Toward the Development of \(\gamma\)-Lactones and \(\gamma\)-Lactams as Protease Inhibitors

Thesis presented for the award of Ph.D degree by

Nessa S. Mullane BSc.

Supervisor: Dr. Timothy Smyth

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Declaration

The work presented in this thesis is the original work of the author and due reference has been made, when necessary, to the work of others. No part of this thesis has previously been submitted to this or any other university.

____________________________
Nessa Mullane
Abstract

A select set of γ-lactones, γ-lactams and related bicyclic γ-lactams - pyrrolothiazole[2,1b]thiazoles - were designed, synthesised and evaluated enzymatically as inhibitors of the serine proteases, elastase, and (to a very limited extent) thrombin. Computational docking was used to predict the suitability of substituent groups on the γ-lactone and γ-lactam scaffolds for occupancy of the primary binding pockets that border the enzyme active site. Weak activity against thrombin was observed for one of the pyrrolothiazoles (108 showed a 40% reduction in enzyme activity when incubated for 40 min at 1 mM), however, the levels of inhibition of elastase were considerably lower for all the structures examined. It was considered that the inherent reactivity of the γ-lactone and γ-lactam scaffolds studied here was too low for effective acylation of the active-site serine of elastase.

The synthetic methodology that was used was based on mucopolysaccharide chemistry that allowed for a modular synthesis of the various scaffolds. For both the mono and bicyclic structures, the C-3 vinylic halogen of mucopolysaccharide was initially replaced using a 1,4-addition-elimination process to give a muco-derivative which, when reacted with D-penicillamine lead to the pyrrolothiazole core structure, and when treated with diphenyldiazomethane was converted to a ring-opened ester that proved to be a key unit for formation of γ-lactams bearing an electron-withdrawing group tosyl group on the lactam nitrogen atom. The formation of a sulfonylimine from the ring-opened ester proceeded smoothly when treated with a tellurodiimide reagent, prepared by reaction of tellurium powder and chloramine-T. Reaction of the sulfonylimine with a hydride reagent generated the monocyclic γ-lactam. Reaction of the ring-opened ester with a hydride reagent proved to be an effective method for formation of the monocyclic γ-lactones, while reaction of this ester with a methyl Grignard reagent led to a monocyclic γ-lactam with a methyl group in the 5-position. In the case of each of these monocyclic units, and the pyrrolothiazole core structure, the remaining vinyl halide was then replaced so as to complete the installation of the desired substituents. This last displacement was effected both by azide ion and via Suzuki-Miyaura coupling using aromatic boronic acid or ester reagents. A Click reaction with the azide derivatives allowed for installation of a phenyltriazole group.
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<tr>
<td>AAT</td>
<td>$\alpha$-1-antitrypsin</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>DIW</td>
<td>De-ionised water (distilled water)</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPM</td>
<td>Diphenylmethyl ester (benzhydryl ester)</td>
</tr>
<tr>
<td>EM</td>
<td>Effective molarity</td>
</tr>
<tr>
<td>ENZ</td>
<td>Enzyme</td>
</tr>
<tr>
<td>EWG</td>
<td>Electron-withdrawing group</td>
</tr>
<tr>
<td>HNE</td>
<td>Human neutrophil elastase</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectroscopy</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LG</td>
<td>Leaving group</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic spectroscopy</td>
</tr>
<tr>
<td>R.T.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>STAB</td>
<td>Sodium triacetoxyborohydride</td>
</tr>
<tr>
<td>TBS</td>
<td>tert-butyl dimethyl silyl</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TFAA</td>
<td>Trifluoroacetic anhydride</td>
</tr>
<tr>
<td>TMG</td>
<td>Tetramethylguanidine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
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Chapter 1

Introduction
Chapter 1 Introduction

The work in this thesis deals with the design, synthesis and enzymatic evaluation of three related structure types as novel templates of protease inhibitors. The structures are novel in that first, they have not been previously prepared, and second, they have the potential to act as inhibitors of serine proteases. The introduction section is organised on the following basis: (i) general information on serine proteases, their mode of action and key aspects of substrate binding; (ii) examples of distinct inhibitors of serine proteases and their mode of action; (iii) specific examples of inhibitors; (iv) background notes on the initial structure type – a bicyclic pyrrolothiazole unit – that was examined in this thesis; (v) notes and observations on the second and third structure types – monocyclic, unsaturated γ-lactones and unsaturated γ-lactams - that formed the core part of the work relating to the development of potential inhibitors of elastase, which is a physiologically relevant target enzyme in the context of diseases such as cystic fibrosis and emphysema.

1.1 Serine proteases

Proteases are one of the largest classes of enzymes in living organisms and selectively catalyse the hydrolytic cleavage of amide bonds in proteins and peptides. There are four main classes of proteases, classified according to their mechanism of action and the key catalytic moiety in the enzyme active site, namely serine, cysteine, aspartic and metallo. The main focus of this work is serine proteases. These are characterised by the presence of a serine residue in the active site of the enzyme.

The mode of action of serine proteases, is based on the catalytic triad, A, at the active site of the enzyme as shown in Scheme 1.1. The triad A is a coordinated structure consisting of three essential amino acids, histidine (His-57), serine (Ser-195) and aspartic acid (Asp-102). The substrate approaches the active site and forms a non-covalent Michaelis complex, B. In the acylation step of the reaction, the hydroxyl of Ser-195 reacts with the carbonyl of the peptide substrate, assisted by His-57 acting as a general base, to yield a tetrahedral intermediate, C. The resulting His-57-H+ is stabilised by the hydrogen bond to Asp-102. The oxanion intermediate is stabilised by interaction with the main chain NH moieties of Gly-193 and Ser-195 – this ensemble is known as the oxanion hole. Interaction with the oxanion hole is essential for effective
substrate hydrolysis. The tetrahedral intermediate collapses with the expulsion of a leaving group, assisted by His-57-H$^+$ acting as a general acid, to yield the acylated enzyme, D. The deacylation half of the reaction involves the following: water attacks the acylated enzyme, assisted by His-57, yielding a second tetrahedral intermediate, E, this intermediate collapses expelling Ser-195 and a carboxylic acid product and the original active site, A, is reformed. This entire reaction occurs extremely rapidly with very fast formation of the acylated enzyme followed by its rapid hydrolysis. The active site, while acylated, remains in a conformation sterically ideal to allow His-57 to continue to act as a general base, while the required molecule of water can enter the active site to facilitate the hydrolysis.\textsuperscript{2}

Scheme 1.1 Mode of action of serine proteases (amino acid numbering for human neutrophil elastase (HNE)).
The salient features of this reaction scheme have been substantiated and are elaborated on here. Crystal structures of transition state analogues, such as trifluoromethyl ketones, affirm the role of the oxyanion hole in providing stabilisation by hydrogen-bonding of the two tetrahedral intermediates on the catalytic cycle. A number of crystal structures of acylenzyme assemblies have been reported. In the work of Wilmouth and co-workers it was observed that a stable acylated enzyme was formed when crystals of PPE and a heptapeptide were grown at pH 5. This acylated enzyme was transformed into products when the crystals were subsequently immersed briefly in buffers at higher pH. That the role of Asp-102 is provision of electrostatic stabilisation of the His-57 imidazolium ion, following deprotonation of Ser-195, has been affirmed by both $^1$H NMR spectroscopy and analysis of neutron diffraction data (neutron diffraction has the capability of locating hydrogen atoms), thereby discounting the postulated transfer of a proton from this imidazolium ion to the carboxylate of Asp-102.

Formation of a tetrahedral intermediate by addition of a nucleophile to a carbonyl group is subject to stereoelectronic constraints. In Figure 1.1 is shown the Bürgi-Dunitz trajectory for approach of a nucleophile occurring at an oblique angle to the p-orbital on carbon, due to repulsion between the nucleophile and the lone pair electrons on oxygen. In addition, the lone pair electrons on the amide nitrogen atom are shown to be antiperiplaner to the nucleophile-carbon bond according to the stereoelectronic theory of Deslongchamps.

![Figure 1.1 Schematic representation of the stereoelectronic aspects of a tetrahedral intermediate by addition of a nucleophile (Nu') to an amide carbonyl group.](image)

The breakdown of the tetrahedral intermediate requires transfer of a proton, from the imidazolium ion of His-57, to the nitrogen atom of the amine leaving group and departure of this leaving group along a Bürgi-Dunitz trajectory (Figure 1.2).
Figure 1.2 Schematic representation of the breakdown of a tetrahedral intermediate involving protonation and departure of the nucleofuge along a Bürgi-Duitz trajectory.

It has been suggested that, following formation of the tetrahedral intermediate, the nitrogen lone pair electrons would not be suitably orientated to accept a proton from the imidazolium ion of His-57, and that either inversion of the nitrogen atom, or rotation about the C-N bond, would be required to facilitate this proton transfer.\textsuperscript{12,13} A possible further difficulty with this proton transfer was also raised in the context of the orientation of the imidazolium ion. Thus, it was argued that, following deprotonation of Ser-195, the imidazolium ion would not be suitably positioned to transfer its proton to the nitrogen atom of the amine leaving group, but would be ideally positioned to only re-protonate Ser-195.\textsuperscript{12,14} As a possible solution to this latter conundrum, a histidine “ring-flip” motion was proposed, which envisaged a significant rotation of the imidazolium unit undoing the hydrogen bond between it and Ser-195 and forming a new hydrogen bond with the nitrogen of the nucleofugic moiety (Figure 1.3).\textsuperscript{15}

![Tetrahedral Intermediate, 1](image1.png)

![Tetrahedral Intermediate, 2](image2.png)

Figure 1.3 Histidine “ring-flip” schematic for proton transfer from the His-57 imidazolium ion to the amine leaving group of a peptide substrate (reconstructed from data given in reference).\textsuperscript{15}

Of the four hydrogen bonds shown for the initial tetrahedral intermediate, 1, (Figure 1.3) two involve C-H bonds (labelled $b$ and $d$,) for which there is experimental evidence from both X-ray data\textsuperscript{16} and from NMR spectroscopy data.\textsuperscript{15} The “ring-flip” process involves breaking these four hydrogen bonds - prior to reforming four new hydrogen bonds - a process which was expected to be energetically unfavourable. This
proposal had proved to be controversial, but has now been discounted on foot of experimental observations and theoretical evaluations. One quantum mechanical evaluation indicated an energy barrier of 15 – 20 kcal/mol for this “ring-flip” process.\textsuperscript{17} Analysis of protease X-ray data by a number of groups pointed to a minimal amount of molecular motion as the reaction progressed along the reaction coordinate from starting peptide substrate to the hydrolysed components; the consensus of their analyses pointed to an “economy of atomic motion” as the system proceeds through the catalytic cycle with migration of the electrophilic carbon atom of the substrate between a leaving group (the amine moiety of the substrate) and a nucleophile (the Ser-195 oxygen atom).\textsuperscript{18,19} This aspect of “economy of atomic motion” has been identified, from analysis of crystal structure data, as occurring along the reaction path in non-protease enzymes also.\textsuperscript{20}

Recent high-level computational analysis of the key stages in the catalytic cycle at a protease active site provides an integrated view of progress along the reaction coordinate.\textsuperscript{21} This analysis showed that only a subtle re-orientation of the imidazolium moiety of His-57 was needed to relay the proton to the nitrogen atom of the leaving group (Figure 1.4). Thus, progression along the reaction coordinate involved a shortening of the newly-formed carbon-oxygen bond of the tetrahedral intermediate together with a lengthening of the carbon-nitrogen bond (3 to 4, Figure 1.4) prior to transfer of the proton to the nitrogen atom of the departing amine entity. The analysis showed that the energy cost for these molecular motions was 1.2 kcal/mol. In addition, their model did not require any inversion of the nitrogen atom of the amine leaving group and required only a small rotation (15°) about the C-N bond between 3 and 4 (Figure 1.4) prior to completion of the proton transfer.
While experimental verification of this level of detail may not be possible it would appear that, in the highly polar environment of an enzyme active site, subtle modulation of the position and orientation of the substrate and enzyme components is sufficient to accommodate the stereoelectronic requirements of all aspects of the bond making/breaking stages.

1.2 Kinetic description of an enzyme-catalysed reaction

The minimal equation to describe a simple one-substrate (S), one-product (P) reaction catalysed by an enzyme (E) is as follows:

\[
E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow[k_2]{\text{ES}} E + P \quad (1)
\]

Where the reaction between P and E is negligible, then the rate of product formation will be determined by the concentration of ES (the enzyme-substrate complex) and \( k_2 \). The rate of reaction \( V \) is therefore given by equation (2):

\[
V = k_2[ES] \quad (2)
\]

Following initiation of the reaction, the Briggs-Haldane model\(^{22}\) assumed that the concentration of ES reaches a steady state, whereby the rate of formation of ES is exactly equal to the rate of loss of ES. This situation can be expressed by equation (3):

\[
k_1[E][S] = (k_{-1} + k_2)[ES] \quad (3)
\]
This leads to equations (4) and (5):  
\[ [ES] = k_1[E][S]/(k_{-1} + k_2) \]  
\[ (4) \]
\[ [ES] = [E][S]/((k_{-1} + k_2)/ k_1) \]  
\[ (5) \]

In the Michaelis-Menton formulation the ratio \((k_{-1} + k_2)/ k_1)\) is given by \(K_M\) so that equation (5) can be written as:

\[ K_M [ES] = [E][S] \]  
\[ (6) \]

The total concentration of enzyme, \([E]_t\), is given by the sum of \([ES]\) and \([E]\) (the concentration of free enzyme at any time \(t\)). The following substitutions are made in order to express the rate of reaction in terms of reaction components that can be quantified, \(viz\). \([E]_t\) and \([S]\):

\[ [E]_t = [E] + [ES] \]  
\[ (7) \]
\[ K_M [ES] = [S][E]_t - [ES][S] \]  
\[ (8) \]
\[ K_M [ES] + [ES][S] = [S][E]_t \]  
\[ (9) \]
\[ [ES](K_M + S) = [S][E]_t \]  
\[ (10) \]
\[ [ES] = [S][E]_t/(K_M + [S]) \]  
\[ (11) \]
\[ V = k_2[S][E]_t/(K_M + [S]) \]  
\[ (12) \]

Equation (12) is known as the Michaelis-Menten equation and \(K_M\) is known as the Michaelis constant. The term \(k_2[E]_t\) is the maximum rate for the reaction, \(V_{max}\), as it corresponds to the case of equation (2) where all of the enzyme is in the form of ES, \(i.e.\) the enzyme is fully saturated with substrate and addition of more substrate cannot drive the reaction any faster. Thus, the Michaelis-Menten equation can also be written as:

\[ V = V_{max}[S]/(K_M + [S]) \]  
\[ (13) \]
A generalised graph of \( V \) versus \([S]\) for equation (13) is shown in Figure 1.5 and this can be viewed as corresponding to three distinct regions. At quite low values of \([S]\) where \( K_M \gg [S] \) with the result that the rate is directly proportional to \([S]\) as equation (13) can be written as:

\[
V = V_{\text{max}}[S]/(K_M)
\]  

(14)

At very high values of \([S]\) where \([S] \gg K_M\), with the result that the rate is now constant and is at its maximum value as equation (13) can be written as:

\[
V = V_{\text{max}}
\]  

(15)

Finally, in the instance where \( K_M = [S] \) then \( V = V_{\text{max}}/2 \). Thus \( K_M \) is equal to the substrate concentration at which reaction velocity has attained half of its maximum value. As such, \( K_M \) is a measure of the substrate concentration required for effective catalysis to occur; an enzyme with a high \( K_M \) value requires a higher concentration of substrate to achieve a given rate of reaction than an enzyme with a low \( K_M \) value. Throughout the above analysis it is assumed that \([ES]\) << \([S]\). In the circumstance where \( k_1 \gg k_2 \) then \( K_M \approx K_S \), where \( K_S \) is the dissociation constant of ES. Whereas, \( K_S \) quantifies how much of the enzyme is present as ES at thermodynamic equilibrium, \( K_M \) relates to \([ES]\) under steady-state conditions.

![Figure 1.5](image.png)

Figure 1.5 A plot of equation (13) showing the variation of reaction rate with substrate concentration. Here \( V_{\text{max}} = 100 \) and \( K_M = 1 \) mM; the units of concentration are taken, arbitrarily, as mM for this illustration.
For a multistep reaction the Michaelis-Menten equation can also be used.

Consider the reaction:

\[
\begin{align*}
E + S & \xrightarrow{k_1} \text{ES} \xrightarrow{k_2} \text{ES}' \xrightarrow{k_3} E + P
\end{align*}
\] (16)

Here ES' could represent the acylated form of a protease enzyme. The form of the Michaelis-Menten given in equation (12) is now written as:

\[
V = k_{\text{cat}}[S][E]/(K_M + [S])
\] (17)

The term \(k_{\text{cat}}\) is a combination of other rate constants and its value depends on which step is rate-determining.

\[
k_{\text{cat}} = \frac{k_2k_3}{k_2 + k_3}
\] (18)

\[
K_M = \frac{k_3(k_1 + k_2)/k_1(k_2 + k_3)}
\] (19)

The value of \(k_{\text{cat}}\) (units of s\(^{-1}\)) gives a direct measure of the catalytic formation of product under optimum conditions (saturated enzyme) and corresponds to the number of substrate molecules turned over per molecule of enzyme per second. The ratio \(k_{\text{cat}}/K_M\) (units of M\(^{-1}\) s\(^{-1}\)) is a measure of enzyme efficiency and specificity. In the case where the rate of an enzyme-catalysed reaction is measured at very low substrate concentration, \(i.e.\ [S] \ll K_M\) and \([E] \cong [E]\) then equation (17) becomes:

\[
V = (k_{\text{cat}}/K_M)([S][E])
\] (20)

Thus, \(k_{\text{cat}}/K_M\) corresponds to a second-order rate constant for conversion of substrate to product by the enzyme (equation (21)) under conditions of very low substrate concentration. The maximum value of \(k_{\text{cat}}/K_M\) is \(10^8-10^9\) M\(^{-1}\) s\(^{-1}\), \(i.e.\) the diffusion-controlled limit whereby every encounter of the substrate with the enzyme leads to formation of product.\(^{24}\)
Comparison of $k_{\text{cat}}/K_M$ values, for turnover of one substrate versus another gives a measure of the specificity of an enzyme for a particular substrate; likewise, comparison of $k_{\text{cat}}/K_M$ values for two related enzymes (e.g. proteases) for given substrates gives a measure of the specificity of the enzymes toward any given substrate.

1.3 Analysis of enzyme kinetics

Kinetic measurements are typically carried out with a fixed concentration of enzyme and variable concentrations of substrate. The initial rate of the reaction is measured as here the change in $[S]$ is linear with time: this is the stage in the reaction where $[ES]$ is almost constant. Equation (13) can be arranged in the form of equation (22) so that a plot of $1/V$ against $1[S]$ produces a straight line (Figure 1.6). This double-reciprocal plot is known as a Lineweaver-Burk plot. A linear regression allows for values of $V_{\text{max}}$ and $K_M$ to be calculated.

$$\frac{1}{V} = \frac{K_M}{V_{\text{max}}}[S] + \frac{1}{V_{\text{max}}}$$

Figure 1.6 Lineweaver-Burk plot of the data from Figure 1.5; some typical error bars (vertical lines) are included here.

Where $1/[S] = 0$, $[S]$ is infinitely large and the reaction rate is at its maximum value. Thus, $1/V_{\text{max}}$ is obtained as the intercept value on the $1/V$ axis where $1/[S] = 0$. Knowing $V_{\text{max}}$ allows determination of $k_{\text{cat}}$ as $k_{\text{cat}} = V_{\text{max}}[E]$. The point on the $1/[S]$ axis where $1/V = 0$ allows for determination of a value for $K_M$. Where the kinetic data is accurate and precise and extends from low to high $[S]$ then a good estimate of $K_M$ and $V_{\text{max}}$ is obtained by a Lineweaver-Burk plot. Where there are errors in $V$ then these are
magnified in $1/V$ (see error bars in Figure 1.6) and so this method of analysis can be unsatisfactory. An alternative straight-line graph can be obtained by the Eadie-Hofstee plot$^{26,27}$ as shown in figure 1.7. Here equation (14) is given in the form of equation (23).

$$V = V_{\text{max}} - \left( K_M V[S] \right)$$  \hspace{1cm} (23)

![Figure 1.7 Eadie-Hofstee$^{26,27}$ plot of the data shown in Figure 1.5.](image)

The problems associated with different methods of analysis of enzyme kinetic data have been reviewed$^{28}$ and has led to the conclusion that the direct linear plot, developed by Eisenthal and Cornish-Bowden,$^{29,30}$ is a rigorous, yet simple, graphical method of evaluating $V_{\text{max}}$ and $K_M$ values and associated confidence limits.$^{31}$ This method is better than a least-squares non-linear regression analysis of the kind of data shown in Figure 1.7, when the associated errors do not follow a normal distribution and are not of a constant magnitude;$^{28}$ in many cases the nature of error distribution is unknown due to the added experimental measurements that would be required to determine this.

A direct linear plot of (some of) the data from Figure 1.5 is shown in Figure 1.8. The plot is constructed by placing each value of $[S]$ on the negative side of the abscissa and the corresponding value of $V$ on the ordinate, and then a straight line is drawn connecting each pair of $[S], V$ values. From the intersection point of these lines the values of $V_{\text{max}}$ and $K_M$ are obtained directly as shown in Figure 1.8. The basis of this approach derives from taking equation (13) and rearranging it in the form of equation (24), which is of the general form shown in equation (25), and is the equation of a straight line in $xy$ space with intercepts $a$ on the $x$ axis and $b$ on the $y$ axis.
\[
\frac{V_{\text{max}}}{V} - \frac{K_M}{[S]} = 1 \tag{24}
\]
\[
x/a - y/b = 1 \tag{25}
\]

Figure 1.8 A direct linear plot of (some of) the data from Figure 1.5. The abscissa is the \(K_M\) axis and the ordinate is the \(V_{\text{max}}\) axis.

The data presented in Figure 1.8 is error free and so all the lines intersect at a single point. More than one intersection point is obtained when error is associated with the data. This results in a set of values for \(V_{\text{max}}\) and \(K_M\) from which the median (middle value) is taken as the best value of each parameter, and confidence limits can be calculated for each parameter.\textsuperscript{29,30} Data treatment can be computerised.

### 1.4 Kinetic analysis of enzyme inhibition

The presence of an inhibitor (I), which competes with the substrate for binding at the enzyme active site, can be represented by (26); \(K_i\) is the equilibrium dissociation constant for the inhibitor.

\[
\begin{align*}
E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES & \overset{k_2}{\rightarrow} E + P \\
E + I & \underset{K_i}{\rightleftharpoons} EI
\end{align*}
\tag{26}
\]

Under these conditions the total concentration of enzyme, \([E]_t\) is the sum of \([ES], [E]\) and \([EI]\) with the result that equation (27) is the form of the Michaelis-Menten equation that is now derived (in place of equation (13)).
\[ V = \frac{V_{\text{max}}[S]}{(K_M((1 + [I]/K_i) + [S]))} \]  

(27)

A generalised graph of \( V \) versus \([S]\) for equation (27) is shown in Figure 1.9 for a situation where \( K_M = 1 \text{ mM}, V_{\text{max}} = 100, K_i = 0.5 \text{ mM} \) and where \([I] = 0, 1 \text{ and } 2 \text{ mM}\).

![Graph showing reaction rate variation](image)

Figure 1.9 A plot of equation (27) showing the variation of reaction rate with substrate concentration in the presence of a competitive inhibitor (I). Here \( K_M = 1 \text{ mM}, V_{\text{max}} = 100, K_i = 0.5 \text{ mM} \), and \([I] = 0 \left( \bullet \right), 1 \left( \Delta \right) \text{ and } 2 \text{ mM } \left( \square \right) \); the units of concentration are taken, arbitrarily, as mM).

Lineweaver-Burk\textsuperscript{25} treatment of the data in Figure 1.9 gives the graphical form shown in Figure 1.10. This shows that \( V_{\text{max}} \) remains unchanged in the presence of the inhibitor; the same is true for \( K_M \). This is so as the presence of the inhibitor does not alter the way in which the substrate interacts with the enzyme – see the upper part of equation (24). On the 1/[S] axis two new intercept values are obtained and these correspond to values of \(-1/((K_M((1 + [I]/K_i) \text{ where } [I] = 1 \text{ and } 2 \text{ mM respectively, which allows for the determination of a value for } K_i. \text{ While the Lineweaver-Burk}\textsuperscript{25} \text{ type plot is a useful graphical representation of the data, treatment of the data by a direct linear plot allows for an accurate evaluation of } K_i.\textsuperscript{29,30}

---

13
Two other modes of enzyme inhibition can occur and these can be recognised by the form of the Lineweaver-Burk plots of the relevant kinetic data. In one case the inhibitor can bind to both E and ES; this is known as mixed inhibition and is referred to as noncompetitive inhibition when the inhibitor binds with equal affinity to E and ES. A typical Lineweaver-Burk plot for such a scenario is shown in Figure 1.11. The lines do not intersect on the $1/V$ axis indicating that $V_{\text{max}}$ is altered by the presence of the inhibitor; this is consistent with the mode of inhibition where I can bind to ES as well as to E.
In the second case the inhibitor binds only to ES; this situation arises, mainly, for multi-substrate enzymes; this is referred to as uncompetitive inhibition. The Lineweaver-Burk\textsuperscript{25} plot in this instance will appear as shown in Figure 1.12.

Figure 1.12 Lineweaver-Burk\textsuperscript{25} plot of the data shown in Figure 1.9 for a case of uncompetitive inhibition where [I] = 0 (●), 1 (▲) and 2 mM (■).

Another measure of the efficacy of an inhibitor that is quoted is its IC\textsubscript{50} value – the concentration of inhibitor required to reduce enzyme activity by 50%. It is a parameter that is frequently given as it is can be obtained experimentally more rapidly than \( K_i \). It is, however, a relative measure of inhibitor potency as its value is dependent on the substrate concentration used in the inhibition assay. This aspect can be qualitatively ascertained by examining equation (26). The IC\textsubscript{50} value is the [I] that is required to reduce [ES] by 50%. When [S] is very low then [ES] is quite low and so the [I] required to reduce enzyme activity by 50% will correspond, very closely, to the [I] required to bind with half of the free enzyme. Note that this is the definition of \( K_i \), and so IC\textsubscript{50} \( \approx \) \( K_i \) when the inhibition assay is carried out with very low [S]. Conversely, when [S] is high, and by default [ES] is high but the concentration of free enzyme is low, then a much larger [I] is needed to reduce [ES] by 50%. In this case IC\textsubscript{50} > \( K_i \). The relationship between IC\textsubscript{50} and \( K_i \), for a competitive inhibitor, is given quantitatively by the Cheng-Prusoff equation:\textsuperscript{32}

\[
K_i = \frac{IC_{50}}{1+(S/K_M)}
\]  

(28)

It is clear from equation (28) that when [S] is low then IC\textsubscript{50} \( \approx \) \( K_i \), whereas, when [S] is high then IC\textsubscript{50} > \( K_i \), and when [S] = \( K_M \) then \( K_i = IC_{50}/2 \). When reporting IC\textsubscript{50} values it is important, therefore, to give the [S] and the \( K_M \) value for S in the inhibition assay. The binding of a competitive enzyme inhibitor may be measured by assaying the residual enzyme activity in the presence of varying amounts of the inhibitor. A plot of
the residual activity, expressed as a percentage of the activity in the absence of inhibitor, will appear as shown in Figure 1.13. The graph appears like a Langmuir binding isotherm and the data points follow equation (29).

\[ \% \text{Residual Activity} = 100 \left( \frac{V_i}{V_o} \right) = 100 \left( \frac{1}{1 + \left( \frac{[I]}{IC_{50}} \right)} \right) \quad (29) \]

Here \( V_i \) and \( V_o \) correspond to the enzyme activity in the presence and absence of inhibitor respectively. It is common practice to show this data in the form of a semi-logarithmic graph as illustrated in Figure 1.14.33

Figure 1.13 Residual enzyme activity (%) as a function \([I]\) where \( I \) is a competitive inhibitor of the enzyme; the units of \([I]\) are taken, arbitrarily, as \( \mu \text{M} \).

Figure 1.14 Residual enzyme activity (%) as a function of \( \log[I] \) where \( I \) is a competitive inhibitor of the enzyme; the units of \([I]\) are taken, arbitrarily, as \( \mu \text{M} \).

An attractive feature of \( IC_{50} \) measurements is that a single substrate concentration is used in combination with a wide range of inhibitor concentrations.
(obtained by serial dilutions). For this reason, it is the method of choice when screening a large number of inhibitors as it requires considerably fewer assays than the number needed to measure $K_i$.

1.5 Binding of Serine Proteases

Almost one third of all proteases can be classed as serine proteases. Serine proteases have a similar mode of action at the active site (the catalytic triad). What accounts then for their varying specificity? The answer lies in the difference in the physical conformation of the enzyme. This difference results in binding pockets that are specific to each protease. The accepted nomenclature of Schechter and Berger is used to describe the subsites of interaction between a protease and its substrate/inhibitor. The amino acid residue located on the carbonyl side of the scissile amide bond is designated P1; the corresponding pocket on the enzyme where this side group is accommodated is designated S1. Subsequent amino acid side groups on this side are labelled P2, P3…etc. and their corresponding enzyme sites S2, S3…etc. The ligands on the amino-terminal side of the substrate are labelled P1', P2' and their corresponding binding pockets S1', S2', and so on (Scheme 1.2).

