J = 6.0 Hz, NHCH₂CH₂NH), 7.70 (2H, d, J = 8.8 Hz, ArH), 7.79 (2H, d, J = 8.8 Hz, ArH); ¹³C NMR (d₆-DMSO): δ (ppm) 39.8 (CH₂), 118.2 (CF₃), 128.8 (2 × CH), 129.9 (2 × CH), 138.1 (quat.), 138.8 (quat.), 161.9 (C=O); ESIMS m/z 257 (MNa⁺, 8 %), 237 (52), 235 (MH⁺, 100), 218 (8).
3.3.2.7 Preparation of tert-butyl \{2-(4-nitrophenyl)-sulfonamido\}ethylcarbamate 4d

4-Nitrophenylsulfonyl chloride (833 mg, 3.75 mmol) was added to a mixture of N-Boc-ethylenediamine (0.49 ml, 3.12 mmol) and diisopropylethylamine (0.65 ml, 4.68 mmol) in DCM (5 ml), and the mixture was stirred for 24 hours at room temperature, under nitrogen. When the reaction was complete (as indicated by TLC), the reaction mixture was diluted with DCM (10 ml) and washed with water (2 × 20 ml), then brine (2 × 25 ml). The organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and evaporated under reduced pressure. The organic extract was purified by column chromatography on silica, eluting with ethyl acetate-hexane (0:100 to 50:50), to yield tert-butyl \{2-(4-nitrophenyl)-sulfonamido\}ethylcarbamate 4d as a yellow solid (687 mg, 82.5 %); mp 158-163 °C; IR (nujol) $\nu_{\text{max}}$/cm$^{-1}$ 3375 (NH), 3276 (NH), 1693 (CO), 1458 (Ar-C), 1338 (S=O); $^1$H NMR (400MHz, CD$_3$COCD$_3$): $\delta$ (ppm) 1.37 (9H, s, C(CH$_3$)$_3$), 3.10 (2H, t, $J = 6.0$ Hz, NHCH$_2$CH$_2$NH), 3.17 (2H, q, $J = 6.0$ Hz, NHCH$_2$CH$_2$NH), 6.07 (1H, s, NH), 6.95 (1H, s, NH), 8.13 (2H, d, $J = 9.0$ Hz, ArH), 8.44 (2H, d, $J = 9.0$ Hz, ArH); $^{13}$C NMR (CD$_3$COCD$_3$): $\delta$ (ppm) 27.6 (CH$_3$)$_3$, 40.1 (CH$_2$), 43.1 (CH$_2$), 78.1 (quat., CMe$_3$), 124.3 (2 ×
CH), 128.3 (2 × CH), 146.3 (quat.), 150.1 (quat.), 155.9 (C=O); ESIMS m/z 369 (18 %), 368 (MNa\(^+\), 100), 311 (13), 246 (3).

### 3.3.2.8 Preparation of N-(2-aminoethyl)-4-nitrophenylsulfonamide trifluoroacetate salt 1d

![Chemical Structure](image-url)

tert-Butyl [2-(4-nitrophenyl)-sulfonamido]ethylcarbamate 4d (120 mg, 0.35 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution. The reaction mixture was stirred in ice for 2 hours until the reaction was complete, the mixture was left to allow the excess of TFA to evaporate to give white crystals of N-(2-aminoethyl)-4-nitrophenylsulfonamide trifluoroacetate 1d (100 mg, 83 %); mp 158-162 °C (Found: MH\(^+\), 246.0544. Calc. for C\(_8\)H\(_{11}\)N\(_3\)O\(_4\)S: MH, 246.0543); IR (nujol) ν\(_{\text{max}}\)/cm\(^{-1}\) 3087 (NH), 1460 (Ar-C), 1340 (S=O); \(^1\)H NMR (400MHz, \(d_6\)-DMSO): δ (ppm) 2.85 (2H, broad s, NHCH\(_2\)CH\(_2\)NH\(_3\)), 2.98 (2H, q, J = 6.4 Hz, NHCH\(_2\)CH\(_2\)NH), 8.04 (2H, d, J = 9.2 Hz, ArH), 8.30 (1H, t, J = 6.4 Hz, NH), 8.43 (2H, d, J = 9.2 Hz, ArH); \(^13\)C NMR (\(d_6\)-DMSO): δ (ppm) 39.8 (CH\(_2\)), 117.9 (CF\(_3\)), 125.1 (2 × CH), 128.8 (2 × CH), 145.2 (quat.), 150.1 (quat.), 161.8 (C=O); ESIMS m/z 247 (19 %), 246 (MH\(^+\), 100), 229 (7).
3.3.2.9 Preparation of tert-butyl \{2-(2-nitrophenyl)-sulfonamido\}ethylcarbamate 4e

2-Nitrophenylsulfonyl chloride (1.25 g, 5.62 mmol) was added to a mixture of N-Boc-ethylenediamine (0.74 ml, 4.68 mmol) and diisopropylethylamine (1.23 ml, 7 mmol) in DCM (10 ml), and the mixture was stirred for 24 hours at room temperature, under nitrogen. When the reaction was complete (as indicated by TLC), the reaction mixture was diluted with DCM (20 ml) and washed with water (2 × 25 ml), then brine (2 × 30 ml) before being dried with anhydrous Na$_2$SO$_4$. The organic extract was purified by column chromatography on silica, eluting with ethyl acetate-hexane (0:100 to 50:50), to yield tert-butyl \{2-(2-nitrophenyl)sulfonamido\}ethylcarbamate 4e as a brown liquid (990 mg, 79 %); IR (nujol) $\nu_{\text{max}}$ /cm$^{-1}$ 3372 (NH), 1688 (CO), 1458 (Ar-C), 1377 (CH$_2$), 1336 (S=O); $^1$H NMR (400MHz, CDCl$_3$): $\delta$ (ppm) 1.42 (9H, s, C(CH$_3$)$_3$), 3.22 (2H, broad s, NHCH$_2$CH$_2$NH), 3.28 (2H, $J = 5.3$ Hz, NHCH$_2$CH$_2$NH), 4.87 (1H, s, NH), 5.74 (1H, s, NH) 7.74 (2H, m, ArH), 7.86 (1H, m, ArH), 8.12 (1H, m, ArH); $^{13}$C NMR (CDCl$_3$): $\delta$ (ppm) 14.1 (CH$_3$)$_3$, 40.3 (CH$_2$), 43.8 (CH$_2$), 60.4 (quat.), 125.4 (CH), 130.9 (CH), 132.2
(CH), 133.6 (CH), 135.3 (quat.), 148.1 (quat.), 156.1 (C=O); ESIMS m/z 369 (17 %), 368 (MNa⁺, 100), 312 (9), 246 (11).

3.3.2.10 Preparation of \( N\)-(2-aminoethyl)-2-nitrophenylsulfonamide trifluoroacetate salt 1e

\[
\text{\begin{center}
\begin{array}{c}
\text{\large SO} \\
\text{\large NH} \\
\text{\large CH}_2\text{NH} \\
\text{\large CF}_3\text{COO}
\end{array}
\end{center}
\]

\( \text{tert-Butyl \{2-(2-nitrophenyl)sulfonamido\}ethylcarbamate 4d, (500 mg, 1.45 mmol) was dissolved in DCM (5 ml) and TFA (0.75ml) was added to the solution. The reaction mixture was stirred in ice for 2 hours until the reaction was complete, the mixture was left to allow the excess of TFA to evaporate to give a brown solid of \( N\)-(2-aminoethyl)-2-nitrophenylsulfonamide trifluoroacetate 1e (400 mg, 80 %); mp 159-163 °C (Found: MH⁺, 246.0543. Calc. for C₈H₁₁N₃O₄S: MH, 246.0543); IR (nujol) \( \nu_{\text{max}}/\text{cm}^{-1} \) 3087 (NH), 1460 (Ar-C), 1377 (CH₂), 1343 (S=O), 1166 (S=O); \(^1\text{H NMR} (400\text{MHz, } d_6\text{-DMSO}): \delta \text{ (ppm)} 2.88 \text{ (2H, t, } J = 6.4 \text{ Hz, NHCH}_2\text{CH}_2\text{NH}), 3.09 \text{ (2H, t, } J = 6.4 \text{ Hz, NHCH}_2\text{CH}_2\text{NH}), 7.89 \text{ (2H, m, ArH), 8.00 \text{ (2H, m, ArH)}; } \text{\(^{13}\text{C NMR} (d_6\text{-DMSO): } \delta \text{ (ppm)} 39.7 \text{ (CH}_2\text{), 117.8 \text{ (CF}_3\text{), 125.2 \text{ (2 } \times \text{ CH), 128.8 \text{ (2 } \times \text{ CH), 145.3 \text{ (quat.)}, 150.1 \text{ (quat.), 161.8 \text{ (C=O}); ESIMS m/z 430 (63), 247 (13 %), 246 (MH}^+\text{, 100), 186 (7).}
\end{array}
\end{center}
\]
3.3.2.11 Preparation of **tert-butyl** \{2-(4-acetamidophenyl)sulfonamide\}ethylcarbamate 4f

