Novel substrates for the improved detection and identification of pathogenic bacteria

Laszlo Andras Kondacs

A thesis submitted in partial fulfilment of the requirements of the University of Sunderland for the degree of Doctor of Philosophy

This research programme was carried out in collaboration with the Freeman Hospital (Newcastle-upon-Tyne) and bioMérieux (La Balme-les-Grottes, France)

October 2018

Acknowledgement

I would like to express my deepest thanks to my supervisors.

To Prof. Roz Anderson, who welcomed, cared for and supervised me even in her hardest times, I wish she would still be with us.

To Dr Mark Gray, who was always gave me the right professional guidance and supervision.

To Prof. John Perry, who tested all my compounds, and gave me good advice in microbiology, adding a real value to my thesis.

To Dr Lewis Bingle, who gave me useful advice in microbiology.

I would like to thank bioMerieux for the financial support and the cheerful meetings. It was great to be in a team with Sylvain, Jan-Marc, Alex, Philippe and all of the others including Steve and the Northumbria University group.

For the professional guidance, I would like to thank Lisa, Peter, Steven, Kelvin, Alex, Suresh, Andrey and Philippa.

For the technical support, I would like to thank Arun, Andrea, Maria, Paul, Byron, Debbs and John, especially thanks for the NMR and MS measurement, and for the borrowed equipment and chemicals.

For the professional advice, I would like to thank Dr Keith Thomas, Dr John Lough, Dr Stephanie Myers and Dr Mark Ashton.

For gossips and fun, I would like to thank my labmates: Golnaz, Clemmens, Kelvin, Rachel, Alex, Andrey, Lisa, Peter, Noha, Philippa, Anna, Amer, Suresh and Aya.

Благодаря за търпението и любовта на Стела. Обичам те!

Külön köszönet Nyerges Miklósnak, hogy ajánlotta nekem ezt a lehetőséget.

Köszönöm Anya, Apa, Lindi, Gábor, Bebóka a támogatást otthonról.

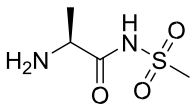
Abstract

Many diseases are caused by pathogenic bacteria. A key example of this is sepsis, which is mostly caused by staphylococci and Gram-negative bacteria. In addition, the highly resistant ESKAPE pathogens are responsible for the majority of hospital acquired infections. In order to treat bacterial infections effectively, and to avoid promoting bacterial resistance against antibacterial drugs, the correct agents must be used, for which in turn the detection and identification of pathogenic strains is essential.

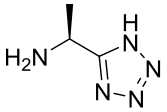
This research aims to develop selective chromogenic culture media, by introducing new antibacterial agents for the improved selectivity and new chromogenic substrates for selective visualisation of certain bacterial strains.

The intention of the major part of this work was to inhibit the growth of commensal bacteria in clinical samples, as they mask the growth of the infection-causing bacteria. New and known compounds were prepared for

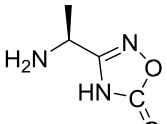
evaluation as alanine racemase inhibitors. The compounds were tested on a range of clinical pathogenic and non-pathogenic bacterial strains. The molecules developed were based on the amino acid alanine and utilised bioisosteres and other replacements for the carboxylic acid moiety. Key compounds targeted included alanylmethanesulfonamide **27-L**, 1-aminoethyl5-oxo-1,2,4-oxadiazole **33-L** and 1-aminoethyltetrazole **32a-L**.



27-L



32a-L



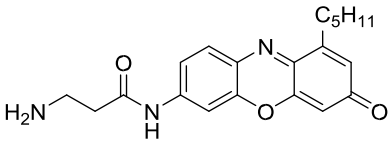
33-L

Each compound was tested initially as the alanyl-X dipeptide form. While most of the alanine bioisosteres were known structures, their novel peptide derivatives required synthetic development using both solution and solid phase techniques. The solid phase synthesis of several *C*-terminal 1aminoethyltetrazole peptides was successfully established by using 2-chlorotrityl chloride resin.

The investigation of the antimicrobial activity of the synthesised compounds identified several clinically applicable selective inhibitors. These compounds were shown to provide differentiation between *Salmonella* and *Escherichia coli*, or enterococci and streptococci. This work also gave a useful comparison between the different alanine bioisosteres, and showed the importance of di- and oligopeptide permease systems in order to reach sufficient bacterial activity. The microbiological activity of 1-aminoethyltetrazole peptide derivatives was studied in more detail, due to their potential in clinical applications for the diagnosis of food poisoning.

In other work, also directed towards the rapid and selective detection and identification of pathogenic bacteria in a clinical environment, new Chromogenic substrates were prepared. Each of these compounds contained a chromogen with a phenoxazin-3-one scaffold linked to an amino acid residue. The purpose of the amino acid is to act as a unit recognised and cleaved by specific hydrolytic bacterial enzymes. Upon liberation, electronic differences between the conjugated and free forms of the chromogen resulted in the development of distinct colour changes, which provide the basis of

bacterial detection and identification. Synthetic methods have been developed for the efficient and economical production of this series of substrates. After preparation, these compounds were tested against a panel of clinically relevant bacteria. The aim of these substrates was to present an alternative substrate for (*N*-β-alanyl)-7-amino-1-pentylphenoxazine-3-one **86a**, which is applied commercially in chromID® *Pseudomonas aeruginosa* chromogenic medium designed for the clinical detection of *P.aeruginosa*. The new substrates are designed to fully explore the chemical space of phenoxazinonebased chromogenic substrates, and to decrease the colour, as substrate **86a** causes significant background colour in culture media.



86a

The future application of these substrates in chromogenic media resides in their potential to advance the identification of specific pathogenic bacteria and to thus facilitate the treatment of bacterial infections.

1 Table of Contents

Abbreviations

A - absorbance

Ala - alanine

AlaR - alanine racemase

API - analytical profile index

Arg - arginine

B - base

Boc - *tert*-butoxycarbonyl

CAN – cerium(IV) ammonium nitrate

Cbz - benzyloxycarbonyl

CDI - 1,1'-carbonyldiimidazole

CFU - colony-forming unit

CHN - elemental analysis d

– doublet

DBU - 1,8-diazabicyclo[5.4.0]undec-7-ene

DCC - *N*,*N*'-dicyclohexylcarbodiimide

DCM - dichloromethane

DIPEA - *N*,*N*-diisopropyl-*N*-ethylamine

DMAP - 4-dimethylaminopyridine

DMF - *N*,*N*-dimethylformamide

DMSO - dimethylsulfoxide

dpp. - dipeptide permease

E - enzyme

EDCI - 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

E-I - enzyme-inhibitor complex

ELISA - enzyme-linked immunosorbent assay

-

eq equivalent

ESI - electrospray ionisation

ESKAPE - (***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****. aeruginosa*, and ***E****nterobacter species*)

Et - ethyl

Fmoc - fluorenylmethyloxycarbonyl

FT-IR - Fourier transform infrared

g - gram

Gly – glycine Glu –

glutamic acid h -

hour

HBTU - (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HPLC - high performance liquid chromatography

HRMS - high resolution mass spectrometry

I - inhibitor

IBCF - isobutyl chloroformate

Ile - isoleucine

IPA - 2-propanol k -

reaction rate constant

Leu - leucine

logD - log of partition of a chemical compound between the lipid and aqueous phases

Lys - lysine M -

mol/dm^3 m/z -

mass/charge

-

MALDI-TOF - Matrix-Assisted Laser Desorption Ionisation-Time of Flight
Me methyl

Met - methionine

MHz – megahertz

MIC - minimal inhibitory concentration mL

- millilitre

Mp - melting point

MRSA - Methicillin-resistant *S. aureus*

MS - mass spectrometry

MW - microwave

NAG - N-acetylglucosamide

NAM - N-acetylmuramic acid

NAPBQI - (N-acetyl-p-benzoquinone imine)

NMM - *N*-methylmorpholine

NMR - nuclear magnetic resonance

Nor - norvaline

PCR - polymerase chain reaction

Ph - phenyl Phe - phenylalanine pK_a - negative

logarithm of acidic dissociation constant PLP -

pyridoxal 5`-phosphate ppm - part per million Prot -

protecting Pyr - pyroglutamine quart -quartet quin -

quintet

R - general group

R_f – retention factor
RT room temperature

-

s- second s -

singlet spp.

- species

t - triplet

^{t}Bu - *tert*-butyl

TEA - triethyl amine

TFA - trifluoracetic acid

THF - tetrahydrofuran

THP - tetrahydropyran

TIS - triisopropylsilane

TNBS - Trinitrobenzenesulfonic acid

Tosyl/Tos - *para*-toluenesulfonyl

TP - 2-chloro-trityl chloride resin

Tr - trityl

Tyr - tyrosine

UV/VIS - ultraviolet/visible

WHO - World Health Organisation

δ - chemical shift (ppm) v

- wave number (cm^{-1})

□ - molar extinction coefficient

□- wave length (nm)

1 Introduction

1.1 Importance of bacterial detection and identification

A significant proportion of known infectious diseases are caused by bacteria. Some important examples include tuberculosis, which is caused by *Mycobacterium tuberculosis,* or pneumonia which can be caused by *Streptococcus pneumoniae*. From the middle of the last century until recently, most bacterial infections were readily treated by an appropriate antibacterial agent. Resistance against most antibacterial drugs has normally been observed a few years after their appearance on the market and introduction into clinical practice.[1] It is estimated by O`Neill that, due to the emergence of resistant and multi-resistant pathogenic bacteria, in 2050 more patients will die due to bacterial infections than by cancer.[2] This shows the seriousness of the impending global problem that antimicrobial resistant bacteria will pose in the decades to come.

The fight against antimicrobial resistance can be supported by discovering new targetable mechanisms that are essential for bacteria. This, along with systematic searches and serendipitous discoveries, will support the invention and development of novel antibacterial drugs and drug mixtures. The rate at which new resistant strains emerge can be minimised by the antibiotic stewardship strategy, which aims to avoid unnecessary use and to promote appropriate use of antimicrobial agents.[3] Targeted treatment with narrowspectrum antibacterials is also essential to avoid increasing selection for resistance. This requires rapid, economic and reliable identification methods for pathogenic bacterial species.[2]

1.1.1 Brief overview of bacterial structure

Bacteria are single-celled microorganisms. They exist on the micrometre size scale, but occur with different morphologies. These microorganisms have DNA as genetic material and ribosomes floating in the cytoplasm, which is surrounded by cell membranes, cell wall and often with a capsule. Where they are motile, this is due to pili or flagellae (Figure 1).

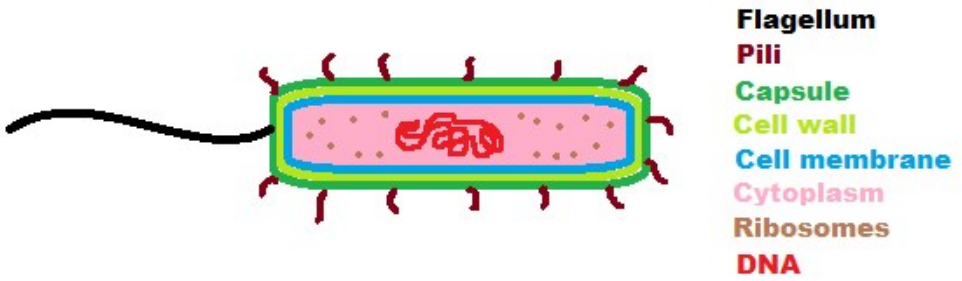


Figure 1 *General structure of bacteria*

Bacteria can be characterised into two main groups by key differences in their cell wall structure through Gram staining. During Gram staining the bacteria is treated with crystal violet dye. This causes Gram-positive bacteria to exhibit purple coloration, but within Gram-negative bacteria this coloration is not visible. The different results of this test are due to the difference between the cell wall of Gram-positive and negative bacteria. The cell wall of Gram-positive bacteria contains a cytoplasmic phospholipid membrane and an outer peptidoglycan layer. The peptidoglycan layer is a network of polysaccharide chains, containing *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), linked with oligopeptides containing L-(Ala, Lys), D-(Glu, Ala) amino acids and a pentaglycine subunit. The cell envelope of Gram-negative bacteria contains 2 layers of phospholipid layers with a thinner peptidoglycan part between them. Due to this extra layer of protection these bacteria are often hardier, and more resistant to threats, such as toxins including antimicrobials, that they encounter in their environment (Figure 2).[4]

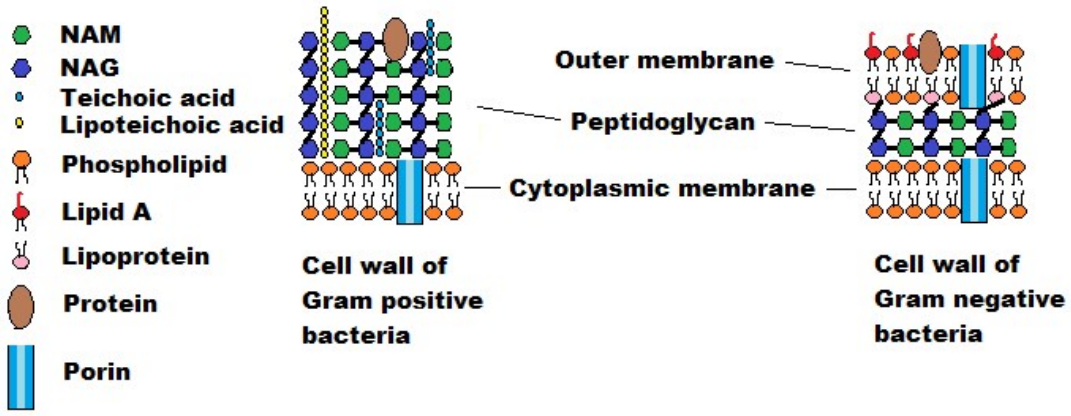


Figure 2 *Structure of the cell wall of Gram-positive and Gram-negative bacteria*

1.1.2 Clinically relevant pathogenic bacteria

Amongst bacterial species, only a small proportion are pathogenic and responsible for infections and diseases: according to a study more than 2000 bacterial species live inside the human body, and less than 100 species were identified as potential pathogens.[5] A high proportion of nosocomial infections are caused by the so-called 'ESKAPE' pathogens (***E****nterococcus faecium*, ***S****. aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****. aeruginosa*, and ***E****nterobacter species*) or *Clostridium difficile.* Some key characteristics associated with these bacteria from a clinical viewpoint are introduced in Table 1; all of these organisms have a potential to develop multi drug resistance, and "escape" from the action of antibacterial drugs.[6,7]

Pathogenic bacterial species	***Gram staining***	***Infection caused***
Enterococcus faecium	positive	urinary tract and soft tissue infections sites in patients with a compromised immune system[8]
Staphylococcus aureus	positive	skin, respiratory infections or food poisoning; released toxins and enzymes are destructive[8]
Klebsiella pneumoniae	negative	lobar pneumonia[8]
Acinetobacter baumannii	negative	opportunistic pathogen in hospitals, affecting patients with a compromised immune system; causing a range of different diseases, such as pneumonia.[9]

Pseudomonas aeruginosa	negative	opportunistic pathogen, mostly affecting immunocompromised people with other diseases, such as cystic fibrosis; causing hospital-acquired infections, such as ventilator-associated pneumonia and sepsis.[8]
Enterobacter spp	negative	opportunistic pathogen in hospitals, affecting patients with a compromised immune system; causing urinary tract and respiratory infections[8]
Clostridium difficile	positive	Affecting patients during antibiotic therapy; toxin producing; causing diarrhoea and inflammation, can be fatal[8]

Table 1 *Main features of the 'ESKAPE' pathogens and Clostridium difficile*

1.2 Current techniques for bacterial detection and identification

Current techniques for bacterial identification comprise several established methods, in addition to traditional Gram staining and identification of colony appearance. These include the recently adopted technique of Matrix-Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectrometry,[10] which is a successful and rapid method for identification of bacterial cells and their biomolecular building blocks, such as ribosomal and other proteins. This usually provides a definitive identification, as the genome and proteome of each species and strain of bacteria will be characteristic to it. Moreover, bacteria can be detected and identified by techniques based on enzyme-linked immunosorbent assay (ELISA), biochemical assays (e.g. analytical profile index (API) strips), or polymerase chain reaction (PCR) methods.[11] However, while powerful, these techniques have disadvantages. For instance, MALDI-TOF requires substantial investment in expensive equipment, while PCR requires trained specialists. Some of these modern techniques are time consuming: methods such as API require the growth of a bacterial culture before the assay procedure. Also, samples with multiple organisms require further purification steps. These barriers can limit the

potential scope of application for these methods. Additionally, these issues are most acutely felt in the developing world, where the frontline battle against the emergence of resistant pathogenic bacteria is already well underway, but also where the requisite investment in expensive cutting-edge technologies is not a viable option.

Another well-established group of techniques for bacterial detection and identification is based on detection of specific bacterial enzyme activity, as exploited by chromogenic and fluorogenic culture media. These culture media provide selective isolation of pathogenic bacteria from clinical samples using one or more antimicrobials with concomitant identification using a chromogenic or fluorogenic substrate, targeted to a specific bacterial enzyme.[12] These targeted enzymes hydrolyse the fluorogenic and chromogenic substrates, resulting in coloured or fluorescent compounds to indicate the specific enzyme activity. Methods of this type are much more suited to deployment in the developing world, as well as in developed nations, as they require much lower up-front costs and ongoing resources, and the training required to carry out such tests is within the capability of a wide range of healthcare employees.

Chromogenic media contains a matrix, such as agar, nutrients and chromogenic substrates that are used to make certain strains visible by special coloration providing the basis of their detection. These media normally contain antibacterial agents to prevent the growth of non-relevant bacteria, which improves selectivity.

In this thesis two distinct groups of components of chromogenic culture media were developed. The main focus was to discover new alanine racemase inhibitors as antibacterial agents, selectively inhibiting the growth of certain bacteria, thereby increasing the selectivity of the chromogenic substrates used in existing media. However, in addition, new chromogenic substrates were also investigated. These compounds were analogues with a well-established substrate applied in chromID Pseudomonas® chromogenic culture medium.

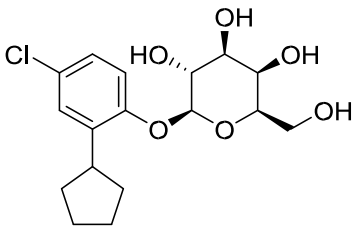
2 Suicide substrates

2.1 Introduction

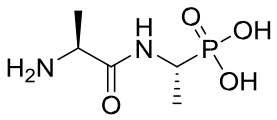
In a clinical sample from a non-sterile body site there are often many other commensal bacterial strains, besides the pathogen. These additional organisms can compromise the detection of the pathogen, because they may affect the growth of the pathogenic bacteria or produce the same target enzyme. To avoid this issue, selective antibiotics or antibacterial agents can be used in the culture medium to prevent the commensal microorganisms in a clinical sample from growing in culture. These added compounds can act on a common enzyme, such as alanine racemase (AlaR), which is potentially present at the genetic level, but not expressed, or not accessible to the targeted pathogen, due to limited transport through the cell-wall.

Suicide substrates can be used as antibacterial agents, which iare defined by the medicinal dictionary as a compound that is not toxic to a cell, but which liberate a closely-related metabolite which undergoes metabolic transformation to a product that does inhibit a crucial enzyme.

A number of enzyme substrates, acting as antibacterial compounds have been reported to be applied successfully in culture media for selective isolation of certain bacterial species from a competitive environment. For example, *Shigella* was selectively enriched in the presence of *Escherichia coli* by using 4-chloro-2-cyclopentylphenyl-□-D-galactopyranoside **1** as a selective substrate (Figure 3). The antibacterial effect of the substrate is based on the □-galactosidase activity of *E. coli*, which is lacking in *Shigella*. The enzyme present in *E. coli* releases 4-chloro-2-cyclopentylphenol by hydrolysis, which is toxic for *E. coli* but any *Shigella* present would be unaffected and continue to be able to grow allowing for their subsequent detection.[13,14] *Salmonella* species were selectively isolated from clinical samples with alafosfalin **2-LL**; further details of the action of alafosfalin **2-LL** are discussed in later chapters (Figure 3).[15] Further research and development is required for the selective identification and isolation of pathogenic bacteria within clinically relevant mixtures of species, as this has only been optimized for a few cases.[12]



1

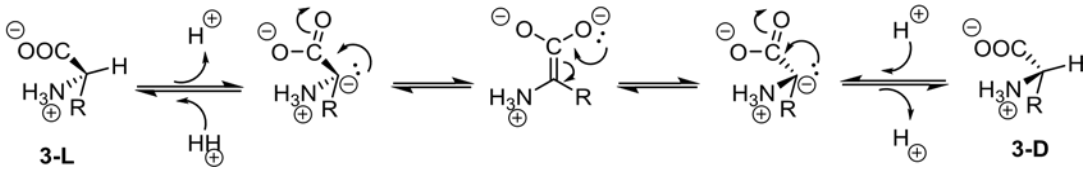


2-LL

Figure 3 *4-Chloro-2-cyclopentylphenyl-□-D-galactopyranoside* ***1*** *and alafosfalin* ***2-LL*** *as selective antibacterial agents*

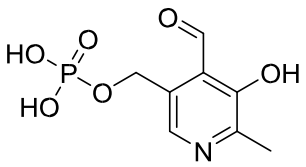
2.1.1 Alanine racemase (AlaR)

AlaR is an amino acid racemase enzyme. Amino acid racemases are responsible for catalysing transformation of the natural L-amino acids to their D-isomer by racemisation (Scheme 1).[16] AlaR acts on L-alanine **3-L** to form a racemic mixture of L- **3-L** and D-alanine **3-D**. The latter is the building block of the bacterial peptidoglycan, and therefore it is essential for all bacteria to build their cell wall.



Scheme 1 *Racemisation of amino acids*

AlaR is a pyridoxal 5`-phosphate (PLP **4**) dependent enzyme, which means PLP **4** is an essential coenzyme for the function of the alanine racemase (Figure 4).[16]



4

Figure 4 *Pyridoxal 5`-phosphate (PLP), shown as the corresponding acid*

There are a number of published X-ray crystal structures of AlaR extracted from different bacteria. Some of these structures included a modified alanine ligand or an inhibitor crystallized with the enzyme, which provides extended knowledge of its mechanism of action and structural features.[17-20] AlaR is a homodimer: the 2 active sites are placed between the segments and each contains the PLP coenzyme and a water molecule inside.

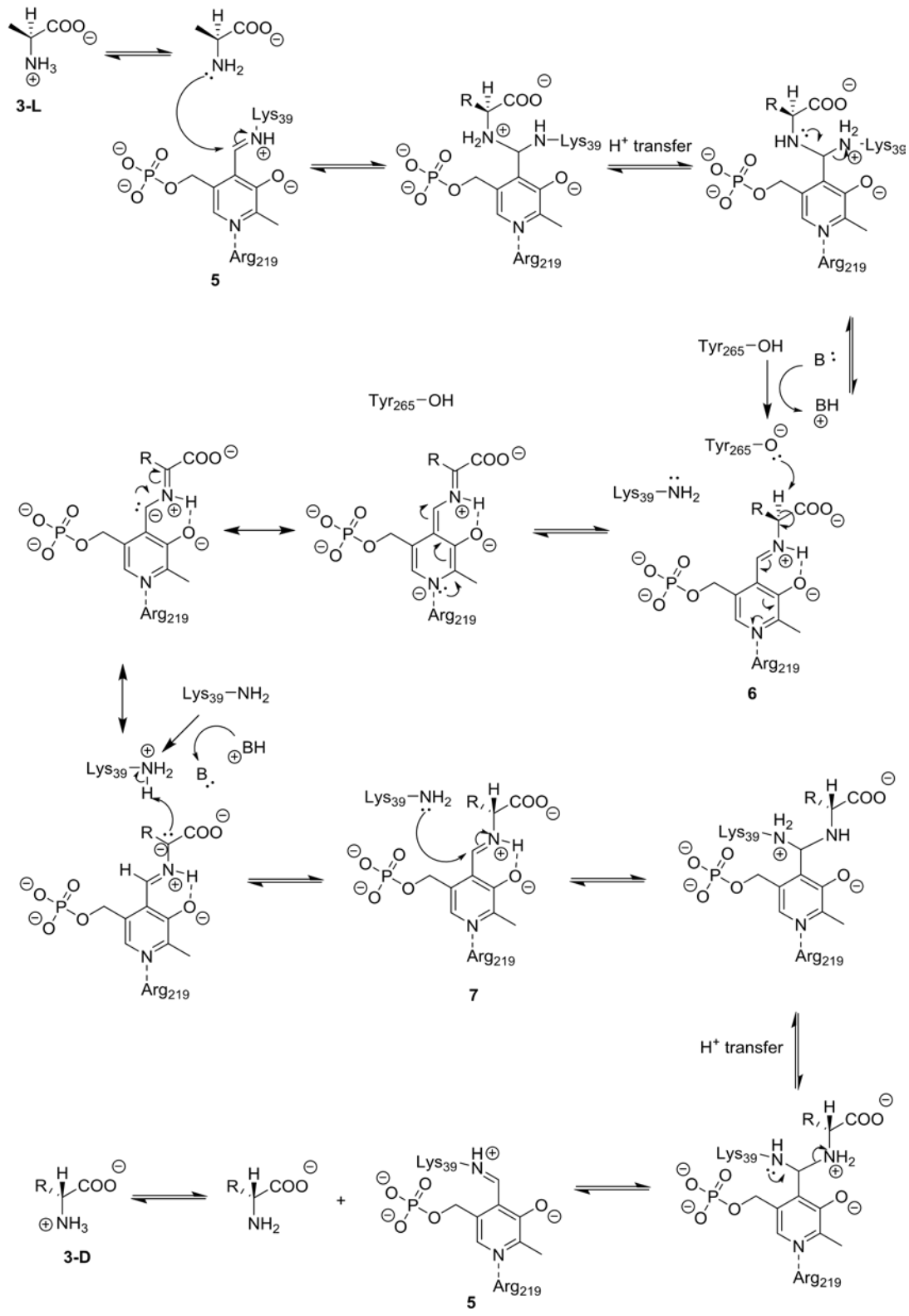
Previous studies mostly support a two-base mechanism of AlaR,[7,21,22] which states that the racemisation is assisted by two amino acid residues (Lys and Tyr). In the active site of AlaR, in most studied bacterial strains, PLP is connected to the Lys_{39} residue as an internal aldimine **5**. Lys_{39} can be replaced

by an L-alanine **3-L**,[18] forming an external aldimine **6.** This species is activated for racemisation, as the pK_a of the α-H of the alanine is decreased from 16.5[23] to approximately 14[24] by the Schiff`s base formation. In other PLP-dependent amino acid racemase enzymes, the nitrogen of the PLP-pyridine is protonated in order to provide a sufficiently low pK_a to facilitate the deprotonation step. In alanine racemase, however, the lone pair of the basic nitrogen of the PLP is involved in a hydrogen bond with Arg_{219}. According to the findings of Dixon & Bruice, a protonated PLP-complex provides a more acidic α-H than an unprotonated analogue.[24] However, due to solvent effects, the PLP displays similar catalytic activity in AlaR as in other amino acid racemases [25-27]. The αposition of the external L-alanine aldimine **6** can be deprotonated by the Tyr_{265} phenolate anion, and reprotonated from the opposite side of the PLPconjugated planar complex by the Lys_{39} side chain ammonium ion. This process results in the formation of the corresponding D-alanine external aldimine **7**. D-Alanine **3-D** is liberated from the active site complex after Lys_{39} attacks the external aldimine **7** and forms the internal aldimine **5** to restart the catalytic cycle (Scheme 2).

At the time of writing there is still discussion in the literature regarding the fine details behind some of these key stages of racemisation. Unresolved questions include: how does the Tyr-OH get deprotonated and how does the Lys-NH_2 get re-protonated? Without these additional steps, the reaction should stop after a single turnover. After no water or other residues were found that would enable the proton transfer between Lys_{39} and Tyr_{265},[18] Esaki *et al*. suggested that the carboxyl moiety is involved in this process[10], although the Major group subsequently reported computational results showing that the carboxyl group did not orientate correctly to Tyr_{265}: the 180° rotation of the carboxylic group between the Lys_{39} and Tyr_{265} residues requires 15 kcal/mol, and it is hindered by hydrogen bonds with Met_{312} and Arg_{136}.[26] The enzyme can also transform D-alanine **3-D** to the L-enantiomer **3-L** using a similar process shown in Scheme 2, although this process is slower than the L to D transformation.[28] In this case, the D-alanine external aldimine is deprotonated by the free amino group of Lys_{39} and reprotonated by the acidic phenolic moiety of Tyr_{265} [22]. There are bacteria that have their catalytic Lys or Tyr

residues in different places and the active site is thus distinct, such as that found in *Streptomyces coelicolor.*[29] This feature of different alanine racemases doesn`t change the mechanism of the racemisation, although it might cause selective action by certain inhibitors.

The suggested mechanism is based on the above cited publications aiming to highlight and summarize their findings (Scheme 2). The mechanism of action and the structure has been mainly studied using *Bacillus stearothermophilus*.



Scheme 2 *Mechanism of action of racemisation of L-alanine by AlaR. (Adapted from references:[7,16,18,21,22,25-27])*

2.1.2 Alanine racemase inhibitors

AlaR is one of the best studied racemases, therefore several AlaR inhibitors have been developed and published. The structures of these inhibitors tend to display obvious similarities to the natural substrate, and could be considered close analogues to it. D-Cycloserine **8-D**[30] and *O*-carbamoyl-Dserine **9-L**[31] can inhibit AlaR in many bacteria. D-Cycloserine **8-D,** also known as Seromycin, is an effective antibacterial agent against *Mycobacterium avium* and *Mycobacterium tuberculosis* and was included on the WHO list of essential medicines required for a basic healthcare system in 2015.[32] Some D-alanine analogues, such as β-haloalanine **10-L**,[33,34] β,β,β-trifluoroalanine **11-L**,[35] and β-halovinylglycine **12-L**,[36] also have remarkable inhibitory effects. L-Fosfalin **13-L**[37] and L-1-aminoethylboronic acid **14-L**[38] are both slow binding and time dependent inhibitors of AlaR (Figure 5).[16,39] L-Fosfalin **13-L** is not an effective antibacterial agent in its unmodified form, because it passes through the cell membrane in insufficient quantities to have an inhibitory effect on AlaR. However, C-terminal fosfalin containing di- and oligopeptides show significant antibacterial activity, presumably by taking advantage of active transport systems. Once successfully inside the bacterial cell, peptidase activity upon these oligopeptides can release active fosfalin in a pro-drug type process.

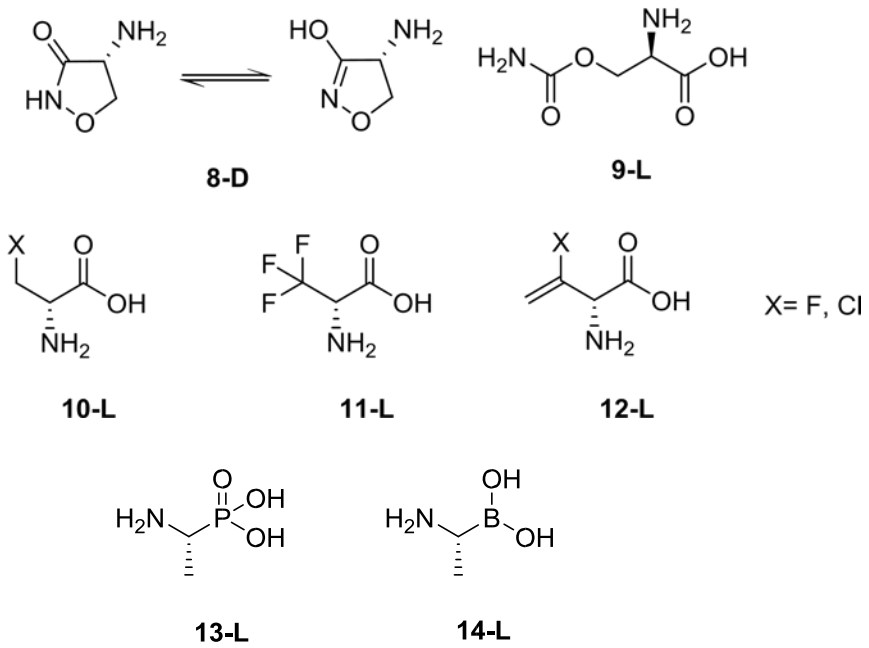
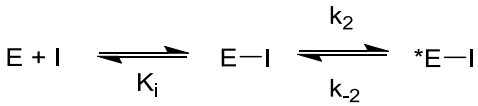


Figure 5 *Known AlaR inhibitors*[38]

From the perspective of the research reported within this thesis, L-fosfalin **13L** is the most relevant inhibitor of alanine racemase from which to continue this discussion. Its mechanism of inhibition of AlaR has been studied in detail. The Walsh group investigated the kinetics of the inhibition of the Gram-positive *Bacillus stearothermophilus* alanine racemase and discovered that it was timedependent.[37] At the beginning of the process, L-fosfalin **13-L** (I) acts as a competitive inhibitor toward the natural substrate L-alanine **3-L**, as both substances compete for the active site of AlaR (E), (K_i = 1 mM). In the case where the inhibitor (I) binds, it forms the enzyme-inhibitor complex (E-I) (Scheme 3). After the initial formation of the enzyme-inhibitor complex (E-I), the inhibition becomes non-competitive, as it was found that the K_m of the native enzyme is the same as the K_m after preincubation with fosfalin. As the fosfalin binds, the amount of free enzymatic sites decreases, thus reduction of V_{max} was observed.[37] The external aldimine **15** changes the structure of the active site (E*-I) making itself unavailable for racemisation or hydrolysis (Figure 6). This is thought to be due to the interactions made by the tetrahedral dianionic phosphate group, which alters the position of Lys39, Tyr265 and Arg136 by hydrogen bonding interactions, most importantly directing Lys39 away from the imine moiety of the PLP-complex.[7,40] The dissociation of the fosfalin–AlaR complex occurs very slowly (k_2>>>k_{-2}), thus inhibition persists for long periods of time once effective binding has taken place ($t_{1/2}$ + 25 days).



Scheme 3 *Reaction scheme of inhibition of Bacillus stearothermophilus alanine racemase with fosfalin* ***13-L****. K_i = 1 mM, k_2 =10 min^{-1}, k_{-2} = 5 x 10^{-5} min^{-1}.[37,40] E: enzyme, I: inhibitor, E-I: enzyme-inhibitor complex, *E-I: modified enzyme-inhibitor complex.*

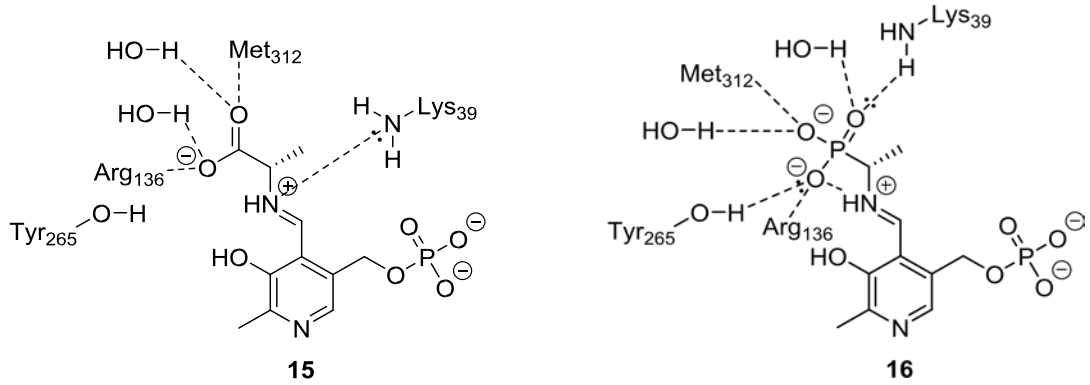


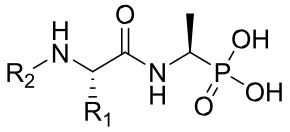
Figure 6 *The external aldimine of alanine* ***15***[20] *and L-fosfalin* ***16***[17] *(*E-I) in the active site of AlaR.*

Amino acid and peptide transport

Bacteria use oligopeptides to provide their amino acid supplies.[41] These molecules are transported mostly by active transport, which is carried out by oligopeptide[42] or dipeptide[43] permease proteins located in the plasma membrane and in periplasmic space (Dpp. in *E. coli*).[43] Once internalised, these oligopeptides are hydrolysed by specific aminopeptidases located in the cytoplasm or in the plasma membrane to release the free amino acids ready for use by the bacterium. The oligopeptide carrier protein recognises only the amino group of the *N*-terminal amino acid of the peptide and largely ignores the carboxylic terminus; this feature allows peptides with different chain lengths to cross the cell wall by this system. However, in the example of transporting homolysine oligopeptides into *E. coli*, it was found that homologues bigger than or equivalent to pentalysine could not be transported into the cell.[44] These data suggest that these permeases are only able to transport oligopeptides with limited chain length . Some single amino acids can be taken up by a specific amino acid permease in parallel with the oligopeptide form, such as arginine by OccD1/OprD protein in *P. aeruginosa*[45] or methionine in *E. coli*.[46] Alanine, which is the most relevant for the project, can only pass bacterial cell walls as a constituent of a di- or oligo- peptide, for example by the DppA dipeptide permease system in *E. coli*.[43]

2.1.2.1 Alanine bioisostere containing peptides as antibacterial agents

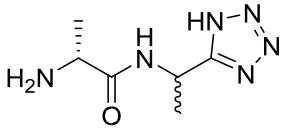
Amino acid analogue drug molecules, such as L-fosfalin **13-L**, can be transported into the cell as di- or oligopeptides containing C-terminal fosfalin to increase the antibacterial activity. A number of di-, tri- and larger oligopeptides **17** were designed for selective action by different research groups (Figure 7).[47-51] Selectivity is provided by the variety of aminopeptidase enzymes and permeases in different species and strains.



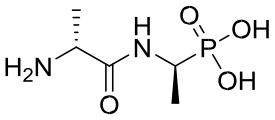
17

Figure 7 *Schematic structure of C-terminal fosfalin containing di- or oligopeptides; R_1: most natural, and some unnatural amino acid side chains, R_2: H or amino acid or peptide block*

As permease systems and aminopeptidases naturally work on L-stereoisomers, the L configuration is necessary for all amino acid building blocks of these di- and oligopeptide derivatives of fosfalin for significant antibacterial action. In the early research in this area, D-alanyl-D,L-1aminoethyltetrazole **18-DD/L** [52] and D-alanyl-D-fosfalin **2-LL**[47] were investigated as synthetic D-alanyl-D-alanine peptidoglycan building block mimetics targeting the inhibition of cell wall biosynthesis (Figure 8). However, these compounds were found to be inactive against all studied bacterial strains.



18-DD/L



2-LL

Figure 8 *D-alanyl-D,L-1-aminoethyltetrazole **18-DD/L**[52] and D-Alanyl-Dfosfalin **2-LL**[47]*

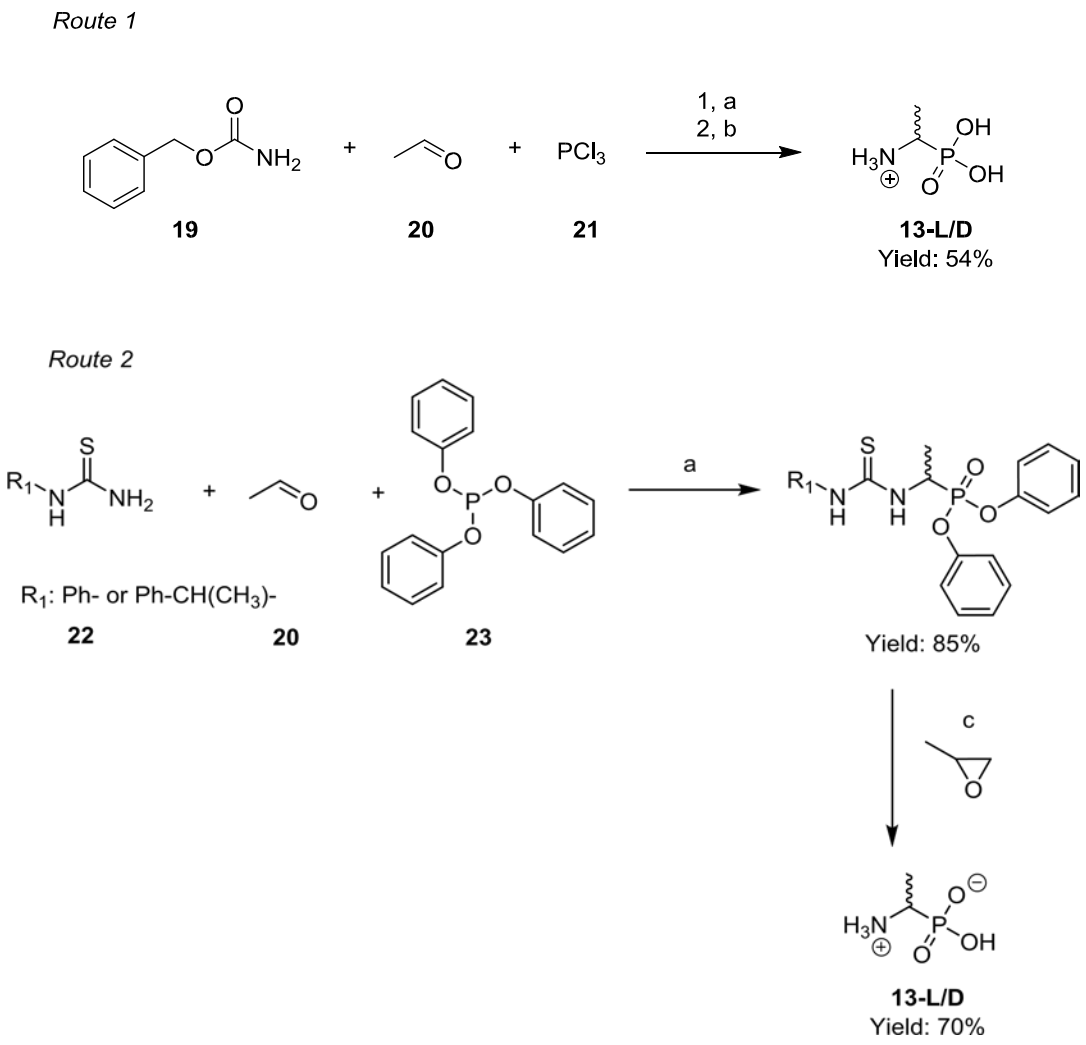
Further investigation of other L-L, D-L, L-D isomers of alanyl-1aminoethyltetrazole **18-LL/D**[53] showed these compounds to be inactive as

antibacterial agents. However, their action was studied against only selected bacteria, focusing on *E. coli* strains. Due to this narrow range of prior biological testing and the insufficiently described purity and characterisation of these peptides within the cited paper, substrate **18-LL/D** and other similar compounds were prepared, fully characterised, and their antibacterial action was investigated against a wide range of clinically relevant pathogenic microbes in this thesis.

2.1.3 Synthetic approaches

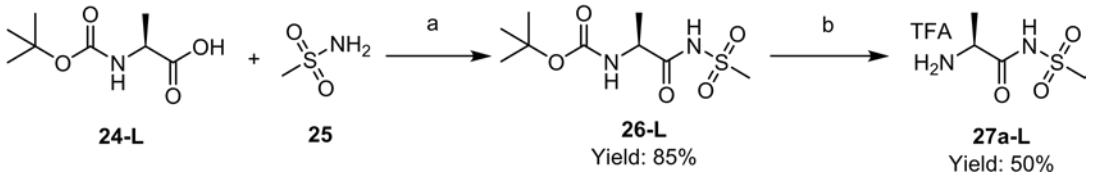
2.1.3.1 Synthesis of alanine bioisosteres

The alanine analogue fosfalin **13-L,** a well investigated AlaR inhibitor, was synthesised by two similar known methods.[54, 55] Both methods used acetaldehyde **20**, a trivalent phosphorous compound containing bonds between a phosphorus atom and three higher electronegativity atoms **21** or **23**, and a carbamate **19** or thiocarbamide **22** as reactants. Both reactions start with Schiff base formation between the aldehyde **20** and the carbamide **22** or thiocarbamate **19**, this is followed by the attack of the lone pair of the phosphorus reagent on the electrophilic imine carbon.[54] Synthetic route 1 is called the Oleksyszyn reaction (Scheme 4).[55] In both routes the protecting groups were removed by acidic hydrolysis.



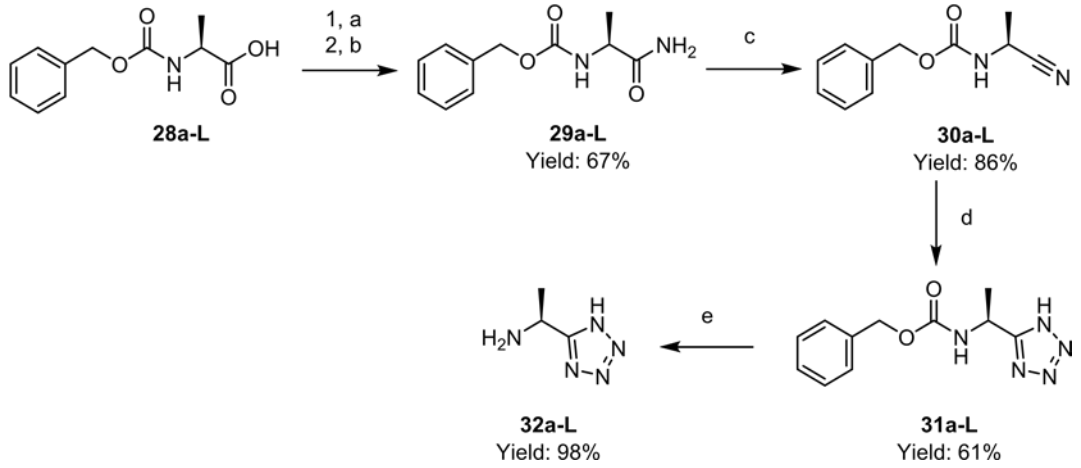
Scheme 4 *Two established methods to make fosfalin* ***13-L/D****.*[54, 55] *Reagents and conditions: (a) CH_3COOH, reflux; (b) H_2O, HCl, reflux; (c) AcOH/HCl (1:10), reflux.*

As well as the phosphonic acid found within fosfalin, there are other potential bioisosteres for replacement of the carboxylic acid moiety within alanine known from other research areas. However, compounds displaying these functional groups have not been systematically evaluated as inhibitors of AlaR. One such molecule is L-alanylmethanesulfonamide **27a-L**. This compound has been made previously by a condensation reaction between methanesulfonamide **25** and Boc-L-alanine **24-L** [56] to form the Boc protected L-alanylmethanesulfonamide **26-L** (Scheme 5), although it does not appear to have been tested for AlaR inhibitory activity.



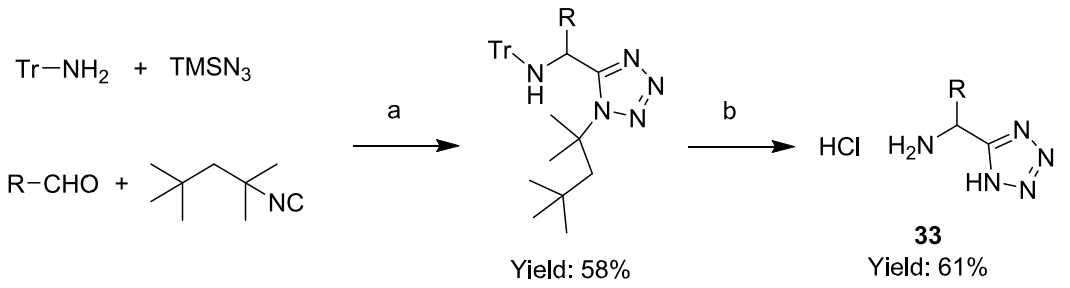
Scheme 5 *Synthesis of L-alanylsulfonamide TFA salt* ***27a-L****[56]. Reagents and conditions: (a) DMAP, EDCI, DCM, r.t.; (b) TFA, DCM, r.t..*

Another possible carboxylic acid bioisosteric analogue of L-Ala is L-1aminoethyltetrazole **32a-L**, which has been made from Cbz-L-alanine **28a-L**. Within this route, the carboxylic moiety was converted to an amide **29a-L**, then dehydrated to produce the corresponding nitrile group found within **30a-L**. The protected tetrazole **31a-L** was synthesised by a bipolar cycloaddition reaction between the Cbz-alanine nitrile **30a-L** and sodium azide, followed by the removal of the Cbz protecting group in the final step by catalytic hydrogenation.[57] (Scheme 6). Tetrazole analogues of other amino acids were prepared by a similar method from the appropriate amino acids by Grzonka and Liberek[58,59] and by the Smissmann group.[52]



Scheme 6 *Synthesis of 1-aminoethyltetrazole* ***32a-L****[57] Reagents and conditions: (a) IBCF, TEA, THF, -15 °C; (b)* NH_3*; (c) pyridine, TosCl, DCM, 0 °C; (d)* NH_4Cl*,* NaN_3*, DMF, 90 °C; (e)* H_2*, Pd/C, ethanol, r.t., atm..*

A library of racemic tetrazole analogues of amino acids **33** were synthesised using the Ugi reaction under microwave irradiation (Scheme 7).[60]



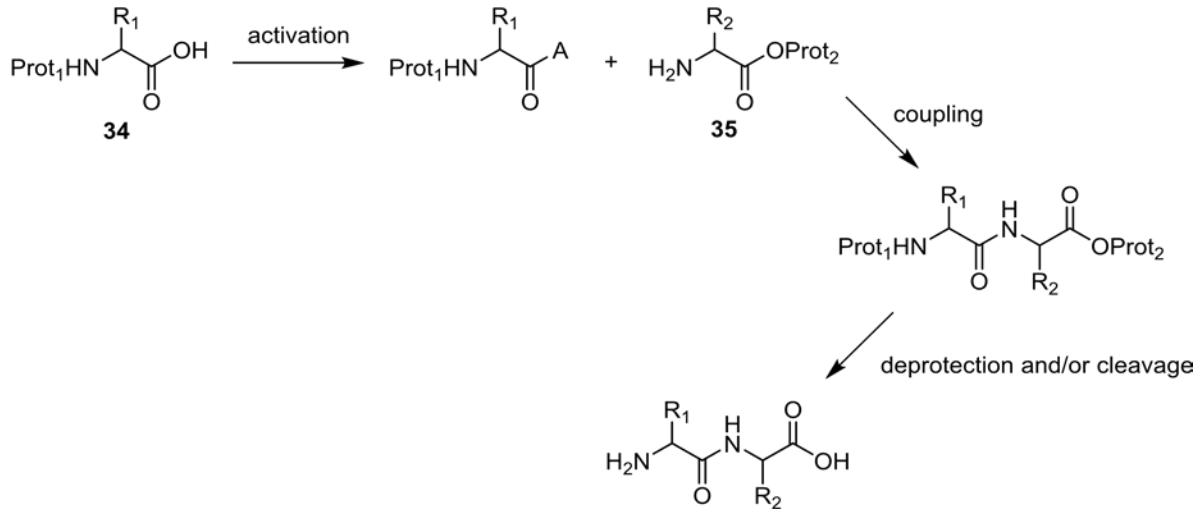
Scheme 7 *Synthesis of racemic tetrazole analogues of amino acids **33** by Ugi reaction.[60] Reagents and conditions: (a) MeOH, MW, 100 °C, 30 mins; (b) 1.25 M HCl, EtOH, reflux. R: amino acid side chains, Tr = trityl.*

2.1.3.2 General methods for peptide synthesis

Di- or oligopeptide forms of these potential AlaR inhibitors are assumed to be necessary for successful transportation, especially if they are alanine analogues employing carboxylic acid bioisosteres. There are two established ways to synthesise peptides: solution phase and solid phase synthesis. While coupling in solution phase is a suitable method to synthesise small peptides, longer peptides usually require solid phase synthesis in order to gain the final peptide in good yield.[61]

In the homogenous solution phase reactions, all reactants are in the same liquid phase, while in heterogeneous solid phase synthesis, one of the reactant amino acids or peptides is attached to a solid phase resin carrier. In both synthetic approaches, reactive groups, such as amino-, hydroxyl- and carboxyl-moieties on non-reacting termini or on side chains, need to be protected by special orthogonal protective groups, if their integrity is expected to be unchanged after completion of all of the required synthetic steps. After activation of the carboxylic motif of one of the amino acid **34** or peptide reactants, it will react with a free amino group of another amino acid **35** or peptide to form a new peptide bond (Scheme 8). Protecting groups for amino moieties are most commonly Boc, Cbz or Fmoc, while carboxylic acids can be protected as an ester, and hydroxyl motifs as silyl- or benzyl ethers. Activation reagents include the classic DCC, CDI, IBCF or HBTU, the latter of which is preferred in solid phase synthesis, because it can activate the carboxylic group very quickly (less than 30 mins) and conveniently.[62] The reaction mixture

normally contains an organic amine base, such as NMM or DIPEA. In solution phase, it is normal to utilise equimolar quantities of peptide and activating reagent. This usually results in good yields. In contrast, in solid phase synthesis, a large excess of reagents and solvent are used in order to achieve near quantitative conversions. The excess is washed away as waste after the reaction. In summary, the solid phase synthetic approach results in the synthetic peptide with nearly quantitative yield even with longer chain peptides, although, due to the large excess of reagents, it creates more waste than solution phase methodology.

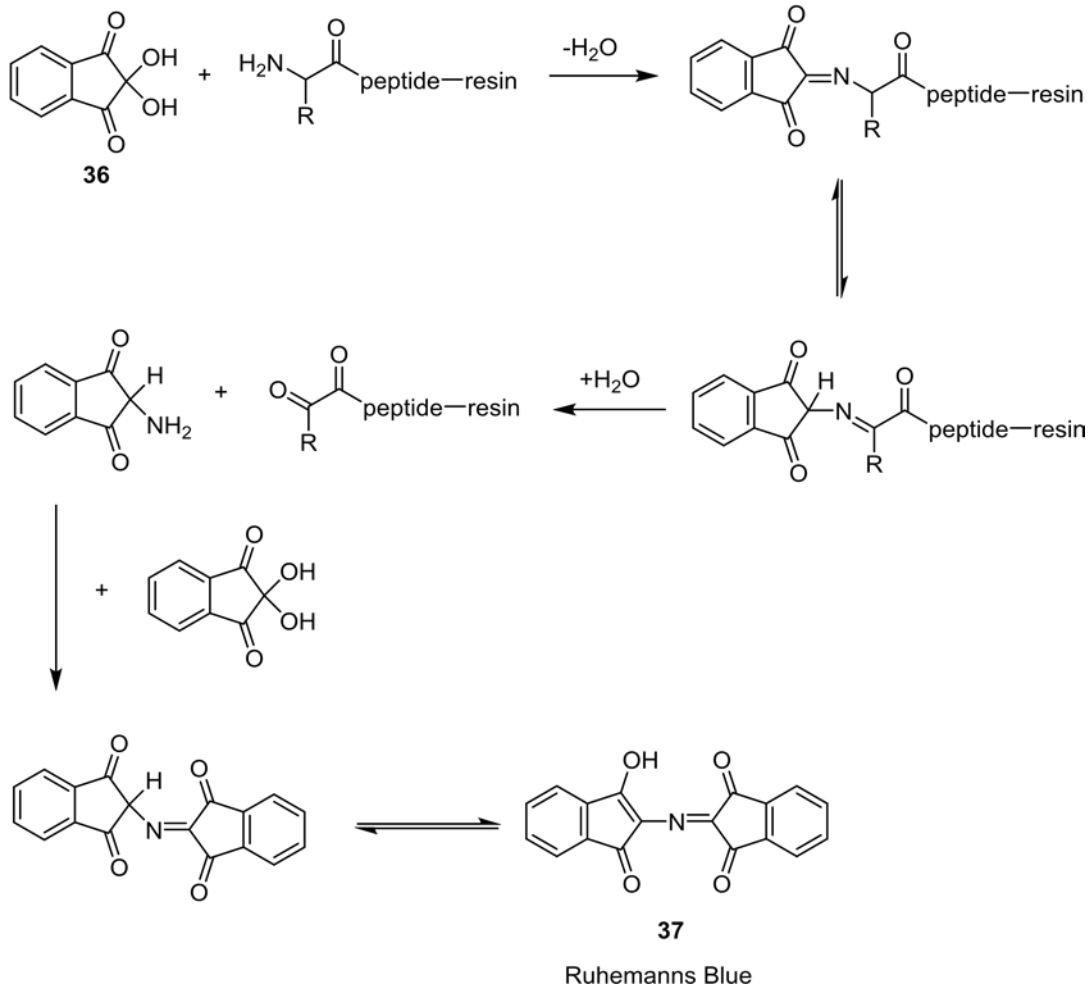


Scheme 8 *General procedure of peptide coupling. R_1 and R_2: amino acid side chains with protecting groups as necessary, $Prot_1$: mostly Cbz-, Fmoc-, Boc-groups, $Prot_2$: alkyl group (solution phase) or resin (solid phase), A: activating group (anhydride, benzotriazolyl ester)*

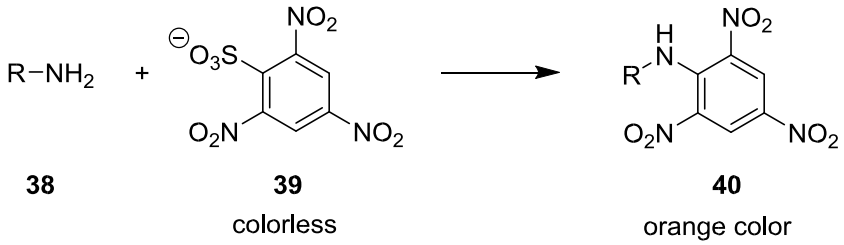
There are two main protecting group approaches used in solid phase peptide synthesis, depending on the orthogonal protecting group strategy. These are Fmoc[63] and Boc[64] based solid phase synthesis. In each approach, reagents are selected that allow for deprotection of the *N*-terminus without cleaving the peptide from the resin, or the removal of other protecting groups from the side chains of the peptide. These methodologies require different resins.[65] In the Fmoc approach, an acid-sensitive and base-resistant resin is required, such as 2-chlorotrityl chloride resin or Wang resin, allowing base-sensitive protecting groups to be readily removed and preserving the integrity of functional groups protected with acid-sensitive groups. In contrast, in the Boc

methodology, the Merrifield resin is commonly employed, as the resin is required to be resistant to milder acidic conditions, so acid-sensitive protecting groups can be removed, while other protecting groups and the resin remain untouched. The Fmoc technique is generally deemed preferable in modern peptide synthesis, due to the milder and safer cleavage conditions for removal of the resin from the synthesised peptide at the end of the synthesis (TFA instead of hydrofluoric acid).

There are a few methods developed to track solid phase peptide synthesis. The detection of free amino groups gives information regarding the extent of completion of the coupling reaction, and Fmoc groups can be detected in order to track the Fmoc deprotection reactions. To detect free primary amino groups, the Kaiser test and the TNBS tests are normally used. The Kaiser test is performed with the ninhydrin reagent **36**, in a phenol, pyridine and ethanol solvent mixture. The test is carried out in the presence of potassium cyanide, which is utilised as a reducing agent; a positive reaction gives a blue colouration **37** on the resin beads (Scheme 9).[66] The TNBS test is a reaction of primary amines **38** with 2,4,6-trinitrobenzenesulfonic acid (TNBS) **39** in the presence of diisopropyl-ethylamine in DMF and water. When primary amino moieties within the resin beads are present an orange or red colour **40** (Scheme 10) is produced.[67] Fmoc groups can be detected by treating a few resin beads with TFA in DCM; the residual cleaved dibenzofulvene is a good chromophore, which can be easily detected under UV light or checked by UV spectroscopy.