Substrates or inhibitors have substituents that can be tailored specifically to exploit the interactions that are specific to that pocket. For example, the primary binding pocket, S1, of thrombin (Scheme 1.3 and Figure 1.15) is quite deep with Asp-189 located at the bottom, which forms ionic interactions with basic P1 ligands such as arginine, cleaving bonds on the carboxy side of these substituents. A wide variety of human diseases, such as thrombosis, are associated with the aberrant activity of thrombin.
<table>
<thead>
<tr>
<th>Thrombin</th>
<th>S3</th>
<th>S2</th>
<th>S1</th>
<th>S1'</th>
<th>S2'</th>
</tr>
</thead>
<tbody>
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<td>Leu 99</td>
<td>Leu 59</td>
<td>Ala 190</td>
<td>His 57</td>
<td>Leu 41</td>
<td></td>
</tr>
<tr>
<td>Ile 174</td>
<td>Tyr 60A</td>
<td>Cys 191</td>
<td>Tyr 60A</td>
<td>Cys 42</td>
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<td>Trp 215</td>
<td>Pro 60B</td>
<td>Glu 192</td>
<td>Trp 60D</td>
<td>Cys 58</td>
<td></td>
</tr>
<tr>
<td>Tyr 60A</td>
<td>Pro 60C</td>
<td>Gly 193</td>
<td>Lys 60F</td>
<td>Lys 60F</td>
<td></td>
</tr>
<tr>
<td>Trp 60D</td>
<td>Trp 60D</td>
<td>Tyr 228</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Scheme 1.3 Amino acid residues of the various binding pockets of thrombin.

![Electrostatic surface view of thrombin](image)

Figure 1.15 Electrostatic surface view of thrombin (PDB 1K22), showing the relative size of the S1 binding pocket. Blue: positive electrostatic potential, Red: negative electrostatic potential, White: neutral electrostatic potential.

Elastase is one the most widely studied serine proteases. The primary binding pocket of elastase (S1) is smaller than thrombin and is hydrophobic and so cleaves peptide bonds on the carboxy side of small amino acids like alanine, glycine and valine (Scheme 1.4 and Figure 1.16). Human neutrophil elastase (HNE) and pancreatic elastase (PE) are two major forms of elastase that cleave the important connective tissue protein elastin. HNE is found in dense granules of polymorphonuclear leukocytes and is essential for phagocytosis and defence against infection by invading microorganisms. PE is stored as an inactive zymogen in the pancreas and is secreted into the intestines where it becomes activated by trypsin.37
Scheme 1.4 Amino acid residues of the various binding pockets of HNE and PPE.

Figure 1.16 Electrostatic surface view of PPE (PDB 1QGF) showing the S1 binding pocket. Blue: positive electrostatic potential, Red: negative electrostatic potential, White: neutral electrostatic potential.

1.6 Inhibition of serine proteases

Under normal circumstances, serine protease activity is strictly regulated by endogenous inhibitors known as serpins (serine protease inhibitors). Serpins are the only protease inhibitor superfamily found in all branches of life, the third most common protein family in blood and the most abundant inhibitors in humans. Thus, serpins are the largest and most diverse family of protease inhibitors. They are involved in many proteolytic cascades as well as being employed for hormone transport, storage functions and blood pressure regulation, however the word serpin is still used to describe the entire family, even those not involved in inhibition.38
The entire superfamily of serpins displays highly conserved architecture throughout. The most significant differences between individual serpins have been observed in the reactive centre loop (RCL), a flexible stretch of approximately 20 residues tethered to the main body of the molecule. The RCL of a serpin contains an amino acid sequence complementary to the active site of the target protease with the specificity of the interaction being primarily dictated by the P1 residue (Figure 1.17). Conformational differences between serpins appear to display a level of plasticity involving incorporation of the RCL into $\beta$-sheet A (Figure 1.18).\textsuperscript{38,39}

Figure 1.17 Differences in P1 residues of serpins is a key feature for target specificity.

Figure 1.18 Crystal structures illustrating conserved architecture with inherent conformational plasticity particular to the serpin family. The RCL, shown in red, is at the top with the main $\beta$-sheet A in blue. The P1 and P1' residues of each serpin are shown as red spheres. (a) Native structure of $\alpha$-1-antitrypsin (PDB code: 1QLP); (b) native structure of murine $\alpha$-1-antichymotrypsin (PDB code: 1YXA). The P15 residue is inserted into the $\beta$-sheet A; (c) native antithrombin (PDB code: 1T1F). Both the P15 and P14 residues are inserted into $\beta$-sheet A, resulting in the RCL being translated further to the left.\textsuperscript{39}
The primary function of serpins is to neutralise over-expressed serine protease. Their mode of action is characterised by an irreversible suicide mechanism whereby a reversible Michaelis complex is initially formed in the rate-determining step. The mechanism proceeds initially in a manner similar to that of normal substrate binding – that is, scission of the scissile bond, located on the RCL.

As the RCL is inserted into the active site and the acylation occurs, a rapid and irreversible conformational translocation occurs resulting in distortion of the active site of the enzyme in such a manner that His57 is dislocated from Ser195 and its action as a general base is inhibited, trapping the protease in the stable acyl-intermediate conformation. Hydrolysis of the acylated enzyme at this point therefore occurs very slowly rendering the protease de-activated (Figure 1.19).\textsuperscript{39,40}

![Figure 1.19 Mode of action of an inhibitory serpin.\textsuperscript{39}](image)

The extent of separation of P1’ Ser195 from P1 Met358 for α-1-antitrypsin/elastase interaction is illustrated in Figure 1.20A while distances apart of Ser195 and His57 are detailed and the removal of the carbonyl oxygen of Met358 from the oxyanion hole is shown in Figure 1.20B.
Figure 1.20 (A) Crystal structure (1EZX) of α-1-proteinase covalently bound to its substrate elastase after the translocation step; the extent of the translocation is illustrated by the distance apart of Ser359 and Met358 of α-1-proteinase; (B) α-1-antitrypsin covalently bound to elastase after the translocation step; the distance shown of His57 from Ser195 is measured in Angstroms.41

The significant conformational change that occurs upon scission of a scissile peptide bond of an endopeptidase by the serpin α-1-antitrypsin (AAT) is illustrated in Figure 1.19. The key stages are as follows: (i) the native structure of AAT is shown on the bottom with the RCL coloured in red, extending upwards and the P1 residue shown in space-filling representation. The protease approaches from above; (ii) the protease encounters the RCL of AAT in a covalent Michaelis complex; (iii) this acylated transitional step in the reaction sequence has not been confirmed but can be deduced from steps (ii) and (iv); (iv) as the acylated enzyme becomes fully incorporated into the RCL, a large conformational shift occurs which distorts the active site of the protease, distending the oxyanion hole and denaturing the protease. The protease is effectively inhibited at this point.

1.6.1 Elastase and α-1-Antitrypsin

In the case of HNE, the natural inhibitor is α1 – antitrypsin (AAT). AAT plays an essential role in protecting extracellular connective tissue from attack by elastase. Under normal conditions, HNE reacts preferentially with AAT over its natural substrates. The turnover number for HNE activity upon elastin can be determined and has been shown to be remarkably low, approximately 200 – 2000 fold lower than that of HNE acting on a soluble peptide substrate, such as AAT.42
Beside its physiological function as an essential host defence, HNE is also one of the most destructive enzymes in the body. Given the nature of the above relationship, imbalances in the body between the natural protease burden and the protective serpin screen, via either an increase in the HNE burden or a decrease in the AAT screen, leads to unchecked proteolytic activity, causing degradation of various connective tissues and has been linked to a number of disease states.

α-1-Antitrypsin deficiency for example is one of the most common lethal hereditary disorders of caucasians of european descent. The disease state, caused by genetic mutations, has been linked to emphysema, liver disease and chronic bronchitis. Normal AAT levels are 20 – 53 μM and when serum levels of AAT are < 11 μM, the lower respiratory tract is vulnerable to the natural amount of HNE present, allowing alveoli destruction which results most commonly, in emphysema. \(^{43-45}\)

Over-expression of HNE leads to a protease/serpin imbalance that can result in life-threatening inherited diseases such as cystic fibrosis (CF). CF patients suffer from over-manufacture of sputum in the lungs and resulting inflammation as a result of a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). \(^{46}\) Due to a combination of hyper-recruitment of neutrophils to the inflamed area, resulting in an overproduction of HNE, and a reduced ability to export water from cells, early stage CF leads to incessant coughing and copious phlegm production. \(^{47}\) In later stages, the cycles of neutrophil recruitment and inflammation, lead to chronic bacterial infection, which in turn lead to changes in the architecture of the lungs, caused by unchecked HNE release, perhaps as much as 5 orders of magnitude greater than in a normal lung while AAT levels may be increased only 4-5 fold resulting in the body’s anti-protease capacity being exceeded, \(^{48}\) leading to destruction of the connective tissue of the lung and chronic difficulties in breathing. \(^{49}\)

1.7 Synthetic inhibitors

Synthetic inhibitors of serine proteases can be broadly classed into two groups based on their mode of inhibition – non-covalent and covalent inhibitors. Traditionally drugs that bind covalently to the active site have been disfavoured, by the major
pharmaceutical companies, due to their potential for off-target reactivity. This can result in either acute tissue injury or it can activate the immune system through hapten formation, nonetheless there are specific examples where covalent inhibitors have provided successful therapies for various indications.\textsuperscript{50}

1.7.1 Non-covalent mode of inhibition

Non-covalent inhibitors do not bind covalently with the Ser-195 in the oxyanion hole. These rely on strong hydrophobic and electrostatic interactions with the oxyanion hole and subsites.

\begin{center}
\includegraphics[width=\textwidth]{images/non_covalent.png}
\end{center}

\textit{N-α-tosylarginine methyl ester (TAME)} is an arginine derivative that in itself is a poor substrate for thrombin.\textsuperscript{51} However, a number of prototypes derived from TAME have been found to be effective inhibitors of thrombin. Argatroban, a low molecular weight molecule, evolved from TAME by modification of the sulfonyl group; the ester group was replaced by an amide group and a carboxylic acid group incorporated was at the \textit{C}-terminus. It is selective for thrombin ($K_i = 19$ nm) and has little effect on related serine proteases, such as trypsin and elastase. In Figure 1.21 is shown the central amino arginine (P1) occupying the S1 pocket and interacting with Asp-189, the C-terminal piperidine amide (P2) and \textit{N}-terminal methyltetrahydroquinolinesulfonyl (MTHQS) group (P3) occupying the lipophilic S2 and S3 pockets.
Melagatran is another reversible non-covalent inhibitor of thrombin ($K_i = 2$ nm).\textsuperscript{51} The crystal structure of melagatran bound to the active site of thrombin shows that it relies on extensive binding through the S1 and S2/S3 pockets. As shown in Figure 1.22 the benzamidino unit forms strong interactions with Asp 189 at the bottom of the S1 pocket, while the azetidine ring and cyclohexyl group extend into S2/S3 pockets.
There are various injectable direct thrombin inhibitors (DTI) currently available on the market and are used for many conditions, such as heparin-induced thrombocytopenia (HIT) and acute coronary syndromes (ACS). Ximelagatran is the prodrug, which is converted in-vivo to the active agent melagatran. Ximelagatran was the first member of the drug class of direct thrombin inhibitors that could be taken orally, without anticoagulant monitoring. However, ximelagatran was withdrawn from the market when safety data indicated that severe hepatic injury in a patient could develop after exposure to the drug.

1.7.2 Covalent mode of inhibition

In Scheme 1.5 is shown three distinct modes of covalent inhibition. In the following section, a number of examples showing these modes of inhibition are illustrated. All of these structures are mechanism-based inhibitors, in that a key step for each of these is analogous to the covalent-binding reaction of a substrate at the enzyme active site.

In mode of inhibition, A, acylation of the active site serine occurs and, in general, where there is minimal active site reorganisation following this reaction, enzyme activity is recoverable via enzyme-catalysed hydrolysis of this acylated active site. In other instances, a significant degree of active site reorganisation can occur following active site acylation with the result, that hydrolysis of the acylated enzyme becomes extremely slow. This may be due to either rotation of His-57 from its normal
position and/or displacement of the water molecule required for hydrolysis. Mode of inhibition B is reversible covalent,\textsuperscript{60-65} which proceeds via equilibrium adduct formation with a reactive carbonyl group that does not contain a leaving group, while mode C, is direct reversible-acylation involving a cyclic structure bearing a leaving group.\textsuperscript{59,66-73}

![Diagram showing covalent modes of inhibition of serine proteases](image)

Scheme 1.5 Covalent modes of inhibition of serine proteases (Lg = leaving group, EWG = electron withdrawing group).

### 1.7.2.1 Covalent mode of inhibition A

One of the earliest inhibitor structures is a peptide chloromethyl ketone, D-Phe-Pro-ArgCH\textsubscript{2}Cl ketone, (PPACK)\textsuperscript{57}. This is an extremely potent irreversible inhibitor of thrombin and acts via a ‘double-hit’ mechanism, which results in cross-linking of the active site and consequently no recovery of enzyme activity. The double-hit mechanism involves hemiketal formation with the hydroxymethyl side-chain of Ser-195, followed by nucleophilic displacement of the $\alpha$-chloro group via the imidazole unit of His-57 as shown in Scheme 1.6. The mode of inhibition was confirmed by X-ray crystallography.\textsuperscript{35,36,54}
Scheme 1.6 Reaction of PPACK with thrombin (condensed reaction scheme – proton transfer steps not explicitly shown).

The 1,2,5-thiadiazolidin-3-one 1,1-dioxide template, 5, was designed to act in a similar double-hit manner (Scheme 1.7). It was anticipated that, following active site acylation, conjugate addition of the His-57 imidazole to the sulfonyl imine resulting in a cross-linked enzyme-adduct would occur. X-ray crystallography\textsuperscript{74} confirmed, however, only the formation of the acylated enzyme. The imidazole nitrogen of His-57 was found to be too far away to bind covalently so the cross-linked species was ruled out (Scheme 1.7).

Scheme 1.7 1,2,5-thiadiazolidin-3-one 1,1-dioxide template 5 and possible reactions at the active site (condensed reaction scheme – proton transfer steps not explicitly shown).

\[ R_1 = \text{\textit{n}-propyl, } R_2 = \text{methyl, } \text{Lg} = 2,6\text{-dichlorobenzoate} \]
Originally discovered as inhibitors of penicillin-binding proteins, \( \beta \)-lactams, such as 6, have also been employed as serine protease inhibitors\(^5^9\) (Scheme 1.8). Compound 6 was found to be an irreversible inhibitor of HNE. Interestingly, X-ray analysis highlighted an unusual form of active site reorganisation. The ester carbonyl group was seen to be rotated out of the oxyanion hole and the consequent displacement of the water molecule required for hydrolysis.

Scheme 1.8 Reaction of \( \beta \)-lactam 6 with HNE showing conformational change\(^5^9\) (condensed reaction scheme – proton transfer steps not explicitly shown).

This mode of inhibition seems to be unique to the \( \beta \)-lactam in that Schofield and co-workers found that closely related \( \gamma \)-lactam structures did not act in the same way.

Sivelestat is an inhibitor that acylates (pivalylates) the enzyme active-site of elastase. Hydrolysis of the pivalylated enzyme restores full activity after 24 h (Scheme 1.9).\(^5^6\) Sivelestat has been used successfully in cases of acute lung injury.\(^5^5,5^8\)
1.7.2.2 Reversible covalent – Mode B

Use of reversible inhibitors is paramount where modulation of the activity of endogenous enzymes on a long-term basis is required to manage a chronic disease state; this minimises modification of other proteins, which could lead to hapten formation.\textsuperscript{75,76} Emphysema and cystic fibrosis are chronic diseases that involve overproduction of elastase, the effect of which could be moderated by use of enzyme inhibitors.\textsuperscript{60,61,65} Low molecular weight, non-peptidyl structures can attain high enzyme affinities via covalent bond formation, while reversibility can be realized when bonding arises by equilibrium adduct formation with the active-site serine.

The typical construct for equilibrium adduct formation with the alcohol sidechain of serine is a boronate, an aldehyde, or a ketone bearing an electron-withdrawing group (EWG).\textsuperscript{65} These transition state analogues form a reversible covalent enzyme-inhibitor complex that resembles the normal enzymatic reaction intermediate. The trifluoromethyl and oxadiazole ketones 7 and 8 ($K_i = 101$ and 12.2 nM respectively for elastase) are examples of ketones with EWGs.\textsuperscript{60-63,65} In the case of 7 and 8 the EWG activates the carbonyl of the peptidyl ketones towards nucleophilic addition by the active site Ser-195; the resultant hemiketal represents an analogue of the normal reaction pathway intermediate.\textsuperscript{77} This is stabilised by interactions with the oxyanion hole (Scheme 1.10). The crystal structure of elastase with 8\textsuperscript{62} covalently bound at the active site confirmed adduct formation, with the hemiketal oxyanion of the tetrahedral intermediate located at the oxyanion hole, stabilised by hydrogen bonds between the hemiketal oxyanion and two amide protons of Ser-195 and Gly-193. The
crystal structure shows the isopropyl group occupying the S1 pocket and the phenyl-aminopyrimidinone unit spanning the S3 pocket. π-π Interaction and/or van der Waals’ interaction was observed between the phenyl ring and the imidazole ring of His-57. The EWG of 8 contributed not only to the reactivity of the ketone moiety but also to the stabilization of the intermediate by forming a hydrogen bond with His-57.

Scheme 1.10 Transition state analogues 7 and 8 as an inhibitor of elastase (condensed reaction scheme – proton transfer steps not explicitly shown).

Peptidyl boronic acids have also been found to be potent inhibitors of elastase. Boronate esters 9 are hydrolyzed in-situ to the active boronic acid species and Ser-195 of the active site forms an adduct with the electrophilic boron. The resultant boronate species is stabilized by interaction at the oxyanion hole (Scheme 1.11), similar to the trifluoromethyl ketones.

Scheme 1.11 Boronate ester 9 at the active site of elastase (condensed reaction scheme – proton transfer steps not explicitly shown).
1.7.2.3 Direct reversible-acylation – Mode C

Few structures have been shown to act as serine protease inhibitors by direct reversible-acylation. Some benzoxazinones (Scheme 1.12) act as reversible inhibitors of chymotrypsin and of elastase by acylation of the active-site, and although deacylation by re-cyclisation can occur, this does not generally regenerate the original structure.\(^{66,68}\) Thus benzoxazinone \(10\), a short-acting elastase inhibitor, is released from the acylated enzyme by formation of the corresponding quinazolinedione. For other benzoxazinones enzyme activity is recovered by hydrolysis of the acylated enzyme, however, for benzoxazinone \(11\) experimental data indicated that reformation of \(11\) from the acylated enzyme was faster than either hydrolysis or formation of the corresponding quinazolinedione indicating that reverse of the acylation step was occurring.

![Scheme 1.12 Benzoxazinones at the active site of elastase (condensed reaction scheme – proton transfer steps not explicitly shown).](image)

A more relevant construct for reversible-acylation is the trans-lactone that is present in the naturally-occurring thrombin inhibitors \(\text{GR133686}\) and \(\text{GR133487}\) \((K_i = 0.83 \text{ and } 0.97 \mu \text{M at pH } 6)\) (Figure 1.23) first identified by GlaxoSmithKline.\(^{70,72}\)
Figure 1.23 Naturally-occurring and synthetic trans-lactone inhibitors of thrombin.

It was unequivocally established that these compounds act by acylation of the active-site serine and that deacylation occurs exclusively by re-formation of the original trans-lactone. In the crystal structure 1AWF (thrombin acylated by GR133487) it is clear that the amino acid residues Trp-60D and Tyr-148 (that arch over the S-1 pocket) constrain the hydroxy and acyl groups of the cleaved γ-lactone to remain in an approximate di-equatorial orientation on ring A (Figure 1.24 and Table 1.1), with the hydroxy oxygen atom at an internuclear separation of 3.04 Å to the acyl carbon atom and at an angle of 100° to the carbonyl group; in terms of a Bürgi-Dunitz trajectory these values are appropriate for reformation of the γ-lactone bond. As a substantial strain energy must be overcome to reform the trans structure, the constraint applied by the enzyme enforces the deacylation path, while the active-site His-57 is positioned to act as the required base for this reaction. In the case of GR133686 the internuclear separation is 3.37 Å and the rate of reformation of the trans-lactone is ten times slower than for GR133487; hydrolysis of the acylated enzyme in each case is impeded by displacement of the required water molecule by the hydroxy group of the cleaved γ-lactone. Synthetic compounds such as GR167088 (Figure 1.23) were developed that retained the trans-lactone unit but were more amenable to synthesis, while analogous trans-lactams were prepared to circumvent the hydrolytic instability (in plasma) of the synthetic trans-lactones.
Figure 1.24 Schematic of ring opened \textit{trans}-lactones and \textit{trans}-lactams (top); \textbf{GR133478} (PDB 1AWF) covalently bound to thrombin showing Trp-148 and Trp-60D arching over the S1 pocket (left); \textbf{GW311616A} (PDB 1HV7) covalently bound to elastase showing the open nature of the binding site (right). Associated data is given in Table 1.1. Blue: positive electrostatic potential, Red: negative electrostatic potential, White: neutral electrostatic potential.

Table 1.1 Data from crystal structures of acylated thrombin and elastase.

<table>
<thead>
<tr>
<th>PDB Code</th>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>D (Å)</th>
<th>⁴Dihe</th>
<th>Enzyme constraint</th>
<th>Reversible acylation</th>
<th>Histidine position/ available as base</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1AWF</td>
<td>Thrombin</td>
<td>GR133487</td>
<td>3.04</td>
<td>85</td>
<td>Yes</td>
<td>Yes</td>
<td>Normal/yes</td>
<td>70,72</td>
</tr>
<tr>
<td>1AWH</td>
<td>Thrombin</td>
<td>GR133686</td>
<td>3.37</td>
<td>87</td>
<td>Yes</td>
<td>Yes</td>
<td>Normal/yes</td>
<td>70,72</td>
</tr>
<tr>
<td>1HV7</td>
<td>Elastase (HNE)</td>
<td>GW311616A</td>
<td>4.10</td>
<td>-163</td>
<td>No</td>
<td>No</td>
<td>Rotated/no</td>
<td>80,81</td>
</tr>
<tr>
<td>1QGF</td>
<td>Elastase (PPE)</td>
<td>12</td>
<td>2.97</td>
<td>-68</td>
<td>No</td>
<td>No</td>
<td>Rotated/no</td>
<td>59,73</td>
</tr>
</tbody>
</table>

⁴Dihedral angle of atom \(a\) (Figure 1.7, top) to the plane of atoms \(b-c-d\). ⁵His-57 for thrombin and PPE, His-60 for HNE.

One such \textit{trans}-lactam, \textbf{GW311616A} (Figure 1.25), was developed as an elastase inhibitor, the mode of action of which involves irreversible acylation of the active-site serine.⁸⁰,⁸¹ In the crystal structure 1HV7 (human neutrophil elastase (HNE)
acylated by GW311616A there are no amino acid residues to constrain the sulfonamide and acyl groups of the cleaved γ-lactam so their orientation is di-axial on ring A with an internuclear separation of 4.10 Å between the sulfonamide nitrogen and the acyl carbon atoms - well outside the distance for re-formation of the trans-lactam bond (Figure 1.24 and Table1.1). The monocyclic γ-lactam 12 (Figure 1.25) was shown by Schofield and co-workers59,73 to be a weak ($K_i = 1.3$ mM) irreversible inhibitor of porcine pancreatic elastase (PPE). Although the crystal structure 1QGF (PPE acylated with 12) shows that the internuclear separation of the sulfonamide nitrogen and acyl carbon atoms of the cleaved γ-lactam band is 2.97 Å, reformation of the γ-lactam was not observed as His-57 was rotated and was not positioned to act as a base for the cyclisation or hydrolysis (Table 1.1). This contributes to the irreversible inhibition of elastase by the γ-lactam.

![Figure 1.25 A synthetic bicyclic trans-lactam and monolactam elastase inhibitors.](image)

Another structure studied by Schofield and co-workers was γ-lactam 13 (Figure 1.26). This was found to be readily hydrolysed by PPE to 14, however, it was shown that PPE could catalyse the cyclisation of 15 to 13, indicating that deacylation by cyclisation could compete with hydrolysis of the acylated enzyme generated from 13.59,73 In an effort to evade this deacylation path (and for other reasons) the fused thiophenes 16 and 17 were prepared and were shown to inhibit PPE (IC$_{50} = 20$ and 1 μM respectively).69 Although the syn geometry of the sulfonamide and acyl groups on the thiophene ring favour cyclisation (in the acylated enzyme) it was considered that, the nucleophilicity of the sulfonamide nitrogen atom would be diminished by conjugation with the thiophene ring thereby retarding cyclisation as a deacylation path. X-ray crystallography confirmed active-site acylation for 16, that His-57 was not rotated and that the sulfonamide nitrogen and acyl carbons were held in close proximity; it was not established, however, if deacylation by cyclisation occurred.
1.8 Development aspects of trans-lactams as elastase inhibitors

At an early stage in the elaboration of the trans-lactone and trans-lactam scaffolds as protease inhibitors it was found that, whereas the racemic trans-lactones 18 and 19 were inhibitors (IC₅₀, 7 and 23 μM respectively) of human leucocyte elastase the racemic trans-lactam 20 showed no significant activity. This was attributed to the low reactivity of the γ-lactam bond.

Trans-lactam structures 20-22 were subsequently prepared and evaluated as inhibitors of human neutrophil elastase (Table 1.2). Comparison of the inhibition data (Table 1.2) for 20 and 21 clearly show the importance of activation of the γ-lactam bond by incorporation of an electron-withdrawing group on the γ-lactam nitrogen atom. The crystal structure of 21 covalently linked to Ser-195 at the active site of PPE shows the methyl group of the sulphonamide pointing out of the S-1' binding pocket, and as such does not contribute to binding with the enzyme. The fact that 22 has, essentially, an almost identical IC₅₀ value as 21, is consistent with the view that the alkyl/aryl component of the sulphonamide does not provide any significant binding. Subsequently, it was found that the 2-naphthyl group was not viable in in-vivo testing, so further development was carried out with the methylsulfonamide structure.
Table 1.2 Inhibition data for selected racemic trans-lactams against HNE.

<table>
<thead>
<tr>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 H</td>
<td>&gt;500</td>
</tr>
<tr>
<td>21 SO$_2$CH$_3$</td>
<td>0.047</td>
</tr>
<tr>
<td>22 SO$_2$-2-Naphthyl</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Reduction of the allyl moiety and installation of a piperidinyl side-chain led to 23. The piperidinyl unit served to provide enhanced water solubility in its hydrochloride form. In further in-vivo tests it was found that 23 was metabolised largely through hydrolysis of the lactam ring. This issue was overcome satisfactorily by replacement of the propyl unit that is adjacent to the lactam carbonyl group, with an isopropyl unit. A serendipitous observation showed that incorporation of unsaturation in the side-chain group was beneficial in terms of in-vivo activity, and this resulted in the preparation of the trans-lactam 24 (HNE IC$_{50}$ 0.022 µM) by the GlaxoSmithKline company.

\[
\text{(rac)-23} \quad \text{24 (GW311616A) (absolute stereochemistry)}
\]

In light of the latter observations above it is evident that an in-silico study, aimed at identifying the optimum ligands for enzyme binding, would not have been overly productive. It may be noted that no low-molecular weight organic structures are known that inhibit elastase merely by non-covalent binding; this is due to the relatively small size of the S-1 binding pocket. Thus, low-molecular weight organic inhibitors of elastase depend on covalent attachment in order to achieve tight binding. This situation is quite different with thrombin, which has an extensive and convoluted S-1 pocket that can partially close over the active site, and for which a number of low-molecular weight organic compounds act as effective inhibitors through non-covalent binding (Argatroban, Section 1.7.1).
A number of back-up structures, based on the generic trans-lactam unit shown below were also prepared; one of these, 25 (HNE, IC_{50} = 0.010 μM), has a five-membered heterocyclic ring side group (an oxazole) with a pendant pyrrolidine.80

![Trans-lactam with generic side-chain](image1)

25 (absolute stereochemistry)

1.9 Development aspects of 5,5-fused thiophene γ-lactams as elastase inhibitors

A study on the development of 5,5-fused thiophene γ-lactams (Scheme 1.13) was carried out by Migaud et al. in conjunction with the GlaxoSmithKline company.69 It was considered that the synthesis of the thiophene γ-lactam would be simpler than that of the trans-lactams such as 24, and that a similar level of potency might be obtained. The initial choice of substituents followed directly from the earlier trans-lactam work. Thus, a methylsulfonamide was chosen for activation of the γ-lactam bond and an ethyl group was chosen to be compatible with the relatively small S-1 binding pocket of elastase. The work, however, did not deliver the desired outcome. It was hoped that, starting from 26, the preparation of structure type 31 could be readily achieved. The bicyclic template 27 was readily prepared from 26, however, monoalkylation of 27 to generate 30 proved to be problematic; 30 was prepared by starting from a version of 26 that already contained the ethyl group. Dialkylation of 27 occurred quite readily and this led to the preparation of the bis-allylated structure 28. Ring-closing metathesis, using a Grubbs’ catalyst, gave 29 containing a spirocyclic side chain. Incorporation of a substituent (R') onto the thiophene ring, that could occupy the S2/S3 binding pocket of elastase, proved not to be a viable option.84
Scheme 1.13 5,5-fused thiophene $\gamma$-lactam structures and an outlined synthetic route.\textsuperscript{69}

Evaluation of the elastase inhibitory potency of some of these structures was carried out using both PPE and HNE. The bis-allylated structure 28 was the least active ($IC_{50} = 120 \mu M$, $>500 \mu M$ for PPE and HNE respectively), while the spiro-thiophene 29 was the most active ($IC_{50} = 1 \mu M$ for HNE). An $IC_{50}$ value of 20 $\mu M$ (for PPE) was obtained for each of 27 and 30. It is interesting to note that development of the most active of these structures, 29, evolved in a fortuitous manner, and without any \textit{in-silico} evaluation of ligand suitability for occupancy of the S-1 binding pocket. Inhibition of PPE by 27 was reversible, however, it was not established if this arose due to hydrolysis of the acylated enzyme or to re-formation of intact 27. It was postulated that, with all these structures, the thiophene ring system would stabilise the acyl-enzyme complex with respect to re-formation of the $\gamma$-lactam, as a result of resonance with the amine lone pair electrons released upon ring-opening. A crystal structure was obtained for PPE acylated with 30 (the coordinates were not made available in the public domain) which shows that the sulphonamide nitrogen atom is suitably positioned for re-formation of the $\gamma$-lactam ring, and that His-57 is positioned to act as a general base to facilitate the ring closure (Figure 1.27). It was postulated that the stability of this acylated structure was, at least in part, due to delocalisation of the nitrogen lone pair electrons onto the thiophene ring.\textsuperscript{69}
Figure 1.27 Schematic of elastase (PPE) acylated with thiophene 30 reconstructed from the crystal structure view given in reference.69

1.10 Clinical status of elastase inhibitors

Sivelestat is the only elastase inhibitor that has been licensed for clinical usage, and then only in Japan. Its use has been reported only in relation to treatment of acute lung injury.55,58,85 There is no report of its use in the treatment of chronic diseases such as cystic fibrosis.

