PHOTOBIOLOGICAL SWITCHES
OF α-CHYMOTRYPSIN

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Andrew J. Harvey

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Abstract

This thesis examines the design, synthesis and testing of photobiological switches of α-chymotrypsin. A photobiological switch is a biologically active compound that incorporates a photoactive group in order that light-triggered switching of the biological target is afforded. The switches described in this thesis incorporate azobenzene as the photochromic group and α-ketoester or α-ketoamide as the inhibitory moiety. A novel component of these switches is the use of peptidyl groups to enhance the specificity of α-chymotrypsin toward these compounds. Thus, there are three fundamental elements to these photobiological switches; the specificity region, the switch and the inhibitory moiety. The target molecules in this thesis result from permutations of these three elements giving compounds of the type A, B and C.

In Chapter One, the concept of the photobiological switch is discussed and existing and potential applications of this technology are outlined. A survey is given of photochromic materials that are suitable for incorporation into photobiological switches. Examples of enzyme bioswitching methodologies are given and the mechanism, specificity and inhibition of α-chymotrypsin are discussed. Finally, as a distillation of the preceding discussions, the design of target compounds of the type A, B and C is outlined.

In Chapter Two, the syntheses of four compounds of the type A (2.1, 2.2, 2.3, 2.4) are detailed. Target compounds 2.1 and 2.2 comprise one amino acid and target compounds 2.3 and 2.4 comprise two amino acids. In addition, the synthesis of 2.52, a compound for the referencing of enzyme kinetic data with reported data, is discussed. The syntheses of five compounds of the type B are discussed in Chapter Three. Compounds 3.1, 3.2 and 3.3 are regioisomeric and allow for structure activity relationship investigations into the substitution of the azobenzene group. In Chapter Four, the syntheses of three compounds of the type C are detailed.

In Chapter Five, the hydration, photoisomerisation and racemisation behaviours of the target compounds are examined. Hydration studies of 3.1 (type B) showed that
this compound was readily hydrated in solutions containing water. Evidence of hydrate formation was gathered for compounds of the type A (2.4) and compounds of the type C (4.2). The photoisomerisation of the target compounds was studied and trends were observed according the substitution of the azobenzene moiety. For example, the most efficient switching was observed for azobenzenes substituted in the para position with methylene groups and poor results were observed for azobenzenes substituted with a para ketone. Racemisation studies showed that 3.1 was prone to deuteration in a HEPES buffer solution at pH 7.8.

In Chapter Six, the bioswitching ability of the target compounds was measured by enzyme assay. The level of α-chymotrypsin inhibition by reference compound 2.52 was measured (K_i = 0.17 μM) to be comparable to the literature value for this compound. The target compounds of the types A and B were in a comparable range (K_i = 0.04-10 μM) of bioactivity to the reference compound and in every case the (Z) isomer was more active against α-chymotrypsin than the (E) isomer. Compounds of the type C, however, did not show activity against α-chymotrypsin at millimolar concentration. As expected, the type of inhibition for all of the active compounds fitted most closely to a competitive model of inhibition. A preliminary study into the viability of an in situ reversible assay for the bioswitches described in this thesis is discussed. From the three buffers tested (HEPES, TRIS, phosphate), the phosphate buffer would be the buffer of choice for an in situ reversible assay.

Chapter Seven is a summary chapter, giving an overview of the utility of the target compounds and a critical discussion of their design. Potential future directions for research into photobiological switches of α-chymotrypsin are discussed.
Abbreviations

\( \Delta \) heat or change (depending on context)
\( \chi_A \) mole fraction of A
Ar aryl
Boc tert-butoxycarbonyl
br broad
c concentration (optical rotation measurement)
ca. circa
calcd for calculated for
Cbz carbobenzyloxy
d doublet or day (depending on context)
DCC dicyclohexylcarbodiimide
DEAD diethylazodicarboxylate
DIEA \( N,N \)-diisopropylethylamine
DMAP 4-dimethylaminopyridine
DMF dimethylformamide
DMSO dimethylsulfoxide
EDCI 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EI electron ionisation
equiv. equivalent(s)
FAB fast atom bombardment
Fmoc 9-fluorenylmethoxycarbonyl
h hour
HEPES \( N-(2\text{-hydroxyethyl})\text{piperazine-2\text{-ethanesulfonic acid}} \)
HOBT 1-hydroxybenzotriazole
HPLC high performance liquid chromatography
HRMS high resolution mass spectrometry
IR infrared
J coupling constant
KHMDS potassium hexamethyldisilazide
lit. literature
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<tr>
<th>Abbreviation</th>
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<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MoOPH</td>
<td>oxodiperoxymolybdenum(pyridine)(hexamethylphosphoric triamide) complex</td>
</tr>
<tr>
<td>mp</td>
<td>melting point</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance-</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PSS</td>
<td>photostationary state</td>
</tr>
<tr>
<td>Pth</td>
<td>phthalimido</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>qu</td>
<td>quantitative yield</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>singlet or second (depending on context)</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetrabutylammonium fluoride</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-tetramethylpiperidin-1-oxyl</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
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CHAPTER ONE

INTRODUCTION
1.1 Light as a Biological Agent

Light has the capability to alter the characteristics of certain molecules. Photochemical reactions in synthetic organic chemistry, such as light-induced pericyclic reactions and cis-trans photoisomerisations, exemplify this phenomenon. The interaction between light and molecules is also of fundamental importance in biological systems. Photosensitive systems in nature exist in rather disparate organisms. Vision processes of vertebrate and invertebrate species rely upon extremely sensitive photoreceptor cells. Changes in the intensity and duration of sunlight can result in leaf senescence in deciduous plants. Certain types of algae have phototactic mechanisms, which enable them to seek or avoid light. Common to each of these mechanisms is a complexity that is beyond the current level of scientific understanding. In trying to understand light-dependent processes in nature, scientists mimic the complex behaviour with simplified artificial models. These models involve the interaction of light with synthetic molecules and the interaction of synthetic molecules with biomolecules.

1.2 Modelled Enzyme Photoregulation

An enzyme is a biological catalyst that increases the rate of conversion of biological substrates to products. The activity of an enzyme can be modified by the physical or chemical action of an agent on that enzyme. Examples of agents that can affect the activity of an enzyme include the medium and external effector molecules. Let us consider a situation in which the characteristic(s) of an agent, upon which an enzyme's activity is dependent, are able to be modified by the action of light. In this situation, light has become an agent for the modification of the activity of the enzyme. Further to this concept, when the incidence of light onto the intermediate agent is controlled by a mechanism external to the system (e.g. an experimenter), the external mechanism is able to regulate the activity of the enzyme with light. When the external mechanism is the experimenter and the intermediate agent is artificial the system is described as modelled enzyme photoregulation.
In the field conceived from an interdisciplinary fusion of organic chemistry, photochemistry and biochemistry one finds the concept of the artificial photobiological switch, a broad categorisation for the entity previously described as the intermediate agent. Willner defines the artificial photobiological switch as “a biologically active material or a biomodel compound that upon chemical modification with photoactive group(s) is transformed into a light-triggered assembly in respect to its functionalities.”

The domain of the artificial photobiological switch is broader than the description of the intermediate agent in modelled enzyme photoregulation, because of the general scope of its activity. The switch is defined as biologically active whereas the intermediate agent is a subset of this category, being active with respect to an enzyme. In this thesis the intermediate agent shall be referred to, depending on the context, as a photobiological switch, switch, photoswitch: these terms are to be understood to be an abbreviation of artificial photobiological switch. The importance of the term “switch” in conceptualising this entity will be more fully discussed in Section 1.4.1.

1.3 Applications of Photobiological Switches

In addition to modelling light-sensitive biological systems, artificial examples of enzyme photoregulation have many potential applications. Examples include photoamplification, phototherapy, enzyme purification and optical data recording. One application of the photobiological switch is the chemical amplification of weak light signals. To understand this idea, some fundamentals of photochemistry need to be discussed. A photochemical reaction comprises two parts: firstly there is the absorption of photons to elevate the energy state of the molecule (light reaction) and secondly there are subsequent reactions (dark reactions). Because the quantum yield of any light reaction cannot exceed unity, signal amplification must arise as a result of coupled dark reactions. Martinek described the use of silver halides in obtaining photographic images as a classic non-biological example of light amplification by chemical means. A silver ion is reduced upon absorption of a photon (light reaction) and the resulting silver atom acts as a localised catalyst for the dark reactions of the development process. Here the signal (a photon) was amplified because it led to the formation of a catalyst, which in turn was able to convert many molecules. The concept of signal amplification
is readily transferable to the action of light on a photobiological switch. The photoconverted switch enables the activation of an enzyme. The activated enzyme is able to convert substrate at an increased rate relative to deactivated enzyme.

Edelson and Parrish discussed the development of therapeutic agents that utilise a mechanism of light activation. Both researchers cited specificity to the site of action as a highly desirable characteristic of effective medicine. Parrish investigated the ability of light of different wavelengths to penetrate skin to different depths and the consequent therapeutic application of this ability. He posited the photon-activated drug delivery system (PADDS). In this system, photoactive medicines or photolabile liposomes containing therapeutic agents would be administered to the patient. The drugs would be activated at the specific site of action by the irradiation of skin with depth-specific and functional group-specific wavelengths of light. Edelson employed a technique involving photoactivation of a naturally occurring compound 8-methoxypsoralen (8-MOP) to treat cutaneous T-cell lymphoma (CTCL), a malignancy of white blood cells. The photoactive compound 8-MOP was a tricyclic molecule comprising furan and isocoumarin moieties that was proposed to act by intercalating with DNA (sliding between base pairs) and then binding with the DNA on irradiation with light. The DNA, once bound to 8-MOP, was not able to undergo replication.

In contrast to the irradiation of tissue, the technique of activating 8-MOP involved the irradiation of blood that has been temporarily removed from the body (photopheresis). Although the interaction of a photoactive agent with DNA does not fit within the category of modelled enzyme photoregulation this example is illustrative of the potential of light therapy in this field.
Step One
The photobiological switch is tethered to a solid support and the assembly is contained within a chromatographic column.

Step Two
Impure enzyme is applied to the column. Enzyme associates with the switch and the impurities are washed through the column.

Step Three
Upon irradiation with light the switch changes such that it has lower affinity for the enzyme. Pure enzyme can proceed down the column.

Figure 1.2 Photoaffinity chromatography promises a mild purification technique for enzymes.