4-Acetamidophenylsulfonyl chloride (730 mg, 3.12 mmol) was added to a mixture of N-Boc-ethylenediamine (0.5 ml, 3.12 mmol) and diisopropylethylamine (0.65 ml, 3.75 mmol) in DCM (10 ml) and the mixture was stirred for 24 hours at room temperature, under nitrogen. When the reaction was complete, the reaction mixture was diluted with DCM (15 ml) and washed with water (2 × 25 ml), then brine (2 × 25 ml), then dried using anhydrous Na$_2$SO$_4$ to yield tert-butyl \{2-(4-acetamidophenyl)sulfonamido\}ethylcarbamate 4f as a yellowish-white solid (700 mg, 95 %); mp 152-155 °C; IR (nujol) $\nu_{\text{max}}$/cm$^{-1}$ 3365 (NH), 3280 (NH), 3197 (NH), 1683 (CONH), 1645 (C=O), 1461 (Ar-C), 1377 (CH$_2$), 1340 (S=O), 1153 (C-O); $^1$H NMR (400MHz, CD$_3$COCD$_3$): $\delta$ (ppm) 1.37 (9H, s, C(CH$_3$)$_3$), 2.12 (3H, s, CH$_3$), 2.75 (1H, s, NH), 2.85 (2H, q, $J = 6.4$ Hz, NHCH$_2$CH$_2$NH), 3.03 (2H, q, $J = 6.4$ Hz, NHCH$_2$CH$_2$NH), 7.65 (2H, d, $J = 9.0$ Hz, ArH), 7.71 (2H, d, $J = 9.0$ Hz, ArH), 9.45 (1H, s, NH); $^{13}$C NMR (CD$_3$COCD$_3$): $\delta$ (ppm) 23.4 (CH$_3$), 27.6 (CH$_3$)$_3$, 40.7 (CH$_2$), 43.6 (CH$_2$), 78.8 (C-O), 118.9 (2 × CH), 127.8 (2 × CH), 134.7 (quat.), 143.4 (quat.), 156.5 (quat.), 169.1 (quat.); ESIMS $m/z$ 381 (19 %), 380 (MNa$^+$, 100), 258 (19), 130 (14).
3.3.2.12 Preparation of \( N\{-4\{-N\{-2\-

\text{aminoethlysulfamoyl}\text{phenyl}\}\text{acetamide trifluoroacetate salt 1f} \)

\[
\begin{align*}
\text{Me} & \quad \text{CONH} \\
\text{N} & \quad \text{SO} \\
\text{O} & \quad \text{NH} \\
\text{CF}_3\text{COO} & \quad \text{NH}_3
\end{align*}
\]

tert-Butyl \{2-(4-acetamidophenyl)sulfonamide\}ethyl carbamate 4f (250 mg, 0.70 mmol) was dissolved in DCM (5 ml) and TFA (0.75 ml) was added to the solution. The reaction mixture was stirred in ice for 2 hours until the reaction was complete, the mixture was left to allow the excess of TFA to evaporate to give a white solid of \( N\{-4\{-N\{-2\-

\text{aminoethlysulfamoyl}\text{phenyl}\}\text{acetamide trifluoroacetate salt 1f} \)

(240 mg, 96 %); mp 86-93 °C; IR (nujol) \( \nu_{\text{max}}/\text{cm}^{-1} \) 3244 (NH), 3176 (NH), 3112 (NH), 1716 (CONH), 1642 (C=O), 1463 (Ar-C), 1377 (CH\(_2\)), 1341 (S=O), 723 (Ar-H); \(^1\text{H NMR} \) (400MHz, CD\(_3\)COCD\(_3\)): \( \delta \) (ppm) 2.10 (3H, s, CH\(_3\)), 3.31 (2H, \text{t}, \text{J} = 5.6 \text{ Hz}, \text{NHCH\(_2\)CH\(_2\)NH}), 3.89 (2H, \text{t}, \text{J} = 5.6 \text{ Hz}, \text{NHCH\(_2\)CH\(_2\)NH}), 7.77 (2H, \text{d}, \text{J} = 8.8 \text{ Hz}, \text{ArH}), 7.81 (2H, \text{d}, \text{J} = 8.8 \text{ Hz}, \text{ArH}); \(^{13}\text{C NMR} \) (CD\(_3\)COCD\(_3\)): \( \delta \) (ppm) 23.3 (CH\(_3\)), 40.4 (CH\(_2\)), 48.3 (CH\(_2\)), 117.8 (CF\(_3\)), 118.2 (2 \times \text{CH}), 128.1 (2 \times \text{CH}), 134.5 (quat.), 143.1 (quat.), 162.5 (C=O), 168.8 (CONH\(_2\)), ESIMS \( m/z \) 280 M\(\text{Na}^+\) (10), 259 (22 %), 258 (MH\(^+\), 100), 216 (9).
3.3.2.13 Preparation of 4-amino-\(N\)-(2-aminoethyl)benzenesulfonamide \(1g\)

Aq. hydrochloric acid (2M, 5 ml) was added to \(N\)-(4-(\(N\)-(2-aminoethyl)sulfamoyl)phenyl)acetamide \(1f\) (200 mg, 0.78 mmol) and the solution was allowed to reflux for 24 hours. When the reaction was complete, the pH of the solution was adjusted to 10, then it was extracted with EtOAc (2 × 25 ml), followed by brine (2 × 25 ml), and dried over anhydrous Na\(_2\)SO\(_4\) to yield 4-amino-\(N\)-(2-aminoethyl)benzenesulfonamide \(1g\), as a pure brown solid (70 mg, 35 %); mp 101-105 °C (Found: M\(^+\), 216.0803 Calc. for C\(_8\)H\(_{13}\)N\(_3\)O\(_2\)S: M\(^+\), 216.0801); IR (nujol) \(\nu\)\(_{\text{max}}\)/cm\(^{-1}\) 3175 (NH), 1460 (Ar-C), 1377 (CH\(_2\)), 1345 (S=O); \(^1\)H NMR (400MHz, CD\(_3\)COCD\(_3\)): \(\delta\) (ppm) 3.02 (2H, q, \(J = 5.6\) Hz, NHC\(_2\)CH\(_2\)NH), 3.19 (2H, t, \(J = 5.6\) Hz, NHCH\(_2\)C\(_2\)NH), 5.41 (1H, s, NH), 5.94 (1H, s, NH), 6.72 (2H, d, \(J = 8.6\) Hz, ArH), 7.51 (2H, d, \(J = 8.6\) Hz, ArH); \(^{13}\)C NMR (CD\(_3\)COCD\(_3\)): \(\delta\) (ppm) 43.8 (CH\(_2\)), 50.2 (CH\(_2\)), 113.0 (2 × CH), 128.7 (2 × CH), 128.9 (quat.), 152.3 (quat.); ESIMS \(m/z\) 238 MNa\(^+\) (47), 217 (11 %), 216 (MH\(^+\), 100), 156 (7).
3.3.3 Coupling of sulfonamides with β-alanine