Clinical trials involving α(1)-antitrypsin (AAT) and secretory leukocyte protease inhibitor (SLPI) have been carried out and have been commented on by McElvaney and co-workers.61 Intravenous administration of purified α(1)-Antitrypsin (AAT) in order to inhibit neutrophil elastase in cystic fibrosis (CF) individuals gave brief inhibition of neutrophil elastase (NE) and then only at quite high dosage levels of AAT. The administration of purified AAT via aerosol was also evaluated with CF patients; while this approach showed promise considerable further development with regard to the supply of purified AAT and the aerosol technology was needed.61 Evaluation of a recombinant form of SLPI via aerosol administration with CF individuals gave some inhibition of neutrophil elastase but only at high dosage levels. Overall it was concluded that considerable challenges would need to be overcome before SLPI administration could provide an effective therapy. The possible benefits of low-molecular weight organic inhibitors were noted by McElvaney and and coworkers, while recognising that considerable research was still required to advance any such structure for use in treating cystic fibrosis.61
There are no reports of clinical trials involving the trans-lactam structure produced by the GlaxoSmithKline company.

1.11 Previous Work in the Group

As part of a research project carried out by Ruddle and Smyth on modified penicillins the Δ7 pyrrolo[2, 1-b]thiazole 32 was obtained (Figure 1.28), and was found to act as a relatively potent irreversible inhibitor (IC₅₀ = 19 μM) of PPE. Screening of 32 for elastase inhibitory activity was undertaken because of its gross structural similarity to the monolactam 33 (Kᵢ = 1.32 mM, PPE) and the trans-lactam 24 (Figure 1.28; note 24 also referred to earlier on page 37). The ring-fused bicyclic core of both 32 and 24 are essentially planar, although 32 lacks an alkyl group α to the γ-lactam carbonyl and only has a carboxylate in place of the piperidinocrotonyl unit of 24. A feature common to both 32 and 33 is the carboxylate group β to the lactam carbonyl.

![Figure 1.28 Pyrrolothiazole structure 32, monolactam 33 and trans-lactam 24.](image)

The benzhydryl group in 32 is sterically demanding and seems, at first sight, to be inappropriate as a ligand of an effective elastase inhibitor. It is relevant to note that the benzhydryl esters of the 7-alkylidenecephalosporins 34 and 35 (Figure 1.29) had been shown to be irreversible inhibitors of HLE (IC₅₀ = 0.26 and 0.39 mM respectively). It was postulated that the alkylidene group extends into the S-1 pocket with the β-lactam carbonyl adjacent to Ser-195 and the benzhydryl group extending above the S-1' pocket. It is plausible to view that the binding and reaction of 32 (Figure 1.28) could occur in a similar manner: placing the γ-lactam carbonyl of 32 adjacent to Ser-195 of PPE would position the benzhydryl group above the S-1' binding pocket and out of contact with amino acid residues of the enzyme.
It should be noted that, unlike 32 (Figure 1.28), 34 and 35 (Figure 1.29) are not soluble in aqueous buffers and this necessitated the use of 40% (v/v) of DMSO in the incubation solution, prior to subsequent dilution for running the assay itself. In the work of Ruddle and Smyth and of O’Dwyer and Smyth it was consistently observed that the activity of PPE diminished dramatically when the enzyme activity was assayed in 10 – 15% DMSO (v/v) aqueous buffers. A question, therefore, arises as to whether the binding and reaction of 34 and 35 occurred with the native conformation of the enzyme or with some other conformation that only occurs in a 40% DMSO solution.

The modified penicillin project of Ruddle and Smyth was primarily focused on triggering release of a side-chain component at the 7-position, following scission of the β-lactam ring. This aspect is illustrated by the reaction of 40 with allyl alcohol and triethylamine (in Scheme 1.14) resulting in the lost of methanol from the ester side-chain and formation of 43 as a co-product. Isomerisation of 42 occurred, presumably, via deprotonation of the bridgehead hydrogen to give a delocalised anion followed by reprotonation to form 43 exclusively as the thermodynamically more stable isomer. Selective deprotection of the allyl ester gave the salt 32.
Scheme 1.14 Modified penicillin research. (i) Isoamyl nitrite, TFA, CH₂Cl₂, R.T. 15 min, not isolated; (ii) propylene oxide, rhodium octanoate, C₆H₆, R.T. under N₂, 15 min, 99%; (iii) methyl(triphenylphosphoranylidene) acetate, CH₂Cl₂, -55 °C, 40 min, 49%; (iv) allyl alcohol, NEt₃, 12 h R.T, 77%; (v) (i) Pd(PPh₃)₄, toluene-5-sulfinic acid sodium salt (tetrahydrate), THF, DIW, 1 h R.T. 91%; (ii) sodium bicarbonate.

A later research programme, undertaken by O’Dwyer and Smyth, explored the possibility of preparing derivatives of 43. The synthetic strategy that was explored to achieve this is shown in Scheme 1.15. A key step required the α-alkylation of 43 at the 6-position. Despite numerous attempts this alkylation step proved to be unachievable. Given this finding, and the fact that the preparation of the modified penicillin type structure was quite a complex process, no further work on the pyrrolo[2,1-b]thiazole Δ⁷ isomer was carried out.

Scheme 1.15 Possible synthetic strategy to functionalised pyrrolo[2,1-b]thiazoles (Δ⁷ isomer).
The focus of the research of O’Dwyer and Smyth then switched to the pyrrolo[2,1-b]thiazole $\Delta^6$ isomer (42) (Scheme 1.14). It was decided to explore an alternative route to this structure type that would allow for ready variation of the ligands at the 6- and 7-positions, and to evaluate these as inhibitors of PPE. The $\Delta^6$ and $\Delta^7$ isomers present subtle differences in topology and of reactivity: the $\Delta^7$ isomer (43) is planar and the position of the double bond allows for resonance delocalisation through to the nitrogen lone pair, while the $\Delta^6$ isomer (42), is non-planar with respect to carbon-8 and the position of the double bond allows resonance interaction to the $\gamma$-lactam carbonyl. The synthetic route that was developed was built around an established reaction for the single-stage assembly of a pyrrolothiazole unit. This was based on bicyclisation of mucohalic acid and $\alpha$-penicillamine (Scheme 1.16) and was first reported by Wasserman and co-workers.91

\[
\begin{align*}
\text{Mucohalic acid (} X = \text{Cl, Br}) & + \quad \text{D-Penicillamine} \\
\to & \quad \text{A pyrrolo[2,1-b]thiazole } \Delta^6 \text{ structure}
\end{align*}
\]

Scheme 1.16 Bicycle reaction with $\alpha$-penicillamine. (i) HOAc, EtOH/H2O, 20 °C, 4h.

The fact that the bicyclic product was a single diastereomer was verified by Moore and Arnold,92 who prepared 51 via this route (Scheme 1.17), and confirmed its structure by X-ray crystallography.

\[
\begin{align*}
\text{Mucochloric acid} & \to \quad \text{49} \\
\text{(i) MeOH/HCl, (ii) NaBH}_4, (b) \text{NaNO}_2/\text{HCl} & \to \quad \text{51}
\end{align*}
\]

Scheme 1.17 Preparation of 51 from 49; (i) $\alpha$-penicillamine; (ii) (a) MeOH/HCl, (b) N$_3$-, (iii) (a) NaBH$_4$, (b) NaNO$_2$/HCl.

A likely mechanism for this bicyclisation involves initial reversible imine formation followed by addition of the thiol to one face or the other to give the monocyclic diastereomers 52 and 52’ (Scheme 1.18). Subsequent general-acid catalysed (HOAc) intramolecular displacement of the hydroxy group generates the bicyclic structures 53 and 53’. If all stages are reversible then the thermodynamically most stable bicycle should be predominately formed – this would be the case where the difference
in thermodynamic stability of 53 and 53' is of the order of 5-6 kcal/mol. As part of our work the thermodynamic stability of 53 and 53', with X = Cl (Figure 1.30), was evaluated at the 6-311G level using a Gaussian programme (Gaussian 03W) and it was found that 53 was 6.4 kcal/mol more stable than 53'. The stereochemical outcome appears to be controlled by an unfavourable endocyclic interaction between the 3-carboxyl group and the γ-lactam carbonyl which occurs in 53' but not in 53 (Figure 1.30). The facile formation of structure type 53, involving H₂O as a leaving group in a weakly acidic aqueous medium at ambient temperature, attests to the strong thermodynamic driving force for cyclisation of structure type 52 (Scheme 1.18).

Scheme 1.18 Mechanism of the bicyclisation reaction between D-penicillamine and a mucohalic acid (X = Cl, Br).

Figure 1.30 The diastereomeric products 53 and 53' (X = Cl (green)). 53 is more stable than 53' by 6.4 kcal/mol as evaluated at the Gaussian 6-311G level.
1.11.1 Exploration of routes to substituted pyrrolothiazoles

For the synthesis of pyrrolothiazoles substituted at the 6- and 7-positions, options arise in terms of introduction of one or both ligands prior to, or after, the bicyclisation stage as outlined in Scheme 1.19.

Scheme 1.19 Overview of routes for the synthesis of pyrrolothiazoles substituted at the 6- and 7-positions.

Route A involves partial functionalisation of mucohalic acid at the C-4 position, followed by bicyclisation and subsequent functionalisation at the C-7 position of the bicycle.

Route B involves full functionalisation of mucohalic acid at both the C-3 and C-4 positions, followed by bicyclisation to yield a functionalised pyrrolothiazole.

Route C involves bicyclisation followed by sequential functionalisations at the C-6 and C-7 positions of the pyrrolothiazole.
Route D involves partial functionalisation of mucohalic acid at the C-3 position, followed by bicyclisation with subsequent functionalisation at the C-6 position of the pyrrolothiazole.

In the work of O’Dwyer and Smyth, it was established that route A was not viable. This was primarily due to the observation that the bicyclisation reaction did not proceed efficiently when the halogen at the C-4 position of mucohalic acid was replaced by a non-electron withdrawing group, such as a 1,2,3-triazole. This process was explored as shown in Scheme 1.20. A chemoselective substitution of the C-4 chloride was achieved by a 1,4-addition-elimination reaction of azide ion with a protected form of mucochloric acid. Subsequent treatment of the azido structure with phenylacetylene generated the triazole 54. The bicyclisation of this triazole with D-penicillamine did not proceed efficiently (6% after chromatography) while a subsequent Suzuki-Miyaura coupling reaction failed to proceed.

![Scheme 1.20 Route A](image)

Scheme 1.20 Route A (i) (a) Na\(^+\) \(\cdot\)N\(_3\); (b) phenylacetylene; (c) HF/pyridine. (ii) D-penicillamine; diphenylidazomethane. (iii) Suzuki-Miyaura coupling.

In the case of route B it was found that selective substitution at the C-6/C-7 position of the bicyclic structure via chemoselective Suzuki-Miyaura coupling reactions was not successful.

Further investigation of routes C and D was carried out by O’Dwyer and Smyth and, contemporaneously, as part of the work in this thesis.
1.12 Aims and Objectives

The first part of the work in this thesis overlapped with and continued on from the earlier work of O’Dwyer and Smyth.\textsuperscript{88} The focus here was on preparation of substituted pyrrolothiazole structures that would complement those prepared by Smyth and O’Dwyer.\textsuperscript{88} The work of Smyth and O’Dwyer followed route D wherein a thioalkyl group (CH\textsubscript{3}CH\textsubscript{2}S- and (CH\textsubscript{3})\textsubscript{2}CH\textsubscript{2}S-) was installed at the 3-position of mucobromic acid (this becomes the 6-position in the pyrrolothiazole), followed by bicyclisation with D- penicillamine and subsequent installation of the C-7 group via Suzuki-Miyaura coupling. The C-7 ligands were chosen to accord with the aromatic/heterocyclic side group of the generic \textit{trans}-lactam structure (page 38), which could occupy the S-2/S-3 binding pocket of elastase, while water solubility was based on the carboxylate group on the bicyclic core unit. A summary of some of the pyrrolothiazole structures that were prepared by O’Dwyer and Smyth is shown in Scheme 1.21.

![Scheme 1.21 Sumary of pyrrolothiazole structures](image)

Scheme 1.21 Summary of pyrrolothiazole structures (i) KOH\textsubscript{aq}, (ii) RSH or ArOH, KOH\textsubscript{aq}, (iii) HCl\textsubscript{aq}; (iv) D-penicillamine, EtOH/H\textsubscript{2}O 1:1, H\textsubscript{2}Ac (1.2 equiv.); (v) Diphenyldiazomethane; (vi) Suzuki-Miyaura coupling; (vii) AlCl\textsubscript{3} in CH\textsubscript{2}Cl\textsubscript{2}/EtNO\textsubscript{2}, -80 °C, 1 h.

In this work, it was aimed to install a set of phenoxy groups at C-6, and following bicyclisation, to install a set of C-7 ligands via Suzuki-Miyaura coupling. In this case the C-7 ligands were also chosen to accord with the aromatic/heterocyclic side group of the generic \textit{trans}-lactam structure (page 38), which could occupy the S-2/S-3 binding pocket of elastase. The installation of a phenoxy ligand at C-6, in place of the ethyl- or isopropylthio group, was pursued in the context of potentially providing some insight on structure-reactivity aspects of elastase inhibition.
The second part of the work in this thesis focused on the preparation of monocyclic \(\gamma\)-lactones (72) and \(\gamma\)-lactams (structure types 73 and 74), and the screening of these for inhibitory activity against elastase. In the case of the \(\gamma\)-lactams it was envisaged to prepare both structures with no activation of the amide bond (73) as well as more reactive structure types by incorporation of an electron-withdrawing group, such as a sulfonyl group, on the amide nitrogen atom (74).
Chapter 2

Results and Discussion
Chapter 2 Results and Discussion

This chapter is divided into two parts. The first part deals with further exploration of the pyrrolothiazole structures, overlapping with and complementing the work of O’Dwyer and Smyth. The second part deals with the preparation of monocyclic γ-lactones and γ-lactams and their potential to act as inhibitors of elastase.

2.1 Results and Discussion – Part 1

Further exploration of pyrrolothiazole synthesis

As discussed in Chapter 1, in the work of O’Dwyer and Smyth a successful synthetic strategy for forming a pyrrolothiazole bicyclic unit that allowed for variation of the R_1 and R_2 substituents had been identified. This involved partial functionalisation of mucocloric acid, followed by bicyclisation with D-penicillamine with subsequent functionalisation at the C-6 position of the pyrrolothiazole (Scheme 1.19, route D); examples are given in Scheme 1.21.

2.1.1 Bicyclisation reaction with L-cysteine

Prior to investigation of Route C and Route D (Scheme 1.19) an initial exploration of the bicyclisation reaction was carried out with L-cysteine in place of D-penicillamine in order to evaluate the generality, or lack thereof, of this bicyclisation process. The reaction between mucocloric acid and L-cysteine yielded only 25% of the bicyclised material, far lower than the same reaction with D-penicillamine. This is in line with the findings of LaLonde and Xie. When this reaction was monitored by ^1^H NMR spectroscopy it was clear that the reaction mixture was a good deal more complex with L-cysteine in place of D-penicillamine, indicating the formation of (unidentified) side products. The cleaner outcome with D-penicillamine may be as a result of a faster thiazolidine ring formation step due to a gem-dimethyl effect, making the bicyclisation path dominant over competing side reactions. Thus, if the thiol group is not rapidly consumed in the intramolecular cyclisation it can undergo intermolecular reactions such as 1,4-addition-elimination, and/or 1,2-addition to the imine functionality (Scheme 2.1). Such reactions would generate dimeric and polymeric structures. One interpretation of the gem-dimethyl effect (Thorpe-Ingold effect) is that there is less
strain about the quarternary carbon in the cyclic structure compared with the acyclic structure. Recent computational analysis has suggested that limited solvation of the nucleophile in the gem-dimethyl acyclic structure may also be a factor in the rate acceleration (over a non-methylated structure).  

Scheme 2.1 Bicyclisation reaction between mucochloric acid and D-penicillamine or L-cysteine. (i) mucochloric acid (12.4 mmol), sodium chloride (15.9 mmol), acetic acid (16.3 mmol), L-cysteine (24.8 mmol), methanol/DIW (2:1) stirred at 22.5°C for 12 hours, 27%. (ii) mucochloric acid (1.92 mmol), sodium chloride (2.46 mmol), acetic acid (2.52 mmol), D-penicillamine (2.11 mmol); ethanol/DIW (1:1) stirred at 22.5°C for 12 hours, 84%.

This cysteine-based pyrrolothiazole (75) was then used to further examine the possibility of selective functionalisation at the C-6 and C-7 positions (Scheme 1.19, Route B). The bicyclic material 75 was first converted to its diphenylmethyl ester (Scheme 2.2) via reaction with diphenylziazomethane; conversion to the ester facilitated chromatographic purification and enhanced solubility of the bicyclic structure, while the diphenylmethyl ester group could be conveniently removed subsequently under mild reaction conditions using AlCl3 at low temperature. Reaction progress was easily monitored by evolution of nitrogen gas concomitant with the dissipation of the purple colour of the diazo compound. The ester product was purified by column chromatography and was characterised by 1H NMR spectroscopy and HRMS.
Scheme 2.2 Diphenylmethyl ester formation by reaction with diphenyl diazomethane. (i) diphenyl diazomethane (approx. 0.65 M) in dichloromethane added dropwise to 75 in dichloromethane; stirred at 22.5 °C until nitrogen evolution ceased; 45%.

The C-7 chloro group should be replaceable via a 1,4-addition-elimination reaction with a nucleophile (Scheme 2.3). Following from earlier work of O’Dwyer and Smyth azide ion was used as the nucleophile. This would allow for the incorporation of a small heterocyclic unit (a 1,2,3-triazole) at the C-7 position via a “Click” reaction using an alkyne (Scheme 2.4). Such a heterocyclic unit would be similar to the side-chain groups identified in the work of the GlaxoSmithKline company on the generic trans-lactam structures (see generic trans-lactam on page 38 in introduction section).

The reaction was carried out using a set reaction protocol. Thus, the reaction was set up at -20 °C in dichloromethane followed by the gradual addition of a cold suspension of sodium azide in acetonitrile (Scheme 2.3). The solution contained 15-crown-5 in order to facilitate solubilisation of the sodium salt. After stirring at -20 °C for 5 h the temperature was raised to 4 °C and stirring continued at this temperature overnight in a cold room. The reaction product was isolated and purified by column chromatography to yield the azido derivative 78. The infra-red spectrum showed a characteristic azide stretch at 2137 cm⁻¹, while a marked upfield shift of chemical shift of H-8, from 5.57 ppm for the dichloro species 77 to 5.39 ppm for azido compound 78 was observed by ¹H NMR spectroscopy.

Scheme 2.3 Formation of azido derivative 78 via 1,4 addition elimination. (i) 77 (3.75 mmol), 15-crown-5 (0.468 mmol), sodium azide (4.13 mmol), acetonitrile; stirred at -20 °C for 5 hr, stirred at 4 °C for 18 hr; 51%.
The term “Click” chemistry was first introduced by Sharpless and refers to a method to generate substances quickly by joining small units together; it is based on the fact that nature also generates compounds by joining small modular units. The copper(I) catalysed 1,2,3-triazole formation from azides and terminal acetylenes is an important example of “Click” chemistry. It is generally, but not exclusively, regioselectivity, uses mild reaction conditions and has wide substrate scope and high yields.

The Cu(I) catalyst is formed in-situ by the reduction of the Cu(II) salt (CuSO₄) by sodium ascorbate. Based on experimental and computational data Sharpless et al. proposed a catalytic cycle as shown in Scheme 2.4. It starts with coordination of the alkyne via a π-bond to the Cu(I) species with loss of one of an acetonitrile (or water) ligand, followed by formation of a Cu(I) acetylide. A stepwise sequence occurs through which the azide bonds to the copper and, via the intermediate formation of a six-membered copper-containing metallacycle, a five-membered 1,2,3-triazole bearing a copper ligand is formed. Finally, substitution of a proton for this copper ligand results in formation of the 1,2,3-triazole product and reformation of the copper catalyst.

The pyrrolothiazole derivative 79 was prepared by addition of phenylacetylene (8 equiv.) in acetonitrile, and solutions of sodium ascorbate (0.4 equiv.) and copper(II)sulphate pentahydrate in water, to a solution of 78 in acetonitrile (Scheme 2.5). The solvent ratio was kept at 9:1 acetonitrile: water and the total volume rarely exceeded 1 mL. In order to drive the reaction, the reaction was stirred vigorously in a vial, and allowed to evaporate overnight. This evaporation feature is unusual but was
found to be essential for product formation here. The basis for this reaction protocol arose from observations made in the work of O’Dwyer and Smyth.\textsuperscript{88} Thus, samples from a normal solution-phase reaction were withdrawn at different time intervals (10, 20, 30 min) and set aside prior to recording of their \textsuperscript{1}H NMR spectra. Prior to preparing these samples for recording the spectra, the solvent had evaporated. The spectra clearly indicated the formation of triazole product: a singlet at 8.78 ppm corresponding to the vinylic hydrogen of a triazole\textsuperscript{88} was obvious and this was accompanied by a downfield shift in the resonance of H-8 from 5.39 to 6.35. However, when a sample from the original reaction system was taken after 1h and analysed immediately by \textsuperscript{1}H NMR the extent of product formation was considerably less than that observed in the earlier samples that had been set aside and from which the solvent had slowly evaporated. Compound \textbf{79} was purified by column chromatography (yield 18\%) and characterised (\textsuperscript{1}H NMR, \textsuperscript{13}C NMR and DEPT 135 spectroscopy and HRMS).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme2.5.png}
\caption{Formation of 1,4-disubstituted 1,2,3 triazole \textbf{79}. (i) \textbf{78} (2.41 mmol), phenylacetylene (19.28 mmol), sodium ascorbate (1M), acetonitrile; 18\%.}
\end{figure}

In parallel with this work O’Dwyer and Smyth\textsuperscript{88} had prepared compound \textbf{80} (Scheme 2.6). This structure is the analogue of \textbf{79}, but has the gem-dimethyl unit derived from \textit{D}-penicillamine. Functionalisation of \textbf{80} at C-6, via a Suzuki-Miyaura coupling was attempted using phenyl boronic acid. Despite repeated attempts, this coupling reaction was not successful. Based on this result no further functionalisation at C-6 of \textbf{79} was carried out.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme2.6.png}
\caption{Functionalisation of \textbf{80} at C-6. (i) \textbf{80} (2.41 mmol), \textit{D}-penicillamine, phenylboronic acid, sodium carbonate, sodium ascorbate, acetonitrile; \textit{X}.}
\end{figure}
Scheme 2.6 (i) Attempted Suzuki coupling reaction of 80. (i) (a) phenyl boronic acid (2 equiv.), CsF (3 equiv.), PdCl₂P(Ph₃)₂ (0.05 equiv.), BnEt₃Na⁺Cl⁻ (0.05 equiv.), 1:1 degassed toluene/water, 105 °C, 4 h. (b) HCl 2M.

Overall it was concluded that: (i) use of L-cysteine in place of D-penicillamine was not productive due to the low yield of the bicyclisation step; (ii) use of azide ion followed by a “Click” reaction with phenylacetylene to install a phenyltriazole ligand at C-7 was feasible, although the yield of purified product was low; (iii) Suzuki-Miyaura coupling at C-6 of pyrrolothiazole structure types 80 (and by analogy 79) is not productive.

All further work in this section was carried out using D-penicillamine for the bicyclisation reaction.

2.1.2 Complete functionalisation of mucohalic component prior to cyclisation

Route C in Scheme 1.19 identifies the possibility of complete functionalisation of mucochloric (or mucobromic) acid prior to the bicyclisation stage. This next section documents my work in relation to evaluating this route.

The route depends on selectively incorporation of ligands at C-4 and C-5 using sequential Suzuki reactions, followed by bicyclisation with D-penicillamine. Suzuki-Miyaura coupling⁹⁹ of an organometallic reagent and a vinyl halide represents one of the most straightforward methods for carbon-carbon formation and so was a potentially viable synthetic method for the incorporation of ligands at the C-3 and C-4 positions of a mucohalic derivative.

The general mechanism for Suzuki coupling reaction, with Pd(Ph₃)₄ as catalyst, is illustrated in Scheme 2.7. The first stage involves oxidative addition of the vinyl halide to the Pd⁰ catalyst to generate a Pd²⁺ species. This then undergoes transmetallation with the aryl boronate; this latter is formed by addition of a base, here fluoride ion, to the aryl boronic acid (or boronate ester when this is used). The final stage involves reductive elimination from the Pd²⁺ complex to formed the coupled product and regenerate the Pd⁰ catalytic entity. Overall, this sequence of oxidative addition, transmetallation and reductive elimination is similar to that of the Stille coupling.¹⁰⁰
Scheme 2.7 General features of the mechanism of Suzuki-Miyaura coupling.

Chemoselective coupling at the C-4 position of mucohalic acid is based on this position being more electron-deficient than the C-3 position, as a result of resonance delocalisation within the conjugated α,β-unsaturated keto unit (Scheme 2.8). Thus, an initial Suzuki-Miyaura coupling should allow incorporation of a ligand at C-4 and a subsequent Suzuki-Miyaura coupling would install a ligand at the C-3 position.

Scheme 2.8 Possible chemoselective suzuki-Miyaura coupling sequence.

Chemoselective Suzuki reactions have been well documented in literature.\textsuperscript{101-104} Zhang and co-workers reported a method for the preparation of Vioxx that relied on chemoselective Suzuki-Miyaura coupling reactions (Scheme 2.8).\textsuperscript{104}
Hussain et al. reported chemoselective Suzuki-Miyaura coupling reactions of 2,3-dibromo-1H-inden-1-one (Scheme 2.10). This involved a one-pot reaction of 87 with two different arylboronic acids, which were sequentially added, affording the unsymmetrical 2,3-diaryl-1H-inden-1-one, 88 containing two different terminal aryl groups.

\[
\begin{align*}
\text{87} & \xrightarrow{1) \text{Ar}_2^1\text{B(OH)}_2} \text{Ar}_2^1\text{Ar}_2^2 \\
& \xrightarrow{2) \text{Ar}_2^2\text{B(OH)}_2} \text{Ar}_2^1\text{Ar}_2^2
\end{align*}
\]

Scheme 2.10 Site-selective Suzuki-Reactions carried out by Hussain et al.\textsuperscript{102} (1) Ar\textsubscript{1}B(OH\textsubscript{2}) (1.0 equiv), Pd(PPh\textsubscript{3})\textsubscript{4} (3 mol %), 2M K\textsubscript{2}CO\textsubscript{3} (aq), dioxane, 45ºC, 4h; (2) Ar\textsubscript{2}B(OH\textsubscript{2}) (1.1 equiv), Pd(PPh\textsubscript{3})\textsubscript{4} (3 mol %), dioxane, 45ºC, 7h.

### 2.1.2.1 Suzuki reaction on protected mucohalic acid derivatives

Prior to evaluation of the chemoselective coupling reactions, the C-5 hydroxyl group of mucochloric acid was protected (Scheme 2.11). A tert-butyldimethylsilyl (TBDMS) unit was chosen as the protecting group due to its established ease of installation\textsuperscript{105} and selective removal.\textsuperscript{103} The protecting group was clearly identified by \textsuperscript{1}H NMR analysis; two singlets at 0.22 and 0.24 ppm ((CH\textsubscript{3})\textsubscript{2}Si-) and a singlet at 0.95ppm ((CH\textsubscript{3})\textsubscript{3}C-) (Figure 2.1, top spectrum).

Scheme 2.11 Protection of mucochloric acid. (i) mucochloric acid (29 mmol), tert-butyldimethylsilylchloride (31.97 mmol) in DMF (35.87 mmol), diisopropylethylamine (34 mmol); 90%.
Evaluation of the chemoselective coupling used phenyl boronic acid with 82 to in an attempt to form the mono-substituted derivative 89 (Scheme 2.12). Variation of reaction time, temperature and using less than 1 equivalent of phenyl boronic acid failed to yield this structure. Consistently, the product formed was the di-substituted derivative 90. Removal of the protecting group from 90 was carried out using HF-pyridine as per literature procedures.\textsuperscript{103} \textsuperscript{1}H NMR analysis showed complete removal of the TBSO protecting group and change in the chemical shift of H-5 shift from 6.40 ppm for the protected species, 90 to 6.51 ppm for the deprotected, 91 (Figure 2.1, middle and bottom spectra). This chemoselective coupling was further examined in a final-year-project by an undergraduate student working with me, however, the mono-substituted product was not obtained. Instead, attention was turned to evaluating the bicyclisation reaction with the di-substituted muco derivative 91.

Scheme 2.12 Suzuki-Miyaura coupling reaction carried out on protected mucochloric acid 82. (i) 82 (0.498 mmol), phenyl boronic acid (0.747 mmol), caesium fluoride (0.966 mmol), PdCl\textsubscript{2}(PPh\textsubscript{3})\textsubscript{2} (0.01245 mmol), benzyldimethylhexadecyl ammonium chloride (0.01245 mmol), toluene/DIW (1:1), refluxed for 2.5 hours, 77 %. (ii) 90 (0.835 mmol), HF/pyridine (0.5 mL) stirred in Teflon beaker for 12 hr, 76%. (iii) 82 (0.498 mmol), phenyl boronic acid (0.125 – 0.498 mmol), caesium fluoride (0.966 mmol), PdCl\textsubscript{2}(PPh\textsubscript{3})\textsubscript{2} (0.01245 mmol), benzyldimethylhexadecyl ammonium chloride (0.01245 mmol), toluene/DIW (1:1).
Figure 2.1 $^1$H NMR spectra for compounds 82, 90, and 91.
2.1.2.2 Bicyclisation reaction with a fully functionalised mucohalic derivative

The bicyclisation reaction was attempted using the diphenyl muco derivative 91 and D-penicillamine under the reaction conditions identified previously. However, no reaction occurred and no bicyclic product was obtained (Scheme 2.13).