It is envisaged that an effective photobiological switch, that is either tethered to a solid support or is integral to a polymeric substance, could be exploited as a means for mild chromatographic purification of a target enzyme (as depicted in Figure 1.2). Such a system would build on the well-established technologies of affinity adsorption chromatography. The photocontrolled system would rely on a change in affinity of the switch for the enzyme that results from irradiation of the support-switch-enzyme assembly with light.
1.4 Photochromism in relation to photobiological switches

1.4.1 Defining Photochromism

To further develop the concept of a photobiological switch as an intermediary agent between a light signal and an enzyme, the following characteristics have been adopted from Willner’s model of a bioswitch.¹

1. The switch shall contain a photoisomerisable chromophore;
2. The photoisomerisable unit shall exhibit reversible properties. Therefore, once the photoisomerisable moiety has undergone photonic excitation and concomitant chemical change, it is able to revert to its original state either by thermal relaxation or by a secondary photonic excitation;
3. A switch that is associated with a certain enzyme will lead to activation of that enzyme as long as the triggered-on state of the enzyme exists. Thus an amplification factor results from a single photonic event;
4. The same bioswitch can lead to different bioreactions with different enzymes.

Conditions 1 and 2 correlate to a category of materials known as photochromic materials. It is true that certain materials exhibiting photochromism have proven to be fundamental building blocks for biological photoswitches.

Brown defines photochromism as “a reversible change of a single chemical species between two states having distinguishably different absorption spectra, such change being induced in at least one direction by the action of electromagnetic radiation.”² This definition of the phenomenon of photochromism can be represented diagrammatically:

\[
\begin{align*}
A & \xrightarrow{\text{hv}_1} \text{hv}_2 \rightarrow B \\
\text{A or hv}_2 &
\end{align*}
\]
The nature of the chemical change between states A and B (e.g. (E)/(Z) isomerism, homolytic cleavage) is not important. Photochromism is exhibited if the reversibility criterion is met. Although A is a single chemical species, B can be either a single chemical species or it may be more than one species. The criteria for photochromism are met if B reverts exclusively to A. The photophysics of photochromism are discussed in Section 5.3.

It is worthwhile exploring the analogy between a switch and the behaviour of photochromic materials. A switch has two possible states – “off” and “on” – that are interconvertible. The “on” state is changed exclusively to “off” and vice versa. Analogously, a photochromic compound has two interconvertible states and the interconversion is reversible. The switch analogy becomes more useful when considering photobiological switches, for which the “off” and “on” states relate to the activity state of the associated enzyme.

1.4.2 Examples of photochromism

An overview of suitable photochromic materials is presented with a view to evaluating potential components of photobiological assemblies. Although photochromic materials, by definition, meet several criteria of photobiological switches, there are additional qualities that are advantageous for effective photoregulation. The criteria for evaluation are:

1. That significant differences in structure and dipole moment exist between the two states of the photochromic material, with a view to incurring maximal activity change on the associated enzyme;
2. That the conversion by thermal relaxation is a sufficiently slow process that the thermally less stable state can have a lasting and measurable effect on the enzyme;
3. That the conversion by thermal relaxation is also possible through photochemical means in order that the total system is under control of the experimenter;
4. That the state of the photochromic material corresponding to the deactivated state of the enzyme (“off”) contains a negligible amount of the activating isomer;
5. That the synthesis of the photochromic material is relatively facile.
These criteria are applied to the photochromic moieties discussed in Sections 1.4.2.1 to 1.4.2.4 to evaluate their potential as components in photobiological switches. In Section 1.4.2.4.2 there is a discussion on why the azobenzene moiety was selected for inclusion into the target compounds in this thesis.

1.4.2.1 Photochemical ring closures and openings (4n+2)

There are two major aspects associated with the photochromic change in (4n+2)π electron systems. Firstly, when the molecule ring-opens there is an increase in steric bulk. Secondly, there is often the creation of a zwitterionic product during ring opening that corresponds to a large change in dipole moment. Coupling this class of compound to enzymes should lead to effective switching. The photochromic processes of (4n+2)π electron systems, being examples of the broader class of pericyclic reactions, adhere to the rules of orbital symmetry as outlined by Woodward and Hoffmann. The ramifications of these rules will be discussed by example.

1.4.2.1.1 1,2-Diarylethlenes

Early examples of this class that were derived from (Z)-hexa-1,3,5-triene exhibited poor photochromic behaviour. The problem with these systems was the high levels of fatigue processes. The useful life of a photochromic system is diminished by the occurrence of such irreversible side reactions. One example of attempted 1,2-diarylethylene photochromism is the photochemical ring closure of (Z)-stilbene 1.2 to afford 4a,4b-dihydrophenanthrene 1.3. The reverse reaction of ring opening yields 1.2 by a thermal or a photochemical mechanism. The ring opening proceeds slowly in the dark as the thermal process is symmetry forbidden. The major fatigue pathway for this system is the dehydrogenation of 1.3 to phenanthrene 1.4.

Scheme 1.1 Photocyclisation of (Z)-stilbene 1.2
More recently, successful photochromic systems have been developed using the (Z)-hexa-1,3,5-triene template. These molecules have a core structure comprising a cyclopentene substituted with two thiophene moieties. Scheme 1.2 shows an example with the 1,2-dithienylperfluorocyclopentene (DTFCP) core unit. Derivatisation of DTFCP yielded excellent photochromes with very high resistance to photofatigue. Compounds of this type have exhibited stability over $10^4$ photocycles. A reversible interconversion occurs between the $\pi$-conjugated closed form 1.6 and the cross-conjugated open form 1.5 when the compounds are irradiated with visible and ultraviolet light, respectively. The ring-closed isomer 1.6 is thermally stable.

Scheme 1.2  Photochromic action of 1,2-dithienylperfluorocyclopentene derivatives

A number of applications have been found for this type of 1,2-diarylethylene. In one example, boronic acids were included in the R-groups to form a photoconvertible “tweezer” system for saccharides. A complex between the diboronic acids and the hydroxyl groups of a sugar was formed when the photoswitch was in the twisted antiparallel open conformation 1.5. The aim of creating electronic assemblies on a molecular scale was the impetus for work on a conducting wire and switch assembly. The assembly included a DTFCP core and thiophene oligomers as the R-groups. Polythiophene and oligomers of thiophene have the properties of a molecular wire.
1.4.2.1.2 **Fulgides**

Fulgides are derivatives of dimethylene succinic anhydride 1.7 that can exhibit photochromism with high thermal stability and high quantum efficiencies.

![Figure 1.3 Dimethylene succinic anhydride as a template for photochromic fulgides](image)

A representative example is the work done on the 3-thienylfulgides (Scheme 1.3).\(^\text{15}\) The (Z)-3-thienylfulgide 1.8 is converted quantitatively to the (E) isomer 1.9 by irradiation with ultraviolet light. Compound 1.9 is set up for a \((4n+2)\) electrocyclic reaction to form the ring-closed isomer 1.10. The photochemical ring closure proceeds via a conrotatary mechanism to give the coloured 7,7a-dihydrobenzothiophene derivative 1.10 and this product undergoes photochemical conrotatary (thermal disrotatary) ring opening to yield the colourless 1.9. The pattern of substitution has proven important to the photochromic utility of these compounds. A problem that exists with fulgides is the occurrence of irreversible side reactions that diminish the lifetime of the system. For example, when \(R_2\) is a hydrogen this group can undergo a thermal \([1,5]\)-shift in the closed ring form.\(^\text{16}\) The 3-thienylfulgide system exhibits optimal photochromic action when \(R_1 = R_2 = R_3 = \text{Me}\). In this case compound 1.10 is thermally stable at 160 °C.

![Scheme 1.3 Photochromic action of a 3-thienylfulgide](image)
Chapter One: Introduction

In order to overcome the fatigue properties of the fulgide system, Yokoyama synthesised 3-indonylfulgides with \( R_1 \) as a methyl group at first, then as a trifluoromethyl group.\(^{17}\) The methyl group was replaced with the trifluoromethyl group to avoid antarafacial [1,5]-sigmatropic rearrangement of the primary hydrogens in the closed form. This change of functional group resulted in improved fatigue resistance (from 10–100 useful cycles with the methyl group to 1000 useful cycles with trifluoromethyl), but the wavelength of light that yielded the closed form gave a low level of enrichment (PSS\(_{405}\)\((E):(Z):(C)\) = 23:44:33)\(^{\dagger}\).

1.4.2.1.3 Spiropyans

The spiropyans constitute a broad class of photochromic materials.\(^{18}\) In Figure 1.4 the components of a spiropyran are shown: a substituted benzopyran and a second heterocycle. The two heterocycles are linked by a common tetrahedral carbon atom, about which the two halves of the molecule lie in orthogonal planes.\(^{19}\)

![Figure 1.4 Schematic diagram of a spiropyran depicting the orthogonal parts of the molecules in planes P and P'. The symbol H depicts a heterocycle.](image)

The variable heterocycle, depicted as H in Figure 1.4 is commonly nitrogen-containing and saturated or benzofused. Common examples of H include indoline, benzothiazoline, benzoxazine and benzodithiole.\(^{1}\) In solution, the spiropyans absorb in the ultraviolet for wavelengths between 320 and 380 nm. Irradiation of a spiropyran with light in this spectral region converts the closed form 1.11 to the coloured merocyanine form 1.12/1.13 (see Scheme 1.4).

\(^{\dagger}\) PSS\(_{405}\) refers to the photostationary state resulting from irradiation at \( \lambda = 405 \) nm.
The merocyanine form consists of resonance contributions from the zwitterionic resonance conformer 1.12 and the quinonoid conformer 1.13. The thermal reversion to the closed form tends to be a fast process. The substitution pattern of the spiropyran is important in increasing the thermal stability of the merocyanine and in improving the quantum yields of the photochemical electrocyclisation. For example, the merocyanine in Scheme 1.4 was found to be most thermally stable with $R_6 = \text{NO}_2$ and $R_8 = \text{OMe}$.\textsuperscript{20}

The masked chelating ability of 1.14 provided a further means for improvement of the merocyanine stabilisation of spiropyrans.\textsuperscript{21} The authors claim to have developed the first thermally stable, dual-wavelength photoactivation spiropyran-merocyanine molecular switch. The merocyanine was stabilised from thermal decay by binding with a metal ion. Upon ultraviolet irradiation ($\lambda = 254$ nm) in the presence of calcium ions, 1.14 exclusively gave the merocyanine-metal complex. This complex was stable for at
least three days in the dark and underwent rapid reversion to the spiropyran form on irradiation with visible light ($\lambda = 520$ nm).

The spiropyran backbone can be functionalised readily. Functionalisation allows for facile conjugation with biomaterials through amino, hydroxyl or carboxyl groups. Spiropyrans are reported to have relatively poor fatigue properties. Typical lifetimes of spiropyrans range from 10 to 300 useful cycles.

1.4.2.1.4 1,5-Electrocyclisation

Photochromism based on 1,5-electrocyclisation is a relatively new field. Dürer used the system outlined in Figure 1.5 for categorising this type of reaction. Although no photochromic compounds of type-1 have been found, type-2 and type-3 have yielded useful photochromic systems. Combinations of these three types have been fruitful, for example a type-1,2 combination gave a $\Lambda^3$-pyrazoline template for photochromic compounds.