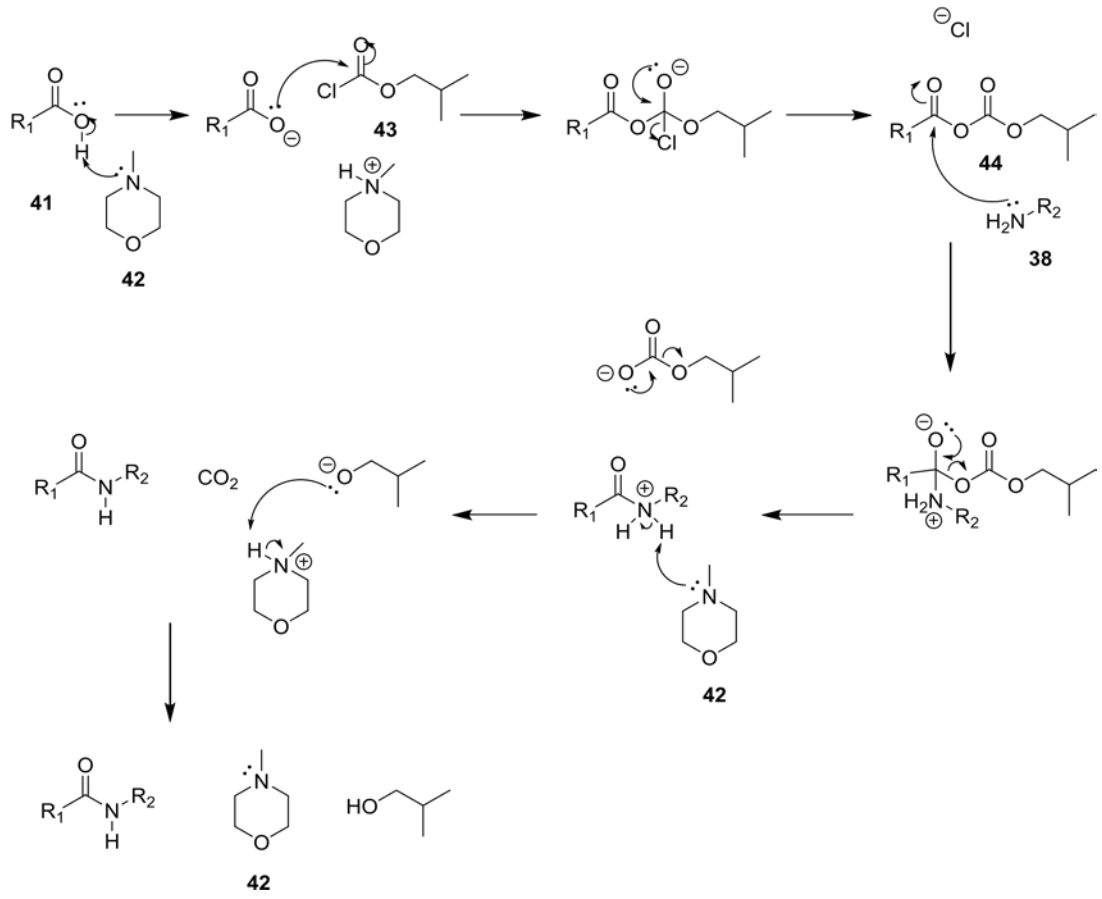


Scheme 9 *Action of ninhydrin reagent* ***36*** *in the Kaiser test*



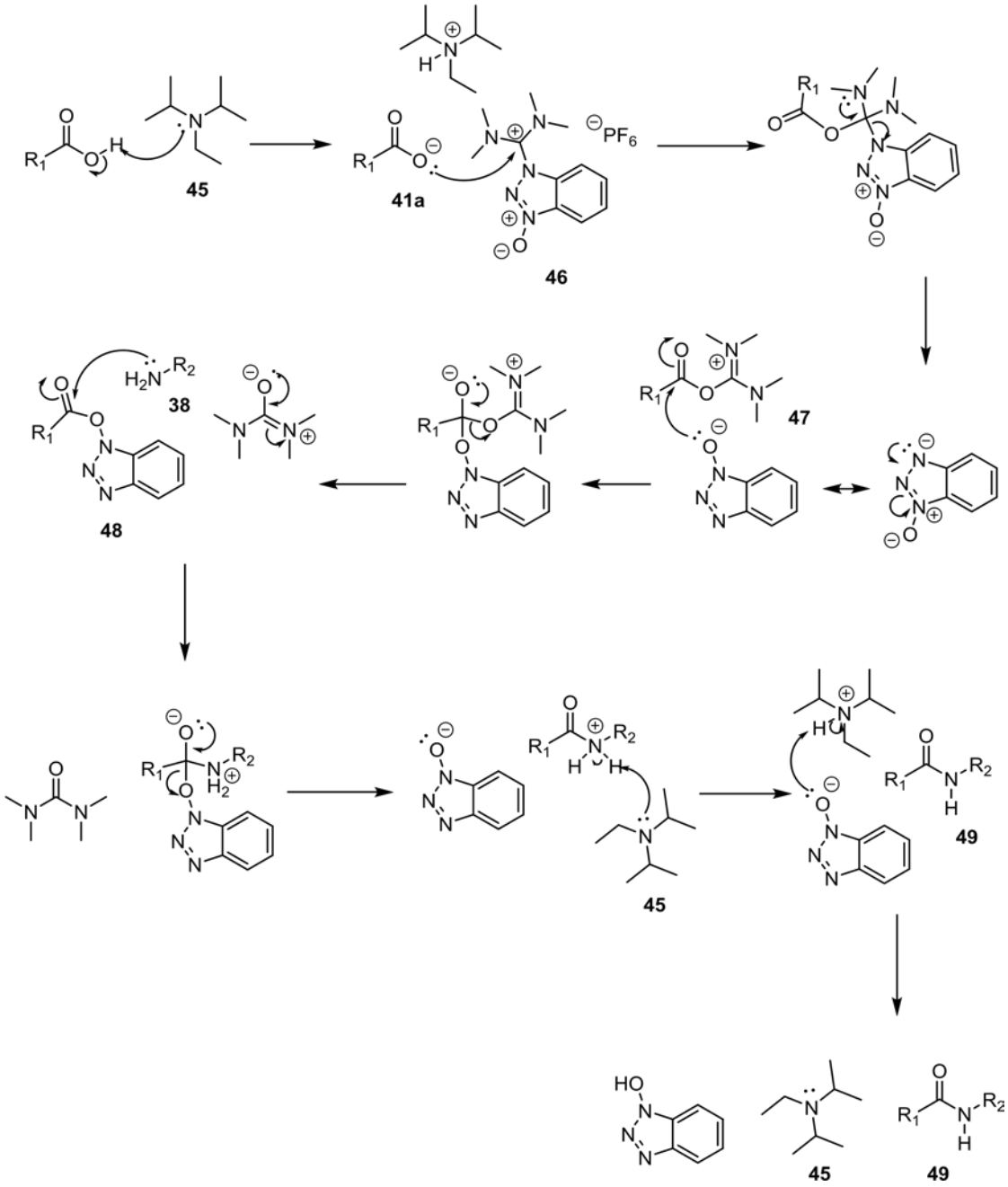
Scheme 10 *Reaction between a primary amine and 2,4,6trinitrobenzenesulfonic in the TNBS test*

In our research, two main coupling methods were used. A popular coupling technique in solution phase uses isobutyl chloroformate (IBCF) **43** as an activating agent and *N*-methylmorpholine (NMM) **42** as base. The additionelimination type reaction of the carboxylic acid **41** and the IBCF **43** results in an anhydride based intermediate **44**, which reacts readily with the amino group of a peptide or an amino acid **38** (Scheme 11).



Scheme 11 *Mechanism of peptide coupling using IBCF and NMM*

In recent years, the preferred solid phase peptide synthesis activating agents have been ammonium or phosphonium-based reactants. One commonly utilised example is 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) **46**, with diisopropylethylamine (DIPEA) **45** providing an ideal base for this reaction. The HBTU **46** reacts with the carboxylate ion **41a** and forms the active benzotriazolyl ester **47** through an *O*-acylisourea intermediate **47**. The nucleophilic amino group of the amino acid or peptide **38** then readily attacks the electrophilic carbon of the active ester **48** to give the peptide **49** as the product (Scheme 12).

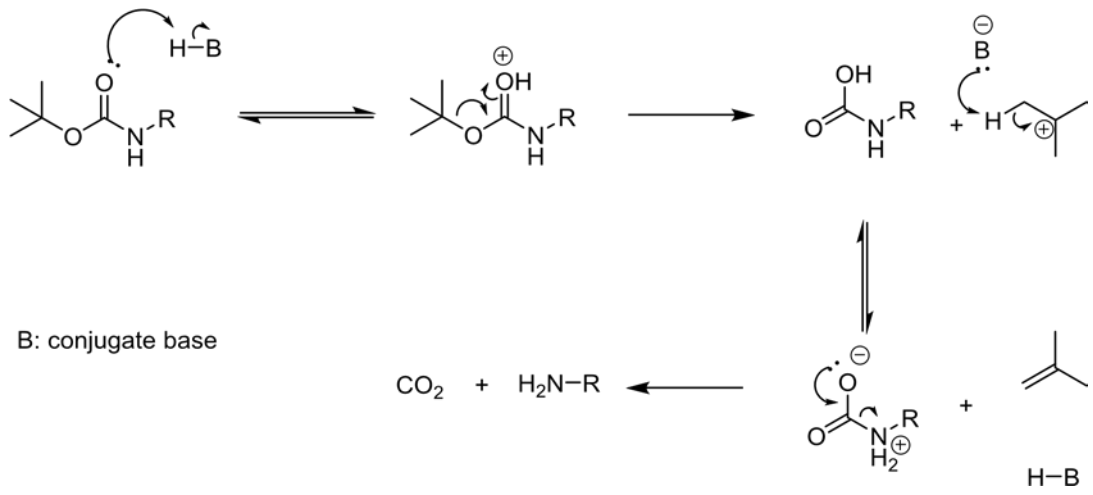


Scheme 12 *Mechanism of amide bond formation using HBTU and DIPEA*

As in peptide synthesis, protecting groups have an important role, it is necessary to discuss the main properties of the three main groups applied to amine protection.

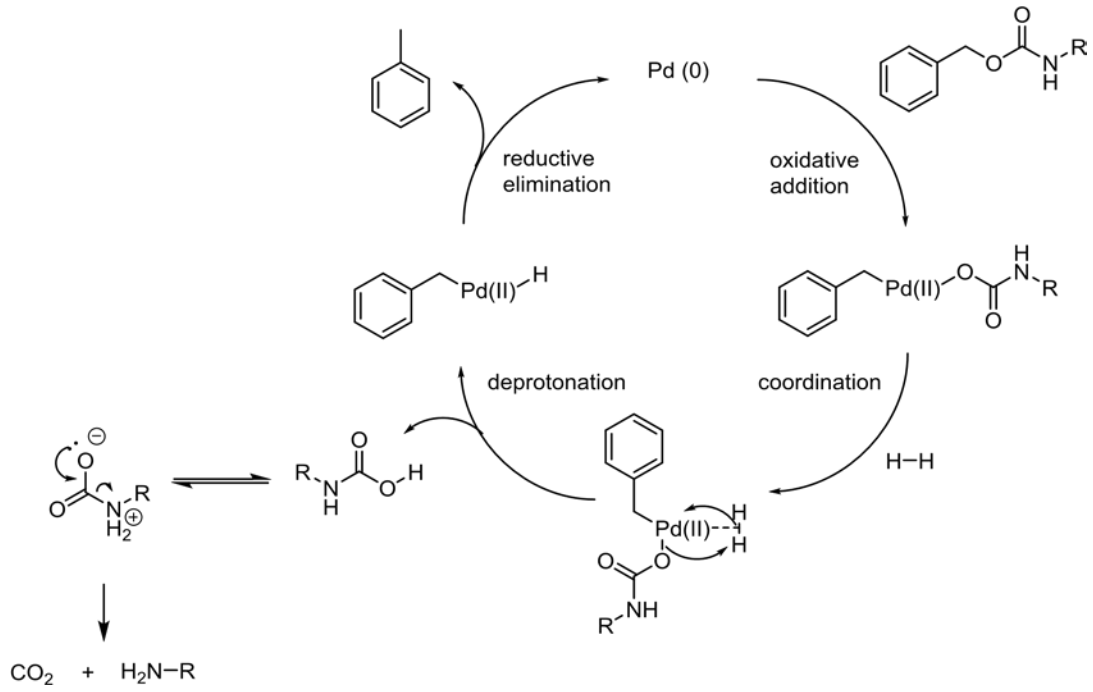
The *tert*-butyloxycarbonyl (Boc) group provides effective protection for amino groups against electrophiles in basic and neutral conditions. It can be removed

under acidic conditions, for example, with hydrochloric acid or TFA (Scheme 13).



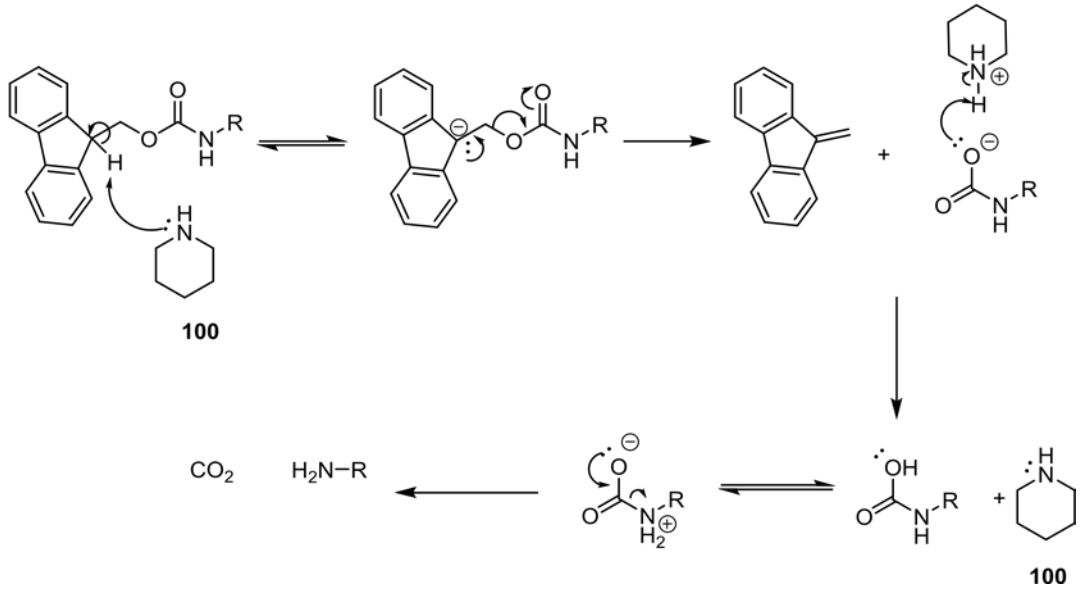
Scheme 13 *Amine deprotection: removal of the Boc group.*

The carboxybenzyl (Cbz) group provides effective protection for amino groups against electrophiles in basic, neutral and weakly acidic conditions. It can be removed under strong acidic conditions, such as hydrobromic acid in acetic acid, or by the more popular catalytic hydrogenation (Scheme 14).



Scheme 14 *Deprotection of Cbz group by catalytic hydrogenation*

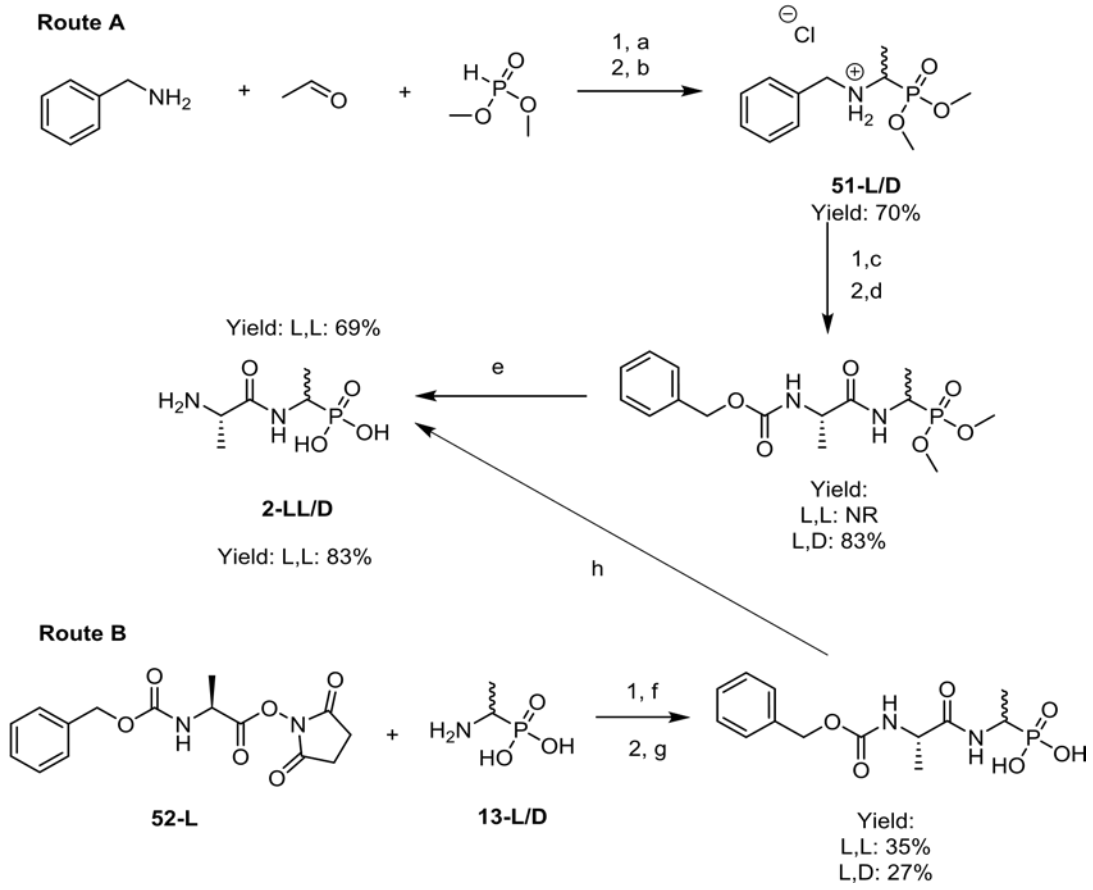
The fluorenylmethyloxycarbonyl (Fmoc) group is mainly applied in solid phase synthesis. It provides effective protection for amino groups against electrophiles in acidic or neutral conditions. It can be removed under basic condition, such as by piperidine **50** (Scheme 15).



Scheme 15 *Deprotection of Fmoc group by piperidine* ***100***

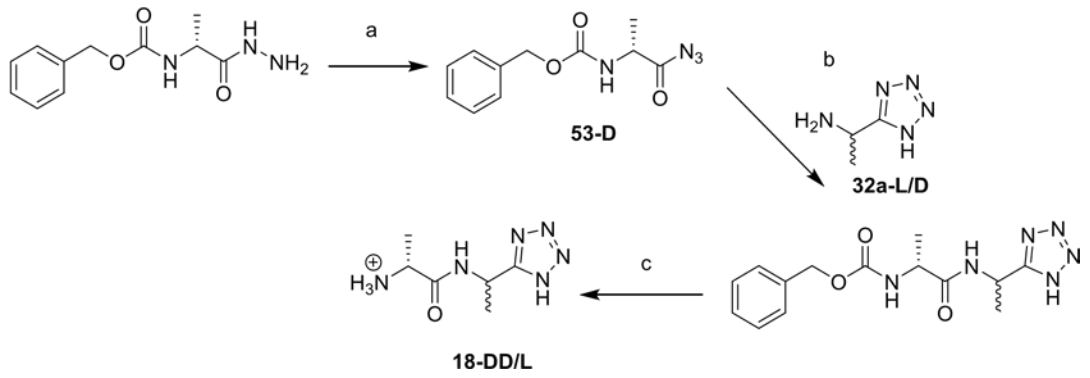
2.1.3.3 Synthesis of alanine bioisostere containing dipeptides

Alafosfalin **2-LL/D** is a dipeptide form of the AlaR inhibitor fosfalin **13-L** with significant antibacterial effects. It has been synthesized by two types of solution phase peptide coupling methods. In one, fosfalin dimethyl ester **51L/D**, which can be made by a Kabanich-Fields reaction,[68] and Cbz-L-alanine were reacted in the presence of DCC and TEA, followed by acid-catalysed deprotection (Route A, Scheme 16). Alternatively, the activated Cbz-L-alanyl hydroxysuccinimide **52-L** can be reacted with L/D fosfalin **13-L/D**, and then the Cbz protecting group removed by catalytic hydrogenation (Route B, Scheme 16).[69] The synthesis of further fosfalin-containing peptides using similar methodology is discussed in the same paper,[69] and in a recent thesis,[51] which also shows classical solution phase methods suitable for phosphonopeptide synthesis.



Scheme 16 *Synthetic route for the preparation of alafosfalin* ***2-LL/D****.*[69] *Reagents and conditions: (a) water; (b) HCl, water(c) H_2, Pd/C, DCM; (d) DCC, Et_3N, Cbz-L-Ala-OH; (e) HBr, AcOH; (f) $NaHCO_3$, H_2O, ethanol; (g) diastereomer separation by: cation exchange resin (RSO_3H), water, EtOH, Bn-NH_2; (h) H_2, Pd/C.*

A few examples of 1-aminoethyltetrazole containing peptides have been synthesised previously, such as D-alanyl-D,L-1-aminoethyltetrazole **18-DD/L**. This was made using a traditional peptide coupling method between D,L-1aminoethyltetrazole **32a-L/D** and Cbz-D-alanine azide **53-D**[52] (Scheme 17).



Scheme 17 *Synthesis of D-alanyl-D,L-1-aminoethyltetrazole* ***18-DDL****.*[52] *Reagents and conditions: (a) acetic acid, 5 M* HCl_{aq}*,* $NaNO_2$*; (b) TEA, DMF; (c) acetic acid, HBr.*

The synthesis of peptide analogues with tetrazole-containing *C*-terminal amino acids using solid phase peptide synthesis is possible by linking the tetrazole ring to the resin and thus keeping it protected during the whole synthesis. Previously, several research groups attached molecules with a tetrazole ring successfully to different resins. The most commonly used resins for this purpose were 2-chlorotrityl- and trityl chloride resins,[70] [71] but methods using the Wang resin,[72] or the vinyl ether type THP resin[73] have also been reported.

2.2 Results and discussion

2.2.1 Research aims

The main goal of this research was to synthesize several alanine analogues, each displaying bioisosteric replacement of the carboxylic acid **3L** unit found within alanine itself, such as L-alanyl-methanesulfonamide **27-L**, L-1-aminoethyltetrazole **32a-L** and L-1-aminoethyloxadiazol-5(4*H*)-one **33-L** (Figure 9). Once such chemical probes were in-hand, their propensity towards alanine racemase inhibition would be evaluated. Subsequently, bioisostere containing peptides would be produced. It was envisaged that the peptides may exhibit better bacterial cell transportation. In all cases the chemical properties of the novel molecules would be characterised, and their antibacterial activities investigated. Some of the target compounds have been reported in the literature, although they have not been tested as alanine

racemase inhibitors, to the best of our knowledge. For the derivatives which were new, a feasible synthetic method needed to be developed.

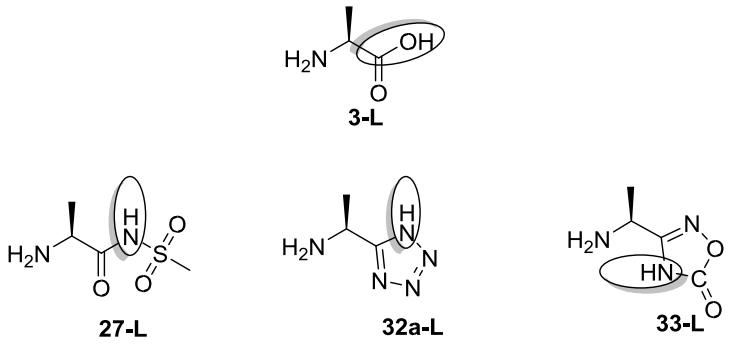


Figure 9 *Alanine bioisosteres as potential alanine racemase inhibitors; the source of the acidic proton is circled within each structure.*

The structure property relationship of established carboxylic acid bioisosteric groups were compared in a recent review.[74] This source provided an invaluable resource for the choice of bioisosteres that we would go on to utilise. Key criteria were similar acidity to normal carboxylic acids (p$K_{a:}$ 4.64 in the given example) and good water solubility. The higher lipophilicity (logD) of tetrazole and oxadiazol-5(4*H*)-one groups was seen as a possible advantage as it would make passive transport into bacteria more likely, reducing the reliance on active transport systems. Physicochemical data for alanine is presented in Table 2 as a reference for prediction of physicochemical properties of alanine bioisosteres. (Table 2).

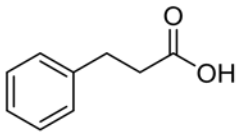
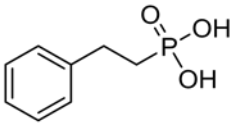
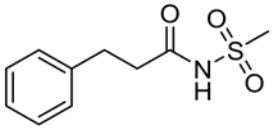
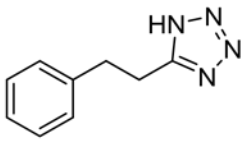
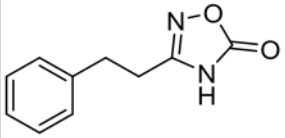
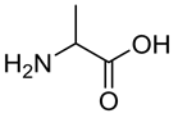
Class	Structure	Aqueous solubility [a] (μM)	$logD_{7.4}$ [b]	pK_a
Carboxylic acid		110.69 ± 3.04	-0.49 ± 0.19	4.64
Phosphonic acid		152.36 ± 1.18	-1.14	2.34
Acylmethanesulfonamide		199.70 ± 0.30	-1.02	4.94
Tetrazole		≥ 200	-0.25 ± 0.10	5.09
Oxadiazol-5(4*H*)-one		≥ 200	0.32	5.73
Alanine		1.87[75] [c]	-2.75[76] [d]	2.35[77]

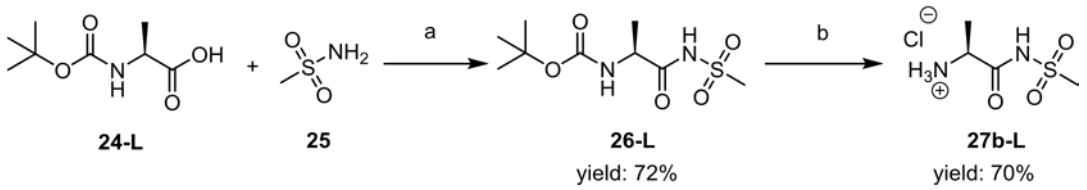
Table 2 *Structure property relationship of carboxylic acid bioisosteric groups, the data is taken from reference [74]; a: Kinetic solubility in aqueous phosphate buffer (pH 7.4) determined by LC/MS after 24 h of incubation. b: Distribution coefficient between n-octanol and aqueous buffer (pH 7.4) determined by LC/MS; c: measured at 25 °C; d: logP in 20 °C.*

2.2.2 Synthesis of alanine bioisosteres

2.2.2.1 Alanylmethanesulfonamide

Alanylmethanesulfonamide **27-L** is a known molecule, although its antibacterial properties have not been studied. It was synthesized following a published method described in chapter 2.1.3. (Scheme 5),[56] although minor

modifications were made towards a more economical synthesis. *N*-Bocalanine **24-L** was activated with CDI in the presence of DBU, then it was coupled with methanesulfonamide **25** to give *N*-Boc-alanylmethanesulfonamide **26-L** as the product. The Boc protecting group was removed by hydrochloric acid in diethyl ether (Scheme 18).



Scheme 18 *Synthesis of L-alanylmethanesulfonamide HCl salt* ***27b-L****. Reagents and conditions: (a) CDI, DBU, DCM, r.t.; (b) 2M HCl in DEE, r.t..*[56]

The main features of alanylmethanesulfonamide compounds are shown on the ^{1}H-NMR spectrum of an example compound *N*-Boc-alanylmethanesulfonamide **26-L**. As shown in Figure 10, alanine bioisosteres and their derivatives have three main distinctive signals (2), (3), (4) in their ^{1}HNMR spectrum. The hydrogen (2) next to carboxylic acid replacement moiety, in this example next to the oxomethanesulfonamide group, is a quintet between 4.00 and 5.00 ppm; its multiplicity can change to a quartet depending on the solvent and whether the neighbouring amino group is acylated or not. Another feature is the methyl side chain (3), which is a doublet around 1.50 ppm. The carbamate N-H hydrogen (4) is a doublet at 7.18 ppm, due to its neighbouring hydrogen. Furthermore, the sulfonamide has an acidic proton (5), which has a slightly broad and very deshielded signal at 11.77 ppm. A specific methanesulfonamide feature is the singlet signal around 3.20 ppm, which belongs to its methyl group (9). The Boc group has a typical singlet signal for nine hydrogens around 1.50 ppm in all Boc-protected compounds (Figure 10).

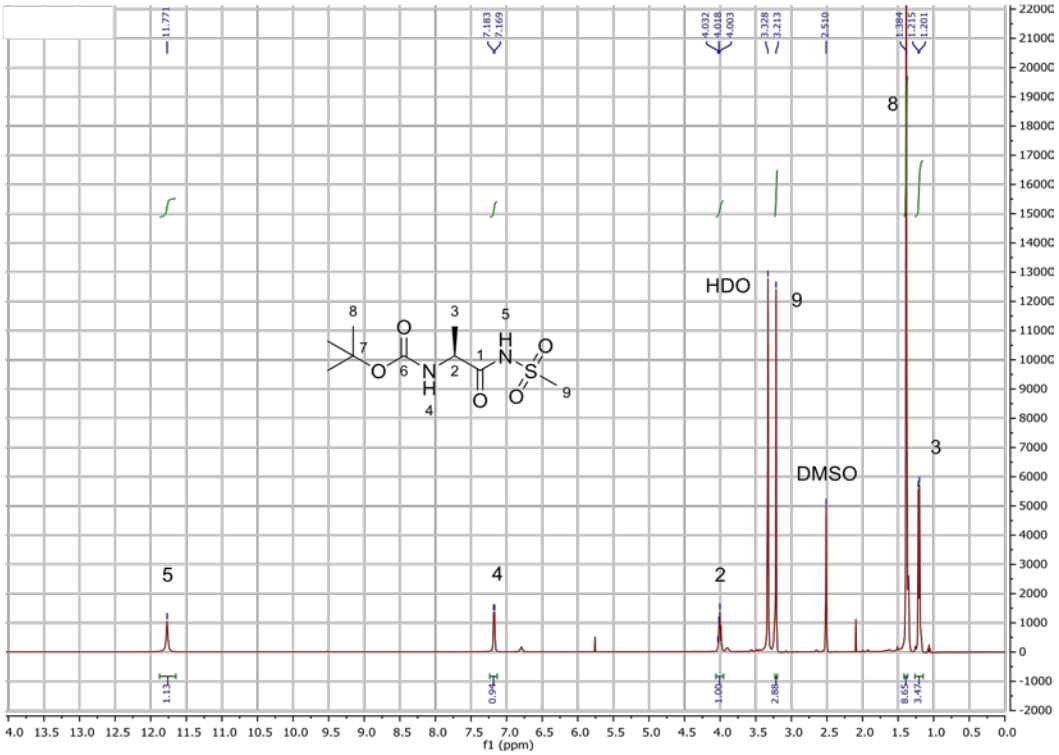
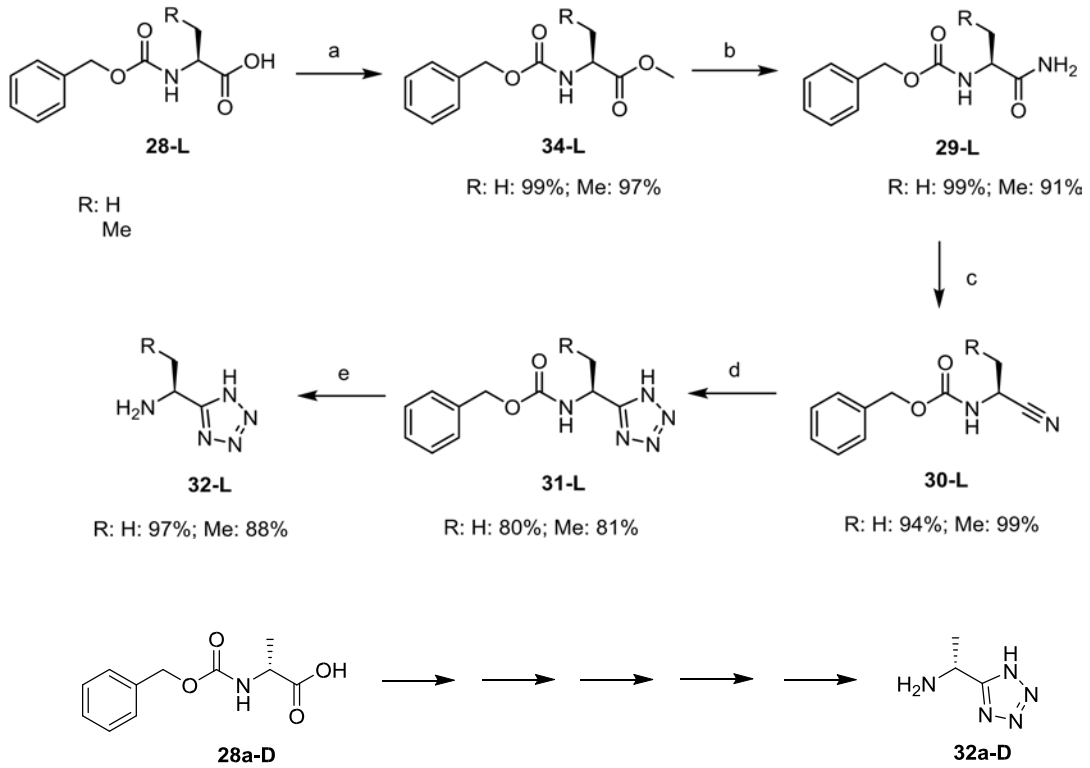


Figure 10 *^{1}H-NMR spectrum of N-Boc-L-alanylmethanesulfonamide* ***26-L*** *in DMSO-d_6.*

2.2.2.2 L-1-aminoalkyltetrazoles

The synthesis of another alanine bioisostere, L-1-aminoethyltetrazole **32a-L**, was published by Bavetsias and introduced in chapter 2.1.3. (Scheme 6).[57] This synthetic route was followed with minor modifications, producing **32a-L** in good overall yield (71%). Cbz-L-Alanine **28a-L** was methylated with methyl iodide in the presence of Cs_2CO_3 in DMF. The resulting methyl ester **34a-L** was transformed to the analogue amide **29a-L** in the presence of 7 M ammonia in methanol by heating at 50 °C in a sealed tube. In the following step, the amide **29a-L** was converted to nitrile **30a-L** using tosyl chloride in pyridine and DCM to perform the dehydration. Cbz-L-1-aminoethyltetrazole **31a-L** was produced by a dipolar cycloaddition of NaN_3 to the nitrile moiety in the presence of NH_4Cl in DMF. L-1-Aminoethyltetrazole **32a-L** was obtained after removing the Cbz-protecting group by Pd-catalysed hydrogenation (Scheme 19). The D-1-aminoethyltetrazole **32a-D** was synthesized by the same methodology starting with Cbz-D-Alanine **28a-D**, and L-1aminopropyltetrazole **32b-L** from Cbz-L-ethylglycine **28b-L**. However, L-

1aminopropyltetrazole **32b-L** is not an alanine bioisostere, it is a homologue that will enable exploration of the steric requirements for tetrazole inhibitors of AlaR. In certain cases, larger amino acid substrates can be accommodated, for example (1-amino-2-propenyl)phosphonic acid **54-L** is an inhibitor of AlaR,[78] which is a homologue of the AlaR inhibitor fosfalin **13-L** (Figure 11), introduced in chapter 2.1.2.



Scheme 19 *Synthesis of 1-amino-alkyltetrazoles **32**. Reagents and conditions: (a) Cs_2CO_3, MeI, DMF, r.t.; (b) NH_3 in MeOH, 50 °C; (c) TosCl, pyridine, DCM, 0 °C; (d) NaN_3, NH_4Cl, DMF, 90 °C; (e) $H_{2\,(g)}$, 5% Pd/C, EtOH, 3 bar, r.t..*[57]

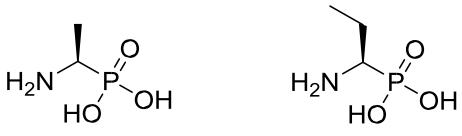
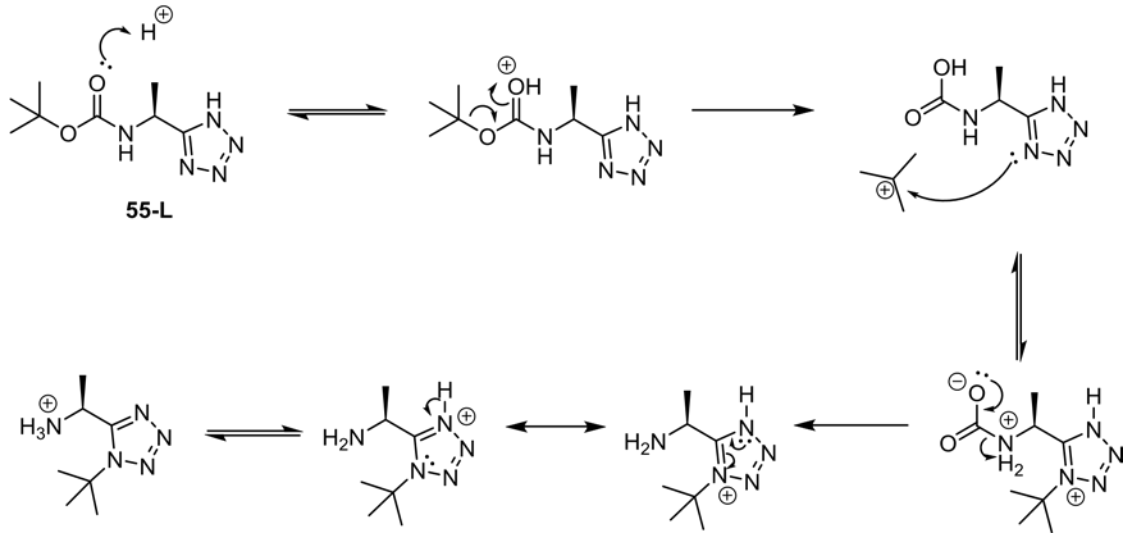


Figure 11 *Fosfalin **13-L** and its homologue (1-amino-2-propenyl)phosphonic acid **54-L***

The same methodology was performed using Boc protection instead of the Cbz, although the first 4 steps worked similarly, deprotection of the Boc group under acidic conditions causes partial *tert*-butyl substitution of the tetrazole proton due to the '*in situ*' liberation of *tert*-butyl cations. The side reaction was

observed even in the presence of a *S*-methyl-thiophenol scavenger (Scheme 20).



Scheme 20 *Side reaction during Boc deprotection of Boc-L-1aminoethyltetrazole* ***55-L***

The ^{1}H-NMR spectroscopic properties of Cbz-L-1-aminoethyltetrazole **31a-L** are shown in Figure 12. As described previously, the specific signals of alanine bioisosteres were (Figure 12): H-2 signal is a quintet at 5.00 ppm, H-3 is a doublet at 1.50 ppm and H-4 is a doublet at 8.00 ppm. The acidic tetrazole proton (5) appears as a deshielded broad peak near 16.00 ppm, it is only visible in DMSO-d_6. The Cbz protecting group has two specific peaks: around 5.00 ppm the deshielded benzyl hydrogens (7) are a singlet, although they often appear as 2 overlapping doublets due to their diastereotopic feature. This effect is illustrated by the example of Cbz-L-Alanyl-L-1-aminoethyltetrazole **69a-LL** in Figure 13. Returning to figure 12, the 5 aromatic hydrogens (9-11) associated with the phenyl unit are represented by a multiplet around 7.30 ppm.

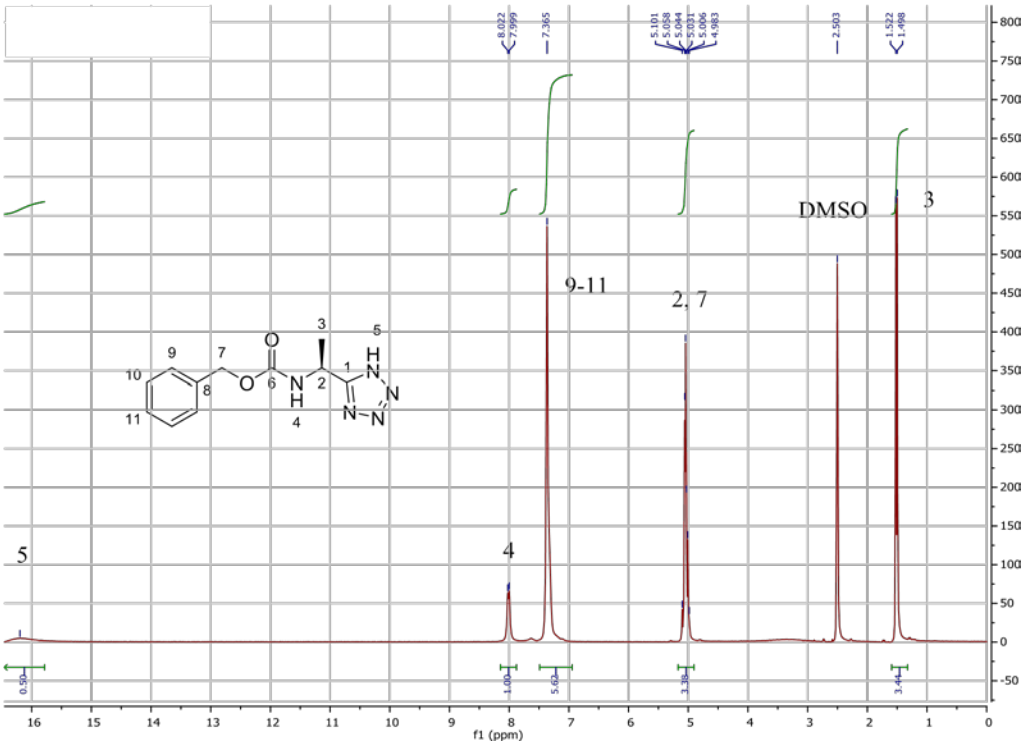


Figure 12 *^{1}H-NMR spectrum of Cbz-L-1-aminoethyltetrazole* ***31a-L*** *in DMSO-d_6.*

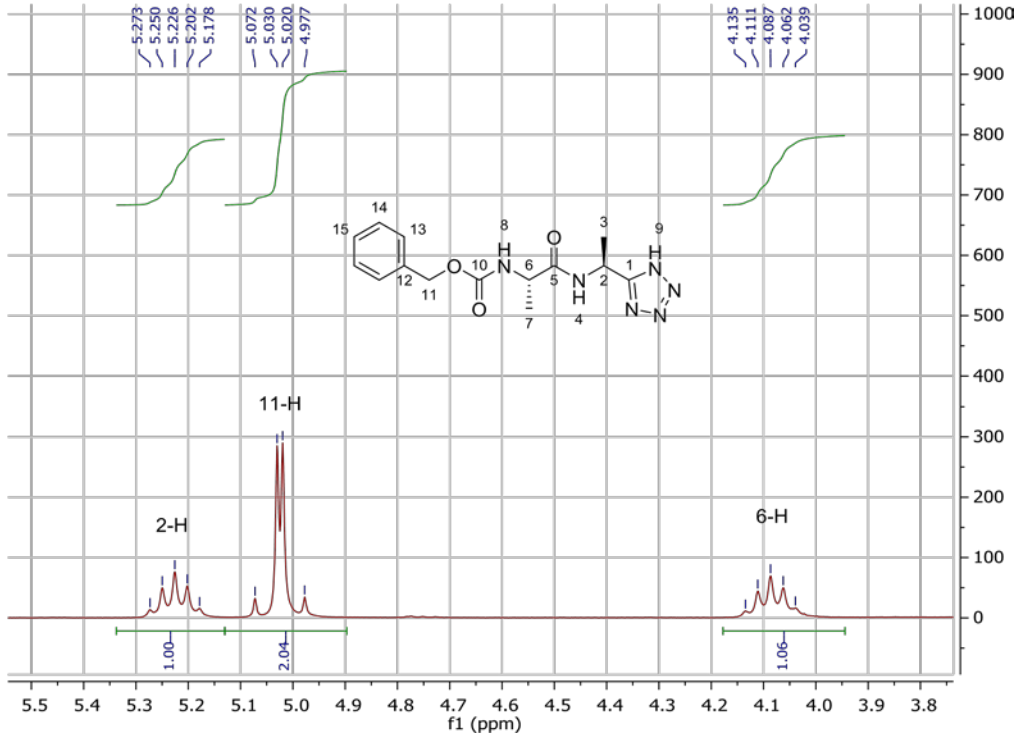
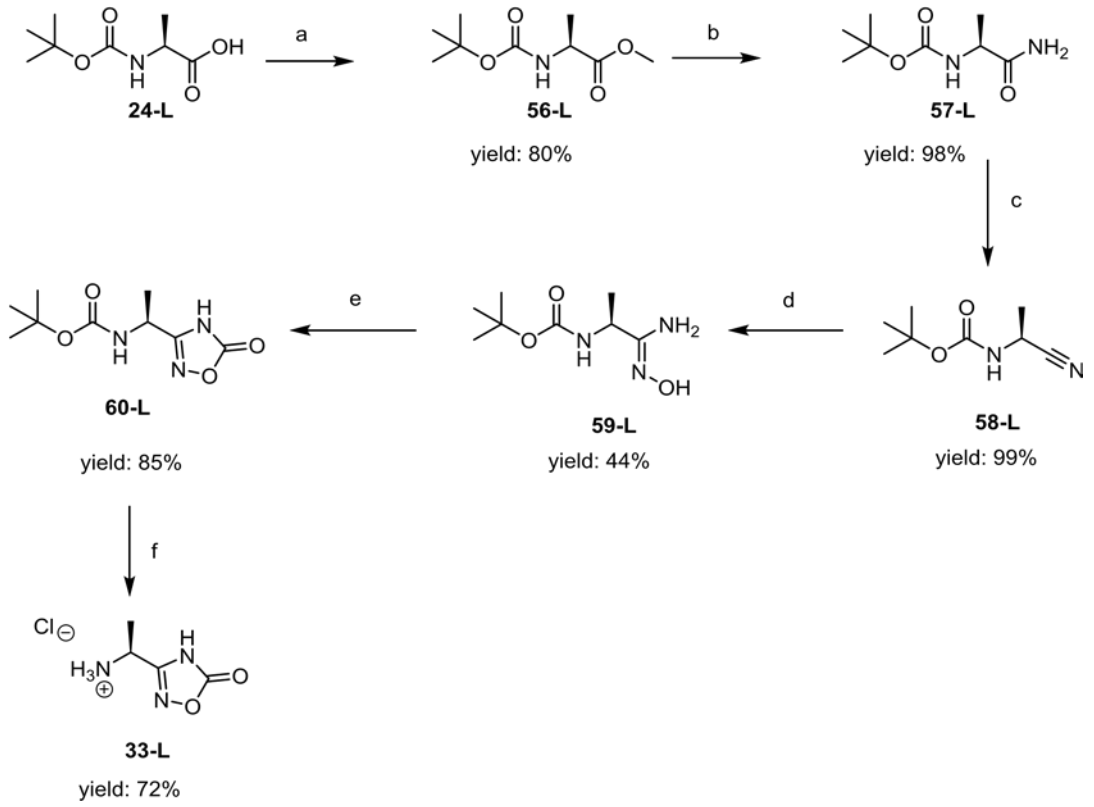


Figure 13 *A part of ^{1}H-NMR spectrum of Cbz-L-Alanyl-L-1aminoethyltetrazole* ***69a-LL*** *in DMSO-d_6.*

2.2.2.3 L-1-aminoethyl-5-oxo-1,2,4-oxadiazole

L-1-Aminoethyl-5-oxo-1,2,4-oxadiazole **33-L** was synthesized as another potential alanine racemase inhibitor. The key intermediate Bocalanine nitrile **58-L** was synthesized from Boc-L-alanine **24-L** through a methyl ester **56-L** and an amide **57-L** intermediate. This constitutes a similar series of reactions to those discussed previously in chapter 2.2.2.2 towards the Cbzalanine nitrile **30a-L** during the preparation of L-1-aminoethyltetrazole **32a-L**. The heterocyclic compound **60-L** was formed from the nitrile **58-L** with hydroxyl amine in ethanol. This was followed by the reaction of the hydroxylcarbimidoyl intermediate **59-L** with CDI in the presence of DBU in order to close the five-membered ring. After removing the Boc protecting group, the product was collected as a hydrochloride salt **33-L** (Scheme 21). However, when the synthesis was attempted using a Cbz protecting group instead of Boc, the heterocyclic ring decomposed during the attempted removal of the Cbz group through standard catalytic hydrogenation, making this approach unsuitable.



Scheme 21 *Synthesis of L-1-aminoethyl-5-oxo-1,2,4-oxadiazole* ***33-L****.*

*Reagents and conditions: (a) Cs_2CO_3, MeI, DMF, r.t.; (b) NH_3 in MeOH, 50 °C; (c) TosCl, pyridine, DCM 0 °C; (d) K_2CO_3, NH_2OH*HCl, ethanol, 70 °C; (e) CDI, DBU, DCM, r.t.; (f) 2M HCl in DEE, r.t..*

The ^{1}H-NMR spectrum of Boc-L-1-aminoethyl-5-oxo-1,2,4-oxadiazole **60-L** is assigned on Figure 14. The specific signals of the protected alanine bioisostere can be seen as H-2 quintet (multiplet) at 4.50 ppm, 3-H doublet at 1.30 ppm, and H-4 amide hydrogen with a broad doublet at 7.30 ppm. H-9 of the Boc-group is a singlet at 1.40 ppm. The acidic hydrogen signal of the 5oxo-1,2,4-oxadiazole moiety (5) appears as a broad singlet at 12.25 ppm in DMSO-d_6.

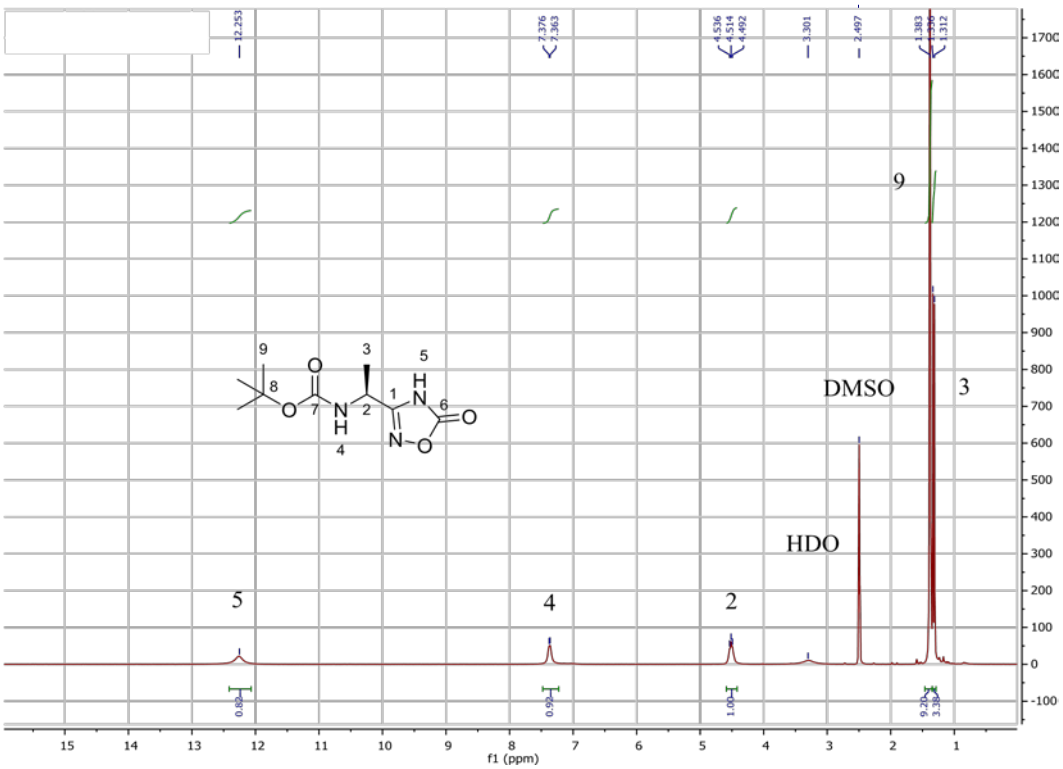


Figure 14 *^{1}H-NMR spectrum of Boc-L-1-aminoethyl-5-oxo-1,2,4-oxadiazole* ***60-L*** *in DMSO-d_6.*

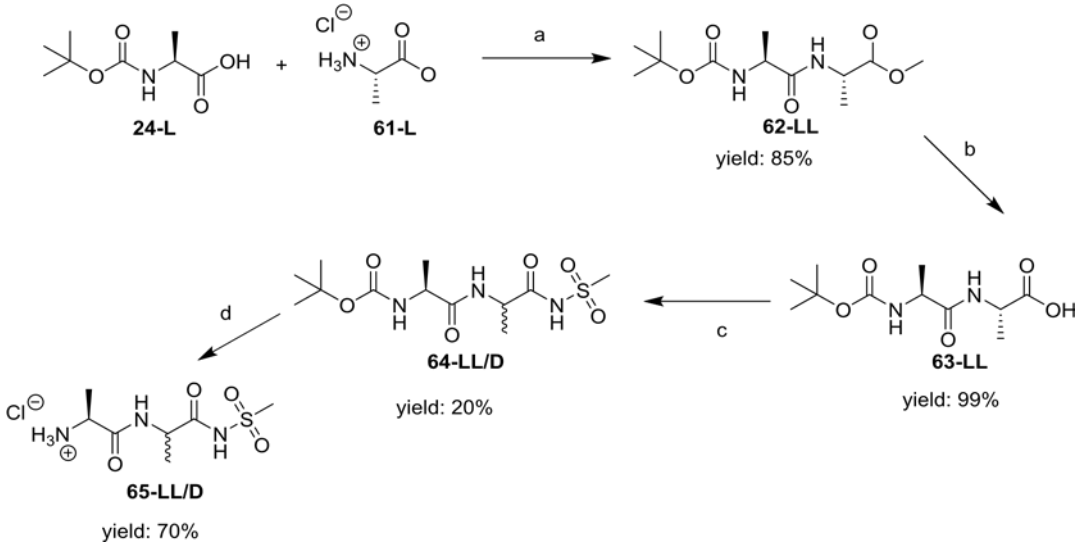
2.2.3 Synthesis of alanine bioisostere-containing oligopeptides

There is no known transport system in bacterial cells that can transport a single alanine, and consequently a single alanine bioisostere molecule, into the cell to reach the target enzyme. Alanine is normally taken in as di- or oligopeptides by di- or oligopeptide permease systems. Following the example of fosfalin containing peptides as efficient antibacterial agents, di- and tripeptide forms of the molecules introduced in chapter 2.1.2. were synthesised and studied.

2.2.3.1 Solution phase approach

2.2.3.1.1 L-Alanyl-alanylmethanesulfonamide

L-Alanyl-alanylmethanesulfonamide **65-LL/D** is a dipeptide derivative of the alanylmethanesulfonamide **27-L** bioisostere**.** After failures in coupling alanylmethanesulfonamide **27-L** with Boc-alanine **24-L** in the presence of IBCF and NMM**,** *N*-Boc-L-alanyl-L-alanine methyl ester **62-LL** was synthesized with a general solution phase peptide coupling method using IBCF and NMM in excellent yield. After a hydrolytic step, *N*-Boc-L-alanyl-Lalanine **63-LL** was coupled with methanesulfonamide in the presence of EDCI and DMAP to form the Boc protected methanesulfonamide compound **64LL/D**. This method was utilised after the previously shown coupling method (Scheme 18) using CDI and DBU was shown not to be successful for these particular substrates. After removal of the Boc protecting group, L-alanylalanylmethanesulfonamide **65-LL/D** was isolated as white crystals (Scheme 22).



Scheme 22 *Synthesis of L-alanyl-alanylmethanesulfonamide hydrochloride* ***65-LL/D****. Reagents and conditions: (a) IBCF, NMM, DCM; (b) 1M NaOH (aq), MeOH; (c) EDCI, DMAP, $MeSO_2NH_2$, DCM; (d) 2M HCl (DEE).*

It is probable that minor racemization of the C-2 centre occurred during the synthesis of *N*-Boc-L-alanyl-alanylmethanesulfonamide **64-LL/D**. This was observed on the ^{1}H-NMR spectrum of L-alanyl-alanylmethanesulfonamide hydrochloride **65-LL/D**: double signals were observed for both the methyl

groups at C-3 and C-7 and for the □-hydrogens at C-2 and C-6, suggesting that it was a diastereomeric mixture (Figure 15), which was proven by LC-MS analysis (ratio 85 : 15).

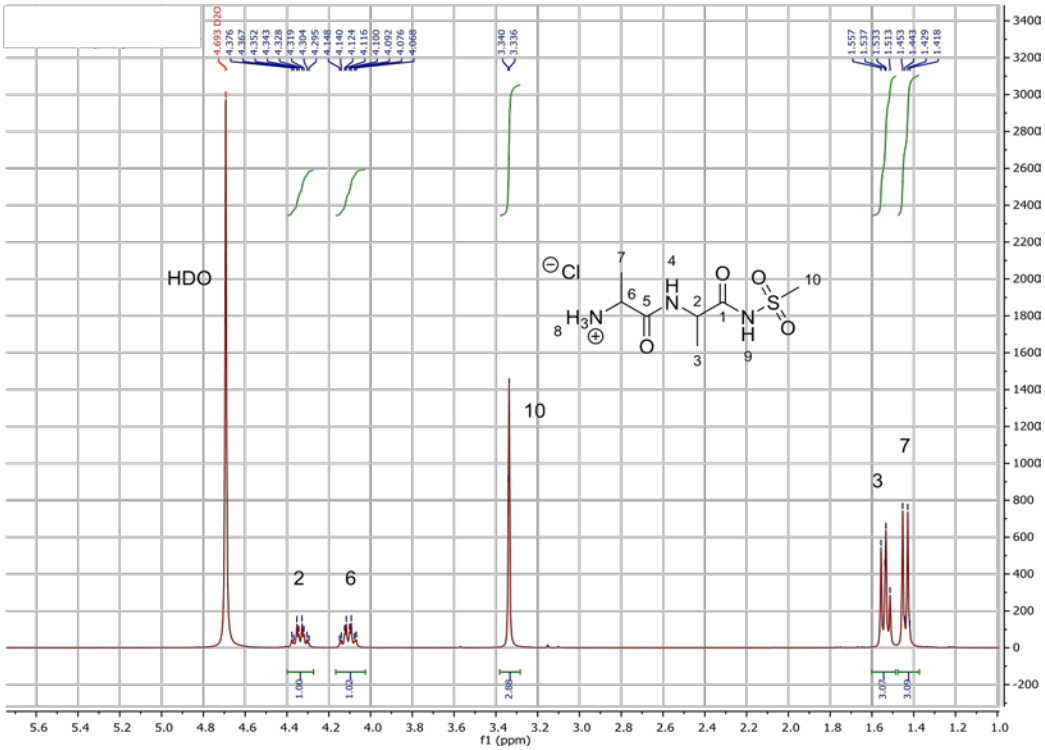
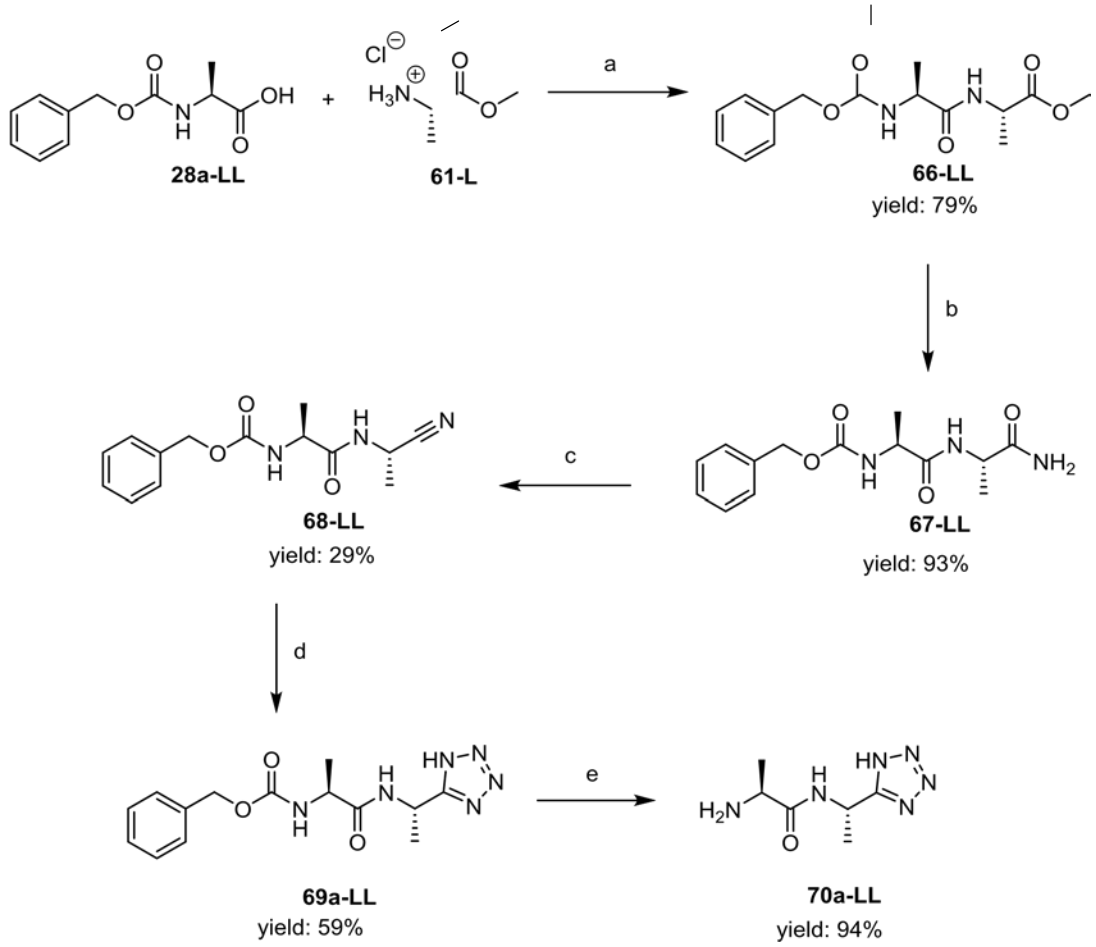


Figure 15 *^{1}H-NMR spectrum of L-alanyl-alanylmethanesulfonamide hydrochloride* ***65-LL/D*** *in D_2O.*

2.2.3.1.2 C-terminal L-1-aminoalkyltetrazole peptides

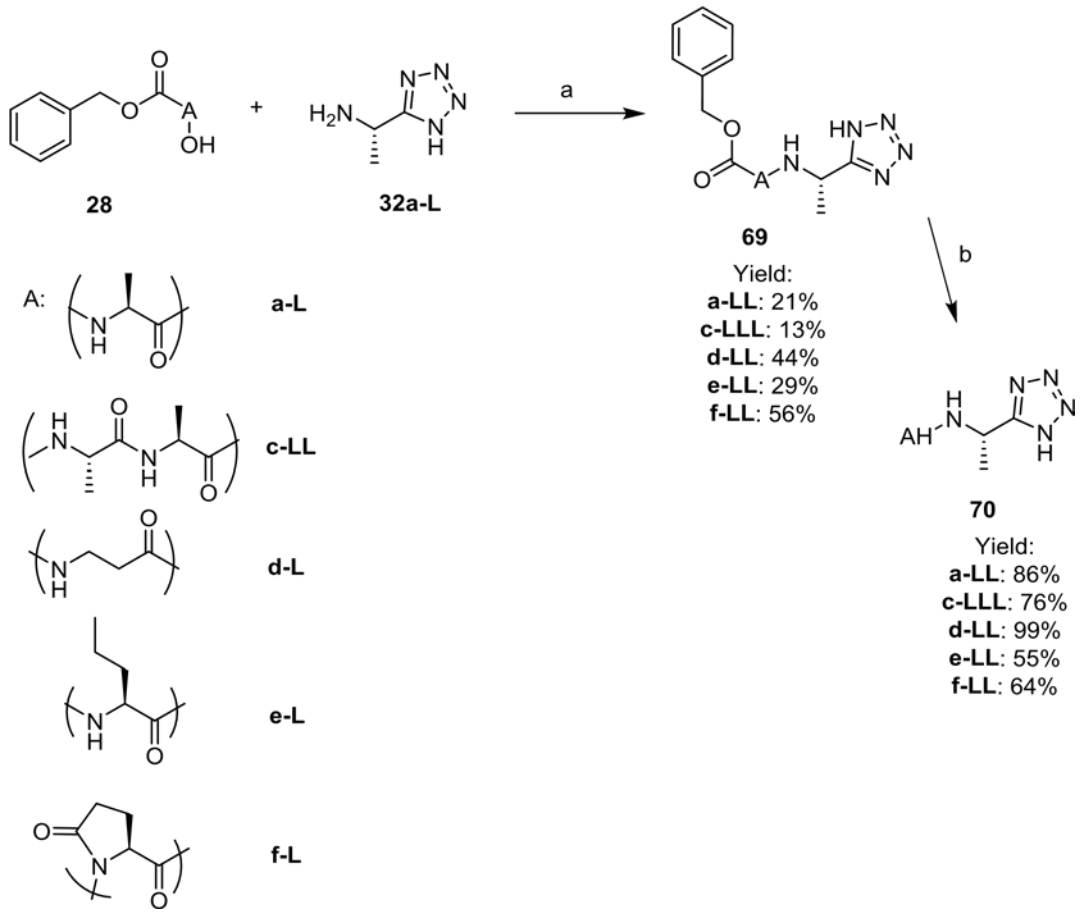
Similar methodology was used in the first synthesis of L-alanyl-L-1aminoethyltetrazole **70a-LL**, in which the dipeptide Cbz-L-alanyl-L-alanine methyl ester **66-LL** was synthesized with a peptide coupling between Cbz-Lalanine **28a-L** and L-alanine methyl ester **61-L**, then the methyl ester **66-LL** was transformed to a tetrazole moiety **69a-LL** in 3 steps, in a similar manner to the synthesis of L-1-aminoethyltetrazole depicted in chapter 2.2.2. (Scheme 19). In this procedure, the nitrile **68-LL** formation was problematic, due to the relatively poor solubility of the amide intermediate **67-LL**. This reaction step was successfully carried out with cyanuric chloride in DMF, albeit in low yield. Finally, the Cbz protecting group was removed by catalytic hydrogenation (Scheme 23).