Scheme 2.13 Bicyclisation attempt of the diphenyl derivative and D-penicillamine (i) 91 (1.92 mmol), sodium chloride (2.46 mmol), D-penicillamine (2.11 mmol), acetic acid (2.52 mmol) in ethanol/water (1:1).

Page et al.\textsuperscript{106,107} had reported a bicyclisation procedure using phthalaldehydic acid and valinol (Scheme 2.14). Phthalaldehydic acid is analogous to mucohalic acid as its ring closed and ring opened forms are in equilibrium with each other. It is similar to the diphenyl derivative 91, as it has no halogen on the ring; this is in contrast to the situation with the mucohalic acids wherein the 4-halo group may provide some additional activation of the aldehyde toward nucleophilic addition. This reaction was carried out in toluene using Dean-Stark conditions. This synthesis was repeated and the product was successfully isolated and purified by chromatography. \textsuperscript{1}H NMR analysis of 93 agreed with the data reported by Page.\textsuperscript{106,107}

Scheme 2.14 Bicyclisation of phthalaldehydic acid and valinol. (i) valinol (1.8 mmol), phthalaldehydic acid (1.8 mmol); toluene (20 mL); reflux (24 hr); 68%.

In an effort to drive the bicyclisation reaction between the diphenyl derivative 91 and D-penicillamine, the reaction was repeated under the same conditions reported by Page, that is, in toluene and heated to reflux under Dean-Stark conditions. The reaction did not proceed. In order to verify that the ring-opened form of 91 and of phthalaldehydic acid could be generated under similar conditions the reactions were monitored by \textsuperscript{1}H
NMR spectroscopy. It was found that in the presence of a base, in this case, tetramethylguanidine (TMG), phthalaldehydic acid and the diphenyl derivative \(91\) existed entirely in their ring opened forms. This was evident by the characteristic aldehydic hydrogen resonance for each of these at approximately 10 ppm. This showed that it was possible for the diphenyl muco derivative \(91\) to ring open. When the phthalaldehydic acid, in the basic solution, was reacted with valinol, and monitored by \(^1\)H NMR spectroscopy, formation of the imine \(94\) was observed by a change of the aldehydic hydrogen resonance from 10.28 ppm for the aldehydic hydrogen to 9.84 ppm for the imine hydrogen. However, when this process was repeated with the diphenyl derivative \(91\) there was no indication by \(^1\)H NMR spectroscopy of imine \(95\) formation (Scheme 2.15); it was not obvious why no reaction occurred in this instance.

Scheme 2.15 Reactions in the presence of TMG.

As this reaction process was unsuccessful, it was concluded that a change of synthetic strategy was necessary. Focus shifted to the route that involved partially functionalisation of a mucohalic acid at C-3, followed by bicyclisation with D-penicillamine, and subsequent installation of a ligand at the C-7 position of the resulting pyrrolothiazole.

**2.1.3 Partial functionalisation of the mucohalic component prior to cyclisation**

This part of the work was carried out in parallel with some of the research of O’Dwyer and Smyth.\(^{88}\) The general synthetic strategy is outlined in Scheme 2.16. The first step was to convert muco bromic acid into its ring-opened form by treatment with aqueous base. Next, an anionic nucleophile compatible with aqueous base would be
used to effect a 1,4-addition-elimination reaction thereby substituting for the 3-bromo group. Following ring closer (by re-acidification), the bicyclisation reaction with D-penicillamine could be used to form the pyrrolothiazole unit. Conversion to the corresponding diphenylmethyl ester, using diphenyldiazomethane, would allow for facile chromatographic purification. Two options then arise for substitution of the 7-bromo group of the pyrrolothiazole structure, viz. Suzuki-Miyaura coupling or installation of the azide unit (via 1,4-addition-elimination) followed by a “Click” reaction using an acetylene. Thiolates and phenoxides were identified as anionic nucleophiles that could be readily formed in aqueous base. In the approach of O’Dwyer and Smyth, ethyl- and isopropylthiolate ions were employed successfully. In my work the phenoxides derived from phenols were used successfully. Each of these ligands on the pyrrolothiazole structure was viewed as potential components that could occupy the S-1 binding pocket of elastase. It was considered that some useful structure-activity information, regarding inhibition of serine proteases, might be obtained from a set of such structures bearing either an alkylthio or a phenoxy group at C-6.

**Scheme 2.16 General synthetic strategy:**

(i) aqueous base; (ii) (a) anionic nucleophile, (b) acid; (iii) (a) bicyclisation with D-penicillamine, (b) diphenyldiazomethane; (iv) Suzuki-Miyaura coupling; (v) (a) azide ion, (b) “Click” reaction.

Incorporation of the phenoxide unit at the C-3 position of mucobromic acid was achieved by reaction with the required phenol in aqueous base. Under these reaction conditions mucobromic acid exists entirely in its anionic ring-opened form and the phenols occur as the phenoxide ions. The 1,4-addition-elimination product precipitated from solution and was identified by $^1$H NMR spectroscopy as the ring opened substituted mucochloric acid on the basis of the aldehydic hydrogen resonance at
9.95 ppm. The precipitate was acidified to pH 2 resulting in ring closure to give the substituted mucohalic acids 96, 97 and 98 (Scheme 2.17). The $^1$HNMR spectra of the ring-opened and ring-closed forms of 96 are shown in Figure 2.2.

Scheme 2.17 Synthesis of partially functionalised mucohalic acid 96. (i) mucochloric acid (3.88 mmol) in aq. KOH (4.56 mmol), phenol (5.82 mmol) in aq. KOH (5.82 mmol); stirred at 22.5°C for 3 hr; 36%. (ii) mucobromic acid (3.88 mmol) in aq. KOH (4.56 mmol), 3-chlorophenol (5.82 mmol) in aq. KOH (5.82 mmol); stirred at 22.5°C for 3 hr; 50%. (iii) mucobromic acid (3.88 mmol) in aq. KOH (4.56 mmol), 3-nitrophenol (5.82 mmol) in aq. KOH (5.82 mmol); stirred at 22.5°C for 3 hr; 52%.

Figure 2.2 $^1$H NMR spectra for compound 96 and its ring-open form.* DMSO-d$_6$
The pyrrolothiazoles 99 and 100 were synthesised from D-penicillamine and muco derivatives 96 and 97 (Scheme 2.18) using the same bicyclisation procedure as before. These pyrrolothiazoles were successfully isolated and characterised (1H and 13C NMR spectroscopy and HRMS). As before, the acids of the bicyclised material were converted to the diphenylmethyl esters 101 and 102 with diphenyldiazomethane. These compounds were purified by column chromatography and confirmed by 1H NMR spectroscopy and HRMS. 1H NMR spectrum for 102 shown in Figure 2.3.

Scheme 2.18 Synthesis of pyrrolothiazole unit 101 and 102. (i) 96/97 (1.92 mmol), sodium chloride (2.46 mmol), D-penicillamine (2.11 mmol), acetic acid (2.52 mmol) in 1:1 ethanol/water; 99 (44%), 100 (76%); (ii) diphenyldiazomethane (approx. 0.65 M) in dichloromethane added dropwise to 99/100 in dichloromethane; stirred at 22.5 ºC until nitrogen evolution ceased; 101 (99%) 102 (63%).

Figure 2.3 1H NMR spectrum of 102 (CDCl₃).

The azide derivative 103 was synthesised, as before, by 1,4-addition of sodium azide (Scheme 2.19). The method was adapted slightly for reasons of solubility. DMSO
was used as a co-solvent to ensure solubility of all materials. This step was problematic and low yielding. The reaction did not go to completion and some of the starter compound 101 remained. $^1$H NMR spectroscopy showed formation of the azide by the characteristic shift of H-8 from 5.89 ppm for 101 to 5.76 ppm for the azide derivative 103. This could not be separated by column chromatography. As a result, the “Click” reaction was carried out on the crude azide 103 and the 1,4 disubstituted 1,2,3-triazole 104 was formed. Chromatography at this stage removed any 101 that still remained in the crude. $^1$H NMR spectroscopy showed the distinctive 1,4-triazole peak at 8.44 ppm.

Scheme 2.19 Synthesis of the 1,4 disubstituted 1,2,3-triazole 104. (i) 101 (3.75 mmol), 15-crown-5 (0.468 mmol), sodium azide (4.13 mmol), acetonitrile, stirred at -20ºC for 5 h, stirred at 4ºC for 18 h; 39% (crude). (ii) 103 (2.41 mmol), phenylacetylene (19.28 mmol), sodium ascorbate (1M), acetonitrile (10.4 mL); 16%.

A Suzuki-Miyaura coupling of the pyrrolothiazole unit 102 with 3-furanyl boronic acid was carried out to yield 105 in 40% yield (Scheme 2.20). This was carried out according to the procedures identified before. Compound 105 was isolated by column chromatography and characterised ($^1$H NMR (Figure 2.4) and $^{13}$C NMR (Figure 2.5) spectroscopy and HRMS).

Scheme 2.20 Suzuki-Miyaura reaction to give 105. (i) 102 (0.498 mmol), 3-furanboronic acid (0.747 mmol), caesium fluoride (0.996 mmol), PdCl$_2$(PPh$_3$)$_2$ (0.01245 mmol), benzyldimethylhexadecyl ammonium chloride (0.01245 mmol); in 1:1 degassed toluene/water; refluxed for 3 hr, 40 %.
Figure 2.4 $^1$H NMR spectrum of 105 (CDCl$_3$).

Figure 2.5 $^{13}$C NMR spectrum of 105 (CDCl$_3$).
2.1.4 Deprotection and salt formation

In order to carry out aqueous enzymatic assays with the functionalised pyrrolothiazole 105, removal of the diphenylmethyl ester protecting group was required followed by salt formation. A method of deprotection was successfully implemented using established methodology (AlCl₃, at -84 °C using nitroethane/dichloromethane as solvent) to generate the free acid 106. This was converted to the sodium salt, 107, using aqueous hydrogen carbonate (0.8 equiv.) followed by freeze-drying (Scheme 2.21).

Scheme 2.21 Deprotection of pyrrolothiazole 105 and salt formation (i) AlCl₃ (1.664 mmol) in nitroethane added to a cold (-84 °C) solution of 14b (0.6748 mmol) in dichloromethane, 1 h, 45%. (ii) 106 (0.1417 mmol) extracted with sodium carbonate (0.8 equiv), 79%.

The salt 108 was also generated in the same way from the ester 104.

2.1.5 Enzymatic evaluation

At this point the pyrrolothiazole salts 107 and 108 were screened for inhibitory activity of elastase, and in addition 107 was also screened for inhibition of thrombin. This work was carried out in parallel with screening by O’Dwyer and Smyth⁸⁸ of the pyrrolothiazole salts 68, 69 and 32 for inhibitory activity of elastase.

Established solution-phase enzyme assay procedures were followed.¹¹⁰,¹¹¹ Thus, the rate of turnover of N-succinyl-Ala-Ala-Ala-p-nitroanilide (𝐾ₑ = 1.15 mM)¹¹¹ by a fixed concentration of enzyme, both in the absence and presence of varying
concentrations of the test structures, was measured by monitoring the increase in absorbance at 410 nm, due to the release of \( p \)-nitroaniline, as a function of time. The concentration of the relevant components of the stock and assay solutions are given in Table 2.1. In the case of thrombin the assay substrate used was Sar-Pro-Arg-\( p \)-nitroanilide dihydrochloride (Sar = sarcosine).

\[
\text{\includegraphics[width=\textwidth]{molecules.png}}
\]

Table 2.1 Enzyme assay solution concentrations.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Solution [mM]</th>
<th>Assay Solution in Cuvette [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous phosphate buffer pH 7.2</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Elastase solution</td>
<td>( 2.45 \times 10^{-2} )</td>
<td>( 2.45 \times 10^{-4} )</td>
</tr>
<tr>
<td>Substrate N-Suc-Ala(3)-( p )-NA solution</td>
<td>1.5</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The enzyme assay results are summarised in Table 2.2; the data for the \( \Delta^7 \) isomeric pyrrolothiazole 32\(^{86} \) is included for reference. It was noted that turbidity developed in the assay cuvette when the concentration of 107 was doubled to 2 mM. The turbidity was detectable by the naked eye and was clearly observable by scanning the contents of the cuvette at any wavelength in the range 250 - 600 nm. The origin of the turbidity could not be ascertained, although, there are literature reports on the observation of non-specific enzyme inhibition that was attributed to agglomerate formation.\(^{112} \) Data is included in Table 2.2 only where it was unambiguous that turbidity did not occur in the assay cuvette. Switching from a phosphate buffer to a tris buffer did not eliminate the problem of turbidity.\(^{112} \)
Table 2.2 Summary of enzyme inhibition data.

<table>
<thead>
<tr>
<th>Entry No.</th>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>[Inhib] mM</th>
<th>Residual enzyme Activity (%)</th>
<th>[Substrate] mM</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PPE</td>
<td>32</td>
<td>0.019</td>
<td>50</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>PPE</td>
<td>107</td>
<td>1</td>
<td>83</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>3</td>
<td>PPE</td>
<td>108</td>
<td>1</td>
<td>90</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>4</td>
<td>PPE</td>
<td>69</td>
<td>5</td>
<td>50</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>PPE</td>
<td>68</td>
<td>5</td>
<td>85</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88</td>
</tr>
<tr>
<td>6</td>
<td>Thrombin</td>
<td>107</td>
<td>1</td>
<td>60</td>
<td>0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

<sup>a</sup>Incubation time = 40 min; error on residual enzyme activity is 5%. <sup>b</sup>N-succinyl-Ala-Ala-Ala-Ala-p-nitroanilide; <sup>c</sup>Sar-Pro-Arg-p-nitroanilide.

It is clear from the data in Table 2.2 (entries 2-5) that all of the Δ<sup>6</sup> isomeric pyrrolothiazoles are quite weak inhibitors of elastase, and that they are considerably less active than the original Δ<sup>7</sup> isomer 32 (entry 1). The inhibitory activity of 107 against thrombin was also quite weak (entry 6), although it showed slightly higher activity compared with elastase (entry 2).

The pyrrolothiazole isomers differ in two ways – the planarity of the ring fused system and non-conjugated lactam carbonyl of the Δ<sup>7</sup> pyrrolothiazole is in contrast to the slightly bevelled ring-fused system and conjugated lactam carbonyl of the Δ<sup>6</sup> isomers. The conjugated lactam carbonyl of the Δ<sup>6</sup> isomers should be inherently less reactive than the corresponding carbonyl in the Δ<sup>7</sup> isomers, and this may be one factor that contributes to the weak inhibitory activity of the Δ<sup>6</sup> isomers. As pointed out earlier (page 36, section 1.8) the efficacy of low-molecular-weight organic structure as inhibitors of elastase is dependent on covalent bond formation at the active site (no low-molecular weight organic structures are known that inhibit elastase merely by non-covalent binding), and furthermore, the rate of covalent bond formation is also an important factor. This reactivity aspect has been studied by Page et al.<sup>79</sup> in relation to some of the trans-lactone and trans-lactam structures that were developed by the Glaxo Smith Kline company as elastase inhibitors.<sup>83</sup>
Reactivity was assessed by determining the rate constant for hydroxide ion catalysed hydrolysis, $k_{OH}$, of relevant structures (equation 30). Where the hydrolysis of a structure is general-base catalysed then the rate of hydrolysis ($k_{obsd}$) in an aqueous buffer is given by equation 31:

$$Y \xrightarrow{k_{obsd}} Aqueous buffer$$

$$k_{obsd} = k_{H2O} [H_2O] + k_B [Base] + k_{OH}[OH^-]$$

(31)

The buffer components consist of a base and its conjugate acid, so $k_B$ is the rate constant for hydrolysis by the buffer base component and $k_{H2O}$ is the rate constant for water acting as a base. Analysis of $k_{obsd}$ data from a number of buffer solutions allows for evaluation of $k_{OH}$. From a survey of acylating agents as covalent enzyme inhibitors Page et al.79 have shown that, for therapeutically useful structures, the $k_{OH}$ values fall into the range $0.01 – 1.0 \text{ M}^{-1} \text{ s}^{-1}$. Structures with $k_{OH}$ values greater than $1 \text{ M}^{-1} \text{ s}^{-1}$ have a rate of background hydrolysis that is too high for these to be useful inhibitors, while structures with $k_{OH}$ values less than $0.01 \text{ M}^{-1} \text{ s}^{-1}$ acylate the enzyme active site at too slow a rate for effective inhibition to occur. Relevant data on the trans-lactone and trans-lactam structures are presented in Chart 2.1.79

Both of the $\gamma$-lactones (rac)-18 and 109 act as elastase inhibitors while the unactivated $\gamma$-lactam (rac)-20 showed no inhibition. Notably the $k_{OH}$ value of $3.07 \times 10^{-6}$ M$^{-1}$ s$^{-1}$ for the simple $\gamma$-lactam 110, and by analogy that for (rac)-20, is very low. In contrast the corresponding value for $\gamma$-lactone 109 is significantly higher at $2.5 \times 10^{-1}$ M$^{-1}$ s$^{-1}$. Attachment of a sulfonyl group to the lactam nitrogen atom provides considerable activation as seen in the $k_{OH}$ value of $0.7 \times 10^{-1}$ M$^{-1}$ s$^{-1}$ for $\gamma$-lactam 24 and, notably, this structure acts as an effective elastase inhibitor with an IC$_{50}$ value of 0.22 $\mu$M. Clearly, the presence of a ligand, to occupy the S-2/S-3 enzyme binding pocket, also plays a role as seen in the IC$_{50}$ values of 7 and 0.34 $\mu$M respectively (without incubation) for the lactones (rac)-18 and 109.
Chart 2.1 Reactivity and inhibition data for selected \( \gamma \)-lactone and \( \gamma \)-lactam structures.

In this work a detailed study of the rates of hydrolysis of the pyrrolothiazole structures was not carried out. Some indication of their hydrolytic stability/reactivity was obtained by monitoring a solution of the salt 107 in D\textsubscript{2}O phosphate buffer (50 mM), pH 8.5 at 20 \( ^{\circ} \)C, by \textsuperscript{1}H NMR spectroscopy. Over a 24 h period no hydrolysis was observed; this was taken as indicative of low reactivity for this structure type.

At this point in the work the view was taken that the \( \Delta^6 \) isomeric form of the pyrrolothiazole structures, identified above, did not warrant further investigation. In particular, it did not appear that this bicyclic unit would readily allow for incorporation of substituents that would significantly enhance the reactivity of the \( \gamma \)-lactam bond. Attention was directed, instead, to the synthesis and enzymatic evaluation of closely related mono-cyclic \( \gamma \)-lactone and \( \gamma \)-lactams (Chart 2.2). It was considered that comparision of monocyclic structures with the bicyclic counterparts would be informative in itself, and also that within the mono-cyclic \( \gamma \)-lactam structure there was scope to activate the lactam bond by incorporation of electron-withdrawing groups on to the lactam nitrogen atom. This work is presented in the following section.
2.1.6 Alternative to bicyclic scaffold

At the outset attention was turned to exploratory work on the synthesis of monocyclic γ-lactams wherein the substituents R₁, R₂ and R₁' could be varied, and where R₁' was not an electron-withdrawing group.

The general strategy chosen for assembly of this structure type had some similarities with that used for the synthesis of the pyrrolo[2,1-b]thiazole Δ⁶ structures (Scheme 2.22). In each case reaction of the neutral ring-opened form of a mucohalic derivative with an amine generates an imine followed by reaction of this with a nucleophile to generate a cyclic structure: where the penicillamine thiol group acts as the nucleophile the bicyclic pyrrolothiazole (Path A) is formed, whereas with hydride ion as the nucleophile the monocyclic γ-lactam unit is formed (Path B). As in the synthesis of the pyrrolothiazole structures, the C-3 substituent was installed prior to the reductive amination step while the C-4 substituent was installed via a Suzuki-Miyaura coupling reaction on the γ-lactam unit.
Scheme 2.22 Overview of synthetic strategies. **Path A**: route to pyrrolothiazoles; **Path B**: route to monocyclic γ-lactams.

Reductive amination of mucohalic acids, with a variety of amines, had been reported by Zhang and co-workers to give moderate-to-good yields (45-92%) of the corresponding γ-lactams. The only instance where they recorded failure of this process was when using either ammonium formate or ammonium acetate as an ammonia equivalent to generate the N-unsubstituted γ-lactam; the finding was attributed to reduction of the aldehyde group of the ring-opened form of the mucohalic acid being faster than imine formation, resulting in the formation of a γ-lactone.

In the exploratory work carried out here a 3-nitrophenoxy group was installed as the R₁ substituent using the 1,4-addition-elimination reaction previously described in the pyrrolothiazole synthesis; the product 98 was obtained in a 52% yield (Scheme 2.17). A 3, 4-dimethoxyphenethyl group was installed as the R₁’ substituent by employing 3, 4-dimethoxyphenethylamine in the reductive amination step; the product 111 was obtained in modest yield of 29% after chromatography (Scheme 2.23). Furanyl and 3-methylpyrazole units were installed as R₂ substituents via Suzuki-Miyaura coupling using PdCl₂(PPh₃)₂ in toluene/water at 100 ºC; a reaction time of 3h was required for 112 (66% after chromatography) and 24h for 113 (75% after chromatography). The nitro substituent was chosen for its potential to be reduced to an amino group and hence provide water solubility in its hydrochloride form. The furanyl unit was the same as that used in the pyrrolothiazole 105 (page 74, section 2.1.6) while the pyrazole unit represented a variation on this five-membered heterocyclic unit. The 3,4-dimethoxyphenethyl moiety was chosen with a view, in the longer term, as a ligand of a
possible structure that could be envisaged as a thrombin inhibitor. The compounds 112, 113 and 114 were characterised by HRMS and by $^1$H NMR and $^{13}$C NMR spectroscopy (Figure 2.6 – 2.10). The $^{13}$C NMR and DEPT 135 spectrum of 114 is given in Figure 2.9 and allows for clear distinction of the methylene and methyl carbons.

Scheme 2.23 (i) 2-(3,4-dimethoxyphenyl)ethylamine hydrochloride (2.06 mmol), STAB (2.58 mmol) in dichloroethane, 20 °C for 12 h, 30% (chromatography), (ii) PdCl$_2$(PPh$_3$)$_2$, 1:1 degassed toluene/water, 100 °C, 3-furanboronic acid, 3h, 112 60% (chromatography); (iii) PdCl$_2$(PPh$_3$)$_2$, 1:1 degassed toluene/water, 100 °C, 1-methylpyrazole-4-boronic acid pinacol ester, 24h, 113 75% (chromatography).
Figure 2.6 $^1$H NMR spectrum of 111 (CDCl$_3$).

Figure 2.7 $^{13}$C NMR spectrum of 111 (CDCl$_3$).
Figure 2.8 $^1$H NMR spectrum of 112 (CDCl₃).

Figure 2.9 $^{13}$C NMR and DEPT 135 spectra of 112 (CDCl₃).
The exploratory phase of the synthesis was terminated at this point in order to focus on related structures that were likely to have a more reactive carbonyl group. Reduction of the nitro group and formation of a water soluble hydrochloride salt was not pursued. In the overall synthesis, two of the steps occurred in poor-to-moderate yields – the 1,4-addition-elimination step for installation of the nitrophenoxy unit (52%) and the reductive amination step (29%). The Suzuki-Miyaura couplings gave good yields, although use of the boronic acid pinacol ester required considerably longer reaction time (24h) compared with the boronic acid (3h).

2.2 Results and Discussion – Part 2

Attention was next turned to the synthesis of monocyclic γ-lactones and γ-lactams with an electron-withdrawing group on the amide nitrogen atom. Both structure types should be more reactive than 112 and 113 as an ester/lactone functionality is generally more reactive than a simple amide/lactam, while incorporation of a sulfonyl group on the nitrogen atom of an amide/lactam is known to enhance its reactivity significantly (page 36, section 1.8). Although both structure types still contain a conjugated carbonyl unit, it was felt that synthesis and enzymatic evaluation of these
would provide a more complete picture on the potential, or lack thereof, of these as inhibitors of elastase.

\[ \text{\textgamma-lactone scaffold} \quad \text{\textgamma-lactam scaffold} \]

2.2.1 Computational docking

Computational docking was used to guide initial substituent selection for \(\textgamma\)-lactone and \(\textgamma\)-lactam compounds. This was carried out using the elastase crystal structure 1FZZ (PPE acylated with 8) and the Molecular Virtual Docking programme (MolDock).\(^{116}\) A structure file (as \(x,y,z\) coordinates) of the proposed inhibitor was generated using the AM1 semi-empirical method for structure optimisation within the Gaussian 03W software package\(^{117}\) The MolDock programme uses this structure file in combination with the elastase crystal structure file (also in the form of \(x, y, z\) coordinates); the elastase file is readily accessible online from the Protein Data Bank (PDB).\(^{78}\) The MolDock programme treats the structure as a flexible unit and the protein as a rigid structure while it generates and evaluates several thousand poses, in any one docking run, between the inhibitor structure and the active site of elastase; the MolDock algorithm takes into account van der Waals interactions, hydrogen bonding (including its directionality) and steric effects, but does not take into account any potential for covalent bond formation between the active site serine sidechain and the inhibitor structure. The docked poses, thus, correspond to virtual Michaelis complexes or juxtapositions of the inhibitor structure within the active site. Up to ten docking runs were carried out for any given inhibitor structure. At the conclusion of each docking run, the top five ranked poses of the inhibitor structure within the active site are stored as an output file. The ranking is given based on a MolDock score that is in arbitrary units; a more negative value here is indicative of a better non-covalent binding of the structure within the active site. The magnitude of the MolDock score increases with increasing molecular weight of the docked inhibitor structure so a more useful guide to ranking within a set of structures is the MolDock score per heavy atom. In Table 2.3,
the results of docking a variety of monocyclic \( \gamma \)-lactam/lactone and bicyclic pyrrolothiazole structures together with data for two known covalent inhibitors 29 and 8, with elastase (PPE), are presented. The MolDock score/heavy atom data indicate that the non-covalent affinity of known inhibitors is not significantly different from those of the other structures. Clearly, the powerful inhibitory activity of 29 (IC\(_{50} = 1 \mu\)M) and of 8 (\(K_i = 12\) nM)\(^6\) is driven by formation of the covalent bond between its carbonyl and Ser-195. In the case of 8 the top ranked pose was seen to be very closely aligned with the covalently-bound form of this within the active site of elastase as seen from the crystal structure (PDB code 1FZZ); the one key difference, of course, being the much shorter internuclear separation of the reactive carbonyl group of 8 and the oxygen atom of the hydroxymethyl sidechain of the active-site serine of elastase as a result of the formation of a covalent bond between these.
Table 2.3 Summary of MolDock scores of the top-ranked pose (non-covalent Michaelis complex) for selected structures with PPE.

<table>
<thead>
<tr>
<th>Structure</th>
<th>MolDock Score</th>
<th>MolDock Score/Heavy Atom</th>
<th>No. of Heavy Atoms</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure" /></td>
<td>-112.16</td>
<td>-6.23</td>
<td>18</td>
<td>This work</td>
</tr>
<tr>
<td><strong>114</strong></td>
<td><img src="image2" alt="Structure" /></td>
<td>-143.40</td>
<td>-4.48</td>
<td>32</td>
</tr>
<tr>
<td><strong>115</strong></td>
<td><img src="image3" alt="Structure" /></td>
<td>-113.28</td>
<td>-6.66</td>
<td>17</td>
</tr>
<tr>
<td><strong>29</strong>: covalent inhibitor IC$_{50} = 1\mu$M$^{ref}$</td>
<td><img src="image4" alt="Structure" /></td>
<td>-136.26</td>
<td>-4.13</td>
<td>33</td>
</tr>
<tr>
<td><strong>8</strong>: covalent inhibitor $K_i = 12n$M$^{ref}$</td>
<td><img src="image5" alt="Structure" /></td>
<td>-120.89</td>
<td>-5.26</td>
<td>23</td>
</tr>
<tr>
<td><strong>68</strong></td>
<td><img src="image6" alt="Structure" /></td>
<td>-130.90</td>
<td>-5.03</td>
<td>26</td>
</tr>
<tr>
<td><strong>69</strong></td>
<td><img src="image7" alt="Structure" /></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$Arbitrary units.
The docked poses of the inhibitor structure within the active site can be visualised in a manner that shows the hydrogen bonding interactions and that allows for key internuclear distances and angles to be determined. In this way, the juxtaposition of the \( \gamma \)-lactone/lactam carbonyl and the active-site serine hydroxymethyl sidechain can be determined as well as getting a qualitative indication of how well the various inhibitor substituents can fit into the corresponding binding pockets around the elastase active site. The top ranked pose of \textit{114} and \textit{115} is shown in Figures 2.11 and 2.12 respectively. The fitness of the docked poses as Michaelis complexes is attested to by (a) the internuclear separation (2.51 and 2.48 Å respectively) and angle of approach of the oxygen atom of Ser-195 sidechain to the reactive carbonyl group (approx. 83º in each case), and (b) the formation of hydrogen bonds by this carbonyl group with the oxyanion-hole amide NH moieties of Gly-193 and Ser-195. In addition, the substituent groups are seen to occupy the S1 and S2 binding pockets without any repulsive steric effects. In the case of \textit{115} the sulfonamide unit occupies the S1’ region (pointing out upwards from the active site) in a similar fashion to that observed in the crystal structure for the methylsulfonyl group of the \textit{trans}-lactam GW311616A\textsuperscript{80,81} (Figure 1.24, section 1.7.2.3).

Figure 2.11 Top ranked pose of \( \gamma \)-lactone \textit{114} docked at the active site of elastase. An electrostatic surface view is shown on the left-hand side. Blue: positive electrostatic potential, Red: negative electrostatic potential, White: neutral electrostatic potential. The view on the right-hand side shows hydrogen bonding (dashed blue line) and the internuclear separation (dashed green line) of the \( \gamma \)-lactone carbonyl carbon atom and the oxygen atom of the active-site serine.
Figure 2.12 Top ranked pose of $\gamma$-lactone 115 docked at the active site of elastase. An electrostatic surface view is shown on the left-hand side. Blue: positive electrostatic potential, Red: negative electrostatic potential, White: neutral electrostatic potential. The view on the right-hand side shows hydrogen bonding (dashed blue line) and the internuclear separation (dashed green line) of the $\gamma$-lactam carbonyl carbon atom and the oxygen atom of the active-site serine.