![Figure 1.5 Templates for 1,5 electrocyclisations](image-url)

**Figure 1.5** Templates for 1,5 electrocyclisations
The mechanism of the 1,5 electrocyclisation is dictated by the conservation of orbital symmetry. By the Woodward-Hoffmann formalism, the pentadienyl anion 1.15 can undergo either conrotatory photochemical or disrotatory thermal ring closure to the cyclopentadienyl anion 1.16.

Examples of a type-2 system are the spiro-1,8a-dihydroindolizines 1.17. These compounds give the coloured betaines 1.18 on irradiation with ultraviolet or visible light. The thermal stability of the betaines can be adjusted by substitution. Half-lives of the betaines range from milliseconds to days. Although 1.18 is drawn as the (E)-isomer, the (E)- and (Z)-isomers are readily interconvertible at room temperature. Resonance structures can be drawn such that bond C2-C3 is a single bond.

**Scheme 1.5** Photochromic action of the spiro-1,8a-dihydroindolizines

Using the method of Fischer, a number of photostationary state ratios of type-2 system compounds were calculated.\(^{23}\) This simple method is based on differences in the ultraviolet spectra of two photostationary states. The ratio of closed form/open form for different compounds at the photostationary state ranged from 1:1 to 1:19.

1.4.2.2 **Cycloaddition reactions**

Photochromic systems involving cycloaddition reactions fall into two categories. By the principles of orbital symmetry, photochemical \((2\pi_s+2\pi_s)\) and \((4\pi_s+4\pi_s)\) cycloadditions are allowed (**Scheme 1.6**).\(^1\)
As shown in Scheme 1.6, cycloaddition proceeds at one wavelength and cyclodissociation occurs at a different, typically shorter, wavelength. Thermal reversion processes are also possible. The cycloaddition reactions can be either intermolecular or intramolecular.

Cycloaddition reactions of norbornadiene derivatives have been the most extensively studied of the \((2\pi_a + 2\pi_a)\) systems.\(^{24}\) The quantum yields of cycloaddition in the norbornadiene system (Scheme 1.7) are typically high \((\Phi = 0.4-0.6)\), because of the proximity of the double bonds, which results in a weak transannular interaction. The cycloreversion of thermally stable \(1.20\) is slow at 180 °C in the gas phase. However in the presence of Rh(I) and Pd(II) catalysts \(1.20\) thermally reverts to \(1.19\) at -26 °C. Photochemical cycloreversion of \(1.20\) occur at short wavelengths \((\lambda = 187 \text{ nm})\), but the reaction suffers from the formation of side products. More facile photochemical cycloreversion occurs in the presence of acceptor sensitisers in non-polar solvents. The quantum yield for \(1.20 \rightarrow 1.19\) in the presence of 1-cyanonaphthalene is near unity.

Scheme 1.6  Photochemically-allowed cycloaddition reactions

Scheme 1.7  Photochromic action of norbornadiene
If the norbornadiene derivatives are the archetypal \((2\pi_s + 2\pi_s)\) system, derivatives of anthracene hold similar status for \((4\pi_s + 4\pi_s)\) systems. More than one hundred derivatives of anthracene are known to photodimerise. The dimers are thermally stable at room temperature. As shown in Scheme 1.8, the anthracene derivative 1.21 undergoes reversible cycloaddition to give the photodimer 1.22.

\[
2 \times \begin{array}{c}
\text{Me} \\
\text{Me} \\
\text{Me} \\
\text{Me}
\end{array} \quad \frac{\text{hv}_1}{\Phi = 0.16} \quad \begin{array}{c}
\text{Me} \\
\text{Me} \\
\text{Me} \\
\text{Me}
\end{array} \quad \frac{\text{hv}_2}{\Phi = 0.81} \quad \begin{array}{c}
\text{Me} \\
\text{Me} \\
\text{Me} \\
\text{Me}
\end{array}
\]

Scheme 1.8  Photochromism of 9,10-dimethylanthracene

A characteristic of cycloaddition reactions that is advantageous in the development of bioswitches is the change in steric bulk on photoaddition. Another characteristic of these reactions is the formation of intramolecular cavities on cycloaddition. This phenomenon has allowed the development of photoreversible molecular carriers. For example, crown ethers conjugated with anthracene moieties, having both photochromic and ion-binding characteristics, have been synthesised.

1.4.2.3  Bond cleavage (homo- & heterolytic)

This class of photochromic systems involves the formation of “more or less separated” species resulting from bond cleavage and the subsequent recombination of these species. Homolytic cleavage gives two radical fragments and heterolytic cleavage gives two ionic fragments. Photochromism involving heterolytic cleavage has been identified as a good model for bioswitching. The charged species resulting from photolysis can form electrostatic interactions with nearby biomaterial components. An example is the leucocyanins 1.23 that were photodissociated to give the corresponding ion pairs 1.24 in excellent quantum yields (\(\Phi = 0.6-1.0\)). The rate of the thermal recombination process was influenced by aryl substitution.
1.4.2.4 Photoisomerism across double bonds (stilbenes, azobenzenes)

This type of photochromic material can be subclassified according to the atomic components of the double bond: carbon-carbon, carbon-nitrogen and nitrogen-nitrogen. Photochromism in these systems arises from reversible $(E) \rightarrow (Z)$ isomerisation. Photochemical isomerisation proceeds via a twisted transition state that is able to relax to either the $(E)$ or $(Z)$ isomer. Therefore, double bond photochromic systems yield photostationary states (PSS) of both isomers upon irradiation. The ratio of $(E)/(Z)$ depends on the degree of absorbance overlap at the wavelength of irradiation. Furthermore the $(Z)$ isomer tends to be less stable than the $(E)$ isomer in double bonded systems. Consequently $(Z)$ undergoes thermal isomerisation in most systems, with half-lives over a great range. The rate of dark isomerisation depends on the compound, temperature, solvent and the presence of catalysts.

1.4.2.4.1 Carbon-carbon double bonds - stilbenes and indigos

The stilbenes are well-studied examples of photochromism involving carbon-carbon double bonds. Fischer has grouped derivatives of stilbene according to their temperature-dependent quantum yield behaviour. Stilbene 1.25 belongs in the group in which $\Phi_E$ (the quantum yield for $(E) \rightarrow (Z)$) increases as temperature increases and $\Phi_Z$ (the quantum yield for $(Z) \rightarrow (E)$) is independent of temperature. Figure 1.6 shows how the quantum yields, $\Phi_E$ and $\Phi_Z$ act in concert to give the mole fraction of $(E)$: $\chi_E$. The results are given for irradiations at $\lambda = 313$ nm over a range of temperatures.
Chapter One: Introduction

![Chemical Structure](image)

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>+25</th>
<th>-65</th>
<th>-105</th>
<th>-140</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Phi_E$</td>
<td>0.50</td>
<td>0.31</td>
<td>0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>$\Phi_Z$</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>$\chi_E$</td>
<td>0.07</td>
<td>0.12</td>
<td>0.27</td>
<td>0.65</td>
</tr>
</tbody>
</table>

**Figure 1.6** Temperature dependence of quantum yields and mole fraction of $E$ for the irradiation ($\lambda = 313$ nm) of stilbene 1.25 in methylcyclohexane/isoctane (MCH/IH 2:1).

Generally $\Phi_E$ can be affected by temperature, solvent and medium viscosity. Both $\Phi_E$ and $\Phi_Z$ depend on the specific structure of the stilbene undergoing photoisomerisation. The thermal reconversion of $(Z) \rightarrow (E)$ for stilbenes has activation barriers in the range 146 to 176 kJmol$^{-1}$.30

A second class of photochromic compounds that isomerise about a carbon-carbon double bond are the indigoes (**Scheme 1.10**).30 Because of the minor alterations in structure and dipole moment upon isomerisation, one would anticipate minimal change of a biomaterial associated with such compounds.

![Scheme 1.10](image)

**Scheme 1.10** Photochromism of indigo 1.26
1.4.2.4.2 Nitrogen-nitrogen double bonds – azobenzenes

Azobenzene 1.27 is a template compound, which has been extensively employed in artificial enzyme photoregulation.\textsuperscript{33} Azobenzenes contain a nitrogen-nitrogen double bond substituted with two aryl moieties. As observed with the stilbenes, azobenzenes undergo photoinduced (E)$\xleftarrow{hv(\lambda_1)}$ (Z) isomerism about the double bond to yield photostationary equilibria. The ratio of (E)/(Z) at the photostationary state depends on the wavelength of light used for the irradiation. Generally, irradiation of an azobenzene with ultraviolet light will yield predominantly (Z) and irradiation with visible light will yield predominantly (E). Fischer investigated the wavelength dependence for azobenzene isomerism.\textsuperscript{34} It was found that the composition of the photostationary state was proportional to the ratio of extinction coefficients at a certain wavelength (Figure 1.7). The final PSS was always independent from the initial PSS prior to irradiation.\textsuperscript{35} For further discussion of this phenomenon and the mechanism of isomerism refer to Section 5.3.

![Diagram of azobenzene isomerism](image)

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>365</th>
<th>405</th>
<th>436</th>
<th>546</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon(E)/\varepsilon(Z)$</td>
<td>9</td>
<td>0.3</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Percent of E</td>
<td>9</td>
<td>88</td>
<td>86</td>
<td>75</td>
</tr>
</tbody>
</table>

Figure 1.7 Wavelength dependence of photostationary states in azobenzene

The features of azobenzene photochromism that lend azobenzenes to biological photocontrol are manifold. Importantly, there are substantial differences in structure and dipole moment between the (E) and (Z) isomers.\textsuperscript{36} The (E) isomer is near planar across any azobenzene group, whereas the (Z) isomer adopts a twisted conformation in order to relieve steric interactions between the ortho hydrogens. (Z)-Azobenzene groups are
shorter in length than the corresponding (E) isomers. Using azobenzene as an example of these differences, the phenyl rings in (Z)-1.27 are twisted out of plane by 53°.37 The dipole moment of (E)-1.27 is almost 0.0 D and that of (Z)-1.27 is 3.0 D.1

Further characteristics of the azobenzene group that makes an azobenzene a worthwhile photobiological switch include relative stability of the isomers, resistance to photodegradation and synthetic versatility. The thermal stability of a (Z) isomer depends on the substitution pattern of the azobenzene. Comparatively high rates of isomerisation are observed for azobenzene derivatives with hydroxyl or amino groups in ortho or para positions and those substituted with both electron donating and withdrawing groups, but azobenzene derivatives that are outside of these two narrow categories have activation energies in solution in the range 88 to 100 kJmol⁻¹.30,38 These activation barriers correspond to thermal half-lives that are suitable for photobiological switches. Azobenzene photosystems are reported to have high stabilities against competitive photodegradation. The photochemical side reactions possible in azobenzenes include photoreduction, photo-oxidation and photocyclisation, but the quantum yields of these processes are negligible (<10⁻³).31 Azobenzene derivatives that enable coupling to biological materials (hydroxy, carboxyl, amino substituents) are synthetically available and azobenzenes are readily synthesised. One inherent disadvantage of azobenzenes is the formation of photostationary states. The ideal photoswitch calls for complete conversion to the “off” position rather than a state consisting of mainly “off” plus residual “on”.