3.3.3.1 Synthesis of tert-butyl [3-oxo-3-{2-(phenylsulfonamido)ethyl}amino]propylcarbamate 7a

\[ \text{N-(2-Aminoethyl)benzenesulfonamide 1a (160 mg, 0.81 mmol), and t-Boc-β-alanine (153.5 mg, 0.81 mmol) were dissolved in DMF (5 ml), then EDC (155.5 mg, 0.81 mmol), HOBT (109.5 mg, 0.81 mmol), and DIPEA (1 ml, 5.6 mmol) were added to the mixture. The clear solution was stirred at room temperature for 24 h and after the reaction was complete, the DMF was removed in vacuo, then the residue was partitioned between EtOAc (200 ml) and water (200 ml). The separated organic layer was washed with 5% aq. NaHCO}_3 (2 × 20 ml), 0.5 M citric acid (2 × 10 ml), then brine (2 × 25 ml). The organic layer was dried over \( \text{Na}_2\text{SO}_4 \) and evaporated to give tert-butyl [3-oxo-3-{(2-phenylsulfonamido)ethyl}amino]propylcarbamate 7a (150 mg, 93 %) as a yellowish-white liquid; IR (nujol) \( \nu_{\text{max}}/\text{cm}^{-1} \): 3341 (NH), 3178 (NH), 1728 (C=O), 1650 (C=O), 1460 (Ar-C), 1377 (CH$_2$), 1342 (S=O), 1156 (C-O); $^1$H NMR (400MHz, d$_6$-DMSO): 1.30 (9H, s, C(CH$_3$)$_3$), 2.69 (2H, q, \( J = 6.0 \text{ Hz} \), NHCH$_2$CH$_2$NH), 2.90 (2H, q, \( J = 6.0 \text{ Hz} \), NHCH$_2$CH$_2$NH), 3.28-3.32 (4H, m broad), 6.74 (1H, t, \( J = 6.0 \text{ Hz} \), NH), 7.60 (3H, m, ...)
ArH), 7.74 (2H, dt, $J = 6.8, 1.6$ Hz, ArH); $^{13}$C NMR ($d_{6}$-DMSO): $\delta$ (ppm) 28.5 (CH$_3$)$_3$, 31.1 (CH$_2$), 38.1 (CH$_2$), 39.5 (CH$_2$), 42.6 (CH$_2$), 78.2 (C=O), 126.8 (2 × CH), 128.8 (2 × CH), 130.3 (CH), 140.7 (quat.), 155.8 (quat.), 165.1 (quat.); ESIMS $m/z$ 395 (18 %), 394 (MNa$^+$, 100), 329 (15), 291 (11).

3.3.3.2 Preparation of 3-amino-N-{2-(phenylsulfonamido)ethyl}propanamide trifluoroacetate 8a

3-amino-N-{2-(phenylsulfonamido)ethyl}propanamide trifluoroacetate 8a (100 mg, 77 %) as a brown solid; mp 124-128 °C; (Found: MH$^+$, 272.0990. Calc. for C$_{11}$H$_{17}$N$_3$O$_3$S: MH, 272.0988); IR (nujol) $\nu$max/cm$^{-1}$ 3284 (NH), 3151 (NH), 1670 (CONH), 1458 (Ar-C), 1377 (CH$_2$),1338 (S=O), 1166 (S=O), 721 (Ar-H); $^1$H NMR (400MHz, $d_6$-DMSO): 2.36 (2H, t, $J = 6.4$ Hz, COCH$_2$CH$_2$), 2.74 (2H, q, $J = 6.4$ Hz, COCH$_2$CH$_2$NH$_3$), 2.91 (2H, q, $J = 6.34$ Hz, NHCH$_2$CH$_2$NH), 3.05 (2H, q, $J = 6.4$ Hz, NHCH$_2$CH$_2$NH), 7.57 (3H, m, ArH), 7.75 (2H, tert-Butyl [3-oxo-3-{(2-phenylsulfonamido)ethyl}amino]carbamate 7a (130 mg, 0.35 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution. The reaction mixture was stirred in ice bath for 2 hours until the reaction was complete, then the mixture was left to allow the excess of TFA to evaporate to give 3-amino-N-{2-(phenylsulfonamido)ethyl}propanamide trifluoroacetate 8a (100 mg, 77 %) as a brown solid; mp 124-128 °C; (Found: MH$^+$, 272.0990. Calc. for C$_{11}$H$_{17}$N$_3$O$_3$S: MH, 272.0988); IR (nujol) $\nu$max/cm$^{-1}$ 3284 (NH), 3151 (NH), 1670 (CONH), 1458 (Ar-C), 1377 (CH$_2$),1338 (S=O), 1166 (S=O), 721 (Ar-H); $^1$H NMR (400MHz, $d_6$-DMSO): 2.36 (2H, t, $J = 6.4$ Hz, COCH$_2$CH$_2$), 2.74 (2H, q, $J = 6.4$ Hz, COCH$_2$CH$_2$NH$_3$), 2.91 (2H, q, $J = 6.34$ Hz, NHCH$_2$CH$_2$NH), 3.05 (2H, q, $J = 6.4$ Hz, NHCH$_2$CH$_2$NH), 7.57 (3H, m, ArH), 7.75 (2H,
dt, $J = 6.8, 1.6$ Hz, ArH), $8.09$ (1H, $t, J = 6.4$ Hz, NH); $^{13}$C NMR ($d_6$-DMSO): $\delta$ (ppm) 32.4 (CH$_2$), 35.6 (CH$_2$), 38.9 (CH$_2$), 42.2 (CH$_2$), 118.2 (CF$_3$), 126.8 (2 × CH), 129.6 (2 × CH), 132.9 (CH), 140.7 (quat.), 158.5 (quat.), 161.8 (C=O); ESIMS $m/z$ 294 (MNa$^+$, 13), 272 (MH$^+$, 100), 201 (18).

3.3.3.3 Synthesis of tert-butyl [3-{2-(4-methylphenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7b

![Structural formula of tert-butyl [3-{2-(4-methylphenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7b]

$N$-(2-Aminoethyl)-4-methylphenylsulfonamide trifluoroacetate 1b (225 mg, 1 mmol), and $t$-Boc-$\beta$-alanine (198 mg, 1 mmol) were dissolved in DMF (5 ml), then EDC (201 mg, 1 mmol), HOBt (142 mg, 1 mmol), and DIPEA (1.5 ml, 8.5 mmol) were added to the mixture. The clear solution was stirred at room temperature for 24 hours, and after the reaction was complete the solvent was removed under vacuum, the residue was partitioned between EtOAc (200 ml) and water (200 ml). The organic layer was washed with 5% aq. NaHCO$_3$ (2 × 20 ml), 0.5 M citric acid (2 × 10 ml), then brine (2 × 25 ml), then dried over Na$_2$SO$_4$ to give tert-butyl [3-{2-(4-methylphenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7b (200 mg, 88 %) as a white solid; mp 123-125 °C; IR (nujol)
ν\textsubscript{max}\textpercm 3334 (NH), 3274 (NH), 3178 (NH), 1635 (CONH), 1340 (S=O), 1458 (Ar-C), 1377 (CH\textsubscript{2}), 1157 (C-O), 721 (Ar-H); \textsuperscript{1}H NMR (400MHz, d\textsubscript{6}-DMSO): 1.31 (9H, s, C(CH\textsubscript{3})\textsubscript{3}), 2.12 (2H, t, J = 7.4 Hz, CH\textsubscript{2}CO), 2.35 (3H, s, CH\textsubscript{3}), 2.69 (2H, q, J = 6.4 Hz, CH\textsubscript{2}), 3.01 (4H, m, CH\textsubscript{2}CH\textsubscript{2}), 6.68 (1H, t, J = 5.4 Hz, NH) 7.35 (2H, d, J = 8.0 Hz, Ar-H), 7.54 (1H, t, J = 6.0 Hz, NH), 7.62 (2H, d, J = 8.0 Ar-H), 7.81 (1H, t, J = 5.6 Hz, NH); \textsuperscript{13}C NMR (d\textsubscript{6}-DMSO): δ (ppm) 21.4 (CH\textsubscript{3}), 28.65 (CH\textsubscript{3})\textsubscript{3}, 36.15 (CH\textsubscript{2}), 37.0 (CH\textsubscript{2}), 38.8 (CH\textsubscript{2}), 42.3 (CH\textsubscript{2}), 77.8 (quat.), 126.9 (2 × CH), 130.0 (2 × CH), 137.8 (quat.), 143.3 (quat.), 155.8 (C=O), 170.8 (C=O); ESIMS m/z 408 (MNa\textsuperscript{+}, 100), 386 (MH\textsuperscript{+}, 40 %), 330 (15), 286 (37).