As part of the docking study, it was also found that a cyclopropyl group could be accommodated within the S-1 binding pocket equally well as the thioethyl unit, and that the S-2/S-3 binding pocket could readily accommodate phenyltriazole, furanyl and 1-methylpyrazolyl substituents. No attempt was made to define the “optimal” ligands by this docking study, as it was felt that this was not likely to be a productive exercise at this point in the work. It was considered that gathering an initial set of data on elastase inhibition was required before a second generation of structures should be considered. Also, as indicated earlier (page 36, section 1.8), low-molecular-weight ($\leq 500$ MW) organic structures need to react covalently with elastase in order to effect a significant degree of inhibition; this is due to the relatively small size of the S-1 binding pocket of elastase compared to other serine proteases such as thrombin. Thus, it was felt that it was sufficient to show that the substituent groups that were chosen for the synthesis of the first generation structures were compatible with the various binding pockets rather than trying to use the docking process in an attempt to identify the putative “optimal” substituents.
2.2.2 γ-Lactone synthesis

The modular synthesis of γ-lactone compound that was identified and successfully implemented in this work is shown in Scheme 2.24. This involved the chemoselective replacement of the bromo group at C-3 of mucohalic acid with ethyl mercaptan as previously carried out in the group.108 The subsequent novel step involved trapping muco structure 116 in its neutral ring-opened form via formation of the diphenylmethyl ester 117, which would then undergo reductive cyclisation to give lactone 53 and incorporation of the C-4 substituents would be achieved via Suzuki-Miyaura coupling reactions to generate 119, 114 and 121.

Scheme 2.24 Overview of the proposed modular synthetic route to γ-lactones. (i) Trapping of a muco-structure in its ring-opened form using diphenyldiazomethane; (ii) reductive cyclisation using STAB; (iii) substituent installation using Suzuki-Miyaura coupling reactions; (iv) reductive cyclisation using methylmagnesium bromide.

An interesting feature of the 1H NMR spectrum of 116 was the splitting pattern of the ethylsulfanyl side-chain, the methylene hydrogens of which are diastereotopic (non-equivalent) due to the chiral centre at C-5. This means that H_A and H_B can potentially have distinct chemical shifts and can couple each other. In fact, they each appear as an overlapping doublet of quartets due to coupling to each other and to the adjacent methyl group (Figure 2.13). The methyl group protons appear as a simple triplet as \( J_{AX} = J_{BX} \).
Figure 2.13 Diastereotopic methylene hydrogens coupled to a methyl group where $J_{AX} = J_{BX}$.

An example of this coupling is shown in Figure 2.14. The spectrum illustrates two highly overlapping doublet of quartets observed for compound 116. The dashed black lines and dashed red lines represent $H_A$ while the solid red and solid black lines represent $H_B$.

Figure 2.14 Ethyl sulfanyl sidechain doublet of quartets for 116.

To minimise the formation of the 3,4-diethylsulfanyl structure (the di-substituted structure) the pH of the reaction system was initially lowered to 8-9 and a steady stream of nitrogen was bubbled through the solution to remove excess ethyl mercaptan (b.p. = 35°C).

The muco-derivative 116 was reacted with diphenylidiazomethane, which readily generated the acyclic diphenylmethyl ester 117 (Scheme 2.25). This reaction proceeded via the neutral ring-opened form of 116 that is present in low equilibrium concentration with the ring-closed form. The reaction progress was monitored by $^1$H NMR.
spectroscopy and the formation of product was clearly identified by the presence of an aldehydic peak at ~ 9 ppm. The increase in intensity of this peak corresponded directly with the decrease in intensity of the H-5 resonance of the starting material 116. There was also a distinctive upfield shift of the ethylsulfanyl methylene hydrogen atoms, which appear as a quartet as they are no longer diastereotopic (Figure 2.15). The reaction of the diphenyldiazomethane was noticeably faster with 116 than with mucobromic acid itself. 117 was purified by column chromatography (yield 74%) and characterised (1H NMR spectroscopy (Figure 2.15), 13C NMR and DEPT spectroscopy, HRMS and elemental analysis).

Scheme 2.25 Benzhydryl ester 117 formation (i) diphenyldiazomethane (approx. 0.65 M) in dichloromethane added dropwise to 116 (2.37 mmol) in acetone (2 mL); stirred at 22.5 ºC until nitrogen evolution ceased; 74 %.

Figure 2.15 1H NMR spectrum of 117 (CDCl3; *H2O residue in CDCl3).
Hydride reduction of mucosalic acids has previously been reported.\textsuperscript{104,118,119} While reduction of 116 with STAB gave the desired product 118 it was in low yield and also generated some of the desbromo form of 118. The desbromo structure is probably formed by 1,4-addition-elimination of hydride ion. The desbromo form of 118 is a known compound (prepared by a different reaction sequence\textsuperscript{120}) and is readily identifiable by its $^1$H NMR spectrum wherein the C-5 methylene hydrogens appear as a doublet ($J = 2.23$ Hz) at 4.89 ppm and the vinylic hydrogen at C-4 appears as a triplet ($J = 2.23$ Hz) at 6.95 ppm). Its formation here confirms the regiochemistry of the ethyl mercaptan addition that it is at the C-3 position. Reductive cyclisation of 117 using sodium triacetoxyborohydride (STAB) yielded the $\gamma$-lactone 118 in much higher yield (Scheme 2.26). Compound 118 was purified by column chromatography (71\% yield) and characterised ($^1$H NMR spectroscopy (Figure 2.16), $^{13}$C NMR and DEPT 135 spectroscopy, HRMS and elemental analysis). $^1$H NMR spectrum of compound 118 (Figure 2.16) shows the appearance of a characteristic peak at $\sim 4.8$ ppm for the H-5 hydrogens and a distinctive downfield shift of the ethylsulfanyl methylene hydrogen atoms.

Scheme 2.26 Reduction with STAB (i) 117 (7.01 mmol), 15-crown-5 (5.6 mmol), STAB (28.03 mmol), refluxed for 1 hr; 71\%.
When the diphenylmethyl ester 117 was treated methylmagnesium bromide, the 5-methyl \( \gamma \)-lactone 120 was obtained as a reductive cyclisation product (Scheme 2.27). In order to ensure anhydrous conditions for this reaction a solution of 117 in toluene was refluxed under Dean-Stark conditions for 1 h to remove any water that may have been present and subsequently this solution was cooled (-80 °C; liquid nitrogen, ethyl acetate slurry) and the Grignard reagent (1M in THF) was added rapidly. The benzhydryl pseudo-ester was formed (~30%) in this process due to the addition of the displaced diphenylmethoxide to 117. It was necessary to add the MeMgCl solution in one addition as the pseudo ester was the predominant product when MeMgCl was added slowly. A chiral centre (C-5) has been introduced into the molecule so the methylene hydrogen atoms of the ethylsulfanyl group are diastereotopic and so each appears as an overlapping doublet of quartets on \(^1\)H NMR spectroscopy (Figure 2.17 for 120 and Figure 2.18 for the pseudo-ester). Compound 120 was purified by column chromatography (yield 40%) and characterised (\(^1\)H NMR spectroscopy (Figure 2.17), \(^{13}\)C NMR and DEPT 135 spectroscopy, HRMS and elemental analysis).
Scheme 2.27 Grignard reaction to give 120. (i) 117 (4.69 mmol) in toluene (50 mL) refluxed under dean stark conditions for 1 hr; MeMgCl (1M in THF, 5.64 mmol); -84°C for 10 mins, quenched with 1M HCl (10 mL); 40%.

Figure 2.17 ¹H NMR spectrum of 120 (CDCl₃; *H₂O residue in CDCl₃). The diastereotopic methylene hydrogen peaks are enlarged in the insert.
Suzuki couplings were carried out as previously described on 118 and 120 using both 4-(4-morpholinomethyl)phenyl boronic acid pinacol ester and 4-carboxyphenyl boronic acid. These reactions required 5.25 and 24 h respectively. Products were isolated by column chromatography and yields varied from 67% (119), 64% (121) to 34% (114) (Scheme 2.28). $^1$H NMR spectra are shown in Figure 2.19 for 119, Figure 2.20 for 114 and Figure 2.21 for 121. Each of the diastereotopic methylene hydrogens of the ethylsulfanyl group for 121 appears as an overlapping doublet of quartets. These have been expanded and inserted onto Figure 2.27.
Scheme 2.28 Suzuki-Miyaura coupling (i) 118 (0.498 mmol), 4-(4-morpholino methyl)phenyl boronic acid pinacol ester (0.747 mmol), caesium fluoride (0.996 mmol), PdCl₂(PPh₃)₂ (0.01245 mmol), benzyldimethylhexadecyl ammonium chloride (0.01245 mmol); 1:1 degassed toluene/water, refluxed for 5.25 h, 67 %. (ii) as for (i) with 4-carboxyphenyl boronic acid (0.747 mmol), refluxed for 24 h; 34 %. (iii) as for (ii) 120 (0.498 mmol), refluxed for 24 h; 64 %.

Figure 2.19 ¹H NMR spectrum of 119 (CDCl₃; *H₂O residue in CDCl₃).
Figure 2.20 $^1$H NMR spectrum of 114 (Acetone-d6).

Figure 2.21 $^1$H NMR spectrum of 121 (Acetone-d6).
2.2.3 γ-Lactam synthesis

Attention was next turned to the γ-lactam scaffold bearing an electron-withdrawing sulphonamide group on the nitrogen atom. The modular synthetic route that was proposed, and successfully implemented, is shown in Scheme 2.29. The ring-opened muco derivative 117, previously used in the γ-lactone synthesis, was viewed as a viable starting material that should lead on to the sulfonylimine 122. Reductive cyclisation of this to give the γ-lactam structure 123 should occur in a similar manner to the successful reductive cyclisation of 117 to the γ-lactone 118 (Scheme 2.25).

Scheme 2.29 Overview of the proposed modular synthetic route to γ-lactams. (i) formation of a sulfonylimine; (ii) reductive cyclisation using STAB; (iii) substituent installation using a Suzuki-Miyaura coupling reaction.

The sulfonylimine is key to the proposed synthesis of the γ-lactam and three potential routes were identified for its formation. These are discussed below.

Kokosinski\textsuperscript{121} reported a method for preparing the muco derivative 125 with an aryl sulphonamide at C-5 shown below (Scheme 2.30). The method involved treatment of mucochloric acid with a sulphonamide in the presence of sulphuric acid, under reflux in toluene. Two possible mechanisms are shown in Scheme 2.30; the lower path involves the formation of an oxocarbonium ion (by dehydration in the strong acid solution) followed by addition of the sulphonamide, whereas the upper path shows formation of another oxocarbonium ion, via protonation of the aldehyde, followed by addition of the sulphonamide. By analogy with a mucochloric acid, it was felt that cyclic structure 57 would exist in equilibrium with its ring-opened form (Scheme 2.20)
Scheme 2.30 Possible mechanisms for the formation of the muco derivative prepared by Kokosinski. 

It was envisaged that this could be used to prepare muco synthon 126 starting from the substituted muco derivative 116 (Scheme 2.31). Thus treatment of 126 with a base would form the sulfonylimine 127 which could be treated with 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). This leaving group was used by Schofield et al. for formation of γ-lactam 17. In our work, we were unable to reproduce the reaction described by Kokosinski with mucobromic acid and p-toluenesulfonamide.

Scheme 2.31 Possible route to a γ-lactam structure from a mucohalic derivative. (i) base; (ii) HBTU; (iii) reductive cyclisation using STAB.
Wu et al.\textsuperscript{122} reported a method for FeCl\textsubscript{3}-catalyzed synthesis of \( N \)-sulfonylimines via the condensation of aldehydes with \( N \)-sulfonamides (130) (Scheme 2.32). This involved iron Lewis acid activation of the carbonyl group of the aldehyde, making it more susceptible to nucleophilic attack by the sulfonamide.

\[
\begin{array}{c}
\text{R}_1\text{CHO} + \text{R}_2\text{SO}_2\text{NH}_2 \xrightarrow{\text{FeCl}_3} \text{N}^+\text{SO}_2\text{R}_2
\end{array}
\]

Scheme 2.32 Iron-catalyzed \( N \)-sulfonylimine synthesis.\textsuperscript{122}

Using the reaction conditions reported, the reaction was repeated with the benzhydryl derivative of mucobromic acid 131 and \( p \)-toluene sulfonamide (Scheme 2.33). It was hoped that once the imine 132 had been formed it could be reduced using STAB and cyclised to give a \( \gamma \)-lactam 133. However, we were unable to form the imine in the presence of the Lewis acid.

\[
\begin{array}{c}
\text{BrBr} \text{O} \text{H} \text{OCHPh}_2 \xrightarrow{\text{FeCl}_3} \text{BrBr} \text{O} \text{N}^+\text{SO}_2\text{PhCH}_3
\end{array}
\]

Scheme 2.33 Possible route to a \( \gamma \)-lactam structure from a mucohalic derivative.

Trost et al.\textsuperscript{123} reported a method for the formation of \( N \)-tosylimines. This involved the in situ formation of the reagent \( N,N' \)-ditosyltellurodiimide, 134, by reaction of tellurium metal with chloramine-T. In Scheme 2.34 is shown a possible mechanism for the formation of the tellurodiimide by reaction of chloramine-T and tellurium metal: this involves formation of a nitrone from the chloramine salt followed by its complexation with the tellurium metal.

\[
\begin{array}{c}
\text{Na}^+ \text{tN-SO}_2\text{Ar} \xrightarrow{\text{Cl}} \text{tN-SO}_2\text{Ar} \quad \text{tN-SO}_2\text{Ar} \xrightarrow{\text{Na}^+ \text{Cl}^-} \text{Nitrone} \\
\text{ArSO}_2\text{N}=\text{Te}^2 \xrightarrow{\text{Nitrone}} \text{ArSO}_2\text{N}=\text{Te}^2=\text{NSO}_2\text{Ar}
\end{array}
\]

Scheme 2.34 Possible mechanism for formation of a tellurodiimide.
The mechanism for the reaction of the aldehyde with the tellurodiimide is presumed to involve cycloaddition to a four-membered ring followed by cycloreversion to the sulfonylimine 135 (Scheme 2.35). Trost and co-workers demonstrated the viability of this reaction with a variety of aldehydes structures, however, no variation of the sulphonamide component was reported.

![Scheme 2.35 Mechanism for reaction with tellurodiimide.](image)

This process was successfully used to generate the required sulfonylimine. On a trial-and-error basis the reaction conditions for reductive cyclisation for the $\gamma$-lactam were developed. The overall successful synthetic strategy is shown in Scheme 2.36.

![Scheme 2.36 $\gamma$-Lactam synthesis (i) Te(=NSO$_2$R$'''$)$_2$; (ii) STAB, 15-crown-5 (iii) Suzuki-Miyaura coupling.](image)

The sulfonylimine 136 was obtained following the reported method (Scheme 2.36). This involved the in situ formation of the reagent, $N,N'$-ditosyltellurodiimide by reaction of tellurium metal and chloramine-T. To this heterogeneous solution was
added 117 and refluxed in toluene for 1 h. The sulfonylimine 136 was obtained quantitatively, as judged by $^1$H NMR spectroscopy. The imine was clearly identifiable by the disappearance of the aldehydic hydrogen at 9 ppm and the appearance of an imine hydrogen resonance at $\sim$ 8.5 ppm (Figure 2.22, spectrum B). This is quite an effective process given that it is heterogeneous throughout. The tellurium, chloramine-T and the diimide are insoluble in toluene. The by-products, sodium chloride and tellurium dioxide, are also insoluble, which means that they can be removed by filtration. The only impurity appears to be tosylamide derived from an impurity in the chloramine-T.

Once formed the sulfonylimine 136 was treated with STAB under reflux in 1,2-dichloroethane for 2 h to give the cyclic $\gamma$-lactam 141 (Scheme 2.36). A clear downfield shift of the tosyl hydrogens, due to N-acylation, was a key signature of the cyclised structure. Another indicator of the cyclised structure was the downfield shift of the methylene hydrogens of the ethylsulfanyl group. This was a characteristic that was also seen in the formation of the $\gamma$-lactone 118. Conversion of the sulfonylimine 136 to 141 occurred via the formation of the acyclic sulfonamide 139. Conversion of 136 to 139 was clearly observed by $^1$H NMR spectroscopy (Figure 2.22) when the reaction with STAB was carried out at room temperature; some acetic acid in the STAB was presumably, the proton source. Attempted chromatographic purification of the acyclic sulfonamide 139 led to a mixture of the acyclic 139 and cyclic 141.
Figure 2.22 Sequence of $^1$H NMR spectra illustrating reaction progress from 118 to $\gamma$-

lactam 141; key peaks and changes in chemical shift values are highlighted.

- **A**: Compound 118
- **B**: Compound 136, $p$-toluenesulfonamide
- **C**: Compound 139, Diphenyldimethanol
- **D**: Compound 141, 15-Crown-5
With a view to installing a more electron-withdrawing sulphonamide on the γ-lactam nitrogen atom it was decided to prepare the tellurodiimide derivative using chloramine-N (this has a nitro group in place of the p-methyl group of chloramine-T). Chloramine-N was prepared by an established procedure and with this reagent the p-nitrophenylsulfonyl derivative 142 was obtained as per formation of 141, although the sulfonylimine formation and reductive cyclisation reactions were both less efficient. In the sulfonylimine formation step, monitoring of reaction progress, by $^1$H NMR spectroscopy, between 118 and the ditosyltellurodiimide was essential, as the formation of side-products as a function of reaction time was a significant feature. A sample spectrum is shown in (Figure 2.23): this shows approx. 46% sulfonylimine (137) formation with 35% unreacted aldehyde and 19% impurities. One of the impurities was identified as the benzhydryl pseudo ester (Scheme 2.27) and the other appeared to be derived from the sulfonylimine. The reaction was typically stopped at this point as longer reaction times led to the formation of more side-products, which proved difficult to remove.

![Figure 2.23 $^1$H NMR spectrum of 137 (CDCl$_3$).](image-url)
Synthesis of the trifluoromethyl imine derivative 138 was also attempted by this route. Chloramine-Tf\textsuperscript{98} (with a trifluoromethyl group in place of the tolyl group of chloramine-T) was prepared from trifluoromethyl sulfonamide, however, the tellurodiimide process failed to produce the required sulfonylimine. It would appear that there are limitations to the tellurodiimide process of Trost and co-workers.\textsuperscript{123} Whereas formation of the sulfonylimine worked very well with chloramine-T, it was far less successful with chloramine-N, indicating that electron-withdrawing groups on the sulphonamide moiety are not helpful. It should be noted that with both these chloramine reagents, the reaction system was always heterogeneous, and this appeared to be required for a successful formation of the tellurodiimide. Thus, with chloramine-Tf it was seen that this material formed a syrupy solution when added to toluene, or indeed to any other solvent, and this feature may have been partly the cause for the failure to form the tellurodiimide structure.

The final step in the synthesis of the \(\gamma\)-lactams 115 and 143 involved the installation of substituents at the C-4 position using a Suzuki-Miyaura coupling reaction (Scheme 2.36). The benzylmorpholino, which was previously installed on the \(\gamma\)-lactones, was also used here. These reactions required 3-8 h and each product was isolated by column chromatography with yields of 33\% and 15\% respectively. \(^1\)H NMR spectra for 115 (Figure 2.24) and 143 (Figure 2.25) are shown.
Figure 2.24 $^1$H NMR spectrum of 115 (CDCl$_3$; *H$_2$O residue in CDCl$_3$).

Figure 2.25 $^1$H NMR spectrum of 143 (CDCl$_3$; *H$_2$O residue in CDCl$_3$).
An alternative route was identified for the formation of $\gamma$-lactam 147, which was based on formation of the $N$-unsubstituted $\gamma$-lactam 145 (Scheme 2.37). It may be recalled that reductive amination of muco derivatives with ammonia equivalents such as ammonium acetate or formate, to give such a $\gamma$-lactam, was reported as not being successful by Zhang and co-workers.\textsuperscript{104} For this reason, an indirect route was proposed that would involve reductive amination with 3,4-dimethoxybenzylamine to produce $\gamma$-lactam 144, and subsequent removal of the dimethoxybenzyl group to produce 145.\textsuperscript{125-127} $\gamma$-Lactam 70 would then be reacted with trifluoromethanesulfonyl chloride to produce structure 146,\textsuperscript{128,129} which could, finally, be functionalised via a Suzuki-Miyaura coupling.

$\gamma$-Lactam 144 was successfully prepared by reductive amination of 118 with 3,4-dimethoxybenzyl amine, as per the procedure discussed in Section 2.1.6. This was characterised by $^1$H NMR spectroscopy (Figure 2.26), $^{13}$C NMR and DEPT 135 spectroscopy (Figure 2.27) and HRMS. Deprotection with trifluoroacetic acid (the established reagent for this)\textsuperscript{128,129} to give 145 was unsuccessful. Reaction at room temperature over 24 h saw no change in the starting material 144. The reaction mixture was refluxed and this led to unidentifiable products. It was concluded that this was not a viable route for the synthesis of the trifluoromethyl-$\gamma$-lactam 147. No further work was carried out on the formation of the $N$-unsubstituted $\gamma$-lactam 145.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{scheme2.png}
\caption{Scheme 2.37 Alternative synthetic route for $\gamma$-lactam (i) reductive amination; (ii) deprotection with TFA; (iii) reaction with trifluoromethanesulfonyl chloride; (iv) Suzuki-Miyaura coupling.}
\end{figure}
Figure 2.26 $^1$H NMR spectrum of 144 (CDCl$_3$; *H$_2$O residue in CDCl$_3$).

Figure 2.27 $^{13}$C NMR and DEPT 135 spectrum of 144 (CDCl$_3$).
2.2.4 Chemoselective Suzuki Reactions

A synthetic strategy was identified as a potential route for the incorporation of a carbon-based ligand at the C-3 position of the \(\gamma\)-lactam (Scheme 2.38). This route was based on chemoselective Suzuki reactions that were discussed previously (Section 2.1.2). The approach envisaged for the installation of a cyclopropyl group at position 3 was via chemoselective Suzuki-Miyaura coupling of the di-bromo-\(\gamma\)-lactam 148, which would involve installation of the 4-substituent first followed by installation of the cyclopropyl group in a subsequent coupling reaction. Preferential coupling in di-halo-\(\alpha,\beta\)-unsaturated ester and ketone systems is known.\(^{101,102,104}\)

![Scheme 2.38 Potential chemoselective Suzuki-Miyaura coupling sequence.](image)

Evaluation of the selective coupling of 148 with a variety of boronic derivatives under our reaction protocol was not successful (Scheme 2.39). The \(^1\)H NMR spectrum of the crude reaction mixture was complex but gave no clear indication for the formation of a structure corresponding to 149. Time did not permit for further evaluation of the Suzuki protocol for this system.

![Scheme 2.39 Site-selective Suzuki-Miyaura coupling attempt.](image)

2.2.5 Enzymatic Evaluation of \(\gamma\)-lactones and \(\gamma\)-lactams

The ability of \(\gamma\)-lactones 114, 119 and 121 and of \(\gamma\)-lactams 115 and 143 to behave as a elastase inhibitors was assessed. Enzyme inhibition assays were carried out in a standard manner\(^{110,111}\) by monitoring the rate of release of \(p\)-nitroaniline from the synthetic substrate suc-(ala)\(_3\)-\(p\)-NO\(_2\)anilide by elastase (recrystallised PPE from Calbiochem) at 410nm in the presence of varying concentrations of the inhibitor
structures. In order to avoid misleading results that could arise because of turbidity issues in the assay cuvette, the maximum inhibitor concentration was limited to 40 μM. The results are summarised in Table 2.4.

Table 2.4 Summary of PPE inhibition data.

<table>
<thead>
<tr>
<th>Entry No.</th>
<th>Inhibitor</th>
<th>[Inhib] μM</th>
<th>Residual enzyme activity (%)</th>
<th>[Substrate] mM</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32- sodium salt</td>
<td>19.0</td>
<td>50</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>119- HCl</td>
<td>40.0</td>
<td>98</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>3</td>
<td>114- sodium salt</td>
<td>40.0</td>
<td>97</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>4</td>
<td>121- sodium salt</td>
<td>40.0</td>
<td>97</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>5</td>
<td>115- HCl salt</td>
<td>40.0</td>
<td>99</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>6</td>
<td>143- HCl salt</td>
<td>40.0</td>
<td>Not determined&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

<sup>a</sup>Incubation time = 40 min; error on residual enzyme activity is 5%. <sup>b</sup>N-succinyl-Ala-Ala-Ala-p-nitroanilide. <sup>c</sup>Insoluble in phosphate buffers and/or buffer solutions with >20% DMSO.

It is clear from the data in Table 2.4 that, neither the γ-lactones 114, 119, 121, nor the γ-lactam 115, showed any activity when incubated with PPE for 40 min at 40 μM. This observation is significantly different from the finding of 50% inhibition of PPE by the Δ<sup>7</sup> pyrrolothiazole 32 (entry 1<sup>86</sup>) and is also very different from the level of inhibition of the thiophene 29 (IC<sub>50</sub> = 1 μM).<sup>69</sup> It was not possible to assay 143 due to lack of solubility in phosphate buffers or in buffers containing over 20% DMSO (by volume). This solubility issue was not anticipated given that 143 was extracted into aqueous acid during the Suzuki-Miyaura reaction work-up. As with the pyrrolothiazoles assayed earlier (Table 2.2) the lack of meaningful inhibition of PPE by the monocyclic γ-lactones and γ-lactams above, is indicative of a structure type that is too unreactive. A feature common to all of these structures is the conjugated carbonyl group.
γ-Lactone 114 was selected for an evaluation of its hydrolytic reactivity in aqueous buffers. HPLC analysis was used to monitor its stability in a 50 mM phosphate buffer of pH 9.17 at room temperature (nominally 20 °C). A chromatograph of a 0.5 mM solution of 114 in this buffer, immediately after preparation, is shown in Figure 2.23 (left-hand side): a single peak with a retention time of 7.92 min was observed. Over a 6 h period no change was seen in this solution, while after 72 h a new peak was observed at 1.99 min with a 20% diminution in the peak at 1.99 min. A 100 mL aliquot of a LiOH solution was added to a second solution of 114 in the phosphate buffer so as to raise the pH to 12.05. The HPLC trace obtained from this solution, immediately following basicification, is shown in Figure 2.28 (right-hand side) and clearly shows the complete disappearance of the peak at 7.99 and the appearance of a single new peak with a retention time of 1.99 min. It was viewed that this new peak corresponds to the ring-opened form of 114. The relatively slow appearance of this peak in the original phosphate buffer at pH 9.17 was taken as a qualitative indication of the low reactivity of the lactone unit in 114.
Figure 2.28 HPLC Analysis Left: Chromatograph of 114 in phosphate buffer at pH 9.17. Right: Chromatograph of a buffer solution of 114 wherein the pH was raised to 12.05 by the addition of LiOH. Recorded without internal reference for clarity.
Chapter 3

Conclusions and Perspective
Chapter 3 Conclusions and Perspective

The work in this thesis forms part of a body of research on the synthesis, and enzymatic evaluation as elastase inhibitors, of a set of bicyclic $\gamma$-lactams (pyrrolothiazoles) and a closely related set of monocyclic $\gamma$-lactone and $\gamma$-lactam structures (Chart 3.1).

Chart 3.1 Bicyclic $\gamma$-lactams (pyrrolothiazoles) and monocyclic $\gamma$-lactone and $\gamma$-lactam structures.

The pyrrolothiazole $\Delta^7$ isomer 32 originated in the doctoral work of C. Ruddle,$^86$ that dealt with the chemistry of modified penicillins, and was found to act as a moderately potent irreversible inhibitor of PPE ($IC_{50} = 19 \mu M$). Following on from this lead the doctoral work of E. O’Dwyer$^88$ looked at possible ways by which substituents could be incorporated into this pyrrolothiazole structure at the 6–position to occupy the S-1 binding pocket of elastase. The work of O’Dwyer showed that the synthesis of such pyrrolothiazoles, starting from penicillin structures, was not viable, and attention was then directed to the $\Delta^6$ isomeric form of the pyrrolothiazole nucleus. In her work O’Dwyer implemented a viable synthetic route for the $\Delta^6$ isomer which allowed for variation of substituents $R_1$ and $R_2$ within certain limits; this route is summarised in Scheme 3.1. Exploitation of mucohalic acid chemistry allowed for incorporation of thiols (ethyl and 2-propylthiol) as the C-3 substituent followed by reaction with $D$-penicillamine to generate a pyrrolothiazole unit with a bromine at the C-7 position. O’Dwyer showed that carbon-based ligands could then be introduced, via a Suzuki-Miyaura coupling, at the C-7 position, and also that a triazole unit could be incorporated at this position via a displacement reaction using azide followed by a click reaction using an acetylene. A water-soluble form was generated by removal of the benzhydryl group and formation of a sodium salt of the substituted pyrrolothiazole. A small set of these structures were screened for their ability to inhibit PPE, however, only very low levels of inhibition were observed.
Scheme 3.1 Summary of preparation of a substituted pyrrolothiazole (i) Addition-elimination reaction using a mercaptan; (ii) cyclisation with D-penicillamine; (iii) Suzuki-Miyaura coupling, or displacement with azide followed by a click reaction, followed by removal of the benzyhydryl group.

Part of the work here, which was carried out in parallel with some of that of E. O’Dwyer, was based on further elaboration of the substituted pyrrolothiazoles. A second part of the work here focused on the monocyclic $\gamma$-lactone and $\gamma$-lactam structure types shown in Chart 3.1.

With regard to the pyrrolothiazoles, work was carried out on: (i) the bicyclisation reaction using L-cysteine in place of D-penicillamine; (ii) the bicyclisation reaction of D-penicillamine with a muco-derivative bearing phenyl rings at C-3 and C-4; (iii) incorporation of phenols at the C-6 position; (iv) evaluation of two novel pyrrolothiazoles for inhibition of elastase (PPE) and one of these for inhibition of thrombin.