The photochromic azobenzene group was chosen for use in the research described in this thesis due to the advantages listed above. One further condition finalised the decision to use azobenzene derivatives as photoswitches. For the purposes of this thesis the selected photochromic moiety was to act as either the amino or the carboxyl protecting group of an amino acid. Reported enzyme inhibitors contain protecting groups such as benzyl esters or benzyloxy carbamates.61 Azobenzene derivatives have the advantage over many other photochromic materials of having similar size and structure to existing protecting groups.
The \((E)\Rightarrow(Z)\) isomerisation of azobenzenes has proven useful in many contexts. Polymers incorporating azobenzenes found use in forming holographic surfaces.\(^{39}\) An azobenzene moiety was incorporated into a cyclic peptide to afford a photoregulated peptide assembly.\(^{40}\) In a second example of the combination of an azobenzene with cyclic peptides, the azobenzene was exocyclic to two cyclic peptides. This assembly had different supramolecular behaviour for the two isomers of the azobenzene derivative.\(^{41}\) An azobenzene was the switch in a light-switchable catalyst for amide bond formation.\(^{42}\) Crown ethers incorporating azobenzenes have been explored widely.\(^{43}\) Many such assemblies have ion host abilities for only one of the azobenzene isomers. Azobenzenes have been tethered to cyclodextrin structures in order to create photoswitchable molecular hosts.\(^{44}\)
1.5 Models for reversible photoregulation of enzymes by photochromic materials

In this section, various methods for the photoregulation of enzymes are outlined. Work in this area is exemplified, where possible, with azobenzene derivatives as photoactive switches. Enzyme activity can be modified by light-induced changes in the environment of the enzyme, the covalent attachment of photochromic moieties and by photochromic effector molecules.

1.5.1 Environment

Solvent properties that influence enzyme activity include pH, ionic strength, polarity and viscosity. Photoswitchable molecules in a solution can afford changes in one or more of these solvent properties and consequently can effect changes in enzyme activity.\(^2\) The immobilisation of an enzyme in a photoswitchable polymer or the encapsulation of an enzyme in a photolabile liposome can enable light-induced control of the enzyme.\(^45\) The permeability, wettability or sol-gel transitions can be controlled by light in photoisomerisable polymer or liposome assemblies. An example of an enzyme embedded in a photoisomerisable polymer is presented in Figure 1.8.\(^{33c}\)

![Figure 1.8](image)

**Figure 1.8** An enzyme is immobilised in a photochromic copolymer
When the polymer, shown in Figure 1.8, is in the “on” state, the substrate can diffuse through the polymer to access the enzyme. The substrate is then converted into product that can diffuse away from the active site through the polymer. However, upon irradiation, the polymer isomerises to the “off” state. In this case, the rate of substrate transfer through the polymer is reduced and consequently, less substrate is converted to product. A photoresponsive copolymer was formed from acrylamide, \( N,N' \)-methylenebis(acrylamide) and 4-(methacryloylamino)azobenzene in the presence of the enzyme, \( \alpha \)-chymotrypsin.\textsuperscript{33a} During this formation process, the enzyme was immobilised in the copolymer. Upon irradiation of the \((E)\) state of the copolymer/enzyme assembly (exclusively \((E)\)-1.28 by UV spectroscopy) with light filtered to wavelengths from 330 to 370 nm, the \((Z)\) state was formed (1:1 \((E)\)-1.28 / \((Z)\)-1.28 by UV spectroscopy). The copolymer/enzyme assembly was irradiated with visible light to restore \((E)\)-1.28.

![Figure 1.9](image)

**Figure 1.9**  Photochromic behaviour of an azobenzene copolymer

The rate of hydrolysis of a substrate (\( N\)-(3-carboxypropionyl)-L-phenylalanine \( p\)-nitroanilide) by the copolymer/\( \alpha \)-chymotrypsin assembly was measured. In the \((E)\) state the substrate was not hydrolysed to measurable levels. In the \((Z)\) state the substrate was hydrolysed with a rate corresponding to \( V_{\text{max}} = 2 \mu \text{Mmin}^{-1} \). Further studies demonstrated that the copolymer permeability had changed on photoswitching. In the “on” \((Z)\) state the copolymer was permeable to substrate and in the “off” \((E)\) state the copolymer’s permeability to substrate was reduced.
1.5.2 Photochromic moieties covalently attached to enzyme

Photoregulation of enzymes has been achieved by covalently attaching photochromic molecules to the enzymes. In most cases, carboxyl derivatives of photochromic moieties have been attached to lysine side chains of the protein. This approach was used in modifying: α-chymotrypsin with azobenzene units;\textsuperscript{33e} α-chymotrypsin with thiophenefulgide units;\textsuperscript{45a} α-chymotrypsin with nitrospiropyran units;\textsuperscript{46} papain with azobenzene units;\textsuperscript{47} and concanavalin A with thiophenefulgide units.\textsuperscript{48} In each of these cases, the photochromic groups were attached at random to the protein. Although the loading degree of the photoswitch into the protein was predetermined by the reaction conditions, there was always a mixture of proteins with the photochromic groups being attached at different sites. Therefore, this style of covalent modification is described as non-specific.

![Diagram of photoregulation](image)

**Figure 1.10** Photoregulation of an enzyme loaded with photochromic groups.

In **Figure 1.10**, a model is provided for the mechanism of action of this type of photoswitchable system. When the photoisomerisable units are in the “on” state, the tertiary (3D) structure of the enzyme allows for substrate binding in the active site. Substrate is able to be converted to product by the enzyme. On photoswitching to the “off” state, the tertiary structure of the enzyme alters, modifying the shape of the active site. Substrate binding affinity is reduced and less product is formed.
Non-specific covalent modification of enzymes does have its limitations. If the loading of the photochromic agent is too low, there will not be sufficient structural perturbation of the enzyme to affect the activity of the enzyme. Alternatively, if the loading is too high, then the enzyme will be deactivated for both states of the photochromic agent. Because a sample of enzymes loaded with photochrome contains a mixture of differently modified biomolecules, it is difficult to correlate specific molecular interactions with macroscopic properties such as enzyme activity. It is difficult to predesign photoswitchable assemblies with pre-programmed "on" and "off" functions using non-specific modification.

Efforts have been directed at specific covalent modification of enzymes. A potential advantage of this approach is a programmed targeting of the active site. In one example, a photochromic agent was directed to a specific site in the enzyme by including an unnatural, azobenzene-containing amino acid in a peptide. An N-terminal peptide was cleaved from ribonuclease by the action of subtilisin. The resulting S-peptide was able to be non-covalently recombined with the S-protein to restore the activity of ribonuclease. Versions of the S-peptide were chemically synthesised incorporating the photochromic amino acid (4-PAP) in different positions. It was found that (Z)-4-PAP-peptide recombined with S-protein gave slightly higher activity than the native enzyme. The (E)-4-PAP-peptide and S-protein assembly exhibited 25% less activity. These states were photoswitchable.

![Figure 1.11](E)-4-phenylazophenylalanine, (E)-4-PAP

**1.5.3 Effector molecules**

Enzyme activity can be modified by low molecular weight compounds. Examples of effector molecules include inhibitors that deactivate an enzyme and
cofactors that are required for the activity of certain enzymes. These effector molecules can be tethered to photochromic groups to enable photoregulation. Figure 1.12 outlines a system involving a photoisomerisable inhibitor. In the “off” state, the photochromic inhibitor binds with and inactivates the enzyme. As in all examples explained thus far, the “off” state refers to the activity of the enzyme. When the effector molecule is switched to the “on” state, it does not bind to the enzyme. As a result the enzyme is active and its activity is “on”.

![Figure 1.12](image)

**Figure 1.12** A photoisomerisable inhibitor is in the “off” state when the bioactivity is off and in the “on” state when the enzyme is active.

A photochromic cofactor was found to influence the oxidation of glucose by glucose oxidase (GOx). GOx requires an flavin adenine dinucleotide (FAD) cofactor and an electron acceptor, typically oxygen, for the oxidation of glucose. A spiropyran derivative was attached to the FAD cofactor (Scheme 1.11) and the resulting semisynthetic photoisomerisable cofactor was combined with apoenzyme to give photoswitchable GOx. The activity of (spiropyran-FAD, GOx) and (merocyanine-FAD, GOx) was measured with different electron acceptors. It was concluded that the state of photochromic unit strongly influenced the penetration path of the electron acceptor to the FAD redox site. As a result the activity of the enzyme was affected by the switching of the cofactor.
Scheme 1.11 A semisynthetic cofactor switches between spiropyran and merocyanine forms

Photoisomerisable inhibitors of enzymes were investigated with a view to modelling light-regulated processes in nature. Various protease and esterase enzymes were targeted by biologically active azobenzene derivatives. Acetylcholineesterase was the target for the inhibitor systems 1.32 and 1.33.[]

Figure 1.13 (E)- and (Z)-N-4-(phenylazo)phenyltrimethylammonium chloride 1.32 and (E)- and (Z)-N-4-(phenylazo)phenylcarbamoylcholine iodide 1.33.

A more recent example employed azobenzene derivatives to inhibit cysteine and serine proteases.[] Through this research the authors hoped to devise a system for the purification of enzymes by affinity chromatography. The boronic acids (E)-1.34 and (Z)-1.34 were interconvertible with light (Scheme 1.12). Irradiation of a solution of 1.34 with light filtered to wavelengths between 330 and 370 nm gave a photostationary state comprising 80% (Z)-1.34 by HPLC. Complete reconversion to (E)-1.34 was
reported by HPLC after irradiation with visible light (wavelengths over 400 nm). The (E) rich PSS was found to competitively inhibit the serine protease α-chymotrypsin (K_i = 11 μM). The (Z) rich PSS mixture was a less effective inhibitor of α-chymotrypsin (K_i = 41 μM).

Scheme 1.12 Boronic acid (E)-1.34 was a stronger inhibitor of α-chymotrypsin than its isomer (Z)-1.34

Although a 2-3 fold difference in enzyme activity between the two equilibrium mixtures was anticipated, attempts at making an in situ reversible photoswitchable inhibitor/enzyme system yielded disappointing results. The authors stated that potassium chloride and bovine serum albumin were added to facilitate inhibitor solubility and to maintain enzyme activity over the course of the experiment. These changes in the enzyme assay led to a ten-fold increase in the K_i values. Although cyclic photoswitchable behaviour was observed, only a minor increase in relative enzyme activity (1.13) was measured.
1.6 Inhibitors of Chymotrypsin

The structure of an enzyme is described in terms of its primary, secondary and tertiary structures. The primary structure of an enzyme alludes to the linear sequence of amino acids that make up the protein. This primary sequence leads to regular patterns, referred to as the secondary structure. Finally the global three-dimensional structure of the protein, the tertiary structure, is determined by the primary and consequently the secondary structures. Factors such as the type of solvent, solvent pH and temperature all influence the global structure of a protein. Structural features of a protein may include binding sites for metal ions or organic molecules. In the case of enzymes, a pertinent structural feature is the active site, in which the catalysed transformation takes place. Most enzymes have binding sites associated with the active site that involve specific interactions with the substrate.