### 3.3.3.4 Preparation of 3-amino-N-[2-(4-methylphenylsulfonamido)ethyl]propanamide trifluoroacetate 8b

\textit{tert-Butyl [3-{2-(4-methylphenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7b} (165 mg, 0.43 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution. The reaction mixture was stirred in ice bath for 2 hours until the reaction was complete, and the mixture was left to allow the excess of TFA to evaporate to give 3-amino-N-[2-(4-methylphenylsulfonamido)ethyl]propanamide trifluoroacetate 8b (100 mg,
60 %) as a brown liquid; (Found: MH⁺, 286.1221. Calc. for C₁₂H₁₉N₃O₃S: MH, 286.1219); IR (nujol) νₑₓₑ/cm⁻¹ 3290 (NH), 1725 (C=O), 1461 (Ar-C), 1377 (CH₂), 1335 (S=O), 721 (Ar-H); ¹H NMR (400MHz, d₆-DMSO): 2.30 (3H, s, CH₃), 2.35 (2H, t, J = 6.8 Hz, NHCH₂CH₂NH), 2.71 (2H, t, J = 6.4 Hz, CH₂CH₂), 2.91 (2H, q, J = 6.8 Hz, CH₂), 3.04 (2H, t, J = 6.8 Hz, NH), 7.34 (2H, d, J = 8.4 Hz, Ar-H), 7.40 (1H, d, J = 8.4 Hz, NH), 7.59 (2H, d, J = 8.4 Hz, Ar-H), 7.73 (1H, d, J = 8.4 Hz, NH); ¹³C NMR (d₆-DMSO): δ (ppm) 21.3 (CH₃), 32.4 (CH₂), 35.6 (CH₂), 38.9 (CH₂), 42.2 (CH₂), 118.1 (CF₃), 126.9 (2 × CH), 139.8 (2 × CH), 137.8 (quat.), 143.5 (quat.), 161.8 (C=O), 169.7 (C=O); ESIMS m/z 308 (MNa⁺, 17 %), 286 (MH⁺, 100), 215 (15).

### 3.3.3.5 Synthesis of tert-Butyl [3{2-(4-chlorophenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7c

```
O
S
H
H
O
7c
```

N-(2-Aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate 1c (230 mg, 0.98 mmol), and t-Boc-β-alanine (185.5 mg, 0.9799 mmol) were dissolved in DMF (7 ml) then EDC (188 mg, 0.98 mmol), HOBt (132.5 mg, 0.98 mmol), and DIPEA (1.5 ml, 8.5 mmol) were added to mixture. The clear solution was stirred at room temperature for 24 hours and after
the reaction was complete, the DMF was removed in vacuo, then the residue was partitioned between EtOAc (200 ml) and water (200 ml). The organic layer was dried over Na₂SO₄ and evaporated and the residue was purified by column chromatography on silica, eluting with ethyl acetate-hexane (0:100) to ethyl acetate-hexane (50:50), to yield tert-butyl [3-{(2-(4-chlorophenylsulfonamido)ethyl)amino}-3-oxopropylcarbamate 7c as a white solid (120 mg, 52 %); mp 118-122 °C, IR (nujol) νmax/cm⁻¹ 3290 (NH), 1725 (C=O), 1692 (C=O), 1461 (Ar-C), 1345 (S=O), 1377 (CH₂), 1160 (S=O), 721 (Ar-H); ¹H NMR (400MHz, d₆-DMSO): 1.31 (9H, s, (CH₃)₃), 2.12 (2H, t, J = 7.2 Hz, CH₂CO), 2.72 (2H, t, J = 6.8 Hz, CH₂), 3.00 (4H, m, CH₂CH₂), 6.68 (1H, s, NH), 7.46 (2H, d, J = 8.8 Hz, ArH), 7.74 (2H, d, J = 8.8 Hz, ArH), 7.83 (1H, t, J = 5.6 Hz, NH); ¹³C NMR (d₆-DMSO): δ (ppm) 28.6 (CH₃)₃, 36.1 (CH₂), 37.05 (CH₂), 38.8 (CH₂), 42.3 (CH₂), 77.9 (quat.), 128.8 (2 × CH), 129.8 (2 × CH), 137.6 (quat.), 139.6 (quat.), 155.8 (C=O.), 170.9 (C=O); ESIMS m/z 429 (35 %), 428 (MNa⁺, 100), 406 (MH⁺, 40 %), 349 (24).
3.3.3.6 Preparation of 3-amino-N-{2-(4-chlorophenylsulfonamido)ethyl}propanamide trifluoroacetate 8c

The tert-butyl [3-{(2-(4-chlorophenylsulfonamido)ethyl)amino]-3-oxopropylcarbamate 7c (100 mg, 0.25 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution, the reaction mixture was stirred in an ice bath for 2 hours until the reaction was complete, and the mixture was left to allow the excess of TFA to evaporate to give 3-amino-N-{2-(4-chlorophenylsulfonamido)ethyl}propanamide trifluoroacetate 8c (100 mg, 100%) as a brown liquid; (Found: MH+, 306.0675. Calc. for C_{11}H_{16}ClN_{3}O_{3}S: MH, 306.0673); IR (nujol) \nu_{\text{max}}/\text{cm}^{-1} 3160 (NH), 1683 (C=O), 1458 (Ar-C), 1375 (CH2), 1346 (S=O), 720 (Ar-H); \textsuperscript{1}H NMR (400MHz, d_{6}-DMSO): 2.36 (2H, t, J = 6.8 Hz, CH\_2), 2.75 (2H, t, J = 6.8 Hz, CH\_2), 2.91 (2H, q, J = 6.8 Hz, CH\_2), 3.04 (2H, t, J = 6.8 Hz, CH\_2), 7.61 (2H, d, J = 8.4 Hz, ArH), 7.67 (1H, d, J = 8.8 Hz, NH), 7.73 (2H, d, J = 8.4 Hz, ArH), 7.88 (1H, d, J = 8.8 Hz, NH); \textsuperscript{13}C NMR (d_{6}-DMSO): \delta (ppm) 32.4 (CH\_2), 35.6 (CH\_2), 38.9 (CH\_2), 42.2 (CH\_2), 118.3 (CF\_3), 128.8 (2 \times CH), 129.8 (2 \times CH), 137.7 (quat.), 139.6
(quat.), 162.5 (C=O), 169.9 (C=O); ESIMS m/z 377 (22), 307 (37 %), 306 (MH+, 100), 235 (10).

3.3.3.7 Synthesis of tert-butyl [3-{2-(4-nitrophenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7d

\[
\text{O} \quad \text{S} \quad \text{N} \quad \text{H} \quad \text{N} \quad \text{H} \quad \text{O} \quad \text{O} \quad \text{CMe}_3
\]

\(N\)-(2-aminoethyl)-4-nitrophenylsulfonamide trifluoroacetate salt 1d (170 mg, 0.7 mmol), and \(t\)-Boc-\(\beta\)-alanine (131 mg, 0.7 mmol) were dissolved in DMF (8 ml), then EDC (132.8 mg, 0.69 mmol), HOBr (93.63 mg, 0.69 mmol), and DIPEA (1.5 ml, 8.5 mmol) were added to the mixture. The clear solution was stirred at room temperature for 24 hours and after the reaction was complete the solvent was removed in vacuo and the residue was partitioned between EtOAc (200 ml) and water (150 ml). The organic layer was washed with 5% aq. NaHCO\(_3\) (2 x 20 ml), 0.5 M citric acid (2 x 10 ml), then brine (2 x 25 ml), then dried over Na\(_2\)SO\(_4\) and evaporated to give tert-butyl [3-{2-(4-nitrophenylsulfonamido)ethyl}amino]-3-oxopropylcarbamate 7d (160 mg, 94%) as a brown liquid; IR (nujol) \(\nu_{\text{max}}/\text{cm}^{-1}\) 3315 (NH), 3262 (NH), 1680 (C=O), 1651 (CONH), 1450 (Ar-C), 1390 (CH\(_2\)), 1341 (S=O); \(^1\)H NMR (400MHz, \(d_6\)-DMSO): 1.31 (9H, s, (CH\(_3\))\(_3\)), 2.12 (2H, t, \(J = 7.4\) Hz, CH\(_2\)), 2.79 (2H, t,
\[ J = 6.8 \text{ Hz}, \ CH_2 \], 3.03 (4H, m, \ CH_2CH_2), 6.68 (1H, t, \ J = 5.2 \text{ Hz}, \ NH), 7.85 (1H, t, \ J = 5.6 \text{ Hz}, \ NH), 7.99 (2H, d, \ J = 9.0 \text{ Hz}, \ ArH), 8.03 (1H, s, \ NH), 8.38 (2H, d, \ J = 9.0 \text{ Hz}, \ ArH); \]
\[ ^{13}\text{C} \text{ NMR (} d_6-\text{DMSO}) \): δ (ppm) 28.6 (CH₃), 36.2 (CH₂), 37.0 (CH₂), 38.8 (CH₂), 42.3 (CH₂), 77.9 (quat.), 125.05 (2 × CH), 128.4 (2 × CH), 139.5 (quat.), 146.3 (quat.), 150.1 (C=O), 171.05 (C=O); ESIMS \text{ } m/z \text{ } 436 (15), 418 (20 \%), 417 (\text{MH}^+, 100). \] 