It was found that the formation of a bicyclic pyrrolothiazole structure was much less efficient when mucochloric acid was reacted with L-cysteine (27%) than when it was reacted with with D-penicillamine (84%). This finding can be rationalised by a gem-dimethyl (Thorpe-Ingold)\(^9^4\) effect if formation of the monocyclic thiazole unit, from the initially formed imine, is the rate-determining step (r.d.s) in the bicyclisation process (Scheme 3.2). The formation of five-membered ring systems is well known to be accelerated by the presence of a gem-dimethyl unit and this is the kind of reaction that is involved in generation of the monocyclic thiazole unit from the acyclic imine. L-Cysteine has a simple methylene group at the corresponding position to that of the gem-dimethyl group in D-penicillamine, so formation of the monocyclic thiazole from the acyclic imine unit should be much slower. Under these circumstances intermolecular reactions involving the thiol group of the acyclic imine could occur competitively with the intramolecular ring closure thereby lowering the yield of the bicyclic pyrrolothiazole structure; intermolecular addition of a thiol to the imine could occur and/or an
intermolecular addition-elimination reaction by a thiol at one of the vinylic positions of the acyclic imine could occur. It would appear therefore that the usefulness of this bicyclisation process for the preparation of a pyrrolothiazole nucleus is restricted by this structural feature of the aminothiol component.

As part of this work, the influence of substituent variation on the muco-structure on the cyclisation reaction was also examined (Scheme 3.3). A diphenyl-substituted muco structure 91, obtained via a Suzuki-Miyaura coupling reaction of mucochloric acid and phenyl boronic acid, was reacted with D-penicillamine using the same reaction conditions as those used with mucochloric acid. The corresponding bicyclised pyrrolothiazole, however, was not formed. Formation of the (anionic) ring-opened form of the diphenyl-muco structure was confirmed by 1H NMR spectroscopy when the intact diphenyl-muco structure was treated with base. This (anionic) ring-opened form failed to generate an imine, however, on addition of D-penicillamine or of valinol. This finding pointed to a second limitation to the bicyclisation-type reaction shown in Scheme 3.2. In a related model reaction the (anionic) ring opened form of phthalaldehydic acid was observed (1H NMR spectroscopy) to readily generate an imine when valinol was added. Reaction of phthalaldehydic acid with valinol is known to occur when these reagents are heated to reflux (Dean-Stark conditions) in toluene to generate the corresponding bicyclised structure – the tricycl lactam 93 (68% yield); this outcome was confirmed in this work. Treatment of the diphenyl muco-derivative 91 with D-penicillamine under these conditions did not result in the formation of the pyrrolothiazole structure. It is not obvious why the cyclisation reaction between D-
penicillamine and the diphenyl-muco structure failed to occur. The overall result, however, points to further restrictions on this process the solution to which may require development of highly specific reaction conditions.

Scheme 3.3 Reactions of the diphenyl-muco derivative 91 and of phthaldehydic aldehyde with D-penicillamine and/or valinol.

Work was then continued on elaborating the preparation of pyrrolothiazole structures, as shown in Scheme 3.1, using phenoxides as the nucleophiles for the addition-elimination reaction on mucobromic acid. Phenoxy, 3-chlorophenoxy and 3-nitrophenoxide were used successfully. The bicyclisation reaction was successfully carried out with the phenoxy and the 3-chlorophenoxy derivatives. The bromopyrrolothiazole (Scheme 3.1) bearing the phenoxy group was furthered modified using azide displacement followed by triazole formation, while the bromopyrrolothiazole bearing the 3-chlorophenoxy group was subjected to a Suzuki-Miyaura coupling; removal of the benzhydryl ester group in each case led to the pyrrolothiazole salts 107 and 108 (Chart 3.2). Both of these salts were evaluated as inhibitors of PPE and the salt 107 was also evaluated as an inhibitor of thrombin. The level of inhibition was very
low; 17% and 10% inhibition of PPE when incubated for 40 minutes with 1 mM of 107 and 108 respectively. Incubation of thrombin with 1 mM of 107 for 40 minutes showed a 40% drop in enzyme activity. These levels of inhibition are considered to be quite low given that many known inhibitor structures, such as the trans-lactam 24, give over 90% inhibition at the low μM concentration range, and the original lead pyrrolothiazole 32 gave 50% inhibition at 19 μM.

![Chart 3.2 Pyrrolothiazole structures.](image)

It was considered that the conjugated nature of the γ-lactam carbonyl group of 107 and 108 might render these insufficiently reactive to be viable acylation inhibitors of serine-proteases such as elastase. It was observed that the salt 107 in a pH 8.5, 50 mM phosphate buffer showed no hydrolysis over a period of 24 hours when monitored by 1H NMR spectroscopy. According to the hypothesis of Page and co-workers a certain minimum reactivity is required in order for acylation-based structures to act as efficient serine-protease inhibitors,79 the half-life for hydrolysis under the above reaction conditions would need to fall between 6-12 hours.

The final part of this work dealt with synthesis of monocyclic γ-lactones and γ-lactams, computation evaluation of the docking of these to the active site of PPE, and the enzymatic evaluation of these as inhibitors of PPE. Initially a set of γ-lactams bearing an N-alkyl group was prepared using a standard procedure of reductive amination of a muco derivative (Scheme 3.4). This reductive cyclisation of the muco-
derivative was found to be low yielding (29%). In this exploratory work the γ-lactams 112 and 113 were prepared as representative examples.

Scheme 3.4 Formation of γ-lactams 112 and 113 using reductive amination of muco-derivative 98. (i) alkylamine, hydride reagent; (ii) Suzuki-Miyaura coupling.

Subsequently, in the preparation of the γ-lactones and γ-lactams bearing an N-sulfonyl group, a novel approach was used to effect the reductive cyclisation. This involved trapping the muco-derivative in its ring-opened form as a benzhydryl ester prior to the cyclisation stage; this is shown in Scheme 3.5 for the γ-lactone synthesis and in Scheme 3.6 for the γ-lactam synthesis. In these structures the ethylthioloate substituent was used at the 3-position in place of the aryloxy groups used in 112 and 113.

Prior to carrying out the synthesis a computational study on the aptness of the planned structures to fit as Michaelis complexes within the active site of PPE was carried out. The commercially available Molecular Virtual Docking programme was used. The results showed that the γ-lactone and γ-lactam structures, bearing the target substituents, could all be juxtaposed as viable Michaelis complexes within the active site of PPE, and with the substituents occupying the various binding pockets (S-1, S-2/S-3 and S-1’) without any restrictive steric interactions. The Michaelis complex juxtapositions that were returned by the docking study were seen to be very similar to those generated from computational docking of known inhibitors. This facet of the work was carried out to obtain some data on the fitness of the targeted structures, prior to synthesis, to bind non-covalently to the active site of PPE, but the study was not aimed at identifying a combination of putative “optimal” substituents.

The γ-lactones 114, 119 and 121 were synthesised as shown in Scheme 3.5, using the strategy of reductive cyclisation of the ring-opened muco derivative 117; this
strategy gave higher yields and a cleaner reaction compared with reductive cyclisation of the muco structure 116.

Scheme 3.5 Formation of γ-lactones via the ring-opened muco-derivative 117. (i) Diphenyldiazomethane; (ii) hydride reagent; (iii) Suzuki-Miyaura coupling; (iv) Methylmagnesium bromide.

The ring-opened derivative also proved to be particularly valuable in the synthesis of the γ-lactams with an N-sulfonyl group (Scheme 3.6). Here a process developed by Trost and co-workers was used to form a sulfonylimine. This involved formation of a tellurium diimide derivative starting from tellurium powder and chloramine-T. Reaction of this with 117 formed the acyclic sulfonylimine 136 which, without purification, was subjected to reductive cyclisation to form the γ-lactam 141, and this was then converted to 115 using a Suzuki-Miyaura coupling reaction.

Scheme 3.6 Formation of γ-lactams 115 and 143 bearing an N-sulfonyl group via the ring-opened muco-derivative 117. (i) Tellurium diimide reagent; (ii) hydride reagent; (iii) Suzuki-Miyaura coupling.
In this work it was found that there were limitations to the tellurium diimide method of sulfonylimine formation. Through careful monitoring of reaction progress the sulfonylimine 137 was obtained, albeit in low yield, by using chloramine-N in place of chloramine-T. When chloramine-Tf (trifluoromethyl in place of the tolyl moiety of chloramine-T) was used the tellurium diimide reaction failed.

The $\gamma$-lactones 114, 119 and 121 as well as the $\gamma$-lactam 115 were evaluated as inhibitors of PPE; the issue of low solubility in the assay buffer precluded the evaluation of the $p$-nitrophenylsulfonyl derivative 143. At the highest concentration evaluated, 40 $\mu$M, no inhibitory activity was detected. This concentration limit was taken so as to avoid any ambiguity regarding inhibition levels resulting from turbidity which was detected in the assay cuvette at higher concentrations (1 mM). This turbidity may have been due to non-specific inhibition of PPE culminating in proteinaceous aggregate formation.\textsuperscript{112} Notwithstanding this maximum concentration level of 40 $\mu$M, it is clear that the above $\gamma$-lactones and $\gamma$-lactams are considerably less active, as PPE inhibitors, compared to 32 (Chart 3.2), which showed 50% inhibition of PPE at a concentration of 19 $\mu$M.

One distinguishing feature of 32 to all of the other structures evaluated here (107, 108, 114, 119, 121 and 115) is the non-conjugated nature of the $\gamma$-lactam carbonyl group of 32. It is also relevant to note that the thiophene-based lactam, 29, of Migaud and co-workers, has a similarly non-conjugated lactam carbonyl; this compound had an IC\textsubscript{50} of 1 $\mu$M against HNE (human neutrophil elastase).\textsuperscript{69} It is argued that the reactivity of the structures with the conjugated carbonyl group is too low, in the context of the hypothesis of Page and co-workers,\textsuperscript{79} for these to act as viable acylation-based inhibitors. As pointed out earlier, it is essential for low-molecular-weight organic structures to react covalently with PPE (acylation of the active-site serine) in order to act as viable inhibitors, as the scope to bind tightly at the active site, through non-covalent binding of substituent groups to the primary binding pocket S-1, is severely limited by the small size of this pocket. Alanine, with a methyl side-group to occupy the S-1 pocket, is the preferred amino acid residue in the case of a typical elastase substrate; the remaining portion of such a protein substrate can generate significant affinity
through extensive non-covalent interactions with enzyme residues that are not directly adjacent to the active site.

The preparation of the \( \gamma \)-lactam 143, bearing a \( p \)-nitrophenylsulfonfyl group, was aimed at enhancing the reactivity of the lactam carbonyl group. Proper evaluation of this was not achieved, however, due to solubility problems. It was also the case that synthesis of this structure was problematic and low yielding. An approach to enhancing the reactivity, that should be considered in future work, would be to reduce the double bond within the tosyl lactam 115 (the synthesis of which was robust). The reduction could be effected by catalytic hydrogenation (using Pd/C) to generate a racemic mixture of 151 (Scheme 3.7). Such a structure would be more akin to the saturated \( trans \)-lactam 24, and to the monocyclic \( \gamma \)-lactam 12 (Figure 1.25).

\[
\begin{align*}
115 & \quad R_1' = -(p)\text{PhCH}_3, \quad R_2 = -(p)\text{PhCH}_3N \quad \text{O} \\
151 & \quad \text{Racemic mixture of a saturated } \gamma \text{-lactam}
\end{align*}
\]

Scheme 3.7 Possible reduction process for the formation of a racemic mixture of a saturated \( \gamma \)-lactam 151 from 115. (i) \( \text{H}_2 \) over Pd/C.

A final point is made here regarding the possibility of reversible acylation via five-and six-membered ring structures – these are the ring sizes most readily formed by intramolecular process of acyclic precursor structures. As outlined in the introduction the \( trans \)-lactone GR133487 (Chart 3.3) was found to act as a reversible inhibitor of thrombin\(^72\) with the reversibility being due to reformation of the \( \gamma \)-lactone unit. In that instance the reversibility was viewed here as resulting from constraints imposed by enzyme residues arching over the active site. The related \( trans \)-lactam 24 was seen to act as an irreversible inhibitor of elastase;\(^80\) in the case of elastase there are no amino acid residues that can impose the same kind of constraint as that present in thrombin. It can be envisioned that the “reversibility” constraint could be part of the inhibitor structure itself. One structural component that should enable this reversibility is the presence of unsaturation within a five- or six-membered ring structure, while another structural feature is the incorporation of a gem-dimethyl group. In the case of one benzoxazinone structure, 11, reversible inhibition of elastase was postulated (but not
unambiguously proven) to occur via reversible acylation.\textsuperscript{66,68} In the case of the thiophene-based lactam, 29, the possibility of reversibility through reformation of the lactam bond was considered a possibility. This aspect was not further elaborated on in that work, although it was apparent that no recovery of enzyme activity was observed.\textsuperscript{69}

\[
\begin{align*}
E + I & \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} E \cdot I \\
E \cdot I & \underset{k_{\text{deacyl}}}{\overset{k_{\text{acyl}}}{\rightleftharpoons}} E - I \quad \text{hyd} \quad \underset{k_1}{\overset{k_2}{\rightleftharpoons}} E + I'
\end{align*}
\]

Chart 3.3 General schematic (top) of reversible acylation of a serine-protease, and (bottom) possible structures for reversible acylation.

In the context of designing a cyclic structure to act as a reversible acylation inhibitor, engineering an appropriate level of reactivity in the first instance, and also engineering a balance between the rate of ring-opening and that of ring-closing would be critical, although achieving this could be quite a challenging task. Here it was found that the hydrolytic reactivity of the bicyclic pyrrolothiazole 102 and of the monocyclic \(\gamma\)-lactone 114 was found to be particularly low, while the facile nature of the ring-closure reactions used here to form the monocyclic \(\gamma\)-lactones/lactams, and the \(\Delta^6\)
isomer of the pyrrolothiazoles, shows that there is a considerable kinetic and thermodynamic driving force toward ring closure. Taken together these observations suggest that the level of reactivity is too low and that the balance of reactivity is strongly on the side of ring closure for these structure types. The $\Delta^7$ isomer of the pyrrolothiazole still remains an interesting lead. Based on the observed level of PPE inhibition it has an appropriate level of reactivity, however, the main challenge here is to develop a robust synthetic route. In the penicillin work the $\Delta^7$ isomer was formed from the $\Delta^6$ isomer under the (basic) reactions conditions used to drive the rearrangement process, however, the work of O’Dwyer showed this is not a viable route for formation of $\Delta^6$ isomers of pyrrolothiazole structures bearing diverse substituents.
Chapter 4

Experimental
Chapter 4 Experimental

General Detail

$^1$H and $^{13}$C NMR spectra were recorded on either on a 90, 270, 300 or 500 MHz NMR instrument. Solvents used for NMR spectra were deuterated choloform (tetramethylsilane (TMS) as internal standard), deuterated acetone (TMS as internal standard), deuterated dimethylsulfoxide (DMSO) and deuterated water (DSS as internal standard). Thin layer chromatography (tlc) was carried out on Kieselgel 60 F$_{254}$ supported on aluminium foil. Column chromatography was carried using Kieselgel S (32-63μm), - the specific solvent ratio in which a pure product was obtained is given in the experimental detail. Melting points were obtained on a Griffin melting point apparatus. The m.p. for all new compounds were not recorded at the time of their preparation due to an oversight. UV measurements were obtained using a UVIKON XL™ spectrophotometer. Elemental analysis was carried out at the Microanalytical Laboratory, Department of Chemistry, University College Dublin, Dublin. High-Resolution Mass Spectroscopy (HRMS) was carried out at University College Dublin, Dublin. High Performance Liquid Chromatographic (HPLC) Analysis was carried out using a Shimadzu LC-10AT pump linked to a Shimadzu SPD-SA UV spectrophotometric detector and a SphereClone (5 µm) C-18 column (250 x 4.6 mm).

1.4 Addition-elimination – general procedure

To a solution of mucohalic acid (3.88 mmol) in water (4 mL), containing potassium hydroxide (256 mg, 4.56 mmol) was added the required alkyl mercaptan, or substituted phenol, (5.82 mmol) in water (3.5 mL) containing potassium hydroxide (392 mg, 5.82 mmol). The mixture was allowed to stir at room temperature for 3 h. The pH was lowered to <2 using 1 M HCl and extracted with dichloromethane (2 x 10 mL). The organic extracts were combined, dried over magnesium sulfate, filtered and concentrated by rotary evaporation.
4-chloro-5-hydroxy-3-phenoxyfuran-2(5H)-one (96)\textsuperscript{109}

![Chemical Structure](image_url)

Precipitate formed while stirring at room temperature, filtered and acidified using 1 M HCl, worked up as outlined by procedure above. Recrystallised from n-hexane to yield a cream solid (317 mg, 1.40 mmol, 36%); \textsuperscript{1}H NMR (270MHz, DMSO-\text{d}\textsubscript{6}) \text{\delta} 6.15 (s, 1H, H-5), 7.13 (app d, 2H, J = 7.7 Hz, Ar-H\textsubscript{2,6}), 7.18 (app d, 1H, J = 6.9 Hz, Ar-H\textsubscript{4}), 7.39 (app t, 2H, J = 7.9 Hz), 8.47 (s broad, 1H, -OH).

4-Bromo-3-(3-chlorophenoxy)-5H-furan-2-one (97)

![Chemical Structure](image_url)

Purification by silica gel column chromatography using 1:1 dichloromethane/hexane containing 5% acetic acid, followed by washing with water to remove acetic acid, gave 97 as a colourless gum (501 mg, 1.93 mmol, 50%); \textsuperscript{1}H NMR (270 MHz, CDCl\textsubscript{3}) \text{\delta} 6.10 (s, 1H, H-5), 6.96 (ddd, 1H, J = 8.2, 2.5, 1.0 Hz, Ar-H\textsubscript{5}), 7.07 (app t, 1H, J = 2.2 Hz, Ar-H\textsubscript{2}), 7.17 (ddd, 1H, J = 7.9, 2.0, 1.0 Hz, Ar-H\textsubscript{3}), 7.28 (t, 1H, J = 8.2 Hz, Ar-H\textsubscript{6}); \textsuperscript{13}C NMR (67.93 MHz, CDCl\textsubscript{3}, DEPT) \text{\delta} 96.2 (C-1), 115.9, 118.2, 123.0, 125.1, 130.4, 134.9, 142.3, 154.3 (C-3, C-4, ArC), 164.5 (C=O); ESI-HRMS: [M+Na]\textsuperscript{+} calcd: 326.9036, found: 326.9046 and 328.9040.
4-Bromo-3-(3-nitrophenoxy)-5H-furan-2-one (98)

Purification by silica gel column chromatography using 1:1 dichloromethane/hexane containing 5% acetic acid, followed by washing with water to remove acetic acid, gave 98 as a pale yellow gum (637 mg, 2.02 mmol, 52%); \(^1\)H NMR (270 MHz, CDCl\(_3\)) \(\delta\) 6.17 (s, 1H, H-5), 7.44 (ddd, 1H, \(J = 8.2, 2.5, 1.2\) Hz, Ar\(\text{H}_3\)), 7.56 (app t, 1H, \(J = 8.2\) Hz, Ar\(\text{H}_2\)), 7.88 (app t, 1H, \(J = 2.0\) Hz, Ar\(\text{H}_6\)), 8.05 (ddd, 1H, \(J = 8.2, 2.0, 1.0\) Hz, Ar\(\text{H}_5\)); \(^{13}\)C NMR (67.9 MHz, CDCl\(_3\), DEPT) \(\delta\) 96.2 (C-1), 112.7, 119.8, 124.0, 124.5, 130.6, 142.3, 148.9, 154.4 (C-3, C-4, ArC), 163.6 (C=O); ESI-HRMS: [M+H\(^+\)] calcd: 337.9276, found: 337.9290 and 339.9274.

4-Bromo-3-ethylsulfanyl-5-hydroxy-5H-furan-2-one (116)

The pH was initially lowered to 8-9 and a steady stream of nitrogen was bubbled through the solution to remove excess ethyl mercaptan (this minimised formation of the 3,4-diethylsulfanyl structure). Following this, the pH of the solution was lowered to <2. (568 mg, 2.38 mmol, 61%); \(^1\)H NMR (270 MHz, CDCl\(_3\)) \(\delta\) 1.32 (app t, 3H, \(J = 7.4\) Hz, CH\(_3\)-CH\(_2\)), 3.23 (dq, 1H, \(J = 13.5, 7.4\) Hz, CH\(_3\)CH\(_2\)S (diastereotopic)) overlapping with 3.29 (dq, 1H, \(J = 13.5, 7.4\) Hz, CH\(_3\)CH\(_2\)S (diastereotopic)), 5.97 (s, 1H, H-5); \(^{13}\)C NMR (67.5 MHz, CDCl\(_3\), DEPT) \(\delta\) 15.5 (CH\(_3\)-CH\(_2\)), 5.97 (s, 1H, H-5); \(^{13}\)C NMR (67.5 MHz, CDCl\(_3\), DEPT) \(\delta\) 15.5 (CH\(_3\)CH\(_2\)S), 25.0 (CH\(_3\)CH\(_2\)), 97.7 (C-1), 131.1, 139.3 (C-3, C-4), 166.5 (C=O); ESI-HRMS [M-H\(^-\)] calcd: 236.9221, found: 236.9216 and 238.9241. This material was used without further purification in the preparation of 117. (The yield on scale-up (10g) was quite poor).
**Bicyclisation – general procedure with D-penicillamine**

To a solution of D-penicillamine (315 mg, 2.11 mmol) and sodium chloride (144 mg) in water (3 mL), containing acetic acid (144 µL, 2.52 mmol) was added a solution of the required mucohalic acid or muco derivative (1.92 mmol) in ethanol (3 mL). The mixture was stirred at room temperature overnight and was then extracted with dichloromethane (10 mL). The organic layer was dried, filtered and concentrated by rotary evaporation to yield the free acid.

6,7-Dichloro-2,2-dimethyl-5-oxo-2,3,5,7a-tetrahydro-pyrrolo[2, 1-b]thiazole-3-carboxylic acid (76)

76 was isolated as an off-white amorphous solid (454 mg, 1.61 mmol, 84%); \(^1\)H NMR (270 MHz, CDCl₃) δ 1.58 (s, 1H, α-CH₃), 1.62 (s, 1H, β-CH₃), 4.70 (s, 1H, H-3), 5.88 (s, 1H, H-8); \(^1^3\)C NMR (67.5 MHz, CDCl₃) δ 26.3 (α-CH₃), 31.3 (β-CH₃), 61.0, 68.4, 69.6 (C-2, C-3, C-8), 122.5, 147.8 (C-6, C-7), 166.8, 171.9 (2 x C=O)

(3S,7aR)-7-chloro-2,2-dimethyl-5-oxo-6-phenoxy-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylic acid (99)

Recrystallised from ethanol:DIW (50:50) to yield a cream solid (287 mg, 0.845 mmol, 44%); \(^1\)H NMR (270MHz, DMSO-d₆) δ 1.52 (s, 6H, α,β–CH₃), 4.39 (s, 1H, H-3), 6.17 (s, 1H, H-8), 7.05 (app d, 2H, J = 8.7 Hz, Ar-H₂,6), 7.15 (app t, 1H, J = 7.7 Hz, Ar-H₄), 7.38 (app t, 2H, J = 7.7 Hz, Ar-H₃,5), δC (67.5 MHz, CDCl₃); 26.5 (α-CH₃), 31.4 (β-CH₃), 60.5, 67.7, 68.0 (C-2, C-3, C-8), 117.2, 124.3, 129.6, 132.0, 154.8 (C-6,C-7, Ar-C), 167.0, 171.5 (2 x C=O); ESI-HRMS: [M-H]⁻ calcd: 338.0254 found:
338.0254. This material was converted into its benzhydryl ester (101) without further purification.

(3S,7aR)-7-Bromo-6-(3-chlorophenoxy)-2,2-dimethyl-5-oxo-2,3,5,7a-tetrahydropyrrololo[2,1-b]thiazole-3-carboxylic acid (100)

A pale yellow oil (611 mg, 1.46 mmol, 76%); \(^1\)H NMR (270 MHz, CDCl\(_3\)) \(\delta\) 1.66 (s, 6H, \(\alpha\)-CH\(_3\), \(\beta\)-CH\(_3\)), 4.62 (s, 1H, H-3), 5.96 (s, 1H, H-8), 6.93 (ddd, 1H, \(J = 7.9, 2.5, 1.0\) Hz, ArH\(_5\)), 7.05 (app t, 1H, \(J = 2.2\) Hz, ArH\(_2\)), 7.11 (ddd, 1H, \(J = 7.9, 2.5, 1.0\) Hz, ArH\(_3\)), 7.25 (app t, 1H, \(J = 7.9\) Hz, ArH\(_6\)). This material was converted into its benzhydryl ester (102) without further purification.

Bicyclisation with \(\text{L-cysteine}\)

(3R,7aS)-6,7-dichloro-5-oxo-2,3,5,7a-tetrahydropyrrololo[2,1-b]thiazole-3-carboxylic acid (75)

To a solution of \(\text{L-cysteine}\) (3.00 g, 24.8 mmol) in methanol:DIW (8 mL:4 mL) was added a concentrated solution of mucochloric acid (2.10 g, 12.4 mmol) in methanol:DIW (4mL:2mL). Yellow slurry formed. Stirred at room temperature for two days and dark orange solution formed. Concentrated by \~50%\ under reduced pressure. Diluted with DCM (20 mLs) and washed with DIW (10 mLs). Aqueous layer washed with DCM (10 mLs), organic layers combined, dried over MgSO\(_4\), filtered and concentrated under reduced pressure. Isolated as a pale yellow solid, (870 mg, 3.4 mmol, 27%); \(^1\)H NMR (270MHz, CDCl\(_3\)) \(\delta\) 3.51 (difficult to resolve dd, 1H, \(J = 6.7, 11.4\) Hz CH\(_{2a}\) (diastereotopic)) overlapping with 3.55 (difficult to resolve dd, 1H, \(J = 2.7, 11.4\) Hz CH\(_{2b}\) (diastereotopic)) 5.23 (dd, 1H, \(J = 2.7, 6.7\) Hz, H-3), 5.63 (s, 1H, H-8). ESI-HRMS: [M-H] calcd: 251.9281, found: 251.9289.
**Bicyclisation with valinol**

3-isopropyl-2,3-dihydrooxazo[2,3-a]isoindol-5(9bH)-one (93)\textsuperscript{106,107}

Valinol (250 µl, 1.80 mmol) and phthalaldehydic acid (270 mg, 1.80 mmol) were slurried in toluene (20 mL). The mixture was heated to reflux under dean stark conditions for 24 hours. Pale yellow solution formed, cooled to room temperature and toluene was removed under reduced pressure. Crude oil was purified by column chromatography (50:50 hexane: ethyl acetate) to give desired compound 93 as a white solid (267 mg, 1.23 mmol, 68%). \textsuperscript{1}H NMR (270MHz, CDCl\textsubscript{3}) $\delta$ 0.99 (d, 3H, $J = 6.7$ Hz, -CH\textsubscript{3a}), 1.12 (d, $J = 6.7$ Hz, 3H, -CH\textsubscript{3b}), 1.80-1.97 (difficult to resolve m, 1H, -CH-(CH\textsubscript{3})\textsubscript{2}), 3.77-3.86 (difficult to resolve m, 1H, H-3), 3.98 (dd, $J = 7.2$, 8.7 Hz, 1H, -CH-(CH\textsubscript{3})\textsubscript{2}), 4.48 (dd, $J = 7.2$, 8.7 Hz, 1H, -CH\textsubscript{2}-), 5.79 (s, 1H, H-8), 7.55-7.75 (difficult to resolve m, 4H, Ar-H).

**Benzhydryl ester formation – general procedure.**

To the required pyrrolothiazole acid or muco derivative in dichloromethane or acetone (2 mL) at room temperature was added dropwise a solution of diphenyldiazomethane (approx. 0.65 M) in dichloromethane (prepared by oxidation of benzophenone hydrazone with activated MnO\textsubscript{2}) until a pale pink colour persisted and the evolution of nitrogen gas was no longer observable (2-3 h). The solution was concentrated by rotary evaporation and purified by silica gel column chromatography as indicated below.
(3R,7aS)-benzhydryl 6,7-dichloro-5-oxo-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylate (77)

![Chemical Structure 77]

Purification using 80:20 hexane/ethyl acetate gave 77 as a white solid (641 mg, 1.53 mmol, 45%). $^1$H NMR (270MHz, CDCl$_3$) $\delta$ 3.47 (difficult to resolve dd, 1H, $J = 6.7, 11.4$ Hz CH$_2$ (diastereotopic)) overlapping with 3.49 (difficult to resolve dd, 1H, $J = 2.7, 11.4$ Hz CH$_2$ (diastereotopic)), 5.31 (dd, $J = 2.7, 6.7$ Hz, 1H, H-3), 5.57 (s, 1H, H-8), 6.95 (s, 1H, -CH$_2$Ph$_2$), 7.31-7.37 (difficult to resolve m, 10H, Ph$_2$-H). $^{13}$C NMR (67.5MHz, CDCl$_3$) $\delta$ 37.4 (C-2), 59.6, 68.9 (C-3, C-8), 79.9 (-CH$_2$Ph$_2$), 127.0, 127.5, 128.2, 128.3, 128.6, 129.0, 139.0, 148.1 (C-6, C-7, Ar-C), 166.8, 167.8 (2 x -C=O).

ESI-HMRS: [M-H]$^+$ calcd: 528.1022 found: 528.1012.