Enzymes are defined and named in terms of the reactions that they catalyse. A proteolytic enzyme (protease) cleaves peptide bonds of proteins, an esterase cleaves carboxylic esters and an oxidase catalyses redox processes. The proteases are classified according to the amino acid or metal cofactor primarily associated with the catalytic process of peptide bond lysis. For instance, the serine proteases possess the activated hydroxyl group of a serine residue that acts as a nucleophile to cleave the scissile amide bond. A well-studied example of the serine proteases is α-chymotrypsin and this enzyme is the target of the photoswitchable inhibitors prepared in this thesis.

1.6.1 Mechanism of α-chymotrypsin

Much information has been gathered on the mechanism of action of α-chymotrypsin. The enzyme-catalysed hydrolysis occurs in three main steps. Substrate binds with the enzyme (E) at a diffusion-controlled rate in the first step to give a non-covalent, enzyme-substrate complex (E•S). An activated alcohol group within the active site (Ser-195) reacts with the carbonyl of the scissile bond to form the tetrahedral acyl-enzyme intermediate (E-Ac) and the free amine (P₁) that is released from the active
site. The acylation step is followed by nucleophilic attack by a water molecule on the
acyl-enzyme to give free enzyme and carboxylic acid ($P_2$). This two-intermediate
mechanism is compatible with Michaelis-Menten kinetics, although it is more complex
than the one-intermediate case discussed in Appendix 1. The form of the Michaelis-
Menten equation is the same in both cases.

\[
E + S \xleftrightarrow{\text{binding}} E \cdot S \xrightarrow{\text{acylation}} E - Ac + P_1 \xrightarrow{\text{deacylation}} E + P_2
\]

Equation 1.1 Hydrolytic cleavage of substrate (S) catalysed by enzyme (E) to give
products ($P_1$ and $P_2$).

In examining the mechanism of amide hydrolysis catalysed by $\alpha$-chymotrypsin,
answers were sought to explain how the amide cleavage was facilitated by the enzyme.
These answers proved elusive, until crystallographic structures of enzymes bound with
inhibitors were able to be solved.$^{50c}$ With direct information on the tertiary structure of
the enzyme, especially the active site and the associated binding sites, the wealth of pre-
existing kinetic data was able to be fitted to well-informed mechanisms. From this work
the principal explanations for catalytic action were: the stabilisation of the tetrahedral
intermediate by the enzyme; the proximity and optimal orientation of the serine
nucleophile and the substrate carbonyl; and the enhanced nucleophilicity of Ser-195.

Pauling presented enzymologists with a theory that the catalytic activity of
enzymes resulted from preferential binding of the activated transition-state complex of a
substrate.$^{52}$ He posited that there is “...an active region on the surface of the enzyme
which is closely complementary in structure not to the substrate itself, in its normal
configuration, but rather to the substrate molecule in a strained configuration,
corresponding to the activated complex for the reaction catalysed by the enzyme.”. Over
fifty years since this original assertion, the theory of enzymic transition-state
stabilisation still has relevance. Various structural aspects of the $\alpha$-chymotrypsin active
site have been identified through X-ray crystallography as providing stabilisation for the
proposed tetrahedral acyl-enzyme intermediate. For instance, the oxyanion binding
site, \(^{50b}\) a structural feature of the active site, comprises two \(-\text{NH}–\) groups from residues 193 and 195 of the enzyme, which are appropriately positioned to form hydrogen bonds with the carbonyl oxygen of the scissile bond.

![Image of chemical structures](image)

**Figure 1.14** Proposed mechanisms for the interaction of the catalytic triad in serine proteases

The activated serine residue (Ser-195) lies in close proximity to a histidine (His-57) and an aspartate (Asp-102) residue. \(^{53}\) These three amino acids, known as the catalytic triad, are highly conserved in the serine proteases. Various theories have been put forward to explain how the catalytic triad activates Ser-195 to enhance substrate turnover. \(^{54}\) An early theory from Blow et al. was that a charge relay system operated during catalysis. By this theory the negative charge of the carboxylate of Asp-102 was
transferred to Ser-195 to form an alkoxide ion (Scheme 1.14: 1.35 → 1.36). This mechanism was discredited on several grounds, including the unlikelihood of His-57 (pKₐ = 7.5) abstracting a proton from Ser-195 (pKₐ = 14),⁵⁴c and lead to reformulation of the charge relay concept. A second model (Scheme 1.14: 1.37 → 1.38), in which the serine hydroxyl attacked the substrate in concert with the transfer of two protons, gained wide acceptance. In spite of this general acceptance, there were subsequent experimental results from ¹H NMR, ¹⁵N NMR and neutron diffraction studies, that could not be explained by the second model. Another model that was put forward to explain the function of the Asp-His-Ser triad in catalysis (Scheme 1.14: 1.39 → 1.40), posited several roles for Asp-102. It was claimed that Asp-102 enabled His-57 to act as a general base catalyst by: (1) stabilising the required tautomeric form of His-57; (2) correctly orienting the imidazole ring for the acceptance of the serine proton; (3) providing electrostatic stabilisation for the developing imidazolium cation. A recent theory for the action of the His-Asp diad is that, on substrate binding, a small conformational change in the protein causes steric strain between His-57 and Asp-102.⁵⁴ This strain is relieved by the abstraction of a proton from the hydroxyl of Ser-195 and concomitant formation of a low-barrier hydrogen bond between His-57 and Asp-102. The mechanism of the catalytic triad is still in dispute and continuing research aims to establish a reliable model for serine protease catalysis.

1.6.2 The substrate specificity of α-chymotrypsin

Polypeptide binding sites within a protease serve to selectively bind with certain peptides and to orient the substrate for optimum proteolysis. In discussing the binding sites of a protease, the nomenclature of Schechter and Berger (Figure 1.15) has proven useful.⁵⁵ By convention the peptide is drawn with the amino terminus on the left. Residue side chains of the substrate or substrate analogue on the amino terminal side of the scissile bond are denoted P₁, P₂...Pₙ and these groups bind with varying specificity into binding pockets (or subsites) of the protease S₁, S₂...Sₙ. On the carboxyl side of the scissile bond side chains P₁', P₂'...Pₙ' find themselves in pockets S₁', S₂'...Sₙ'.

Figure 1.15 A peptidic substrate of α-chymotrypsin lying in the active site has its amino acid side chains bound in the specific enzyme binding sites. The side chains of the substrate are denoted $P_n$ or $P_n'$ and sit in binding pockets denoted $S_n$ or $S_n'$. The amide bond that is cleaved by the enzyme is indicated by an arrow.

The general substrate specificity of serine proteases was established with the aid of crystallographic studies of natural peptide inhibitors bound with the enzymes. Measuring the efficacy of inhibition of these inhibitors combined with analysis of the crystal data provided information on the shape and polarity of $S_n$ and $S_n'$ sites of serine proteases. Hydrolysis studies gave information on the $P_n/S_n$ specificity of α-chymotrypsin. The leaving group specificity ($P_n'/S_n'$) of α-chymotrypsin was established through acyl transfer studies and through enzyme kinetic studies with fluoromethylketone inhibitors. The acyl transfer results showed that $S'$ specificity was considerably enhanced with at least five residues on the carboxyl side of the scissile bond.

As a result of these studies, the specificity of α-chymotrypsin is well understood. It is known that the primary specificity pocket is $S_1$. This site requires that
P₁ be a large aromatic group. Consequently, the primary specificity of α-chymotrypsin is to cleave peptides on the carboxyl side of a phenylalanine, tyrosine or tryptophan residue. Other substrate-subsite interactions have little effect on binding and catalysis. Because of the singular importance of P₁ to substrate specificity, the fit in the S₁ subsite of α-chymotrypsin is crucial for orienting the substrate in the active site. The types of compound for which α-chymotrypsin is considered to be most specific are presented in Table 1.1.

<table>
<thead>
<tr>
<th>S₂ specificity (dipeptides)</th>
<th>Large side chains e.g. Leu</th>
<th>L-aromatic e.g. Tyr, Phe</th>
<th>Esters e.g. OAr, OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁′ specificity (dipeptides)</td>
<td>L-aromatic e.g. Tyr, Phe</td>
<td>Basic side chains e.g. Arg, Lys</td>
<td></td>
</tr>
<tr>
<td>S₀′ specificity (tetrapeptides)</td>
<td>L-aromatic e.g. Tyr, Phe</td>
<td>Small hydrophilic Side chains e.g. Ser, Gly</td>
<td>Hydrophobic side chains e.g. Leu, Ala</td>
</tr>
</tbody>
</table>

**Table 1.1** Substrate specificity of α-chymotrypsin

**1.6.3 Electrophilic carbonyl derivatives as inhibitors of serine proteases**

The design of inhibitors of serine proteases has been heavily influenced by enzymic transition-state stabilisation theory. The premise being that molecules, which mimic the transition-state to the tetrahedral intermediate, will be tightly bound by the enzyme. If these mimetic molecules are unable to undergo normal catalytic turnover while in the active site, the enzyme will be inactivated. The tetrahedral intermediate is a common structural model for inhibitors of serine proteases. In Figure 1.16, examples of tetrahedral transition-state analogues and proposed adducts with the enzyme are presented. Aldehydes and trifluoromethylketones are reversible inhibitors of serine proteases. A tightly bound tetrahedral adduct is formed between the compound and the protease. When the R-group of these inhibitors is an amino acid or an oligopeptide the
inhibitors can be targeted to a specific serine protease. Although peptidyl aldehydes and trifluoromethylketones exist in aqueous solution predominantly as hydrates, experimental evidence suggests that the compounds lose water to bind to the active site as carbonyls. The compounds are then transformed to the respective hemiacetals and hemiketals by the nucleophilic attack of Ser-195.

![Tetrahedral transition state analogues as inhibitors of serine proteases.](image)

"Tight-binding" inhibition is observed with some electrophilic carbonyl inhibitors of serine proteases. This delayed onset of full inhibition occurs with certain peptidyl aldehydes, peptidyl fluoromethylketones, and peptidyl α-ketoesters. It is theorised that the "tight-binding" inhibition resulted from a pre-binding equilibrium involving dehydration of the hydrated electrophilic carbonyl moiety. There is considerable evidence from the TFMK inhibitors to support this theory.
Another class of electrophilic carbonyl inhibitor is the peptidyl-α-ketoicarboxylic acid derivatives. In Table 1.2, the inhibition constants ($K_i$) for the inhibition of α-chymotrypsin by various compounds are presented. The α-ketoesters $1.41$ and $1.42$ are more effective inhibitors of α-chymotrypsin than the corresponding peptidyl aldehyde $1.53$ and peptidyl trifluoromethylketones $1.54$ and $1.55$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (μM)</th>
<th>Compound</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /> 1.41</td>
<td>0.13</td>
<td><img src="image2.png" alt="Image" /> 1.43</td>
<td>360</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /> 1.42</td>
<td>0.06</td>
<td><img src="image4.png" alt="Image" /> 1.44</td>
<td>54</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /> 1.45</td>
<td>2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 The apparent dissociation constants ($K_i$) of peptidyl-α-ketoesters, aldehyde and trifluoromethylketones for the inhibition of α-chymotrypsin. <sup>a</sup> "Tight-binding" inhibition.