Scheme 23 *Synthesis of L-alanyl-L-1-aminoethyltetrazol* ***70a-LL*** *in solution phase: peptide coupling followed by functional group modification. Reagents and conditions: (a) IBCF, NMM, DCM, -5 °C; (b)* NH_3 *in water, THF, 65 °C; (c) cyanuric chloride, DMF, r.t.; (d)* NaN_3*,* NH_4Cl*, DMF, 90 °C; (e)* $H_{2\,(g)}$*, 5% Pd/C, EtOH, 3 bar, r.t..*

Due to the selective and promising bacterial growth inhibition results of Lalanyl-L-1-aminoethyltetrazole **70a-LL**, which are discussed in chapter 2.2.3., more tetrazole analogues **70c-f** were synthesized and tested for new possible applications as antibacterial agents with different selectivity. The method shown in Scheme 23 was not sufficiently robust, with low yielding steps, preventing preparation of a library of new dipeptides. These peptides **70c-f** were synthesized by a solution phase coupling method using IBCF and NMM in the solvent DMF. Although these reactions resulted in the expected products, the conversion of the reactions was not quantitative and byproducts, such as tetrazole coupled compounds, appeared in the reaction mixture due

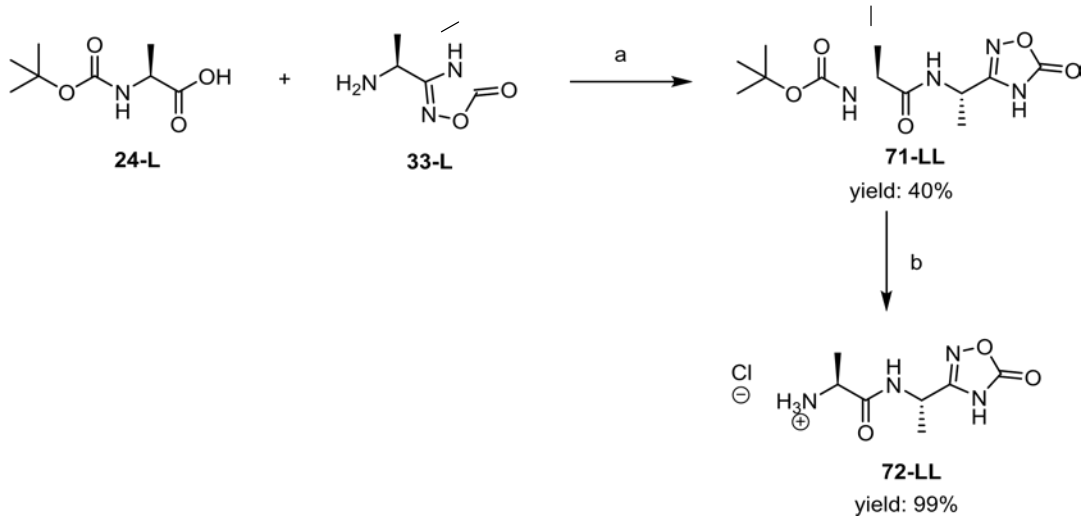
to the unprotected tetrazole ring. Removal of the Cbz protecting group was readily achieved through hydrogenation (Scheme 24).



Scheme 24 *Synthesis of L-alanyl-L-1-aminoethyltetrazoles* ***70c-f*** *in solution phase coupling of L-1-aminotetrazole* ***32a-L****. Reagents and conditions: (a) IBCF, NMM, DMF, -5 °C; (b) $H_{2\,(g)}$, 5% Pd/C, EtOH, 3 bar, r.t..*

2.2.3.1.3 L-Alanyl-L-1-aminoethyl-5-oxo-1,2,4-oxadiazole

Boc-L-Alanyl-L-1-aminoethyl-5-oxo-1,2,4-oxadiazole **71-LL** was synthesized by coupling L-1-aminoethyl-5-oxo-1,2,4-oxadiazole **33-L** with Boc-L-alanine **24-L** in solution phase using IBCF and NMM. The hydrochloride salt **72-LL** was obtained in quantitative yield after acid-catalysed removal of the Boc protecting group (Scheme 25).



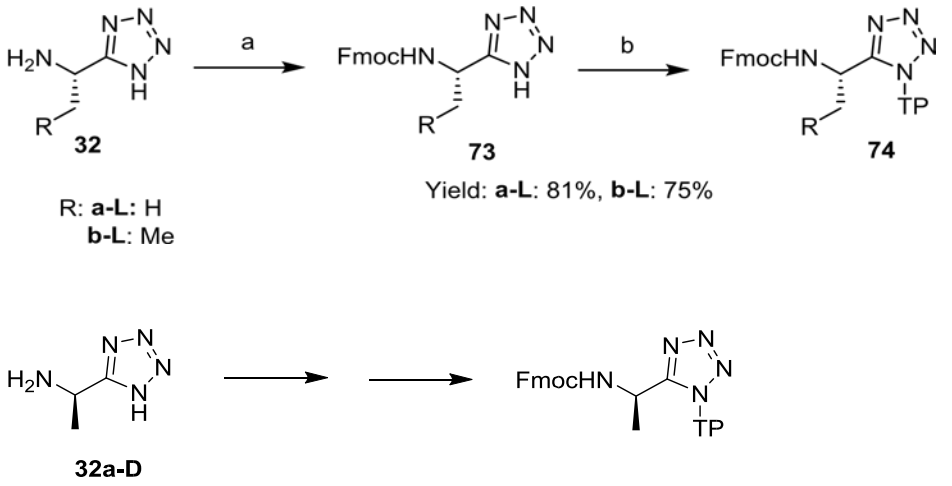
Scheme 25 *Synthesis of L-alanyl-L-1-aminoethyl-5-oxo-1,2,4-oxadiazole* ***72LL****. Reagents and conditions: (a) IBCF, NMM, DMF, -5 °C; (b) 2M HCl inDEE, r.t..*

2.2.3.2 Solid phase approach for the synthesis of C-terminal L-1aminoalkyltetrazole peptides

More di- and oligopeptides containing C-terminal L-1-aminoethyltetrazole were prepared by a solid phase synthetic approach, after solution phase coupling of unprotected L-1-aminoethyltetrazole **32a-L** with Cbz-protected amino acids using IBCF and NMM as coupling agents proved ineffective, presumably due to the reactivity of the unprotected tetrazole moiety. Instead of applying protecting groups on the tetrazole ring, solid phase synthesis was chosen, as it has been reported that the tetrazole ring can be attached to and cleaved from different resins efficiently, and that this linkage would act as a surrogate protecting group in and of itself.[70-73] The 2-chlorotrityl chloride resin employed here required only mild cleavage conditions,[70] avoiding racemization and other side reactions.

During the solid phase synthesis, the Fmoc approach was used,[63] as it is suitable for the chlorotrityl resin and is an established method in peptide synthesis. Fmoc-L-1-aminoethyltetrazole **73-L** was prepared by reacting L-1aminoethyltetrazole **32a-L** with Fmoc chloride in the presence of Na_2CO_3 in a 2 phase heterogenic reaction,[79] and later attached to the 2-chloro-trityl chloride resin following what would become a general technique (Scheme

26).[63] The D-isomer **32a-D** and L-1-aminopropyltetrazole **32b-L** were prepared by the same method. The resin was not loaded with an excess quantity of Fmoc-1-aminoalkyltetrazole **73-L** as is usually seen for other linking amino acids and their mimics in general practice. The synthetic procedure with fully loaded resin resulted in Fmoc peptides as minor impurities at the end, causing further purification steps to be required. Loading with a theoretically equivalent ratio resulted in reasonable yields after cleavage of the peptides (50-70%) and either no or only low amounts of Fmoc peptide impurities (Table 3).

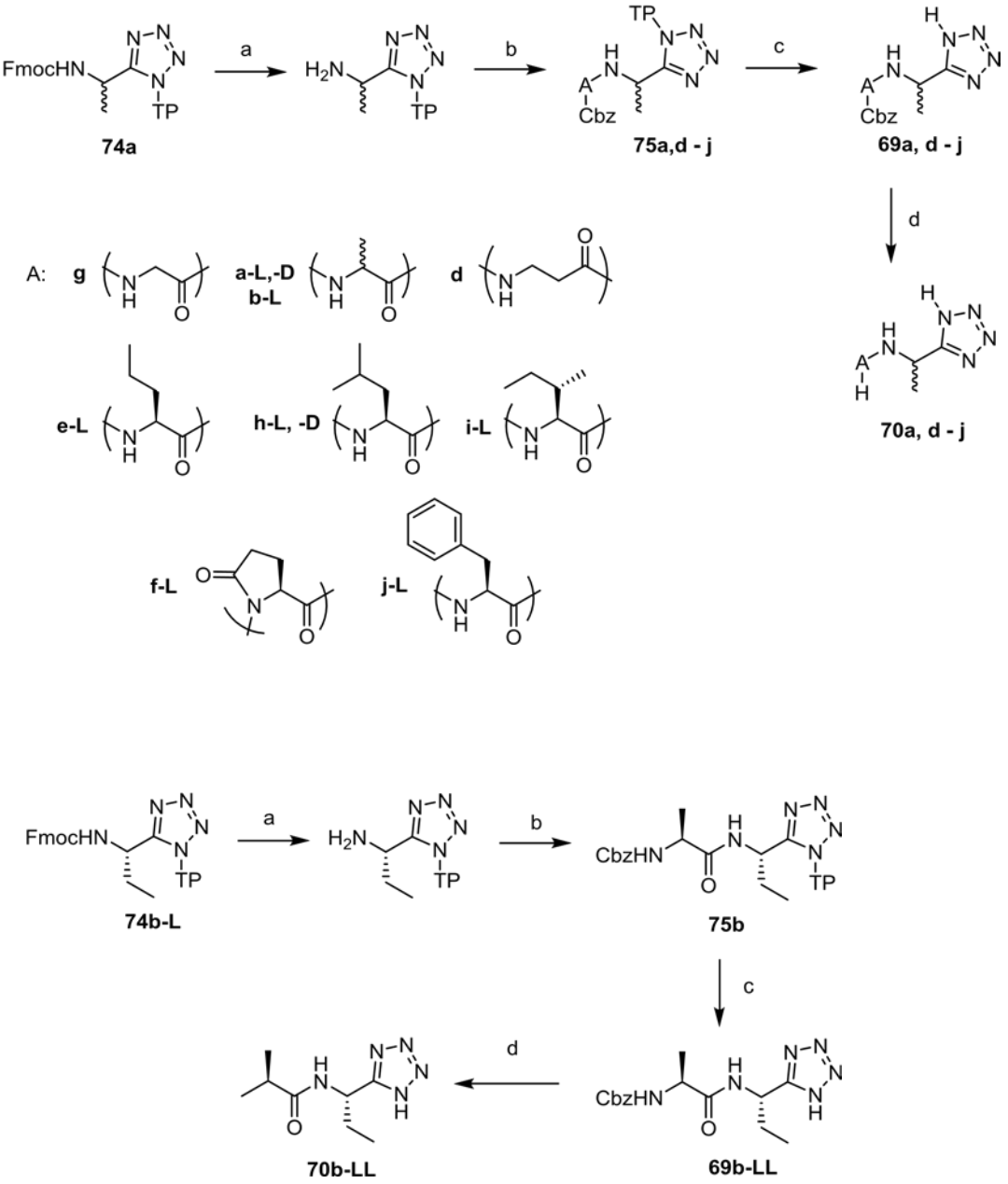


Scheme 26 *Synthesis of Fmoc-L-1-aminoalkyltetrazole **73-L** and its subsequent attachment to the 2-chloro-trityl chloride resin. Reagents and conditions: (a) Fmoc-Cl, $Na_2CO_{3\,(aq)}$, dioxane, 0 °C; (b) 2-chloro-trityl chloride resin (TP-Cl), DIPEA, DCM, r.t..*

Several dipeptide derivatives of 1-aminoalkyltetrazole **70a,b,d-j** were synthesized using the same solid phase synthetic approach. Firstly, the Fmoc protection of resin-attached Fmoc-L-1-aminoalkyltetrazole **74a,b** was removed with 25% piperidine solution in DMF, this part required at least 3 washing steps in order to remove the Fmoc group sufficiently. The free amino group was coupled with the appropriate Cbz protected amino acid using HBTU and DIPEA in DMF. The resin-bound dipeptide **75a,b,d-j** was cleaved from the resin by a TFA/DCM/triisopropylsilane (TIS) mixture in the ratio 5:95:1eq

(equivalent with the theoretical loading of the resin) and resulted in the protected dipeptides **69a,b,d-j** in good to excellent yields (Table 3). The Cbz-

protecting group was removed by Pd-catalysed hydrogenation (Scheme 27); the yield of these reactions depended on the purity of the protected starting compound. HPLC-MS purity measurements revealed the final compounds were diastereomeric mixtures, containing a major and a minor diastereomer in many cases. This suggested that minor racemization of 1-aminoethyltetrazole occurred during either its initial synthesis or the subsequent coupling procedures. The exact ratio of the products are not discussed here as we did not have the time or financial support to engage in a HPLC method development study.



Scheme 27 *Solid phase synthesis of dipeptide derivatives of 1aminoethyltetrazole* ***70a, d - j*** *and L-1-aminopropyltetrazole* ***70b-LL****. Reagents and conditions: (a) piperidine, DMF; (b) Cbz-A-OH, HBTU, DIPEA, DMF; (c) 5% TFA, 95% DCM, 1 eq TIS (equivalent with the theoretical loading of the resin); (d)* $H_{2\,(g)}$*, 5% Pd/C, EtOH, 3 bar, r.t..*

As already mentioned earlier in this section, residual fluorenyl impurities can occur in the Cbz-protected dipeptide products after cleavage from the resin (Table 3). The occurrence of these by-products mainly depends on 3 conditions. Overloaded resin might sterically hide some Fmoc groups from

cleavage, as tetrazole can be bound in 2 possible positions to the trityl moiety, causing some steric hindrance (Figure 16). Loading in bigger batches in a wide necked glass sinter can be inhomogeneous. Deprotection of the Fmoc group requires washing the resin with a 25% DMF solution of piperidine several times (3-4), leading to less Fmoc group-containing byproducts at the end of the synthesis. The completion of Fmoc deprotection was monitored by observation of a cleaved sample on a TLC plate under UV illumination (254 nm), although UV spectroscopic measurement is recommended for more accurate results in potential industrial use in the future.

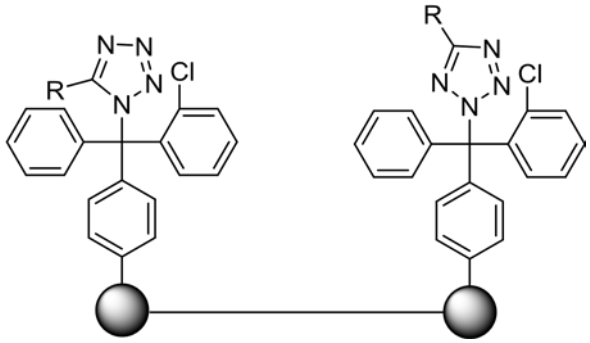


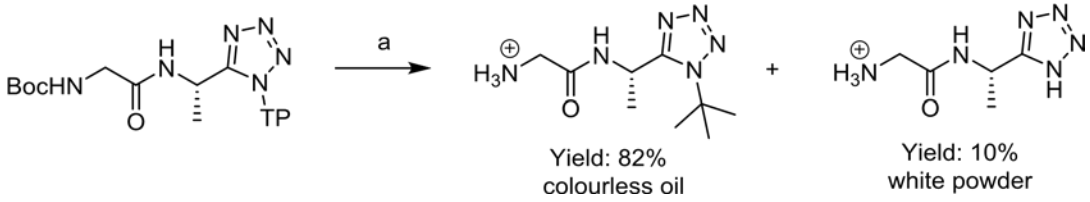
Figure 16 *Possible attachments of tetrazole compounds on 2-chlorotrityl resin*

	abbreviation	loading ratio (Fmoc-1-aminoethyltetrazole **73** : resin)	Theoretical yield of **69** (%) [p]	Percentage of Fmoc residue in **69** %	yield of **70** (%)
70g-L	Gly-L-Ala(T)	1:1	59	n.o.	99
70a-LL	L-Ala-L-Ala(T)	1:1	58	5	86
70a-LD	L-Ala-D-Ala(T)	1:1	89	<1	58
70d-LL	□-Ala-L-Ala(T)	1:1	67	2	78
70e-LL	L-Nor-L-Ala(T)	1:1	51	14[a]	77
70h-LL	L-Leu-L-Ala(T)	2:1	84	6	81
70h-LD	L-Leu-D-Ala(T)	1:1	69	<1	53
70i-LL	L-Ile-L-Ala(T)	1:1	62	2	86
70f-LL	L-Pyr-L-Ala(T)	2:1	89	12	84
70j-LL	L-Phe-L-Ala(T)	1:1	79	15[a]	20
70b-LL	L-Ala-L-EtGly(T)	1:1	52	3	86

Table 3 *Attachment for Scheme 27: yields and conditions. Abbreviations: p: the cleaved mixture was triturated and washed with petroleum ether 40-60*

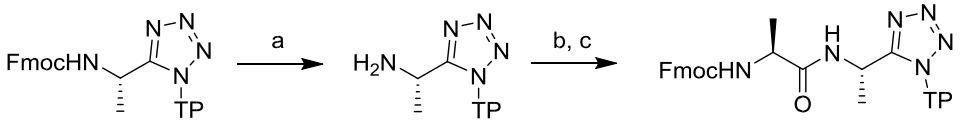
°C before yield and impurity calculation; n.o.: not observed; a: the loading was possibly inhomogeneous, and might have been washed with an insufficient amount of piperidine in DMF.

The use of a Boc- instead of a Cbz-protecting group was a reasonable development, with the aim being to remove a step from the synthesis. However, in our hands the last cleavage and Boc-deprotecting step with TFA caused *tert*-butyl substitution on the tetrazole ring, even in the presence of the cation scavenger TIS (Scheme 28).

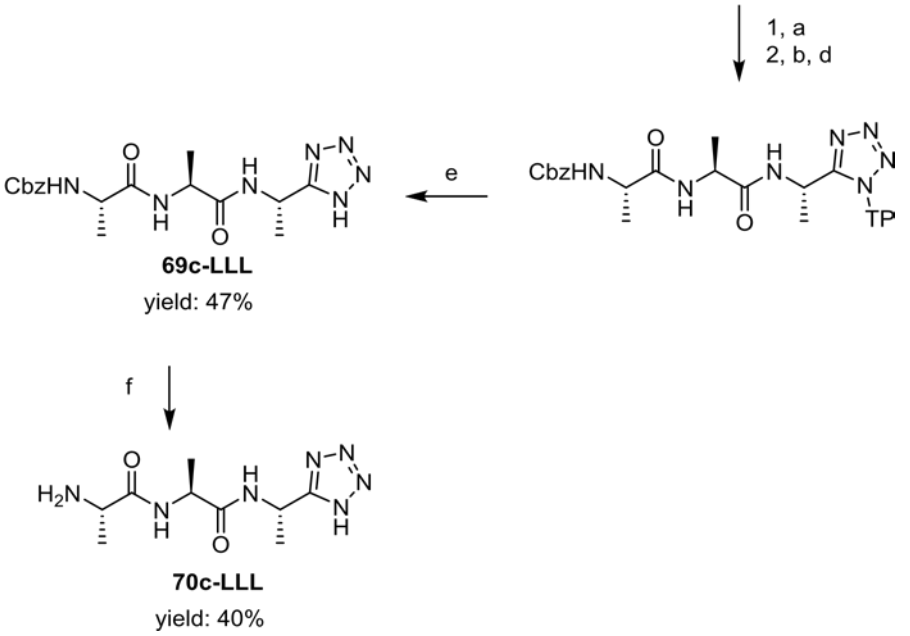


Scheme 28 *Synthetic issues relating to the use of Boc-protecting group with the tetrazoles. Reagents and conditions: (a) 95% TFA, 2.5% H_2O, 2.5% TIS, r.t..*

A tripeptide analogue, L-alanyl-L-alanyl-L-1-aminoethyltetrazole **70c-LLL** was accessed by coupling the attached L-1-aminoethyltetrazole **76-L** firstly with an Fmoc-L-alanine, followed by Fmoc deprotection with piperidine in DMF, and then coupling with a Cbz-L-alanine. Cbz-L-alanyl-L-alanyl-L-1-aminoethyltetrazole **69c-LLL** was cleaved from the resin with a mixture of 5% TFA, 95% DCM, 1 eq. TIS. Subsequent Cbz deprotection by catalytic hydrogenation resulted in L-alanyl-L-alanyl-L-1-aminoethyltetrazole **70c-LLL** (Scheme 29).



76



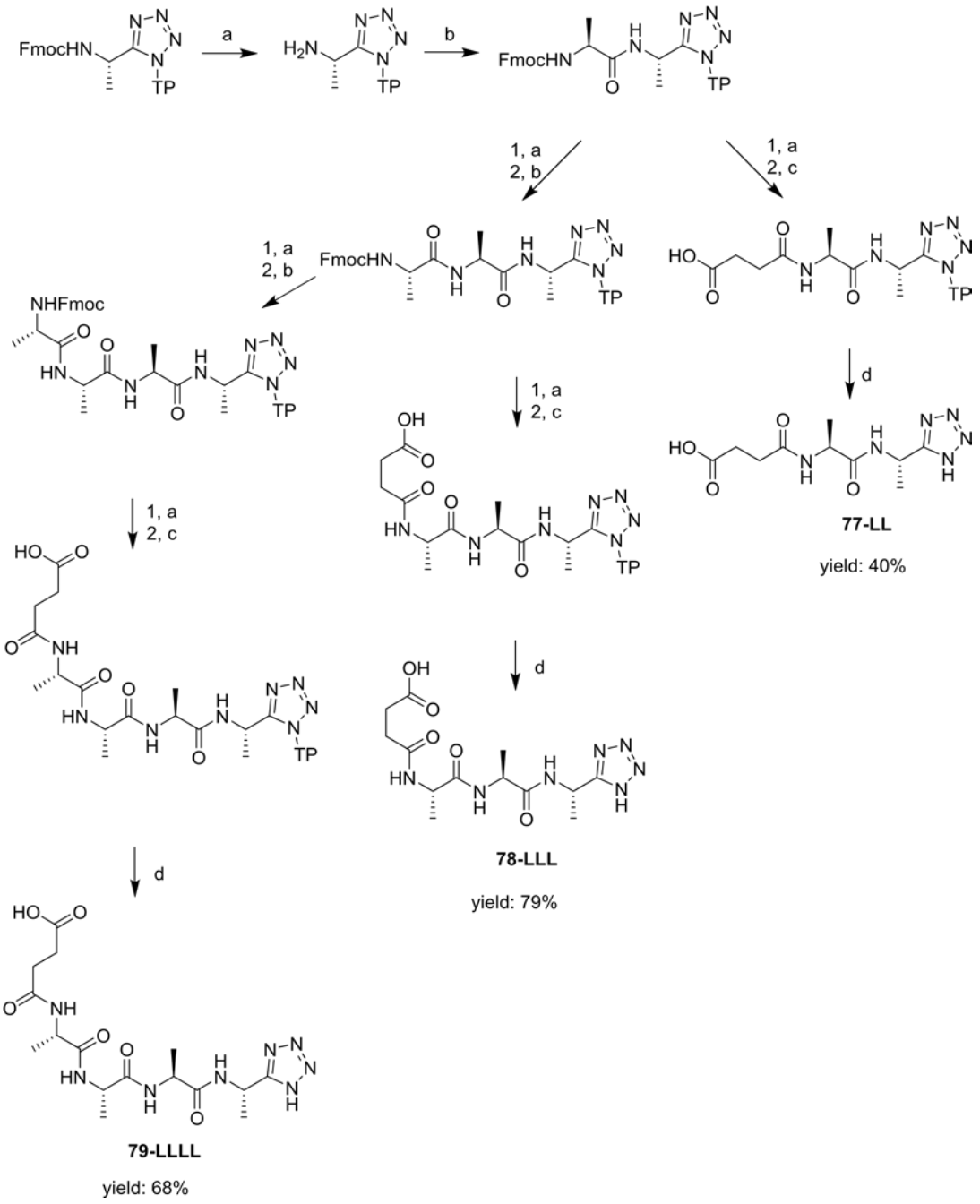
Scheme 29 *Solid phase synthesis of L-alanyl-L-alanyl-L-1aminoethyltetrazole* ***70c-LL****. Reagents and conditions: (a) piperidine, DMF, r.t.; (b) HBTU, DIPEA, DMF, r.t.; (c) Fmoc-L-alanine; (d) Cbz-L-alanine; (e)*
5% TFA, 95% DCM, 1 eq TIS (equivalent with the theoretical loading of the resin), r.t.; (f) $H_{2\,(g)}$*, 0.5% Pd/C, EtOH, 3 bar, r.t..*

A series of di-, tri- and tetra-peptidyl derivatives of *N*-terminal succinyl-, *C*terminal L-1-aminoethyltetrazole **77-LL**, **78-LLL**, **79-LLLL** were synthesized by the same strategy. After the introduction of the appropriate number of alanine residues (1 - 3), the peptides were furnished with a succinyl group using succinic anhydride and DIPEA in DMF (Scheme 30).

Although *N*-Cbz protected peptides were successfully cleaved from the resin by a mixture of 5% TFA, 95% DCM, 1eq TIS, with good solubility in the cleavage medium, the succinyl peptides **77-LL**, **78-LLL**, **79-LLLL** were not sufficiently soluble in this organic solvent mixture and were instead cleaved from the resin by a mixture of 95% TFA, 2.5% H_2O, 2.5% TIS, being soluble in this aqueous mixture. This method was convenient and more effective, due to the lack of a *N*-terminal protecting group.

These substrates were aimed at targeting bacterial elastase enzymes in order to achieve a different pattern of selective inhibition. It has beendescribed in the literature that succinyl-tripeptides were cleaved from chromogenic

peptidase substrates by *P. aeruginosa*.[80] Based on this information, succinyloligopeptides can be potential selective antibacterial agents against *P.*
aeruginosa.



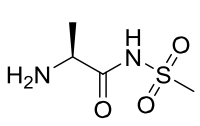
***Scheme 30**. Solid phase synthesis of series of N-terminal succinyl-, Cterminal L-1-aminoethyltetrazole oligopeptides **77-LL**, **78-LLL**, **79-LLLL**. Reagents and conditions: (a) piperidine, DMF, r.t.; (b) Fmoc-L-alanine, HBTU, DIPEA, DMF, r.t.; (c) Succinic anhydride, DIPEA, DMF, r.t.; (d) 95% TFA, 2.5% H_2O, 2.5% TIS, r.t..*

2.2.4 Microbiological evaluation

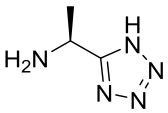
All synthesized alanine bioisostere derivatives were tested against a panel of clinically relevant pathogenic bacteria, by or with the supervision of Prof. John Perry, to investigate their selective antibacterial properties. The inhibition tests were carried out in antagonist (peptone-free), blood supplemented agar media containing the appropriate substrate in different concentrations (1-128 mg/L). These tests were suitable to measure minimal inhibitory concentration (MIC), by observing the growth of bacteria in the presence of the appropriate antibacterial agent. As only small amount of bacteria are inoculated onto the plates, actual bactericidal activity cannot be identified.

L-Alanyl-L-alanyl-L-1-aminoethyltetrazole **70c-LLL** and L-alanyl-L-1aminoethyltetrazole **70a-LL** have been synthesized and tested against a few bacteria in the past, such as *E. coli, S. aureus* and *Salmonella dublin*. In this previous work they were reported to be inactive, although it is hard to compare our new data to these earlier published results due to lack of information regarding the exact experimental protocols that were followed in the earlier studies. For instance, results may differ due to differences in the media, such as peptone content, specificity of the bacterial strain or the difference between the inoculum.[52,53,*52*] In our more detailed investigation which focused upon clinically relevant strains of pathogenic bacteria, and using protocols that have now been established as clinical standard over many years we obtained results that provide a significant contrast to the earlier body of work.

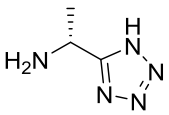
Single alanine bioisosteres have only limited activity (Table 4), as the natural compound alanine is transported as oligopeptide forms into bacterial cells, and not generally as a single amino acid.[41] This result suggests that the increase in lipophilicity associated with changing the carboxyl in the natural compound to other acidic moiety, such as tetrazole, in our analogue does not produce effective passive diffusion at an extent that would lead to significant antimicrobial activity. Furthermore, L-1-aminopropyltetrazole **32b-L** was also inactive; this result could be explained either by issues of transportation into the bacterial cells, or by inactivity against the AlaR enzyme.



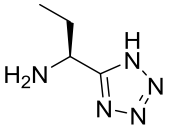
27b-L



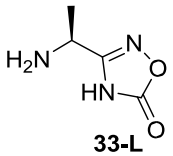
32a-L



32a-D



32b-L



33-L

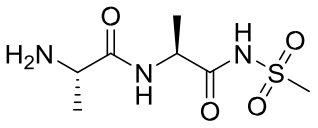
		MIC (mg/L)				
	Strain ID	**27b-L[a]**	**32a-L[a]**	**32a-D[a]**	**32b-L[a]**	**33-L[a]**
Acinetobacter baumannii	ATCC 19606	>128	>128	>128	>128	>128
Burkholderia cepacia	ATCC 25416	>128	>128	>128	>128	>128
Enterobacter cloacae	NCTC 11936	>128	>128	>128	>128	>128
Escherichia coli	NCTC 10418	>128	>128	>128	>128	>128
Klebsiella pneumoniae	NCTC 9528	>128	>128	>128	>128	>128
Providencia rettgeri	NCTC 7475	>128	>128	>128	>128	>128
Pseudomonas aeruginosa	NCTC 10662	>128	>128	>128	>128	>128
Salmonella Typhimurium	NCTC 74	>128	>128	>128	>128	>128
Serratia marcescens	NCTC 10211	>128	>128	>128	>128	>128
Yersinia enterocolitica	NCTC 11176	>128	>128	>128	>128	>128
Escherichia coli	NCTC 12241	>128	>128	>128	>128	>128
Salmonella Enteritidis	NCTC 6676	>128	>128	>128	>128	>128
Enterococcus faecalis	NCTC 775	>128	>128	>128	>128	>128
Enterococcus faecium	NCTC 7171	>128	>128	>128	>128	>128
Listeria monocytogenes	NCTC 11994	>128	>128	>128	>128	>128
Staphylococci epidermidis	NCTC 11047	>128	>128	>128	>128	>128
Staphylococcus aureus	NCTC 6571	>128	>128	>128	>128	>128
Staphylococcus aureus (MRSA)	NCTC 11939	>128	>128	>128	>128	>128

Table 4 *MIC values of different alanine bioisosteres; a: inoculum 150000 CFU/spot*

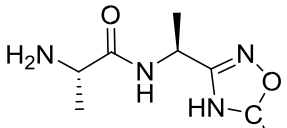
In marked contrast to the results with the single amino acid analogues, targeting the dipeptide permease systems of bacteria proved to be successful in the case of L-alanyl-L-1-aminoethyltetrazole **70a-LL**, and represents the potential of intracellular liberation of L-1-aminoethyltetrazole **32a-L**. L-AlanylL-1-aminopropyltetrazole **70b-LL** inhibited only *Enterobacter cloacae*, which provides a possible selectivity to investigate in the future. The contrast between the results obtained with L-alanyl-L-1-aminopropyltetrazole **70b-LL** and L-alanyl-L-1-aminoethyltetrazole **70a-LL** represents the difference between L-1-aminoethyltetrazole **32a-L** and L-1-aminopropyltetrazole **32b-L** homologues, as L-1-aminoethyltetrazole **32a-L** displays a bioisosteric

replacement of the natural substrate of AlaR and can inhibit the enzyme in different species. The methylene group larger homologue **32b-L** probably has limited access to the active site of the enzyme in most bacterial AlaR. Other analogues either did not inhibit the AlaR enzyme, either possibly decomposed before they could act on AlaR or they may have been impervious to hydrolysis, which would mean the active substance might not be liberate in these species (Table 5).

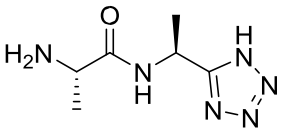
In this research, there was no resources or time to investigate the mechanism of action in depth. Further enzymatic studies can be done in the future to understand these results, by investigating the interaction of these compounds with alanine racemase, alanine aminopeptidase and permease systems.



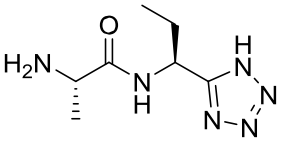
65-LL/D



72-LL



70a-LL



70b-LL

		MIC (mg/L)			
	Strain ID	**65-LL/D[ab]**	**70a-LL[ab]**	**70b-LL[ac]**	**72-LL[ab]**
Acinetobacter baumannii	ATCC 19606	>128	>128	>128	>128
Burkholderia cepacia	ATCC 25416	>128	>128	>128	>128
Enterobacter cloacae	NCTC 11936	>128	≤ 1	8	>128
Escherichia coli	NCTC 10418	>128	≤ 1	>128	>128
Klebsiella pneumoniae	NCTC 9528	>128	≤ 1	>128	>128
Providencia rettgeri	NCTC 7475	>128	>128	>128	>128
Pseudomonas aeruginosa	NCTC 10662	>128	64	>128	>128
Salmonella Typhimurium	NCTC 74	>128	>128	>128	>128
Serratia marcescens	NCTC 10211	>128	2	>128	>128
Yersinia enterocolitica	NCTC 11176	>128	≤ 1	>128	>128
Escherichia coli	NCTC 12241	>128	≤ 1	>128	>128
Salmonella Enteritidis	NCTC 6676	>128	>128	>128	>128
Enterococcus faecalis	NCTC 775	>128	>128	>128	>128
Enterococcus faecium	NCTC 7171	>128	>128	>128	>128
Listeria monocytogenes	NCTC 11994	>128	>128	>128	>128
Staphylococci epidermidis	NCTC 11047	>128	>128	>128	>128
Staphylococcus aureus	NCTC 6571	>128	>128	>128	>128
Staphylococcus aureus (MRSA)	NCTC 11939	>128	>128	>128	>128

Table 5 *MIC values of C-terminal alanine bioisostere containing dipeptides; a: inoculum 150000 CFU/spot; b: the tested compound was prepared by solution phase peptide coupling; c: the tested compound was prepared by solid phase peptide coupling.*

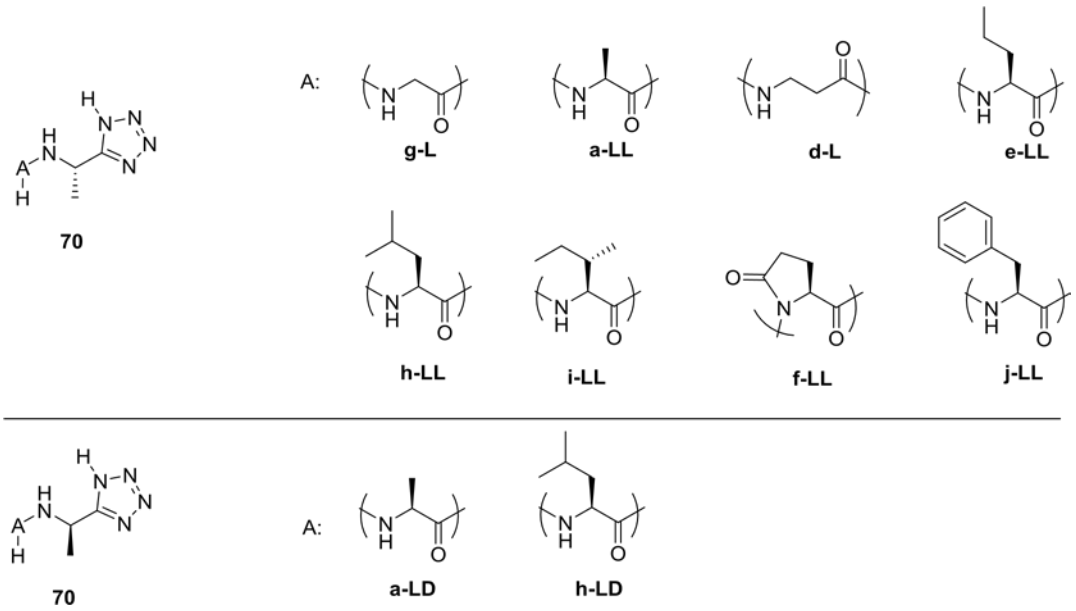
Depending upon the *N*-terminal amino acid, the growth of different Gramnegative and Gram-positive bacteria can be inhibited, thus selectivity can be tuned by choosing specific natural *N*-terminal amino acids within the dipeptide analogues.

Some of the compounds have an inhibition profile that allows them to be applied as selective antibacterial agents in culture media or potentially evaluated for clinical treatment for combating infections after further investigation. The glycyl peptide **70g-L** was only active against a few species, and even then only in higher concentrations. In addition the □-alanyl **70d-L** substrate did not show any antibacterial activity. This compound was aiming to target the □-alanyl aminopeptidase, which only presents in a few bacteria, such as *P. aeruginosa,*[81] *Serratia marcescens* and *Burkholderia cepacia*.[82] This result suggests that either the side chain or the chirality of the N-terminal amino acid might have an important role in their transportation.

Importantly, it was shown that L,L-alanyl peptide **70a-LL** was effective in inhibiting the growth of many Gram-negative species, but did not display inhibition against *Salmonella*. This pattern of activity leads to a possible application for this compound; it could be used in *Salmonella* selective media to improve the clinical detection of *Salmonella* from stool samples, in which overgrowth by commensal gut bacteria, such as *E. coli*, is problematic.[12]

With longer lipophilic side chains L,L-norvalyl **70e-LL**, leucyl **70h-LL**, isoleucyl **70i-LL** and phenylalanyl **70j-LL** peptides had additional antibacterial activity against Gram-positive bacteria, introducing new wide spectrum and selective antibacterial agents. This activity against Gram-positive bacteria may possibly occur due to the lipophilic side chains of the substrates. This could enable passive diffusion as well as, or instead of, active transport into bacteria. The enhanced activity of these peptides with longer side chains might be the result of the reported affinity for longer lipophilic larger side chains of the bacterial proton-dependent oligopeptide transporter proteins.[83] As L,L-leucyl **70h-LL** isoleucyl **70i-LL** and phenylalanyl **70j-LL** peptides selectively allow the growth *Salmonella* species in smaller concentrations, they also have potential use in a new improved *Salmonella* selective medium. Another useful finding is the selective inhibition of enterococci by L-pyroglutamyl derivative **70f-LL**. This pyroglutamyl derivative was selected and synthesized to target enterococci, as they are known to have pyroglutamyl aminopeptidase enzyme.[84] The great selectivity displayed by this compound might due to the amide motif on the

*N*terminus, as the *N*-terminal amino group is necessary for the transport carried out by permease systems.[41] Interestingly, the D-1-aminoethyltetrazole containing peptides **70a-LD**, **70h-LD** were totally inactive, although their L-1aminoethyltetrazole containing diastereomers **70a-LL**, **70h-LL** inhibited many different species (Table 6). This again suggests an active transport mode of entry wherein the transporter would potentially display chiral selectivity.



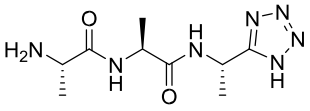
		MIC(mg/L)										
	Strain ID	**70g-L**[bd]	**70a-LL**[ac]	**70a-LD**[ad]	**70d-L**[ac]	**70e-LL**[ac]	**70h-LL**[bc]	**70h-LD**[ad]	**70i-LL**[ad]	**70f-LL**[bd]	**70f-LL**[ac]	**70j-LL**[bd]
Acinetobacter baumannii	ATCC 19606	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Burkholderia cepacia	ATCC 25416	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	≤ 1
Enterobacter cloacae	NCTC 11936	32	≤ 1	>128	>128	≤ 1	≤ 1	>128	4	>128	>128	8
Escherichia coli	NCTC 10418	>128	≤ 1	>128	>128	≤ 1	≤ 1	>128	8	>128	>128	4
Klebsiella pneumoniae	NCTC 9528	>128	≤ 1	>128	>128	≤ 1	2	>128	>128	>128	8	16
Providencia rettgeri	NCTC 7475	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Pseudomonas aeruginosa	NCTC 10662	>128	64	>128	>128	64	64	>128	128	>128	>128	>128
Salmonella Typhimurium	NCTC 74	>128	>128	>128	>128	2	64	>128	128	>128	>128	>128
Serratia marcescens	NCTC 10211	>128	2	>128	>128	≤ 1	≤ 1	>128	16	8	8	8
Yersinia enterocolitica	NCTC 11176	128	≤ 1	>128	>128	≤ 1	≤ 1	>128	2	4	4	NT
Escherichia coli	NCTC 12241	64	≤ 1	>128	>128	≤ 1	≤ 1	>128	2	>128	>128	4
Salmonella Enteritidis	NCTC 6676	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Enterococcus faecalis	NCTC 775	>128	>128	>128	>128	≤ 1	2	>128	>128	≤ 1	2	2
Enterococcus faecium	NCTC 7171	>128	>128	>128	>128	2	16	>128	64	≤ 1	2	32
Listeria monocytogenes	NCTC 11994	>128	>128	>128	>128	4	>128	>128	>128	>128	>128	>128
Staphylococci epidermidis	NCTC 11047	>128	>128	>128	>128	2	2	>128	>128	>128	>128	>128
Staphylococcus aureus	NCTC 6571	64	>128	>128	64	4	16	128	128	>128	>128	≤ 1
Staphylococcus aureus (MRSA)	*NCTC 11939*	*>128*	*>128*	*>128*	*128*	*>128*	*8*	*>128*	*>128*	*>128*	*>128*	*>128*

Table 6 *MIC values of C-terminal 1-aminoethyltetrazole containing dipeptides* ***70a,d-j****; NT: not tested; a: inoculum 150000 CFU/spot; b: inoculum 10000 CFU/spot; c: the tested compound was prepared by solution phase peptide coupling; d: the tested compound was prepared by solid phase peptide coupling.*

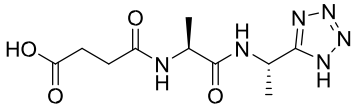
L-Alanyl-L-alanyl-L-1-aminoethyltetrazole **70c-LLL** displayed a similar profile to the previously discussed dipeptides **70a-LL** and **70h-LL**, which also raises its potential in *Salmonella* detection.

The *N*-terminal succinyl containing peptides did not produce significant inhibitory effects against most bacteria (Table 7). These results support the Gilvarg review, which stated that peptide permease systems recognise the *N*terminal amino group of the peptide for the peptide transport.[41]

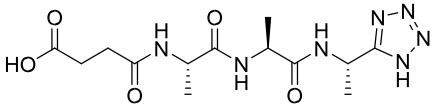
Different substrates were prepared and tested in different phases of the research programme. In general the earlier compounds were prepared using a solution phase approach, while the later compounds were prepeared using solid phase peptide coupling, as it became apparent that these methods were more efficient. However, the different synthetic techniques employed resulted in different impurity profiles, including different diastereomeric excess of the final compounds. Moreover, during the progress of the research programme the applied inoculum also changed from 150000 CFU/spot to 10000 CFU/spot and 1000 CFU/spot. In the initial phases of the research programme the main purpose was to conduct general screening, later the interest focused on finding more active substrates. These factors produce slight apparent differences in the microbiological activities. These factors are best represented using **70f-LL** (Table 6) and **70c-LLL** (Table 7) as examples. Due to these factors both of these compounds were tested with the two different inocula. It is apparent that some MIC values are higher with the higher inoculum. This is relatively easy to rationalise in that the inhibition of more bacteria in the higher inoculum requires a correponding higher substrate concentration, as the experiments were done in plates with the same size. The differences between inhibited species are presumably due to differences in the diastereomeric ratio of the antibacterial compound. As an example of this latter effect, the inhibiton of *K. pneumoniae* by the sample of **70f-LL** (MIC: 8 mg/L) synthesized by solution phase or **70c-LLL** (MIC: 2 mg/L) synthesized by solid phase technique.



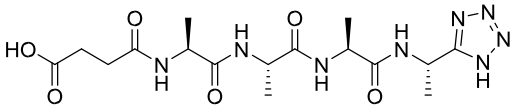
70c-LLL



77-LL



78-LLL



79-LLLL

		MIC (mg/L)				
	Strain ID	**70c-LLL**[bd]	**70c-LLL**[ac]	**77-LL**[bd]	**78-LLL**[bd]	**79-LLLL**[ad]
Acinetobacter baumannii	ATCC 19606	> 128	> 128	> 128	> 128	>128
Burkholderia cepacia	ATCC 25416	> 128	> 128	> 128	> 128	>128
Enterobacter cloacae	NCTC 11936	≤ 1	≤ 1	> 128	> 128	128
Escherichia coli	NCTC 10418	2	2	> 128	> 128	>128
Klebsiella pneumoniae	NCTC 9528	2	> 128	> 128	> 128	>128
Providencia rettgeri	NCTC 7475	> 128	> 128	> 128	> 128	>128
Pseudomonas aeruginosa	NCTC 10662	32	> 128	> 128	> 128	>128
Salmonella Typhimurium	NCTC 74	> 128	> 128	> 128	> 128	>128
Serratia marcescens	NCTC 10211	≤ 1	8	> 128	> 128	>128
Yersinia enterocolitica	NCTC 11176	> 128	4	NT	NT	>128
Escherichia coli	NCTC 12241	2	2	> 128	> 128	>128
Salmonella Enteritidis	NCTC 6676	> 128	> 128	> 128	> 128	>128
Enterococcus faecalis	NCTC 775	> 128	> 128	> 128	> 128	>128
Enterococcus faecium	NCTC 7171	> 128	> 128	> 128	> 128	>128
Listeria monocytogenes	NCTC 11994	> 128	> 128	> 128	> 128	>128
Staphylococci epidermidis	NCTC 11047	32	> 128	> 128	> 128	>128
Staphylococcus aureus	NCTC 6571	> 128	> 128	≤ 1	≤ 1	>128
Staphylococcus aureus (MRSA)	NCTC 11939	> 128	> 128	> 128	> 128	>128

Table 7 *MIC values of C-terminal 1-aminoethyltetrazole containing oligopeptides; NT: not tested; a: inoculum 150000 CFU/spot; b: inoculum 10000 CFU/spot; c: the tested compound was prepared by solution phase peptide coupling; d: the tested compound was prepared by solid phase peptide coupling.*

Selective inhibition of *E. coli* by L-leucyl-L-1-aminoethyltetrazole **70h-LL** and L-alanyl-L-alanyl-L-1-aminoethyltetrazole **70c-LLL** was studied in more detail (Figure 17, 18). L-Leucyl-L-1-aminoethyltetrazole **70h-LL** and L-alanyl-Lalanyl-L-1-aminoethyltetrazole **70c-LLL** were tested against 42-43 *Salmonella* spp. and 16-18 *E. coli* at a range of concentrations between 0.125

and 8 mg/L. Substrate **70c-LLL** was tested at inoculum 10000 CFU/spot and **70h-LL** at inoculum 1000 CFU/spot, they were studied at different stages of the research programme. The lower inoculum aims to mimic a general faecal clinical sample, in which *E. coli* species normally overgrow relative to the *Salmonella* species present. L-Leucyl-L-1-aminoethyltetrazole **70h-LL** can be applied most selectively at a concentration of 0.5 mg/L. At this critical concentration most *E. coli* (n=16), but only a few *Salmonella* spp. (n=7) were found to be inhibited. L-Alanyl-L-alanyl-L-1-aminoethyltetrazole **70c-LLL** is effective at higher concentrations due to the applied higher inoculum, most *E. coli* (n=12) and only 1 *Salmonella* spp. were inhibited at 8 mg/L. There is thought to be great potential in a clinical application for either of these two compounds as supportive antibacterial agents in a new *Salmonella* selective medium. A medium such as this would need development, as these compounds only have their activity in peptone free media. It is thought that peptones probably compete with these substrates, thereby making them less effective.

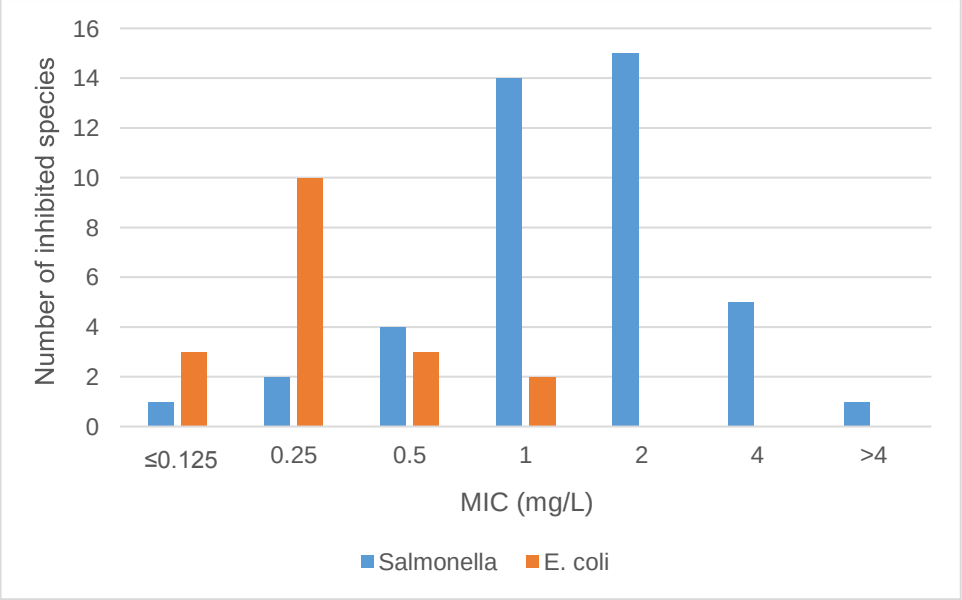


Figure 17 *Overlap of MIC values of L-leucyl-L-1-aminoethyltetrazole* ***70h-LL*** *(synthesis: solid phase peptide coupling) for 42 Salmonella spp. and 18 E. coli tested at low inoculum (1000 CFU/spot). The graph values are cumulative; the species inhibited iat a certain concentration are also inhibited in higher concentrations.*

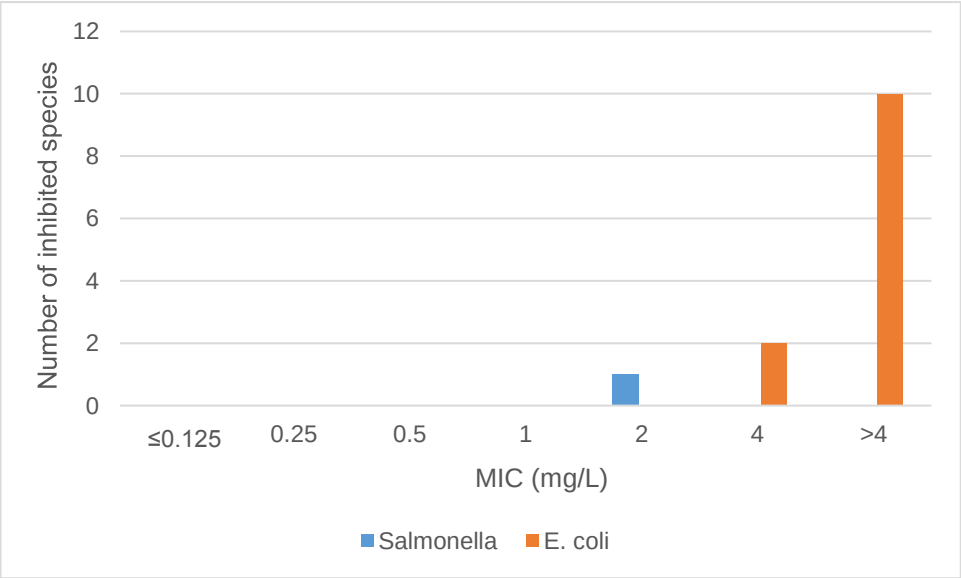


Figure 18 *Overlap of MIC values of L-alanyl-L-alanyl-L-1-aminoethyltetrazole* ***70c-LLL*** *(synthesis: solid phase peptide coupling) for 43 Salmonella spp. and 16 E. coli tested at high inoculum (10000 CFU/spot). The graph values are cumulative; the species inhibited at a certain concentration are inhibited in higher concentrations.*

2.2.5 Conclusion

Research was conducted to find new antibacterial agents for medicinal and diagnostic applications. Three types of alanine carboxylate bioisosteres were synthesized as potential alanine racemase inhibitors (Figure 19). All molecules were tested against a collection of clinically relevant pathogenic bacteria. As the transportation of single alanine into the cell is not provided by any bacteria, these bioisosteres did not have growth inhibitory effects.

Figure 19 *Alanine bioisosteres as potential alanine racemase inhibitors*

Further peptide derivatives were designed, synthesised, and their antibacterial activity was tested, as amino acids are transported as di- and oligopeptides into bacterial cells by permease enzymes and internally liberated by amino peptidase enzymes. L-Alanyl-L-alanylmethanesulfonamide **65-LL/D** and L-1aminoethyl-5-oxo-1,2,4-oxadiazole **72-LL** showed no significant inhibition against the strains tested (Figure 20); one possible reason for this could be due to their inactivation by hydrolysis by bacterial enzymes.

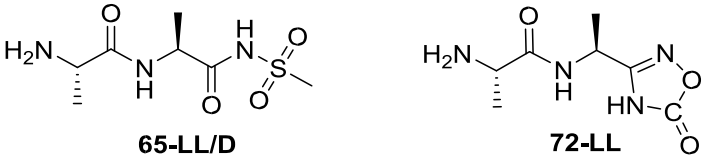


Figure 20 *L-Alanyl-alanylmethanesulfonamide* ***65-LL/D*** *and L-1-aminoethyl-5-oxo-1,2,4-oxadiazole* ***72-LL***

A library of di- and tri- and succinyl oligo-peptide analogues of L-1aminoethyltetrazole was synthesized by a combination of solution and solid phase peptide coupling methods, substrates with antibacterial activity are displayed in Figure 21. The solid phase synthetic method is a novel development for the production of peptides with a C-terminal tetrazole moiety, these peptides were made on 2-chlorotrityl resin by a classic Fmoc peptide coupling strategy using the standard reagents HBTU and DIPEA. Many of

these peptides shown significant antibacterial activity. Depending upon the attached amino acids, the substrates inhibited the growth of bacteria with different levels of selectivity. Some of the findings in this chapter have potential in real world applications. Probably the most important of these is the selective inhibition of *E. coli* and some other *Enterobacteriaceae* by L-alanyl- **70a-LL**, L-leucyl- **70h-LL** and dialanyl- **70c-LLL** substrates. These compounds should be explored further in a clinical setting as they could well form the basis for a *Salmonella* selective culture medium, as they had no or small effect on the growth of most *Salmonella* spp. Another valuable result is the selective inhibition of enterococci beside the growth of other cocci by L-pyroglutamyl dipeptide **70f-LL**. The clinical and commercial potential for this particular compound has already been proven by a patent application that has now been submitted by bioMérieux.[85]

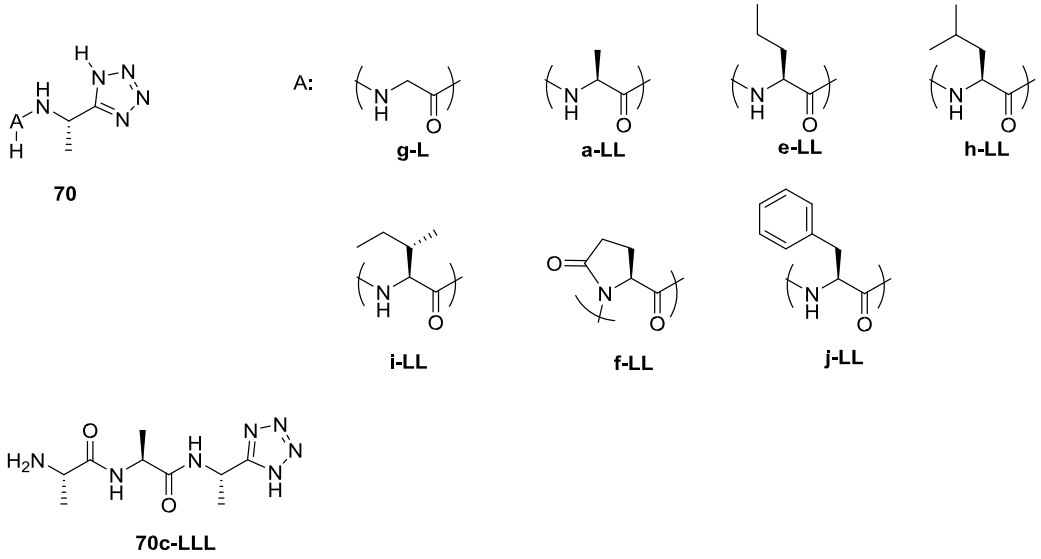


Figure 21 *Di and tripeptide analogues of L-1-aminoethyltetrazole with significant antibacterial activity*

2.3 Experimental section

2.3.1 General information

All commercially available reagents and solvents were obtained from Sigma-Aldrich, Apollo Scientific, Alfa Aesar, Fluorochem, or Fischer Scientific and were used without further purification. Melting points were recorded on a Reichart-Kofler hot-stage microscope apparatus and are uncorrected. Infrared spectra were recorded in the range 4000 – 600 cm^{-1} using a Perkin Elmer Spectrum BX FT-IR spectrophotometer. NMR spectra were obtained using a Bruker Ultrashield 300 spectrometer at 300 MHz for ^{1}H spectra or at 75 MHz for ^{13}C spectra and Bruker Avance III Ultrashield spectrometer 500 MHz for ^{1}H spectra or at 125 MHz for ^{13}C spectra. Low-resolution mass spectra were recorded on a Bruker Esquire 3000plus analyser using an electrospray source in either positive or negative ion mode. High resolution accurate mass measurements were collected by the EPSRC National Mass Spectrometry Facility at Swansea University. Elemental analyses were performed using an Exeter Analytical CE-440 Elemental Analyzer. Thin layer chromatography was performed on Merck silica gel $60F_{254}$. Fischer silica gel 60 (35-70 micron) was used for column chromatography; the samples were pre-absorbed onto silica 60 (35-70 micron). LC-MS analysis was performed using an Agilent 1290 Infinity Series HPLC system and an Agilent 6120 Quadrupole LC-MS detector. LC-MS data was analysed by Agilent ChemStation. Where required, compounds were purified by Agilent 1260 preparative HPLC. Hydrogenation reactions were performed in a Parr mini top bench reactor 4560. Air and moisture sensitive reactions were carried out in oven dried glassware under a nitrogen atmosphere in solvents dried over molecular sieves. Thin layer chromatography results were analysed by UV lamp (254 nm), ninhydrin stain (amine, amide content) and/or ceric ammonium molybdate stain (oxidising agent).

2.3.2 Chemistry

General method A: removal of the Boc protecting group with hydrochloric acid:

N-Boc protected compound (1.13 mmol, 1.00 eq) was stirred at room temperature in 2M HCl (11.3 mmol, 5.7 mL, 10.00 eq) containing diethyl ether overnight. After the reaction was complete the mixture was filtered and washed with diethyl ether.

General method B: synthesis of protected amino acid methyl ester:

To a solution of *N*-protected-amino acid (26.4 mmol, 1.00 eq) in dry DMF (70 mL) cesium carbonate (4.72 g, 14.5 mmol, 0.55 eq) was added and the mixture was stirred for 30 min at room temperature. After dropwise addition of methyl iodide (1.73 mL, 27.8 mmol, 1.07 eq), the reaction was stirred overnight. Ethyl acetate (50 mL) was then added to the mixture and it was washed with water (3 x 50 mL), then the combined aqueous phase was extracted with ethyl acetate (50 mL). The combined organic phase was washed with 10% K_2CO_3 solution (2 x 30 mL), then with brine (30 mL), dried over $MgSO_4$, and subsequently filtered. The solvent was removed under reduced pressure to give the product as a white solid.

General method C: synthesis of protected amino amide:

The protected amino acid methyl ester (420 mmol, 1.00 eq) was dissolved and stirred in 7M solution of ammonia in methanol (60 mL) overnight at 50 °C in a sealed tube. After the reaction was complete, as determined by TLC, the mixture was evaporated to dryness under reduced pressure.