(3S,7aR)-benzhydryl 7-chloro-2,2-dimethyl-5-oxo-6-phenoxy-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylate (101)

![Chemical Structure 101]

Purification using 60:40 hexane/ethyl acetate gave 101 as a white solid (426 mg, 0.843 mmol, 99%). $^1$H NMR (270MHz, CDCl$_3$) $\delta$ 1.29 (s, 3H, $\alpha$-CH$_3$), 1.55 (s, 3H, $\beta$-CH$_3$), 4.77 (s, 1H, H-3), 5.89 (s, 1H, H-8), 6.98 (s, 1H, -CH$_2$-Ph$_2$), 7.20 (difficult to resolve m, 1H, OArH$_{2,4,6}$), overlapping with 7.20-7.51 (difficult to resolve m, 12H, Ar-H$_{1,5, Ph_2-H}$). $^{13}$C NMR (67.5 MHz, CDCl$_3$) $\delta$ 26.1 ($\alpha$-CH$_3$), 32.2 ($\beta$-CH$_3$), 61.0, 67.8, 68.1 (C-2, C-3, C-8), 78.5 (-CH$_2$Ph$_2$), 115.2, 117.8, 120.8, 124.9, 126.7, 127.8, 128.4, 128.9, 129.6, 129.7, 138.9, 139.0, 154.8 (C-6, C-7, ArC, Ph$_2$C) 166.5, 167.3 (2 x C=O). ESI-HMRS: [M-H]$^+$ calcd: 528.1022 found: 528.1012.
(3S,7aR)-7-Bromo-6-(3-chlorophenoxy)-2,2-dimethyl-5-oxo-2,3,5,7a-tetrahydro-pyrrolo[2,1-b]thiazole-3-carboxylic acid benzhydryl ester (102)

Purification using 1:1 dichloromethane/hexane gave 102 as a pale yellow solid (535 mg, 0.92 mmol, 63%); ¹H NMR (270 MHz, CDCl₃) δ 1.29 (s, 3H, α-CH₃), 1.55 (s, 3H, β-CH₃), 4.76 (s, 1H, H-3), 5.95 (s, 1H, H-8), 6.92 (dd, 1H, J = 8.2, 2.5 Hz, ArH₃), 7.00 (s, 1H, CHPh₂), 7.05 (app t, 1H, J = 2.2 Hz, ArH₂), 7.11 (dd, 1H, J = 8.2, 2.2 Hz, ArH₅), 7.25 (app t, 1H, J = 8.2 Hz, ArH₆), 7.29-7.38 (difficult to resolve m, 10H, Ph₂H); ¹³C NMR (67.93 MHz, CDCl₃, DEPT) δ 26.2 (α-CH₃), 32.1 (β-CH₃), 60.8, 68.4, 69.1 (C-2, C-3, C-8), 78.7 (CHPh₂), 115.3, 117.8, 122.4, 124.5, 126.9, 127.8, 128.2, 128.5, 128.6, 130.4, 135.1, 139.0, 139.144.3, 1559.3 (C-6, C-7, ArC), 165.9, 167.3 (2 x C=O); ESI-HRMS: [M+Na]⁺ calcd: 606.0117, found: 606.0129 and 608.0104.

(Z)-3-Bromo-2-ethylsulfanyl-4-oxo-but-2-enoic acid benzhydryl ester (117)

The reaction mixture was washed with aqueous sodium bicarbonate (5% w/v), the organic layer was dried (magnesium sulfate) and the solvent was removed under reduced pressure to leave a yellow-brown oil which was purified by column chromatography (silica) using hexane/ethyl acetate 4:1 to leave 117 as a pale yellow solid (713 mg, 1.76 mmol, 74%); Rₕ 0.30 (silica, hexane/ethyl acetate 4:1); νₘₐₓ (neat)/cm⁻¹ 3069, 3033, 2973, 2925, 2845, 2737, 1731, 1675, 1535; δ_H (270 MHz, CDCl₃) 1.12 (t, 3H, J = 7.4 Hz, CH₃CH₂S), 2.57 (q, 2H, J = 7.4 Hz, CH₃CH₂S), 7.14 (s, 1H, CHPh₂), 7.29-7.42 (difficult to resolve m, 10H, Ph₂H), 9.01 (s, 1H, CHO); δ_C (67.93 MHz, CDCl₃, DEPT) 13.9 (CH₃CH₂S), 26.4 (CH₃CH₂S), 80.2 (CHPh₂), 119.9,
127.4, 128.8, 138.3, 138.9 155.3 (C-2, C-3, Ph₂C) 162.1 (ester C=O), 180.3 (H⁻C=O); Anal. Calcd for C₁₉H₁₇BrO₃S: C, 56.31; H, 4.23; Found C, 56.01; H, 4.01%.

(Z)-3,4-Dibromo-4-oxo-but-2-enoic acid benzhydryl ester (131)

![Structural formula of 131](image)

Worked up as described for 117; a pale yellow oil (657 mg, 1.55 mmol, 45%); Rₜ 0.83 (silica, hexane/ethyl acetate 4:1); λ_max (neat)/cm⁻¹ 3083, 3060, 3026, 2962, 2841, 1765, 1758, 1620; δ_H (270 MHz, CDCl₃) 7.00 (s, 1H, CHPh₂), 7.28-7.46 (difficult to resolve m, 10H, Ph₂H), 9.56 (s, 1H, CHO); δ_C (67.93 MHz, CDCl₃, DEPT) 80.8 (CHPh₂), 127.1, 128.6, 128.8, 129.1, 138.4, 138.6 (C-2, C-3, Ph₂C), 160.9 (ester C=O), 181.4 (CHO); Anal. Calcd for C₁₇H₁₂Br₂O₃: C, 48.15; H, 2.85; Found C, 48.52; H, 2.84%.

1,4 Addition-elimination – general procedure using sodium azide

To a cooled (-20 °C) solution of the appropriate pyrrolothiazole starting material (3.75 mmol) and 15-crown-5 (93 μL, 0.468 mmol) in acetonitrile (20 mL) was slowly added a cooled (4 °C) suspension of sodium azide (268 mg, 4.13 mmol) in acetonitrile (3mL). The reaction mixture was stirred for 5 h at -20 °C before being allowed to warm to 4 °C and stirred for a further 18 h. The reaction mixture was allowed to warm to R.T., filtered and concentrated via rotary evaporation.

(3R,7aS)-benzhydryl 7-azido-6-chloro-5-oxo-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylate (78)

![Structural formula of 78](image)

Purification using 75:25 hexane/ethyl acetate gave 78 as a dark brown oil (144 mg, 0.338 mmol, 51%). ^1H NMR (270MHz, CDCl₃) δ 3.39 (dd, 1H, J = 6.68, 11.38 Hz, CH₂a (diastereotopic)), 3.47 (dd, 1H, J = 2.72, 11.38 Hz, CH₂b (diastereotopic)), 5.31
(dd, 1H, H-3, $J = 2.72$Hz, $6.68$ Hz), 5.39 (s, 1H, H-8), 6.93 (s, 1H, -CH-Ph₂), 7.30-7.40 (m, 10H, Ar-H). This material was used without further purification in the Click reaction to generate 79.

(3S,7aR)-benzhydryl 7-azido-2,2-dimethyl-5-oxo-6-phenoxy-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylate (103)

DMSO (3 mL) used as co-solvent. Dark brown oil (623mg, 1.46 mmol, 39% (crude)). $^1$H NMR (270MHz, CDCl₃) $\delta$ 1.26 (s, 3H, $\alpha$-CH₃), 1.53 (s, 3H, $\beta$-CH₃), 4.77 (s, 1H, H-3), 5.76 (s, 1H, H-8), 6.97 (s, 1H, -CH-Ph₂), 7.03 (difficult to resolve m, 3H, ArH₂,4,6) overlapping with 7.10 -7.37 (difficult to resolve m, 12H, ArH₃,5, Ph₂H). This crude material was used without further purification in the Click reaction to generate 104.

Click Chemistry – 1,3 Huisgen Dipolar Cycloaddition – general procedure

To a solution of the required azido derivative (2.41 mmol) in acetonitrile(10.4 mL) was added phenylacetylene (19.28 mmol) and a 1M solution of sodium ascorbate (191 mg, 0.964 mmol) in DIW (964 μL) and copper(II)sulfate pentahydrate (24 mg, 0.0964 mmol) in DIW (186 μL). The reaction mixture was stirred vigorously overnight and allowed to evaporate to yield a dark, sticky crude material, which was purified by column chromatography using 7:3 hexane/ethyl acetate.

(3R,7aS)-benzhydryl-6-chloro-5-oxo-7-(4-phenyl-1H-1,2,3-triazol-1-yl)-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylate (79)

Purification using 80:20 hexane/ethyl acetate gave 79 as an orange oil (229 mg, 0.434 mmol, 18%). $^1$H NMR (270 MHz, CDCl₃) $\delta$ 3.47 (difficult to resolve dd, 1H, $J =$
6.7, 11.4 Hz CH$_{2a}$ (diastereotopic)) overlapping with 3.51 (difficult to resolve dd, 1H, J = 2.7, 11.4 Hz CH$_{2b}$ (diastereotopic)), 5.42 (dd, 1H, H-3, J = 2.7, 6.7 Hz), 6.35 (s, 1H, H-8), 6.99 (s, 1H, -CH-Ph$_2$), 7.32-7.51 (difficult to resolve m, 13H, triazoleArH$_{3,4,5}$, Ph$_2$H), 7.93 (app dd, 2H, J = 1.7, 6.0 Hz, triazoleArH$_{2,6}$), 8.78 (s, 1H, triazoleH-5'). $^{13}$C NMR (67.5 MHz, CDCl$_3$) $\delta$ 37.3 (C-2), 59.1, 64.7 (C-3, C-8), 79.0 (-CH-Ph$_2$), 110.0, 117.8, 126.3, 127.2, 128.1, 128.3, 128.4, 128.6, 138.9, 139.1 146.38, 149.4 (C-6, C-7, triazoleC-4', triazoleC-5', triazoleArC, Ph$_2$C) 167.0, 167.82 (2 x C=O). ESI-HRMS: [M+H]$^+$ calcd: 529.1075 found: 529.1101.

(3S,7aR)-benzhydryl 2,2-dimethyl-5-oxo-6-phenoxy-7-(4-phenyl-1H-1,2,3-triazol-1-yl)-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylate (104)

Purification using 2:1 he xane/ethyl acetate gave 104 as an orange oil (237mg, 0.386 mmol, 16%). $^1$H NMR (270 MHz, CDCl$_3$) $\delta$ 1.34 (s, 3H, $\alpha$-CH$_3$), 1.55 (s, 3H, $\beta$-CH$_3$), 4.82 (s, 1H, H-3), 6.67 (s, 1H, H-8), 7.01 (s, 1H, -CH-Ph$_2$), 7.14 (difficult to resolve m, 3H, ArH$_{2,4,6}$), overlapping with 7.16-7.65 (m, 15H, ArH$_{3,5}$, Ph$_2$H, triazoleArH$_{3,4,5}$). 7.85 (app dd, 2H, J = 1.7, 6.0 Hz, triazoleArH$_{2,6}$), 8.44 (s, 1H, triazoleH-5'). $^{13}$C NMR (67.5 MHz, CDCl$_3$) $\delta$ 24.5 ($\alpha$-CH$_3$), 31.2 ($\beta$-CH$_3$), 59.0 (C-2), 61.5, 65.9 (C-3,C-8), 77.0 (-CH-Ph$_2$), 115.1, 116.9, 124.0, 124.3, 124.5, 126.2, 127.1, 127.4, 127.9, 128.1 128.3, 126.6, 137.4, 137.7, 147.6, 153.1 (C-6, C-7, triazoleC-4', triazoleC-5', ArC, triazoleArC, Ph$_2$C) 164.5, 166.7 (2 x C=O). ESI-HRMS: [M+H]$^+$ calcd: 615.2084 found: 615.2066.

**Silylations-general procedure**

To a cooled solution of mucohalic acid (29 mmol) and tert-butyldimethylsilyl chloride (31.97 mmol) in DMF (35.87 mmol) was added diisopropylethylamine (34 mmol) dropwise over 5 min. Following complete addition, ice-water (30 mL) was added dropwise over 5 min and the resulting precipitate was collected by vacuum filtration and allowed to air dry before being dried under vacuum for 4 h.
5-(tert-Butyl-dimethyl-silanyloxy)-3,4-dichloro-5H-furan-2-one (82)

82 was collected as a white, free-flowing solid m.p. 89 – 91 °C (lit. 89–91 °C) (7.4 g, 26.1 mmol, 90%). ^1H NMR (270 MHz, CDCl₃) δ 0.20 (s, 3H, CH₃), 0.22 (s, 3H, CH₃), 0.92 (s, 9H, 3CH₃), 6.20 (s, 1H, H-5); ^13C NMR (67.5 MHz, CDCl₃) δ -5.4, -4.6, 18.0, 25.2 (OSi(CH₃)₂C, Si(CH₃)₂C(CH₃)₃, Si(CH₃)₂C(CH₃)₃), 98.1 (C-5), 109.3 (C-3), 147.1 (C-4), 159.0 (C=O).

3,4-Dibromo-5-(tert-butyl-dimethyl-silanyloxy)-5H-furan-2-one (83)

83 was collected as a beige, free-flowing solid m.p. 90-92 °C (lit. 93-94 °C) (9.75 g, 26.1 mmol, 90 %); ^1H NMR (270 MHz, CDCl₃) δ 0.21 (s, 3H, CH₃), 0.24 (s, 3H, CH₃), 0.93 (s, 9H, 3CH₃), 5.99 (s, 1H, H-5); ^13C NMR (67.5 MHz, CDCl₃) δ -5.4, -4.6, 17.8, 25.2 (OSi(CH₃)₂C, Si(CH₃)₂C(CH₃)₃, Si(CH₃)₂C(CH₃)₃), 98.0 (C-5), 108.8 (C-3), 147.2 (C-4), 158.8 (C=O).

**Reductive Amination – general procedure**

4-Bromo-3-(3-nitrophenoxy)-1-phenethyl-1,5-dihydropyrrol-2-one (111)

To a stirred solution of 98 (412 mg, 1.72 mmol) and 2-(3,4-dimethoxyphenyl)ethylamine hydrochloride (448mg, 2.06 mmol), in dichloroethane (5 mL) was gradually added sodium triacetoxyborohydride (547 mg, 2.58 mmol). Stirred
at room temperature for 12 h. The mixture was diluted with dichloroethane (10 mL) and washed twice with DIW (10 mL). The organic extracts were combined, dried, filtered and concentrated by rotary evaporation. Purification by silica gel column chromatography using 1:1 hexane/ethyl acetate gave 111 as a yellow oil (231 mg, 0.516 mmol, 30%); \(^1\)H NMR (270 MHz, CDCl\(_3\)) \(\delta\) 2.89 (app t, 2H, \(J = 7.2\) Hz, NCH\(_2\)CH\(_2\)), 3.71 (app t, 2H, \(J = 7.2\) Hz, NCH\(_2\)CH\(_2\)), 3.86 (s, 3H, OCH\(_3\)), 3.88 (s, 3H, OCH\(_3\)), 3.91 (s, 2H, CH\(_3\)NC(O)), 6.73 (difficult to resolve m, 2H, dimethoxyAr\(_{5,6}\)), 6.86 (app d, \(J = 5.4\), 1H, dimethoxyAr\(_{2}\)), 7.305 (ddd, 1H, \(J = 8.2, 2.5, 1.0\) Hz, ArH\(_3\), 7.50 (app, t, 1H, \(J = 8.2\) Hz, ArH\(_2\)), 7.80 (app t, 1H, \(J = 2.5\) Hz, ArH\(_6\)), 7.98 (ddd, 1H, \(J = 8.2, 2.2, 1.0\) Hz, ArH\(_5\) ); \(^1\)\(^3\)C NMR (67.93 MHz, CDCl\(_3\), DEPT) \(\delta\) 34.1 (NCH\(_2\)C\(_\text{H}_2\)), 44.6, 53.0 (NCH\(_2\)CH\(_2\), CH\(_2\)NC(O); 55.9 (2 x OCH\(_3\)), 111.4, 111.6, 117.7, 114.9, 118.6, 120.6, 123.2, 130.2, 130.4, 144.7, 147.9, 148.9, 149.1, 155.7 (C-3, C-4, ArC, dimethoxyArC), 163.0 (C=O); ESI-HRMS: [M+H\(^+\)] calcd: 463.0505, found: 463.0490 and 465.0471.

**Suzuki-Miyaura coupling – general procedure for benzhydryl ester or mucohalic compound**

To a solution of the required the muco derivatives 82 and 111, or benzhydryl ester 102, (0.498 mmol) in degassed 1:1 toluene/water (4 mL each) was added the required boronic acid (0.747 mmol), caesium fluoride (151 mg, 0.996 mmol), PdCl\(_2\)(PPh\(_3\))\(_2\) (8.74 mg, 0.01245 mmol) and benzyl(dimethylhexadecyl) ammonium chloride (4.93 mg, 0.01245 mmol). The mixture was heated to reflux under N\(_2\) for 2 - 48 h during which time the colour changed from yellow-brown to either pink or brown, reaction progress was monitored by \(^1\)H NMR spectroscopy. The mixture was allowed to cool to R.T., quenched with 0.5 M HCl (50 mL) and diluted with toluene (20 mL) and separated. The aqueous layer was re-extracted with toluene (20 mL); the organic extracts were combined, washed with DIW (30 mL), dried, filtered and concentrated by rotary evaporation to yield a red orange gel, which was purified by silica gel column chromatography as indicated below.
5-(tert-Butyl-dimethyl-silanyloxy)-3,4-diphenyl-5H-furan-2-one (90)

![Chemical Structure]

2.5 h reaction time using phenyl boronic acid with 82; purification using 1:1 ethyl acetate/hexane gave 90 as a white solid (135 mg, 0.382 mmol, 77%); δ_H (270 MHz, CDCl_3); 0.19 – 0.20 (d, J = 2.0 Hz, 6H, 2 x CH_3), 0.85 (s, 9H, 3CH_3), 6.45 (s, 1H, H-5), 7.34 – 7.44 (m, 10H, ArH); δ_C (67.5 MHz, CDCl_3); - 5.1, -4.1, 17.9, 25.4 (OSi(CH_3)2C, Si(CH_3)2C(CH_3)_3, Si(CH_3)2C(CH_3)_3), 97.3 (C-5), 127.1 – 29.4 (ArC), 141.2 (C-3), 156.3 (C-4), 170.5 (C=O). ESI-HRMS for C_{22}H_{26}O_{3}Si: [M + H]^+ calcd: 367.1745, found: 367.1729.

(3S,7aR)-6-(3-Chlorophenoxy)-7-furan-3-yl-2,2-dimethyl-5-oxo-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylic acid benzhydryl ester (105)

![Chemical Structure]

3 h reaction time using 3-furanboronic acid with 102; purification using 3:1 hexane/ethyl acetate gave 105 as a yellow oil (116 mg, 0.199 mmol, 40%); ^1H NMR (270 MHz, CDCl_3) δ 1.34 (s, 3H, α-CH_3), 1.58 (s, 3H, β-CH_3), 4.72 (s, 1H, H-3), 6.11 (s, 1H, H-8), 6.62 (d, 1H, J = 1.7 Hz, furanyl-H), 6.93 (ddd, 1H, J = 8.2, 2.5, 1.0 Hz, ArH), 6.99 (s, 1H, CCHPh_2), 7.06 (difficult to resolve m, 2H, ArH), 7.22 (app t, 1H, J = 8.2 Hz, ArH), 7.27-7.40 (difficult to resolve m, 10H, Ph_2H), 7.45 (d, 1H, J = 1.3 Hz, furanyl-H), 7.74 (s, 1H, furanyl-H); ^13C NMR (67.93 MHz, CDCl_3, DEPT) δ 26.2 (α-CH_3), 32.0 (β-CH_3), 60.8, 68.4, 69.1 (C-2, C-3, C-8), 78.6 (CH(Ph)_2), 115.3, 117.8, 122.4, 124.5, 126.9, 127.8, 128.2, 128.4, 128.6, 128.6, 130.4, 135.1, 138.9, 139.0, 144.3, 155.3 (ArC, Ph_2C furanyl-C, C-6, C-7), 167.8, 167.8 (2 x C=O); ESI-HRMS: [M+Na]^+ calcd: 594.1118, found: 594.1133.
4-Furan-3-yl-3-(3-nitrophenoxy)-1-phenethyl-1,5-dihydropyrrol-2-one (112)

![Chemical Structure](image)

3 h reaction time using 3-furanboronic acid with 111; purification using 1:3 hexane/ethyl acetate gave 112 as a pale yellow solid (130 mg, 0.299 mmol, 60%); $^1$H NMR (270 MHz, CDCl₃) $\delta$ 2.92 (app t, 2H, $J = 7.4$ Hz, NCH₂CH₂), 3.75 (app t, 2H, $J = 7.4$ Hz, NCH₂CH₂), 3.85 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 4.08 (s, 2H, CH₂NC(O)), 6.56 (app d, 1H, $J = 1.98$ Hz, furanyl-İ), 6.76-6.85 (difficult to resolve m, 3H, dimethoxyAr-H), 7.31 (ddd, 1H, $J = 8.41$, 2.27, 0.74 Hz, ArH₃), 7.44 (d, 1H, $J = 2.0$ Hz, furanyl-İ), 7.49 (app t, 1H, $J = 8.4$ Hz, ArH₂), 7.70 (s, 1H, furanyl-İ), 7.81 (app t, 1H, $J = 8.8$ Hz, ArH₆); $^{13}$C NMR (67.93 MHz, CDCl₃, DEPT) $\delta$ 34.2 (NCH₂C₆H₂), 44.5, 48.6 (NCH₂CH₂, CH₂NC(O); 55.9 (2 x OCH₃), 108.1, 111.1, 111.3, 111.7, 116.3, 118.1, 120.6, 122.6, 127.8, 130.2, 130.7, 139.3, 141.5, 144.2, 147.8, 149.0, 156.4 (ArC, C-furanyl, dimethoxyAr-C, C-3, C-4), 164.9 (C=O); ESI-HRMS: [M+H]+ calcd: 451.1505, found: 451.1501.

4-(1-Methyl-1H-pyrazol-4-yl)-3-(3-nitrophenoxy)-1-phenethyl-1,5-dihydropyrrol-2-one (113)

![Chemical Structure](image)

24 h reaction time using the 1-methylpyrazole-4-boronic acid pinacol ester with 111; purification using ethyl acetate then methanol gave 113 as a pale yellow oil (167 mg, 0.374 mmol, 75%); $^1$H NMR (270 MHz, CDCl₃) $\delta$ 2.92 (app t, 2H, $J = 7.2$ Hz, NCH₂CH₂), 3.74 (app t, 2H, $J = 7.2$ Hz, NCH₂CH₂), 3.85 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 4.09 (s, 2H, CH₂NC(O)), 6.76-6.84 (difficult to resolve m, 3H, dimethoxyArH₂,5,6), 7.31 (ddd, 1H, $J = 8.41$, 2.3, 0.7 Hz, ArH₃), 7.49 (t, 1H, $J = 8.4$ Hz, ArH₂), 7.59 (s, 1H, pyrazole-İ), 7.60 (s, 1H, pyrazole-İ), 7.81 (app t, 1H, $J =
HF removal of silyl protecting group

5-Hydroxy-3,4-diphenyl-5H-furan-2-one (91)

90 (0.835 mmol) was dissolved in THF (9 mL). To this was added dropwise HF/Pyridine (0.5 mL) and the reaction mixture covered and allowed to stir overnight in a Teflon beaker. Reaction mixture was quenched with DIW (7 mL) and diluted with diethyl ether (7 mL). Allowed to stir for 20 min. The layers were split and the aqueous layer was extracted with diethyl ether (3 x 20 mL). The organic extracts were combined and washed with 1M HCl (~ 60 mL). The organic extract was separated and washed with brine (~ 60 mL), dried, filtered and concentrated via rotary evaporation. 38 was isolated as a pale yellow, glassy solid (0.635 mmol, 76%) 1H NMR (270 MHz, DMSO-D6) δ 6.65 (d, 1H, J = 6.7 Hz, H-5), 7.29-7.41 (difficult to resolve m, 10H, ArH), 7.95 (d, 1H, J = 7.4 Hz, -OH).

Benzhydryl removal using AlCl₃

(3S,7aR)-6-(3-chlorophenoxy)-7-(furan-3-yl)-2,2-dimethyl-5-oxo-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylic acid (106)

The benzhydryl ester 105 (0.6748 mmol) was dissolved in dichloromethane (15 mL) and cooled under nitrogen to -84 °C. A solution of aluminum trichloride (222 mg, 1.664 mmol) in nitroethane (1.39 mL) was added in one portion to the cooled pyrrolothiazole solution, at which point the solution changed colour from pale to intense yellow. The reaction mixture was allowed to stir at -84 °C for 1 h at which point ethyl
acetate (70 mL) and 5% sodium hydrogen carbonate (45 mL) were added successively while maintaining the temperature at -84 °C. The reaction mixture was allowed to warm to R.T. at which point the aqueous layer was separated and filtered through celite before being extracted with ethyl acetate (20 mL). The aqueous portion was layered with ethyl acetate (30 mL) and the pH lowered to 2.2 using 1 M HCl. The organic extract was separated and the aqueous portion extracted with a further portion of ethyl acetate (30 mL). The organic extracts were combined, dried, filtered and concentrated by reduced pressure to yield a solid (123 mg, 0.304 mmol, 45%), which was further dried under vacuum.

\[ ^{1} \text{H NMR (270 MHz, CDCl}_3) \delta 1.66 \text{ (s, 3H, } \alpha\text{-CH}_3), 1.70 \text{ (s, 3H, } \beta\text{-CH}_3), 4.56 \text{ (s, 1H, H-3), 6.08 \text{ (s, 1H, H-8), 6.64 \text{ (d, 1H, J = 1.7 Hz, furanyl-H), 6.94 \text{ (dd, 1H, J = 8.2, 2.5, 1.0 Hz, ArH}_3), 7.04-7.10 \text{ (difficult to resolve m, 2H, ArH}_2), 7.22 \text{ (difficult to resolve t, 1H, J = 8.2 Hz, ArH}_6), 7.47 \text{ (d, 1H, J = 1.73 Hz, furanyl-H), 7.78 \text{ (s, 1H, furanyl-H).} \]

\[ ^{13} \text{C NMR (67.93 MHz, CDCl}_3, \text{ DEPT) } \delta 26.8 \text{ (} \alpha\text{-CCH}_3), 30.8 \text{ (} \beta\text{-CCH}_3), 60.7, 68.1, 68.9 \text{ (C-2, C-3, C-8), 114.2, 117.2, 122.4, 124.2, 127.8, 128.3, 129.4, 130.4, 135.1, 144.3, 146.9 155.3 \text{ (ArC, furanyl-C, C-6, C-7), 168.8, 171.9 (2 x C}=O).} \]


\[(3S,7aR)-2,2\text{-dimethyl-5-oxo-6-phenoxy-7-(4-phenyl-1H-1,2,3-triazol-1-yl)-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylic acid (104a)}\]

As per 106. Off-white solid (98mg, 0.22 mmol, 33%) \[ ^{13} \text{H NMR (270 MHz, CDCl}_3) \delta 1.34 \text{ (s, 3H, } \alpha\text{-CH}_3), 1.35 \text{ (s, 3H, } \beta\text{-CH}_3), 4.67 \text{ (s, 1H, H-3), 6.63 \text{ (s, 1H, H-8), 7.18 \text{ (difficult to resolve m, 3H, ArH}_2), 7.20-7.62 \text{ (difficult to resolve m, 5H, ArH}_2, \text{ triazoleArH}_3), 7.79 \text{ (app dd, 2H, J = 1.7, 6.0 Hz, triazoleArH}_2), 8.44 \text{ (s, 1H, triazoleH-5').} \]

\[ ^{13} \text{C NMR (67.5 MHz, CDCl}_3) \delta 24.5 \text{ (} \alpha\text{-CH}_3), 31.2 \text{ (} \beta\text{-CH}_3), 59.0 \text{ (C-2), 61.5, 65.9 \text{ (C-3, C-8), 117.2, 117.3, 118.4, 122.9 124.3, 124.6, 126.1, 127.5, 128.7, 129.1, 147.6, 153.1 \text{ (C-6, C-7, triazoleC-4’, triazoleC-5’, ArC, triazoleArC) 167.5, 172.7 (2 x C}=O).} \]

ESI-HRMS: [M-H] calcd: 447.1127, found: 447.1139
Salt formation – using sodium hydrogen carbonate

(3S,7aR)-6-(3-chlorophenoxy)-7-(furan-3-yl)-2,2-dimethyl-5-oxo-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylic acid anion (107)

The pyrrolothiazole free acid 106 was dried under high vacuum to remove all traces of solvent. The remaining material was carefully weighed and dissolved in ethyl acetate and extracted with a solution of sodium hydrogen carbonate (0.8 equiv) in DIW. The aqueous layer was separated and freeze dried to yield the required material (103 mg, 0.240 mmol, 79%). $^1$H NMR (270 MHz, D$_2$O) $\delta$ 1.57 (s, 3H, $\alpha$-CH$_3$), 1.79 (s, 3H, $\beta$-CH$_3$), 4.14 (s, 1H, H-3), 6.19 (s, 1H, H-8), 6.65 (d, 1H, $J = 1.7$ Hz, furanyl-H), 6.94 (ddd, 1H, $J = 8.2$, 2.5, 1.0 Hz, ArH$_3$), 7.10-7.19 (difficult to resolve m, 2H, ArH$_{2,5}$), 7.25 (app t, 1H, $J = 8.2$ Hz, ArH$_6$), 7.51 (d, 1H, $J = 1.7$ Hz, furanyl-H), 7.81 (s, 1H, furanl-H).