3.3.3.8 Preparation of 3-amino-\(N\)-\{2-(4-nitrophenylsulfonamido)ethyl\}propanamide 8d

\[ \text{tert-Butyl \{3-\{2-(4-nitrophenylsulfonamido)ethyl\}amino\}3-oxopropylcarbamate 7d (150 mg, 0.36 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution, the reaction mixture was stirred in ice bath for 2 hours until the reaction was complete, then the mixture was left to allow the excess of TFA to evaporate to give 3-amino-\(N\)-\{2-(4-nitrophenylsulfonamido)ethyl\}propanamide trifluoroacetate 8d (130 mg, 86 %) as a brown liquid; (Found: MH}^+, 317.0916. Calc. for C₁₁H₁₆N₄O₅S: MH, 317.0914); IR (nujol) \nu_{\max}/\text{cm}^{-1} \text{ } 3200 (\text{NH}), 1727 (C=O), 1461 (\text{Ar-C}), 1377 (CH₂), 1336 (S=O), 721 (Ar-H); \] \[ ^1\text{H} \text{NMR (} 400\text{MHz, } d_6-\text{DMSO}) \): 2.35 (2H, t, \ J = 6.8 \text{ Hz}, \ CH₂), 2.81 (2H, t, \ J = 6.4 \text{ Hz}, \ CH₂), 2.90 (2H, t, \ J = 6.8 \text{ Hz}, \ CH₂), 3.04 (2H, t, \ J = 6.4 \text{ Hz}, \ CH₂), 7.97 (2H, d, \ J = 8.8 \text{ Hz}, \]
ArH), 8.36 (2H, d, J = 8.8 Hz, ArH); $^{13}$C NMR (d$_6$-DMSO): $\delta$ (ppm) 32.4 (CH$_2$), 35.6 (CH$_2$), 39.0 (CH$_2$), 42.2 (CH$_2$), 117.9 (CF$_3$), 125.1 (2 × CH), 128.4 (2 × CH), 146.3 (quat.), 150.0 (quat.), 161.8 (C=O), 170.0 (C=O); ESIMS $m/z$ 428 (MNa$^+$, 100), 318 (15 %), 317 (MH$^+$, 100), 246 (12).

3.3.3.9 Synthesis of tert-butyl {3-{2-(2-nitrophenylsulfonamido)ethyl}amino}-3-oxopropylcarbamate 7e

\[
\begin{align*}
\text{O} & \quad \text{S} & \quad \text{N} & \quad \text{H} & \quad \text{O} & \quad \text{O} \\
\text{H} & \quad \text{N} & \quad \text{CMe}_3 \\
\text{NO}_2 & \quad \text{O} & \quad \text{N} & \quad \text{O}
\end{align*}
\]

$N$-(2-aminoethyl)-2-nitrophenylsulfonamide trifluoroacetate salt 1e (200 mg, 0.81 mmol), and $t$-Boc- $\beta$-alanine (155 mg, 0.81 mmol) were dissolved in DMF (10 ml), then EDC (156 mg, 0.82 mmol), HOBt (110.16 mg, 0.81 mmol), and DIPEA (2 ml, 11.33 mmol) were added to the mixture. The clear solution was stirred at room temperature for 24 hours and after the reaction was complete the solvent was removed in vacuo. The residue was partitioned between EtOAc (200 ml) and water (150 ml), then the organic layer was washed with 5% aq. NaHCO$_3$ (2 × 20 ml), 0.5 M citric acid (2 × 10 ml), then brine (2 × 25 ml), dried over Na$_2$SO$_4$ and evaporated to give tert-butyl{3-{2-(2-nitrophenylsulfonamido)ethyl}amino}-3-oxopropylcarbamate 7e (170 mg, 85 %) as a
yellowish-white liquid; IR (nujol) $v_{\text{max}}$ cm$^{-1}$ 3310 (NH), 1689 (C=O), 1410 (Ar-C), 1374 (CH$_2$); $^1$H NMR (400MHz, CDCl$_3$): 1.37 (9H, s, (CH$_3$)$_3$), 2.30 (2H, t, $J = 5.8$ Hz, CH$_2$), 3.20 (2H, q, $J = 5.8$ Hz, CH$_2$), 3.25 (2H, q, $J = 6.4$ Hz, CH$_2$), 3.32 (2H, q, $J = 6.4$ Hz, CH$_2$), 5.86 (1H, s, NH), 6.81 (1H, s, NH), 7.30 (1H, s, NH), 7.89 (2H, m, ArH), 7.94 (1H, m, ArH), 8.10 (1H, m, ArH); $^{13}$C NMR ($d_6$-DMSO): $\delta$ (ppm) 28.6 (CH$_3$)$_3$, 36.2 (CH$_2$), 37.1 (CH$_2$), 38.8 (CH$_2$), 42.3 (CH$_2$), 77.9 (quat.), 124.8 (2 $\times$ CH), 129.7 (2 $\times$ CH), 146.2 (quat.), 138.6 (quat.), 159.8 (C=O), 171.1 (C=O); ESIMS $m/z$ 436 (17), 418 (18 %), 417 (MH$^+$, 100), 361 (60).

3.3.3.10 Preparation of 3-amino-N-[2-(2-nitrophenylsulfonamido)ethyl]propanamide 8e

$t$-Butyl{3-{2-(2-nitrophenylsulfonamido)ethyl}amino}-3-oxopropylcarbamate 7e (160 mg, 0.0.38 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution. The reaction mixture was stirred in ice bath for 2 hours until the reaction was complete, then the mixture was left to allow the excess of TFA to evaporate to give 3-amino-N-[2-(2-nitrophenylsulfonamido)ethyl]propanamide trifluoroacetate 8e (100 mg, 62.5 %) as a brown liquid; (Found: MH$^+$, 317.0915. Calc. for C$_{11}$H$_{16}$N$_4$O$_5$S: MH,
317.0914); IR (nujol) ν_{max}/cm^{-1} 3229 (NH), 1461 (Ar-C), 1160 (S=O), 725 (Ar-H); \textsuperscript{1}H NMR (400MHz, d_{6}-DMSO): 2.35 (2H, t, J = 6.8 Hz, CH\textsubscript{2}), 2.89 (4H, m, CH\textsubscript{2}CH\textsubscript{2}), 3.09 (2H, t, J = 6.4 Hz, CH\textsubscript{2}), 7.81 (2H, m, ArH), 7.91 (2H, m, ArH); \textsuperscript{13}C NMR (d_{6}-DMSO): δ (ppm) 32.4 (CH\textsubscript{2}), 35.6 (CH\textsubscript{2}), 39.1 (CH\textsubscript{2}), 42.3 (CH\textsubscript{2}), 118.1 (CF\textsubscript{3}), 124.7 (CH), 129.7 (CH), 133.0 (CH), 134.5 (CH), 148.2 (quat.), 158.5 (quat.), 161.9 (C=O), 170.1 (C=O); ESIMS m/z 339 (13), 318 (14 %), 317 (MH\textsuperscript{+}, 100), 246 (23).