General method D: synthesis of protected amino nitrile:

To a solution of the required *N*-protected-alanine amide (19.0 mmol, 1.00 eq) in DCM (9 mL), pyridine (14 mL) was added. After cooling to 0 °C, tosyl chloride (4.7 g, 24.7 mmol, 1.30 eq) was added to the solution. Stirring was continued for 30 min at 0 °C and then overnight at room temperature. After the

reaction was complete, as determined by TLC, the system was cooled to 0 °C and quenched with water (70 mL) and ethyl acetate (70 mL). After separation, the aqueous layer was extracted with ethyl acetate (4 x 30 mL) and the combined organic phase was washed with 1.2 M HCl (3 x 40 mL), and saturated $NaHCO_3$ (50 mL), then brine (50 mL). The whole solution was dried over $MgSO_4$, and evaporated under reduced pressure.

General method E: synthesis of Cbz-1-aminoalkyltetrazole:

To a suspension of NH_4Cl (2.03 g, 38 mmol, 1.00 eq) and NaN_3 (2.62 g, 38 mmol, 1.00 eq) in dry DMF (60 mL) in a two-necked round bottom flask equipped with reflux condenser and drying tube, the required Cbz-amino nitrile (38 mmol, 1.00 eq) was added. The mixture was stirred and heated to 90 °C for 1 h. To the cooled mixture, another portion of NH_4Cl (1.04 g, 19 mmol, 0.50 eq) and NaN_3 (1.31 g, 19 mmol, 0.50 eq) were added and the mixture was heated at 90 °C overnight. When the reaction was complete, as determined by TLC, the mixture was filtered and the residue was washed with ethyl acetate. The filtrate was evaporated under reduced pressure. Water (180 mL) was added to the residue and it was acidified to pH 1 with 2.5 M HCl aqueous solution. The precipitated solid was filtered off, and washed with water to give the product.

General method F: removal of the Cbz protecting group:

To the solution of the protected amino acid or peptide derivative (1 meq) in methanol, 5% palladium on activated carbon (0.1 meq) was added portion wise. The mixture was stirred at room temperature under 2 bar pressure of hydrogen overnight in an autoclave. After the reaction was complete determined by TLC, the mixture was filtered through celite and then the solvent removed in vacuum.

General method G: Fmoc protection:

10% Aqueous Na_2CO_3 (13 mL) was added to the suspension of 1-aminolakyltetrazole (4.87 mmol, 1.00 eq) in 1,4-dioxane (7 mL). After the addition of Fmoc chloride (1.38 g, 5.35 mmo, 1.10 eq) solution in 1,4-dioxane

(7 mL), the mixture was stirred for 30 min at 0 °C, then overnight at room temperature. When the reaction was complete, as determined by TLC, it was acidified with 2.5 M HCl aqueous solution (11 mL). The mixture was filtered and the solid product was washed with water before drying.

General method H: solution phase peptide coupling:

To a solution of *N*-protected amino acid (1.77 mmol, 1.00 eq) in dry DMF (7 mL), NMM (0.20 mL, 1.77 mmol, 1.00 eq) was added at -5 °C. After the dropwise addition of IBCF (0.23 mL, 1.77 mmol, 1.00 eq), the mixture was stirred at -5 °C for 1 h. The solution of the amino acid derivative (1.77 mmol, 1.00 eq) and NMM (0.20 mL, 1.77 mmol, 1.00 eq) for a free amine or 0.39 mL, 3.54 mmol, 2.00 eq for an ammonium salt) were added to the mixture. This mixture was stirred at -5 °C for 10 min, then at room temperature overnight. After the reaction was complete, as determined by TLC, the mixture was evaporated under reduced pressure.

General method I: solid phase peptide coupling:

Swelling: The resin was bubbled for 30 min in a long-necked glass sinter in a solvent (DMF or DCM) with a volume 3x that of the bed volume. Subsequently the solvent was removed by filtration.

Washing off: The resin was washed with DMF (3 x 8 mL for 30 s), IPA (2 x 8 mL for 30 s) and finally with petroleum ether (40 – 60 °C) (2 x 8 mL for 30 s). The resin was air-dried, then dried in a vacuum oven at 40 °C overnight. The solvents were removed by filtration after each step.

Attachment to 2-chlorotrityl chloride resin: After swelling the resin in DCM the solution of Fmoc-L-1-aminoethyltetrazole **22** and DIPEA in DCM was added to the resin, and the mixture was bubbled for 2 h. After filtration, the resin was bubbled with DCM, DIPEA and methanol mixture for 1 h. Later the resin was washed with DMF (3 x 8 mL for 30 s), methanol (3 x 8 mL for 30 s) then with DCM or petroleum ether (40 – 60 °C) (3 x 8 mL for 30 s) depending on whether the resin needed to be swelled or dried. The solvents and solutions were removed by filtration after each step.

Fmoc deprotection: The resin swelled in DMF was treated with a mixture of piperidine and DMF (1:4 ratio, 10 mL/g resin, 1 x 5 min and 3 x 10 min), then it was alternately washed with DMF and IPA (2-2 x 8 mL for 30 s) finished with washing with DMF (8 mL for 30 s). The solvents and solutions were removed by filtration after each step.

Fmoc test: A sample consisting of a few resin beads was washed with DMF (4 x 1 mL for 30 s), IPA (5 x 1 mL for 30 s) and with methanol (2 x 1 mL for 30 s), followed by transfer into a separate flask by washing with 1% TFA in DCM (2 x 1 mL for 30 s). The filtrate was placed on a TLC plate with a capillary and observed under UV light (254 nm). A positive test shows fluorescence on the TLC plate. The solvents and solutions were removed by filtration after each step.

Coupling: DIPEA (1 eq) was added to the solution of HBTU (3 eq) and the protected amino acid (3 eq) in DMF (10 mL). The solution was stirred for 8 min and then it was added to the swelled resins. After 20 min bubbling with nitrogen, more DIPEA (0.5 eq) was added to the mixture, then it was bubbled until the TNBS test showed a negative result. After the reaction was complete the resin was washed off using the procedure mentioned above.

Coupling with succinic anhydride: The solution of succinic anhydride (3 eq) in DMF (10 mL) was added to the swelled resins, followed by the addition of DIPEA (1 eq). It was bubbled until the TNBS test showed a negative result (typically 1h). After the reaction was complete the resin was washed off using the procedure mentioned above.

TNBS test: The solution of DIPEA in DMF (10%, 3 drops) and the aqueous solution of TNBS (1 M, 3 drops) were mixed with sample consisting of a few resin beads. After a waiting period of 10 min, the resin beads were inspected under a light microscope (magnification 4x). The result was positive if the beads were coloured red, orange or yellow and it was negative if the beads were colourless.

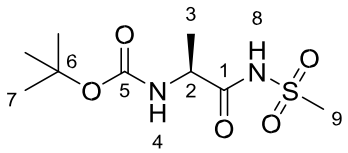
Cleavage from resin: The resin swelled in DCM was treated with 1% TIS containing TFA, DMF mixture (5:95 ratio, 10 mL/g) for 1 h. After washing the

resin with the same TFA, DCM mixture, the filtrate was evaporated under reduced pressure.

HPLC method A: column: ACE 5 C18 (150 x 3 mm i.d.); eluent: isocratic: water (0.1% formic acid) - methanol (0.1% formic acid) 95 : 5 ratio. The purity of all the compounds tested using this method were ≥95%.

Prep HPLC method A: column: Vydac protein and peptide 218TP510; eluent: gradient: water (0.1% formic acid) - methanol (0.1% formic acid) (0 – 90%).

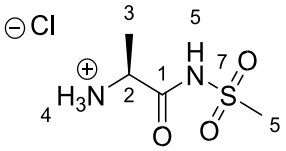
***tert*-Butyl *N*-[(1*S*)-1-(methanesulfonylcarbamoyl)ethyl]carbamate 26-L [56]**:



To a solution of Boc-L-alanine **24-L** (1.00 g, 5.28 mmol, 1.00 eq) in DCM (50 mL) CDI (1.03 g, 6.33 mmol, 1.20 eq) was added portionwise. The solution was stirred at 40 °C for 45 min. After cooling back to room temperature, DBU (2.16 mL, 21.1 mmol, 4.00 eq) and methanesulfonamide **25** (0.61 g, 6.33 mmol, 1.20 eq) were added to the reaction mixture and this was stirred overnight. After the reaction was complete, water (10 mL) then 2.5 M HCl_{aq} (18 mL) were added and the two layers were separated. The aquatic phase was extracted with DCM (3 x 20 mL), then the combined organic phase was washed with brine (20 mL), dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was precipitated from DCM – petroleum ether (40 – 60 °C) to give the desired product as a white solid (1.01 g, 72%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate, product R_f: 0.64). Mp: 123–124 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 11.77 (1H, s, 8-H), 7.17 (1H, d, *J*=7.5 Hz, 4-H), 4.00 (1H, quin, *J*=7.0 Hz, 2-H), 3.21 (3H, s, 9-H), 1.38 (9H, s, 7-H), 1.21 (3H, d, *J*=7.0 Hz, 3-H) ppm; ^{13}C NMR (126 MHz, DMSO-d_6) δ_C 173.7 (1-C), 155.6 (5-C), 78.8 (6-C), 50.5 (2-C), 41.3 (9C), 28.6 (7-C), 17.5 (3-C) ppm**;** ν_{max}/cm^{-1} 3365 (N-H), 3256 (N-H), 1730 (C=O), 1680 (C=O), 1521 (N-H), 1166 (C-O), 1137 (S=O); MS(ESI) *m/z* 289.1 $(M+Na)^+$**.**

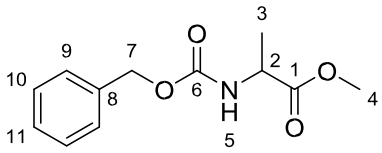
(2*S*)-2-Amino-*N*-methanesulfonylpropanamide hydrochloride salt 27b-L

56:



Boc-L-Alanylmethanesulfonamide **26-L** (0.30 g, 1.13 mmol) was deprotected using general method A. The filtered solid was washed with DCM, and dissolved in water, which was subsequently removed by freeze drying to give the product **27b-L** as a white amorphous hygroscopic solid (0.16 g, 70%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate – methanol, 4:1 ratio, R_f: 0.06). ^{1}H NMR (500 MHz, D_2O) δ_H 4.11 (1H, quart, *J*=7.5 Hz, 2-H), 3.33 (3H, s, 5-H), 1.58 (3H, d, *J*=7.2 Hz, 3-H); ^{13}C NMR (126 MHz, D_2O) δ_C 169.5 (1-C), 49.4 (2-C), 40.2 (5-C), 15.5 (3-C); ν_{max}/cm^{-1} 3377 (N-H), 3222 (N-H), 3006 (C-H), 2864 (C-H), 1717 (C=O), 1146 (S=O); MS(MALDI) *m/z* 167.14 $(M+H)^+$.

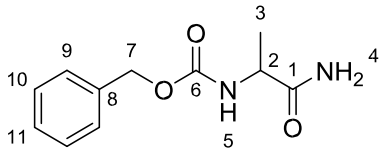
Methyl-(2*S* or *R*)-2-{[(benzyloxy)carbonyl]amino}propanoate 34a [86]:



Cbz-L/D-alanine **28a** (10.0 g, 45.0 mmol) was esterified and worked up using general method B, yielding the desired product as a white powder (10.6 g, 99%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate, 2:1 ratio, R_f: 0.51). Mp: 41 - 43 °C (literature: 43 – 44 °C, light petrol)[87]; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.74 (1H, d, *J*=7.5 Hz, 5-H), 7.34 (5H, m, 9,10,11-H), 5.03 (2H, s, 7-H), 4.11 (1H, quint, *J*=7.5 Hz, 2-H), 3.63 (3H, s, 4-H), 1.27 (3H, d, *J*=7.2 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 173.8 (1-C), 156.3 (6-C), 137.4 (8-C), 128.8 (2C, 10-C), 128.3

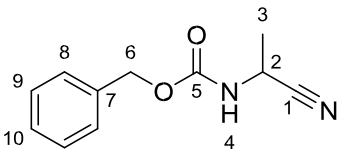
(11-C), 128.2 (2C, 9-C), 65.9 (7-C), 52.3 (4-C), 49.8 (2-C), 17.4 (3-C); ν_{max}/cm^{1} 3335 (N-H), 1703 (C=O), 1668 (C=O), 1524 (N-H), 1209 (C-O); MS(ESI) m/z 260.1 $(M+Na)^{+}$.

Benzyl-*N*-[(1*S* or *R*)-1-carbamoylethyl]carbamate 29a [57]:



Cbz-L/D-alanine methyl ester **34a** (10.0 g, 42 mmol) was reacted and worked up using general method C, to give the product as a white powder (9.40 g, 99%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate, 1:1 ratio, R_f: 0.11). Mp: 131 - 132 °C (literature: 130 – 131 °C, methanol – diethyl ether)[88]; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.35 (6H, m, 5,9,10,11-H), 7.26 (1H, s, 4-H_a), 6.93 (1H, s, 4-H_b), 5.02 (2H, s, 7-H), 3.98 (1H, quint, *J*=7.2 Hz, 2-H), 1.21 (3H, d, *J*=7.2 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 174.9 (1-C), 156.1 (6-C), 137.5 (8-C), 128.76 (2C, 10-C), 128.2 (11-C), 128.1 (2C, 9-C), 65.8 (7-C), 50.4 (2-C), 18.7 (3-C); ν_{max}/cm^{-1} 3386 (N-H), 3308 (N-H), 3196 (N-H), 1651 (C=O), 1537 (NH), 1247 (C-O); MS(ESI) m/z 223.1 $(M+H)^{+}$.

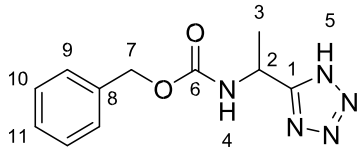
Benzyl-N-[(1*S* or *R*)-1-cyanoethyl]carbamate 30a[57]:



Cbz-L/D-alanine amide **29a** (3.48 g, 15.7 mmol) was reacted and worked up using general method D. The crude product was washed with heptane to give the product as white crystals (3.12 g, 97%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate 1:1 ratio, R_f: 0.41). Mp: 79 - 80 °C (literature: 82 – 83 °C [89]); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.14 (1H, d, *J*=5.4 Hz, 4-H), 7.37 (5H, m, 8,9,10-H), 5.08 (2H, s, 6-H), 4.59 (1H, quint, *J*=7.2 Hz, 2-H), 1.43 (3H, d, *J*=7.2 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 155.7 (5-C), 137.0 (7-C), 128.9 (9-C), 128.5 (10-C),

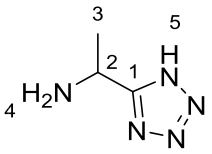
128.4 (8-C), 120.9 (1-C), 66.5 (6-C), 38.2 (2-C), 18.8 (3-C); ν_{max}/cm^{-1} 3332 (N-H), 2356 (C≡N), 1686 (C=O), 1521 (N-H), 1253 (C-O); MS(ESI) m/z 227.1 $(M+Na)^+$.

Benzyl-*N*-[(1*S* or *R*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)ethyl]carbamate 31a [57]:



Cbz-L/D-alanyl nitrile **30a** (7.80 g, 38.0 mmol) was reacted and worked up using general method E. The product was gained as a white solid (7.55 g, 80%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate 1:1, R_f: 0.09). Mp: 134 - 138 °C (literature: 139 - 141 °C [59]); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.99 (1H, d, *J*=6.9 Hz, 4H), 7.33 (5H, m, 9,10,11-H), 5.01 (3H, m, 2,7-H), 1.49 (3H, d, *J*=7,2 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 158.6 (1-C), 155.7 (6-C), 136.8 (8-C), 128.31 (2C, 10-C), 127.8 (3C, 9,11-C), 65.7 (7-C), 41.9 (2-C), 19.3 (3-C); ν_{max}/cm^{-1} 3308 (N-H), 2980, 2866, 2738, 2614 (tetrazole), 1686 (C=O), 1524 (N-H), 1255 (C-O); MS(ESI) m/z 248.1 $(M+H)^+$.

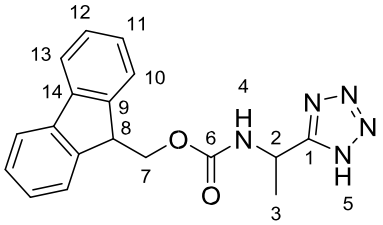
(1*S* or *R*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)ethan-1-amine 32a [57]:



Cbz-L/D-aminoethyltetrazole **31a** (0.75g, 3.0 mmol) was deprotected using general method F. Following filtration, the solid residue was washed with ethanol and the filtrate was evaporated under reduced pressure to give the product as a white solid (0.33 g, 97%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate - ethanol 8:1 ratio, R_f: 0.03). Mp: 230 °C (decomposed) (literature: 268 – 270 °C, (decomposed) **[57]**). ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 4.55 (1H, quart, *J*=6.6 Hz, 2-H), 1.54 (3H, d, *J*=6.9 Hz, 3H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 160.4 (1-C), 44.1 (2-C), 19.8 (3-C);

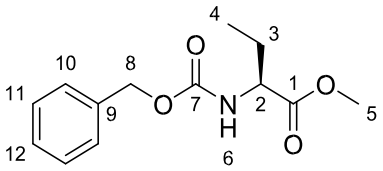
ν_{max}/cm^{-1} 3388 (N-H), 2916, 2719, 2634, 2522 (tetrazole); MS(ESI) m/z 114.1 $(M+H)^+$.

9*H*-Fluoren-9-ylmethyl-*N*-[(1*S* or *R*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamate 73[90]:



L/D-1-Aminoethyltetrazole **32a** (2.00 g, 17.7 mmol) was reacted using general method G. The crude product was purified by recrystallization from acetonitrile to give the pure product as white crystals (0.92 g, 56%). The reaction and the work up was monitored by TLC (eluent: dichloromethane – ethanol 20:1 ratio, R_f: 0.07). Mp: 197 - 199 °C (literature: 200 – 202 °C (decomposed) [90]); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.05 (1H, d, *J*=7.5 Hz, 4-H), 7.88 (2H, d, *J*=7.5 Hz, 13-H), 7.71 (2H, t, *J*=6.6 Hz, 10-H), 7.41 (2H, t, *J*=7.2 Hz, 12-C), 7.31 (2H, m, 11-H), 5.01 (1H, quint, *J*=7.2 Hz, 2-H), 4.32 (1H, m, 8-H), 4.24 (2H, m, 7-H), 1.51 (3H, d, *J*=6.9 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 159.0 (1C), 156.0 (6-C), 144.3 (9a-C), 144.2 (9b-C), 141.2 (14-C), 128.1 (12-C), 127.5 (11-C), 125.7 (10-C), 120.6 (13-C), 66.2 (7-C), 47.1 (8-C), 42.3 (2-C), 19.8 (3C); ν_{max}/cm^{-1} 3310 (N-H), 2965, 2868, 2740, 2617 (tetrazole), 1686 (C=O), 1523 (N-H), 1258 (C-O); MS(ESI) m/z 336.2 $(M+H)^+$.

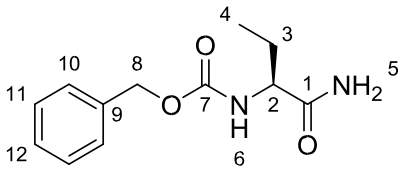
Methyl-(2*S*)-2-{[(benzyloxy)carbonyl]amino}butanoate 34b-L [91]:



Cbz-L-ethylglycine **28b-L** (5.0 g, 21.0 mmol) was reacted and worked up using general method B, yielding the product as colourless oil (5.1 g, 97%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate, 2:1 ratio, R_f: 0.77). ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.69 (1H, d, *J*=7.5 Hz, 6-H), 7.36 (5H, m, 10,11,12-H), 5.04 (2H, s, 8-H), 3.97

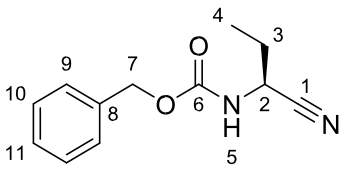
(1H, td, *J*=5.4, *J*=8.4 Hz, 2-H), 3.63 (1H, s, 5-H), 1.66 (2H, m, 3-H), 0.89 (3H, t, *J*=7.2 Hz, 4-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 173.2 (1-C), 156.6 (7-C), 137.4 (9-C), 128.8 (11-C), 128.3 (12-C), 128.2 (10-C), 65.9 (8-C), 55.8 (2-C), 52.2 (5-C), 24.6 (3-C), 10.9 (4-C); ν_{max}/cm^{-1} 3345 (N-H), 1703 (C=O), 1520 (NH), 1206 (C-O); MS(ESI) m/z 250.8 $(M-H)^-$.

Benzyl-*N*-[(1*S*)-1-carbamoylpropyl]carbamate 29b-L [92]:



Cbz-L-ethylglycine methyl ester **34b-L** (2.0 g, 8.0 mmol) was reacted and worked up using general method C. The crude product was washed with diethyl ether to give the product as white crystals (1.72 g, 91%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate, 1:1 ratio, R_f: 0.07). Mp: 140 - 142 °C (literature: 141 °C [93]); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.36 (5H, m, 10,11,12-H), 7.29 (1H, s, 5a-H), 7.20 (1H, d, *J*=8.4 Hz, 6-H), 6.95 (1H, s, 5b-H), 5.03 (2H, s, 8-H), 3.87 (1H, td, *J*=5.4, *J*=8.4 Hz, 2H), 1.67 (1H, m, 3a-H), 1.55 (1H, m, 3b-H), 0.86 (3H, t, *J*=7.2 Hz, 4-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 174. 2 (1-C), 156.4 (7-C), 137.6 (9-C), 128.8 (11-C), 128.2 (12-C), 128.1 (10-C), 65.8 (8-C), 56.3 (2-C), 25.6 (3-C), 10.8 (4-C); ν_{max}/cm^{-1} 3380 (N-H), 3308 (N-H), 3196 (N-H), 1649 (C=O), 1535 (N-H), 1240 (C-O); MS(ESI) m/z 237.1 $(M+H)^+$.

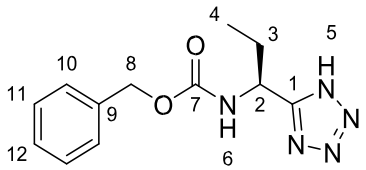
Benzyl-N-[(1*S*)-1-cyanopropyl]carbamate 30b-L [94]:



Cbz-L-ethylglycine amide **29b-L** (4.25 g, 18.0 mmol) was reacted and worked up using general method D to give the product as white crystals (3.88 g, 99%). The reaction and the work up were monitored by TLC (eluent: petroleum ether

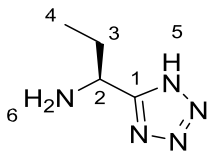
(40 – 60 °C) - ethyl acetate 1:2 ratio, R_f: 0.64). Mp: 43 – 45 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.15 (1H, d, *J*=7.8 Hz, 5-H), 7.37 (5H, m, 9,10,11-H), 5.09 (2H, s, 7-H), 4.48 (1H, quart, *J*=7.8 Hz, 2-H), 1.78 (2H, pentd, *J*=1.5, *J*=7.5 Hz, 3-H), 0.96 (3H, t, *J*=7.2 Hz, 4-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 156.0 (6-C), 137.0 (8-C), 128.9 (10-C), 128.5 (11-C), 128.5 (9-C), 120.1 (1-C), 66.6 (7-C), 44.2 (2-C), 25.8 (3-C), 10.3 (4-C); ν_{max}/cm^{-1} 3312 (N-H), 1694 (C=O), 1524 (N-H), 1263 (C-O); MS(ESI) m/z 219.1 $(M+H)^+$.

Benzyl-*N*-[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)propyl]carbamate 31b-L [95]:



Cbz-L-ethylglycyl nitrile **30b-L** (3.9 g, 18 mmol) was reacted and worked up using general method E. The product was gained as a white solid (3.83 g, 81%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate 1:2, R_f: 0.20). Mp: 142 – 144 °C ; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.98 (1H, d, *J*=7.2 Hz, 6-H), 7.36 (5H, m, 10,11,12H), 5.08 (1H, d, *J*=12.3 Hz, 8a-H), 5.02 (1H, d, *J*=12.6 Hz, 8b-H), 4.83 (1H, quart, *J*=8.1 Hz, 2-H), 1.89 (2H, m, 3-H), 0.89 (3H, t, *J*=7.2 Hz, 4-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 158.4 (1-C), 156.4 (7-C), 137.3 (9-C), 128.8 (11-C), 128.3 (12-C), 128.2 (10-C), 66.1 (8-C), 48.2 (2-C), 26.7 (3-C), 10.7 (4-C); ν_{max}/cm^{-1} 3296 (N-H), 2976, 2880, 2714, 2615, (tetrazole), 1686 (C=O), 1528 (N-H), 1263 (C-O); MS(ESI) m/z 262.1 $(M+H)^+$.

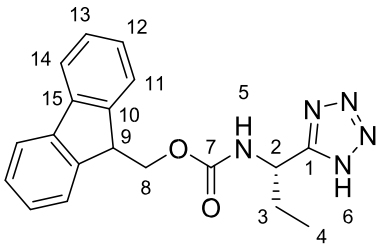
(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)propane-1-amine 32b-L [58]:



Cbz-L-aminopropyltetrazole **31b-L** (3.72 g, 14.0 mmol) was deprotected using general method F. Following filtration, the solid residue was washed with ethanol and the filtrate was evaporated under reduced pressure to give the

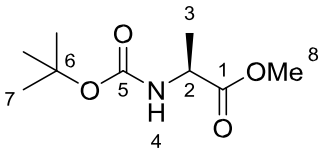
crude, which was washed to gain the pure product as a white solid (1.60 g, 88%). The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.18). Mp: 260 °C, decomposed (literature: 273– 274 °C [58]). ^{1}H NMR (300 MHz, D_2O) δ_H 4.62 (1H, t, *J*=7.2 Hz, 2-H), 2.05 (2H, quint, *J*=7.5 Hz, 3-H), 0.82 (3H, t, *J*=7.5 Hz, 4-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 159.6 (1-C), 48.7 (2-C), 26.0 (3-C), 8.9 (4-C); ν_{max}/cm^{-1} 2820, 2716, 2613, 2561 (tetrazole), 1530 (NH); MS(ESI) m/z 128.1 $(M+H)^+$.

9*H*-Fluoren-9-ylmethyl-*N*-[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)propyl]carbamate 73b-L

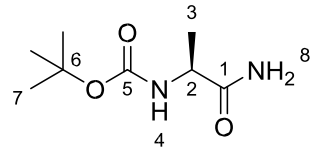


L-1-Aminoethyltetrazole **32b-L** (1.79 g, 6.9 mmol) was reacted using general method G. The crude product was purified by recrystallization in 2 generations from acetonitrile to give the pure product as white crystals (1.66 g, 75%). The reaction and the work up were monitored by TLC (eluent: dichloromethane – ethanol 10:1 ratio, R_f: 0.21). Mp: 209 - 211 °C (melted and decomposed); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 16.26 (6-H), 8.04 (1H, d, *J*=7.8 Hz, 5-H), 7.89 (2H, d, *J*=7.5 Hz, 14-H), 7.72 (2H, t, *J*=7.2 Hz, 11-H), 7.42 (2H, t, *J*=7.5 Hz, 13-H), 7.33 (2H, m, 12-H), 4.81 (1H, quart, *J*=8.1 Hz, 2-H), 4.34 (2H, m, 8-H), 4.23 (1H, m, 9-H), 1.91 (2H, m, 3-H), 0.88 (3H, t, *J*=7.2 Hz, 4-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 156.4 (7-C), 144.3 (10a-C), 144.2 (10b-C), 141.2 (15-C), 128.1 (13-C), 127.5 (12-C), 125.7 (11-C), 120.6 (14-C), 66.2 (8-C), 48.1 (2-C), 47.1 (9-C), 26.6 (3-C), 10.7 (4-C); ν_{max}/cm^{-1} 3300 (N-H), 2978, 2876, 2717, 2617 (tetrazole), 1682 (C=O), 1533 (N-H), 1265 (C-O); MS(ESI) m/z 350.1 $(M+H)^+$; CHN [Found: C, 65.08; H, 5.53; N, 19.86. $C_{19}H_{19}N_5O_2$ requires C, 65.32; H, 5.48; N, 19.86 %].

Methyl (2*S*)-2-{[(tert-butoxy)carbonyl]amino}propanoate 56-L [96]:

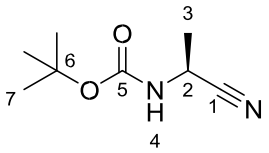


Boc-L-alanine **24-L** (9.8 g 26.4 mmol) was reacted and worked up by general method B to give the product as white powder (9.2 g, 85%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate, 1:1 ratio, R_f: 0.64). Mp: 33 °C (literature 31 - 33 °C [97]): ; ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 7.26 (1H, d, *J*=7.5 Hz, 4-H), 4.01 (1H, quint, *J*=7.5 Hz, 2-H), 3.62 (3H, s, 8-H), 1.38 (9H, s, 7-H), 1.23 (3H, d, *J*=7.5 Hz, 3-H); ^{13}C NMR (126 MHz, DMSO-d_6) δ_C 174.1 (1-C), 155.7 (5-C), 78.6 (6-C), 52.2 (8C), 49.4 (2-C), 28.6 (7-C), 17.4 (3-C); ν_{max}/cm^{-1} 3387 (N-H), 1745 (C=O), 1686 (C=O), 1512 (N-H), 1159 (C-O); MS(ESI) m/z 226.1 $(M+Na)^+$. ***tert*-Butyl-*N*-[(*1S*)-1-carbamoylethyl]carbamate 57-L [98]:**



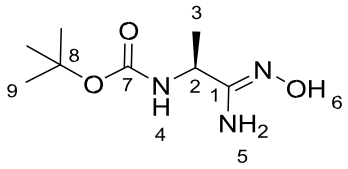
To a solution of Boc-L-alanine methyl ester **56-L** (4.1 g, 20.2 mmol, 1.00 eq) in THF, concentrated aqueous ammonium hydroxide (20 mL, 404 mmol, 20 eq) was added and the mixture was stirred for 2 weeks at room temperature. After the reaction was complete the mixture was evaporated under reduced pressure to give the product as a white solid (3.72 g, 98%). The reaction and the work up was monitored by TLC (eluent: ethyl acetate, R_f: 0.29). Mp: 113 - 116 °C (literature: 123.5 °C [98]); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 7.20 (1H, s, 8-H), 6.90 (1H, s, 8-H), 6.75 (1H, d, *J*=7.5 Hz, 4-H), 3.88 (1H, quint, *J*=7.5 Hz, 2-H), 1.37 (9H, s, 7-H), 1.16 (3H, d, *J*=7.0 Hz, 3-H); ^{13}C NMR (126 MHz, DMSO-d_6) δ_C 174.2 (1-C), 154.4 (5-C), 77.3 (6-C), 49.0 (2-C), 27.7 (7-C), 17.8 (3-C); ν_{max}/cm^{-1} 3388 (N-H), 3352 (N-H), 1780 (C=O), 1642 (C=O), 1517 (NH), 1163 (C-O); MS(ESI) m/z 211.1 $(M+Na)^+$.

***tert*-Butyl *N*-[(1*S*)-1-cyanoethyl]carbamate 58-L [99]**:



Boc-L-alanine amide **57-L** (5.75 g, 30.6 mmol) was reacted and worked up by general D to give the product as white crystals (5.17 g, 99%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate 1:3 ratio, R_f: 0.71). Mp: 108 – 110 °C (literature: 105 – 106 °C [99]); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 7.73 (1H, d, *J*=7.0 Hz, 4-H), 4.50 (1H, quint, *J*=7.5 Hz, 2-H), 1.42 (9H, s, 7-H), 1.39 (3H, d, *J*=7.0 Hz, 3-H); ^{13}C NMR (126 MHz, DMSO-d_6) δ_C 155.0 (5-C), 121.2 (1-C), 79.6 (6-C), 37.8 (2-C), 28.5 (7-C), 18.8 (3-C); ν_{max}/cm^{-1} 3388 (N-H), 2979 (C-H), 2937 (C-H), 2360 (C□N), 1679 (C=O), 1523 (N-H), 1160 (C-O); MS(ESI) m/z 193.1 $(M+Na)^+$.

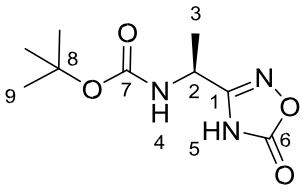
***tert*-Butyl-*N*-[(1*S*)-1-[*N*'-hydroxycarbamimidoyl]ethyl]carbamate 59-L [100]:**



To the solution of Boc-L-alanine nitrile **58-L** (3.00 g, 17.6 mmol, 1.00 eq) in ethanol, hydroxylamine hydrochloride (1.59 g, 22.9 mmol, 1.30 eq), and potassium carbonate (1.59 g, 22.9 mmol, 1.30 eq) were added. The mixture was stirred and heated for 3 h at reflux. After the reaction was complete, the mixture was filtered, and washed with ethanol. The filtrate was evaporated under reduced pressure. The crude product was purified by column chromatography (silica gel, eluent: petroleum ether (40 – 60 °C) - ethyl acetate 3:1, 1:1 then 1:2 ratio), then the product was recrystallized from petroleum ether (60 – 80 °C) - ethyl acetate mixture to give the product as white crystals (1.15 g, 32%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - ethyl acetate 1:1 ratio, R_f: 0.15). Mp: 136 – 138 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.91 (1H, s, 6-H), 6.76 (1H, d, *J*=8.4 Hz, 4-H), 5.21 (2H, s, 5-H), 4.03 (1H, quint, *J*=8.1 Hz, 2-H), 1.38 (9H, s, 9-H), 1.18 (3H, d, *J*=7.2 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 155.1 (7-C), 154.3 (1-C), 78.4 (8-C), 47.1 (2-C), 28.7 (3C, 9-C), 20.0 (3-C); ν_{max}/cm^{-1} 3468 (O-H),

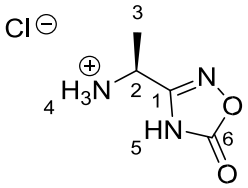
3355 (N-H), 1685 (C=O), 1517 (N-H), 1173 (C-O); MS(ESI) m/z 204.1 $(M+H)^+$.

tert-Butyl-_N_-[(1_S_)-1-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3yl)ethyl]carbamate 60-L:



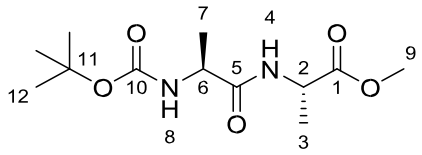
To the suspension of Boc-L-alanylamidoxime **59-L** (0.75 g, 3.69 mmol, 1.00 eq) in dichloromethane (40 mL) CDI (0.78 g, 4.80 mmol, 1.30 eq) was added and the mixture was stirred for 30 min at room temperature. After the addition of DBU (1.47 mL, 7.38 mmol, 2.00 eq) the reaction mixture was stirred overnight at room temperature. After the reaction was complete, water (15 mL), then 2.5 M HCl_{aq} (15 mL) was added. The two phases were separated, and the aqueous phase was extracted with DCM (3 x 20 mL). The combined organic phase was washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 1:1 then 1:2 ratio) to give the product as a white solid (0.72 g, 85%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate and 1 drop of glacial acetic acid, $R_{f:}$ 0.65). Mp: 136 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 12.26 (1H, br s, 5-H), 7.36 (1H, d, *J*=2.1 Hz, 4-H), 4.51 (1H, quint, *J*=7.2 Hz, 2-H), 1.38 (9H, s, 9-H), 1.33 (3H, d, *J*=6.9 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSOd_6) δ_C 162.0 (1-C), 160.3 (6-C), 155.2 (7-C), 79.0 (8-C), 42.9 (2-C), 28.6 (3C, 9-C), 18.0 (3-C); ν_{max}/cm^{-1} 3374 (N-H), 3257 (N-H), 3102 (N-H), 2986 (C-H), 2938 (C-H), 1796 (C=N), 1749 (C=N), 1686 (C=O), 1664 (C=O), 1520 (N-H), 1155 (C-O); MS(ESI) m/z 227.8 $(M-H)^-$ CHN [Found: C, 47.78; H, 6.63; N, 18.24. $C_9H_{15}N_3O_4$ requires C, 47.16; H, 6.60; N, 18.33 %].

3-[(1_S_)-1-aminoethyl]-4,5-dihydro-1,2,4-oxadiazol-5-one 33-L:



Boc-L-1-aminoethyloxadiazolone **60-L** (0.20 g, 0.87 mmol) was deprotected and worked up by general method A. The filtered solid was washed with diethyl ether to give the product as white crystals (0.10 g, 72%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate, R_f: 0.04) Mp: 130 °C (decomposed). ^{1}H NMR (300 MHz, D_2O) δ_H 4.65 (1H, quart, *J*=7.2 Hz, 2-H), 1.64 (3H, d, *J*=7.2 Hz, 3-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 161.6 (1-C), 157.9 (6-C), 42.7 (2-C), 16.0 (3-C); v_{max}/cm^{-1} 3042 (N-H), 2966 (C-H), 2901 (C-H), 1792 (C=O); MS(ESI) m/z 127.8 $(M-H)^-$; CHN [Found: C, 28.95; H, 5.01; N, 25.31. $C_4H_7N_3O_2$ requires C, 29.02; H, 4.87; N, 25.38 %].

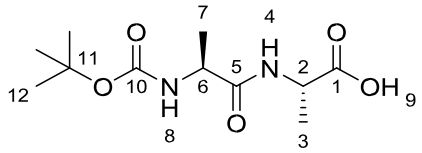
Methyl (2S)-2-[(2S)-2-{[(tertbutoxy)carbonyl]amino}propanamido]propanoate 63-LL [101]:



To a solution of Boc-L-alanine **24-L** (1.00 g, 5.3 mmol, 1.00 eq) in DCM (20 mL), NMM (0.87 mL, 7.95 mmol, 1.50 eq) was added at -5 °C. After the careful addition of IBCF (0.69 mL, 5.3 mmol, 1.00 eq), the mixture was stirred at -5 °C for 1 h. A solution of L-alanine methyl ester hydrochloride **61-L** (0.74 g, 5.3 mmol, 1.00 eq) and NMM (0.58 mL, 5.3 mmol, 1.00 eq) was added to this mixture which was subsequently stirred at -5 °C for 10 min, then at room temperature overnight. After the reaction was complete, the suspension was filtered and the filtrate was evaporated under reduced pressure. The residue was dissolved in DCM (30 mL) and washed with 10% citric acid aqueous solution (20 mL), then 10% K_2CO_3 aqueous solution (20 mL) and finally with brine (20 mL). The organic phase was dried over $MgSO_4$, then evaporated under reduced pressure to give the crude product as a white solid (1.08 g), which was purified by column chromatography (petroleum ether (40 – 60 °C) – ethyl acetate 1:1, then 1:2 ratio) to give the product as a white solid (1.00 g, 69%).The reaction and the work up were monitored by TLC (eluent: ethyl acetate, R_f: 0.57). Mp: 109 °C (literature: 109 – 110 °C, hexane – ethyl acetate [101]); ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ_H 8.11 (1H, d, *J*=7.2 Hz, 4-H), 6.79 (1H, d, *J*=7.2 Hz, 8-H), 4.26 (1H, quint, *J*=7.2 Hz, 2-H), 3.97 (1H, quint, *J*=7.2 Hz,

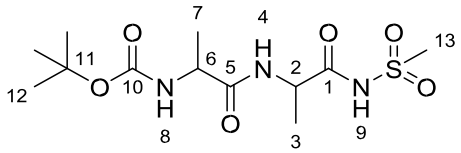
6-H), 3.60 (3H, s, 9-H), 1.36 (9H, s, 12-H), 1.26 (3H, d, *J*=7.2 Hz, 3-H), 1.15 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 173.0 (1-C), 172.7 (5-C), 155.0 (10-C), 77.9 (11-C), 51.8 (9-C), 49.2 (6-C), 47.4 (2-C), 28.2 (12C), 18.0 (7-C), 16.9 (3-C); v_{max}/cm^{-1} 3308 (N-H), 3263 (N-H), 1740 (C=O), 1681 (C=O), 1654 (C=O), 1522 (N-H), 1166 (C-O); MS(ESI) *m/z* 275.2 $(M+H)^+$.

(2S)-2-[(2S)-2-{[(*tert*-Butoxy)carbonyl]amino}propanamido]propanoic acid 63-LL [102]:



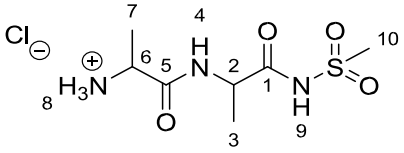
A solution of Boc-L-alanyl-L-alanine methyl ester **63-LL** (2.40 g, 8.80 mmol, 1.00 eq) in methanol (48 mL) was treated with 1M NaOH aqueous solution (36 mL) at room temperature for 4 h. After the reaction was complete, the mixture was evaporated under reduced pressure. The residual aqueous solution was diluted with water (24 mL), then washed with DCM (2 x 24 mL). The aqueous phase was cooled in an ice bath and acidifed with 1M HCl aqueous solution to pH~3. The mixture was stored at 4 °C overnight and filtered to give the product as white crystals (2.27 g, 99%). The reaction and the work up were monitored by TLC (Eluent: ethyl acetate, R_f: 0.57). Mp: 115 - 117 °C (literature: 109 – 110 °C [103]) ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 12.49 (1H, br s, 9-H), 7.95 (1H, d, *J*=7.5 Hz, 4-H), 6.82 (1H, d, *J*=7.8 Hz, 8-H), 4.19 (1H, quint, *J*=7.2 Hz, 2-H), 3.98 (1H, quint, *J*=7.5 Hz, 6-H), 1.37 (9H, s, 12-H), 1.26 (3H, d, *J*=7.2 Hz, 3-H), 1.16 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, DMSO-*d*6) δ_C 174.4 (1-C), 172.9 (5-C), 155.4 (10-C), 78.4 (11-C), 49.8 (6-C), 47.8 (2-C), 28.7 (12-C), 18.6 (7-C), 17.8 (3-C); v_{max}/cm^{-1} 3349 (N-H), 1666 (C=O), 1538 (N-H), 1157 (C-O); MS(ESI) *m/z* 283.1 $(M+Na)^+$.

***tert*-Butyl-N-(1-{[1-(methanesulfonylcarbamoyl)ethyl]carbamoyl}ethyl)carbamate 64-LL/D:**



To a solution of Boc-L-alanyl-L-alanine **63-LL** (0.50 g, 1.92 mmol, 1.00 eq) in DCM (30 mL), EDCI hydrochloride (0.57 g, 2.89 mmol, 1.50 eq) was added dropwise. DMAP (0.59 g, 4.35 mmol, 2.27 eq) and methanesulfonamide **25** (0.20 g, 2.12 mmol, 1.10 eq) were added to the reaction mixture and the mixture was stirred overnight. After the reaction was complete, water (10 mL) then 2.5 M HCl_{aq} (10 mL) were added and the two layers were separated. The aqueous phase was extracted with DCM (3 x 15 mL), then the combined organic phases were washed with brine (15 mL), dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by column chromatography (petroleum ether (40 – 60 °C) – ethyl acetate – glacial acetic acid (0.1%) 1:1 then 1:2 ratio) to give the product as a white solid (0.132 g, 20%). The reaction and the work up were monitored by TLC (Eluent: ethyl acetate – drop of glacial acetic acid, R_f: 0.29). Mp: 156 -158 °C; ^{1}H NMR (300 MHz, CD_3OD-d_4) δ_H 4.32 (1H, m, 2-H), 4.05 (1H, quint, *J*=6.9 Hz, 6-H), 3.22 (3H, s, 13-H), 1.44 (9H, s, 12-H), 1.38 (3H, d, *J*=7.2 Hz, 3-H), 1.31 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, CD_3OD) δ_C 175.9 (1-C), 174.0 (5-C), 157.8 (10-C), 80.9 (11-C), 50.8 (6-C), 49.4 (2-C), 41.3 (13-C), 28.7 (12-C), 18.2 (7-C), 17.3 (3-C); ν_{max}/cm^{-1} 3386 (N-H), 3276 (N-H), 1732 (C=O), 1680 (C=O), 1650 (C=O), 1556 (N-H), 1530 (N-H), 1163 (C-O), 1125 (S=O); MS(ESI) *m/z* 338.2 $(M+H)^+$; CHN [Found: C, 42.96; H, 6.96; N, 12.10. $C_{12}H_{23}N_3O_6S$ requires C, 42.72; H, 6.87; N, 12.45 %]

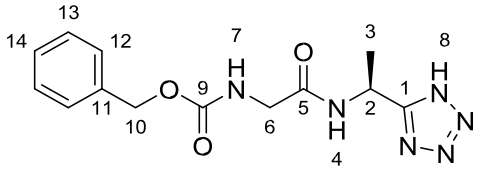
2-amino-N-[1-(methanesulfonylcarbamoyl)ethyl]propanamide 65-LL/D:



Boc-alanylalanylmethanesulfonamide **65-LL/D** (0.10 g, 0.3 mmol) was deprotected and worked up by general method A. The filtered solid was washed with DCM to give the product as white crystals (0.6 g, 70%). Further purification was performed by Prep HPLC method A. The reaction and the

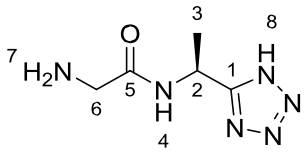
work up were monitored by TLC (eluent: ethanol, R_f: 0.07). Mp: 160 °C (decomposed). ^{1}H NMR (300 MHz, D_2O) δ_H 4.33 (1H, quart, *J*=7.2 Hz, 2-H), 4.11 (1H, quart, *J*=7.2 Hz, 6-H), 3.34 (3H, s, 10-H), 1.54 (3H, d, *J*=7.2 Hz, 3H), 1.43 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 173.9 (1-C), 170.8 (5-C), 50.3 (6-C), 48.9 (2-C), 40.6 (10-C), 16.5 (7-C), 15.8 (3-C); ν_{max}/cm^{-1} 3321 (N-H), 3224 (N-H), 1712 (C=O), 1659 (C=O), 1547 (N-H), 1119 (S=O); MS(ESI) *m/z* 238.1 $(M+H)^+$; HRMS (Found $(M+Na)^+$ 260.0677. Calcd. for $C_7H_{15}O_4N_3SNa$: $(M+Na)^+$ 260.0675.); (Purity test: HPLC method A).

Benzyl-*N*-({[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)ethyl]carbamoyl}methyl) carbamate 69g-L



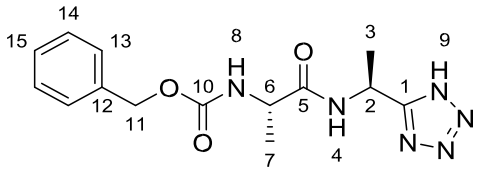
Benzyl-*N*-({[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)ethyl]carbamoyl}methyl) carbamate **69g-L** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to 2-chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.3 mmol) was removed, this was monitored by the Fmoc and TNBS tests. After the coupling of Cbz-glycine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure. The residue washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.24 g, 59%). The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.56). Mp: 134 - 136 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.56 (1H, d, *J*=7.2 Hz, 4-H), 7.35 (6H, m, 7,12,13,14-H), 5.24 (1H, quint, *J*=7.2 Hz, 2-H), 5.03 (2H, s, 10-H), 3.68 (2H, d, *J*=6.3 Hz, 6-H), 1.49 (3H, d, *J*=6.9 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 169.3 (5-C), 158.9 (1-C), 156.9 (9-C), 137.5 (11-C), 128.8 (13-C), 128.2 (14-C), 128.1 (12-C), 65.9 (10C), 43.8 (6-C), 40.3 (2-C), 19.8 (3-C); ν_{max}/cm^{-1} 3289 (N-H), 3269 (N-H), 1697 (C=O), 1661 (C=O), 1530 (N-H), 1244 (C-O); MS(ESI) m/z 305.1 $(M+H)^+$; CHN [Found: C, 51.57; H, 5.41; N, 26.15. $C_{13}H_{16}N_6O_3$ requires C, 51.31; H, 5.30; N, 26.15 %].

2-Amino-*N*-[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)ethyl]acetamide 70g-L:



Cbz-glycyl-1-L-aminoethyltetrazole **69g-L** (0.18 g, 0.57 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with 30 mL methanol, which was discarded. The solid residue was washed into a separate flask with water, which was freeze dried to give the product as a white solid (0.01 g, 99%). Further purification was performed by Prep HPLC method A. Mp: 180 °C (decomposed); ^{1}H NMR (300 MHz, D_2O) δ_H 5.27 (1H, quart, *J*= 6.9 Hz, 2-H), 3.75 (2H, s, 6-H), 1.52 (3H, d, *J*= 6.9 Hz, 3-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 167.3 (5-C), 164.3 (1-C), 42.3 (2-C), 41.0 (6-C), 19.3 (3-C); ν_{max}/cm^{-1} 3215 (N-H), 1672 (C=O) 1547 (N-H); MS(ESI) m/z 171.1 $(M+H)^+$; HRMS (Found $(M+H)^+$ 171.0987. Calcd. for $C_5H_{11}ON_6$: $(M+H)^+$ 171.0989.); (Purity test: HPLC method A).

Benzyl-*N*-[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamate 69a-LL:



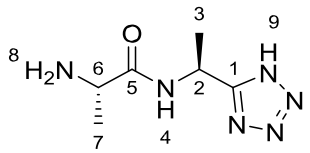
Method 1: Cbz-L-Alanine (0.40 g, 1.77 mmol) and 1-L-aminoethyltetrazole **32a-L** (0.20 g, 1.77 mmol) were coupled by general method H. To the evaporated mixture, ethyl acetate (15 mL) and 0.05M HCl_{aq} (5 mL) were added and the aqueous phase was extracted with ethyl acetate (3 x 5 mL). The combined organic phases were dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was triturated with ethyl acetate and the precipitated solid was filtered to give the title compound as white crystals (0.12g, 21%).

Method 2: Benzyl-*N*-[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamate **69a-LL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to 2chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.3 mmol) was removed,

this was monitored by the Fmoc and TNBS tests. After the coupling of Cbz-L-alanine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure and washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.23 g, 58%).

The reaction and the work up were monitored by TLC (eluent: ethyl acetate and 1 drop of glacial acetic acid, R_f: 0.07). Mp: 186 - 187 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 16.18 (1H, br s, 9-H), 8.54 (1H, d, *J*=7.5 Hz, 4-H), 7.35 (6H, m, 8,13-15-H), 5.23 (1H, quint, *J*=7.2 Hz, 2-H), 5.05 (1H, d, *J*=12.6 Hz, 11-H_a), 5.00 (1H, d, *J*=12.6 Hz, 11-H_b), 4.09 (1H, quint, *J*=7.2 Hz, 6-H), 1.50 (3H, d, *J*=7.2 Hz, 3-H), 1.20 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 172.1 (5-C), 158.3 (1-C), 155.6 (10-C), 137.0 (12-C), 128.3 (14C), 127.7 (15-C), 127.7 (13-C), 65.3 (11-C), 49.8 (6-C), 40.3 (2-C), 19.2 (7-C), 17.9 (3-C); ν_{max}/cm^{-1} 3287 (N-H), 3264 (N-H), 1684 (C=O), 1655 (C=O), 1535 (N-H), 1230 (C-O); MS(ESI) m/z 319.2 $(M+H)^+$; CHN [Found: C, 53.06; H,
5.64; N, 26.58. $C_{14}H_{18}N_6O_3$ requires C, 52.82; H, 5.70; N, 26.40 %].

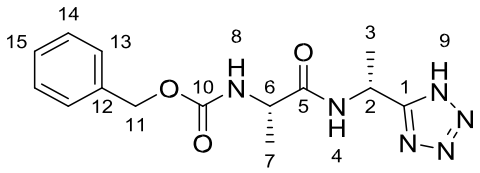
(2*S*)-2-amino-*N*-[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)ethyl]propanamide 70aLL [53]:



Cbz-L-alanyl-1-L-aminoethyltetrazole **69a-LL** (0.58 g, 1.83 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with methanol, which was discarded. The solid residue was washed into a separate flask with water, then freeze dried to give the product as a white solid (0.29 g, 86%). Further purification was performed by Prep HPLC method A. The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.07). Mp: 200 °C (decomposed); ^{1}H NMR (300 MHz, D_2O) δ_H 5.25 (1H, quart, *J*=6.9 Hz, 2-H), 3.57 (1H, quart, *J*=6.9 Hz, 6-H), 1.52 (3H, d, *J*=7.2 Hz, 3-H), 1.25 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C

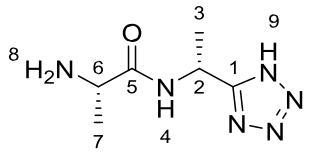
176.2 (5-C), 164.3 (1-C), 49.9 (6-C), 42.0 (2-C), 19.4 (3-C), 19.2 (7-C); v_{max}/cm^{-1} 3247 (N-H), 1643 (C=O) 1561 (N-H); MS(ESI) m/z 185.1 $(M+H)^+$; HRMS (Found $(M+H)^+$ 185.1145. Calcd. for $C_6H_{13}ON_6$: $(M+H)^+$ 185.1145.); (Purity test: HPLC method A).

Benzyl-*N*-[(1*S*)-1-{[(1*R*)-1-(1H-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamate 69a-LD [52]:



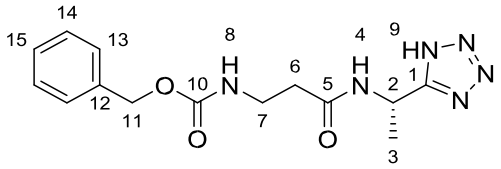
Benzyl-*N*-[(1*S*)-1-{[(1*R*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamate **69a-LD** was prepared using general method I. The Fmoc group of Fmoc-1-D-aminoethyltetrazole attached to 2chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.2 mmol) was removed, this was monitored by the Fmoc and TNBS tests. After the coupling of CbzL-alanine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure, and the residue washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.34 g, 89%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate and 1 drop of glacial acetic acid, R_f: 0.07). Mp: 166-168 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.56 (1H, d, *J*=7.5 Hz, 4-H), 7.35 (6H, m, 8,13-15-H), 5.23 (1H, quint, *J*=7.2 Hz, 2-H), 5.04 (1H, d, *J*=12.9 Hz, 11-H_a), 5.00 (1H, d, *J*=12.6 Hz, 11-H_b), 4.10 (1H, quint, *J*=7.5 Hz, 6-H), 1.50 (3H, d, *J*=7.2 Hz, 3-H), 1.23 (3H, d, *J*=6.9 Hz, 7-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 172.6 (5-C), 158.0 (1-C), 156.1 (10-C), 137.4 (12-C), 128.8 (14-C), 128.2 (15-C), 128.2 (13-C), 65.9 (11-C), 50.4 (6-C), 40.3 (2-C), 19.6 (3-C), 18.8 (7-C); v_{max}/cm^{-1} 3289 (N-H), 2988, 2874, 2756, 2612 (tetrazole), 1682 (C=O), 1651 (C=O), 1528 (N-H), 1224 (C-O); MS(ESI) m/z 319.1 $(M+H)^+$; CHN [Found: C, 53.10; H, 5.78; N, 26.01. $C_{14}H_{18}N_6O_3$ requires C, 52.82; H, 5.70; N, 26.40 %].

(2*S*)-2-amino-*N*-[(1*R*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)ethyl]propanamide 70aLD [52]:



Cbz-L-alanyl-1-D-aminoethyltetrazole **69a-LD** (0.58 g, 1.83 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with ethanol, which was discarded. The solid residue was washed into a separate flask with water, which was freeze dried and purified by column chromatography (eluent: ethyl acetate – ethanol 4:1, 2:1, 1:1 then 0:1 ratio) to give the pure product as white solid (98 mg, 58%). The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.13). Mp: 69 °C (phase change), 177 °C (melted); ^{1}H NMR (300 MHz, D_2O) δ_H 5.24 (1H, quart, *J*=6.6 Hz, 2-H), 4.00 (1H, quart, *J*=6.6 Hz, 6-H), 1.52 (3H, d, *J*=7.5 Hz, 3-H), 1.48 (3H, d, *J*=7.8 Hz, 7-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 170.5 (5-C), 164.0 (1C), 49.3 (6-C), 42.3 (2-C), 19.1 (3-C), 16.7 (7-C); ν_{max}/cm^{-1} 3204 (N-H), 1667 (C=O), 1539 (N-H); MS(ESI) m/z 185.1 $(M+H)^+$; HRMS (Found $(M+H)^+$ 185.1145. Calcd. for $C_6H_{13}ON_6$: $(M+H)^+$ 185.1145.); (Purity test: HPLC method A).

(*S*)-Benzyl-(3-((1-(1*H*-tetrazol-5-yl)ethyl)amino)-3-oxopropyl)carbamate 69d-L:

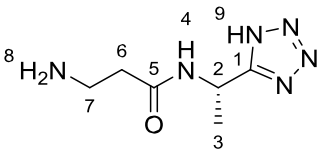


(*S*)-Benzyl-(3-((1-(1*H*-tetrazol-5-yl)ethyl)amino)-3-oxopropyl)carbamate **69dL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to the 2-chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.3 mmol) was removed, this was monitored by the Fmoc and TNBS tests. After the coupling of Cbz-□-alanine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure, and the residue washed with petroleum ether (40 – 60 °C) to obtained the product as white crystals (0.28 g, 67%). The reaction and the work up were monitored by TLC (eluent:

ethyl acetate and 1 drop of glacial acetic acid, R_f: 0.39). Mp: 147-148 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 16.08 (1H, brs, 9-H), 8.56 (1H, d, *J*=7.5 Hz, 4H), 7.35 (5H, m, 13-15-H), 7.21 (1H, m, 8-H), 5.22 (1H, quint, *J*=7.2 Hz, 2-H),

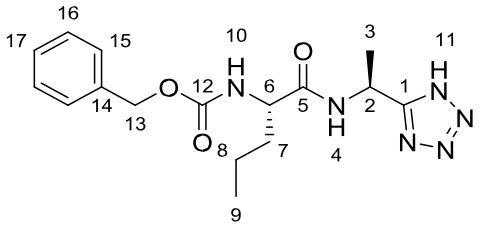
5.02 (2H, s, 11-H), 3.24 (2H, quart, *J*=6.9Hz, 7-H), 2.34 (2H, t, *J*=6.9 Hz, 6-H), 1.48 (3H, d, *J*=7.2 Hz, 3-H); ^{13}C NMR (75,5 MHz, DMSO-d_6) δ_C 170.6 (5-C), 158.8 (1-C), 156.5 (10-C), 137.6 (12-C), 128.8 (2C, 14-C), 128.2 (15-C), 128.1 (2C, 13-C), 65.7 (11-C), 40.9 (2-C), 37.3 (7-C), 35.9 (6-C), 19.6 (3-C); v_{max}/cm^1 3323 (N-H), 3273 (N-H), 2994, 2882, 2746, 2615 (tetrazole), 1692 (C=O), 1645 (C=O); MS(ESI) $(M+Na)^+$ found 341.2 m/z; CHN [Found: C, 53.62; H, 5.83; N, 25.62. $C_{14}H_{18}N_6O_3$ requires C, 52.82; H, 5.70; N, 26.40 %].

3-Amino-*N*-[(1*S*)-1-(1H-1,2,3,4-tetrazol-5-yl)ethyl]propanamide 70d-L:

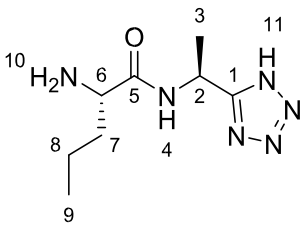


Cbz-□-alanyl-1-L-aminoethyltetrazole **69d-L** (0.20 g, 0.63 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with methanol, which was discarded. The solid residue was washed into a separate flask with water, which was freeze dried to give the product as a light brown hygroscopic amorphous solid (0.09 g, 78%). Further purification was performed by Prep HPLC method A. ^{1}H NMR (300 MHz, D_2O) δ_H 5.21 (1H, quart, *J*=6.9 Hz, 2-H), 3.17 (2H, m, 7-H), 2.63 (2H, t, *J*=6.6 Hz, 6-H), 1.48 (3H, d, *J*=6.9 Hz, 3-H); ^{13}C NMR (75,5 MHz, DMSO-d_6) δ_C 171.6 (5-C), 164.5 (1-C), 40.0 (2-C), 35.8 (7-C), 32.5 (6-C), 19.3 (3-C); v_{max}/cm^{-1} 3232 (N-H), 1641 (C=O); MS(ESI) $(M+H)^+$ found 185.1 m/z; HRMS (Found $(M+H)^+$ 185.1145. Calcd. for $C_6H_{13}ON_6$: $(M+H)^+$ 185.1145.); (Purity test: HPLC method A).

Benzyl-*N*-[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}butyl]carbamate 69e-LL:



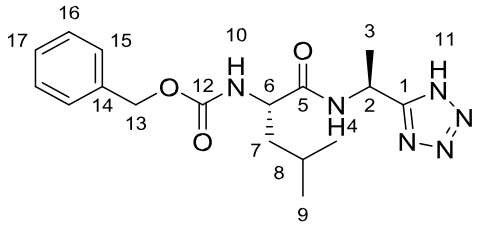
Benzyl-*N*-[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}butyl]carbamate **69e-LL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to 2chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.2 mmol) was removed, this was monitored by the Fmoc and TNBS tests. After the coupling of Cbz-Lnorvaline with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure, and the residue washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.21, 51%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate and 1 drop of glacial acetic acid, R_f: 0.2). Mp: 175 – 178 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 16.22 (1H, brs, 11-H), 8.55 (1H, d, *J*=7.5, 4-H), 7.35 (6H, m, 10, 15-17-H), 5.23 (1H, quint, *J*=7.2 Hz, 2-H), 5.02 (2H, s, 13-H), 4.04 (1H, m, 6-H), 1.59 (2H, m, 7-H), 1.49 (3H, d, *J*=6.9 Hz, 3-H), 1.27 (2H, m, 8-H), 0.84 (3H, t, *J*=7.2 Hz, 9-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 172.2 (5-C), 159.1 (1-C), 156.4 (12-C), 137.5 (14C), 128.8 (2C, 16-C), 128.2 (17-C), 128.1 (2C, 15-C), 65.8 (13-C), 54.6 (6-C), 40.9 (2-C), 34.3 (7-C), 19.7 (3-C), 19.1 (8-C), 14.0 (9-C); v_{max}/cm^{-1} 3310 (NH), 3271 (N-H), 2959, 2877, 2752, 2609 (tetrazole), 1682 (C=O), 1651 (C=O), 1533 (N-H), 1234 (C-O); MS(ESI) m/z 369.2 $(M+Na)^+$; CHN [Found: C, 55.56; H, 6.40; N, 23.35. $C_{16}H_{22}N_6O_3$ requires C, 55.48; H, 6.40; N, 23.35 %]. **(*S*)-*N*-((*S*)-1-(1*H*-tetrazol-5-yl)ethyl)-2-aminopentanamide 70e-LL:**



Cbz-L-norvalyl-1-L-aminoethyltetrazole **69e-LL** (0.10 g, 0.29 mmol) was deprotected by general method F. Following filtration, the solid residue was

washed with methanol, which was discarded. The residue was washed with water, then freeze dried to give the product as a white solid (47 mg, 77%). Further purification was performed by Prep HPLC method A.The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.16). Mp: 251 – 254 °C; ^{1}H NMR (300 MHz, D_2O) δ_H 5.28 (1H, quart, *J*=7.2 Hz, 2-H), 3.79 (1H, t, *J*=6.6 Hz, 6-H), 1.70 (2H, m, 7-H), 1.54 (3H, d, *J*=6.9 Hz, 3-H), 1.21 (2H, m, 8-H), 0.81 (3H, t, *J*=7.2 Hz, 9-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 171.2 (5C), 164.7 (1-C), 53.5 (6-C), 42.0 (2-C), 33.8 (7-C), 19.1 (3-C), 17.6 (8-C), 12.8 (9-C); ν_{max}/cm^{-1} 3210 (N-H), 1687 (C=O), 1551 (N-H); MS(ESI) m/z 213.1 $(M+H)^+$; HRMS (Found $(M+H)^+$ 213.1458. Calcd. for $C_8H_{17}ON_6$: $(M+H)^+$ 213.1460.); (Purity test: HPLC method A).