(3S,7aR)-2,2-dimethyl-5-oxo-6-phenoxy-7-(4-phenyl-1H-1,2,3-triazol-1-yl)-2,3,5,7a-tetrahydropyrrolo[2,1-b]thiazole-3-carboxylic acid anion (108)

As per 107 gave the desired material 108, (81 mg, 0.172 mmol, 78%). $^{13}$H NMR (270 MHz, CDCl$_3$) $\delta$ 1.35 (s, 3H, $\alpha$-CH$_3$), 1.36 (s, 3H, $\beta$-CH$_3$), 4.32 (s, 1H, H-3), 6.51 (s, 1H, H-8), 7.21 (difficult to resolve m, 3H, ArH$_{2,4,6}$), 7.23-7.59 (difficult to resolve m, 5H, ArH$_{3,5}$, triazoleArH$_{3,4,5}$), 7.81 (app dd, 2H, $J = 1.7$, 6.0 Hz, triazoleArH$_{2,6}$), 8.39 (s, 1H, triazoleH-5').
**γ-Lactone synthesis**

**4-Bromo-3-ethylsulfanyl-5H-furan-2-one (118)**

![Chemical Structure](image)

To 117 (2.81 g, 7.01 mmol) in 1,2-dichloroethane (40 mL) containing 15-crown-5 (1.1 mL, 5.6 mmol) was added sodium triacetoxyborohydride (5.94 mg, 28.03 mmol). The mixture was heated under reflux for 1 h. The reaction mixture was washed with deionised water (2 x 50 mL), the organic layer was dried (magnesium sulfate) and the solvent was removed under reduced pressure to leave a deep-brown oil which was purified by chromatography (silica) using ethyl acetate/hexane 3:1 to leave 118 as an orange oily liquid (1.11 g, 4.98 mmol, 71%); Rf 0.82 (silica, ethyl acetate/hexane 3:1); ν_{max}(neat)/cm^{-1} 2970, 2928, 2872, 1769, 1762, 1589; ¹H NMR (270 MHz, CDCl₃) δ 1.29 (app t, 3H, J = 7.4 Hz, CH₃CH₂S), 3.19 (q, 2H, J = 7.4 Hz, CH₃CH₂S), 4.85 (s, 2H, H-5); ¹³C NMR (67.93 MHz, CDCl₃, DEPT) δ 15.5 (CH₃CH₂S), 25.0 (CH₃CH₂S), 73.5 (CH₂O), 127.5, 140.9 (C-3, C-4), 168.1 (C=O); ESI-HRMS: [M+H]⁺ calcd: 222.9428, found: 222.9420 and 224.9410 (1:1); Anal. Calcd for C₆H₇BrO₂S: C, 32.30; H, 3.16; Found C, 32.68; H, 3.20%.

**3-Ethylsulfanyl-5H-furan-2-one (desbromo lactone of (118))**

![Chemical Structure](image)

Purified using 75:25 ethyl acetate/hexane gave a yellow oil; ¹H NMR (270 MHz, CDCl₃) δ 1.37 (t, 3H, J = 7.4 Hz, CH₃CH₂), 2.94 (q, 2H, J = 7.4 Hz, CH₃CH₂S), 4.89 (d, 2H, J = 2.23 Hz, H-5), 6.95 (t, 1H, J = 2.2 Hz, H-4); ESI-HRMS: [M+H]⁺ calcd: 145.0323, found: 145.0324.
4-Bromo-3-ethylsulfanyl-5-methyl-5H-furan-2-one (120)

A solution of 117 (1.9 g, 4.69 mmol) in toluene (50 mL) was refluxed in a Dean-Stark system for 1 h. The solution was cooled to –84 °C (liquid N2/ethylacetate slurry). MeMgCl (1M in THF) (1.88 mL, 5.64 mmol) was added in one go. After stirring for 10 min 1M HCl (10 mL) was added and the mixture was allowed to warm to room temperature. The organic layer was separated, dried (magnesium sulfate) and the solvent was removed under reduced pressure to leave a yellow oil which was purified by chromatography (silica) using dichloromethane/hexane 3:1 to leave 120 as a yellow oil (446 mg, 1.88 mmol, 40%); Purified (silica, dichloromethane/hexane 3:1); ¹H NMR (270 MHz, CDCl₃) δ 1.29 (app t, 3H, J = 7.4 Hz, CH₃CH₂S), 1.54 (d, 3H, J = 6.7 Hz, CH₃), 3.18 (m, 2H, J = 7.4, 1.5 Hz, CH₃CH₂S (two sets of overlapping dq for diastereotopic methylene hydrogen atoms), 5.00 (q, 1H, J = 6.7 Hz, H-5); ¹³C NMR (67.93 MHz, CDCl₃, DEPT) δ 15.5 (CH₃CH₂S), 18.8 (CH₃), 25.1 (CH₃CH₂S), 80.6 (CH(CH₃)O), 127.4, 146.5 (C-3, C-4), 167.8 (C=O); ESI-HRMS: [M+H]⁺ calcd: 236.9585, found: 236.9576 and 238.9553 (1:1); Anal. Calcd for C₇H₉BrO₂S: C, 35.46; H, 3.83; Found C, 35.50; H, 3.81%.

5-Benzhydryloxy-4-bromo-3-ethylsulfanyl-5H-furan-2-one (benzhydryl pseudo-ester)

A yellow oil. ¹H NMR (270 MHz, CDCl₃) δ 1.29 (t, 3H, J = 7.42 Hz, CH₃CH₂), 3.19 (dq, 2H, J = 13.5, 7.4 Hz, CH₃CH₂A (diastereotopic)) overlapping with 3.26 (dq, 2H, J = 13.5, 7.4 Hz, CH₃CH₂B (diastereotopic)), 5.73 (s, 1H, CHPh₂), 5.96 (s, 1H, H-5), 7.25-7.50 (difficult to resolve m, 10H, ArH)); ESI-HRMS: [M+Na]⁺ calcd: 426.9979, found: 426.9979 and 428.9987 (1:1).
γ-Lactam synthesis

4-Bromo-3-ethylsulfanyl-1-(toluene-4-sulfonyl)-1,5-dihydro-pyrrol-2-one (141)

A suspension of anhydrous (freshly dried by heating the required amount to 60°C under vacuum for 4 h) chloramine-T (899 mg, 3.95 mmol) in toluene (80 mL) was refluxed in a Dean-Stark system for 1 h. To the cooled suspension was added tellurium powder (535 mg, 4.20 mmol; freshly prepared by grinding tellurium granules) which was then refluxed for 1 h to give a greyish suspension (the formation of some p-toluenesulfonylimide was always observed by 1H NMR spectroscopy at this stage). After cooling, 117 (1 g, 2.47 mmol) was added and the mixture was heated to reflux for 1 h after which time 1H NMR spectroscopy indicated quantitative conversion to the sulfonylimine 136 (1H NMR (270 MHz, CDCl3) δ 1.08 (app t, 3H, J = 7.4 Hz, CH₃CH₂S), 2.41 (s, 3H, PhCH₃), 2.53 (q, 2H, J = 7.4 Hz, CH₃CH₂S), 7.11 (s, 1H, CHPh₂), 7.25 (d, 2H, J = 8.4 Hz, SO₂ArH₃,5), 7.30 – 7.47 (m, 10H, Ph₂), 7.69 (d, 2H, J = 8.4 Hz, SO₂ArH₂,6), 8.49 (s, 1H, HC═NSO₂). The cooled solution was filtered (without any filter aid) and the toluene removed under reduced pressure. The residue was dissolved in 1,2-dichloroethane (20 mL) to which were added sodium triacetoxyborohydride (2.09 g, 9.86 mmol) and 15-crown-5 (390 µL, 1.97 mmol) and the mixture was heated under reflux for 2 h, after which time 1H NMR spectroscopy indicated the complete disappearance of 136 and the formation of diphenylmethanol and of 141. Trifluoroacetic anhydride (343 µL, 2.47 mmol) was added to convert the diphenylmethanol to its trifluoroacetate (this facilitated subsequent chromatographic purification). The solution was washed with water (10 mL) to remove trifluoroacetic acid. The organic layer was separated, dried, concentrated and the residue was purified by column chromatography (silica) using ethyl acetate/hexane 2:1, to give 141 as a yellow solid (325 mg, 0.864 mmol, 35%); mp 110-112 °C; 1H NMR (270 MHz, CDCl₃) 1.23 (app t, 3H, J = 7.4 Hz, CH₃CH₂S), 2.44 (s, 3H, PhCH₃), 3.10 (q, 2H, J = 7.4 Hz, CH₃CH₂S), 4.51 (s, 2H, H-5), 7.35 (d, 2H, J = 8.0 Hz, SO₂ArH₃,5), 7.95 (d, 2H, J = 8.0 Hz, SO₂ArH₂,6); 13C NMR (67.93 MHz, CDCl₃, DEPT) 15.5 (CH₃CH₂S), 21.7 (ArCH₃), 25.1 (CH₃CH₂S), 55.2 (CH₂N), 128.1, 129.9, 132.5, 134.7, 136.5, 145.6 (ArC, C=C), 163.8 (C=O); ESI-HRMS: [M+H]⁺ calcd: 375.9677, found: 375.9692 and
4-Bromo-3-ethylsulfanyl-1-(4-nitrobenzensulfonyl)-1,5-dihydro-pyrrol-2-one (142)

As per 141 using Chloramine-N (4 eq.) and tellurium powder (2eq). Reaction mixture stirred at 90 °C for 5 h max. (monitored by 1H NMR spectroscopy). Triturated with Et2O (3 x 10 mL); pale yellow solid (141 mg, 0.348 mmol 14 %); m.p. 177-179 °C (dec); 1H NMR (270 MHz, CDCl3) δ 1.24 (app t, 3H, J = 7.4 Hz, CH3CH2S), 3.11 (q, 2H, J = 7.4 Hz, CH3CH2S), 4.56 (s, 2H, H-5), 8.29 (d, 2H, J = 9.3 Hz, SO2ArH3,5), 8.41 (d, 2H, J = 9.3 Hz, SO2ArH2,6); 13C NMR (67.93 MHz, CDCl3, DEPT) δ 15.5 (CH3CH2S), 25.1 (CH3CH2S), 55.2 (CH2N), 124.5, 129.7 (ArC), 132.5, 137.1, 143.0, 151.0 (ArC, C-3/C-4), 163.9 (C=O); ESI-HRMS: [M+Na]⁺ calcd: 428.9190, found: 428.9192 and 430.9203 (1:1); Anal. Calcd for C12H11BrN2O5S2: C, 35.39; H, 2.72; N, 6.88%; Found C, 35.31; H, 2.38; N, 6.53 %. Trituration gave solid of adequate purity that was carried onto Suzuki-Miyaura coupling (143). Column chromatography (silica, 2:1 hexane/ethyl acetate) was carried out to get analytically pure sample.

**Suzuki-Miyaura coupling – general procedure for γ-lactone and γ-lactam**

To a solution of the required γ-lactone 118, 120 or γ-lactam 141, 142 (2.045 mmol) in a degassed toluene/water/ethanol mixture (5/7.5/7.5 mL) was added the required boronic acid or pinacol ester (3.06 mmol), caesium fluoride (621 mg, 4.09 mmol), PdCl2(PPh3)2 (36 mg, 0.0511 mmol) and benzyltrimethylhexadecyl ammonium chloride (20.2 mg, 0.0511 mmol). The mixture was heated at reflux under N2 for 5 - 24 h during which time reaction progress was monitored by 1H NMR spectroscopy.
5.25 h reaction time using 4-(4-morpholino methyl)phenyl boronic acid pinacol ester and 118. Extracted with 1M HCl (2x 5 mL), aqueous layer separated and pH adjusted to ~8 using sodium bicarbonate (5% w/v). Extracted into dichloromethane (2x 10 mL), organic layer dried over anhydrous magnesium sulfate, filtered and concentrated. This was purified by column chromatography (silica) using ethyl acetate to give 119 as an orange oily gum (437 mg, 1.37 mmol 67%); purified (silica, ethyl acetate); \( \nu_{\text{max}}(\text{neat})/\text{cm}^{-1} \) 2961, 2929, 2857, 2811, 1750, 1675; \(^1\)H NMR (270 MHz, CDCl\(_3\)) \( \delta \) 1.26 (app t, 3H, \( J = 7.4 \) Hz, CH\(_3\))CH\(_2\)), 2.48 (app t, 4H, \( J = 4.7 \) Hz, (N(CH\(_2\))\(_2\))(CH\(_2\))), 3.21 (q, 1H, \( J = 7.4 \) Hz, CH\(_3\))CH\(_2\)S), 3.56 (s, 2H, NCH\(_2\)Ph), 3.73 (app t, 4H, \( J = 4.7 \) Hz, O(CH\(_2\))\(_2\))(CH\(_2\))), 5.15 (s, 2H, H-5), 7.47 (d, 2H, \( J = 8.1 \) Hz, ArH\(_{3,3}\)), 7.73 (d, 2H, \( J = 8.1 \) Hz, ArH\(_{2,6}\)); \(^{13}\)C NMR (67.93 MHz, CDCl\(_3\), DEPT) \( \delta \) 15.3 (CH\(_3\))CH\(_2\)S), 26.0 (CH\(_3\))CH\(_2\)S), 53.6 (NCH\(_2\)Ph)), 62.9, 67.0, 70.7 (C-5, N(CH\(_2\))\(_2\)), O(CH\(_2\))\(_2\))(C-120.3, 127.7, 129.5, 141.3, 156.3 (C-3, C-4, ArC ), 171.9 (C=O); ESI-HRMS: \([M+H]^+\) calcd: 320.1320, found: 320.1318; Anal. Calcd for C\(_{17}\)H\(_{21}\)NO\(_3\)S: C, 63.92; H, 6.63; N, 4.39; Found C, 63.60; H, 6.58, N 4.41%. The hydrochloride salt was obtained by addition of HCl (1.25M in MeOH) to the free amine in dichloromethane, removal of the organic solvent, taking up the residue in water and freeze-drying to leave a pale yellow solid: m.p. 78-80 °C; Anal. Calcd for C\(_{17}\)H\(_{22}\)ClNO\(_3\)S\(_2\)O: C, 54.61; H, 6.47, N, 3.75%; Found C, 54.42; H, 6.09; N, 3.71 %.
4-(4-Ethylsulfanyl-5-oxo-2,5-dihydrofuran-3-yl)-benzoic acid (114)

24 h reaction time using 4-carboxyphenylboronic acid and 118. Ethanol was removed under reduced pressure, methylene chloride was added, the aqueous phase was acidified to pH 2 and the organic phase was separated. This organic phase was extracted with aqueous bicarbonate (5% w/v) which was then acidified to pH 2 and extracted with methylene chloride. The methylene chloride was removed under reduced pressure to leave a tan-coloured solid. This was purified by column chromatography (silica) using ethyl acetate/hexane 2:1 with 5% acetic acid, followed by washing with water to remove acetic acid, to give 114 as a light tan solid (184 mg, 0.695 mmol, 34%); mp 182 °C (dec); Purified (silica, 2:1 ethyl acetate/hexane and 5% acetic acid); $\nu_{\text{max}}$(neat)/cm$^{-1}$ 3230-3050, 2962, 2972, 2872, 1736, 1706, 1607, 1585; $^1$H NMR (270 MHz, Acetone-d$_6$) $\delta$ 1.22 (app t, $J = 7.4$ Hz, 3H, CH$_3$CH$_2$), 3.20 (q, 1H, $J = 7.4$ Hz, CH$_3$C$_6$H$_2$S), 5.37 (s, 2H, H-5), 7.98 (d, 2H, $J = 8.9$ Hz, ArH$_{3,5}$), 8.17 (d, 2H, $J = 8.9$ Hz, ArH$_{2,6}$); $^{13}$C NMR (67.93 MHz, Acetone-d$_6$, DEPT) $\delta$ 15.7 (C$_{CH_3CH_2}$), 26.2 (CH$_3$C$_6$H$_2$S), 71.7 (C$_{H_2O}$), 122.8, 129.0, 130.7, 132.8, 136.0, 157.1 (C-3, C-4, ArC), 166.8, 171.8 (2 x C=O); ESI-HRMS: [M+H]$^+$ calcd: 265.0535, found: 265.0544; Anal. Calcd for C$_{13}$H$_{12}$O$_4$S: C, 59.08; H, 4.58; Found C, 59.00; H, 4.28%.

4-(4-Ethylsulfanyl-2-methyl-5-oxo-2,5-dihydrofuran-3-yl)-benzoic acid (121)

24 h reaction time using 4-carboxyphenylboronic acid and 120. Ethanol was removed under reduced pressure, methylene chloride was added, the aqueous phase was acidified to pH 2 and the organic phase was separated. This organic phase was extracted
with aqueous bicarbonate (5% w/v) which was then acidified to pH 2 and extracted with methylene chloride. The methylene chloride was removed under reduced pressure to leave a tan-coloured solid. This was purified using by column chromatography (silica) using ethyl acetate/hexane 2:1 with 5% acetic acid, followed by washing with water to remove acetic acid, to give 121 as a light tan solid (364 mg, 1.31 mmol, 64%); mp 160-162 °C; \( ^1H \) NMR (270 MHz, Acetone-\( d_6 \)) 1.19 (app t, \( J = 7.4 \) Hz, 3H, CH\(_3\)CH\(_2\)2), 1.38 (d, \( J = 6.7 \) Hz, 5-CH\(_3\)), 3.08 (q, 1H, \( J = 13.5, 7.4 \) Hz, CH\(_3\)CH\(_2\)A(S (diastereotopic)), 3.21 (q, 1H, \( J = 13.5, 7.4 \) Hz, CH\(_3\)CH\(_2\)B(S (diastereotopic)), 5.81 (q, 1H, \( J = 6.7 \) Hz, H-5), 7.81 (d, 2H, \( J = 8.6 \) Hz, ArH\(_{3,5}\)), 8.18 (d, 2H, \( J = 8.6 \) Hz, ArH\(_{2,6}\)); \( ^{13}C \) NMR (67.93 MHz, Acetone-\( d_6 \), DEPT) \( \delta \) 15.6 (CH\(_3\)CH\(_2\)S), 19.7 (2-C), 26.0 (CH\(_3\)CH\(_2\)S), 79.1 (CH(CH\(_3\))O), 123.2, 129.6, 130.6, 132.5, 136.1, 162.6 (C-3, C-4, ArC), 166.9, 170.5 (2 x C=O); ESI-HRMS: [M+H]\(^+\) calcd: 279.0691, found: 279.0693; Anal. Calcd for C\(_{14}\)H\(_{14}\)O\(_4\)S: C, 60.42; H, 5.07; Found C, 60.35; H, 5.02%.

3-Ethylsulfanyl-4-(4-morpholin-4-ylmethyl-phenyl)-1-(toluene-4-sulfonyl)-1,5-dihydro-pyrrol-2-one (115)

\[
\text{EtS}^+ \quad \text{N=S}^–_2 \quad \text{O}^+ \quad \text{Ph}^+ \quad \text{S}^–_2 \quad \text{N}^–
\]

\( ^{3} \)H reaction time using 4-(4-morpholino methyl)phenyl boronic acid pinacol ester and 141. Extracted with 1M HCl (2x 5 mL), aqueous layer separated and pH adjusted to ~8 using sodium bicarbonate (5% w/v). Extracted into dichloromethane (2x 10 mL), organic layer dried over anhydrous magnesium sulfate, filtered and concentrated. This was purified by column chromatography (silica) using ethyl acetate to give 115 as a pale yellow solid (396 mg, 0.838 mmol 41%); m.p. 108-110 °C; \( ^{1}H \) NMR (270 MHz, CDCl\(_3\)) \( \delta \) 1.19 (app t, 3H, \( J = 7.4 \) Hz, CH\(_3\)CH\(_2\)2), 2.43 (s, 3H, PhCH\(_3\)) overlapping with 2.46 (app t, 4H, \( J = 4.7 \) Hz, (N(CH\(_2\)CH\(_2\))\(_2\)), 3.11 (q, 1H, \( J = 7.4 \) Hz, CH\(_3\)CH\(_2\)S), 3.54 (s, 2H, NCH\(_2\)Ph), 3.72 (app t, 4H, \( J = 4.7 \) Hz, (O(CH\(_2\)CH\(_2\))\(_2\)), 4.75 (s, 2H, H-5), 7.35 (d, 2H, \( J = 7.9 \) Hz, SO\(_2\)ArH\(_{3,5}\)), 7.44 (d, 2H, \( J = 7.9 \) Hz, ArH\(_{2,6}\)), 7.70 (d, 2H, \( J = 7.9 \) Hz, ArH\(_{3,5}\)), 7.99 (d, 2H, \( J = 7.9 \) Hz, SO\(_2\)ArH\(_{2,6}\)); \( ^{13}C \) NMR (67.93 MHz, CDCl\(_3\), DEPT) 15.2 (CH\(_3\)CH\(_2\)S), 21.7 (ArCH\(_3\)), 26.1 (CH\(_3\)CH\(_2\)S), 51.7 (C-5), 53.6
(N(CH₂CH₂-)₂), 62.9 (NCH₂Ph), 66.9 (O(CH₂CH₂-)₂), 125.6, 128.0, 128.1, 129.4, 129.9, 130.4, 135.1, 141.1, 145.3, 152.1 (C-3, C-4, ArC), 166.9 (C=O); ESI-HRMS: [M+H]⁺ calcd: 473.1569, found: 473.1586; Anal. Caled for C₂₄H₂₈N₂O₄S₂: C, 60.99; H, 5.97; N, 5.93%; Found C, 60.76; H, 5.89; N, 5.88 %. The hydrochloride salt was obtained by addition of HCl (1.25M in MeOH) to the free amine in dichloromethane, removal of the organic solvent, taking up the residue in water and freeze-drying to leave a pale yellow solid: m.p. 206-208 °C (dec); Anal. Caled for C₂₄H₂₉ClN₂O₄S₂·H₂O: C, 54.69; H, 5.93, N, 5.31%; Found C, 54.86; H, 5.69; N, 5.32 %.

3-Ethylsulfanyl-4-(4-morpholin-4-ylmethyl-phenyl)-1-(4-nitrobenzenesulfonyl)-1,5-dihydro-pyrrol-2-one (143)

5 h reaction time using 4-(4-morpholino methyl) phenyl boronic acid pinacol ester and 38b. Extracted with 1M HCl (2x 5 mL), aqueous layer separated and pH adjusted to ~8 using sodium bicarbonate (5% w/v). Extracted into dichloromethane (2x 10 mL), organic layer dried over anhydrous magnesium sulfate, filtered and concentrated This was purified by column chromatography (silica) using ethyl acetate to give 143 as a light tan solid (216 mg, 0.429 mmol 21%); m.p. 140-142 °C; ¹H NMR (270 MHz, CDCl₃) δ 1.20 (app t, 3H, J = 7.4 Hz, CH₃CH₂), 2.46 (app t, 4H, J = 4.4 Hz, (N(CH₂CH₂-))₂), 3.09 (q, 1H, J = 7.4 Hz, CH₃CH₂S), 3.54 (s, 2H, NCH₂Ph), 3.72 (app t, 4H, J = 4.4 Hz O(CH₂CH₂-)₂), 4.80 (s, 2H, H-5), 7.45 (d, 2H, J = 8.3 Hz, ArH₃,5), 7.71 (d, 2H, J = 8.3 Hz, ArH₂,6), 8.33 (d, 2H, J = 8.9 Hz, NO₂ArH₃,5), 8.41 (d, 2H, J = 8.9 Hz, NO₂ArH₂,6); δC (67.93 MHz, CDCl₃, DEPT) 15.2 (CH₃CH₂S), 26.2 (CH₃CH₂S), 51.6 (C-5), 53.6 (N(CH₂CH₂-)₂), 62.9 (NCH₂Ph), 66.9 (O(CH₂CH₂-)₂) 124.4 (ArC), 125.4 (C-3/C-4), 127.9, 129.4, 129.6 (ArC), 130.0, 141.5, 143.4, 150.8, 152.6, (ArC, C-3/C-4), 166.85 (C=O); ESI-HRMS: [M+H]⁺ calcd: 504.1263, found: 504.1278; Anal. Caled for C₂₃H₂₅N₃O₆S₂: C, 54.86; H, 5.00; N, 8.34%; Found C, 54.92; H, 4.89; N, 8.29 %. The hydrochloride salt was obtained by addition of HCl (1M in ether) to give a tan
coloured solid; m.p. 202-204 °C (dec); Anal. Calcd for C$_{23}$H$_{26}$ClN$_3$O$_6$S$_2$: C, 51.15; H, 4.85; N, 7.78 %; Found C, 51.32; H, 4.74; N, 7.93 %.

4-bromo-1-(3,4-dimethoxybenzyl)-3-(ethylthio)-1H-pyrrol-2(5H)-one (144)

\[
\begin{align*}
\text{Br} & \quad \text{EtS} \\
& \quad \text{NCH}_2\text{Ar}
\end{align*}
\]

144 Ar = -3,4-dimethoxybenzene

Prepared as per 111 using 3,4-dimethoxybenzylamine and \(p\)-toluenesulfonic acid (5 mol%). Purification using 75:25 hexane/ethyl acetate gave 144 as a yellow oil (64 mg, 0.17 mmol, 10%). \(\delta\) 1.29 (app t, 3H, \(J = 7.4\) Hz, \(\text{CH}_3\text{CH}_2\text{S}\)), 3.20 (q, 2H, \(J = 7.4\) Hz, \(\text{CH}_3\text{CH}_2\text{S}\)), 3.86 (s, 3H, \(\text{OCH}_3\)), 3.87 (s, 3H, \(\text{OCH}_3\)), 4.52 (s, 2H, H-5), 6.79 (m, 3H, \(\text{ArH}_{2,5,6}\)); \(^{13}\)C NMR (67.93 MHz, CDCl$_3$, DEPT) \(\delta\) 15.5 (\(\text{CH}_3\text{CH}_2\text{S}\)), 25.1 (\(\text{CH}_3\text{CH}_2\text{S}\)), 46.4, 55.9 (\(\text{NCH}_2\text{Ar}, \text{CH}_2\text{NC(O)}\)), 55.9 (2 x \(\text{OCH}_3\)), 111.1, 111.3, 120.6, 128.8, 132.4, 148.7 (ArC, C-3/C-4), 166.9 (C=O); ESI-HRMS: \([\text{M+H}]^+\) calcd: 372.0269, found: 372.0280 and 374.0278 (1:1).

**Enzyme inhibition assays**

**Thrombin assays:**

Thrombin catalyses the conversion of Sar-Pro-Arg-\(p\)-nitroanilide. The rate of this reaction can be monitored by the increase in \(A_{410\text{nm}}\) caused by production of \(p\)-nitroaniline (\(p\)NA). In this assay, the effect of 107 on the rate of \(p\)NA production is used as a measure of inhibitory activity.

**Reagents:**

- Phosphate buffer: 0.05 M, pH = 7.2
- Thrombin: 7.5 mg (2.04 x 10\(^{-7}\) mol) in 0.5 mL phosphate buffer to yield a 0.409 mM solution. This was then diluted for individual assay use to yield 0.0409 mM thrombin stock.
- Substrate stock: Sar-Pro-Ala-\(p\)-nitroanilide (4.02 mg, 7.5 x 10\(^{-6}\) mol) was dissolved in phosphate buffer (0.5 mL) to yield a 15 mM solution.

**Inhibitor stock solution:**

107 pyrrolothiazole chlorophenoxy furanyl 100 mM diluted to 50 mM
Control Assay:

Stock substrate solution (15 mM) (10 μL), phosphate buffer (970 μL) and thrombin stock solution (0.0409 mM) (10 μL) were combined and mixed in a 1 mL quartz cuvette. The change in A<sub>410nm</sub> was monitored continuously for 2 min. Control assays were carried out before and after each experimental trial to assess the change in activity of the enzyme over the course of the experiment.

Inhibition Assay:

Phosphate buffer (960 - 950 μL), stock inhibitor solution (50 mM) (10 - 20 μL) and stock substrate solution (15 mM) (10 μL) were combined in a 1 mL quartz cuvette. To this was added stock elastase solution (0.0025 mM) (100 μL). The change in A<sub>410nm</sub> was monitored continuously for 2 min.

Elastase assays:

Elastase catalyses the conversion of N-succinyl-ala-ala-ala-p-nitroanilide. The rate of this reaction can be monitored by the increase in A<sub>410nm</sub> caused by production of p-nitroaniline (pNA). In this assay, the effect of 114, 119, 121 and 115 on the rate of pNA production is used as a measure of inhibitory activity.

Reagents:

Phosphate buffer: 0.05 M, pH = 7.2
Elastase: 1.7 mg (6.8 x 10<sup>-8</sup> mol) in 1.36 mL phosphate buffer to yield a 0.05 mM solution. This was then diluted for individual assay use to yield 0.0025 mM elastase stock.
Substrate stock: N-succinyl-ala-ala-ala-p-nitroanilide (7.2 mg, 16 mmol) was dissolved in phosphate buffer (2.12 mL) to yield a 7.5 mM solution. This was then diluted for individual assay use to yield 3.0 mM substrate stock.
Inhibitor stock solutions:

- **119** γ-lactone benzylmorpholino ethyl sulfanyl 1 mM diluted to 0.1 mM
- **114** γ-lactone carboxyphenyl ethyl sulfanyl 1 mM diluted to 0.1 mM
- **121** methyl-γ-lactone carboxyphenyl ethyl sulfanyl 1 mM diluted to 0.1 mM
- **115** tosyl-γ-lactam benzylmorpholino ethyl sulfanyl 1 mM (50% DMSO) diluted to 0.1 mM

**Control Assay:**

Stock substrate solution (3.0 mM) (100 μL), phosphate buffer (300 μL) and elastase stock solution (0.0025 mM) (100 μL) were combined and mixed in a 0.5 mL quartz cuvette. The change in A_{410nm} was monitored continuously for 2 min. Control assays were carried out before and after each experimental trial to assess the change in activity of the enzyme over the course of the experiment.

**Inhibition Assay:**

Phosphate buffer (100 μL), stock inhibitor solution (0.1 mM) (200 μL) and stock substrate solution (3.0 mM) (100 μL) were combined in a 0.5 mL quartz cuvette. To this was added stock elastase solution (0.0025 mM) (100 μL). The change in A_{410nm} was monitored continuously for 2 min.

**High Performance Liquid Chromatographic (HPLC) Analysis**

HPLC analysis was carried out using a Shimadzu LC-10AT pump linked to a Shimadzu SPD-SA UV spectrophotometric detector and a SphereClone (5 μm) C-18 column (250 x 4.6 mm). The eluant was a mixture of aqueous ammonium acetate (50 mM, pH 4.49) and acetonitrile (25% by volume); a flow rate of 1 mL/min was used and the detector was set at 265 nm. γ-Lactone 114 eluted with a retention time of 7.92 min while its hydrolysis product eluted with a retention time of 1.99 min. Sodium p-toluenesulfinate was used as internal standard: this eluted with a retention time of 2.85 min.
Chapter 5

References
Chapter 5 References


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Appendix I

Publication
Due to copyright restrictions the full text of the following article is not included in the electronic version of this Ph.D

Please see below link to published version

Journal of Heterocyclic Chemistry

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Modular Synthesis of Pyrrolo[2,1-b]thiazoles and Related Monocyclic Pyrrolo Structures

Emma E. O’Dwyer, Nessa S. Mullane, and Timothy P. Smyth

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