3.4.1 Coupling of Ciprofloxacin with t-Boc-β-alanine 10

![Chemical Structure](image)

Ciprofloxacin (262.6 mg, 0.79 mmol) and t-Boc-β-alanine (150 mg, 0.79 mmol) were dissolved in DMF (5 ml), then EDC (152 mg, 0.79 mmol), and HOBt (107 mg, 0.79 mmol) were added to the mixture. The clear solution was cooled to 0 °C for 20 minutes then the mixture was stirred at room temperature for 24 hours. After the reaction was complete, the solvent was removed in vacuo, then the residue was partitioned between EtOAc (200 ml) and water (150 ml), the organic layer was washed with 5 % aq. NaHCO\textsubscript{3} (2 × 20 ml), 0.5 M citric acid (2 × 10 ml), then brine (2 × 25 ml), dried over Na\textsubscript{2}SO\textsubscript{4} and evaporated to give
N"-(Boc-β-alanyl)ciprofloxacin 10 (240 mg, 91 %) as a white solid; mp 201-206 °C; IR (nujol) ν\text{max}/\text{cm}^{-1} 3423 (NH), 3176 (OH), 1782 (C=O), 1456 (Ar-C), 1377 (CH₂), 1160 (CO) 730 (Ar-H); ¹H NMR (400MHz, d₆-DMSO): 1.14 (2H, d, J = 6.8 Hz, CH₂), 1.27 (2H, m, CH₂), 1.33 (9H, s, (CH₃)₃), 2.49 (2H, m, CH₂), 3.13 (2H, q, J = 6.4 Hz, CH₂), 3.27 (2H, m, CH₂), 3.62 (4H, m, (CH₂)₂), 3.87 (1H, m, CH), 6.71 (1H, t, J = 6.0 Hz, NH), 7.52 (1H, d, JFH = 7.2 Hz, Ar-H), 7.87 (1H, d, JFH = 13.2 Hz, Ar-H), 8.63 (1H, s, Ar-H); ¹³C NMR (d₆-DMSO): δ (ppm) 8.6 (CH₂), 28.6 (3 × CH₃), 33.2 (CH₂), 36.3 (CH), 36.8 (CH₂), 44.9 (CH₂), 49.5 (CH₂), 49.9 (CH₂), 78.1 (quat.), 107.0 (quat.), 107.7 (quat.), 111.3 (CH), 115.5 (CH), 119.2 (2 × CH), 139.5 (quat.), 145.3 (CH), 148.5 (quat.), 152.1 (quat.), 153.4 (quat.), 155.8 (C=O), 166.3 (C=O), 169.7 (C=O.), 176.8 (C=O). ESIMS m/z 503 (MH⁺, 100), 494 (12), 476 (10).
3.4.2 Synthesis of $N''$-($\beta$-alanyl)ciprofloxacin 11

$N''$-($\text{Boc}$-Alanyl)ciprofloxacin 10 (200 mg, 0.4 mmol) was dissolved in DCM (3.5 ml) and TFA (0.5 ml) was added to the solution. The reaction mixture was stirred in an ice bath for 2 hours until the reaction was complete, then the mixture was left to allow the excess of TFA to evaporate to give $N''$-($\beta$-alanyl)ciprofloxacin 11 (190 mg, 95 %) as a yellow liquid; (Found: MH$^+$, 385.1670. Calc. for C$_{20}$H$_{23}$FN$_4$O$_4$: MH, 385.1670); IR (nujol) $\nu_{\text{max}}$/cm$^{-1}$ 3402 (NH), 3176 (OH), 1782 (C=O), 1456 (Ar-C), 1377 (CH$_2$), 1168 (CO), 723 (Ar-H); $^1$H NMR (400MHz, $d_6$-DMSO): 1.15 (2H, m, CH$_2$), 1.28 (2H, q, $J$ = 6.8 Hz, CH$_2$), 2.71 (2H, t, $J$ = 6.4 Hz, CH$_2$), 3.01 (2H, m, CH$_2$), 3.29 (2H, m, CH$_2$), 3.34 (2H, m, CH$_2$), 3.62 (2H, m, CH), 3.68 (2H, m, CH), 3.78 (1H, m, CH), 7.53 (1H, d, $J_{HF}$ = 7.6 Hz, Ar-H), 7.71 (1H, broad s, NH$_3$), 7.89 (1H, d, $J_{HF}$ = 13.2 Hz, Ar-H), 8.63 (1H, s, Ar-H); $^{13}$C NMR ($d_6$-DMSO): $\delta$ (ppm) 8.0 (CH$_2$)$_2$, 30.1 (CH), 35.6 (CH$_2$), 36.2 (CH$_2$), 41.1 (CH$_2$), 44.7 (CH$_2$), 107.1 (CH), 111.5 (quat.), 114.3 (CH), 117.2 (quat.), 118.4 (CF$_3$), 120.1 (quat.), 139.5 (quat.), 158.5 (CH), 158.9 (quat.), 159.2 (quat.), 162.2 (C=O), 166.3 (C=O), 168.8 (C=O), 176.7 (C=O); ESIMS m/z 403 (MH$^+$, 100), 332 (12).
3.5.1 Preparation of $N^7$-(3-phthalimidopropyl)theophylline 14

Theophylline (3.6 g, 20 mmol) was added to dry DMF (50 ml) then $K_2CO_3$ (4.15 g, 30 mmol) and $N$-(3-bromopropyl)phthalimide (6.7 g, 25 mmol) were added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was cooled in ice for 2 hours, the white precipitate was collected by vacuum filtration then recrystallized from 50% $H_2O$ / acetic acid to yield $N^7$-(3-phthalimidopropyl)theophylline 14 (5 g, 74 %) as a pure white solid; mp 260-262 °C; IR (nujol) $\nu_{\text{max}}$/cm$^{-1}$ 1782 (C=O), 1685 (C=O), 1456 (Ar-C), 1377 (CH$_2$), 1168 (CO), 723 (Ar-H); $^1$H NMR (400MHz, CDCl$_3$): 2.29 (2H, q, $J$ = 6.4 Hz, CH$_2$), 3.36 (3H, s, CH$_3$), 3.55 (3H, s, CH$_3$), 3.71 (2H, t, $J$ = 6.0 Hz, CH$_2$), 4.31 (2H, t, $J$ = 6.4 Hz, CH$_2$), 7.72 (2H, d, $J$ = 8.6 Hz, Ar-H), 7.77 (1H, s, Ar-H), 7.83 (2H, d, $J$ = 8.6 Hz, Ar-H); $^{13}$C NMR (CDCl$_3$): $\delta$ (ppm) 27.9 (CH$_2$), 29.6 (CH$_3$), 29.9 (CH$_3$), 34.6 (CH$_2$), 44.4 (CH$_2$), 106.7 (quat.), 123.2 (2 × CH), 131.7 (2 × CH), 134.0 (2 ×quat.), 141.4 (CH),
148.9 (quat.), 151.6 (quat.), 168.5 (2 × quat.); ESIMS m/z 431 (32), 390 MNa⁺ (38), 369 (29), 368 (MH⁺, 100).

3.5.2 Preparation of N⁷-(3-aminopropyl)theophylline 13

To a solution of N⁷-(3-phthalimidopropyl)theophylline 14 (1g, 2.72 mmol) in ethanol (10 mL), hydrazine hydrate (98%, 2.5 mL) was added and the reaction mixture was stirred at room temperature for 48 h. The solvent was removed under vacuum, then 1M aq. HCl (20 ml) was added and the solution extracted with CHCl₃ (2 × 10 ml). The aqueous layer was basified then extracted with CHCl₃ (2 × 10 ml). The organic layers were dried over Na₂SO₄, then evaporated to give N⁷-(3-aminopropyl)theophylline 13 (150 mg, 15 %) as a yellow solid; mp 80-85 °C; (Found: MH⁺, 238.1300. Calc. for C₁₀H₁₅N₅O₂: MH, 238.1298); IR (nujol) νmax/cm⁻¹ 3310 (NH), 1720 (C=O), 1692 (C=O), 1377 (CH₂); ¹H NMR (400MHz, CDCl₃): 1.97 (2H, q, J = 6.8 Hz, CH₂), 2.69 (2H, t, J = 6.8 Hz, CH₂), 3.39 (3H, s, CH₃), 3.57 (3H, s, CH₃), 4.39 (2H, t, J = 6.8 Hz, CH₂), 7.57 (1H, s, CH); ¹³C NMR (CDCl₃): δ (ppm) 27.9 (CH₂), 29.7 (CH₃), 34.0 (CH₃), 38.3 (CH₂), 44.4 (CH₂), 106.9
(quat.), 141.1 (CH), 148.8 (quat.), 151.6 (quat.), 155.1 (2 × quat.); ESIMS m/z 279 (15), 239 (35), 238 (MH⁺, 100), 221 (70).
CHAPTER FOUR

BIOLOGICAL TESTS
4. Biological activity tests

4.1 Antimicrobial susceptibility tests

Disc diffusion and micro-dilution assays were conducted in order to evaluate the antimicrobial activities of the synthesized compounds.

4.1.1 Disc diffusion assay

The disc diffusion assay is a simple and fast method used to test whether a specific bacterium is susceptible to an antimicrobial agent by measuring the zones of inhibition, which is the distance from the edge of a disc impregnated with the test agent to the edge of confluent growth, Figure 22.