Although evidence for the binding mode of α-ketoesters with the active site of serine proteases is more equivocal than for aldehydes and trifluoromethylketones, it is widely assumed that each of these types of compound act in the same manner. As
shown in Figure 1.17, the hydrate of an \( \alpha \)-ketoester 1.46 loses water to give the 1.47 before binding to the enzyme active site. The proposed binding interactions of the \( \alpha \)-ketoester 1.49 include an interaction with the oxyanion binding site and a hydrogen bond between His-57 and the ester carbonyl oxygen. The brackets around the hydrogen of the hemiketal 1.49 indicate that the ionisation state of the hemiketal is unknown. It is known, however, from NMR and pH-dependency studies that trifluoromethylketones form hemiketal adducts with \( \alpha \)-chymotrypsin (pK\(_a\) = 4.0) that are fully ionised at the pH of most kinetic experiments (ca. 7.5).\(^60\) An alternative binding mode that was suggested for \( \alpha \)-ketoesters, evidenced by preliminary X-ray crystallographic studies, was transesterification by the activated Ser-195 to give a stable \( \alpha \)-ketoacylenzyme.\(^63\)

![Figure 1.17 Proposed binding mode of \( \alpha \)-ketoesters with serine proteases.](image.png)

To conclude, derivatives of \( \alpha \)-ketocarboxylic acids are effective reversible inhibitors of \( \alpha \)-chymotrypsin. There are several synthetic routes available for these types of compound.\(^64\) One disadvantage implicit with the peptidyl-\( \alpha \)-ketoester inhibitors of serine proteases is the instability of the ester group to metabolic and hydrolytic degradation.\(^60\) Peptidyl-\( \alpha \)-ketoamides are considerably more stable with respect to
hydrolysis. The hydrolysis of an α-ketoacid derivative would be problematic because the α-ketoacids tend to be considerably less active than the corresponding acid derivatives.

1.7 Work described in this thesis

We have developed photobiological switches that target α-chymotrypsin. The switches incorporate azobenzene as the photochromic group and α-ketoester or α-ketoamide as the inhibitory moiety. A novel component of these switches is the use of peptidyl groups to enhance the specificity of α-chymotrypsin toward these compounds. Thus, there are three fundamental components to these photobiological switches; the specificity region, the switch and the inhibitory moiety. The design of target molecules in this thesis results from permutations of these three elements (Table 1.3).

<table>
<thead>
<tr>
<th>Order of elements in target compounds</th>
<th>Examples of target compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type A</strong></td>
<td><img src="image" alt="Compound" /></td>
</tr>
<tr>
<td>PEPTIDYL</td>
<td>(E)-2.1</td>
</tr>
<tr>
<td>AZOBENZENE</td>
<td></td>
</tr>
<tr>
<td>α-KETO AMIDE</td>
<td></td>
</tr>
<tr>
<td><strong>Type B</strong></td>
<td><img src="image" alt="Compound" /></td>
</tr>
<tr>
<td>AZOBENZENE</td>
<td>(E)-3.1</td>
</tr>
<tr>
<td>PEPTIDYL</td>
<td></td>
</tr>
<tr>
<td>α-KETO ESTER</td>
<td></td>
</tr>
<tr>
<td><strong>Type C</strong></td>
<td><img src="image" alt="Compound" /></td>
</tr>
<tr>
<td>AZOBENZENE</td>
<td>(E)-4.2</td>
</tr>
<tr>
<td>α-KETO AMIDE</td>
<td></td>
</tr>
<tr>
<td>PEPTIDYL</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3  Design of the target compounds in this thesis
The syntheses of compounds of the type A is discussed in Chapter 2, compounds of the type B in Chapter 3 and compounds of the type C in Chapter 4. In Chapter 5 the results from the (E)\rightleftharpoons(Z) isomerisation studies of the target compounds are presented. The photostationary states of each compound at 330<\lambda<370 nm and \lambda>400 nm have been measured. In addition, evidence of hydrate formation is discussed for each class of target compound.

The enzyme kinetic analyses of both photostationary states of each target compound are presented in Chapter 6. The kinetic data for bioactive target compounds have been analysed with graphical techniques to yield kinetic parameters for each photostationary state. The type of inhibition for the active compounds was determined.
1.8 References for Chapter One


Chapter One: Introduction


53 Tsukada, H.; Blow, D. M. J. Mol. Biol. 1985, 184, 703.


59 Imperiali, B.; Abeles, R. H. Biochem. 1987, 26, 4474.


CHAPTER TWO

THE SYNTHESIS OF COMPOUNDS
OF THE TYPE A
2.1 Introduction: Design of the Target Compounds

The compounds 2.1 to 2.4 are compounds of the type A and are designed to be photoswitchable inhibitors of \( \alpha \)-chymotrypsin (Figure 2.1). As discussed in Section 1.7, compounds of the type A comprise three main components, which read from the N-terminus: peptidyl (enzyme specificity), \( \alpha \)-ketoamide (enzyme inhibition), azobenzene (photoswitch). The peptidyl components of these target compounds are designed to interact with the S-subsites of \( \alpha \)-chymotrypsin. The side chain of \( L \)-phenylalanine in each of the target compounds, being on the N-terminal side of the electrophilic, amide bond mimic, corresponds with the \( P_1 \) site of an \( \alpha \)-chymotrypsin substrate. For the target compounds, 2.3 and 2.4 the side chain of \( L \)-leucine corresponds with the \( P_2 \) site of an \( \alpha \)-chymotrypsin substrate.

\[
\text{Ph} \quad \text{Cbz-N} \quad \text{O} \quad \text{N} \quad \text{N} \quad \text{Ph,} \\
\text{Cbz-N} \quad \text{O} \quad \text{N} \quad \text{N} \quad \text{Ph,} \\
\text{Cbz-Leu-N} \quad \text{O} \quad \text{N} \quad \text{N} \quad \text{Ph} \quad \text{Cbz-Leu-N} \quad \text{O} \quad \text{N} \quad \text{N} \quad \text{Ph}
\]

\((E)-2.1 \quad (E)-2.2 \quad (E)-2.3 \quad (E)-2.4\)

**Figure 2.1** Target compounds of the Type A

The target compounds in Figure 2.1 represent two types of compound, the aryl amides (2.1, 2.3) and alkyl amides (2.2, 2.4), which are distinct in that they have different substituents on azobenzene. The absorption characteristics, the \((E)/(Z)\) isomerisation and the rate of \((Z) \rightarrow (E)\) thermal conversion of azobenzene derivatives are dependent on the nature of the substitution of the azobenzene moiety.\(^1\)
Consequently, different PSS compositions at a specific wavelength and different rates of thermal reconversion would be expected for, say, compounds 2.1 and 2.2. Furthermore, the alkyl amides are expected to have more conformational freedom about the amide-azo benzene portion of the molecule than the aryl amides. Thus, 2.1 would adopt different conformations in solution than 2.2 and it would interact with the active site of an enzyme in a different way from 2.2.

Peptidyl-α-ketoamides are often prepared from the amidation of α-hydroxyacids, followed by oxidation. The retrosynthetic scheme (Scheme 2.1) shows that the α-hydroxyesters 2.5 and 2.44 play a pivotal role in the synthesis of the peptidyl-α-ketoamides. In Section 2.2, several routes to 2.5 (R=Cbz) are discussed and evaluated. A recent innovation in the synthesis of peptidyl-α-ketoamides is the oxidation of a cyanoketophosphorane and subsequent trapping of the intermediate with an amine (Wasserman route). We decided to pursue the former option in the synthesis of compounds of the type A following preliminary investigations of the Wasserman route that gave poor results (see Section 2.4).

Scheme 2.1 Retrosynthetic analysis of compounds of the type A to the key intermediates 2.5 and 2.44
2.2 Synthesis of the key intermediates 2.5a and 2.5b

Because of the pivotal importance of 2.5 in the stereospecific synthesis of compounds of the type A, three synthetic routes to 2.5 were investigated. In the first route the methodology of Wasserman et al. was used to provide a simple and effective synthesis, which is shown in Scheme 2.2.3a

\[
\begin{align*}
\text{Cl} & \quad \begin{array}{c}
\text{Cl} \\
\text{CN}
\end{array} & \quad \begin{array}{c}
\text{PPh}_3 \\
\text{CN}
\end{array} & \quad \begin{array}{c}
PPh_3 \\
\text{CN}
\end{array} \\
2.6 & \quad 2.7 & \quad 2.8
\end{align*}
\]

\[
\begin{align*}
\text{Cbz-} & \quad \begin{array}{c}
\text{OH} \\
\text{Ph}
\end{array} & \quad \begin{array}{c}
\text{PPh}_3 \\
\text{CN}
\end{array} \\
2.9 & \quad 2.10
\end{align*}
\]

\[
\begin{align*}
\text{Cbz-} & \quad \begin{array}{c}
\text{O} \\
\text{Me}
\end{array} & \quad \begin{array}{c}
\text{Ph} \\
\text{O}
\end{array} \\
2.11 & \quad 2.5a & \quad 2.5b
\end{align*}
\]

Scheme 2.2 Synthesis of 2.5a and 2.5b by Wasserman methodology

(i) PPh₃, MeNO₂, reflux, 5 h, 73%; (ii) 10% NaOH, 0 °C, 97%; (iii) EDCI, DMAP, 2.8, CH₂Cl₂, 20 h, 78%; (iv) O₃, MeOH, CH₂Cl₂, -78 °C, 10 min, 77%; (v) NaBH₄, MeOH, 20 min, 2.5a 27%, 2.5b 24%. 
Cbz-L-phenylalanine 2.9 was coupled with the cyanophosphorane 2.8 to afford the ketocyanophosphorane 2.10 in 78% yield. Compounds such as 2.10 are thought to form α,β-diketonitrile intermediates when treated with ozone at reduced temperature.\(^{3a}\) These intermediates are susceptible to attack by a nucleophile with displacement of a cyanide ion. Thus, when 2.10 was treated with ozone at \(-78\) °C in the presence of methanol, the α-ketoester 2.11 was formed (77%). The key intermediates 2.5a and 2.5b were isolated after chromatography in 27% and 24% yield, respectively, from the reduction of 2.11 with sodium borohydride. The absolute configurations of diastereomers 2.5a and 2.5b were assigned by comparison of \(^1\)H NMR and optical rotation data with that of the equivalent literature compounds.\(^{Error! Bookmark not defined.}\)

Most of the published routes to 2.5 use the cyanohydrin 2.14 as a key intermediate.\(^4\) In a representative route (Scheme 2.3), Cbz-L-phenylalanine 2.9 is transformed to the corresponding aldehyde 2.13 with retention of configuration at the α-carbon. The aldehyde is treated with cyanide to form the cyanohydrin 2.14, which is hydrolysed in the presence of methanol to form the methyl-α-hydroxyester 2.5.