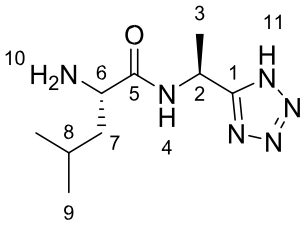
Benzyl-*N*-[(1*S*)-3-methyl-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}butyl]carbamate 69h-LL:



Benzyl-*N*-[(1*S*)-3-methyl-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}butyl]carbamate **69h-LL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to 2chlorotrityl chloride resin (resin:loading; 1:2 ratio; 1.5 mmol) was removed, this was monitored by the Fmoc and TNBS tests. After the coupling of Cbz-Lleucine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure, and the residue washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.45, 84%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate – ethanol – drop of glacial acetic acid 1:1 ratio, R_f: 0.64). Mp: 149 - 150 °C; ^{1}H NMR (300 MHz, DMSOd_6) δ_H 16.1 (1H, brs, 11-H), 8.55 (1H, d, *J*=7.5 Hz, 4-H), 7.36 (6H, m, 10,15,16,17-H), 5.22 (1H, quint, *J*=6.9 Hz, 2-H), 5.04 (1H, d, *J*=12.6, 13a-H), 5.00 (1H, d, *J*=12.6 Hz, 13b-H), 4.07 (1H, m, 6-H), 1.60 (1H, m, 8-H), 1.49 (3H, d, *J*=7.2 Hz, 3-H), 1.42 (2H, m, 7-H), 0.85 (3H, d, *J*=6.6 Hz, 9a-H), 0.84 (3H, d, *J*=6.6

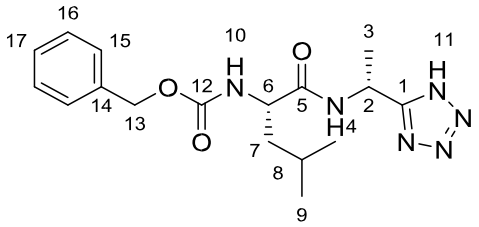
Hz, 9b-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 172.6 (5-C), 156.4 (12-C), 137.5 (14-C), 128.8 (16-C), 128.2 (17-C), 128.1 (15-C), 65.8 (13-C), 53.3 (6-C), 41.0 (2-C), 40.3 (7-C), 24.6 (8-C), 23.6 (9a-C), 21.8 (9b-C), 19.6 (3-C); ν_{max}/cm^{-1} 3316 (N-H), 3271 (N-H), 2961, 2876, 2743, 2615 (tetrazole), 1682 (C=O), 1653 (C=O), 1530 (N-H), 1233 (C-O); MS(ESI) m/z 360.8 $(M+H)^+$; CHN [Found: C, 56.82.; H, 6.68; N, 23.05. $C_{17}H_{24}N_6O_3$ requires C, 56.65; H, 6.71; N, 23.32 %].

(2*S*)-2-Amino-4-methyl-*N*-[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]pentanamide 70h-LL:



Cbz-L-leucyl-1-L-aminoethyltetrazole **69h-LL** (0.95 g, 2.6 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with ethanol, which was discarded. The residue was washed with water, then freeze dried and washed with diethyl ether to give the product as a white solid (0.48 g, 81%). Further purification was performed by Prep HPLC method A. The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.3). Mp: 210 °C (decomposed); ^{1}H NMR (300 MHz, D_2O) δ_H 5.28 (1H, quart, *J*=7.2 Hz, 2-H), 3.49 (1H, t, *J*=7.2Hz, 6-H), 1.53 (3H, d, *J*=7.2 Hz, 3-H), 1.44 (3H, m, 7,8-H), 0.82 (3H, d, *J*=4.8 Hz, 9a-H), 0.80 (3H, d, *J*=5.1 Hz, 9b-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 175.6 (5-C), 169.7 (1-C), 52.9 (6C), 42.7 (7-C), 41.8 (2-C), 24.0 (8-C), 21.9 (9a-C), 21.4 (9b-C), 16.8 (3-C); ν_{max}/cm^{-1} 3219 (N-H), 1653 (C=O), 1562 (N-H); MS(ESI) m/z 227.2 $(M+H)^+$; HRMS (Found $(M+Na)^+$ 249.1436. Calcd. for $C_9H_{18}ON_6Na$: $(M+Na)^+$ 249.1434.); (Purity test: HPLC method A).

Benzyl-*N*-[(1*S*)-3-methyl-1-{[(1*R*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}butyl]carbamate 69h-LD:

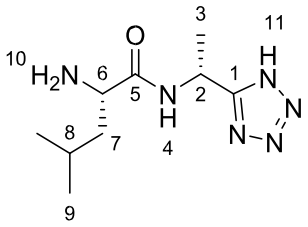


Benzyl-*N*-[(1*S*)-3-methyl-1-{[(1*R*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}butyl]carbamate **69h-LD** was prepared using general method I. The Fmoc group of Fmoc-1-D-aminoethyltetrazole attached to 2chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.2 mmol) was removed, this was monitored by Fmoc and TNBS tests. After the coupling of Cbz-L-leucine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure, and washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.30 g, 69%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate – ethanol 1:1 ratio, R_f: 0.63). Mp: 143 – 144 °C; ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ_H 16.2 (1H, brs, 11-H), 8.61 (1H, d, *J*=7.5 Hz, 4-H), 7.35 (6H, m, 10,15,16,17-H), 5.26 (1H, quint, *J*=7.5 Hz, 2-H), 5.04 (1H, d, *J*=13.2, 13a-H), 5.00 (1H, d, *J*=12.9 Hz, 13b-H), 4.11 (1H, m, 6-H), 1.61 (1H, m, 8-H), 1.45 (3H, d, *J*=6.9 Hz, 3-H), 1.42 (2H, m, 7-H), 0.88 (3H, d, *J*=6.0 Hz, 9a-H), 0.86 (3H, d, *J*=5.7 Hz, 9b-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C

172.4 (5-C), 158.5 (1-C), 156.4 (12-C), 137.5 (14-C), 128.8 (16-C), 128.2 (17C), 128.1 (15-C), 65.9 (13-C), 53.4 (6-C), 41.4 (2-C), 40.3 (7-C), 24.7 (8-C), 23.5 (9a-C), 21.9 (9b-C), 19.6 (3-C); v_{max}/cm^{-1} 3312 (N-H), 3269 (N-H), 2959, 2876, 2756, 2621 (tetrazole) 1690 (C=O), 1649 (C=), 1528 (N-H), 1234 (C-O); MS(ESI) m/z 361.2 $(M+H)^+$; CHN [Found: C, 57.20; H, 6.78; N, 23.18.

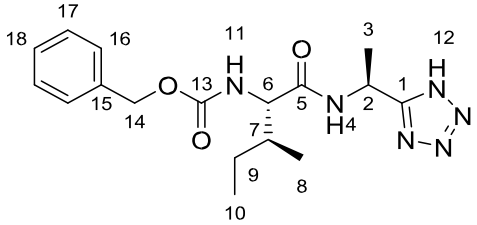
$C_{17}H_{24}N_6O_3$ requires C, 56.65; H, 6.71; N, 23.18 %].

(2*S*)-2-Amino-4-methyl-*N*-[(1*R*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]pentanamide 70h-LD:



Cbz-L-leucyl-1-D-aminoethyltetrazole **69h-LD** (0.25 g, 0.7 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with ethanol, which was discarded. The residue was washed with water, which was freeze dried and purified by column chromatography (eluent: ethyl acetate – ethanol 1:1 – 1:2 – 0:1) to give the pure product as a white solid (83 mg, 53%). The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.21). Mp: 136 °C (decomposed), 161 °C (melted); ^{1}H NMR (300 MHz, D_2O) δ_H 5.27 (1H, quart, *J*=6.9 Hz, 2-H), 3.95 (1H, t, *J*=6.9Hz, 6-H), 1.71 (3H, m, 7,8-H), 1.53 (3H, d, *J*=7.2 Hz, 3-H), 0.93 (3H, d, *J*=6.0 Hz, 9a-H), 0.91 (3H, d, *J*=6.0 Hz, 9b-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 169.8 (5-C), 164.7 (1-C), 52.2 (6-C), 42.4 (2-C), 39.9 (7-C), 24.0 (8-C), 21.6 (9a-C), 21.3 (9b-C), 19.1 (3-C); ν_{max}/cm^{-1} 3194 (N-H), 1667 (C=O), 1543 (N-H); MS(ESI) m/z 227.2 $(M+H)^+$; HRMS (Found $(M+H)^+$ 227.1625 Calcd. for $C_9H_{19}ON_6$: $(M+H)^+$ 227.1620.); (Purity test: HPLC method A).

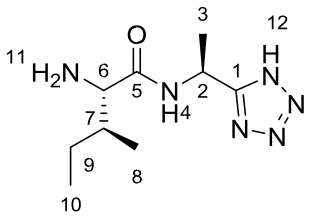
Benzyl-*N*-[(1*S*,2*S*)-2-methyl-1-{[(1*S*)-1-(1H-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}butyl]carbamate 69i-LL:



Benzyl-*N*-[(1*S*,2*S*)-2-methyl-1-{[(1*S*)-1-(1H-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}butyl]carbamate **69i-LL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to 2chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.8 mmol) was removed, this was monitored by the Fmoc and TNBS tests. After the coupling of Cbz-Lisoleucine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under

reduced pressure, and washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.40 g, 62%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate – ethanol 10:1 ratio, R_f: 0.13). Mp: 186 - 189 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.58 (1H, d, *J*=7.2 Hz, 4-H), 7.35 (5H, m, 16,17,18-H), 7.24 (1H, d, *J*=9.0 Hz, 11-H), 5.24 (1H, quint, *J*=6.9 Hz, 2-H), 5.05 (1H, d, *J*=12.6 Hz, 14a-H), 5.00 (1H, d, *J*=12.6 Hz, 14b-H), 3.92 (1H, t, *J*=8.1 Hz, 6-H), 1.70 (1H, m, 7-H), 1.50 (3H, d, *J*=6.9 Hz, 3-H), 1.35 (1H, m, 9a-H), 1.07 (1H, m, 9b-H), 0.77 (3H, t, *J*=7.5 Hz, 10-H), 0.75 (3H, d, *J*=7.5 Hz, 8-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 171.4 (5-C), 158.3 (1-C), 156.5 (13-C), 137.5 (15-C), 128.8 (17-C), 128.2 (18-C), 128.1 (16-C), 65.9 (14-C), 59.3 (6-C), 39.9 (2-C), 36.9 (7-C), 24.7 (9-C), 19.5 (3-C), 15.7 (8-C), 11.3 (10-C); ν_{max}/cm^{-1} 3279 (N-H), 2963, 2878, 2693, 2608 (tetrazole), 1690 (C=O), 1651 (C=O), 1535 (N-H), 1234 (C-O); MS(ESI) m/z 361.1 $(M+H)^+$; CHN [Found: C, 56.61.; H, 6.77; N, 23.01. $C_{17}H_{24}N_6O_3$ requires C, 56.65; H, 6.71; N, 23.32 %].

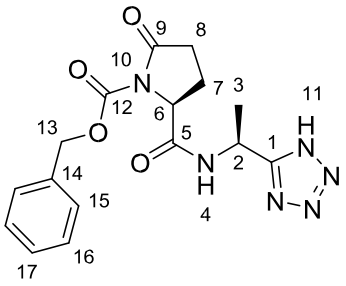
(2*S*,3*S*)-2-amino-3-methyl-*N*-[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]pentanamide 70i-LL:



Cbz-L-isoleucyl-1-L-aminoethyltetrazole **69i-LL** (0.35 g, 0.9 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with ethanol, which was discarded. The residue was washed with water, which was freeze dried to give the product as white solid (190 mg, 86%). Due to hygroscopicity, the corresponding hydrochloride salt was made with 2M HCl in diethyl ether solution (20 mL) for characterization. Further purification was performed by Prep HPLC method A. The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.31). Mp: 164 - 167 °C (melted and decomposed); ^{1}H NMR (300 MHz, D_2O) δ_H 5.39 (1H, quart, *J*=7.2

Hz, 2-H), 3.85 (1H, d, *J*=5.7 Hz, 6-H), 1.92 (1H, m, 7-H), 1.65 (3H, d, *J*=7.2 Hz, 3-H), 1.31 (1H, m, 9a-H), 1.14 (1H, m, 9b-H), 0.89 (3H, d, *J*=6.9 Hz, 8-H), 0.83 (3H, t, *J*=7.5 Hz, 10-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 169.1 (5-C), 158.1 (1-C), 57.7 (6-C), 40.7 (2-C), 36.3 (7-C), 23.9 (9-C), 17.6 (3-C), 14.1 (8C), 10.4 (10-C); ν_{max}/cm^{-1} 3240 (N-H), 1682 (C=O), 1574 (N-H); MS(ESI) m/z 227.2 $(M+H)^+$; HRMS (Found $(M+Na)^+$ 249.1436. Calcd. for $C_9H_{18}ON_6Na$: $(M+Na)^+$ 249.1434.); (Purity test: HPLC method A).

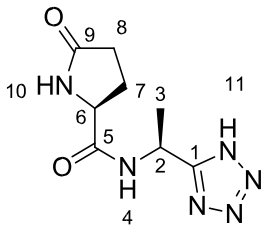
Benzyl-(5*S*)-2-oxo-5-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}pyrrolidine-1-carboxylate 69f-LL:



Benzyl-(5*S*)-2-oxo-5-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}pyrrolidine-1-carboxylate **69f-LL** was prepared using general method I. The Fmoc group of the Fmoc-1-L-aminoethyltetrazole attached to 2-chlorotrityl chloride resin (resin:loading; 1:2 ratio; 2.0 mmol) was removed. After coupling of Cbz-L-pyroglutamine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was subsequently removed by evaporation under reduced pressure. The crude product was purified by column chromatography (eluent: ethyl acetate – ethanol 10:1, 6:1 then 1:1 ratio) to obtain the product as white crystals (0.46g, 65%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate – ethanol 10:1, R_f: 0.09). Mp: 195 °C (decomposed); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 16.05 (1H, brs, 11-H), 8.93 (1H, d, *J*=7.5 Hz, 4-H), 7.36 (5H, m, 15-17-H), 5.17 (3H, m, 2,13-H), 4.64 (1H, dd, *J*=2.7 Hz, *J*=9.0 Hz, 6-H), 2.41 (2H, m, 8-H), 2.26 (1H, m, 7-H_a), 1.94 (1H, m, 7-H_b), 1.42 (3H, d, *J*=6.9 Hz, 3-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 174.1 (9-C), 171.0 (5C), 158.3 (1-C), 150.9 (12-C), 136.0 (14-C), 128.8 (2C, 16-C), 128.5 (17-C), 128.0 (15-C), 67.5 (13-C), 59.3 (6-C), 40.2 (2-C), 31.3 (8-C), 22.3 (7-C), 19.6 (3-C); ν_{max}/cm^{-1} 3289 (N-H), 2993, 2868, 2739, 2615 (tetrazole), 1780 (C=O), 1701

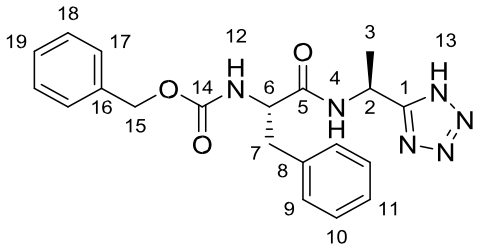
(C=O), 1655 (C=O), 1545 (N-H), 1236 (C-O); MS(ESI) m/z 381.2 $(M+Na)^+$. CHN [Found: C, 53.63; H, 5.10; N, 23.18. $C_{16}H_{18}N_6O_4$ requires C, 53.63; H, 5.06; N, 23.45 %].

(*S*)-*N*-((*S*)-1-(1*H*-tetrazol-5-yl)ethyl)-5-oxopyrrolidine-2-carboxamide 70fLL:



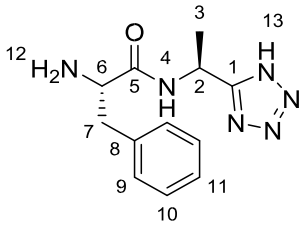
Cbz-L-pyroglutamyl-1-L-aminoethyltetrazole **69f-LL** (0.44 g, 1.2 mmol) was reacted by general method F. Following filtration, the solid residue was washed with methanol and the filtrate was evaporated under reduced pressure. The residual oil was triturated with diethyl ether to give the title compound as a white solid (0.23 g, 84%). Further purification was performed by Prep HPLC method A. The reaction and the work up were monitored by TLC (ethanol, R_f: 0.3). Mp: 160 °C (decomposed); ^{1}H NMR (300 MHz, D_2O) δ_H 5.35 (1H, quart, *J*=7.2 Hz, 2-H), 4.34 (1H, dd, *J*=4.8 Hz, *J*=8.4 Hz, 6-H), 2.52 (1H, m, 7-H_a), 2.40 (2H, m, 8-H) 2.04 (1H, m, 7-H_b), 1.61 (3H, d, *J*=6.9 Hz, 3-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 182.3 (9-C), 174.6 (5-C), 159.0 (1-C), 56.9 (6-C), 41.0 (2-C), 29.3 (8-C), 25.0 (7-C), 18.1 (3-C); ν_{max}/cm^{-1} 3262 (NH), 1649 (C=O), 1547 (N-H); MS(ESI) m/z 225.1 $(M+H)^+$. HRMS (Found $(M+H)^+$ 225.1096. Calcd. for $C_8H_{13}O_2N_6$: $(M+H)^+$ 225.1095.).

Benzyl-*N*-[(1*S*)-2-phenyl-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamate 69j-LL:



Benzyl-*N*-[(1*S*)-2-phenyl-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamate **69j-LL** was prepared using general

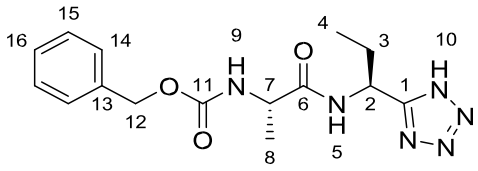
method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to 2chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.0 mmol) was removed, this was monitored by the Fmoc and TNBS tests. After the coupling of Cbz-Lphenylalanine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure, and washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.32 g, 79%). Further purification was performed using a Teledyne-ISCO CombiFlash EZ Prep system (eluent: gradient: dichloromethane – methanol 0 – 30%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate, R_f: 0.11). Mp: 191 - 193 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 16.21 (1H, brs, 8.72 (1H, d, *J*=7.5 Hz, 4-H), 7.44 (1H, d, *J*=8.7 Hz, 12-H), 7.33 - 7.24 (10H, m, 9,10,11,17,18,19-H), 5.27 (1H, quint, 6.9 Hz, 2-H), 4.96 (1H, d, *J*= 13.5 Hz, 15a-H), 4.92 (1H, d, *J*= 13.5 Hz, 15bH), 4.30 (1H, m, 6-H), 3.03 (1H, dd, *J*=3.3 Hz, *J*=13.8 Hz, 7a-H), 2.72 (1H, dd, *J*=10.8 Hz, *J*=13.5 Hz, 7b-H), 1.52 (3H, d, J=6.9 Hz, 3-H); ^{13}C NMR (75.5 MHz,
DMSO-d_6) δ_C 171.7 (5-C), 158.7 (1-C), 156.3 (14-C), 138.5 (8-C), 137.5 (16-C), 129.6, 128.7, 128.5, 128.1, 127.9, 126.7 (9,10,11,17,18,19-C), 65.7 (15C), 56.5 (6-C), 40.0 (2-C), 37.8 (7-C), 19.7 (3-C); ν_{max}/cm^{-1} 3300 (N-H), 2984, 2874, 2741, 2615 (tetrazole), 1684 (C=O), 1651 (C=O), 1531 (N-H), 1234 (CO); MS(ESI) m/z 417.2 $(M+Na)^+$; CHN [Found: C, 60.78; H, 5.68; N, 20.92. $C_{20}H_{22}N_6O_3$ requires C, 60.90; H, 5.62; N, 21.31 %]. **(2*S*)-2-Amino-3-phenyl-*N*-[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]propanamide 70j-LL:**



Cbz-L-phenylalanyl-1-L-aminoethyltetrazole **69j-LL** (0.30 g, 0.8 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with ethanol, which was discarded. The residue was washed with water, which was freeze dried, then purified by column chromatography (eluent: dichloromethane – ethanol 10:1, 8:1, 6:1, 1:1 then 0:1 ratio) to give

the pure product as a white solid (40 mg, 20%). Further purification was performed by Prep HPLC method A. The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.39). Mp: 152 – 155 °C; ^{1}H NMR (300 MHz, D_2O) δ_H 7.15 (3H, m, 10,11-H), 7.01 (2H, m, 9-H), 5.24 (1H, quart, *J*=6.9 Hz, 2-H), 3.77 (1H, m, 6-H), 2.97 (1H, dd, *J*=6.0, *J*=13.5 Hz, 7a-H), 2.89 (1H, dd, *J*= 7.8, *J*=13.5 Hz, 7b-H), 1.43 (3H, d, *J*=7.2 Hz, 3-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 163.5 (1-C), 150.6 (5-C), 135.5 (8-C), 129.2 (9-C), 128.6 (10-C), 127.1 (11-C), 55.5 (6-C), 41.3 (2-C), 39.3 (7-C), 19.3 (3-C); ν_{max}/cm^{-1} 3217 (NH), 1651 (C=O), 1528 (N-H); MS(ESI) m/z 283.2 $(M+Na)^+$. HRMS (Found $(M+H)^+$ 261.1462. Calcd. for $C_{12}H_{17}ON_6$: $(M+H)^+$ 261.1458.); (Purity test: HPLC method A).

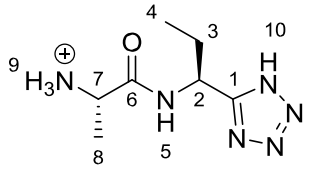
Benzyl-*N*-[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)propyl]carbamoyl}ethyl]carbamate 69b-LL:



Benzyl-*N*-[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)propyl]carbamoyl}ethyl]carbamate **69b-LL** was prepared using general method I. The Fmoc group of the Fmoc-1-L-aminopropyltetrazole attached to 2-chlorotrityl chloride resin (resin:loading; 1:1 ratio; 2.9 mmol) was removed. After coupling of Cbz-L-alanine with the resin, the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure. The crude product was subsequently washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.49 g, 52%). Mp: 174 – 175 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.44 (1H, d, *J*=7.5 Hz, 5-H), 7.41 (1H, d, *J*=7.2 Hz, 9-H), 7.34 (5H, m, 14,15,16H), 5.06 (1H, m, 2-H), 5.02 (2H, s, 12-H), 4.10 (1H, quint, *J*=6.9 Hz, 7-H), 1.88 (2H, m, 3-H), 1.19 (3H, d, *J*=7.2 Hz, 8-H), 0.87 (3H, t, *J*=7.2 Hz, 4-H); ^{13}C NMR
(75.5 MHz, DMSO-d_6) δ_C 173.0 (6-C), 156.8 (1-C), 156.1 (11-C), 137.5 (13-

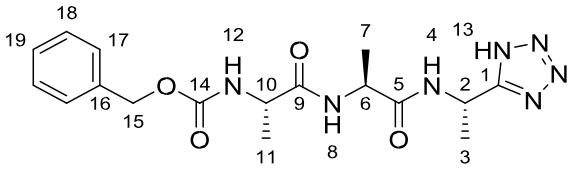
C), 128.8 (15-C), 128.2 (16-C), 128.1 (14-C), 65.8 (12-C), 50.4 (7-C), 45.8 (2C), 26.8 (3-C), 18.4 (8-C), 10.6 (4-C); v_{max}/cm^{-1} 3300 (N-H), 3265 (N-H), 2976,
2876, 2735, 2617 (tetrazole), 1691 (C=O), 1651 (C=O), 1533 (N-H), 1246 (CO); MS(ESI) m/z 333.1 $(M+H)^+$; CHN [Found: C, 54.20; H, 6.11; N, 24.91. $C_{15}H_{20}N_6O_3$ requires C, 54.21; H, 6.07; N, 25.29 %].

(2*S*)-2-Amino-*N*-[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5-yl)propyl]propanamide 70b-LL:

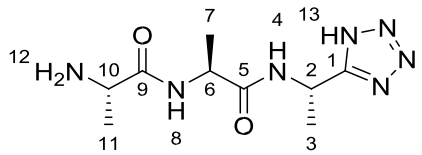


Cbz-L-alanyl-1-L-aminopropyltetrazole **69b-LL** (0.40 g, 1.2 mmol) was deprotected by general method F. Following filtration, the solid residue was washed with ethanol, which was discarded. The residue was washed with water, which was freeze dried to give the product as a white solid (206 mg, 86%). Due to poor solubility, the corresponding hydrochloride salt was prepared using 2M HCl in diethyl ether solution (20 mL) for characterization (hygroscopic solid). Further purification was performed by Prep HPLC method A. The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.12). ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 9.17 (1H, d, *J*=7.6 Hz, 5-H), 8.34 (3H, brs, 9-H), 5.09 (1H, quart, *J*=7.8 Hz, 2-H), 3.93 (1H, quart, *J*=6.7 Hz, 7-H),
1.92 (2H, m, 3-H), 1.38 (3H, d, *J*=7.0 Hz, 8-H), 0.90 (3H, t, *J*=7.4 Hz, 4-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 170.0 (6-C), 158.0 (1-C), 48.6 (7-C), 46.4 (2C), 26.8 (3-C), 17.4 (8-C), 10.6 (4-C); IR; MS(ESI) m/z 199.1 $(M+H)^+$; HRMS (Found $(M+H)^+$ 199.1302. Calcd. for $C_7H_{15}ON_6$: $(M+H)^+$ 199.1302.); (Purity test: HPLC method A).

***N*-[(1*S*)-1-{[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamoyl} ethyl]carbamate 69c-LLL:**



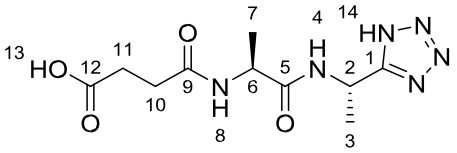
N-[(1*S*)-1-{[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamoyl} ethyl]carbamate **69c-LLL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to the 2-chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.0 mmol) was removed, this was monitored by the Fmoc and TNBS tests. After the coupling of Fmoc-L-alanine with the resin, the Fmoc group was removed again. Finally, Cbz-L-alanine was attached to the resin, and the title compound was cleaved off. The cleaving mixture (TFA/DCM/TIS, ratio 5:95:1 eq) was removed by evaporation under reduced pressure, and washed with petroleum ether (40 – 60 °C) to obtain the product as white crystals (0.19 g, 47%). Mp: 199 – 201 °C (melted and decomposed); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 16.09 (13-H), 8.48 (1H, d, *J*=6.9 Hz, 4-H), 7.93 (1H, d, *J*=7.5 Hz, 8-H), 7.42 (1H, d, *J*=7.2 Hz, 12-H), 7.35 (5H, m, 17-19-H), 5.21 (1H, quint, *J*=6.9 Hz, 2H), 5.02 (2H, s, 15-H), 4.29 (1H, quint, *J*=7.2 Hz, 6-H), 4.06 (1H, quint, *J*=7.2 Hz, 10-H), 1.49 (3H, d, *J*=6.9 Hz, 3-H), 1.19 (6H, d, *J*=6.9 Hz, 7,11-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 172.6 (9-C), 172.3 (5-C), 159.1 (1-C), 156.1 (14-C), 137.5 (16-C), 128.8 (18-C), 128.2 (19-C), 128.1 (17-C), 65.8 (15-C), 50.4 (10-C), 48.4 (6-C), 40.9 (2-C), 19.7 (3-C), 18.5 (7-C), 18.4 (11-C); ν_{max}/cm^{-1} 3298(N-H), 1694 (C=O), 1643 (C=O), 1526 (N-H), 1219 (C-O); MS(ESI) m/z 390.2 $(M+H)^+$; CHN [Found: C, 52.42; H, 6.02; N, 23.26. $C_{17}H_{23}N_7O_4$ requires C, 52.43; H, 5.95; N, 25.18 %]. **(*S*)-*N*-((*S*)-1-(1*H*-tetrazol-5-yl)ethyl)-2-((*S*)-2aminopropanamido)propanamide 70c-LLL:**



Cbz-L-alanyl-L-alanyl-1-L-aminoethyltetrazole **69c-LLL** (0.12 g, 0.31 mmol) was reacted by the general method F. Following the filtration, the solid residue was washed with methanol, then the filtrate was evaporated under reduced pressure to give the crude product (1.11 g, 95%), further recrystallization from

a methanol - ethyl acetate mixture provided the pure product as white crystals (0.477 g, 40%). Further purification was performed by Prep HPLC method A. The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.13). Mp: 135 °C (melted and decomposed); ^{1}H NMR (300 MHz, D_2O) δ_H 5.25 (1H, quart, *J*=7.2 Hz, 2-H), 4.30 (1H, quart, *J*=7.2 Hz, 6-H), 3.95 (1H, quart, *J*=6.9 Hz, 10-H), 1.52 (3H, d, *J*=7.2 Hz, 3-H), 1.43 (3H, d, *J*=7.2 Hz, 11-H), 1.33 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 173.6 (5-C), 171.7 (9-C), 164.1 (1-C), 49.8 (6-C), 49.0 (10-C), 42.0 (2-C), 19.4 (3-C), 17.0 (11C), 16.5 (7-C); ν_{max}/cm^{-1} 3252 (N-H), 1639 (C=O), 1539 (N-H); MS(ESI) m/z 256.1 $(M+H)^+$; HRMS (Found $(M+H)^+$ 256.1520. Calcd. for $C_9H_{18}O_2N_7$: $(M+H)^+$ 256.1516.); (Purity test: HPLC method A).

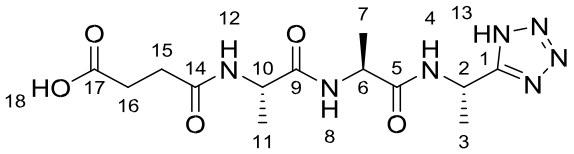
3-{[(1S)-1-{[(1S)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamoyl}propanoic acid 77-LL:



3-{[(1S)-1-{[(1S)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamoyl}propanoic acid **77-LL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to the 2-chlorotrityl chloride resin (resin:loading; 1:2 ratio; 1.4 mmol) was removed. After the coupling of Fmoc-L-alanine with the resin, the Fmoc group was removed again. Finally, a succinyl group was attached to the resin using succinic anhydride, and the title compound was cleaved off. The cleaving mixture (TFA/water/TIS, ratio 95:2.5:2.5) was removed by evaporation under reduced pressure, and recrystallized from a water – acetone solvent mixture to obtain the product as white crystals (0.16 g, 40%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate – ethanol – drop of glacial acetic acid 1:1 ratio, R_f: 0.21). Mp: 170 – 171 °C; ^{1}H NMR (300 MHz, D_2O) δ_H 5.32 (1H, quart, *J*=7.2 Hz, 2-H), 4.28 (1H, quart, *J*=7.2 Hz, 6-H), 2.64 (2H, m, 11-H), 2.54 (2H, m, 10-H), 1.62 (3H, d, *J*=6.9 Hz, 3-H), 1.35 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 176.9 (12-C), 175.0 (9-C), 174.9

(5-C), 158.5 (1-C), 49.7 (6-C), 40.7 (2-C), 29.9 (10-C), 29.0 (11-C), 17.8 (3C), 16.4 (7-C); v_{max}/cm^{-1} 3358 (N-H), 3254 (O-H), 2992, 2945, 2886, 2778 , 2712 (tetrazole), 1713 (C=O), 1636 (C=O), 1543 (N-H), 1526 (N-H), 1231 (CO); MS(MALDI) m/z 285.30 $(M+H)^+$; HRMS (Found $(M+H)^+$ 285.1310. Calcd. for $C_{10}H_{17}O_4N_6$: $(M+H)^+$ 285.1306.).

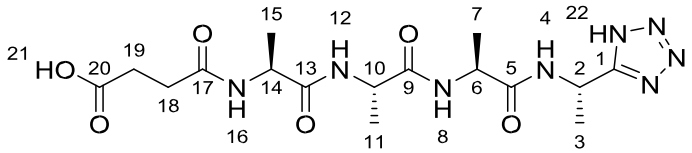
3-{[(1S)-1-{[(1S)-1-{[(1S)-1-(1H-1,2,3,4-tetrazol-5-yl)ethyl]carbamoyl}ethyl]carbamoyl}ethyl]carbamoyl}propanoic acid 78LLL



3-{[(1S)-1-{[(1S)-1-{[(1S)-1-(1H-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamoyl}ethyl]carbamoyl}propanoic acid **78-LLL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to the 2-chlorotrityl chloride resin (resin:loading; 1:2 ratio; 2.8 mmol) was removed. After the coupling of Fmoc-L-alanine with the resin, the Fmoc group was removed, and the free amino group was coupled with Fmoc-L-alanine again. After removing the Fmoc protection, a succinyl group was attached to the resin using succinic anhydride, and the title compound was cleaved off. The cleaving mixture (TFA/water/TIS, ratio 95:2.5:2.5) was removed by evaporation under reduced pressure, and triturated with diethyl ether to obtain the product as white crystals (0.39 g, 79%). Further purification was performed by Prep HPLC method A. The reaction and the work up were monitored by TLC (eluent: ethyl acetate – ethanol – drop of glacial acetic acid 1:1 ratio, R_f: 0.22). Mp: 186 °C (melted and decomposed); ^{1}H NMR (300 MHz, D_2O) δ_H 5.32 (1H, quart, *J*=7.2 Hz, 2H), 4.29 (1H, quart, J=7.2 Hz, 6-H), 4.21 (1H, quart, *J*=7.2 Hz, 10-H), 2.66 (2H, m, 16-H), 2.55 (2H, m, 15-H), 1.62 (3H, d, *J*=6.9 Hz, 3-H), 1.35 (3H, d, *J*=7.2 Hz, 7-H), 1.34 (3H, d, *J*=7.2 Hz, 11-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 177.0 (17-C), 175.3 (14-C), 175.2 (9-C), 174.6 (5-C), 158.1 (1-C), 50.0 (10-C), 49.6

(6-C), 40.7 (2-C), 29.8 (16-C), 29.0 (15-C), 17.8 (3-C), 16.3 (7-C), 16.2 (11C); ν_{max}/cm^{-1} 3343 (N-H), 3381 (O-H), 1707 (C=O), 1651 (C=O), 1634 (C=O), 1539 (N-H), 1201 (C-O); MS(MALDI) m/z 356.30 $(M+H)^+$. HRMS (Found $(M+H)^+$ 356.1682. Calcd. for $C_{13}H_{22}O_5N_7$: $(M+H)^+$ 356.1677.) (Purity test: HPLC method A).

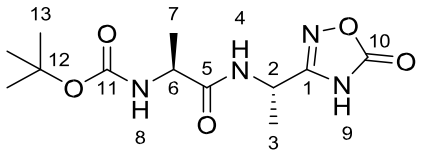
3-{[(1*S*)-1-{[(1*S*)-1-{[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamoyl}ethyl]carbamoyl}ethyl]carbamoyl} p ropanoic acid 79-LLLL:



3-{[(1*S*)-1-{[(1*S*)-1-{[(1*S*)-1-{[(1*S*)-1-(1*H*-1,2,3,4-tetrazol-5yl)ethyl]carbamoyl}ethyl]carbamoyl}ethyl]carbamoyl}ethyl]carbamoyl}propanoic acid **79-LLLL** was prepared using general method I. The Fmoc group of Fmoc-1-L-aminoethyltetrazole attached to the 2-chlorotrityl chloride resin (resin:loading; 1:1 ratio; 1.5 mmol) was removed, and resin was coupled with Fmoc-L-alanine, the Fmoc deprotection and coupling with Fmoc-L-alanine procedure was repeated 2 more times. After removing the Fmoc protection for the final time, a succinyl group was attached to the resin using succinic anhydride, and the title compound was cleaved off. The cleaving mixture (TFA/water/TIS, ratio 95:2.5:2.5) was removed by evaporation under reduced pressure. The residue was triturated with diethyl ether to obtain the product as a white solid (0.44 g, 68%). Further purification was carried out by column chromatography (eluent: ethyl acetate – ethanol 4:1, 1:1 then 0:1 ratio) and by Prep HPLC method A. The reaction and the work up were monitored by TLC (eluent: ethanol, R_f: 0.21). Mp: 231 °C (decomposed); ^{1}H NMR (300 MHz, D_2O) δ_H 5.32 (1H, quart, *J*=7.0 Hz, 2-H), 4.26 (3H, m, 6,10,14-H), 2.64 (2H, m, 19-H), 2.58 (2H, m, 18-H), 1.63 (3H, d, *J*=7.0 Hz, 3-H), 1.36 (9H, d, *J*=7.2 Hz, 7,11,15-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 177.9 (20-C), 175.5 (17-C), 175.3, 174.9, 174.4 (5,9,13-C), 158.7 (1-C), 50.1, 49.8, 49.6 (6,10,14-C), 40.9 (2-C), 29.9 (18-H), 29.2 (19-H), 18.0 (3-C), 16.4, 16.3, 16.2 (7, 11, 15-C); ν_{max}/cm^{-1} 3265 (N-H), 1690 (C=O), 1653 (C=O), 1531 (N-H), 1223 (C-O);

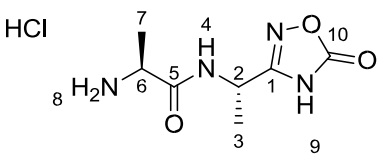
MS(ESI) m/z 425.1 $(M-H)^-$; HRMS (Found $(M+H)^+$ 427.2047. Calcd. for $C_{16}H_{27}O_6N_8$: $(M+H)^+$ 427.2048.); (Purity test: HPLC method A).

***tert*-Butyl ((*S*)-1-oxo-1-(((*S*)-1-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3yl)ethyl)amino)propan-2-yl)carbamate :**



Boc-L-alanine **24-L** (0.21 g, 1.09 mmol) and 1-L-aminoethyloxadiazolone **33L** (0.18g, 1.09 mmol) were coupled by general method H. To the evaporated mixture, ethyl acetate (10 mL) and 0.01M HCl_{aq} (10 mL) were added and the aqueous phase was extracted with ethyl acetate (2 x 15 mL). The combined organic phases were dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 1:1, 1:2, 1:4 then 0:1 ratio) to give the title compound as a white solid (0.13 g, 40%). The reaction and the work up were monitored by TLC (eluent: ethyl acetate – ethanol 4:1 ratio, R_f: 0.26). Mp: 153-154 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 12.27 (1H, br s, 9-H), 8.30 (1H, d, *J*=7.5 Hz, 4-H), 6.87 (1H, d, *J*=7.2 Hz, 8-H), 4.81 (1H, quint, *J*=7.2 Hz, 2-H), 3.97 (1H, m, 6-H), 1.38 (9H, s, 13-H), 1.36 (3H, d, *J*=7.2 Hz, 3-H), 1.18 (3H, d, *J*=7.2 Hz, 7-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 173.0 (5-C), 161.5 (10-C), 160.3 (1-C), 155.5 (11-C), 78.5 (12-C), 50.0 (6-C), 41.2 (2-C), 28.6 (13-C), 18.4 (7-C), 17.8 (3-C); ν_{max}/cm^{-1} 3354 (N-H), 2987 (C-H), 1782 (C=O), 1703 (C=O), 1643 (C=O), 1514 (N-H), 1161 (C-O); MS(ESI) m/z 323.2 $(M+Na)^+$; HRMS (Found $(M+H)^+$ 301.1511. Calcd. for $C_{12}H_{21}O_5N_4$: $(M+H)^+$ 301.1506.).

(*S*)-2-amino-*N*-((*S*)-1-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3yl)ethyl)propanamide hydrochloride 72-LL:



Boc-L-alanyl-L-1-aminoethyloxadiazolone **71-LL** (0.10 g, 0.3 mmol) was deprotected and worked up by general method A. The filtered solid was washed with diethyl ether and dichloromethane to give the product as a white hygroscopic solid (0.060 g, 99%). ^{1}H NMR (300 MHz, D_2O) δ_H 4.98 (1H, quart, *J*=7.2 Hz, 2-H), 4.10 (1H, quart, *J*=7.2 Hz, 6-H), 1.52 (6H, d, *J*=6.9 Hz, 3,7-H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 170.7 (5-C), 161.5 (10-C), 160.8 (1-C), 49.1 (6C), 42.0 (2-C), 16.4 (3-C), 16.3 (7-C); ν_{max}/cm^{-1} 2991 (br, N-H), 1757 (C=O), 1674 (C=O), 1543 (N-H); MS(ESI) m/z 201.0 $(M+H)^+$.

2.3.3 Microbiology

Determination of MICs:

Microbiological tests were done by or under the supervision of Professor John Perry at the Freeman Hospital in Newcastle. All MICs were determined using an agar dilution method.[104] This necessitated the use of a defined antagonistfree medium (peptone-free), prepared with the inclusion of 2% saponin-lysed horse blood, 25 µg/ml NAD and 25 µg/ml haemin.[46] Test compounds were dissolved in sterile deionised water and incorporated into the antagonist-free medium at a final concentration range of 1 – 128 mg/l. All bacteria were prepared from fresh (18 h) subcultures on blood agar. Each isolate was suspended in sterile deionised water to a density equivalent to 0.5 McFarland units using a densitometer (approx. 1.5 x 10^8 CFU/ml) and then diluted 1 in 15. A 1 µl aliquot of each diluted suspension was then delivered onto plates with a multipoint inoculator to give a final inoculum of approximately 1000, 10 000 or 150 000 CFU/spot as recommended.[104] All plates (including antimicrobial-free controls) were incubated for 22 h at 37°C. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration of test compound that resulted in complete inhibition of bacterial growth.

3 Chromogenic substrates

3.1 Introduction

Chromogenic culture media provide rapid and inexpensive tools for bacterial detection and identification that may be readily applied in a clinical environment.[12] Chromogenic substrates are the key components of chromogenic culture media, and are responsible for the detection. These weakly chromogenic or colourless compounds are substrates for bacterial enzymes and contain a chromogen linked to a targeting unit known to be prone to reaction in the presence of a specific enzyme. Upon enzymatic action, the chromogenic molecule is liberated, which produces colour within the bacterial colonies (Figure 22).

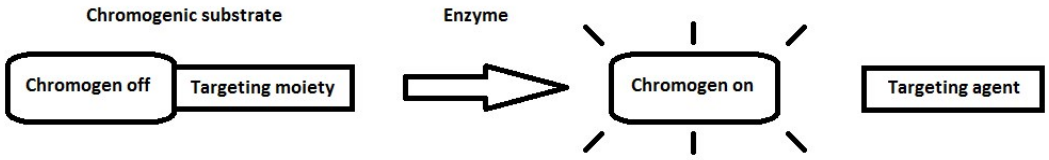


Figure 22 *Action of an enzyme on a chromogenic substrate*

3.1.1 Target enzymes: hydrolases

A number of chromogenic substrates are now available for bacterial identification.[12] These have been designed for selective bacterial detection by targeting different enzymes. Most of the chromogenic substrates target specific bacterial hydrolases. The main classes are introduced here, focusing on the identification of some of the ESKAPE pathogens, and a few other clinically relevant species and strains.

3.1.1.1 Esterases and lipases

Esterases catalyse the hydrolysis and formation of ester groups, with those that hydrolyse esters of long chain fatty acids being commonly referred to as lipases. Esterase hydrolysing esters of short chain organic acids are common enzymes and appear within all living organisms. C8-esterase is produced by

Salmonella spp. and has been targeted by chromogenic substrates **80a** to differentiate it from other *Enterobacteriaceae* strains (Figure 23).[12,105-107]

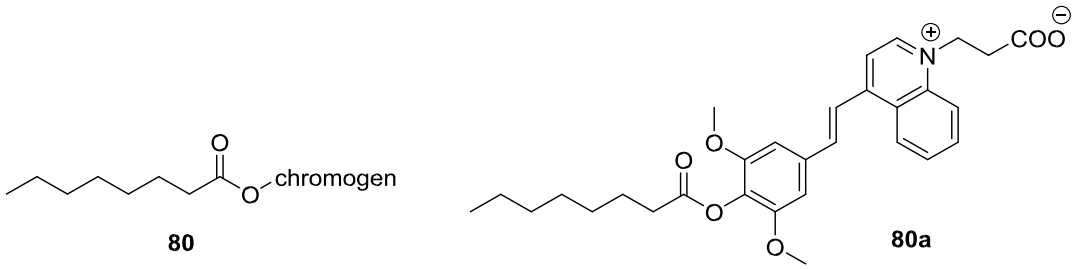


Figure 23 *General structure of C8-esterase substrate* ***80,*** *and an example octanoic acid substrate* ***80a***[100]

3.1.1.2 Glycosidases

Glycosidase enzymes are responsible for the catabolism of complex carbohydrates to their component simpler sugars. They perform this feat by hydrolysing the glycosidic linkages of the polysaccharides to the appropriate sugar building blocks. As there are many hexose isomers, and nature demands precise levels of control over the reactions it allows, evolution has provided a host of specific enzymes. Hexoses are essential for each living organism. Due to the variety of food sources, different species can have different sets of glycosidases. This provides the opportunity for selective detection of many bacterial species. Glycosidases are among the targets for chromogenic substrates in many chromogenic media.[108] A few important examples are described below.

The enzyme□□□-D-glucuronidase is expressed by *E*. *coli* and some *Shigella* spp., and consequently it is a target for chromogenic substrates **81** in selective media aiming for their detection. It has been shown that *E*. *coli* can be identified from urine samples by substrates **81** formed from glucuronic acid and a suitable chromogen, of the form shown in Figure 24.[109,110]

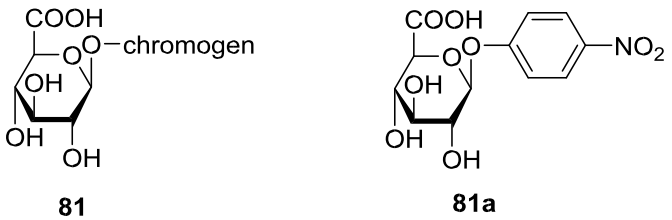
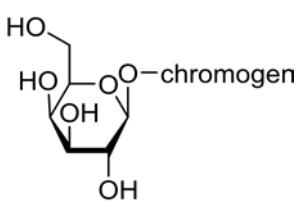


Figure 24 *General structure of a □-D-glucuronidase chromogenic substrate* ***81*** *and an example 4-nitrophenoxy-□-D-glucuronide* ***81a***[111]

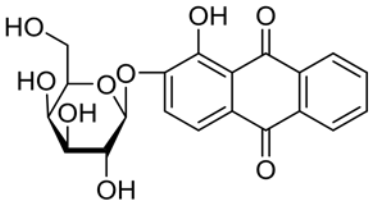
□-D-Galactosidase is expressed by most Enterobacteriaceae, including *E. coli*, but not by *Salmonella* spp., and has been used for differentiation of these species by □-D-galactosidase substrates **82**.[5]

Importantly□|□-D-glucosidase is most commonly present in enterococci, and also in certain Enterobacteriaceae, e.g. the KES group (*Klebsiella-Enterobacter-Serratia*). Consequently, targeting substrates **83a** can identify them in, for example, urine samples.[112]

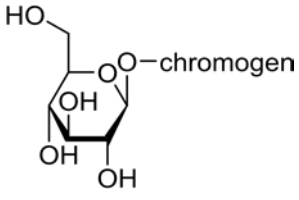
The presence of □-D-glucosidase enables certain related bacterial strains to be distinguished. Substrate **84a** enables the differentiation of *Bacillus anthracis* from other *Bacillus* spp., as its □-D-glucosidase activity is significantly lower due to the lower permeability to substrate **84a** (Figure 25).[10] Selective differentiation of *S. aureus* from other staphylococci was also reported using □-D-glucosidase substrates **84**.[113,114]



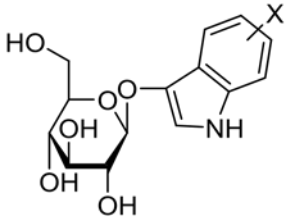
82



82a

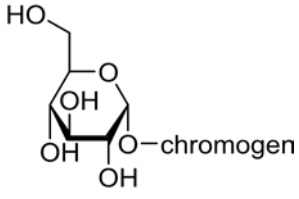


83

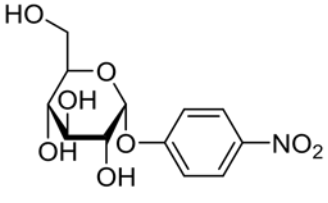


X: halogen

83a



84

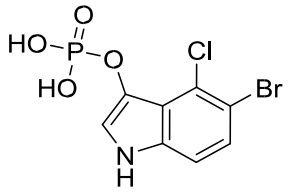


84a

Figure 25 *General structure of substrate structures for □-D-galactosidase* ***82*** *and □-D-glucosidase* ***83****, an □-D-glucosidase* ***84*** *chromogenic substrate and examples alizarin-□-D-galactoside* ***82a***[115]*,halo-indoxyl-□-D-glucoside* ***83a***[112]*(****83a*** *is an indoxyl substrate, there is no more information in the reference) and 4-nitrophenoxy-□-D-glucosid* ***84a***[10]

3.1.1.3 Phosphatases

Phosphatases are responsible for the hydrolysis of phosphate derived groups. The transfer of phosphate groups on and off biological molecules plays a key role in many biological processes. These include the transport of metabolites by kinases, the phosphorylation of glucose in the glycolysis process, energy metabolism and nucleic-acid synthesis.[116] They are essential enzymes for all microorganisms. Phosphate hydrolysing phosphatases are targeted by chromogenic substrates to identify *S. aureus*,[117] group B streptococci and *Candida* spp., for example by an indoxyl substrate **85** (Figure 26).[117] The basis for the selectivity of the phosphatase substrates has not been reported in detail to the best of our knowledge, although substrate transport and permeability probably have a key role, as it is an essential enzyme for all bacteria.



85

Figure 26 *An example of a phosphatase chromogenic substrate 5-bromo-4chloroindoxyl phosphate* ***85***[117]

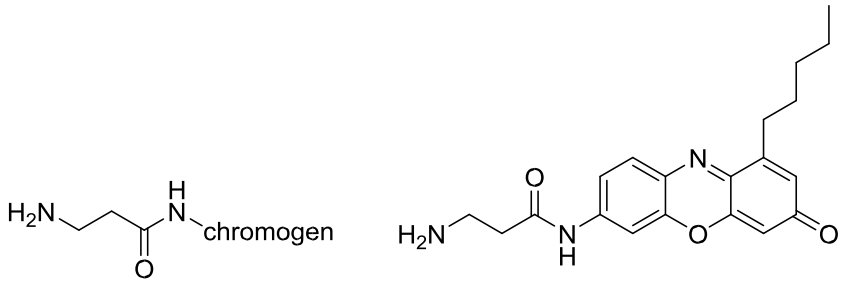
3.1.1.4 Peptidases

Peptidases catalyse the hydrolysis of peptide bonds in every living cell. They can be classified into two main groups. These are the endopeptidases, which can act within the peptide chain, and exopeptidases, which work on *C*- or *N*terminal amino acids.

Aminopeptidases are exopeptidases that can hydrolyse the *N*-terminal amino acid from a peptide chain. They are localised in various places within the cell,

such as in the cytoplasm, the cell-membrane, the periplasmic space and in the extracellular surface.[118]

As well as the 20 natural □-amino acids commonly occurring in peptides and proteins, there are many other lesser known amino acids, each of which is required for specific but crucial functions in different living organisms. Thus, a wide variety of aminopeptidases can be found in bacteria. However, in chromogenic media applications only a few specific peptidases, such as Lalanyl aminopeptidase and □-alanyl aminopeptidase, have been successfully targeted by commercial chromogenic substrates such as **86** (Figure 27).[119] □alanyl aminopeptidase is responsible for the hydrolysis of the □-alanyldipeptides and -oligopeptides. It was isolated from *Pseudomonas* species,[81] but it is also known to be present in both *Serratia marcescens* and in *Burkholderia cepacia,* albeit at lower levels of expression.[82] □-Alanyl **86a** substrate is applied in the clinical detection of *Pseudomonas aeruginosa.*[12]



86 **86a**

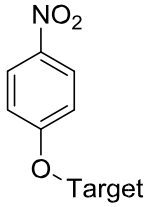
Figure 27 *General structure of a □-alanyl aminopeptidase chromogenic substrate* ***86****, and an example 7-□-alanylamido-1-pentylphenoxazin-3-one* ***86a****[122]*

3.1.2 Main types of chromogenic substrates

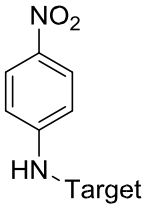
There are numerous chromogenic substrates that have been the basis of published research within the literature. Of these, a smaller number have gone to successful commercial exploitation, where they have been employed in routine tests within chromogenic media utilised within clinical settings.

Chromogenic substrates are built up of two main parts: the targeting linker molecule, discussed above, and the chromogen, which is responsible for the detection by producing or changing colour. Ideally, while the targeting linker molecule is attached to the chromogen, the substrate has no colour (or limited colour). Upon action of the target enzyme, the coloured chromogen is released (Figure 22). Possible targeted enzymes and targeting linker parts have been introduced earlier in the previous chapter (section 3.1.1). The structure of the chromogens can vary significantly, although all of those in current use are aromatic compounds with an extended conjugated □-electron system, or precursors of such molecules. As these substrates most commonly target hydrolase enzymes, they all have a hydrolysable ester, glycosidic or amide bond, which usually liberates a phenolic or amino group that had previously been masked within the substrate. The most commonly encountered chromogenic substrates are introduced below, classified by their structural similarities. In accordance with the relevance of this thesis, only chromogenic substrates for hydrolase enzymes are discussed in this chapter.