Figure 22 The zone of inhibition
4.1.2 Experimental procedure

Sensitest agar (Oxoid, CM0409) medium was prepared and sterilized as per the manufacturer's instructions. 20 ml of the prepared medium was added to each sterile plastic Petri dish to ensure consistent plate depths, and was allowed to set for 20-30 minutes at room temperature. The prepared plates were stored at 2-8 °C and used within 4 weeks of preparation.

Bacteria to be tested were streaked onto the Sensitest agar plates which were then incubated overnight at 37 °C.

The sterile discs (Sigma, 74146) were placed onto a sterile glass Petri dish using sterile forceps, then 60 µl of the compound dissolved in acetone (highest volume which saturated the disc) at different concentrations (10, 50, and 100 mg/ml) was loaded onto the sterile discs, Figure 23.
The bacterial suspensions for this work were prepared as follows:

Sterile Phosphate Buffered Solution (PBS) was pipetted into a sterile falcone tubes, and inoculated with an inoculation loop of culture from overnight plate cultures.

The bacterial suspension was poured onto a plate, which was gently rotated in order to ensure that full surface was covered, and the suspension was then poured onto the next
plate to inoculate it. This was repeated until all the plates were inoculated, the excess solution on the plates was removed using a 1ml pipette, and the inoculated plates were left to dry in a laminar flow cabinet for 5 minutes.

The inoculated plates were labelled after the discs with different compound concentrations were placed onto segments, by gently pressing down to ensure that the discs were in place, and the plates were incubated at 37 ºC for 24-48 h.

The zone of inhibition was measured at 24 h and 48 h time points.

4.1.3 Results

Twenty-seven synthesized compounds were tested in the disc diffusion assay against *Pseudomonas aeruginosa* strain PAO1 in order to evaluate their antibacterial effect, Figure 24, and only six compounds showed anti-pseudomonal activity, although with different efficacy, Table 11.
Figure 24  Disc diffusion test for compounds 4a, 4b, 4c, 4d, (first row), 1a, 1c, 1b, 4e, (second row), 4f, 1d, 1e (third row) against PAO1 at 0, 10, 50, and 100 mg/ml.
Table 11  Disc diffusion assay results for the active compounds at 6 mg/ml on disc

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Structure</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>No growth on sterile disc</td>
</tr>
<tr>
<td>1b</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>2</td>
</tr>
<tr>
<td>1c</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>6</td>
</tr>
<tr>
<td>1d</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>9</td>
</tr>
<tr>
<td>1e</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>5.5</td>
</tr>
</tbody>
</table>
N-(2-Aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate salt 1d, 3-amino-N-(2-(2-nitrophenylsulfonamido)ethyl)propanamide trifluoroacetate 8e, and N-(2-aminoethyl)-4-chlorobenzenesulfonamide 1c were the most effective compounds, while N-(2-aminoethyl)benzenesulfonamide 1a was only slightly active, it was cytotoxic to the bacteria on the disc but it did not affect the bacteria in the medium, possibly due to poor diffusion from the disc. The six active compounds were also tested against the Gram negative *Proteus vulgaris* and Gram-positive *Bacillus subtilis* in order to evaluate the selectivity of their inhibition of the growth of *P. aeruginosa*, Table 12. For these tests, the concentrations of the solutions applied to the discs were 100 mg/ml.
Table 12  Disc diffusion results for active compounds against PAO1, *B. subtilis*, and *P. vulgaris*

<table>
<thead>
<tr>
<th>ID</th>
<th>Molecular Structure</th>
<th>Zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td>8e</td>
<td><img src="image1" alt="Molecular Structure" /></td>
<td>6.8</td>
</tr>
<tr>
<td>1c</td>
<td><img src="image2" alt="Molecular Structure" /></td>
<td>5.2</td>
</tr>
<tr>
<td>1b</td>
<td><img src="image3" alt="Molecular Structure" /></td>
<td>2</td>
</tr>
<tr>
<td>1d</td>
<td><img src="image4" alt="Molecular Structure" /></td>
<td>9</td>
</tr>
</tbody>
</table>
$N$-(2-Aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d had the greatest antimicrobial effect against *P. aeruginosa* and the other organisms, while 3-amino-$N$-{2-(2-nitrophenylsulfonamido)ethyl}propanamide 8e, $N$-(2-Aminoethyl)-4-methylbenzenesulfonamide 1b and $N$-(2-aminoethyl)-4-chlorobenzenesulfonamide 1c trifluoroacetates exhibited the greatest selectivity against *P. aeruginosa* over *B. subtilis* and *P. vulgaris* Figure 25. $N$-(2-Aminoethyl)-4-methylbenzenesulfonamide 1b, and $N$-(2-aminoethyl)benzenesulfonamide 1a had very low anti-pseudomonal effect, with a zone of inhibition of 2 mm and clear disc, respectively.
Figure 25  Disc diffusion test for compounds 1c (first row), and 1b (second row) against B. subtilis (first column), P. vulgaris (second column), and PAO1 (third column) at 100 mg/ml concentration.
4.2.1 Minimum inhibitory concentration (MIC) or micro-dilution assay

The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that inhibits the growth of an organism and the MIC test determines the antimicrobial activity of a compound against a specific bacterium.

4.2.2 Experimental procedure

The bacteria to be tested were cultured onto Sensitest agar plates (Oxoid, CM0409) and incubated overnight at 37 °C.

The active compounds were prepared at serial dilution in a final volume of 100 μl using iso-sensitest broth.

The bacterial suspension was prepared at a turbidity of 0.5 Macfarland standard (approximately OD$_{600} = 0.05-0.1$) which was obtained from a plate incubated overnight.

100 μl of bacterial suspension was added into the appropriate wells of 96-well tissue culture plates, and 100 μl of the substance to be tested was added, so that compounds were tested at concentrations of 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.063, 19.531, 9.7656, 4.8828, 0 μg/ml, and the plates were incubated at 37 °C for 24-48 h. The MIC was determined as the lowest concentration of antimicrobial agent at which there is no visible growth. Figure 26.
Figure 26  Micro-dilution assay of $N$-(2-aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d against PAO1
4.2.3 Results

The six active compounds and the β-alanyl ciprofloxacin were tested against three different organisms; PAO1, *B. subtilis*, and *P. vulgarus*. The effect of the solvent was evaluated, Figure 27 and it is clear that there is no effect of acetone on the bacterial growth. The micro-dilution assay data highlighted that into three classes:

- *N*-((2-Aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d has the greatest effect against PAO1, with an MIC of 78.125 µg/ml, Figure 28.
- *N*-((2-Aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d also has the greatest effect against *B. subtilis*, with an MIC of 156.25 µg/ml, Figure 29.
- Compounds *N*-((2- aminoethyl)-2-nitrophenylsulfonamide trifluoroacetate 1e, *N*-((2-aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate 1c, and *N*-((2-aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d have MIC of 156.25 µg/ml against *P. vulgaris*. Figure 30.

A micro-dilution assay was also conducted in order to evaluate the effect of the coupling with β-alanyl on the selectivity of ciprofloxacin towards the previously mentioned strains, and the results demonstrate that there is no selectivity effect, but that the coupled ciprofloxacin has a greater effect than the other six compounds, Figure 31.
Figure 27   Effect on bacterial growth of PAO1 of 1d (A) and acetone solvent (B)
Figure 28  Micro-dilution assay in PAO1 at concentrations of 0-5000 µg/ml
Figure 29  Micro-dilution assay in *B. subtili* at concentrations of 0-5000 µg/ml
**Figure 30**  Micro-dilution assay in *P. vulgaris* at concentrations of 0-5000 µg/ml
Figure 31  Micro-dilution for ciprofloxacin and its β-alanyl analog 11 in PAO1, B. subtilis, and P. vulgaris
4.3 Cytotoxic effect of compounds on a human cell line

The MTT assay is widely used to evaluate the *in vitro* cytotoxic activity of drugs on cell lines. MTT assay is a colorimetric assay based on conversion of the yellow MTT to purple formazan crystals by cellular reductase enzyme in living cells **Scheme 9**. Formazan is largely impermeable to the cell membrane, thus resulting in its aggregation within living cells. The number of surviving cells is directly correlated to the degree of the formazan generated, the color can then be quantified using a simple colorimetric assay.

**Scheme 9** Reaction scheme for the MTT assay
### 4.3.1 Preparation of MTT solution

The MTT stock solution 12 mM was prepared by dissolving \(3-[4,5\text{-dimethylthiazol-2-yl}]-2,5\text{-diphenyltetrazolium bromide}\) (Sigma Aldrich) in phosphate buffered saline (5 mg/mL, Sigma Aldrich) then filtering through a 0.2 µm syringe filter (Corning).