![Scheme 2.3](image)

**Scheme 2.3** A reported synthesis of 2.5 via the cyanohydrin 2.14.\(^4\)

This procedure was found, in our hands, to be low yielding and to suffer from problems associated with the formation of undesired side products. For instance, a crude mixture containing the aldehyde 2.13 was obtained, but was not purified because of literature
reports that the aldehyde was susceptible to racemisation by silica.\textsuperscript{2a} In order to synthesise active inhibitors of \(\alpha\)-chymotrypsin it is imperative that the target compounds of the type A retain the stereochemistry of the original amino acid \textsuperscript{2.9} because \(\alpha\)-chymotrypsin is particularly sensitive to the stereochemistry of the \(P_1\) position.\textsuperscript{5}

A third route to key intermediate \textbf{2.5} was investigated. This approach was inspired by work on the synthesis of the C13 side chain of taxol \textbf{2.19} (\textbf{Scheme 2.4}).\textsuperscript{6} In the key step of this synthesis, the enolate derived from \textbf{2.18} was treated with an electrophilic source of oxygen (\textbf{Scheme 2.4}). Two reagents were employed for this transformation. Hanessian \textit{et al.} used MoOPH\textsuperscript{†} \textbf{2.15} to convert the potassium enolate of \textbf{2.18} to the \(\alpha\)-hydroxyesters \textbf{2.19} in 83% yield. Davis \textit{et al.} found that, in the presence of LiCl (1.6 equiv.), the stereospecific oxidant, (+)-(camphorylsulfonyl)oxaziridine \textbf{2.17}, transformed the lithium enolate of \textbf{2.18} to the product \textbf{2.19} in 53% yield. A route to the key intermediate \textbf{2.5} based on this sequence requires the preparation of the phenylalanine analogue \textbf{2.21}, which would then be treated as per \textbf{2.18} in \textbf{Scheme 2.4}.

\textsuperscript{†} MoOPH = oxodiperoxymolybdenum(pyridine)(hexamethylphosphoric triamide) complex
Compound 2.21, a precursor to the key intermediate 2.5, was prepared as detailed in Scheme 2.5. The mixed anhydride of Cbz-L-phenylalanine was formed and was subsequently treated with diazomethane to give the diazoketone 2.20 in 79% yield. In the presence of silver benzoate and methanol, 2.20 underwent a Wolff rearrangement to give the β-amino acid 2.21 in 64% yield. The Wolff rearrangement is known to occur with retention of configuration at C3.10 Methyl-N-(benzylxycarbonyl)-(3S)-3-amino-4-phenylbutanoate 2.21 is set up for enolate hydroxylation to form 2.5. The reagent developed by Davis 2.16 was explored as an electrophilic source of oxygen in this conversion. The reaction did not give the desired product. Under strictly anhydrous conditions, 2.21 was treated with potassium hexamethyldisilazide (KHMDS, 6 equiv., -78 °C to -25 °C, 1 h) to give the potassium enolate and (±)-trans-2-(phenylsulfonyl)-3-phenyloxaziridinone 2.16 (3 equiv., -70 °C, 3 h) was added to the reaction mixture. After workup the crude reaction mixture comprised only the returned starting materials 2.16 and 2.21 by 1H NMR.
Scheme 2.5 Synthesis of homophenylalanine 2.21 as a precursor to 2.5
(i) NEt₃, EtOCOCl, THF, ether, -5 °C, 30 min, then CH₂N₂, ether, 0 °C, 1 h, then 4 °C, 3 d, 79%; (ii) MeOH, AgOCOPh, THF, 90 min, 64%.

The preparation of key intermediate 2.5 was next attempted with the MoOPH complex as presented by May et al., who achieved this conversion in 44% yield (66% by returned starting material).¹¹ The reported results of this conversion, however, in our hands were not reproducible. For example, under strictly anhydrous conditions, 2.21 (112 mg) was treated with potassium hexamethyldisilazide (KHMDS, 6 equiv., -78 °C to -25 °C, 1 h) to give the potassium enolate. To the reaction mixture MoOPH complex 2.15 was added from a pre-assembled addition arm (3 equiv., -70 °C, 2 h). After two hours the reaction was quenched and worked up to give a mixture of 2.21 / 2.5 (85 mg, 4:1 by ¹H NMR).

These investigations on the preparation of 2.5 showed that the first route described above, that derived from the work of Wasserman, was the most efficient and most reproducible of the three considered.
2.3 Synthesis of the key intermediate (E)- and (Z)-4-(phenylazo)benzylamine 2.22

Most of azobenzene derivatives reported in this thesis were synthesised predominantly as the (E) isomer with traces of the (Z) isomer present as observed by $^1$H NMR. When placed in solution, however, and allowed to stand in ambient lighting for a certain time (typically reaching equilibrium within minutes to hours) the azobenzene derivative would come to a photostationary state (PSS) comprising both (E) and (Z) isomers as indicated by $^1$H NMR. In this thesis, the photostationary state that comprises the (Z) isomer arising from exposure to daylight is named the ambient light PSS. The phenomenon is more fully described in Section 5.3 and in the Experimental Section (Chapter 8) data on the PSS has been recorded when possible. Ambient light photostationary states have rarely been reported in the literature.

The amine 2.22 (or its amine salt 2.23) was envisioned as a key intermediate in the synthesis of target compounds 2.1 and 2.3. A review of the literature revealed that the amine had not been prepared previously. In the only reported attempt to prepare the amine 2.22, the corresponding N-acetyl derivative was treated with potassium hydroxide in ethanol to yield a hydrazo compound.

![Figure 2.2](image)

**Figure 2.2** Key intermediate, 4-(phenylazo)benzylamine 2.22, its hydrochloride salt 2.23 and a reported analogue 2.24
In a separate piece of research the synthesis of the N-FMOC protected amino acid 2.24, a compound related to the key intermediate 2.22, was reported.\textsuperscript{15} In this report, the experimental detail on the synthesis of 2.24 was very limited.

In spite of the scant information available about the synthesis of 2.24, the synthesis of the key intermediate 2.22 was based on this route. As shown in Scheme 2.6, the hydrochloride salt 2.25 was reacted with 9-fluorenylmethyl chloroformate to form the Fmoc-protected amine 2.26 in 83% yield. The nitro group of 2.26 was reduced with hydrogen in the presence of Adam’s catalyst (platinum (IV) oxide) to afford the amine 2.27 in 70% yield. Nitrosobenzene and 2.27 were then reacted in glacial acetic acid to form N-Fmoc-4-(phenylazo)benzylamine 2.28 in 50% yield. The Fmoc protecting group was removed by the treatment of 2.28 with tetrabutylammonium fluoride in THF to give the amine 2.28 in 90% yield.

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {2.25};
\node (b) at (1.5,0) {2.26};
\node (c) at (3,0) {2.27};
\node (d) at (0,-1.5) {2.28};
\node (e) at (3,-1.5) {\textit{(E)}-2.22};
\node (f) at (1.5,-1.5) {\textit{(E)}-2.28};
\draw [->] (a) -- (b) node[midway,above] {i};
\draw [->] (b) -- (c) node[midway,above] {ii};
\draw [->] (c) -- (d) node[midway,above] {iii};
\draw [->] (d) -- (f) node[midway,above] {iv};
\end{tikzpicture}
\end{center}

\textbf{Scheme 2.6} Synthesis of key intermediate 2.22.
\begin{itemize}
\item[(i)] 9-fluorenylmethyl chloroformate, DIEA, CH\textsubscript{2}Cl\textsubscript{2}, 2 h, 83%;
\item[(ii)] H\textsubscript{2}, PtO\textsubscript{2}, EtOAc, 16 h, 70%;
\item[(iii)] PhNO\textsubscript{2}, g. AcOH, 48 h, 50%;
\item[(iv)] TBAF, THF, 5 min, 90%.
\end{itemize}

In an earlier route, the free amine 2.22 was prepared in poor yield by the alkaline deprotection of the phthalimide 2.30 (see Scheme 2.7). It was found, however, that a modification of the conditions of the deprotection afforded the corresponding amine salt
2.23 in good yield. As shown in Scheme 2.7, compound 2.23 was prepared in two steps from the alcohol 2.29. The hydroxyl group of 2.29 was substituted with a phthalimido group under Mitsunobu conditions to give N-phthalimido-4-(phenylazo)benzylamine 2.30 in 82% yield.¹⁶

\[ \text{(E)-2.22} \rightarrow \text{(E)-2.23} \]

**Scheme 2.7** Synthesis of key intermediate 4-(phenylazo)benzylamine.HCl 2.23.

(i) DEAD, TPP, phthalimide, THF, 20 h, 82%; (ii) + (iii) aqu. MeNH₂, THF, 4 h, 2.31 (18%), 2.22 (62%); (iv) + (v) aqu. MeNH₂, THF, 5 min, 60 °C, conc. HCl, 5 min, 2.23 (80%).

Attempts to cleave the phthalimido group of 2.30 with an aqueous solution of 40% methylamine met with mixed success (Scheme 2.7, Steps ii + iii).¹⁷ The N-substituted phthalimide 2.30 underwent fast (less than 10 min at rt) cleavage to the ring-opened intermediate 2.31. However further hydrolysis to the desired amine was much slower, requiring long reaction times and giving mixtures of 2.31 and 2.22. Typical yields of 2.22 after flash chromatography were in the range of 30 to 60%.

² The synthesis of 2.29 is presented in this section (Scheme 2.8).
Chapter Two: Synthesis of compounds of the type A

Literature reports on the kinetics of the cleavage of phthalimido groups from nitrogen provided a resolution to this problem.\textsuperscript{18} It was reported that alkaline hydrolysis of unreactive \textit{N}-substituted phthalimides occurred rapidly to the intermediate ($t_{1/2}<1$ s, 0.1 M NaOH, 30 °C), but the second hydrolysis to the free amine was considerably slower under these basic conditions ($t_{1/2} >17000$ h, 0.1 M NaOH, 30 °C). Conversely, the first step of the acid cleavage of unreactive \textit{N}-substituted phthalimides was very slow ($t_{1/2}=13750$ h, 0.1 M HCl, 30 °C) and the second hydrolysis step was much faster ($t_{1/2}=2$ h, 0.1 M HCl, 30 °C). This information was applied to the deprotection of the \textit{N}-substituted phthalimide 2.30 (Scheme 2.7, Steps iv + v). Accordingly, a solution of 2.30 was treated with 40% aqueous methylamine at 60 °C for five minutes followed by the addition of concentrated hydrochloric acid to give an orange precipitate. The reaction mixture was filtered to give the amine salt 2.23 in 80\% yield.