The simplest and oldest chromogenic substrates are 4-nitrophenol **87** and 4nitroaniline **88** derivatives (Figure 28).[57,67] As an example, the L-alanyl substrate of 4-nitroaniline **88** is used to differentiate between Gram-negative and Gram-positive bacteria, as L-alanyl aminopeptidase is expressed by all Gram-negative bacteria, but only by a few of the Gram-positives in sufficient quantities for the enzymatic reaction (Scheme 31).[120]

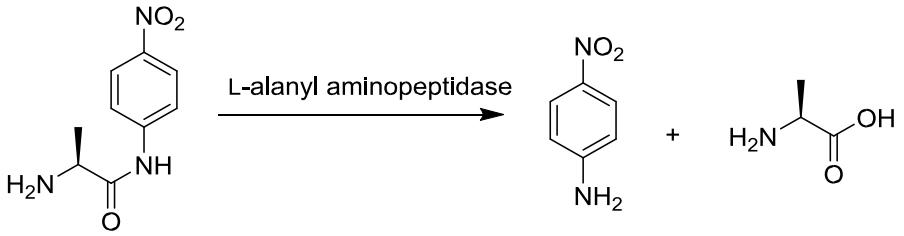


87



88

Figure 28 *Structure of chromogenic substrates with a single aromatic ring, a 4-nitrophenol* ***87*** *and a 4-nitroaniline based substrate* ***88****.*



88a-L **89** yellow **3-L**

Scheme 31 *The action of L-alanyl aminopeptidase on L-N-alanyl-4nitroaniline substrate* ***88a-L*** *to give chromogen 4-nitroanililne* ***89***[120]

Several fused aromatic systems are suitable as chromogens. Popular examples in this category include 1-naphthol **90** and 2-naphthylamine **91**.[57] Indole based systems **92**, **93**, and especially indoxyl substrates, are particularly popular and have been widely applied in bacterial detection (Figure 29). These compounds are precursors of indigoid dyes **95**, which are known for their bright colours. Substrates that incorporate these molecules are hydrolysed by the target enzymes, liberating the corresponding indoxyl compound **94**. This in turn goes through an oxidative dimerization and liberates the colourful indigo derivative **95** (Scheme 32). The colour of these compounds depends on the R_1 and R_2 substituents and can vary in a wide spectrum from blue to red. With the appropriate moiety attached, they can target a variety of enzymes, including esterases, phosphatases, aryl sulfatase, and a range of glycosidases. Consequently, they are used in chromogenic media to detect a wide range of microorganisms. Their low water solubility is their main disadvantage, and they require aerobic conditions for colour development in culture media.[12,108,121,122]

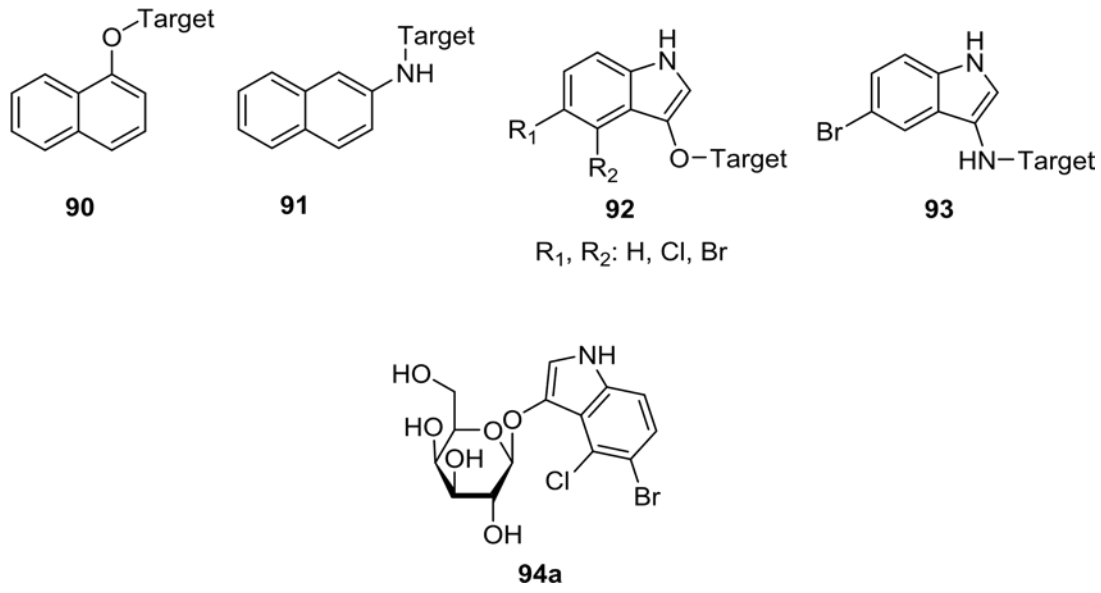
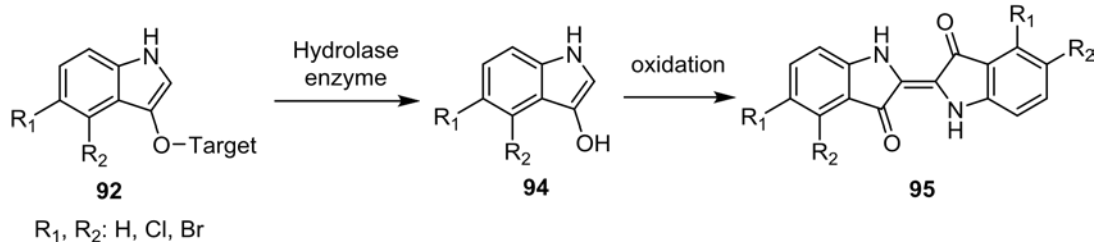
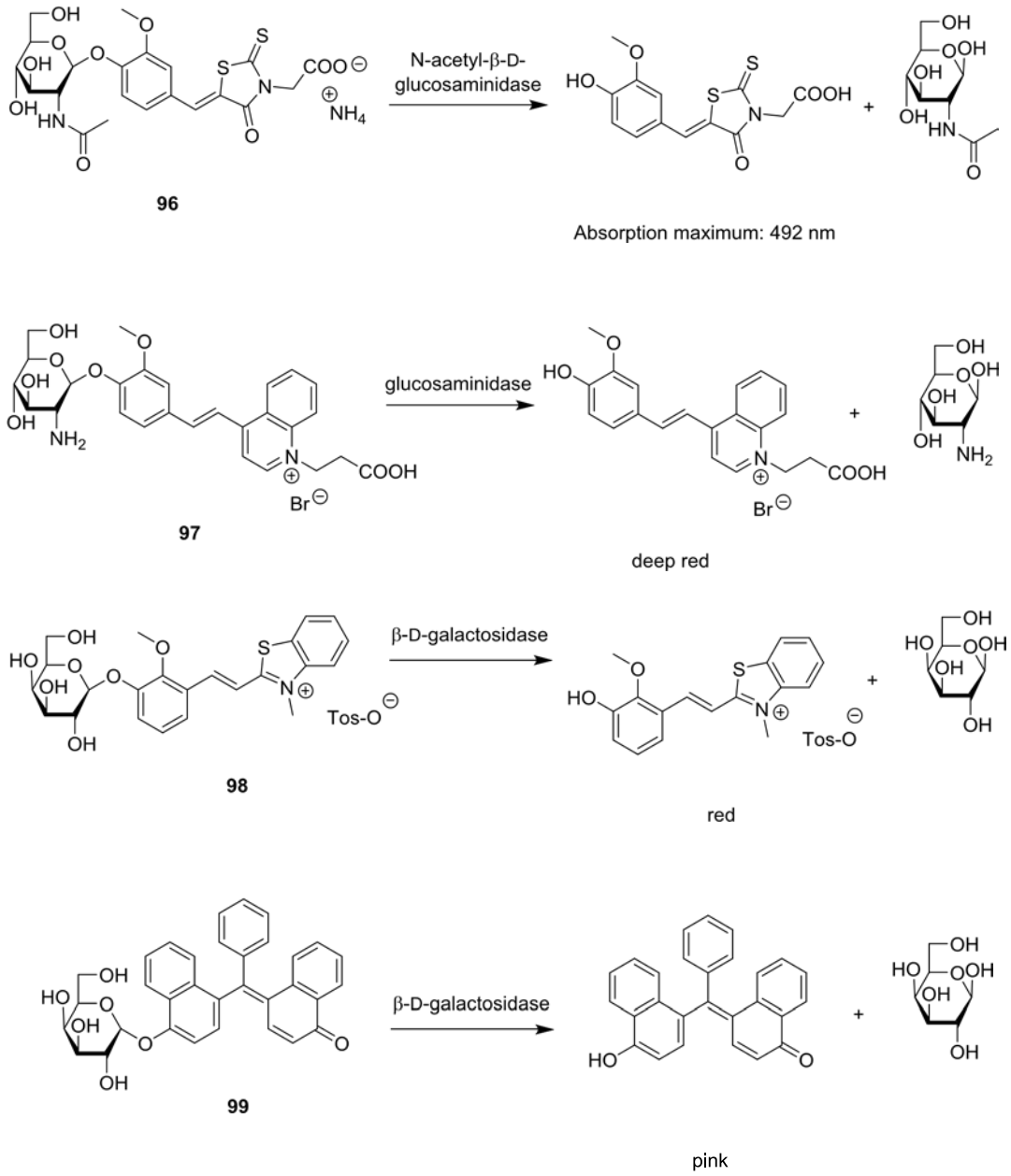


Figure 29 *Structures of chromogenic substrates with fused bicyclic aromatic ring systems, such as napht-1-ol* ***90****, napht-2-yl amine* ***91****, indoxyl* ***92****, 3-aminoindol* ***93*** *based substrates and an example □-D-galactosidase indoxyl substrate 5-bromo-4-chloroindoxyl-□-D-galactoside* ***94a***



Scheme 32 *Action of bacterial hydrolase on indoxyl substrate* ***92****.*

Some substrates have a common styrene core, which is connected with more unsaturated bonds or aromatic rings for greater extended □-electron delocalisation, in order to produce enhanced colour due to the chromogenic unit (Scheme 33). Compound **96** is an *N*-acetyl-□-D-glucosaminidase substrate which is utilised clinically for urine analysis.[123] The glucosaminidase substrate **97** is applied in a chromogenic medium for the identification of *Candida* spp..[124] Compound **98** is used as a □-galactosidase substrate for the detection of *E. coli*.[125] As a final example, *p*-naphtholbenzein-□-D-galactoside **99** is an effective substrate to differentiate species of *Enterobacteriaceae*.[126]



Scheme 33 *Examples of chromogenic substrates with a styrene core rhodanine and styrene motif containing* N-*acetyl-□-D-glucosaminidase substrate* ***96****, conjugated quinolinium and styrene motif containing glucosaminidase substrate* ***97****, benzothiazol and styrene moiety containing □-galactosidase substrate* ***98****, p-naphtolbenzein-□-D-galactoside* ***99****.*

Aminopeptidase substrates with a 9-(4`-aminophenyl)-10-acridinium chromophore scaffold were reported as useful diagnostic tools, e.g. the Lalanyl derivatives **100** and **101** were successfully applied for differentiation between Gram-negative and Gram-positive bacteria (Figure 30), (Scheme 34).[127,128]

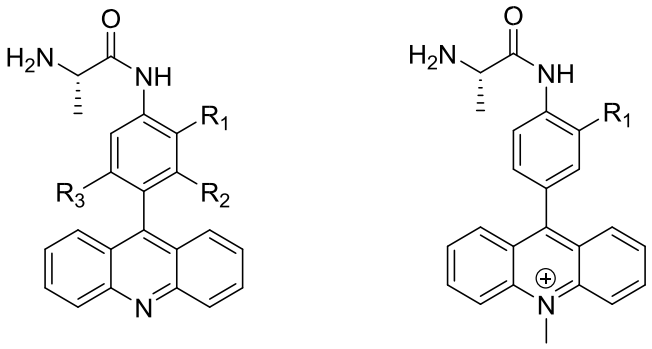
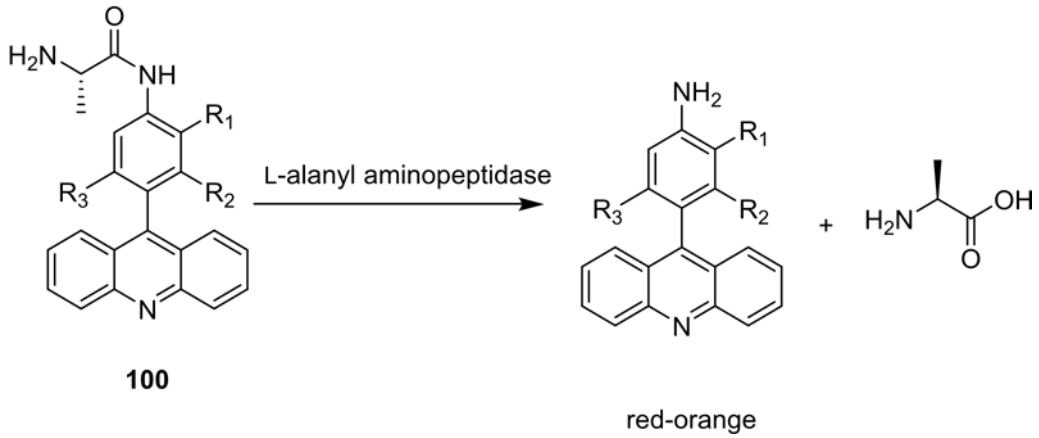
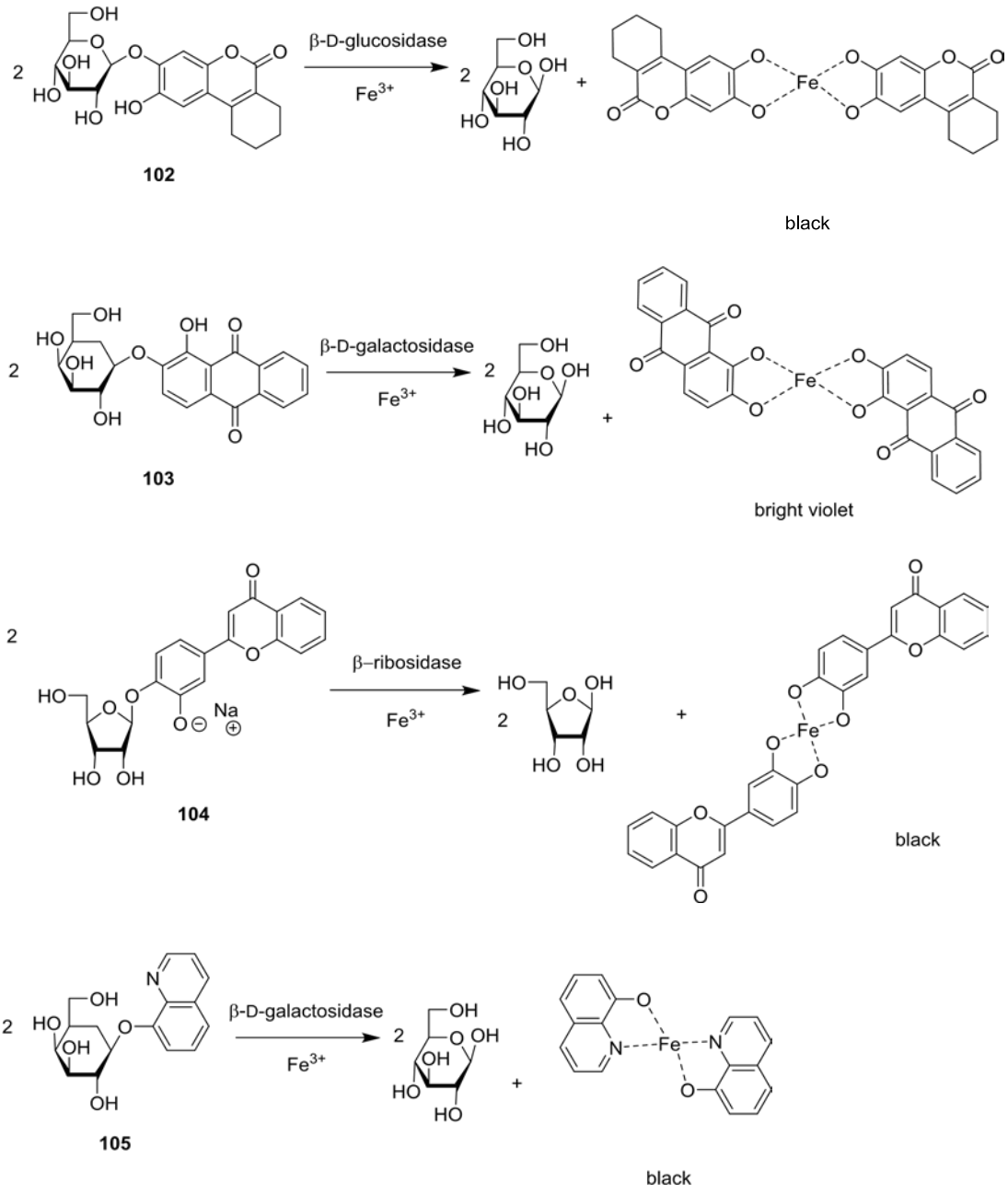


Figure 29 *Structure of chromogenic substrates with a 9-(4`-aminophenyl)-10-acridine scaffold. Examples are L-alanyl-9-(4`-aminophenyl)-10-acridine* ***100*** *and L-alanyl-9-(4`-aminophenyl)-10-methyl-acridinium salt* ***101.***



Scheme 34 *Action of L-alanyl aminopeptidase on chromogenic substrate Lalanyl-9-(4`-aminophenyl)-10-acridine* ***100*** *to give the chromogen 9-(4`aminophenyl)-10-acridine derivative.*

Certain chromogenic substrates form dark coloured iron chelates in the presence of target hydrolases. Such a chromogen is provided by cyclohexenoesculetin, which is used as the signalling unit found within some □-galactosidase[129] and □-D-glucosidase substrates **102**.[130] Alizarin-□-Dgalactoside is another useful □-galactosidase substrate **103**.[115] A □-ribosidase substrate **104** based on dihydroxyflavone has been used for differentiation of Gram-negative bacteria.[121] As a final example, 8-hydroxyquinoline has been used as a chromogen for detection of □-galactosidase **105** and □-glucuronidase (Scheme 35).[129,131]



Scheme 35 *Action of various glycosidases on different chelating chromogenic substrates with selected examples cyclohexenoesculetin □-Dglucosidase* ***102****, alizarin-□-D-galactoside* ***103****, a flavon based □-riboside* ***104****, 8-hydroxyquinoline-□-D-galactoside* ***105****.*

The □-alanyl aminopeptidase substrate, 7-amino-1-pentylphenoxazin-3-one **86a**, developed at the University of Sunderland, is now applied within the commercially available chromID® *P. aeruginosa* chromogenic medium (Figure

32), which is used to differentiate *P. aeruginos*a from other common bacteria within clinical samples.[12,132-134] *P. aeruginosa* forms dark purple colonies with a metallic sheen on the orange background of the medium (Scheme 36).

While **86a** works well in practice, there is always scope for improvement. In order to enhance the colour of the chromogen, decrease the background colour of the substrate, and provide comparable substrates that would be more economical to produce, a series of similar phenoxazin-3-one derivatives **106**, **107** were subsequently designed and synthesised. However, none of the newer substrates provided sufficient improvement upon biological testing to justify application in clinical practice (Figure 31).[132,135] This collection of substrates provides a starting point for this chapter of this thesis.

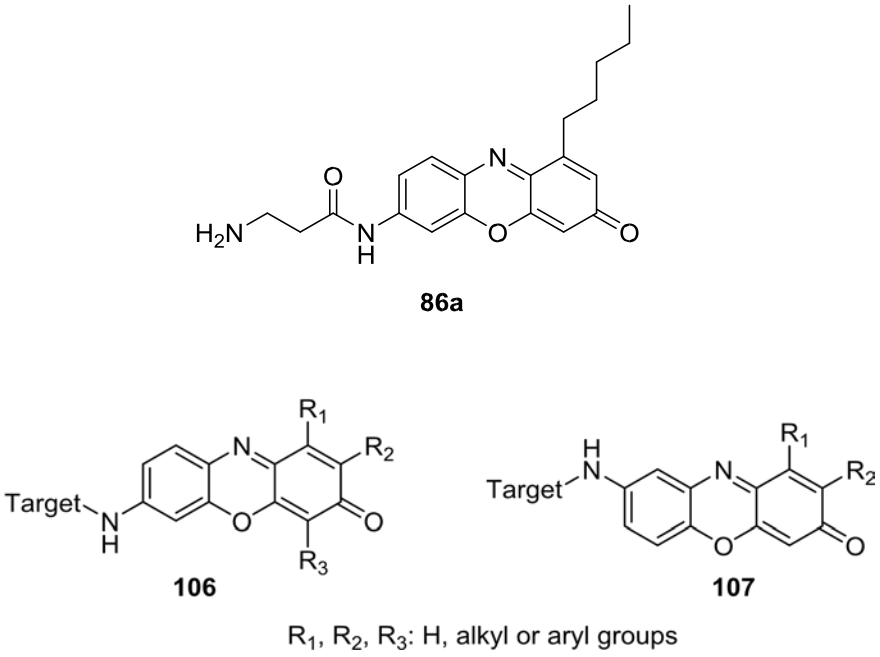
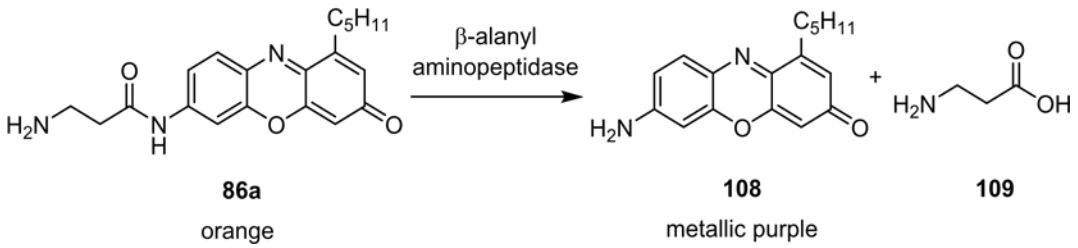


Figure 30 *Structure of different chromogenic substrates with phenoxazin-3one scaffold.*



Scheme 36 *Action of β-alanyl aminopeptidase on 7-N-(β-alanyl)amido-1pentylphenoxazin-3-one* ***86a***[27] *to give 7-amino-1-pentylphenoxazin-3-one chromogen.*

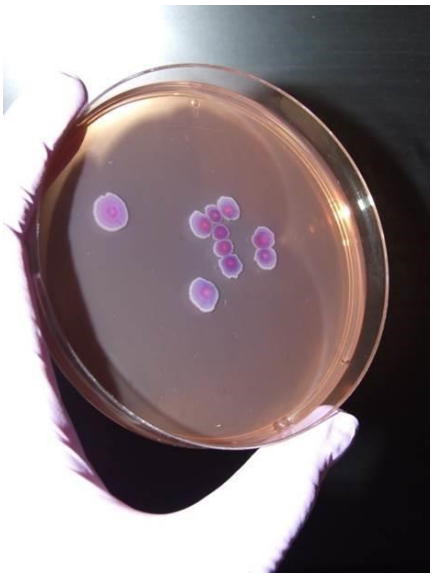
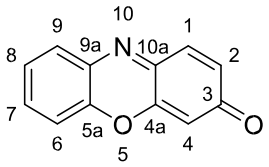


Figure 31 *A plate of chromID® P. aeruginosa chromogenic medium and P. aeruginosa colonies*[27-29]

3.1.3 Natural molecules with a phenoxazin-3-one scaffold

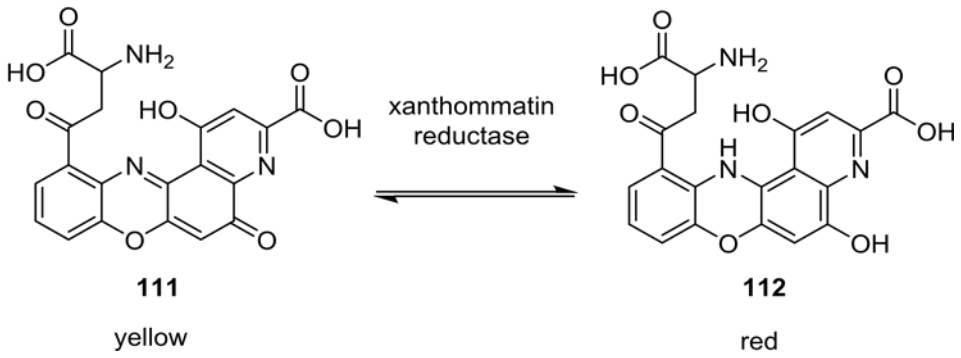
The phenoxazin-3-one core **110** occurs within several natural molecules (Figure 33), such as ommochromes, various fungal metabolites and actinomycins.[82,136]



113

Figure 32 *The phenoxazin-3-one core* ***110***

Ommochromes are natural pigments and have been isolated from the eyes of different arthropod species. For example, xanthommatin **111** is a yellow pigment, which can be reduced by a reductase enzyme to a red form **112**. These 2 forms are responsible for the eye coloration of *Drosophila melanogaster* (Scheme 37).[137]



Scheme 37 *Xanthommantin* ***111*** *and its enzymatic reduction to 5,12dihydroxanthommantin*

Some pigments, such as cinnabarin **113**, are also now known to display the same core scaffold and have been isolated from different fungi. Cinnabarin **113** was found in, amongst others, *Polystictus sanguineus* (Figure 34).[138] It has remarkable antibacterial activity against *Bacillus cereus* and *Leuconostoc plantarum* (MIC: 62.5 mg/L).[139]

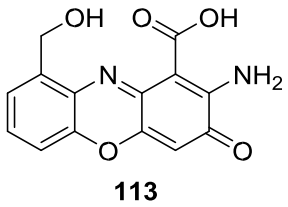
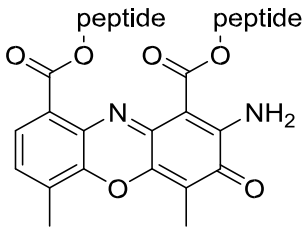


Figure 33 *The structure of cinnabarin* ***113***

The most studied group of natural phenoxazinon-3-one based molecules are the actinomycins **114**, which are produced by *Streptomyces* species (Figure 35). Actinomycins **114** have antibacterial activity against a range of Gramnegative and Gram-positive bacteria. Synthetic and natural actinomycins also have anticancer properties, due to their good binding ability with DNA, actinomycin D is employed clinically for the treatment of several cancer types. Thus, since compounds bearing this core scaffold are known to display good penetration into the target bacteria, it is logical that they should also be explored as the basis of chromogenic scaffolds for bacterial detection and identification. These are red or orange coloured molecules due to their chromogenic phenoxazinone scaffold.[140]

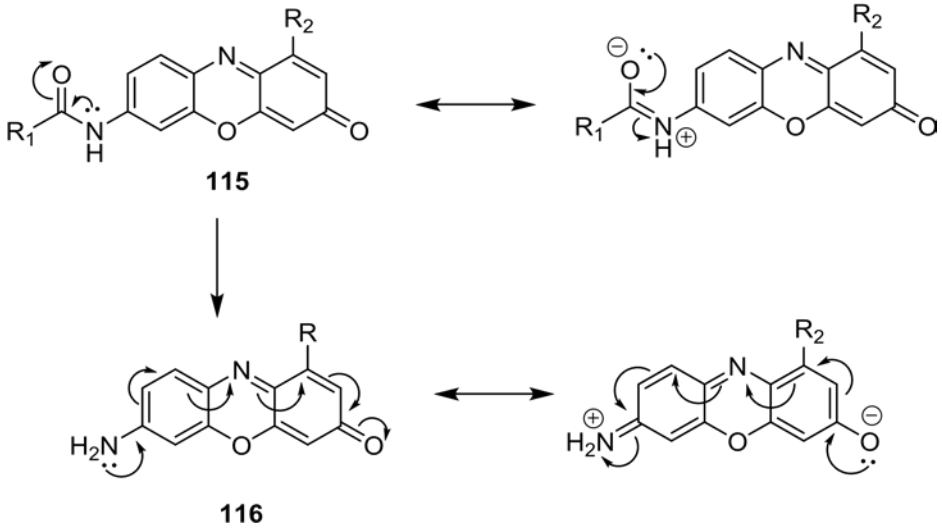


114

Figure 34 *General structure of actinomycins* ***114***

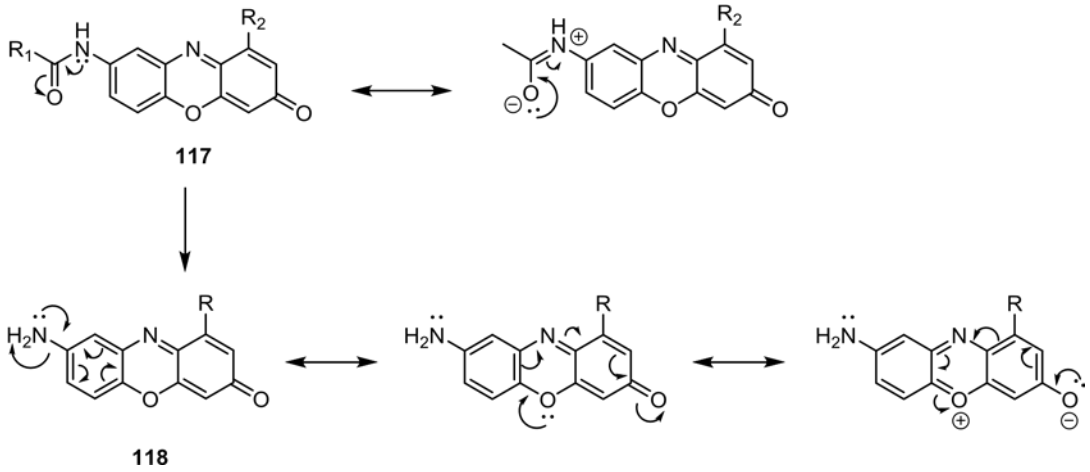
3.1.4 Background behind the chromogenic properties of 7 and 8-amidophenoxazine-3-one substrates

7-Aminophenoxazine-3-one substrates **115**, such as the above mentioned 7*N*-(β-alanyl)amino-1-pentylphenoxazin-3-one **86a**, liberate strongly coloured (purple **108**) chromogens **116** in the presence of the appropriate bacterial enzyme (□-alanyl aminopeptidase). The amino group of a 7-aminophenoxazine-3-one **116** can delocalise its electron pair through the whole molecule core resulting strong colouration. In substrates **115** the lone pair of the nitrogen is delocalised in the amide bond, preventing it from being able to participate in the electron system of the phenoxazin-3-one part. This phenomenon causes the limited colour of the substrate (light orange **86a**) (Scheme 38).



Scheme 38 *Action of 7-amino-phenoxazinone-3-one substrates* ***115***

The amino group of 8-aminophenoxazinone-3-one **118** contrasts with the 7aminophenoxazine-3-one **116** in that it cannot delocalise its lone pair throughout the core of the whole molecule. Instead, it only has a strong electron donating effect on its individual ring. As a consequence, 8-amino chromogens **116** exhibit less intense colouration than the corresponding 7amino chromogens **118**. Furthermore, the 8-amino substrates **117** are expected to have a smaller colour contrast to their chromogens **118**, than the equivalent 7-amino substrates **115** (Scheme 39).



Scheme 39 *Action of 8-amino-phenoxazinone-3-one substrates* ***117***

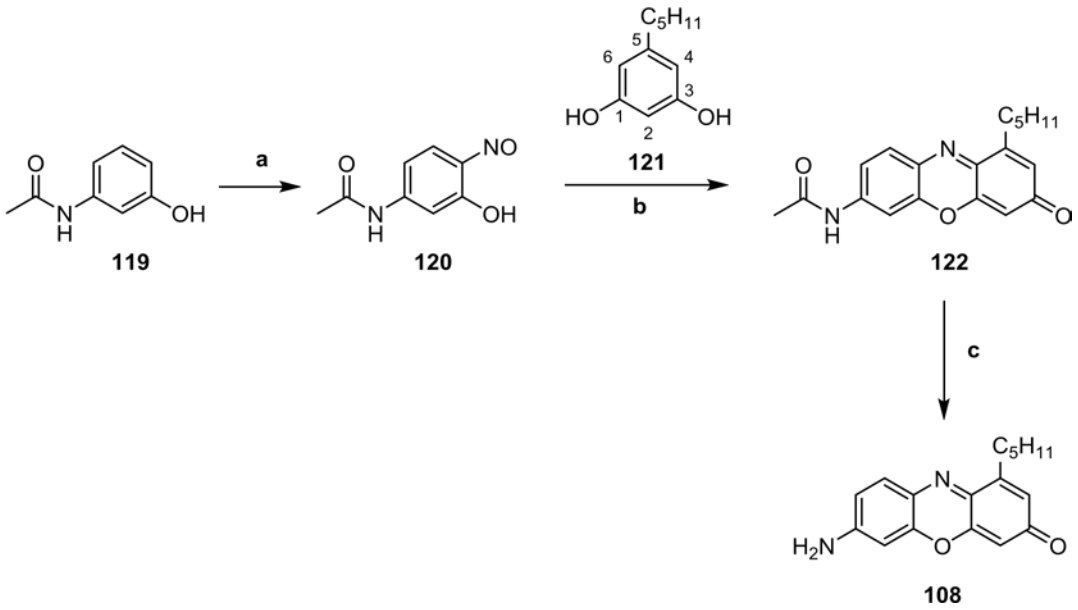
3.1.5 Synthesis of enzymatic substrates with a phenoxazinone scaffold

As well as the natural phenoxazinone based molecules, a number of synthetic derivatives have also been reported. The synthetic compounds were either made by derivatisation of the natural compounds, or by condensation of unicyclic aromatic rings furnished with appropriately reactive functional groups. Reactions, such as Schiff base formation, water elimination, nucleophilic aromatic substitution, or oxidative multistep condensation reactions, have been used to build up the phenoxazin-3-one core in a variety of molecules.[82] Due to the specific focus of this project, only the synthesis of the phenoxazin-3-one based hydrolase targeting chromogenic substrates are

discussed further, with the examples chosen to illustrate the most common synthetic approaches deployed.

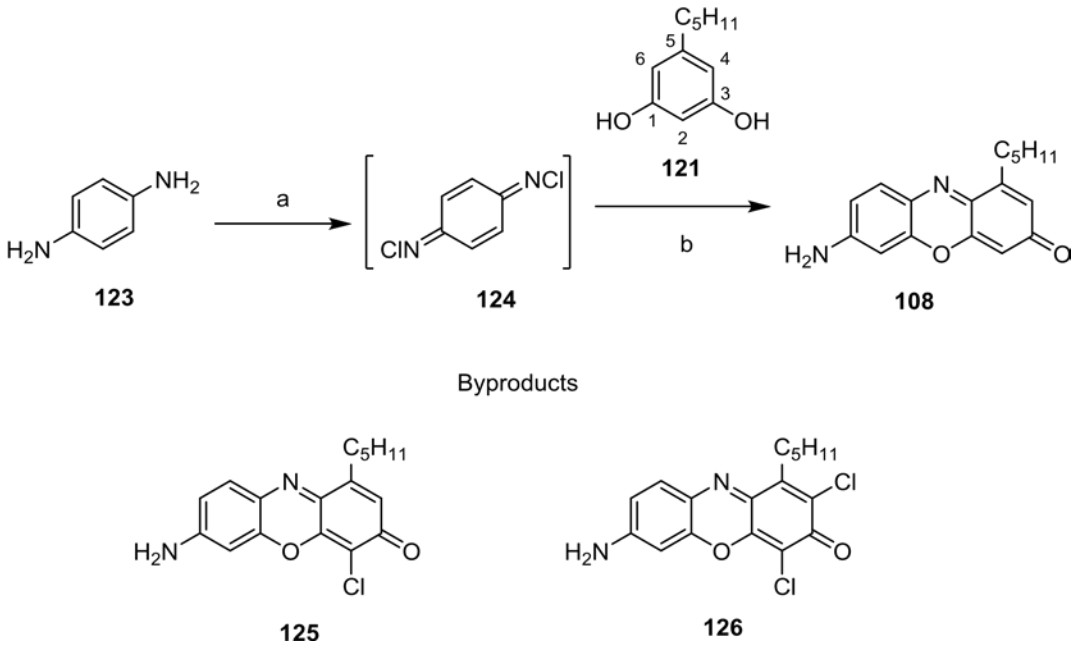
7-Amino-1-pentylphenoxazin-3-one **86a** was the first reported member of the phenoxazin-3-one chromogenic substrate series.[28] Moreover, this molecule has seen the most significant use in clinical applications. To date, there are 3 reported preparation procedures for this key molecule.[28,141]

In the earliest reported approach,[141] 3-acetamidophenol **119** was nitrosylated, then the product **120** was reacted with olivetol **121** in a mixture of butanol and concentrated sulfuric acid. The latter condensation is a multistep reaction. These individual steps are the nucleophilic aromatic substitution of the phenolic group of compound **120** by the nucleophilic 1-phenolic moiety of olivetol **121**, and an electrophilic aromatic substitution of the 6-hydrogen of olivetol unit by the electrophilic nitroso motif. The chromogen phenoxazin-3one **108** was gained after an acidic hydrolysis of the amide within **122**. Although this method contains only 3 steps, its overall poor yield and the high price of the olivetol **121** makes this synthesis unsuitable for industrial production (Scheme 40).



Scheme 40 *Synthetic method I for 7-amino-1-pentylphenoxazin-3-one* ***108****;*[141] *Reagents and conditions: (a) $NaNO_2$, H_2SO_4; (b) nBuOH, c. H_2SO_4; (c) 85% H_2SO_4, ethanol.*

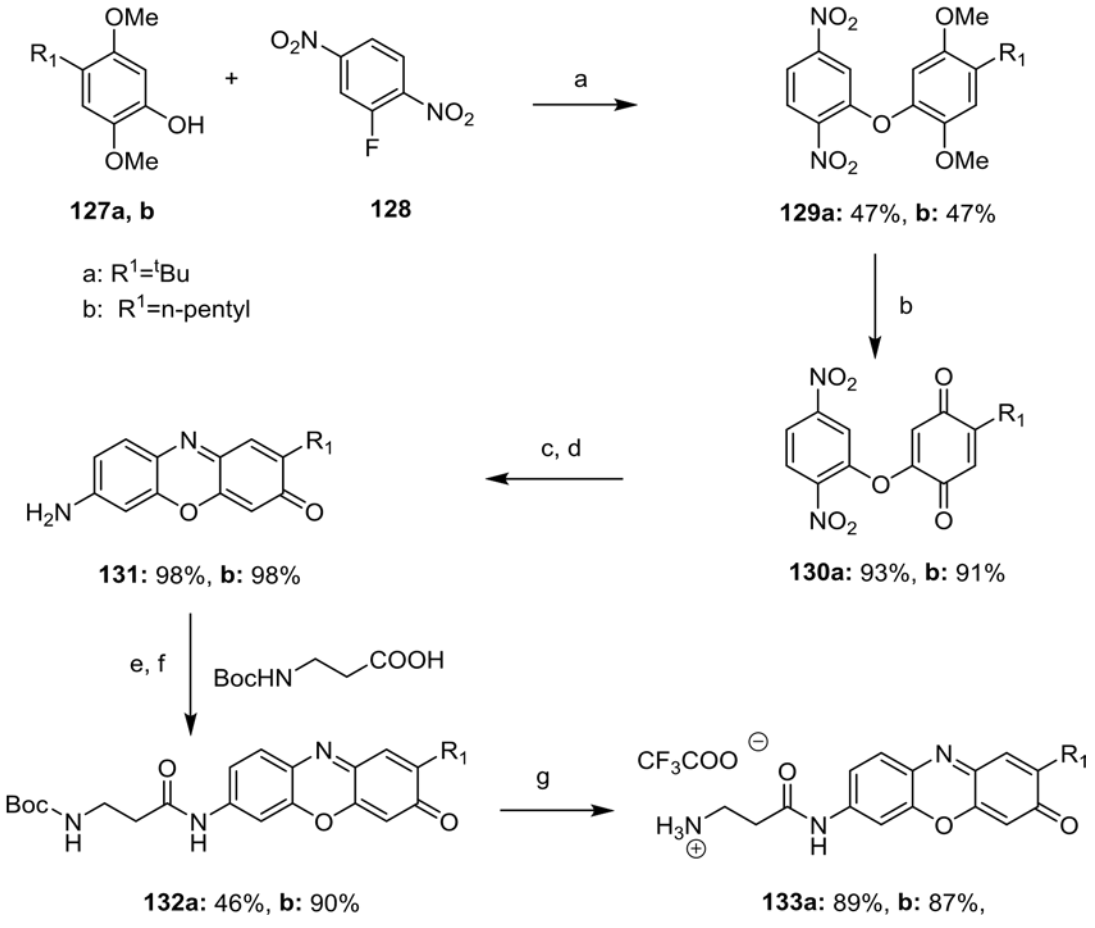
Phenoxazin-3-one **108** was also prepared by an oxidative chlorination method, in which 1,4-dichlorobenzoquinonediimine **124,**[142] prepared *in situ* from 1,4-dimethylaminobenzene **123**, was reacted with olivetol **121**. This onepot synthesis results in formation of the desired chromogen **108** with poor yield. In addition, this method also gave two chlorinated phenoxazine-3-one byproducts **125** and **126**, with a difficult purification of the resorufamine **108** from the final reaction mixture (Scheme 41). [141]



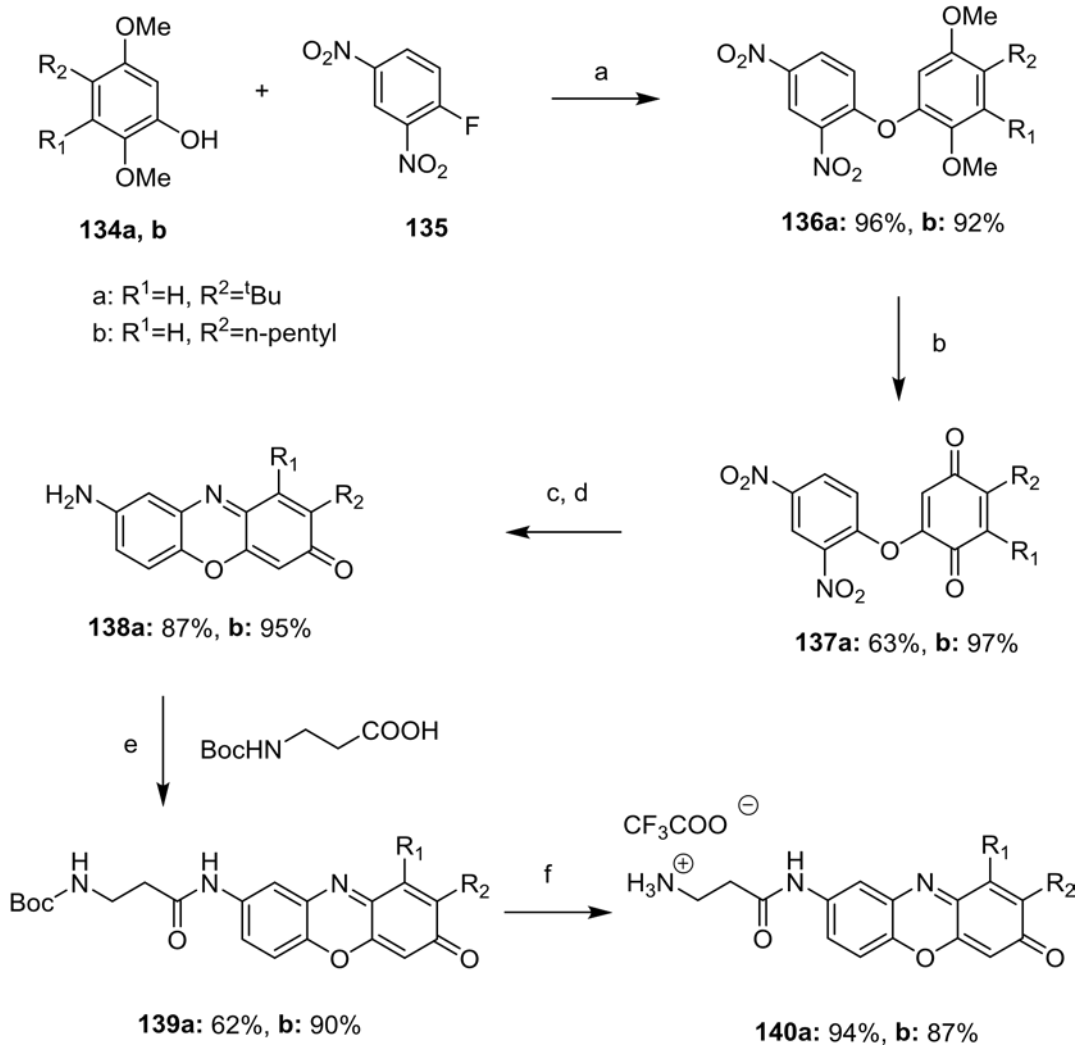
Scheme 41 *Synthetic method II for 7-amino-1-pentylphenoxazin-3-one* ***108***;[141] *Reagents and conditions: (a) Cl_2, $CO(NH_2)_2$, methanol; (b) reflux.*

Our research group has been targeting phenoxazin-3-one based chromogenic substrates for several years. A common synthetic strategy was developed for both 7- and 8-aminophenoxazin-3-one derivatives **133** and **140**, starting with the nucleophilic aromatic substitution of dinitrofluorobenzenes **128** and **135** with the appropriately substituted dimethoxyphenol derivatives **127** and **134**. This step was carried out in two different ways depending on the target molecule. If the target was a 7-aminophenoxazin-3-one **133**, the phenolate form was required for the nucleophilic substitution, which was prepared from the corresponding phenol using NaH as a base. If 8-aminophenoxazin-3-one **140** was targeted, the reaction required less basic conditions, due to the more electrophilic dinitrofluorobenzene **135**. The role of triethylamine was more

important for trapping the liberated hydrofluoric acid rather than the deprotonation of the phenol. The methyl ether moieties of the diphenylether product **129** and **136** were removed in an oxidative demethylation step by cerium(IV) ammonium nitrate. Following reduction of the nitro groups, immediate spontaneous oxidative cyclisation led to the phenoxazin-3-one chromogen **131** and **138.** The Boc-protected □-alanine was coupled to the chromogen by a solution phase peptide synthetic approach using IBCF as the activating agent and *N*-methylpiperidine as a base. Deprotection of the Bocgroup using TFA comprised the final step of the synthesis, producing the required substrate as the corresponding trifluoroacetate salt (Scheme 42, 43).[132,135]



Scheme 42 : *Synthetic method for 7-N-(□-alanyl)-aminophenoxazin-3-one* ***133a,b****;[28] Reagents and conditions: (a) NaH, DMF, RT; (b) cerium(IV) ammonium nitrate, CH_3CN, H_2O, RT; (c) H_2, Pd/C 5%, EtOAc/MeOH; (d) Ag_2O, MeOH; (e) H_2, Pd/C 5%, DMF; (f) IBCF, dry THF, N-methylpiperidine, 0 °C; (g) TFA, RT.*



Scheme 43 : *Synthetic method for 8-N-(□-alanyl)-aminophenoxazin-3-one* ***140a,b****;[28] Reagents and conditions: (a) DMSO, Et_3N, RT; (b) cerium(IV) ammonium nitrate, CH_3CN, H_2O, RT; (c) H_2, Pd/C 5%, EtOAc/MeOH; (d) Ag_2O, MeOH; (e) IBCF, dry THF, N-methylpiperidine, 0 °C; (f) TFA, RT.*

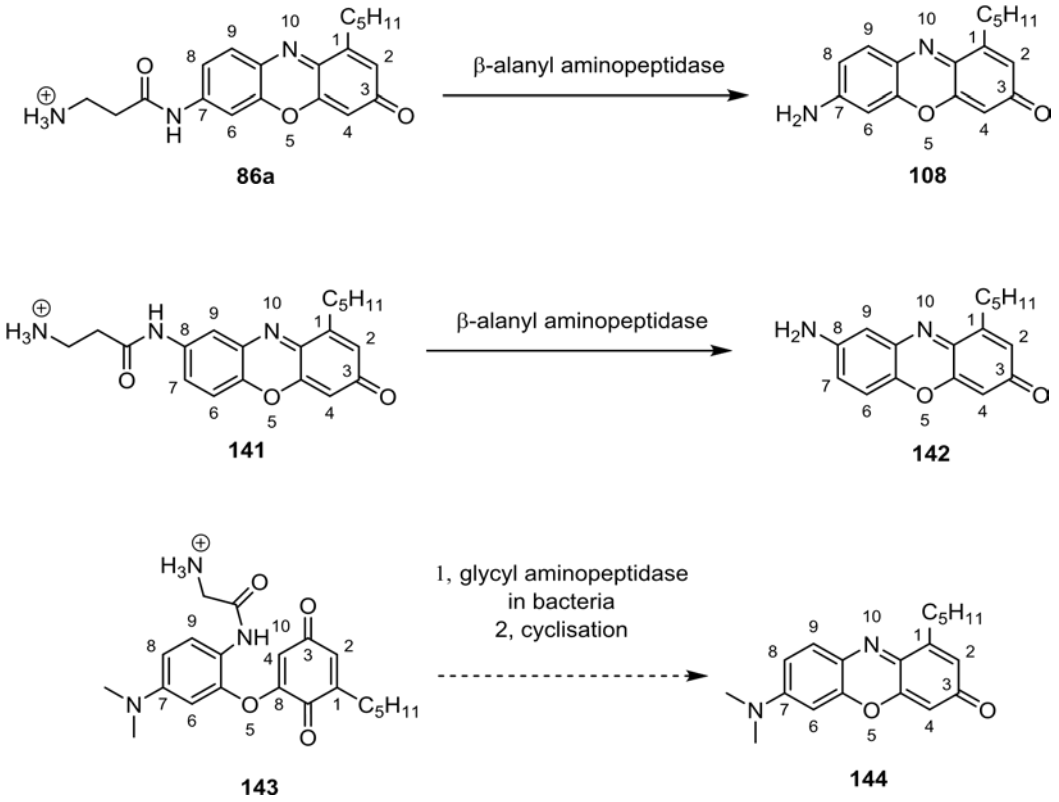
3.2 Results and discussion

3.2.1 Research aims

The research described here aimed to synthesize, characterize and microbiologically evaluate certain chromogenic substrates with phenoxazinones providing the basis of the chromogen scaffold (Scheme 44). A study of the candidates would extend and complete a decade of research work done in this research group. The main goal was to improve a □-alanyl aminopeptidase substrate, 7-amino-1-pentylphenoxazin-3-one **86a** applied in chromID® *P. aeruginosa* chromogenic medium, by chemical modifications

focusing on decreasing the background colour provided by the substrate, thus enabling greater contrast and clarity for the end user in the clinic.[12,132-134]

A novel chromogenic substrate **143** with a possibly liberating phenoxazin-3one chromogen was planned to be investigated as glycyl peptide to explore its properties as a less specific substrate. This substrate was expected to have decreased background colour, as the two rings are not connected through a conjugated electron system, in contrast to the envisaged chromogen liberated from it.



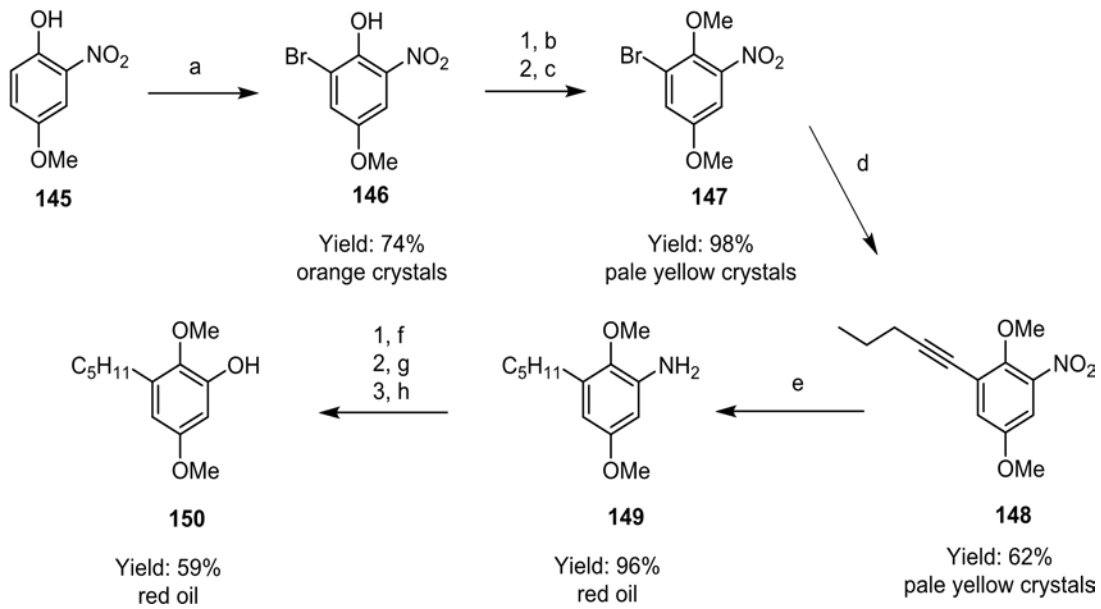
86a 108 141 142 143 144

Scheme 44 Reference **86a** and planned chromogenic substrates **141**, **143** and their action in bacteria

3.2.2 Synthesis of chromogenic substrates

In order to synthesise substrates **141** and **143**, a feasible route was developed for production of a common intermediate, 2,5-dimethoxy-3-pentylphenol **150**. The whole synthesis started with the bromination of 2-nitro-4-methoxyphenol **145** with bromine in glacial acetic acid. [143]

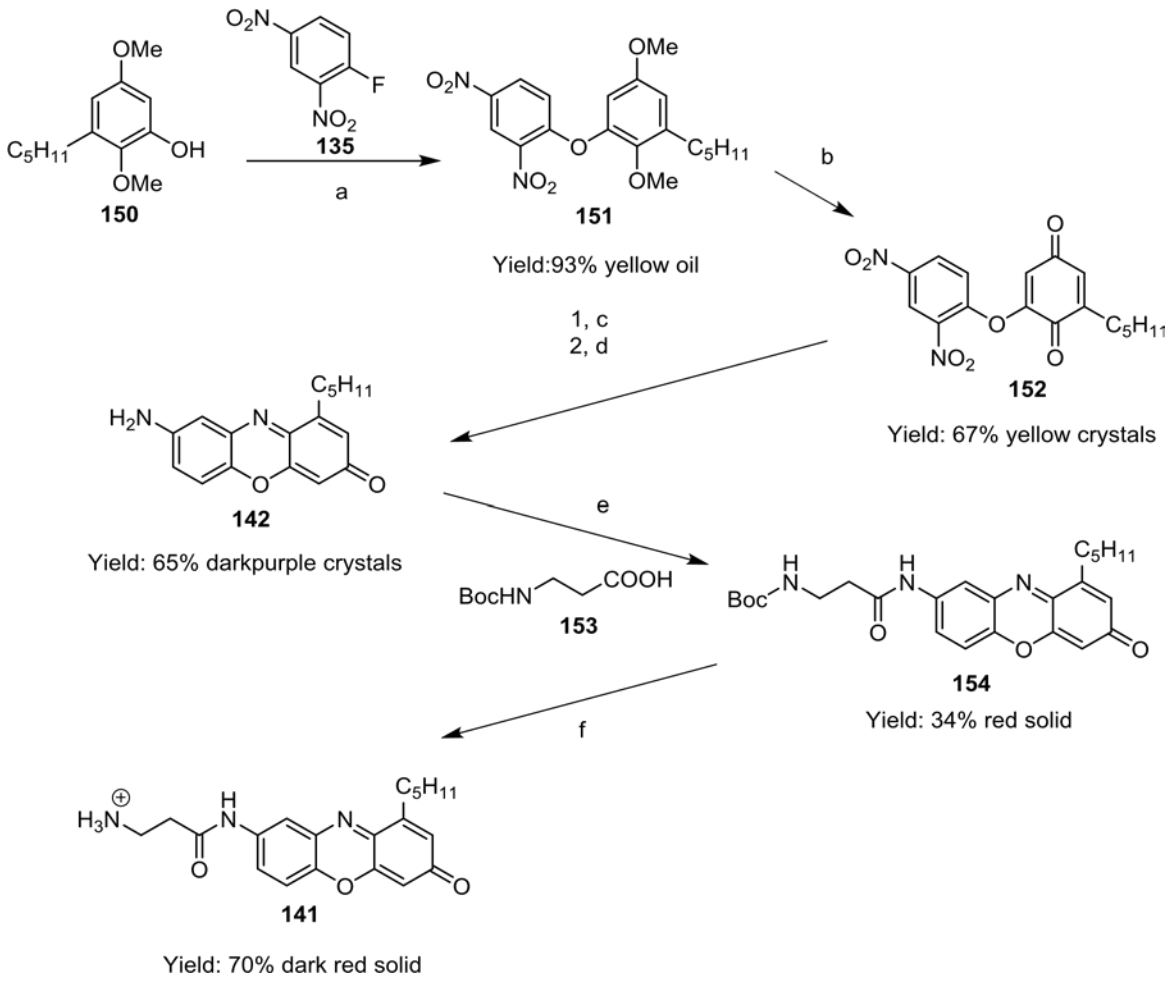
The phenolic group of the product **146** was methylated with iodomethane in DMF, after the phenolic moiety was activated as a cesium salt in order to be a better nucleophile. Later, molecule **147** was reacted with pent-1-yne in a Sonogashira-type cross coupling reaction. The subsequent reduction of the nitro moiety by catalytic hydrogenation resulted in 2,5-dimethoxy-3pentylaniline **149**. The amino group was transformed to a phenolic hydroxyl motif by a Sandmeyer reaction (Scheme 45). The total synthesis was carried out on a 10 g scale in 5 steps with an overall 25% yield. Moreover, only the last 3 steps required chromatographic purification. These factors suggest that these materials could be produced economically on a relatively large scale if the results of the biological testing showed improvement over existing technologies.



Scheme 45 *Synthesis of 2,5-dimethoxy-3-pentylphenol intermediate* ***150****.*

Reagents and conditions: (a) Br_2, KBr, glacial acetic acid, water; (b) Cs_2CO_3, DMF; (c) Me_2SO_4, DMF; (d) pent-1-yne, $Pd(PPh_3)_2Cl_2$, Cu(I)I, Et_3N, DMF, N_2; (e) $H_{2\,(g)}$, 5% Pd/C, EtOH, 4 bar; (f) $NaNO_2$, 5M H_2SO_4; (g) urea; (h) 5M H_2SO_4, 90 °C.

8-β-Alanylamido-1-pentyl phenoxazinone **141** was synthesised following a published synthetic route.[132,135] 2,4-Dinitro-fluorobenzene **135** was reacted with 2,5-dimethoxy-3-pentylphenol **150** in a nucleophilic aromatic substitution reaction to give the diphenyl ether compound **151**. The methoxy-groups were demethylated using cerium ammonium nitrate (CAN), in which the oxidative properties of CAN produced the quinone **152**. The chromogen **142** was prepared by the catalytic hydrogenation of the nitro-groups followed by immediate selective re-oxidation by silver(I) oxide and a spontaneous cyclisation step. The Boc-protected □-alanine was coupled to the chromogen **142** by a solution phase peptide synthesis approach using IBCF as the activating agent and *N*-methylmorpholine as a base. Deprotection of the Bocgroup using TFA gave the final substrate as a trifluoroacetate salt **141** (Scheme 46).



Scheme 46 *Synthesis of substrate* ***141****. Reagents and conditions: (a) Et_3N, DMSO; (b) CAN, acetonitrile; (c) $H_{2\,(g)}$, 5% Pd/C, MeOH, ethyl acetate, 4 bar; (d) Ag_2O; (e) IBCF, NMM, THF; (f) TFA.*

Here, the ^{1}H-NMR spectra of 8-amino-1-pentylphenoxazine-3-one **142** chromogen is compared with the corresponding □-alanyl-8-amido-1pentylphenoxazine-3-one **141** chromogenic substrate. The spectra displayed in Figures 37 and 38 were made from solutions with different solvents, so the different solvent effects are not included in the comparison. However, the effect of the lone pair of the 8-amino group is clearly seen through the shift to lower ppm of the ortho hydrogens (H-7: 6.88 ppm, H-9: 7.08 ppm) of chromogen **142**, when compared to the substrate. In the substrate **141** these hydrogens are deshielded (H-7: 7.70 ppm, H-9: 8.15 ppm) due to the occupation of the lone electron pair in the amide bond. The 6-H hydrogen in the meta position has similar chemical shift in both molecules, as that position

is less effected by the +M effect by the nitrogen lone pair. Unchanged signals of H-2 and H-4 shows the lack of the total delocalisation of the amino group of the 8-amino-chromogen **142**, into the centre and right and rings. This is in keeping with the predictions made in chapter 3.1.4 (Figure 36 and 37).

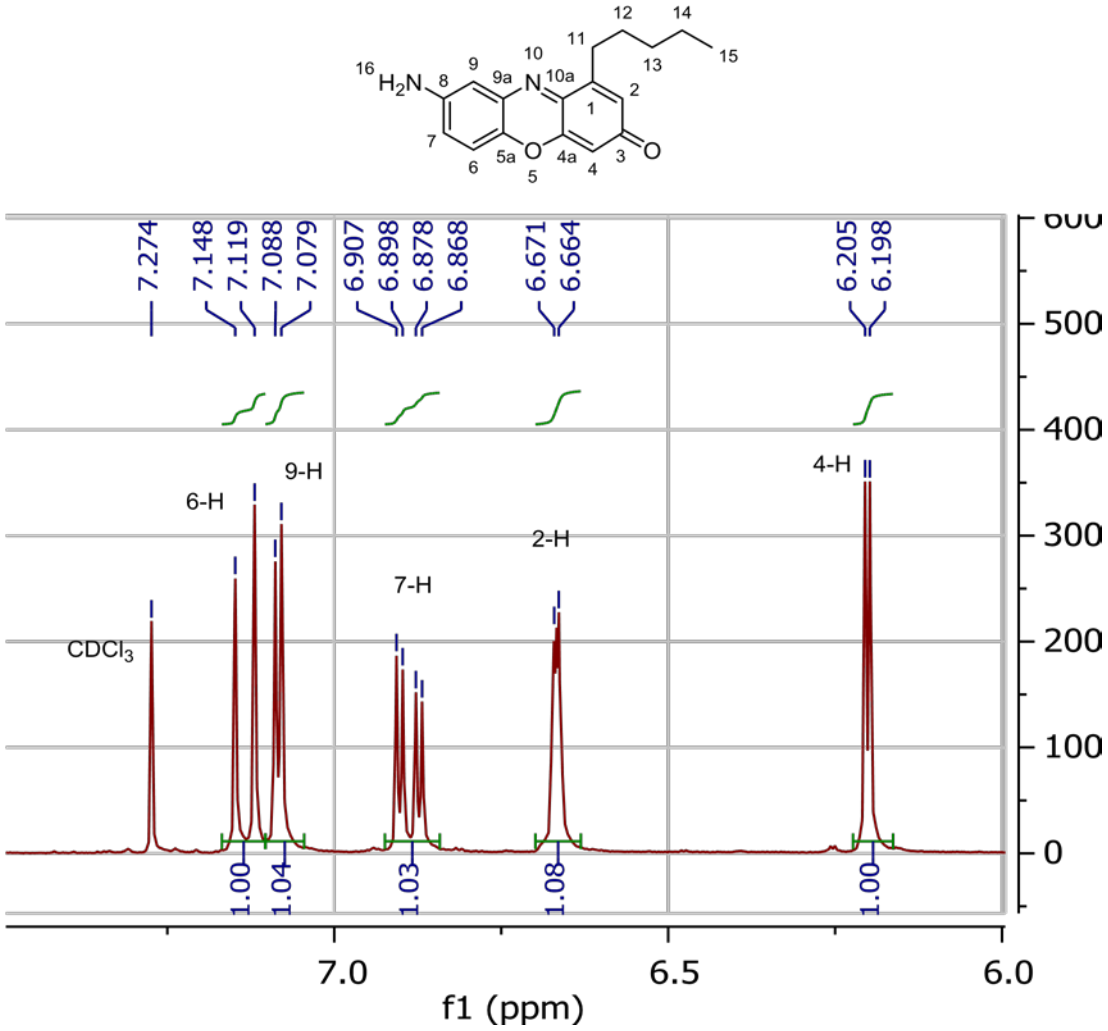


Figure 35 *Aromatic region of ^{1}H-NMR spectra of 8-amino-1pentylphenoxazine-3-one* ***142*** *in $CDCl_3$*

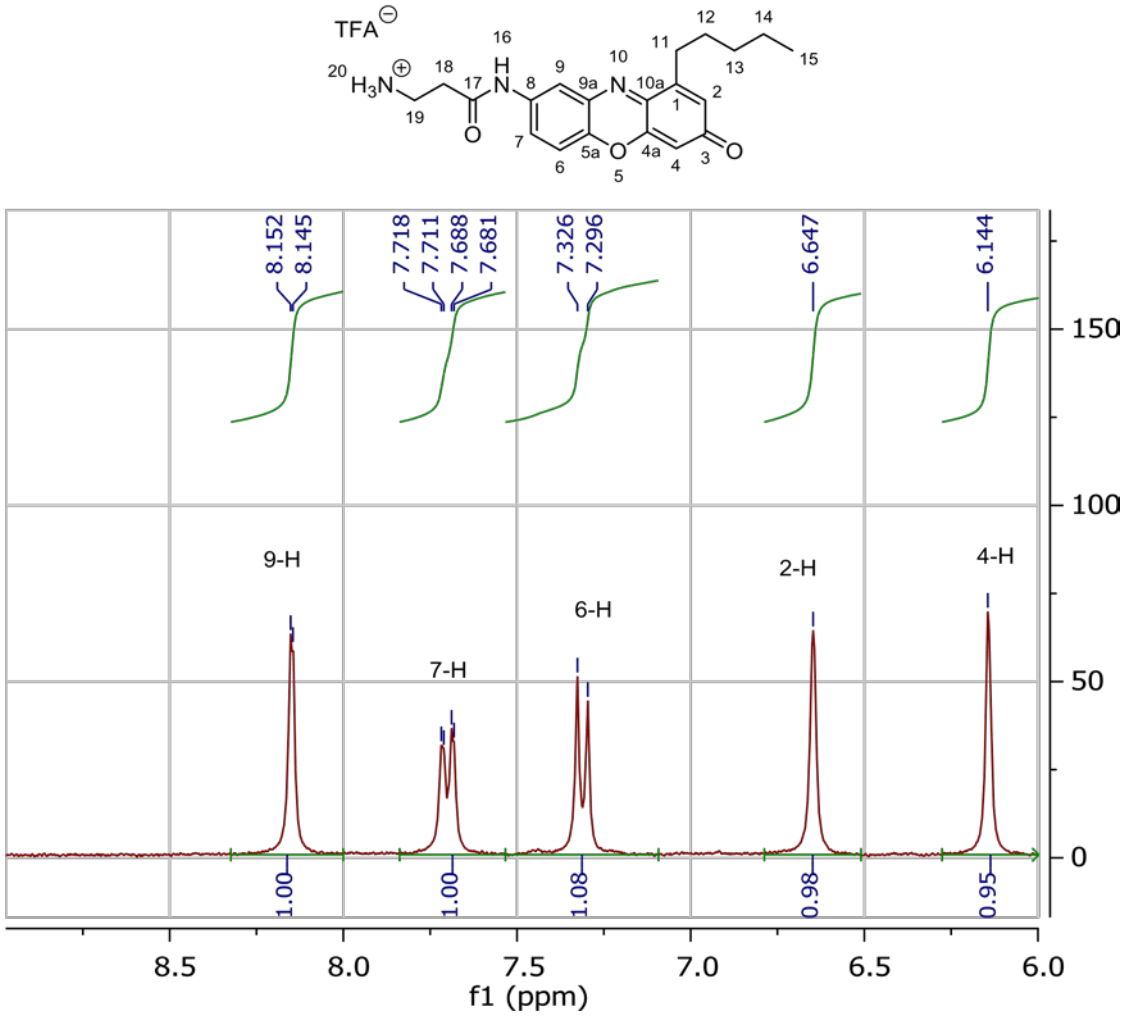
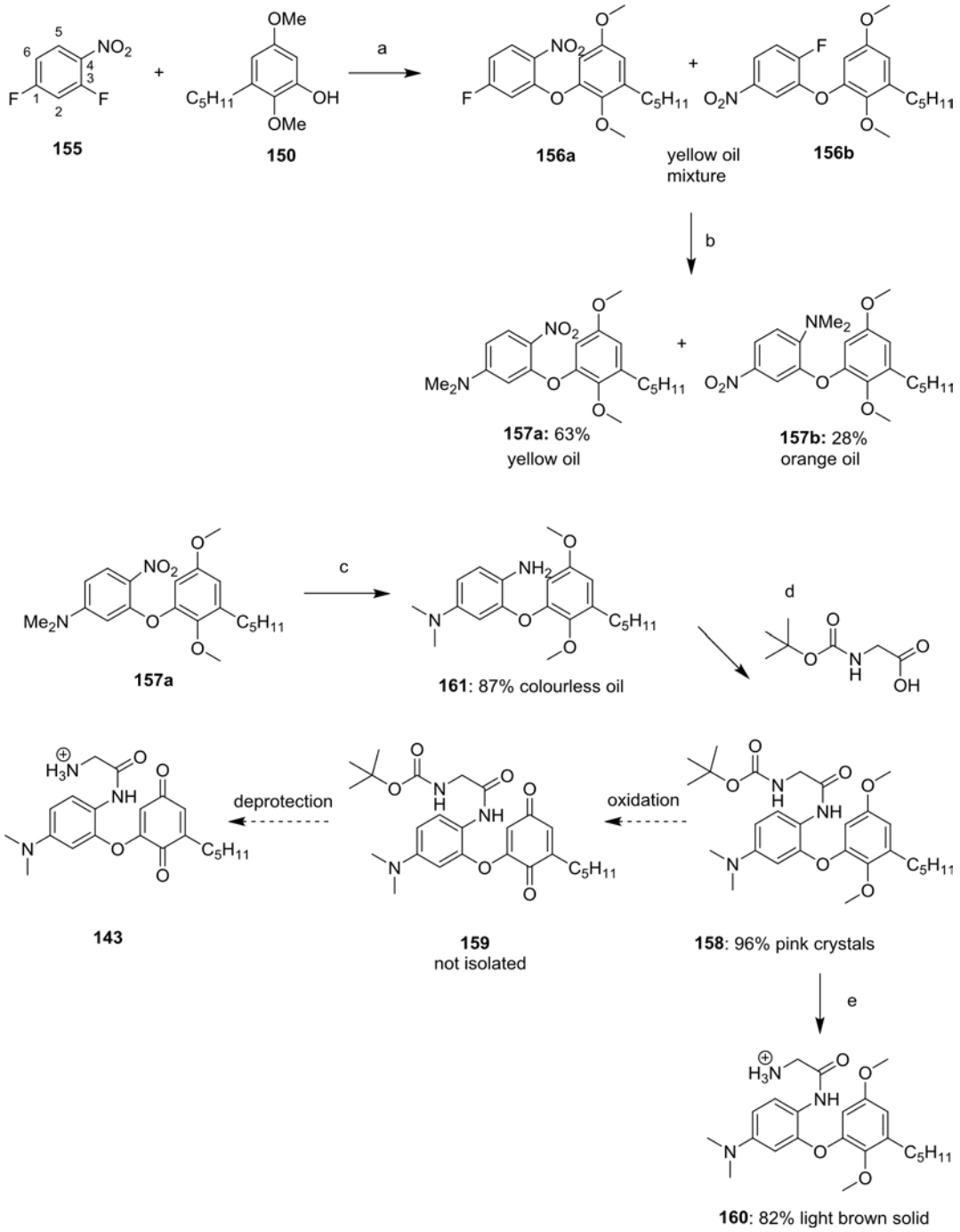


Figure 37 *Aromatic region of ^{1}H-NMR spectra of □-alanyl-8-amido-1pentylphenoxazine-3-one* ***141*** *in D_2O*

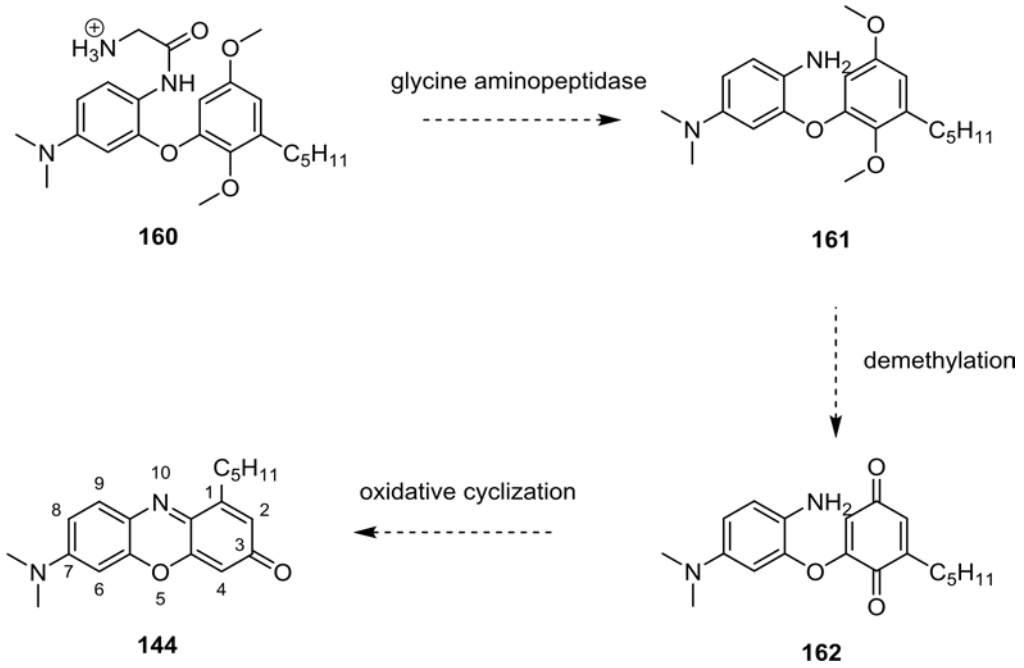
A novel of phenoxazin-3-one liberating substrate **143** was designed. The novelty of this compound lay in the requirement for two separate and distinct enzymatic reactions to occur in the bacteria, which would enhance the selectivity of this potential substrate. Moreover, as the substrate does not in and of itself contain a fused aromatic ring system, the background signal from this type of substrate should be negligible in comparison to substrates that already contain intact phenoxazinone cores. As it is a novel chromogenic substrate, a glycyl linker was chosen instead of □-alanyl to investigate its possibilities against a wider range of bacteria, as □-alanyl aminopeptidase is

a very specific enzyme, glycine may fit in many aminopeptidase pockets, and the substrate is expected to be cleaved internally in most bacteria.