### 4.3.2 Prostate cancer (PC3 cell line) assay

PC3 cells were grown to ~70-80% confluence, seeded into 96-well plates at 3000 cells/well and incubated for 24 h. The seven previously identified active compounds were dissolved in DMSO and prepared as 200 mM stock solutions. The solutions were then diluted with RPMI 1640 media supplemented with 10 % foetal bovine serum and filtered through a 0.2 µm syringe filter, then added into the 96-well plate. The final concentrations of the compounds were 100, 50 and 10 µM and each concentration was added to 6 wells. 0.1% of DMSO in media was also prepared and added to 6 wells of the plate, and this was used as a standard control for each plate. The plates were then incubated for a further 72 hours \(T_{72}\). The medium was then removed from each well and was replaced with 10 % MTT stock solution in serum and phenol red-free medium (100 µL/ well). The plates were then incubated for 3 hours, the medium was then removed and replaced with DMSO (50 µL/ well). After mixing, the plates were placed in the microplate reader (FLUOstar Omega) and the absorbance was read at 540 nm. A control plate was prepared in the same way as above and read 24 hours \(T_0\) after seeding; this was used as a control to give an indication of the
number of cells before drug treatment. The cell viability was then calculated as a percentage using the following formula:

\[
\text{Viability} = \left( \frac{\text{Abs} (T_2) - \text{Abs} (T_0)}{\text{Abs} (\text{DMSO}) - \text{Abs} (T_0)} \right) \times 100
\]

4.3.3 Preparation of HeKa (primary human keratinocytes-adult) cells

The cells were purchased from Gibco Invitrogen and were cultured according to the manufacturer’s recommendations using a defined culture system. The assays were conducted using the same method as above, except that cells were seeded at 2000 cells/well.

4.3.4 Results from cytotoxicity tests

The MTT assay on the prostate cancer (PC3) cell line shows that the all compounds have little effect on the growth of prostate cancer cell, except \(N\)-(2-aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate \(1d\), which is highly cytotoxic at high concentration (100 µM), Figure 32.
The viability assay on human keratinocytes cells line shows that only \( N\)-(2-aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d, results in the inhibition of growth at 50 \( \mu \text{M} \) and 100 \( \mu \text{M} \), while the rest of the synthesized compounds have no effect, Figure 33.
**Figure 33** Cell viability (MTT assay) for the active compounds in HeKa cells
CHAPTER FIVE

CONCLUSIONS
5. Conclusions

This project was based upon previous work on the synthesis of new chromogenic substrates for the identification of the Gram negative multi-drug resistant *Pseudomonas aeruginosa*, which suggested the presence of β-alanyl aminopeptidase in this bacterium. In this project, a 3-D model of β-alanyl aminopeptidase has been constructed and evaluated. Virtual database screening was then conducted in an attempt to discover novel inhibitors of this target enzyme. Twenty-seven novel compounds were synthesized, purified, and characterized and β-alanine was linked to these synthesized compounds. The fluoroquinolone antibiotic ciprofloxacin was also coupled to β–alanine and all compounds were evaluated for anti-pseudomonal activity.

In disc diffusion assays, N-(2-aminoethyl)benzenesulfonamide trifluoroacetate salt 1a, N-(2-aminoethyl)-4-methylbenzenesulfonamide trifluoroacetate salt 1b, N-(2-aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate 1c, N-(2-aminoethyl)-4-nitrophenylsulfonamide trifluoroacetate 1d, N-(2-aminoethyl)-2-nitrophenylsulfonamide trifluoroacetate 1e, and 3-amino-N-{2-(2-nitrophenylsulfonamido)ethyl}propanamide trifluoroacetate 8e showed the greatest anti-pseudomonal activity, Table 13.
**Table 13**  Disc diffusion assay for the active compounds against PAO1 (at 100 mg/ml concentration)

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Compound name</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>N-(2-aminoethyl)benzenesulfonamide trifluoroacetate salt</td>
<td>No growth on sterile disc</td>
</tr>
<tr>
<td>1b</td>
<td>N-(2-aminoethyl)-4-methylbenzenesulfonamide trifluoroacetate salt</td>
<td>2</td>
</tr>
<tr>
<td>1c</td>
<td>N-(2-aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate</td>
<td>6</td>
</tr>
<tr>
<td>1d</td>
<td>N-(2-aminoethyl)-4-nitrophenylsulfonamide trifluoroacetate</td>
<td>9</td>
</tr>
<tr>
<td>1e</td>
<td>N-(2-aminoethyl)-2-nitrophenylsulfonamide trifluoroacetate</td>
<td>5.5</td>
</tr>
<tr>
<td>8e</td>
<td>3-amino-N-{2-(2-nitrophenylsulfonamido)ethyl}propanamide trifluoroacetate</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*N-(2-Aminoethyl)-4-nitrophenylsulfonamide trifluoroacetate 1d, 3-amino-N-{2-(2-nitrophenylsulfonamido)ethyl}propanamide trifluoroacetate 8e, and N-(2-aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate 1c* had the greatest anti-pseudomonal activity. In order to determine the selectivity of their antibacterial activity, the active compounds were
also tested against the Gram negative *Proteus vulgaris* and Gram positive *Bacillus subtilis*. 3-Amino-N-{2-(2-nitrophenylsulfonamido)ethyl}propanamide trifluoroacetate 8e had the greatest selectivity, followed by N-(2-aminoethyl)-4-chlorophenylsulfonamide trifluoroacetate 1c and N-(2-aminoethyl)-4-methylbenzenesulfonamide trifluoroacetate salt 1b, while N-(2-aminoethyl)-2-nitrophenylsulfonamide trifluoroacetate 1e and N-(2-aminoethyl)-4-nitrophenylsulfonamide trifluoroacetate 1d had no selectivity, with strong inhibitory effects against all three bacterial species, *Table 14*.

**Table 14** Disc diffusion assay for the active compounds at 100 mg/ml concentration against PAO1, *B. subtilis*, and *P. vulgaris*

<table>
<thead>
<tr>
<th>Compound ID</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zone diameter (mm)</strong></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td>8e</td>
<td>6.8</td>
</tr>
<tr>
<td>1c</td>
<td>5.2</td>
</tr>
<tr>
<td>1b</td>
<td>2</td>
</tr>
<tr>
<td>1d</td>
<td>9</td>
</tr>
<tr>
<td>1a</td>
<td>Clear disc</td>
</tr>
<tr>
<td>1e</td>
<td>5.5</td>
</tr>
</tbody>
</table>

As shown in *Table 14*, the 3-amino-N-{2-(2-nitrophenylsulfonamido)ethyl}propanamide 8e showed some selectivity, with a 6.8 mm zone of inhibition for PAO1 with only 0.3 and 1.3 mm zones for *B. subtilis* and *P. vulgaris* respectively. In addition, the N-(2-aminoethyl)-4-chlorophenylsulfonamide 1c produced a zone of inhibition of 5.2 mm
against PAO1, and 0.7 and 1.8 mm zones for B. subtilis and P. vulgaris, respectively. In contrast, in a micro-dilution assay, compound 1d showed the greatest anti-pseudomonal effect, with an MIC of 78.125 µg/ml, although this effect was not specific as it also had the greatest effect against B. subtilis and P. vulgaris, with an MIC of 156.25 µg/ml for both species.

In the micro-dilution assay, ciprofloxacin coupled to β-alanyl 11 showed greater effect than the other six compounds, with no selectivity against P. aeruginosa. An MTT assay using PC3 and HeKa cells showed that all compounds have little effect on the growth of the prostate cancer (PC3) and HeKa cells, except N-(2-aminoethyl)-4-nitrobenzenesulfonamide trifluoroacetate 1d which was cytotoxic to the PC3 cell line at high concentration (100 µM), and cytotoxic to HeKa cells at 50 µM and 100 µM. Although compounds 8e, 1c, and 1b have selective effects upon PA01, their anti-pseudomonal activities are not sufficient for the development of potent anti-pseudomonal agents, suggesting that β-alanyl aminopeptidase has no physiological role in the growth of Pseudomonas aeruginosa. The results obtained in this work do, however, support further work to enhance the activity of these non-classical sulphonamides, as well as proteomics and protein purification in order to isolate and study β-alanyl aminopeptidase crystallographically.
6. References


90. Discover studio visualizer v. 1.7; Accerlyrs Inc SD, USA. (2007).