The synthesis began with a nucleophilic aromatic substitution reaction between 1,3-difluoro-4-nitrobenzene **155** and 2,5-dimethoxy-3-pentylphenol **150**. The 2 products **156a**, **156b** were reacted with dimethyl amine without separation, as the dimethyl amino isomers **157a**, **157b** were found to be easier to separate chromatographically. The nitro group of isomer **157a** was reduced to the corresponding aniline by catalytic hydrogenation. As the aromatic amine product **161** was unstable under air, which was observed as a rapid coloration, it was coupled immediately with Boc-glycine in the presence of IBCF and *N*methylpiperidine to give a stable product **158**. Oxidative demethylation of compound **158** resulted in a quinone intermediate, which could not be isolated due to the high reactivity of this molecule. An alternative substrate **160** was subsequently designed from molecule **158** with the intention being that an extra enzymatic demethylation step may occur in certain bacteria of interest (Scheme 47). Substrate **160** supposedly can undergo an aminopeptidase enzymatic step, the liberated intermediate **161** is observed to be decomposing to blue and light purple coloured compounds during its synthesis. This therefore could possibly cause coloration in bacteria with glycine aminopeptidase activity. Further demethylation and oxidative cyclization expected to result the phenoxazinone chromophore **144**, although occurrence of enzymatic demethylation of *para*-dimethoxyaromatic compounds to quinone in bacteria is undiscovered, but possible (Scheme 48).



Scheme 47 *Synthesis of substrate* ***160****. Reagents and conditions: (a) NaH, DMF; (b) HNMe$_2$*HCl, DMSO; (c) H$_{2\,(g)}$, 0.5% Pd/C, MeOH, ethyl acetate, 4 bar; (d) IBCF, NMM, THF, immediately with crude* ***161****; (e) DEE, HCl.*



Scheme 48 *Expected action of substrate* ***160*** *in bacteria*

^{1}H-NMR spectrum of substrate **160** in D_2O is displayed in Figure 38. Aromatic ring A has two hydrogens 4-H and 6-H in a meta position with respect to C-2. The two doublets these hydrogen atoms produce (6.81 ppm, 6.62 ppm) occur at relatively low ppm, due to the electron rich A ring. Due to this it is relatively easy to distinguish between these hydrogens and those associated with the A' ring. 3'-H has interaction with only 5'-H in a relatively meta position. This causes a doublet signal (6.97 ppm) with a small *J* value (2.4 Hz). 5'-H can interact with 3'-H in meta and 6'-H in ortho positions causing a doublet-doublet signal (7.35 ppm, *J*=2.4 Hz, *J*=8.7 Hz). 6'-H has a strong interaction with 5'-H, causing a doublet (8.05 ppm) with the higher *J* value (8.7 Hz) typically associated with neighbouring hydrogens on an aromatic ring. Glycine □hydrogens have singlet signal at the typical 4.06 ppm. All methyl groups are represented by singlets. As expected the methoxy groups are more deshielded (3.77 ppm, 3.68 ppm) than the dimethylamino moiety (3.16 ppm) due to the higher electronegativity of oxygen in comparison to nitrogen. The pentyl group has a set of signals, which appear in a similar manner in all of the pentyl compounds mentioned in this thesis. 9-H has a positively shifted triplet at 2.63 ppm, due to the electron withdrawing effect of the neighbouring aromatic unit. More signals associated with the pentyl group are a quintet for

10-H (1.58 ppm), while 11-H and 12-H overlap and appear as a multiplet (1.29 ppm), and finally the terminal 13-H appears as a triplet at 0.84 ppm.

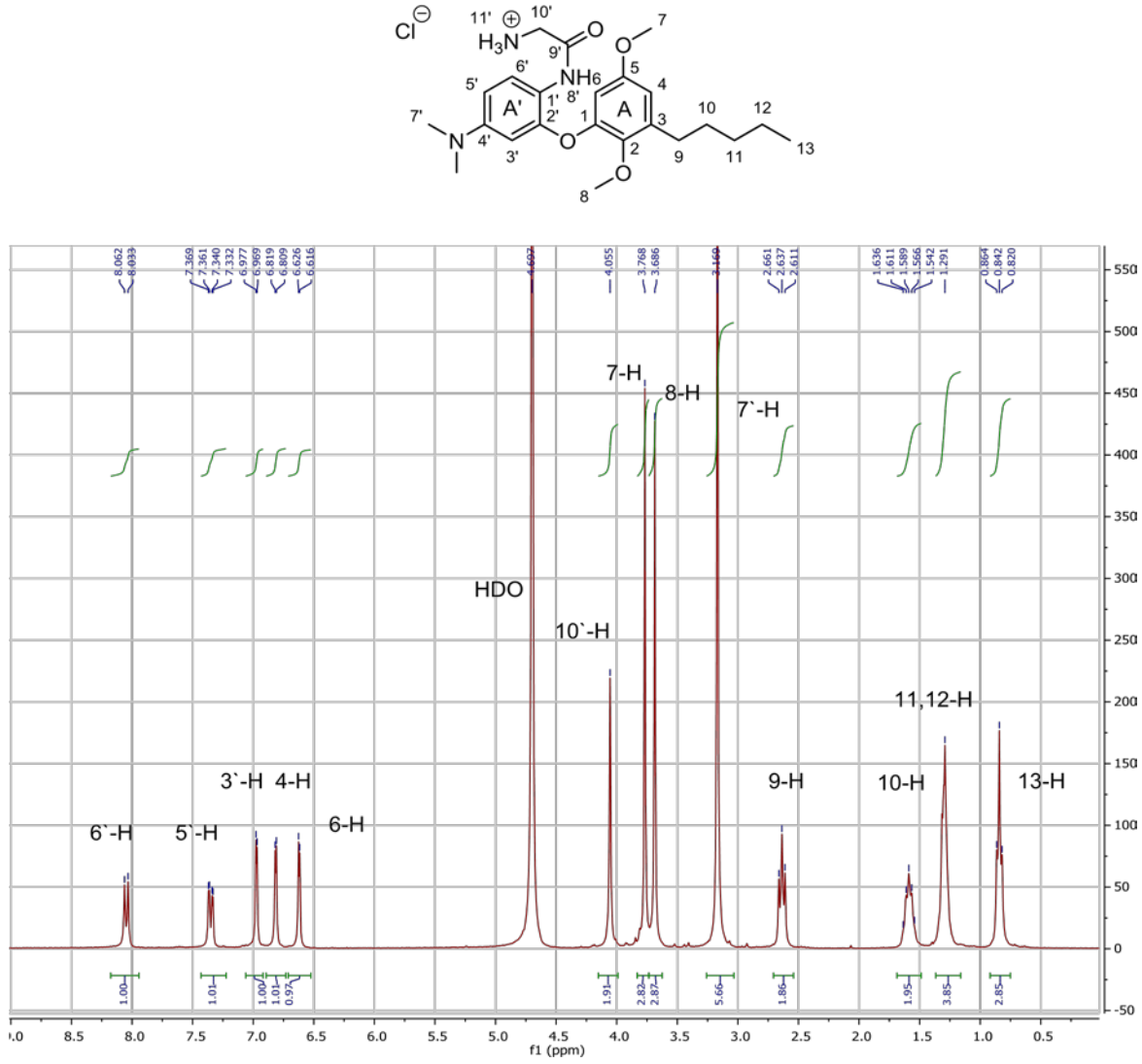
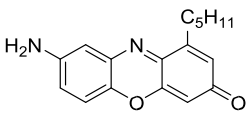
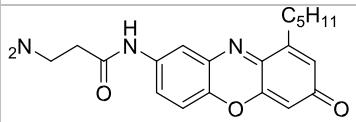
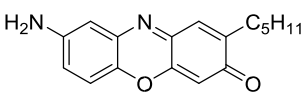
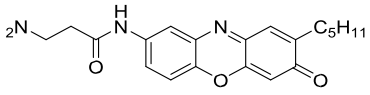


***Figure 38**[1]H-NMR of substrate **160** in D_2O.*

3.2.3 Chromogenic study

The chromogenic properties of substrate **141** and chromogen **142** were compared with our previously published pentyl-amino-phenoxazin-3-ones and substrates.[135] An ideal chromogenic substrate liberates a strongly absorbing chromogen (large □), while itself only absorbs weakly (small □) or shows no absorption at all. Another important factor is the contrast between the chromogen and the substrate, which is represented by □□, where a larger □□

means greater contrast and better differentiation between chromogen and substrate. The chromogen with a 7-amino substituent **166** has a significantly more intense colour. Indeed, the molar extinction coefficient [□] is more than 3 times greater than the 8-amino phenoxazinone chromogens **142** and **163**. In comparison, the chromogenic substrate **166** displays only twice the colour intensity of substrates **141** and **164** by the same measure. Moreover, the difference between the absorption maxima of the chromogen and chromogenic substrate of the 7-amino derivatives **165**-**166** is larger than the 8-amino derivatives **142**-**141**, **163**-**164**. These results support the theory regarding the relative extent of delocalisation occurring within these compounds, as discussed in section 3.1.4.This factor is important for the easy differentiation between the chromogen and the chromogenic substrate if UV/VIS spectroscopy is used for tracking the bacterial aminopeptidase enzyme activity. These data show that 7-amido compounds have better chromogenic properties for application in chromogenic culture medium than 8-amido-phenoxazinones (Table 8).

Chromogen(□, A, □: $dm^3*mol^{-1}*cm^{-1}$)	□□□□	**Chromogenic substrate**
 **142** 1-pentyl-8-amino		 **141**
544.5 nm, 0.457 A, □: 9140	61.5 nm	483 nm, 0.438 A, □: 8760
 **163** 2-pentyl-8-amino		 **164**

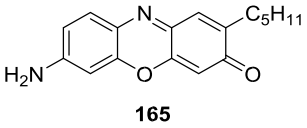
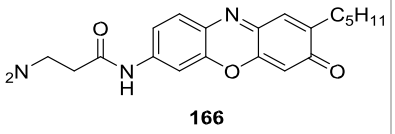
540 nm, 0.322 A, □ 6400	65 nm	475 nm, 0.388 A, □ 7760
 **165** 2-pentyl-7-amino		 **166**
528 nm, 1.554 A, □ 31080	71 nm	457 nm, 0.861 A, □ 17220

Table 8 *Chromogenic properties of differently substituted phenoxazinone chromogenic substrates and their parent chromogens in 50 µM methanol solution.*

3.2.4 Microbiological evaluation

8-□-Alanylamido-1-pentylphenoxazinone **141** was tested against *P. aeruginosa, Serratia marcescens,* and *Burkholderia cepacia*, all of which contain the enzyme □-alanyl aminopeptidase. In addition, *Enterobacter cloacae* was used as a control, due to its lack of □-alanyl aminopeptidase. Colour formation was clearly observed with *Serratia marcescens* and weakly observable with *P. aeruginosa*. In comparison with the reference substrates, substrate **141** gave similar, but less intense reactions (Table 9), and produced a similarly strong brownish background colour in the culture media (Figure 39).[132,135]

	Strain ID	**164**	**141**	**166**	**86a**
Enterobacter cloacae	NCTC 11936	NT	- (-)	NT	NT
Enterobacter cloacae	ATCC 13047	- (-)	NT	- (-)	- (-)
Pseudomonas aeruginosa	NCTC 10662	- (++)	(±)	- (+)	+ (++)
Pseudomonas aeruginosa	NA	+ (+)	NT	++ (++)	++ (++)
Pseudomonas aeruginosa	NA	+ (++)	NT	++ (++)	++ (++)
Serratia marcescens	NA	++ (++)	NT	+ (++)	+ (++)
Serratia marcescens	NCTC 10211	+ (++)	(+)	+ (+)	++ (++)
Serratia marcescens	ATCC 264	- (++)	NT	+ (+)	+ (++)
Serratia marcescens	ATCC 43861	+ (++)	NT	+ (++)	+ (++)
Burkholderia cepacia	ATCC BA-246	- (-)	NT	- (-)	- (-)
Burkholderia cepacia	LMG 1222	- (+[a])	NT	- (-)	- (++[e])
Burkholderia cepacia	ATCC BAA-1911	- (++[e])	NT	- (+[e])	+ (++[e])
Burkholderia cepacia	NCTC 10743	NT	- (-)	NT	NT

Table 9 *Spot test screening of substrates against a range of clinically relevant Gram-negative bacteria. Comparison of new substrate* ***141*** *with reference* ***164***, ***166*** *and* ***86a****. Substrates were tested at a concentration of 40 mg/L,* ***141*** *substrate was tested at a concentration of 50 mg/L. Results after 24 h and 48 h of incubation at 37 °C. "–" Means no coloration, " ± " means coloration with weak intensity, "+" means coloration with medium intensity,*
"++" means coloration with strong intensity. NG, no growth. [a]This strain produces a yellow pigment. NT, the substrate has not been tested.

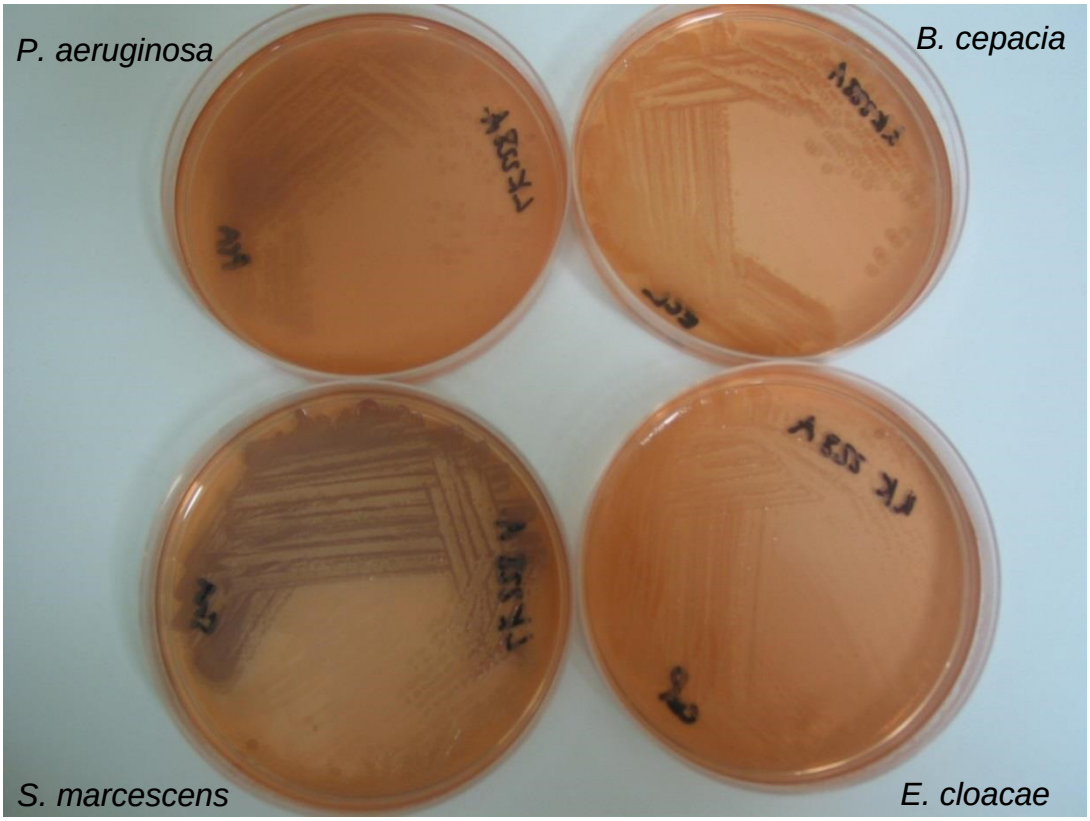
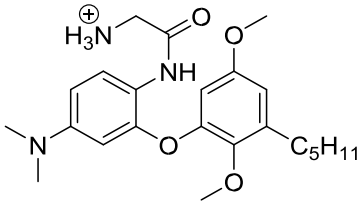


Figure 39 *Microbiological test results of 8-β-alanylamido-1pentylphenoxazinone* ***141****. Tests were carried out in Columbia agar at 50 mg/L concentration.*

Substrate **160** was tested against a panel of clinically relevant bacteria and fungi. The expected purple coloration was only observed weakly with *P. aeruginosa* as pale pink colonies. On the other hand, substrate **160** inhibited the growth of all tested Gram-positive bacteria and fungi and a few of the Gram-negative bacteria (Table 10). This might be the effect of the reactive moieties within the liberated molecule, such as the nucleophile aromatic primer amino or the electrophilic quinone keto units. The latter units in

particular could be subject to nucleophilic attack by biological macromolecules essential to the bacteria, which would impair their normal function and inhibit the growth of the bacteria as a result. This compound could be useful as an antibacterial agent after further investigation, although it may display toxicity against humans cells in the same way as against the bacteria.



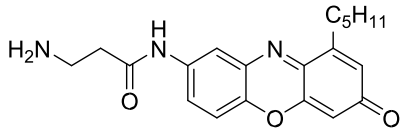
160

Substrate **160** tested at 100 mg/L			
	Strain ID	Growth	Colour
Escherichia coli	NCTC 10418	-	-
Klebsiella pneumoniae	NCTC 9528	-	-
Providencia rettgeri	NCTC 7475	+/-	-
Enterobacter cloacae	NCTC 11936	-	-
Serratia marcescens	NCTC 10211	+	-
Salmonella Typhimurium	NCTC 74	+	-
Pseudomonas aeruginosa	NCTC 10662	+	Trace pink
Yersinia enterocolitica	NCTC 11176	+	-
Burkholderia cepacia	NCTC 10743	+	-
Acinetobacter baumannii	ATCC 19606	-	-
Streptococcus pyogenes	NCTC 8306	-	-
Staphylococcus aureus (MRSA)	NCTC 11939	-	-
Staphylococcus aureus	NCTC 6571	-	-
Staphylococcus epidermidis	NCTC 11047	-	-
Listeria monocytogenes	NCTC 11994	-	-
Enterococcus faecium	NCTC 7171	-	-
Enterococcus faecalis	NCTC 775	-	-
Bacillus subtilis	NCTC 9372	-	-
Candida albicans	ATCC 90028	-	-
Candida glabrata	NCPF 3943	-	-

Table 10 *Spot test screening of substrates against a range of clinically relevant bacteria and fungi.* ***160*** *substrate was tested at a concentration of 100 mg/L. Results after 24 h of incubation at 50 °C. '+' Means growth, '+/- ' means weak growth, '-' means no growth was observed.*

3.3 Conclusion

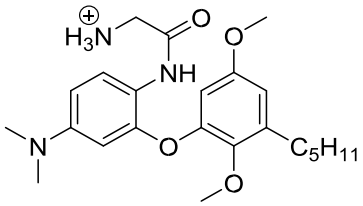
7-β-Alanylamido-1-pentylphenoxazin-3-one **86a** is a chromogenic substrate, which is applied in chromID Pseudomonas® chromogenic culture medium. A library of differently substituted phenoxazin-3-ones has been prepared previously for the development and optimization of chromogenic properties of the substrates and chromogens. These studies also explored different synthetic strategies which were investigated for more economical and cost effective production. As a missing element of this research, chromogenic substrate 8-□-alanylamido-1-pentylphenoxazinone **141** was synthesised and tested against bacteria known to express □-alanyl aminopeptidase (Figure 40). Substrate **141** responded to *Serratia marcescens* and more weakly to *P. aeruginosa* with significant background colour. Investigation of substrate **141** completed a decade of research work done by this research group.



144

Figure 40 *Chromogenic substrate 8-□-alanylamido-1-pentylphenoxazinone* ***141***

A novel chromogenic substrate **143** based on the liberation of a chromogen phenoxazin-3-one core **144** was designed aiming a decreased background colour in culture medium. Unfortunately, the designed substrate **143** could not be synthesized due to stability reasons. Instead an alternative substrate **160** was made and tested (Figure 41). The effectiveness of compound **160** required a complex enzymatic activity cascade. It did not display significant coloration in any species, although it had strong antibacterial activity against Gram-positive and a few Gram-negative bacteria, which was possibly caused by the reactive electrophilic parts of the enzymatically liberated molecule.

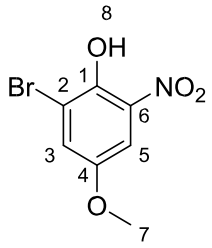


163

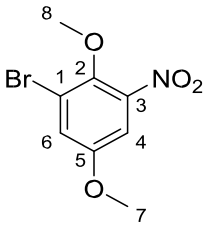
Figure 36 *Substrate* ***160***

3.4 Experimental section

2-Bromo-4-methoxy-6-nitrophenol 146 [143]:

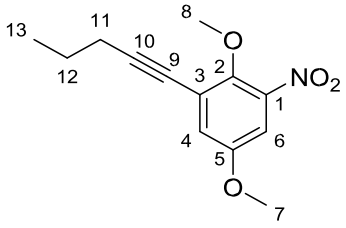


To a solution of 4-methoxy-2-nitrophenol **145** (5.00 g, 29.6 mmol, 1.00 eq) in a mixture of glacial acetic acid (100 mL) and water (40 mL) potassium bromide (3.52 g, 29.6 mmol, 1.00 eq), then bromine (1.52 mL, 29.6 mmol, 1.00 eq) was added dropwise at room temperature. Precipitation of orange crystals was observed a few min after the addition of bromine. After 3 h of stirring at room temperature, the mixture was filtered and washed once with a small amount of water. The filtered crystals were dried in a desiccator using phosphorus pentoxide at atmospheric pressure and room temperature. Water (30 mL) was added to the filtrate and additional product was extracted into ethyl acetate (2 x 50 mL), then washed with brine (40 mL), dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) - DCM, 1:2 ratio, R_f: 0.50). The residue was recrystallized from petroleum ether (60 – 80 °C) to give the product **146** as orange crystals (5.89 g, 74%). Mp: 114 - 115 °C (literature: 116 °C **[143]**); ^{1}H NMR (300 MHz, $DMSOd_6$) δ_H 10.49 (1H, br s, 8-H), 7.61 (1H, d, *J*=3.0 Hz, 5-H), 7.48 (1H, d, *J*=3.0 Hz, 3-H), 3.78 (3H, s, 7-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 152.2 (4-C), 143.7 (1-C), 137.8 (6-C), 126.5 (3-C), 114.9 (2-C), 108.7 (5-C), 56.8 (7-C); v_{max}/cm^{-1} 3238 (O-H), 1529 (-NO_2), 1412 (C-O), 1329 (-NO_2), 1212 (C-O), 678 (C-Br); MS(ESI) m/z 245.8 (M^{79}Br-H)$^-$, 247.8 (M^{81}B-H)$^-$. **1-Bromo-2,5-dimethoxy-3-nitrobenzene 147 [143]:**



To a solution of 2-bromo-4-methoxy-6-nitrophenol **146** (3.5 g, 14 mmol, 1.00 eq) in dry DMF (50 mL) Cs_2CO_3 (6.90 g, 21 mmol, 1.50 eq) was added turning the initially orange coloured solution to dark red. After 30 min stirring, dimethyl sulfate (2.01 mL, 21 mmol, 1.50 eq) was added dropwise at room temperature and then the solution was stirred at 70 °C over night. After cooling to room temperature, water (300 mL) was added, and the precipitated pale yellow solid was filtered, and washed with water. The filtered crystals were dried in a desiccator using phosphorus pentoxide at atmospheric pressure and room temperature (3.73 g, 74%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 9:1 ratio, R_f: 0.32). Mp: 97 - 98 °C (literature: 106 °C **[143]**); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.60 (1H, d, *J*=3.0 Hz, 6-H), 7.53 (1H, d, *J*=3.0 Hz, 4-H), 3.85 (3H, s, 7-H), 3.83 (3H, s, 8-H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ_C 155.5 (2-C), 145.0 (3-C), 142.9 (5C), 123.2 (6-C), 118.9 (1-C), 109.6 (4-C), 62.5 (8-C), 56.5 (7-C); ν_{max}/cm^{-1} 1524 (-NO_2), 1475 (C-O), 1340 (-NO_2), 1228 (C-O), 597 (C-Br); MS(ESI) m/z 284 ($M^{79}Br+H)^+$, 286 ($M^{81}Br+H)^+$.

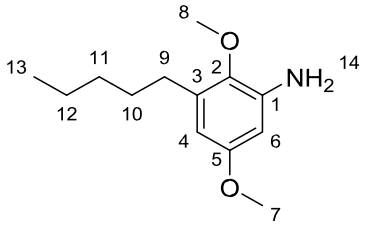
2,5-Dimethoxy-1-nitro-3-(pent-1-yn-1-yl)benzene 148:



A dry Schlenk tube was charged with 1-bromo-2,5-dimethoxy-3-nitrobenzene **147** (2.00 g, 7.63 mmol, 1.00 eq) $(Ph_3P)_2PdCl_2$ (0.21 g, 0.31 mmol, 0.04 eq), Cu(I)I (0.06 g, 0.31 mmol, 0.04 eq), dry trimethylamine (20 mL) and dry DMF (20 mL). The resulting mixture was heated at 80 °C for 15 min, before the addition of the solution of pent-1-yne (1.51 mL, 15.3 mmol, 2.00 eq) in dry DMF (10 mL). The reaction mixture was heated at 80 °C over night. After

cooling to room temperature water (60 mL) and petroleum ether (40 – 60 °C) (60 mL) was added to the mixture, and the two phases were separated. The aqueous phase was extracted with petroleum ether (40 – 60 °C) (2 x 60 mL), then the combined organic phases were washed with 2.5 M HCl_{aq} (30 mL) and brine (50 mL), and it was dried over $MgSO_4$. After evaporating the solvent under reduced pressure, the crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 40:1 ratio). Further purification was carried out by recrystallization from hexane to give the title compound as butter coloured crystals (1.19 g, 62%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 9:1 ratio, R_f: 0.45). Mp: 43 – 44 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 7.21 (1H, d, *J*=3.0 Hz, 6-H), 7.12 (1H, d, *J*=3.3 Hz, 4-H), 4.02 (3H, s, 7-H), 3.82 (3H, s, 8-H), 2.47 (2H, t, *J*=6.9 Hz, 11-H), 1.69 (2H, sext, *J*=7.2, 12-H), 1.09 (3H, t, *J*=7.5, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 154.6 (2-C), 148.1 (5-C), 144.6 (1-C), 123.2 (4-C), 121.9 (3-C), 109.1 (6-C), 97.5 (10-C), 75.1 (9-C), 62.4 (7-C), 56.0 (8-C), 21.9 (12-C), 21.6 (11-C), 13.5 (13C); ν_{max}/cm^{-1} 2234 (C≡C), 1531 (-NO_2), 1352 (-NO_2), 1246 (C-O); MS(ESI) m/z 272.1 $(M+Na)^+$; CHN [Found: C, 62.47; H, 6.16; N, 5.41. $C_{13}H_{15}NO_4$ requires C, 62.64; H, 6.07; N, 5.62 %].

2,5-Dimethoxy-3-pentylaniline 149:



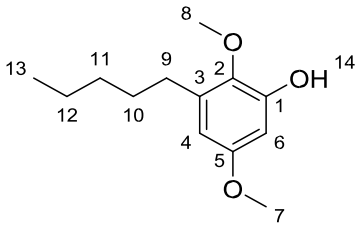
To the solution of 2,5-dimethoxy-1-nitro-3-(pent-1-yn-1-yl)benzene **148** (0.50 g, 2.00 mmol, 1.00 eq) in propanol, 10% palladium on activated carbon (0.05 g, 0.10 meq) was added portion wise. The mixture was stirred at room temperature under 4 bar pressure of hydrogen over night in an autoclave. After the reaction was complete, the mixture was filtered through celite, washed with ethanol and the filtrate was evaporated under reduced pressure. The crude product was purified by column chromatography (eluent:

petroleum ether (40 – 60 °C) – ethyl acetate 7:1 ratio) to give the pure product as a red oil (0.44 g, 96%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 6:1 ratio, R_f: 0.15). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 6.16 (1H, d, *J*=3.0 Hz, 6-H), 6.13 (1H, d, *J*=3.0 Hz, 4-H), 3.77 (2H, br s, 14-H), 3.73 (3H, s, 7-H), 3.70 (3H, s, 8-H), 2.56 (2H, m, 9-H), 1.57 (2H, m, 10-H), 1.36 (4H, m, 11,12-H), 0.90 (3H, t, *J*=6.9 Hz, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 156.3 (5-C), 140.4 (1-C), 139.7 (2-C), 136.6 (3-C), 104.3 (4-C), 99.4 (6-C), 59.9 (8-C), 55.3 (7-C),

31.9 (11-C), 30.4 (10-C), 29.7 (9-C), 22.6 (12-C), 14.0 (13-C); ν_{max}/cm^{-1} 3363 (N-H), 1215 (C-O); MS(ESI) m/z 224.2 $(M+H)^+$; HRMS (Found $(M+H)^+$

224.1645. Calcd. for $C_{13}H_{22}O_2N$: $(M+H)^+$ 224.1645.).

2,5-Dimethoxy-3-pentylphenol 150:

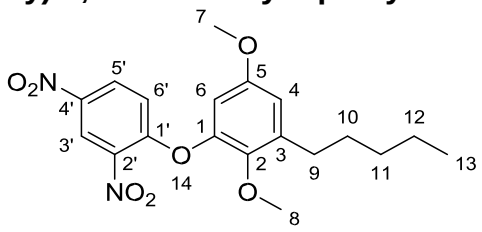


The suspension of 2,5-dimethoxy-3-pentylaniline **149** (2.11 g, 9.06 mmol, 1.00 eq) in 5 M sulfuric acid (140 mL) was heated to 80 °C until it became a clear solution **A**. After cooling to 0 °C, sodium nitrite (0.75 g, 10.9 mmol, 1.20 eq) was poured as an aqueous solution (10 mL) to solution **A**, and stirred for 1.5 h at 0 °C followed by the addition of urea (0.19 g, 3.17 mmol, 0.35 eq). The reaction mixture was added dropwise to 5 M sulfuric acid (70 mL) at 90 °C and it was stirred for a further 1 h. When the reaction was complete, the reaction mixture was allowed to cool to room temperature and extracted with diethyl ether (3 x 100 mL). The combined organic phases were washed with brine (60 mL), dried over $MgSO_4$, and it was evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 10:1 ratio) to give the pure product as a red oil (1.19 g, 59%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 10:1 ratio, R_f: 0.18). ^{1}H

NMR (300 MHz, $CDCl_3$) δ_H 6.41 (1H, d, *J*=2.7 Hz, 6-H), 6.29 (1H, d, *J*=3.0 Hz, 4-H), 5.67

(1H, s, 14-H), 3.76 (6H, s, 7,8-H), 2.61 (2H, m, 9-H), 1.61 (2H, m, 10-H), 1.39 (4H, m, 11,12-H), 0.92 (3H, t, *J*=7.2 Hz, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 156.5 (5-C), 149.4 (1-C), 139.2 (2-C), 136.3 (3-C), 106.6 (4-C), 98.9 (6-C), 61.4 (8-C), 55.4 (7-C), 31.8 (11-C), 30.1 (10-C), 29.6 (9-C), 22.5 (12-C), 14.0 (13-C); ν_{max}/cm^{-1} 3428 (O-H), 1145 (C-O); MS(ESI) m/z 263.1 $(M+K)^+$; HRMS (Found $(M+H)^+$ 225.1487. Calcd. for $C_{13}H_{21}O_3$: $(M+H)^+$ 225.1485.).

1-(2',4'-Dinitrophenoxy)-2,5-dimethoxy-3-pentylbenzene 151:

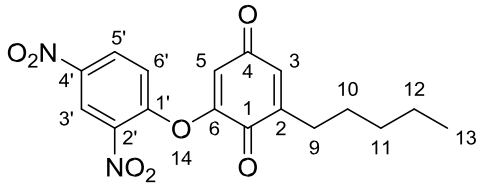


To a solution of 2,5-dimethoxy-3-pentylphenol **150** (0.40 g, 1.80 mmol, 1.00 eq) in DMSO (8 mL) triethyl amine (0.25 mL, 1.80 mmol, 1.00 eq) was added, and the mixture was stirred for 5 min before the addition of 2,4- dinitro-1fluorobenzene **135** (0.23 mL, 1.80 mmol, 1.00 eq). After stirring the reaction mixture for 5 h at room temperature, it was quenched with water (40 mL), and extracted with dichloromethane (3 x 20 mL). The combined organic layers were washed with aqueous 2 M NaOH solution (3 x 20 mL), water (20 mL) and brine (20 mL). The organic portion was subsequently dried over $MgSO_4$, and the solvent evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 15:1 ratio) to give the pure product as a yellow oil (0.65 g, 93%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 15:1 ratio, Rf: 0.22). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 8.84 (1H, d, *J*=2.7 Hz, 3'-H), 8.27 (1H, dd, *J*=2.7 Hz,

J=9.3 Hz, 5'-H), 6.90 (1H, d, *J*=9.3 Hz, 6'-H), 6.72 (1H, d, *J*=3.0 Hz, 4-H), 6.57 (1H, d, *J*=3.0 Hz, 6-H), 3.78 (3H, s, 8-H), 3.70 (3H, s, 7-H), 2.62 (2H, m, 9-H),

1.61 (2H, m, 10-H), 1.35 (4H, m, 11,12-H), 0.91 (3H, t, *J*=6.6 Hz, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 156.3 (5-C), 155.8 (1'-H), 145.9 (1-H), 143.2 (2C), 141.2 (4'-C), 139.5 (3-C), 138.5 (2'-C), 128.7 (5'-C), 121.9 (3'-C), 117.4 (6'-C), 113.8 (4-C), 105.3 (6-C), 61.7 (7-C), 55.7 (8-C), 31.7 (11-C), 30.3 (10C), 29.8 (9-C), 22.4 (12-C), 14.0 (13-C); v_{max}/cm^{-1} 1533 (-NO_2), 1340 (-NO_2), 1263 (C-O), 1236 (C-O); MS(ESI) m/z 391.1 $(M+H)^+$; HRMS (Found $(M+H)^+$ 391.1499. Calcd. for $C_{19}H_{23}O_7N_2$: $(M+H)^+$ 391.1500.).

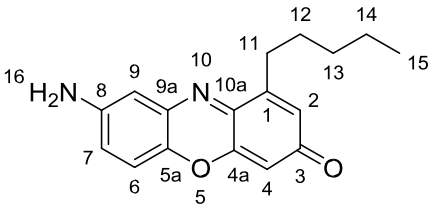
2-(2,4-Dinitrophenoxy)-6-pentylcyclohexa-2,5-diene-1,4-dione 152:



A solution of CAN (2.53 g, 4.60 mmol, 3.00 eq) in water (13 mL) was added dropwise with a dropping funnel to a solution of 1-(2',4'-dinitrophenoxy)-2,5dimethoxy-3-pentylbenzene (0.60 g, 1.54 mmol, 1.00 eq) **151** in acetonitrile (20 mL), and the mixture was stirred for 1 h. After the reaction was complete, acetonitrile was removed under reduced pressure. The aqueous residue was extracted with dichloromethane (3 x 20 mL), the combined organic phases were washed with brine (20 mL), dried over $MgSO_4$, and evaporated under reduced pressure. The crude product (0.53 g) was purified by recrystallization in two generations to give the title compound as yellow crystals (0.37 g, 67%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 10:1 ratio, R_f: 0.27). Mp: 65-67 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 8.98 (1H, d, *J*=2.7 Hz, 3'-H), 8.52 (1H, dd, *J*=2.7 Hz, *J*=9.0 Hz, 5'-H), 7.38 (1H, d, *J*=9.0 Hz, 6'- H), 6.61 (1H, d, *J*=2.1 Hz, 5-H), 6.08 (1H, d, *J*=2.4 Hz, 3-H), 2.48 (2H, td, *J*=1.5 Hz, *J*= 6.6 Hz, 9-H), 1.55 (2H, m, 10H), 1.37 (4H, m, 11,12-H), 0.92 (3H, t, *J*=6.9 Hz, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 186.3 (4-C), 180.3 (1-C), 155.7 (2-C), 151.9 (1'-C), 148.4 (6-C), 144.4 (4'-C), 140.6 (2'-C), 132.9 (5-C), 129.3 (5'-C), 123.4 (6'-C), 122.5 (3'C), 115.7 (3-C), 31.4 (11-C), 28.8 (9-C), 27.4 (10-C), 18.4 (12-C), 13.9 (13-

C); v_{max}/cm^{-1} 1681 (C=O), 1653 (C=O), 1533 (-NO_2), 1344 (-NO_2), 1229 (C-O); MS(ESI) m/z 383.1 $(M+H)^+$; CHN [Found: C, 56.96; H, 4.55; N, 7.69. $C_{17}H_{16}N_2O_7$ requires C, 56.67; H, 4.48; N, 7.77 %].

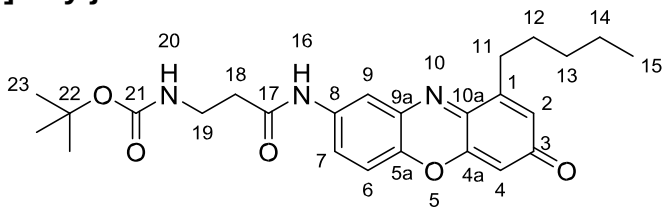
8-amino-1-pentyl-3*H*-phenoxazin-3-one 142:



An autoclave reactor was charged with a solution of 2-(2,4-dinitrophenoxy)-6pentylcyclohexa-2,5-diene-1,4-dione **152** (0.35 g, 0.97 mmol, 1.00 eq) in methanol (15 mL) and ethyl acetate (15 mL). After the portion wise addition of 5% palladium on activated charcoal (0.035 g, 0.10 meq), it was hydrogenated under 3 bar at room temperature over night. The reaction mixture was filtered through celite and the solid residue was washed with methanol into a round bottom flask preloaded with Ag_2O (0.68 g, 2.92 mmol, 3.00 eq). The filtrate was stirred over night. After the reaction was complete, it was filtered through celite, and washed with methanol. After the filtrate was evaporated under reduced pressure, the crude product (0.33 g) was purified by column chromatography (eluent: dichloromethane – methanol 100:0 then 80:1 ratio) to give the pure product as dark purple crystals (0.18 g, 65%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 1:1 ratio, R_f: 0.25). Mp: 151 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 7.14 (1H, d, *J*=8.7 Hz, 6-H), 7.09 (1H, d, *J*=3.0 Hz, 9-H), 6.89 (1H, dd, *J*=2.7 Hz, *J*=8.7 Hz, 7-H), 6.68 (1H, d, *J*=2.1 Hz, 2-H), 6.21 (1H, d, *J*=2.1 Hz, 4-H), 3.84 (2H, br s, 16-H), 2.84 (2H, t, *J*=8.4 Hz, 11-H), 1.68 (2H, m, 12-H), 1.41 (4H, m, 13,14-H), 0.93 (3H, t, *J*=6.9 Hz, 15-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 186.3 (3-C), 150.4 (4a-C), 148.5 (10a-C), 146.8 (1-C), 144.0 (5a-C), 137.2 (8-C), 133.6 (9a-C), 132.0 (2-C), 120.4 (7-C), 116.3 (6-C), 114.0 (9-C), 105.3 (4-C), 31.5 (13-C), 29.7 (11-C), 28.8 (12-C), 22.4 (14C), 14.0 (15-C); v_{max}/cm^{-1} 3439 (N-H), 3356 (N-H), 1639 (C=O); UV/Vis (50 □M

methanol) λ_{max1} = 361.5 nm, ε = 19560 $dm^3*mol^{-1}*cm^{-1}$, λ_{max2} = 544.5 nm, ε = 9140 $dm^3*mol^{-1}*cm^{-1}$; MS(ESI) m/z 283.1 $(M+H)^+$; CHN [Found: C, 72.47; H, 6.62; N, 9.59. $C_{17}H_{18}N_2O_2$ requires C, 72.32; H, 6.43; N, 9.59 %].

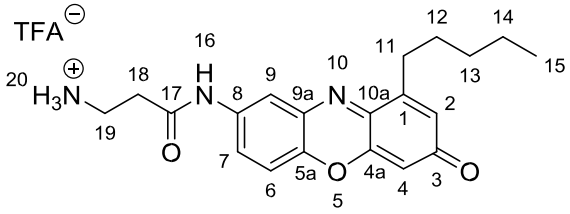
tert-Butyl-_N_-{2-[(3-oxo-1-pentyl-3_H_-phenoxazin-8yl)carbamoyl]ethyl}carbamate 154:



To a solution of Boc-β-alanine **153** (0.26 g, 1.38 mmol, 3.00 eq) in dry THF (7 mL), NMM (0.18 mL, 1.61 mmol, 3.50 eq) was added at 0 °C, and stirred for 5 min. After the careful addition of IBCF (0.18 mL, 1.38 mmol, 3.00 eq), the mixture was stirred at 0 °C for 1 h. The solution of 8-amino-1-pentyl-3*H*phenoxazin-3-one **142** (0.13 g, 0.46 mmol, 1.00 eq) was added to the mixture at 0 °C and it was stirred at room temperature over night. After the reaction was complete, the mixture was quenched with 5% $NaHCO_3$ aqueous solution (30 mL) and extracted with ethyl acetate (2 x 30 mL). The combined organic layers were washed with 5% $NaHCO_3$ aqueous solution (40 mL), water (40 mL) and brine (40 mL). The organic portion was dried over $MgSO_4$, and the solvent evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 4:1, 2:1 then 1:2 ratio) to give the pure product as a red solid (0.07 g, 34%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 1:2 ratio, R_f: 0.16). Mp: 184 - 186 °C ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 8.40 (1H, br s, 16-H), 8.17 (1H, d, *J*=2.1 Hz, 9-H), 7.66 (1H, dd, *J*=2.4 Hz, *J*=8.7 Hz, 7-H), 7.25 (1H, d, *J*=9.0 Hz, 6-H), 6.67 (1H, d, *J*=2.1 Hz, 2-H), 6.23 (1H, d, *J*=2.1Hz, 4-H), 5.20 (1H, m, 16-H), 3.54 (2H, quart, *J*=5.7 Hz, 19-H), 2.84 (2H, t, *J*=7.5 Hz, 11-H), 2.68 (2H, t, *J*=6.0 Hz, 18-H), 1.67 (2H, m, 12-H), 1.40 (4H, m, 13,14-H), 0.93 (3H, t, *J*=6.9 Hz, 15-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 186.4 (3-C), 169.7 (17-C), 156.6 (21-C), 150.0 (4a-C), 148.9 (10a-C), 147.2 (1-C), 140.4 (5a-C), 135.2 (8-C), 133.0 (9a-C), 132.1 (2-C), 124.3 (7-C), 120.8 (9-C), 116.0 (6-C), 106.0 (4-C),

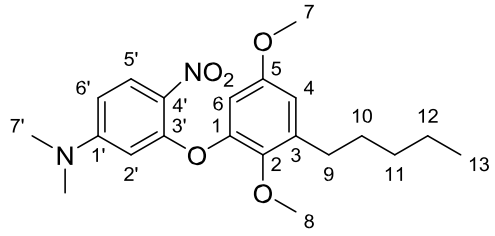
80.0 (22-C), 38.0 (18-C), 36.5 (19-C), 31.6 (13-C), 29.7 (11-C), 28.8 (12-C), 28.4 (23-C), 22.4 (14-C), 13.9 (15-C); ν_{max}/cm^{-1} 3225 (N-H), 1694 (C=O), 1645 (C=O), 1564 (N-H); MS(ESI) m/z 454.2 $(M+H)^+$; HRMS (Found $(M+H)^+$ 454.2331. Calcd. for $C_{25}H_{32}O_5N_3$: $(M+H)^+$ 454.2336.).

2-[(3-oxo-1-pentyl-3*H*-phenoxazin-8-yl)carbamoyl]ethan-1-aminium trifluoroacetate 141:



tert-Butyl-*N*-{2-[(3-oxo-1-pentyl-3*H*-phenoxazin-8yl)carbamoyl]ethyl}carbamate **154** (60 mg, 0.13 mmol, 1.00 eq) was swirled with neat TFA (3 mL) for 1 min, then the mixture was evaporated under reduced pressure. Methanol (10 mL) was added to the residue, and evaporated under reduced pressure, this process was repeated 3 times. The residue was dissolved in minimal amount of methanol, and diethyl ether was added until a purple solid precipitated from the solution. The solid compound was filtered and washed with diethyl ether to give the title compound as a red powder (38 mg, 63%). Mp: 200 °C (decomposed); ^{1}H NMR (300 MHz, methanol-d_4) δ_H 8.15 (1H, d, *J*=2.1 Hz, 9-H), 7.70 (1H, dd, *J*=2.1 Hz, *J*=9.0 Hz, 7-H), 7.31 (1H, d, *J*=9.0 Hz, 6-H), 6.65 (1H, s, 2-H), 6.14 (1H, s, 4-H), 3.31 (2H, m, 19-H), 2.85 (4H, m, 11,18-H), 1.69 (2H, m, 12-H), 1.43 (4H, m, 13,14H), 0.95 (3H, m, 15-H); ^{13}C NMR (75.5 MHz, methanol-d_4) δ_C 186.9 (3-C),
169.2 (17-C), 150.5 (4a-C), 148.7 (10a-C) 147.8 (1-C), 140.1 (5a-C), 136.0 (8-C), 132.6 (9a-C), 131.3 (2-C), 124.5 (7-C), 119.8 (9-C), 115.8 (6-C), 104.8 (4-C), 35.4 (19-C), 32.3 (18-C), 31.4 (13-C), 29.3 (11-C), 28.7 (12-C), 22.1 (14C), 12.9 (15-C); UV/Vis (50 □M methanol) □$_{max1}$ = 351.0 nm, □□= 13860 $dm^3*mol^{-1}*cm^{-1}$, □$_{max2}$ = 483.0, □□= 8760 $dm^3*mol^{-1}*cm^{-1}$; ν_{max}/cm^{-1} 1676 (C=O), 1643 (C=O), 1555 (N-H); MS(ESI) m/z 354.2 $(M+H)^+$; HRMS (Found $(M+H)^+$ 354.1810. Calcd. for $C_{20}H_{24}O_3N_3$: $(M+H)^+$ 354.1812.).

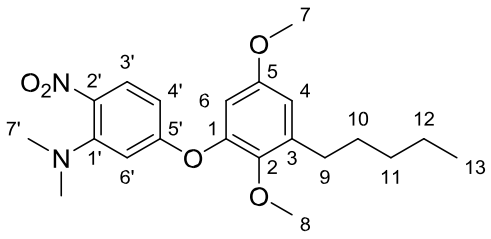
3-(2,5-dimethoxy-3-pentylphenoxy)-*N*,*N*-dimethyl-4-nitroaniline 157a:



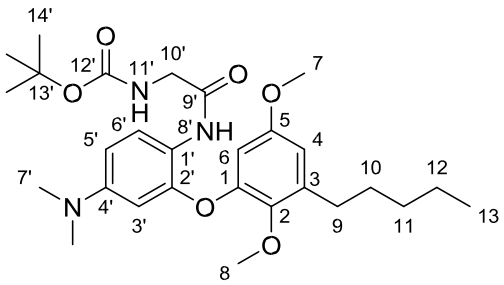
To a solution of 2,5-dimethoxy-3-pentylphenol **150** (0.80 g, 3.57 mmol, 1.00 eq) in DMF (10 mL) sodium hydride (60% oil dispersion, 0.16 g, 3.93 mmol, 1.10 mmol) was added portionwise. Once, the gas formation had stopped 1,3difluroro-4-nitrobenzene (0.57 g, 3.57 mmol, 1.00 eq) **155** was added, and the reaction mixture was stirred for 4 h at room temperature. After the reaction was complete, the mixture was quenched with brine (20 mL) and extracted with dichloromethane (3 x 15 mL). The combined organic layers were washed with water (15 mL), and brine (15 mL). The organic portion was subsequently dried over $MgSO_4$, and the solvent then evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 6:1 then 4:1 ratio) to give a regioisomeric mixture of intermediate **156** as a yellow oil (0.74 g), which was used without separation and characterization in the following reaction step.

To a solution of dimethylammonium hydrochloride (0.32 g, 3.97 mmol, 1.00 eq) and K_2CO_3 (1.10 g, 7.93 mmol, 2.00 eq) in DMSO (10 mL) a DMSO (5 mL) solution of isomer mixture **156** was added, and the reaction was heated at 70 °C over night. After the reaction was complete, the mixture was quenched with water (20 mL) and extracted with ethyl acetate (2 x 25 mL). The combined organic layers were washed with saturated $NaHCO_3$ aqueous solution (25 mL), and brine (25 mL). The organic portion was then dried over $MgSO_4$, and the solvent evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 20:1, 10:1 then 5:1 ratio) to give the pure isomers **157a** as yellow crystals (0.47 g, 61%) and **157b** as yellow oil (0.25, 33%). Full characterization of 3-(2,5-dimethoxy-3-pentylphenoxy)-*N*,*N*-dimethyl-4-nitroaniline **157a**: Mp: 69 -71 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 8.02 (1H, d, *J*=9.3 Hz, 5'H), 6.48 (1H, d, *J*=2.7 Hz, 4-H), 6.32 (1H, d, *J*=3.0 Hz, 6-H), 6.29 (1H, dd, *J*=2.7 Hz,

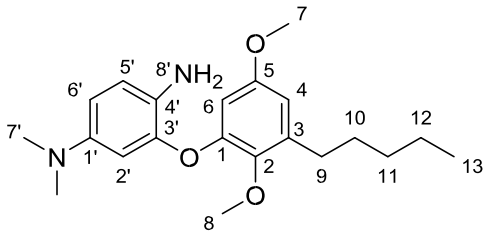
J=9.3 Hz, 6'-H), 5.88 (1H, d, *J*=2.7 Hz, 2'-H); 3.73 (3H, s, 8-H), 3.65 (3H, s, 7-H), 2.87 (6H, s, 7'-H), 2.55 (2H, t, *J*=7.5 Hz, 9-H), 1.55 (2H, quint, *J*=7.5 Hz, 10-H), 1.27 (4H, m, 11,12-H), 0.82 (3H, t, *J*= 6.6 Hz, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 155.7 (5-C), 154.8 (1'-C), 153.8 (3'-C), 148.3 (1-C), 143.0 (2-C), 138.3 (3-C), 128.7 (5'-C), 128.7 (4'-C), 111.0 (4-C), 105.6 (6'-C), 104.0 (6C), 99.9 (2'-C), 61.4 (8-C), 55.6 (7-C), 40.0 (7'-C), 31.7 (11-C), 30.4 (10-C), 30.1 (9-C), 22.5 (12-C), 14.0 (13-C); ν_{max}/cm^{-1} 1605 (benzene), 1564 (-NO_2), 1383 (-NO_2), 1273 (C-O), 1207 (C-N); MS(ESI) m/z 389.2 $(M+H)^+$; CHN [Found: C, 65.25; H, 7.31; N, 7.19. $C_{21}H_{28}N_2O_5$ requires C, 64.93; H, 7.27; N, 7.19 %].



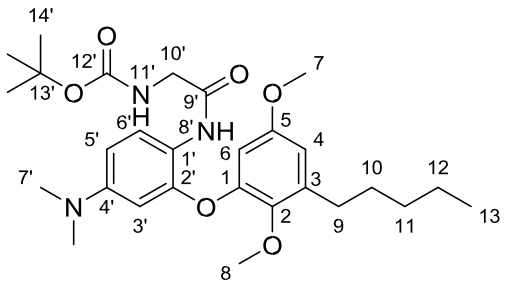
Characterisation of 5-(2,5-dimethoxy-3-pentylphenoxy)-*N*,*N*-dimethyl-2nitroaniline **157b:** ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 7.76 (1H, d, *J*=9.3 Hz, 3'-H), 6.54 (1H, d, *J*=3.0 Hz, 4-H), 6.43 (1H, d, *J*=2.4 Hz, 6'-H), 6.39 (1H, d, *J*=3.0 Hz, 6-H), 6.24 (1H, dd, *J*=2.4 Hz, *J*=9.0 Hz, 4'-H), 3.67 (3H, s, 7-H), 3.64 (3H, s, 8-H), 2.78 (6H, s, 7'-H), 2.55 (2H, t, *J*=7.8 Hz, 9-H), 1.53 (2H, quint, *J*=7.2 Hz, 10-H), 1.27 (4H, m, 11,12-H), 0.83 (3H, t, *J*=6.9 Hz, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 161.9 (5'-C), 155.8 (5-C), 148.6 (1'-C), 147.4 (1-C), 143.9 (2-C), 138.6 (3-C), 133.9 (2'-C), 129.4 (3'-C), 112.2 (4-C), 106.0 (4'-C), 105.3 (6C), 104.6 (6'-C), 61.2 (8-C), 55.6 (7-C), 42.5 (7'-C), 31.7 (11-C), 30.3 (10-C), 30.1 (9-C), 22.5 (12-C), 14.0 (13-C); ν_{max}/cm^{-1} 1607 (benzene), 1562 (-NO_2), 1339 (-NO_2), 1288 (C-O), 1222 (C-N); MS(ESI) m/z 389.2 $(M+H)^+$. **tert-Butyl-N-({[2-(2,5-dimethoxy-3-pentylphenoxy)-4(dimethylamino)phenyl]carbamoyl}methyl)carbamate 158**:



An autoclave reactor was charged with a solution of 3-(2,5-dimethoxy-3pentylphenoxy)-*N*,*N*-dimethyl-4-nitroaniline **157a** (0.38 g, 0.98 mmol, 1.00 eq) in methanol (15 mL) and ethyl acetate (15 mL). After the portion wise addition of 5% palladium on activated charcoal (0.076 g, 0.20 meq), it was hydrogenated under 3 bar at room temperature over night. The reaction mixture was filtered through celite and the solid residue was washed with methanol and ethyl acetate and the filtrate was evaporated under reduced pressure. The crude intermediate **161** was used in the following reaction without purification due to its sensitivity to air.



Characterisation of 3-(2,5-dimethoxy-3-pentylphenoxy)-*N*1,*N*1dimethylbenzene-1,4-diamine **161:** ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 6.68 (1H, brs, 5'-H), 6.43 (1H, brs, 6'-H), 6.35 (1H, d, *J*=3.0 Hz, 4-H), 6.31 (1H, brs, 2'H), 6.18 (1H, d, *J*=3.0 Hz, 6-H), 3.78 (3H, s, 8-H), 3.59 (3H, s, 7-H), 3.09 (2H, brs, 8'-H), 2.70 (6H, brs, 7'-H), 2.56 (2H, t, *J*=7.8 Hz, 9-H), 1.55 (2H, quint, *J*=7.5 Hz, 10-H), 1.29 (4H, m, 11,12-H), 0.84 (3H, t, *J*=7.2 Hz, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 155.7 (5-C), 150.3 (1-C), 145.1 (3'-C), 144.2 (1'-C), 142.3 (2-C), 137.7 (3-C), 129.6 (4'-C), 117.7 (5'-C), 110.8 (6'-C), 108.8 (4-C), 106.4 (2'-C), 102.1 (6-C), 61.2 (8-C), 55.5 (7-C), 41.9 (7'-C), 31.8 (11-C), 30.4 (10-C), 30.2 (9-C), 22.5 (12-C), 14.0 (13-C); MS(ESI) m/z 358.8 $(M+H)^+$.

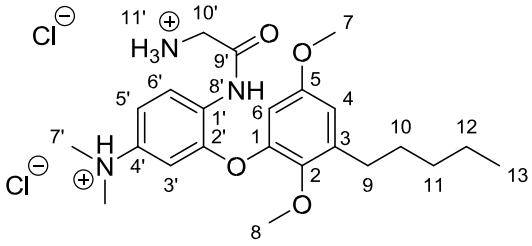


To a solution of Boc-glycine (0.38 g, 2.18 mmol, 3.00 eq) in dry THF (10 mL), NMP (0.31 mL, 2.54 mmol, 3.50 eq) was added at 0 °C, and stirred for 5 min. After the careful addition of IBCF (0.28 mL, 2.18 mmol, 3.00 eq), the mixture was stirred at 0 °C for 1 h. The solution of the crude 3-(2,5-dimethoxy-3pentylphenoxy)-*N*1,*N*1-dimethylbenzene-1,4-diamine **161** (0.26 g, 0.73 mmol, 1.00 eq) was added to the mixture at 0 °C and it was stirred at room temperature over night. After the reaction was complete, the mixture was quenched with saturated $NaHCO_3$ aqueous solution (30 mL) and extracted with ethyl acetate (2 x 30 mL). The combined organic layers were washed with saturated $NaHCO_3$ aqueous solution (30 mL), water (30 mL) and brine (30 mL). The organic portion was dried over $MgSO_4$, and the solvent then evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 4:1 then 3:1 ratio) to give the pure product as pale purple crystals (0.31 g, 82%). The reaction and the work up were monitored by TLC (eluent: petroleum ether (40 – 60 °C) – ethyl acetate 2:1 ratio, R_f: 0.17). Mp: 83 - 86 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 8.10 (1H, s, 8'-H), 8.04 (1H, d, *J*=9.0 Hz, 6'-H), 6.42 (2H, m, 5',4-H), 6.27 (1H, d, *J*=3.0 Hz, 6-H), 6.22 (1H, d, *J*=1.8 Hz, 3'-H), 5.11 (1H, m, 11'-H), 3.81 (2H, d, *J*=5.7 Hz, 10'-H), 3.73 (3H, s, 8-H), 3.61 (3H, s, 7-H), 2.78 (6H, s, 7'-H), 2.56 (2H, t, *J*=6.9 Hz, 9-H), 1.54 (2H, quint, *J*=7.5 Hz, 10-H),

1.33 (9H, s, 14'-H), 1.29 (4H, m, 11,12-H), 0.84 (3H, t, *J*=6.9 Hz, 13-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 167.0 (9'-C), 155.8 (5,12'-C), 149.5 (1-C), 147.4 (4'-C), 142.8 (2-C), 138.1 (3-C), 122.6 (6'-C), 119.1 (1'-C), 110.4 (4-C), 108.2 (5'-C), 103.5 (6-C), 102.9 (3'-C), 80.2 (13'-C), 61.5 (8-C), 55.6 (7-C), 45.0 (10'C), 40.8 (7'-C), 31.7 (11-C), 30.4 (10-C), 30.1 (9-C), 28.2 (14'-C), 22.5 (12-C), 14.0 (13-C); ν_{max}/cm^{-1} 3399 (N-H), 3321 (N-H), 1719 (C=O), 1688 (C=O),

1583 (benzene), 1526 (N-H), 1229 (C-O), 1142 (C-N); MS(ESI) m/z 516.3 $(M+H)^+$; CHN [Found: C, 65.51; H, 8.08; N, 8.17. $C_{28}H_{41}N_3O_6$ requires C, 65.22; H, 8.01; N, 8.15 %].

{[2-(2,5-dimethoxy-3-pentylphenoxy)-4-(dimethylamino)phenyl]carbamoyl}methanaminium chloride 160:



tert-utyl-N-({[2-(2,5-dimethoxy-3-pentylphenoxy)-4-(dimethylamino)phenyl]carbamoyl}methyl)carbamate **158** (0.25 g, 0.49 mmol) was reacted using general method A. The product was obtained as a hygroscopic solid, so it was dissolved in water, and the solution was freeze dried to give the non-hygroscopic product as light brown solid (0.18 g, 82%). Mp: 94 - 96 °C; ^{1}H NMR (300 MHz, D_2O) δ_H 8.05 (1H, d, *J*=8.7 Hz, 6'-H), 7.35 (1H, dd, *J*=2.4 Hz, *J*=8.7 Hz, 5'-H), 6.97 (1H, d, *J*=2.4 Hz, 3'-H), 6.81 (1H, d, *J*=3.0 Hz, 4-H), 6.62 (1H, d, *J*=3.0 Hz, 6-H), 4.06 (2H, s, 10'-H), 3.77 (3H, s, 7-H), 3.69 (3H, s, 8-H), 3.17 (6H, s, 7'-H), 2.64 (2H, t, *J*=7.2 Hz, 9-H), 1.59 (2H, quint, *J*=6.6 Hz, 10-H), 1.31 (4H, m, 11,12-H), 0.84 (3H, t, *J*=6.6 Hz, 13H); ^{13}C NMR (75.5 MHz, D_2O) δ_C 166.2 (9'-C), 155.9 (5-C), 150.3 (1'-C), 147.8 (1-C), 142.6 (2-C), 140.3 (4'-C), 139.6 (3-C), 127.2 (2'-C), 126.1 (3'-C), 115.0 (5'-C), 112.2 (4-C), 108.3 (6'-C), 105.2 (6-C), 61.7 (8-C), 55.9 (7-C), 46.1 (7'-C), 41.1 (10'-C), 30.9 (11-C), 29.8 (10-C), 29.3 (9-C), 21.9 (12-C), 13.3 (13-C); v_{max}/cm^{-1} 2602 ($-NH_3^+$), 1697 (C=O), 1609 ($-NH_3^+$), 1543 (N-H), 1215 (CO); MS(ESI) m/z 416.3 $(M+H)^+$; CHN (*2HCl+H_2O) [Found: C, 54.02; H, 7.12; N, 8.13. $C_{23}H_{37}Cl_2N_3O_5$ requires C, 54.55; H, 7.36; N, 8.30 %].

3.4.1 Microbiology

*Microbiological tests were done Professor John Perry at the Freeman Hospital in Newcastle. Spot test screening of chromogenic substrate **141** against a range of clinically relevant Gram-negative bacteria was performed in Columbia agar at a concentration of 50 mg/L. Results were examined after 24*

*h of incubation at 37 °C. Substrate **160** was tested at a concentration of 100 mg/L.*

Results were examined after 24 h of incubation at 50 °C.

4 Summary, Conclusions and Future Work

4.1 Conclusion

4.1.1 Suicide substrates

Bacterial infections represent a major and ongoing problem in healthcare. For a good portion of the past century they have been successfully treated with a good degree of success with a variety of available antibacterial agents. However, resistance against most antibacterial drugs was observed a few years after the introduction of these drugs into clinical practice. Over time, the prevalence of resistant strains that do not respond to a wide range of drugs has become such that patients now succumb to infections that would have been readily treated a decade or two ago. This trend is expected to continue in the decades ahead, until we reach a point in 2050 where potentially more people will die due to bacterial infections than succumb to cancer.

One way to delay, or hopefully even halt this emerging problem is through the evermore selective treatment of bacterial infections. This is possible by targeting only the pathogenic bacteria by narrow spectrum antibacterials. For selective treatment, pathogenic strains causing an infection need to be rapidly and accurately identified in order to deploy the most selective and effective antibacterial agent. Many diagnostic tools exist to identify and detect bacteria, however all of these techniques have their own pros and cons, making a suite of complementary methods desirable in clinical practice. Within the available methods chromogenic culture media provide an inexpensive and relatively rapid method for selective bacterial identification, which does not require specialists or highly trained professionals for operation. Chromogenic culture media are convenient tools, especially in developing countries, where there are less available resources for acquisition and maintenance of high end equipment. This is especially important as these developing nations are at the front-line of the ongoing battle against antimicrobial resistance.

Chromogenic media contains a matrix, which is usually agar, nutrients and food sources depending on the requirements of the targeted bacteria. Chromogenic substrates are used to make certain strains visible by specific

coloration in the presence of specific bacteria providing the basis of the detection. These media also often contain antibacterial agents to prevent the growth of non-relevant bacteria, which improves selectivity.

In this thesis two distinct groups of components of chromogenic culture media were developed. The main focus was to discover new antibacterial agents selectively inhibiting the growth of certain bacteria, thereby increasing the selectivity of the chromogenic substrates used in existing media. However, in addition, new chromogenic substrates were also investigated. These compounds were analogues with a well-established substrate applied in chromID Pseudomonas® chromogenic culture medium.

In the main portion of this research, antibacterial agents targeting the common bacterial enzyme alanine racemase were developed and investigated. Three types of alanine carboxylate bioisosteres were synthesized as potential alanine racemase inhibitors (Figure 42). All molecules were tested against a collection of clinically relevant pathogenic bacteria. As the transportation of a single alanine unit into the cell is not provided by any bacteria, these bioisosteres did not inhibit the growth of any bacteria when deployed as the simple amino acid analogue.

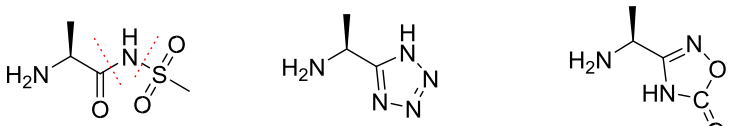


Figure 42 *Alanine bioisosteres as potential alanine racemase inhibitors*

Further peptide derivatives were designed, synthesised, and their antibacterial activity was established. The rationale behind this is that amino acids are known to be transported as di- and oligopeptides into bacterial cells by permease enzymes and subsequently internally liberated by amino peptidase enzymes. L-Alanyl-L-alanylmethanesulfonamide **65-LL/D** and L-1-aminoethyl5-oxo-1,2,4-oxadiazole **72-LL** showed no significant inhibition against the strains tested (Figure 43). One possible reason for this could be due to hydrolytic inactivation by bacterial enzymes at the site of the carboxylate isostere, possible hydrolytic sites on an acylmethanesulfonamide

are shown with red lines on **27-L** (Figure 42). Alternatively, a lack of hydrolysis by peptidase enzymes at the linking peptide bonds in the di and tri peptides would mean that the active substance would never be released, which would also explain activity. Obviously, lack of permeability through a mismatch with the required transporter or the rapid export of the compounds through efflux mechanisms provide alternative potential explanations that would account for the lack of activity too.

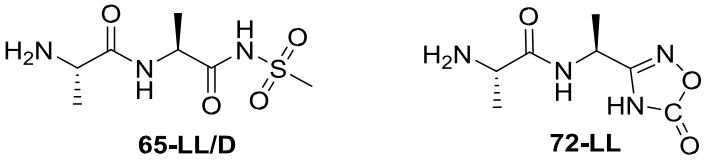


Figure 43 *L-Alanyl-alanylmethanesulfonamide* ***65-LL/D*** *and L-1-aminoethyl-5-oxo-1,2,4-oxadiazole* ***72-LL***

Selected di- and tri- and succinyl oligo-peptide analogues of L-1aminoethyltetrazole were synthesized by both solution and solid phase peptide coupling methods, substrates with antibacterial activity are shown in Figure 44. A novel solid phase synthetic method was introduced for the production of peptides with a C-terminal tetrazole moiety. A Fmoc peptide coupling strategy was found to be successful using 2-chlorotrityl resin and the standard reagents HBTU and DIPEA (Scheme 49). Many of these peptides displayed significant antibacterial activity. Depending upon the attached amino acids, the substrates caused growth inhibition of bacteria with different levels of selectivity. The patterns of activity seen have potential in real world applications. The most significant example is the selective inhibition of *E. coli* and some *Enterobacteriaceae* by L-alanyl- **70a-LL**, L-leucyl- **70h-LL** and dialanyl- **70c-LLL** substrates. These compounds should be investigated in clinical applications, as they could provide a selective antibacterial ingredient in a *Salmonella* selective culture media, as they had no or small effect of the growth of most *Salmonella* spp. The selective inhibition of enterococci while not affecting the growth of other cocci by L-pyroglutamyl dipeptide **70f-LL** is also potentially a valuable result.

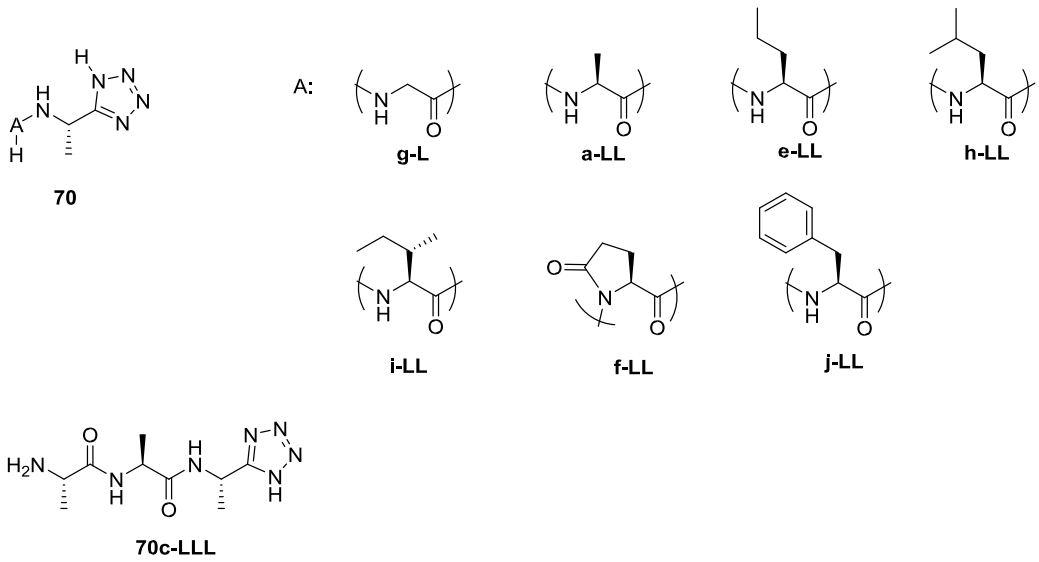
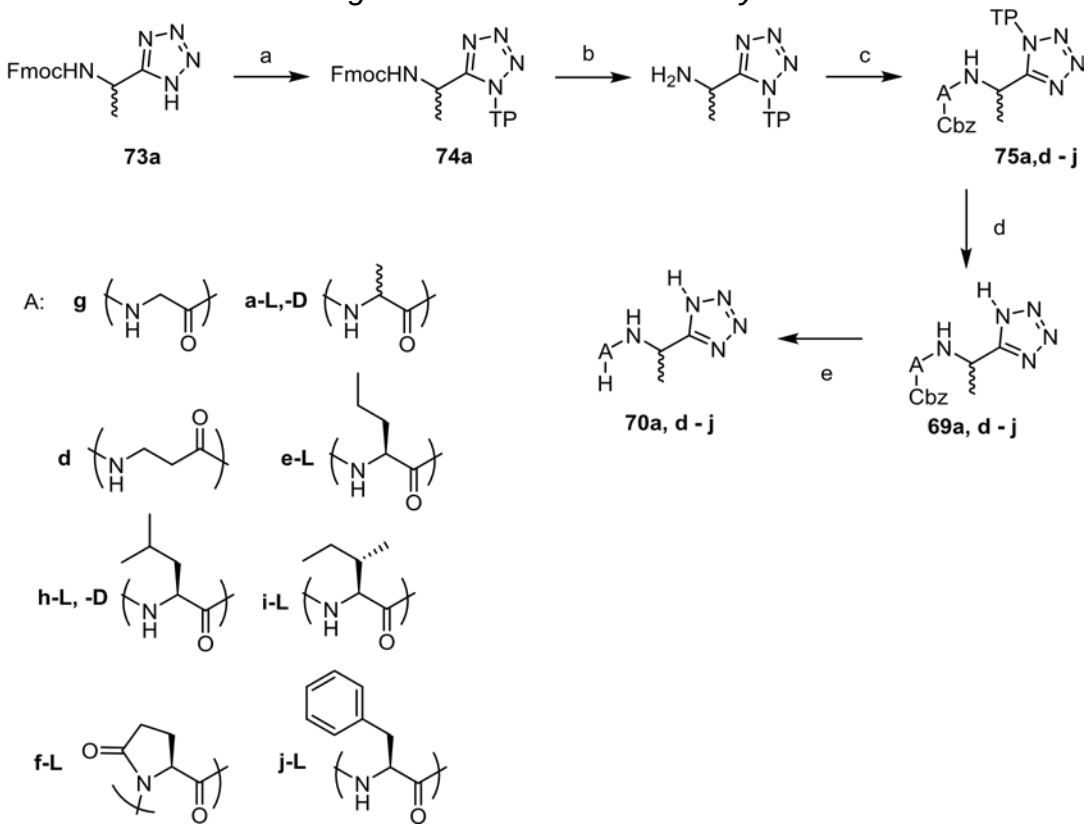


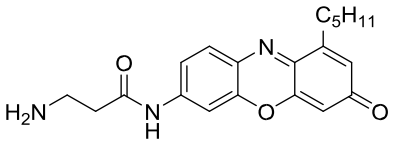
Figure 44 *Di and tripeptide analogues of L-1-aminoethyltetrazole with significant antibacterial activity*



Scheme 49 *Solid phase synthesis of dipeptide derivatives of 1aminoethyltetrazole* ***70a, d - j****. Reagents and conditions: (a) 2-chloro-trityl chloride resin (TP-Cl), DIPEA, DCM (b) piperidine, DMF; (c) Cbz-A-OH, HBTU, DIPEA, DMF; (d) 5% TFA, 95% DCM, 1 eq TIS (equivalent with the theoretical loading of the resin); (e)* $H_{2\,(g)}$*, 5% Pd/C, EtOH, 3 bar.*

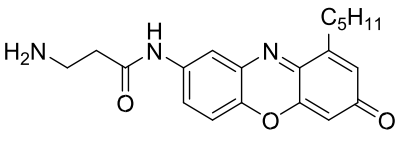
4.1.2 Chromogenic substrates

In the other significant theme within this thesis, new chromogenic substrates for bacterial detection and identification were developed. 7-β-Alanylamido-1pentylphenoxazin-3-one **86a** is a chromogenic substrate (Figure 45), which is applied in chromID Pseudomonas® chromogenic culture medium. A library of differently substituted phenoxazin-3-ones has been prepared previously for the development and optimization of chromogenic properties of the substrates and chromogens. These studies also explored different synthetic strategies which were investigated for more economical and cost effective production. As a missing element of this research, chromogenic substrate 8-□alanylamido-1-pentylphenoxazinone **141** was synthesised by adaption of a well-established synthetic route. The compound obtained was tested against bacteria known to express □-alanyl aminopeptidase (Figure 46). Substrate **141** responded clearly to *S. marcescens* and more weakly to *P. aeruginosa,* albeit with significant background colour. This chromogenic study pointed out that 7amido substrates are preferable to 8-amido substrates in terms of the intensity of the chromogen and the contrast between the chromogen and the chromogenic substrates. Investigation of substrate **141** completed a decade of research work performed by this research group in this area of study.



86a

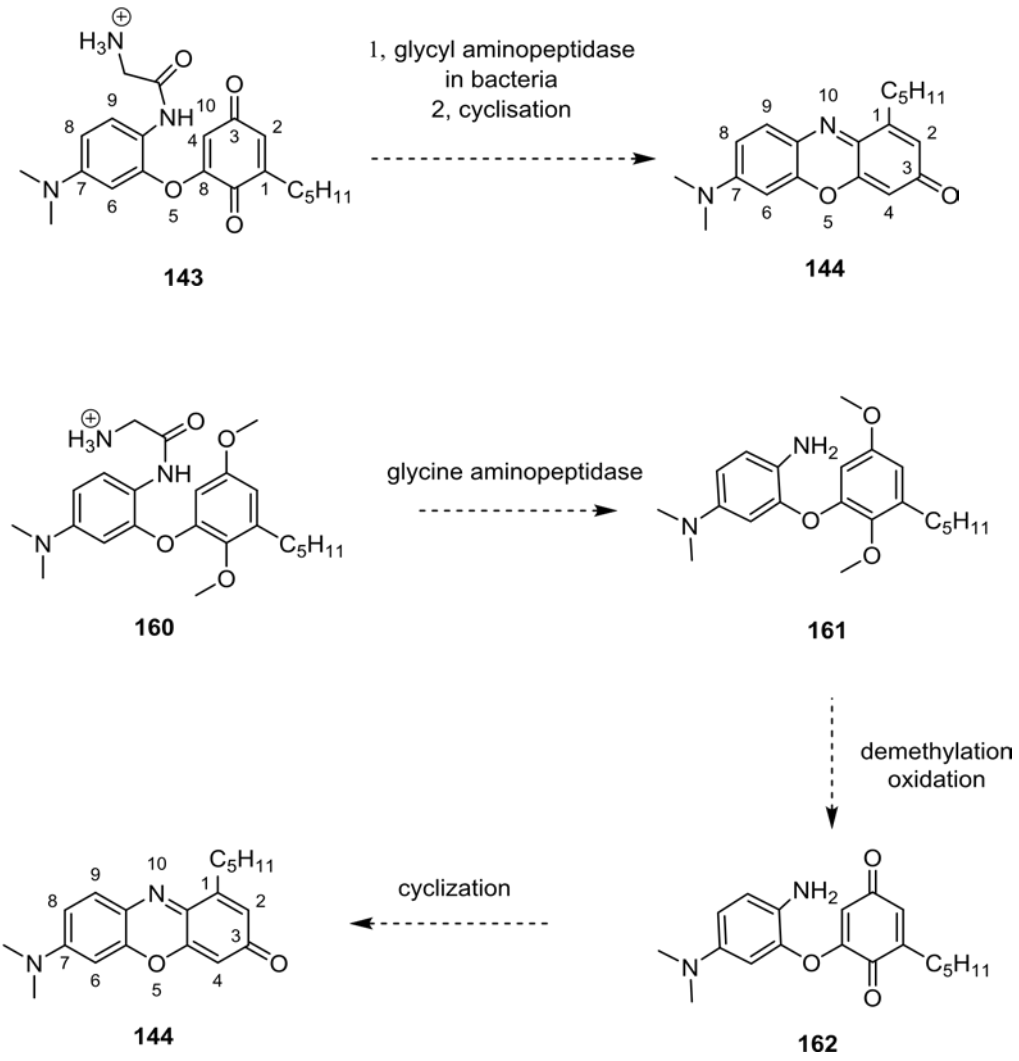
Figure 45 *Reference chromogenic substrate 7-□-alanylamido-1pentylphenoxazinone* ***86a***



141

Figure 46 *Chromogenic substrate 8-□-alanylamido-1-pentylphenoxazinone* ***141***

A more novel type of chromogenic substrate **143**, based on the enzymatically triggered *in situ* formation of a chromogenic phenoxazin-3-one core **144** was designed (Scheme 50). The key aim of this study was a decreased background colour in culture medium in comparison to the chromogenic substrates in utilised in current clinical practice. Unfortunately, the designed substrate **143** could not be isolated under ambient conditions due to stability reasons, which would preclude its use in a clinical setting. Instead, an alternative substrate **160** was made and tested. The effectiveness of compound **160** required a complex enzymatic activity cascade (Scheme 50). This compound did not display significant coloration in any species, although it had relatively strong antibacterial activity against Gram-positive organisms and a few Gram-negative bacteria. This activity could be rationalised by the reactive electrophilic parts displayed transiently by the enzymatically liberated molecule.

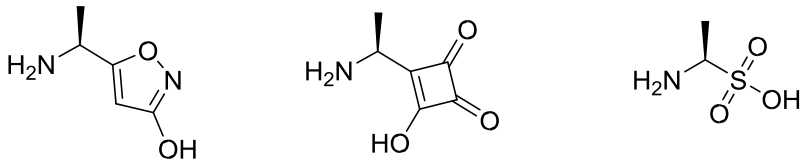


Scheme 50 *Proposed action of planned substrate* ***143*** *and synthesized substrate* ***160***

4.2 Future work

4.2.1 Alalanine bioisosteres

The library of di-, tri- and succinyl oligo-peptide analogues of L-1aminoethyltetrazole can be extended with more peptides, although the candidates thought to have the greatest potential are mentioned in this thesis. More alanine bioisosteres and their peptide derivatives can be investigated, examples of which may include isoxazole **167**, squaric acid **168** or sulfonic acid **169** derivatives (Figure 47).

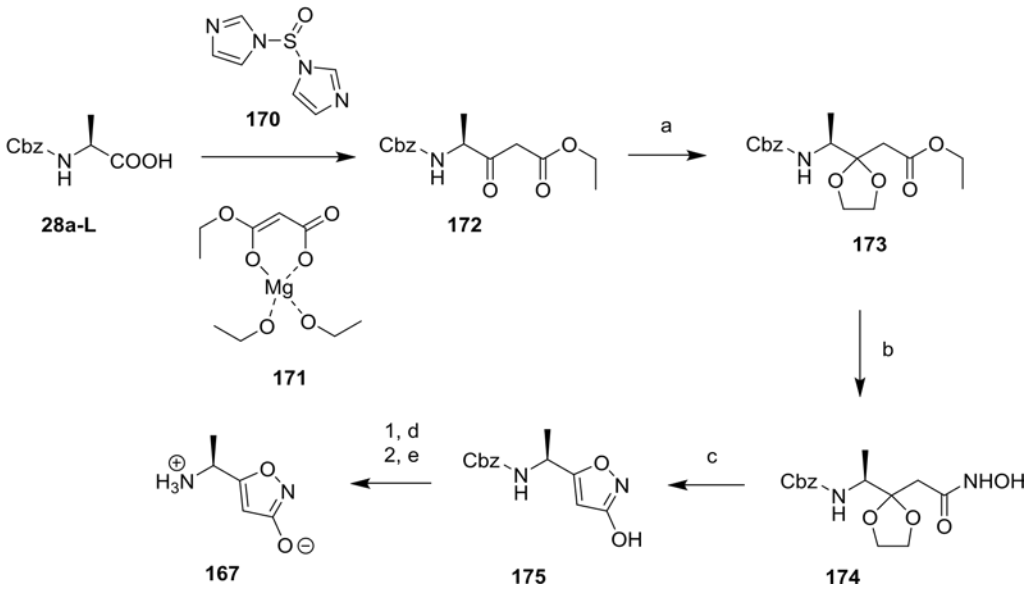


167 **168** **169**

Figure 47 *Alanine bioisosteres for future investigation*

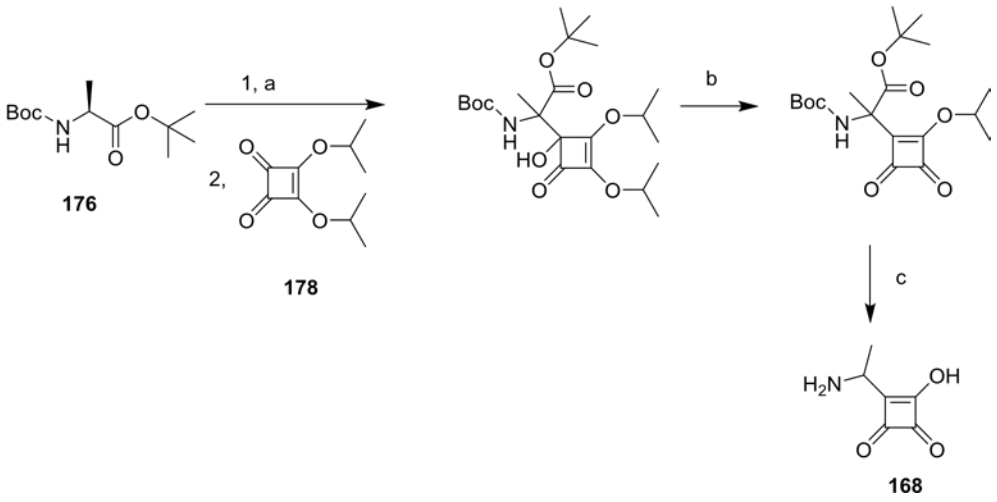
All 3 potential bioisosteres are already known, and have been synthesised by different research groups, albeit for potential applications unrelated to inhibition of AlaR.

In the first step of a known synthesis of L-5-(1-aminomethyl)-3-isoxazole **167**, Cbz-L-alanine **28a-L** was activated with N,N'-thionyldiimidazole **170**, which readily reacted with monoethylmalonate diethoxy magnesium enolate **171**. The addition-elimination type reaction was followed by decarboxylation to give the core molecule of the synthesis, containing a defined stereo centre **172**. After protection of the free keto moiety as a ketal, the ester group in intermediate **173** was transformed to a hydroxylamide motif (**174**). Liberation of the keto group results in a spontaneous intramolecular ring closure giving Cbz-L-5-(1-aminomethyl)-3-isoxazole **175** as the product. The Cbz protection was removed with hydrobromic acid (Scheme 51).[144] The same research group published another method, although this results in the production of the product **167** in a racemic form. [145] This compound was synthesised as a potential GABA agonist, and we are at present unaware of any attempts to test this compound against bacteria.



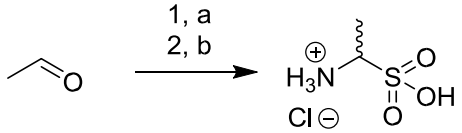
Scheme 51 *Synthesis of L-5-(1-aminomethyl)-3-isoxazole* ***167****.*[144] *Reagents and conditions: (a) ethylene glycol, para-toluenesulfonic acid; (b) NH_2OH, KOH; (c) HCl, MeOH; (d) HBr, acetic acid; (e) TEA.*

1-Aminoethyl squaric acid **168** was prepared as an alanine bioisostere by the Ohfune group for its "general biological potential", such as protease inhibition.[146] The starting compound Boc-L-alanine *tert*-butyl ester **167** was transformed to the corresponding dianionic enolate with *sec*-butyl lithium, which was reacted with 3,4-diisopropoxy-3-cyclobutene-1,2-dione **178**. Further isopropanol elimination, then protecting group hydrolysis and decarboxylation resulted in the isolation of 1-aminoethyl squaric acid **168** as the racemic mixture (Scheme 52).[146]



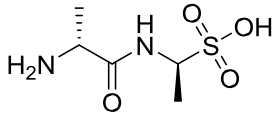
Scheme 52 *Synthesis of 1-aminoethyl squaric acid* ***168****.*[146] *Reagents and conditions: (a) 2 eq sec-BuLi, THF, -78 °C, 1 h; (b) catalytic HCl, DCM, r.t., 12 h; cc HCl, acetone, r.t., 12 h.*

Aminomethanesulfonic acid **169** was synthesised by the reaction of acetaldehyde **20** and sodium bisulfite in an aqueous ammonia solution (Scheme 53).[147,148] D-alanyl-D-1-aminoethanesulfonic acid **179** were investigated as synthetic D-alanyl-D-alanine peptidoglycan building block mimetics targeting the inhibition of cell wall biosynthesis, although it found to be inactive against studied bacterial strains (Figure 48).[147]



20 **169**

Scheme 493 *Synthesis of aminomethanesulfonic acid* ***169****.* [147,148] *Reagents and conditions: (a) $NaHSO_3$, NH_4OH; (b) HCl.*



179

Figure 48 *D-alanyl-D-1-aminoethanesulfonic acid* ***179***

4.2.2 Kinetic and stereochemical study of AlaR inhibitors

Our working assumption is that all of these molecules would exhibit their action through AlaR inhibition. A kinetic profile of our alanine bioisosteres can be investigated on a purified sample of the target enzyme AlaR in a manner similar to investigations done with fosfalin (Chapter 2.1.2). A time dependent, qualitative study of the transformation of L-alanine to D-alanine in the presence of the isolated AlaR enzyme and the chosen inhibitor would be possible by a chiral HPLC technique, and would provide an accurate kinetic profile of the inhibition. As many of these compounds, including the natural substrate, do not contain a useful chromophore, MS would be a better means of following the reactions and inhibition rather than using UV-vis detectrion.

Di- and oligopeptide derivatives need to be cleaved by aminopeptidase reactions to liberate the appropriate bioisostere. The selectivity and sensitivity of these enzymes towards the different amino acid substrates can be investigated for the better understanding of these results. L-1aminoethyltetrazole substrates can be exposed to chosen examples of isolated alanine, □-alanine, leucine or pyroglutamine aminopeptidase enzymes. The time dependent liberation of L-1-aminoethyltetrazole could be tracked by HPLC-MS.

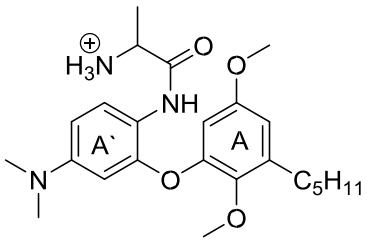
The success of the active transport of these molecules can be studied by filter binding assays. A selected permease, for example Dpp, can be treated with a solution of a selected substrate. The change in the substrate concentration after filtration would indicate the extent of binding, which could be followed by HPLC measurements.

In additional future work, quantitative and qualitative stereochemical information can be investigated and confirmed, focusing on the stereochemical outcome of the solid phase synthesis of L-1-aminoethyltetrazole containing peptides, and a detailed analysis of the relative extent of biological activity for each stereoisomer. As all natural enzymes are chiral objects, isomers in enantiomeric or diastereomeric relationships are expected to have different effects on permeases, aminopeptidases and AlaR. Furthermore, as diastereomers may exhibit different physicochemical properties in terms of log*P* and pK_a, differences in ionisation or lipophilicity may play non-specific roles affecting passive diffusion, or protein binding. Experimental data to establish these physicochemical properties would be useful for understanding the differences between the biological effects and for further application of these molecules. Taken together, this set of studies could help to explain the differences in biological results between the same compounds **70f-LL**, **70c-LLL** that were observed when they were made through different synthetic methods. The stereochemical effects relating to activity can largely be studied by chiral HPLC techniques. The enantiomeric excess of the synthesised L- and D-1-aminoethyltetrazoles **32** and the starting compounds **28** would show the extent of stereoselectivity of the two main

synthetic routes. Using knowledge gleaned relating to the configuration of 1aminoethyltetrazole **32** the stereo selectivity of the solid phase synthesis can be investigated by measuring the diastereomeric excess of the Cbz protected peptides. An X-ray study of 1-aminoethyltetrazole **32** and some dipeptides would also help to prove the stated configurations. Where enantiomerically pure compounds are required for further study, they can be separated by resolution using chiral acids, such as the widely used tartaric acid. Dipeptide diastereomers can be separated using either chromatography or recrystallization.

4.2.3 Antibacterial investigation of certain chromogenic substrates

The scaffold of **160** can be attached to other linkers, to investigate more specific antibacterial activity (Figure 49). One such example would be the corresponding L-alanine **179** derivative, which is likely to display differences in activity between Gram-positive and Gram-negative organisms. However, significant chromogenic activity is not expected from such derivatives based on the results of this research. In addition, the molecule required is time consuming and relatively expensive to produce, therefore, if any interesting antibacterial effects were to be discovered, the logical next steps would be to establish the mechanism of action of this compound through structure activity relationship studies. This would potentially allow us to produce simple antibacterial agents that would be cost effective to produce, while allowing scope for elaboration in a further wave of studies to tailor these compounds towards selective antibacterial action.



179

Figure 49: Substrate **179**

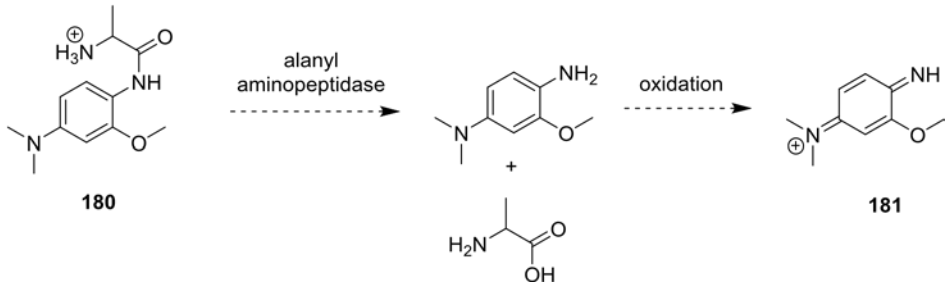
Further investigations regarding the mechanism of antibacterial action of these molecules (**160, 179**) would help us to understand their biological effects. This in turn would allow us to rationally optimize the structure for reaching the best biological effects. This type of study would be good to pursue in the years ahead as there is a critical need for new antimicrobial agents due to the emerging threat of resistance to the antimicrobial agents currently in routine use.

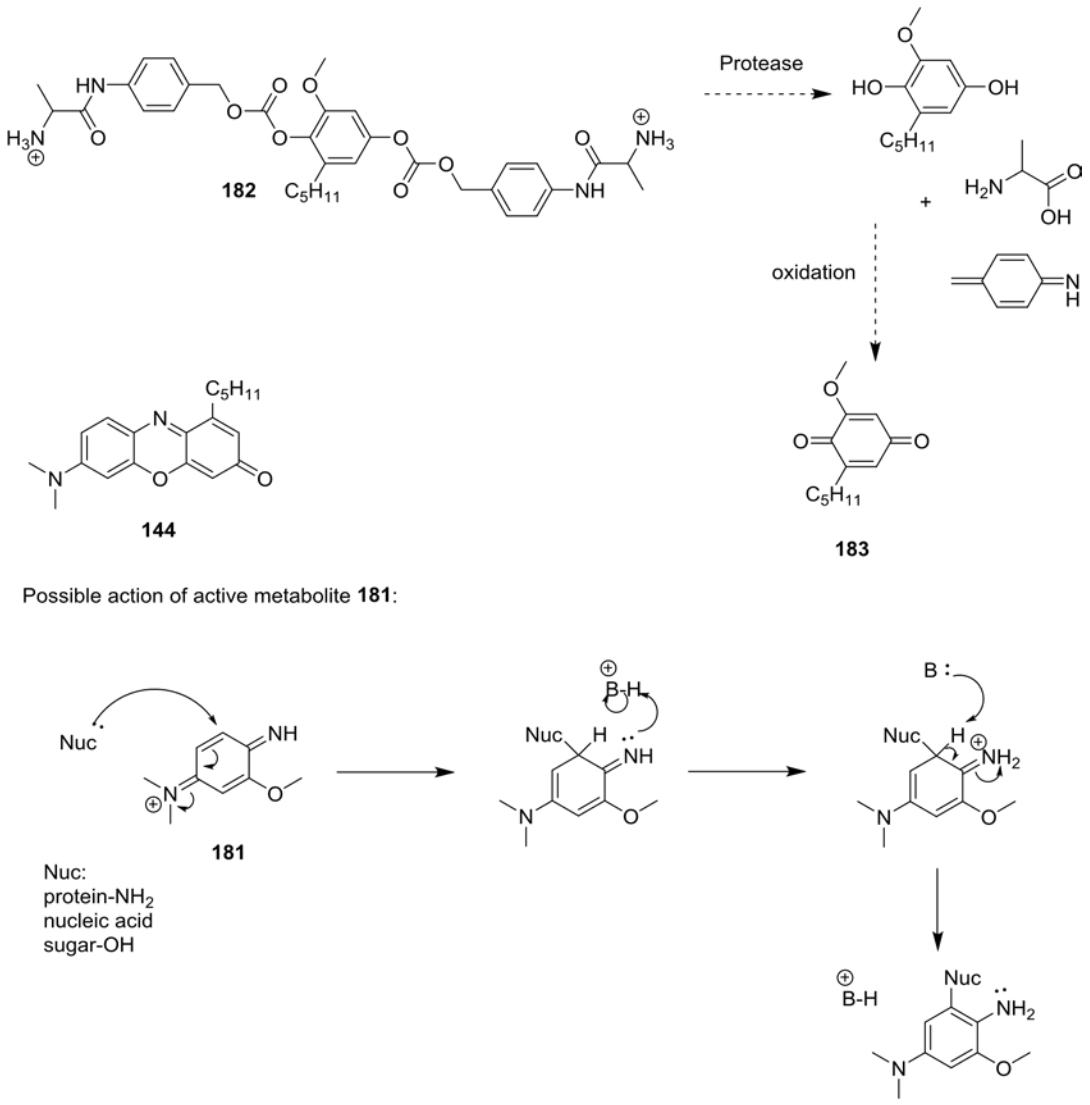
Several research questions can be posed to be answered in such an investigation. What is the active molecule form; is it the fully formed phenoxazinone, or a reactive intermediate? If the latter, which unit is responsible for the antimicrobial effect? Some suggestions include an electrophilic quinone or a quinone-like iminium salt. These questions could be answered by biological trials of certain modified molecules (Scheme 54). The toxicity of ‘A`’ ring can be investigated by using substrate **180**, which would liberate a potentially toxic iminium salt **181** in the presence of alanyl aminopeptidase and oxidase enzymes. In a separate study, the toxicity of ring ‘A’ can be studied with substrate **182**, in the presence of protease and oxidase activity a potentially toxic agent **183** would be liberated. In further variations introducing a 4-amidobenzyloxy linker would be interesting to pursue. This is a well-established strategy[149] used to mask hydroxyl or amino groups, and would allow us to apply these substrates in a prodrug form. Active metabolites **181** and **183** are both electrophiles, which can easily react with natural nucleophiles, such as amino groups or nucleophilic side-chains of proteins, sugars, or perhaps most crucially, nucleic acids. These metabolites can react in similar way to that shown in the example of **181** (Scheme 54), which is based on the analogous reactive intermediate NAPQI, which is thought to be the reactive metabolite that causes most harm in cases of paracetamol overdose.[150]

These compounds might also have toxic effects on humans, as these electrophilic metabolites could also react with mammalian biomolecules, in a manner directly related to the toxicity of paracetamol in cases of overdose. If this is indeed the case, it would significantly limit their application as medicinal

products intended to treat bacterial infections. However, this in no way precludes their other potential utility in diagnosis of bacterial infections. That being said, if an effective and selective prodrug strategy is found it may be possible for the liberated electrophile to be present only in bacterial cells, which would potentially allow for them to be used in treating infections, but probably only in cases of last resort.

At this stage we cannot rule out the case where the formation of the final phenoxazinone **144** from substrate **160** or **179**, may have toxic effects too. One reason why this may indeed be the case are the existence of the antimicrobial natural products known as the actinomycins **114.** These compounds bind to the bacterial DNA, although it should be pointed out that these molecules have two large peptide units which can also interact with the DNA as well as the phenoxazinone moiety.[140] However, it should also be mentioned that a similar antibacterial profile was observed for some other individual phenoxazinone based substrates, such as substrates **86a** and **163** which were prepared and investigated in earlier studies by our research group.[135]





***Scheme 54**: Compounds **180**,**182**, **144** designed for the investigation of the mechanism of antibacterial action and the proposed active metabolites **181**, **183** and the proposed reactivity of active metabolite **181**.*

To conclude, we now find ourselves in the midst of an ongoing battle with new pathogenic strains of bacteria that are increasingly showing themselves to be impervious to the effects of some of our most effective antibacterial agents. This is largely brought about by Darwinian evolution, wherein inappropriate bacteria are exposed to relatively low doses of these drugs. One way to combat this is through rapid and economical diagnosis of bacterial infections, with the aim being that the correct drug is given to each patient. The work described in this thesis outlines two distinct methods by which improved diagnosis may be possible, through selective inhibition of certain bacterial

strains and species, and through the development of selective chromogens that respond to enzymes found only in certain bacterial species. The war we are fighting may never be won without a paradigm change in the technology available, but through efforts such as this we may stem the tide of resistance, and thereby help to save untold numbers of lives.

5 References

(1) Dantas, G.; Sommer, M. O. A. *Am. Sci.* **2014**, *102*, 42.

(2) O`Neill, J. “Tackling drug-resistant infections globally: final report and recommendations,” 2016.

(3) Bartlett, J. G. *Clin. Infect. Dis.* **2011**, *53*, S4.

(4) Murray, P. R.; Rosenthal, K. S.; Pfaller, M. A. *Medical Microbiology*; Elsevier, 2013.

(5) McFall-Ngai, M. *Nat.* **2007**, *445*, 153.

(6) Rice, L. B. *J. Infect. Dis.* **2008**, *197*, 1079.

(7) Pendleton, J. N.; Gorman, S. P.; Gilmore, B. F. *Expert Rev. AntiInfect. Ther.* **2014**, *11*, 297.

(8) Ryan, K. J.; Ray, C. G. *Sherris MEDICAL MICROBIOLOGY*; 4th ed., 2004.

(9) Antunes, L. C. S.; Visca, P.; Towner, K. J. *Pathog. Dis.* **2014**, *71*, 292.

(10) Hsieh, S.-Y.; Tseng, C.-L.; Lee, Y. S.; Kuo, A. J.; Sun, C. F.; Lin, Y. H.; Chen, J. K. *Mol. Cell. Proteomics* **2008**, *7*, 448.

(11) Yousef, A. E. *Detection of Bacterial Pathogens in Different Matrices: Current Practices and Challenges: Principles of Bacterial Detection.*; Springer Science, 2008.

(12) Perry, J. D. *Clin. Microbiol. Rev* **2017**, *30*, 449.

(13) Park, C. E.; Rayman, M. K.; Szabo, R.; Stankiewicz, Z. K. *Can. J. Microbiol.* **1976**, *22*, 654.

(14) Park, C. E.; Rayman, M. K.; Stankiewicz, Z. K. *Can. J. Microbiol.* **1977**, *23*, 563.

(15) J. D. Perry; Riley, G.; Gould, F. K.; Perez, J. M.; Boissier, E.; Ouedraogo, R. T.; Freydiere, A. M. *J. Clin. Microb.* **2002**, *40*, 3913.

(16) Conti, P.; Tamborini, L.; Pinto, A.; Blondel, A.; Minoprio, P.; Mozzarelli, A.; Micheli, C. D. *Chem. Rev.* **2011**, *111*, 6919.

(17) Stamper, C. G. F.; Morollo, A. A.; Ringe, D. *Biochemistry* **1998**, *37*, 10438.

(18) Shaw, J. P.; Petsko, G. A.; Ringe, D. *Biochemistry* **1997**, *36*, 1329.

(19) Morollo, A. A.; Petsko, G. A.; Ringe, D. *Biochemistry* **1999**, *38*, 3293.

(20) Watanabe, A.; Yoshimura, T.; Mikami, B.; Hayashi, H.; Kagamiyama, H.; Esaki, N. *J. Biol. Chem.* **2002**, *277*, 19166.

(21) Yoshimura, T.; Goto, M. *FEBS Journal* **2008**, *275*, 3527.

(22) Sun, S.; Toney, M. D. *Biochemistry* **1999**, *38*, 4058.

(23) Stroud, E. D.; Fife, D. J.; Smith, G. G. *J. Org. Chem* **1983**, *48*, 5368.

(24) Dixon, J. E.; Bruice, T. C. *Biochemistry* **1973**, *12*, 4762

(25) Rubinstein, A.; Major, D. T. *Biochemistry* **2010**, *49*, 3957.

(26) Major, D. T.; Gao, J. *J. Am. Chem. Soc.* **2006**, *128*, 16345.

(27) Spies, M. A.; Woodward, J. J.; Watnik, M. R.; Toney, M. D. *J. Am. Chem. Soc.* **2004**, *126*, 7464.

(28) Wu, D.; Hu, T.; Zhang, L.; Chen, J.; Du, J.; Ding, J.; Jiang, H.; Shen, X. *Prot. Sci* **2008**, *17*, 1066.

(29) Tassoni, R.; Aart, L. T. v. d.; Ubbink, M.; Wezel, G. P. v.; Pannu, N. S. *Biochemical and Biophysical Research Communications* **2017**, *483*, 122.

(30) Lee, H. K. *Chest* **1960**, *37*, 378.

(31) Neuhaus, F. C. *Antimicrob. Agents Chemother.* **1967**, *7*, 304.

(32) http://www.who.int/medicines/publications/essentialmedicines/EML_2015_FINAL_amended_NOV2015.pdf?ua=1; 19th ed. 2015.

(33) Kollonitsch, J.; Barash, L.; Kahan, F. M.; Kropp, H. *Nat.* **1973**, *243*, 346.

(34) Badet, B.; Roise, D.; Walsh, C. T. *Biochemistry* **1984**, *23*, 5188.

(35) Faraci, W. S.; Walsh, C. T. *Biochemistry* **1989**, *28*, 431.

(36) Thornberry, N. A.; Bull, H. G.; Taub, D.; Greenlee, W. J.; Patchett, A. A.; Cordes, E. H. *J. Am. Chem. Soc.* **1987**, *109*.

(37) Badet, B.; Inagaki, K.; Soda, K.; Walsh, C. T. *Biochemistry* **1986**, *25*, 3275.

(38) Duncan, K.; Faraci, W. S.; Matteson, D. S.; Walsh, C. T. *Biochemistry* **1989**, *28*, 3541.

(39) Azam, M. A.; Jayaram, U. *J. Enzyme Inhib. Med. Chem* **2016**, *31*, 517.

(40) Copie, V.; Faraci, W. S.; Walsh, C. T.; Griffin, R. G.

Biochemistry **1988**, *27*, 4966.

(41) Gilvarg, C. In *Ciba Foundation Symposium 4 - Peptide Transport in Bacteria and Mammalian Gut*; Gilvarg, C., Ed. 1972.

(42) Diddens, H.; Zahner, H. *Eur. J. Biochem.* **1976**, *66*, 11.

(43) Smith, M. W.; Tyreman, D. R.; Payne, G. M.; Marshall, N. J.; Payne, J. W. *Microbiology* **1999**, *145*, 2891.

(44) Gilvarg, C.; Katchalski, E. *J. Biol. Chem.* **1965**, *240*, 1965.

(45) Parkin, J.; Khalid, S. *Biophysic. J.* **2014**, *107*, 1853

(46) Kadner, R. J.; Watson, W. J. *J. Bacteriol.* **1974**, *119*, 401.

(47) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 677.

(48) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Holmes, S. W.; Lambert, R. W.; Lloyd, W. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.*

1980, *18*, 897.

(49) Allen, J. G.; Atherton, F. R.; Hall, M. J. *Nat.* **1978**, *272*, 56.

(50) Atherton, F. R.; Hassall, C. H.; Lambert, R. W. *J. Med. Chem.*

1986, *29*, 29.

(51) NG, K. T. *PhD thesis* **2016**, *University of Sunderland*.

(52) Smissman, E. E.; Terada, A.; El-Antably, S. *J. Med. Chem.*

1976, *19*, 165.

(53) Morley, J. S.; Payne, J. W.; Hennessey, T. D. *J. Gen. Microb.*

1983, *129*, 3701.

(54) Kudzin, Z. H.; Stec, W. J. *Synthesis* **1978**, 469.

(55) Oleksyszyn, J.; Tyka, R.; Mastalerz, P. *Synthesis* **1978**, 479.

(56) Roberts, T. C.; Smith, P. A.; Campbell, D.; Duron, S. G.;

Higuchi, R. I. In *WO2012/166665 A2* 2012; Vol. WO2012/166665 A2.

(57) Bavetsias, V.; Marriott, J. H.; Melin, C.; Kimbell, R.; Matusiak, Z. S.; Boyle, F. T.; Jackman, A. L. *J. Med. Chem.* **2000**, *43*, 1910.

(58) Grzonka, Z.; Liberek, B. *Rocz. Chem.* **1971**, *45*, 967.

(59) Grzonka, Z.; Liberek, B. *Tetrahedron* **1971**, *27*, 1783.

(60) Zhao, T.; Kurpiewska, K.; Kalinowska-Tluscik, J.; Herdtweck, E.; Domling, A. *Chem. Eur. J.* **2016**, *22*, 3009.

(61) Jaradat, D. M. M. *Amino Acids* **2018**, *50*, 39

(62) Han, Y.; Albericio, F.; Barany, G. *J. Org. Chem* **1997**, *62*, 4307.

(63) Chan, W. C.; White, P. D. *Fmoc solid phase peptide synthesis*, 2000.

(64) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

(65) Moss, J. A. *Curr. Protoc. Protein. Sci.* **2005**, *40*, 18.7.1

(66) Troll, W.; Cannan, R. K. *J. Biol. Chem.* **1952**, *200*, 803.

(67) Hancock, W. S.; Battersby, J. E. *Anal. Biochem* **1976**, *71*, 260.

(68) Keglevich, G.; Balint, E. *Molecules* **2012**, *17*, 12821.

(69) Atherton, F. R.; Hassall, C. H.; Lambert, R. W. *J. Med. Chem.* **1986**, *29*, 29.

(70) Severinsen, R.; Lau, J. F.; Bondensgaard, K.; Hansen, B. S.; Begtrup, M.; Ankersen, M. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 317.

(71) Matthews, D. P.; Green, J. E.; Shuker, A. J. *J. Comb. Chem.* **2000**, *2*, 19.

(72) Cousaert, N.; Willand, N.; Gesquiere, J.-C.; Tartar, A.; Deprez, B.; Deprez-Poulain, R. *Tetrahedron Lett.* **2008**, *49*, 2743.

(73) Yoo, S.-e.; Seo, J.-s.; Yi, K.-y.; Gong, Y.-d. *Tetrahedron Lett.* **1997**, *38*, 1203.

(74) Lassalas, P.; Gay, B.; Lasfargeas, C.; James, M. J.; Tran, V.; Vijayendran, K. G.; Burden, K. R.; Kozlowski, M. C.; Thomas, C. J.; Smith, A. B.; Huryn, D. M.; Ballatore, C. *J. Med. Chem.* **2016**, *59*, 3183.

(75) Dey, B. P.; Lahiri, S. C. *Ind. J. Chem. Section A* **1988**, *27*, 297.

(76) Naef, R. *Molecules* **2015**, *20*, 18279.

(77) Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M. *Data for Biochemical Research*, 1989.

(78) Vo-Quang, Y.; Carniato, D.; Vo-Quang, L.; Lacoste, A.-M.; Neuzil, E.; Goffic, F. L. *J. Med. Chem.* **1986**, *29*, 579.

(79) Ishiyama, H.; Yoshizawa, K.; Kobayashi, J. *Tetrahedron* **2012**, *68*, 6186.

(80) Viglio, S.; Luisetti, M.; Zanaboni, G.; Doring, G.; Worlitzsch, D.; Cetta, G.; Iadarola, P. *J. Chrom. A* **1999**, *846*, 125.

(81) Komeda, H.; Asano, Y. *FEBS Journal* **2005**, *272*, 3075.

(82) Bedernjak, A. F. *PhD thesis* **2010**, *University of Sunderland*.

(83) Doki, S.; Kato, H. E.; Solcan, N.; Iwaki, M.; Koyama, M.; Hattori,

M.; Iwase, N.; Tsukazaki, T.; Sugita, Y.; Kandori, H.; Newstead, S.; Ishitani, R.; Nureki, O. *PNAS* **2013**, *110*, 11343.

(84) Mineyama, R.; Saito, K. *Microbios* **1998**, *94*, 47.

(85) Unpublished to date; Vol. FR1758830.

(86) Mundinger, S.; Jakob, U.; Bannwarth, W. *Chem. Eur. J.* **2014**, *20*, 1258.

(87) Mijovic, M. P. V.; Walker, J. *J. Chem. Soc.* **1960**, 909.

(88) Chen, S.-T.; Wu, S.-H.; Wang, K.-T. *Synthesis* **1989**, *1*, 37.

(89) Yamada, T.; Suegane, K.; Kuwata, S.; Watanabe, H. *Bull. Chem. Soc. Jpn* **1977**, *50*, 1088.

(90) Manturewicz, M.; Kosson, P.; Grzonka, Z. *Pol. Chem. J.* **2007**, *81*, 1327.

(91) Itaya, T.; Shimizu, S.; Nakagawa, S.; Morisue, M. *Chem. Pharm. Bull.* **1994**, *42*, 1927

(92) Jia, Z. J.; Song, Y.; Xu, Q.; Kane, B.; Bauer, S. M.; Pandey, A. 2012; Vol. WO2012061418 (A2).

(93) Mengelberg *Chem. Ber.* **1956**, *89*, 1185.

(94) Das, B.; Damodar, K.; Shashikanth, B.; Srinivas, Y.; Kalavthi, I. *Synlett* **2008**, *20*, 3133.

(95) Tsuyoshi, M.; Taisaku, T.; Hiroshi, M.; Seiichi, I.; Yoshiaki, T.; Koji, S.; Tsutomu, Y. 2009; Vol. EP2025672 (A1).

(96) Kuttan, A.; Nowshudin, S.; Rao, M. N. A. *Tetrahedron Lett.* **2004**, *45*, 2663.

(97) Surprenant, S.; Lubell, W. D. *Journal of Organic Chemistry* **2006**, *71*, 848

(98) Houssin, R.; Lohez, M.; Bernier, J. L.; Henichart, J. P. *J. Org. Chem* **1985**, *50*, 2787

(99) Zhu, J. L.; Lee, F. Y.; Wu, J. D.; Kuo, C. W.; Shia, K. S. *Synlett* **2007**, *8*, 1317

(100) Sureshbabu, V. V.; Hemantha, H. P.; Naik, S. A. *Tetrahedron Lett.* **2008**, *49*, 5133.

(101) Ariyoshi, Y. *Bull. Chem. Soc. Jpn* **1984**, *57*, 3197.

(102) Jahani, F.; Tajbakhsh, M.; Golchoubian, H.; Khaksar, S.

Tetrahedron Lett. **2011**, *52*, 1260.

(103) Su-Sun, W. 1978; Vol. US4116951 (A).

(104) Andrews, J. M. *J. Antimicrob. Chemother.* **2001**, *48*, 5.

(105) Varadi, L. *PhD thesis* **2012**, *University of Sunderland*. (106) Varadi, L.; Luo, J. L.; Hibbs, D. E.; Perry, J. D.; Anderson, R. J.; Orenga, S.; Groundwater, P. W. *Chem. Soc. Rev.* **2017**, *46*, 4818.

(107) Cooke, V. M.; Miles, R. J.; Price, R. G.; Richardson, A. C. *Appl. Env. Microbiol.* **1999**, *65*, 807.

(108) Orenga, S.; James, A. L.; Manafi, M.; Perry, J. D.; Pincus, D. H. *J. Microbiol. Methods* **2009**, *79*, 139.

(109) Mazoyer, M. A.; Orenga, S.; Doleans, F.; Freney, J. *J. Clin. Microb.* **1995**, *33*, 1025.

(110) Dijk, S. v.; Bruins, M. J.; Ruijs, G. J. H. M. *J. Clin. Microb.* **2009**, *47*, 456.

(111) Delisle, G. J.; Ley, A. *J. Clin. Microb.* **1989**, *27*, 778.

(112) Perry, J. D.; Butterworth, L. A.; Nicholson, A.; Appleby, M. R.; Orr, K. E. *J. Clin. Pathol.* **2003**, *56*, 528.

(113) Sadler, D. F.; Ezzell, J. W.; Keller, K. F.; Doyle, R. J. *J. Clin. Microb.* **1984**, *19*, 594.

(114) Perry, J. D.; Rennison, C.; Butterworth, L. A.; Hopley, A. L. J.; Gould, F. K. *J. Clin. Microb.* **2003**, *41*, 5695.

(115) James, A. L.; Perry, J. D.; Chilvers, K.; Robson, I. S.; Armstrong, L.; Orr, K. E. *Lett. Appl. Mycrobiol.* **2000**, *30*, 336.

(116) Basacomb, S. *Methods Microbiol.* **1988**, *19*, 105.

(117) Wolf, P. L.; Horwitz, J.; Mandeville, R.; Vazquez, J.; Muehll, E. v. d. *Tech. Bull. Reg. Med. Tech* **1969**, *39*, 83.

(118) Gonzales, T.; Robert-Baudouy, J. *FEMS Microbiol. Rev.* **1996**, *18*, 319.

(119) Laine, L.; Perry, J. D.; Lee, J.; Oliver, M.; James, A. L.; Foata, C. D. L.; Halimi, D.; Orenga, S.; Galloway, A.; Gould, F. K. *J. Cystic Fibrosis* **2009**, *8*, 143.

(120) Cerny, G. *Eur. J. Appl. Microbiol.* **1976**, *3*, 223.

(121) Butterworth, L. A.; Perry, J. D.; Davies, G.; Burton, M.; Reed, R. H.; Gould, F. K. *J. Appl. Microbiol.* **2004**, *96*, 170.

(122) Kiernan, J. A. *Biotech. histochem.* **2007**, *82*, 73.

(123) Pocsai, I.; Taylor, S. A.; Richardson, A. C.; Aamlid, K. H.; Smith, B. V.; Price, R. G. *Clin. Chem.* **1990**, *36*, 1884.

(124) Cooke, V. M.; Miles, R. J.; Price, R. G.; Midgley, G.; Khamri, W.; Richardson, A. C. *Appl. Env. Microbiol.* **2002**, *68*, 3622.

(125) Bainbridge, B. W.; Mathias, N.; Price, R. G. *FEMS microbil. lett.* **1991**, *80*, 319.

(126) James, A. L.; Chilvers, K. F.; Perry, J. D.; Armstrong, L.; Gould, F. K. *Appl. Env. Microbiol.* **2000**, *66*, 5521.

(127) Anderson, R. J.; Groundwater, P. W.; Huang, Y.; James, A.; Orenga, S.; Rigby, A.; Roger-Dalbert, C.; Perry, J. D. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 832.

(128) James, A. L.; Perry, J. D.; Rigby, A.; Stanforth, S. P. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1418.

(129) James, A. L.; Perry, J. D.; Ford, M.; Armstrong, L.; Gould, F. K. *Appl. Env. Microbiol.* **1996**, *62*, 3868.

(130) James, A. L.; Perry, J. D.; Ford, M.; Armstrong, L.; Gould, F. K. *J. Appl. Microbiol.* **1997**, *82*, 532.

(131) James, A. L.; Yeoman, P. *Zbl. Bakt. Hyg. A* **1988**, *267*, 316.

(132) Zaytsev, A. V.; Anderson, R. J.; Bedernjak, A.; Groundwater, P. W.; Huang, Y.; Perry, J. D.; Orenga, S.; Roger-Dalbert, C.; James, A. In *Org. Biomol. Chem.* 2008; Vol. 6, p 682.

(133) Anderson, R. J.; Groundwater, P. W.; James, A.; Monget, D.; Zaytsev, A. V. In *PTC Patent EP 2012/1786828 B1.* 2012.

(134) Desmoceaux, M.; Monget, D. In *PTC Patent EP 2006/1235927 B1.* 2006.

(135) Bedernjak, A. F.; Zaytsev, A. V.; Babolat, M.; Cellier, M.; James, A. L.; Orenga, S.; Perry, J. D.; Groundwater, P. W.; Anderson, R. J. *J. Med. Chem.* **2016**, *59*, 4476.

(136) Zaytsev, A. *PhD thesis* **2006**.

(137) Santoro, P.; Parisi, G. *J. Exp. Zool.* **1986**, *239*, 169.

(138) Grienberg, J.; Honkanen, E.; Patoharju, O. *Acta Chem. Scand.* **1957**, *11*, 1485.

(139) Smânia, E. d. F. A.; Smânia Júnior, A.; Loguercio-Leite, C. *Revista de Microbiologia* **1998**, *29*, 317.

(140) Hollstein, U. *Chem. Rev.* **1974**, *74*, 625.

(141) Anderson, R. J.; Groundwater, P. W.; James, A.; Monget, D.; Zaytsev, A. V. In *WO 2006/030119 A1* 2006.

(142) Willstatter, R.; Mayer, E. *Ber. Dtsch. Chem. Ghes.* **1904**, 1498.

(143) Ramesh, S.; Nagarajan, R. *J. Org. Chem* **2013**, *78*, 545.

(144) Larsen, P. K.; Larsen, A. L. N.; Thyssen, K. *Acta Chem. Scand.* **1978**, *B 32*, 469

(145) Krogsgaard-Larsen, P.; Christensen, A. B. *Acta Chem. Scand.* **1974**, *28*, 636.

(146) Ishida, T.; Shinada, T.; Ohfune, Y. *Tetrahedron Lett.* **2005**, *46*, 311.

(147) Shiba, T.; Miyoshi, K.; Kusumoto, S. *Bull. Chem. Soc. Jpn* **1977**, *50*, 254.

(148) Yang, K.-W.; Golich, F. C.; Sigdel, T. K.; Crowder, M. W. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5150.

(149) Chang, P. V.; Dube, D. H.; Sletten, E. M.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2010**, *132*, 9516.

(150) Lemke, T. L.; Williams, D. A.; Roche, V. F.; Zito, S. W. *Foye`s Principles of Medicinal Chemistry*; 7th ed., 2013.