Apart from the use of the alcohol 2.29 as a precursor to the key intermediate 2.23, the alcohol serves as a precursor to target compounds in Section 3.3 and Section 4.2. Compound 2.29 was prepared by the route shown in Scheme 2.8. The synthesis proceeds via the condensation reaction of nitrosobenzene 2.33 and ethyl-4-aminobenzoate 2.35 (Step iii, Scheme 2.8). Nitrosobenzene 2.33 was prepared from nitrobenzene 2.32 in 42\% yield and 2.35 was prepared from the acid 2.34 in good yield. These precursor compounds, 2.33 and 2.35, were set to reflux in glacial acetic acid to afford the azobenzene derivative 2.36 in 69\% yield. Compound 2.36 was then reduced with lithium aluminium hydride to afford 4-(phenylazo)benzylalcohol 2.29 in excellent yield.
Scheme 2.8 Synthesis of 4-(phenylazo)benzylalcohol 2.29.  
(i) Zn, NH₄Cl, water, 15 min, then Na₂Cr₂O₇, conc. H₂SO₄, water, 0 °C, 42%; (ii) HCl, EtOH, reflux, 24 h, 74%; (iii) g. AcOH, reflux, 4 h, 69%; (iv) LiAlH₄, ether, 20 min, 99%.
2.4 Synthesis of target compounds (E)- and (Z)-N-[4-(phenylazo)benzyl]-(3S)-3-[(benzyloxy)carbonyl]amino]-2-oxobenzenebutanamide 2.1 and (E)- and (Z)-N-[4-(phenylazo)phenyl]-(3S)-3-[(benzyloxy)carbonyl]amino]-2-oxobenzenebutanamide 2.2

Although the formation of peptidyl-α-ketoamides from acylcyanophosphoranes offered a simple and effective synthesis of compounds of the type \( \text{A} \),\(^{3}\) the methodology was not reproducible in our model experiment. In this model experiment (Figure 2.3) a solution of the cyanophosphorane 2.10 in dichloromethane was treated with ozone at low temperature to form a diketone reactive intermediate.\(^ {3a} \) Excess benzylamine was then added and the reaction mixture was stirred at reduced temperature for 90 minutes. Unfortunately, none of the desired product 2.37 was evident in the resulting crude product. One component of the reaction mixture was identified as 2.38 by \(^1\)H NMR and HRMS. The result of this model study, combined with the fact that the amine ultimately required for this reaction 2.28 was not soluble under the reaction conditions, led to the investigation of alternative routes to peptidyl-α-ketoamides.

![Figure 2.3](image)

**Figure 2.3** The acylcyanophosphorane 2.10 did not give the desired α-ketoester 2.37.

The synthesis of α-ketoamides directly from α-ketoacids appears to present a more facile route than from α-hydroxyacids, but literature precedent suggests that α-ketoamides should not be formed by the coupling of α-ketoacids with amines. Slee et al. reported an attempt at coupling the α-ketoacid of Cbz-L-phenylalanine with a proline derivative.\(^ {2b} \) A complex mixture resulted from the reaction, including multiple side
products from nucleophilic attack at the electrophilic ketone moiety. Consequently, the \(\alpha\)-ketoamides discussed in this section were prepared from the \(\alpha\)-hydroxyacids 2.39.

\[
\begin{align*}
\text{Cbz} & \text{N} \backslash \text{H} \backslash \text{OH} \quad \text{OR} \\
\text{Ph} & \text{O} \\
\text{R} &= \text{Me} \\
\text{2.39} & \text{R} = \text{H}
\end{align*}
\]

Scheme 2.9 Synthesis of the \(\alpha\)-ketoamides 2.1 and 2.2

(i) LiOH, MeOH, water, 4 h, 99%; (ii) 2.23, EDCI, HOBT, DIEA, DMF, CH\(_2\)Cl\(_2\), 16 h, 61%; (iii) TEMPO, KBr, NaOCl, NaHCO\(_3\), water, CH\(_2\)Cl\(_2\), 0 °C, 10 min, qu; else DMPO, CH\(_2\)Cl\(_2\), 30 min, qu; (iv) 4-(phenylazo)aniline, EDCI, HOBT, DIEA, DMF, CH\(_2\)Cl\(_2\), 16 h, 46%; (v) TEMPO, KBr, NaOCl, NaHCO\(_3\), water, CH\(_2\)Cl\(_2\), 0 °C, 10 min, qu; else DMPO, CH\(_2\)Cl\(_2\), 30 min, qu.

As shown in Scheme 2.9, a mixture (1:1 by \(^1\)H NMR) of the key intermediates 2.5a and 2.5b was hydrolysed with lithium hydroxide to give a mixture (1:1 by \(^1\)H
NMR) of the acids 2.39a and 2.39b (99%). This mixture was then coupled with 4-(phenylazo)benzylamine hydrochloride 2.23 to give a mixture (2:3 by $^1$H NMR) of diastereomers A and B of 2.40 (61%). The acids 2.39a and 2.39b were also coupled with 4-(phenylazo)aniline to give a mixture (1:1 by $^1$H NMR) of diastereomers A and B of 2.41 (46%).

Specific and mild oxidants such as Dess-Martin periodinane, $^{19}$ TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl)$^{20}$ and oxaly chloride/dimethylsulfoxide (Swern conditions)$^{21}$ are available for the conversion of secondary alcohols to ketones. There are examples of each of these reagent systems being employed for the oxidation of peptidyl-$\alpha$-hydroxyamides. Swern conditions were reported to give poor yields of $\alpha$-ketoamides and $\alpha$-ketoesters.$^{22}$ Oxidations with TEMPO$^{2a}$ and periodinane$^{22}$ were both reported to proceed in good yield with excellent retention of chiral integrity.

It was decided to use both periodinane and TEMPO in the syntheses of target compound 2.1 and to compare the optical rotation measurements of the products obtained from the different reagents. The $\alpha$-hydroxyamide 2.40 was oxidised with TEMPO to give the ketone 2.1 in excellent yield ([α]$_D$ = + 4±2 °, c 0.23 acetonitrile). When 2.40 was oxidised with periodinane to afford 2.1 quantitatively the optical rotation of the product was measured at ([α]$_D$ = + 5±5 °, c 0.10 acetonitrile). The TEMPO oxidation was also used in the synthesis of 2.4, a chain-extended example of 2.2, which has two stereocentres (Scheme 2.13, Section 2.6). In this synthesis, the $\alpha$-ketoamide 2.4 was prepared as a single diastereomer by $^{13}$C NMR from the oxidation of the corresponding $\alpha$-hydroxyamide with TEMPO. Consequently, it is apparent that each reagent (TEMPO and periodinane) is equally efficacious at avoiding racemisation of the ketone.

As shown in Scheme 2.9, the $\alpha$-hydroxyamide 2.41 was oxidised with TEMPO to give the ketone 2.2 in excellent yield ([α]$_D$ = + 9±3 °, c 0.35 acetonitrile).
2.5 Extension by one amino acid: the synthesis of (2S,3S)-3-[[N-Cbz-L-leucinyl]amino]-2-hydroxybenzenebutanoic acid 2.46b

The single diastereomer 2.5b was extended by one amino acid en route to the target compounds 2.3 and 2.4. The first part of this synthesis required the preparation of (2S,3S)-3-[[N-Cbz-L-leucinyl]amino]-2-hydroxybenzenebutanoic acid 2.46b (Scheme 2.10).

![Scheme 2.10](image)

Scheme 2.10 Synthesis of 2.46b from key intermediate 2.5b
(i) HBr, AcOH, 20 min, mixture (12:1 by $^1$H NMR) of 2.42b (86%) and 2.43b (6%); (ii) 2.42b/2.43b (9:1 by $^1$H NMR), N-Cbz-L-leucine, EDCI, HOBT, DIEA, DMF, CH$_2$Cl$_2$, 16 h, gave 2.44b/2.45b (9:1 by $^1$H NMR); (iii) 2.44b/2.45b (9:1 by $^1$H NMR), LiOH, MeOH, water, 4 h, gave exclusively 2.46b, 69% over 2 steps.

Hydrogen bromide in acetic acid (33% v/v) has been reported as an effective agent for the removal of benzylloxycarbonyl (Cbz) groups. When compound 2.5b was treated with this reagent a mixture of the alcohol 2.42b and its acetoxy derivative 2.43b

\[ \text{Absolute configuration of 2.5b assigned by comparison of } ^1\text{H NMR and optical rotation data with literature compounds.} \]
was formed. On varying the conditions of this transformation, it became apparent that the ratio of products $2.42b/2.43b$, as determined by $^1H$ NMR, was dependent on the concentration of starting material (see results in Table 2.2). From these results, it is clearly apparent that an increased concentration of starting material leads to decreased acetoxy derivative $2.43b$ relative to the alcohol $2.42b$. The presence of the acetoxy derivative was not troublesome, because the lithium hydroxide-mediated methyl ester cleavage two steps along in the synthesis acted to cleave the acetate as well, resulting in a single product ($2.46$). Thus, the free acid, $N$-Cbz-$L$-leucine was coupled with a mixture (9:1 by $^1H$ NMR) of the amine salts $2.42b/2.43b$ by EDCI coupling methodology to give a mixture (9:1 by $^1H$ NMR) of $2.44b/2.45b$ (Step ii, Scheme 2.10). This mixture was treated with lithium hydroxide to afford the free acid $2.46b$ as a single diastereomer, by $^{13}C$ NMR, in 69% yield over two steps.

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>Mass SM (mg)</th>
<th>$2.42b/2.43b$ (by $^1H$ NMR)</th>
<th>Mass P (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.26</td>
<td>14</td>
<td>4:1</td>
<td>3</td>
</tr>
<tr>
<td>0.97</td>
<td>100</td>
<td>9:1</td>
<td>65</td>
</tr>
<tr>
<td>1.47</td>
<td>500</td>
<td>12:1</td>
<td>397</td>
</tr>
<tr>
<td>2.00</td>
<td>206</td>
<td>19:1</td>
<td>117</td>
</tr>
</tbody>
</table>

Table 2.2 Concentration dependence of O-acetate formation in the Cbz-deprotection of $2.5b$.

At this point, it is worth noting that the Cbz-protected compound $2.5a$ (diastereomeric to $2.5b$ at C2) behaved differently to $2.5b$ when reacted with hydrogen bromide (33% v/v) in acetic acid (Section 3.2). At various concentrations of $2.5a$ in the reagent, the only product was the desired alcohol. There was no acetate formation observed in this case.
2.6 Synthesis of the target dipeptides 2.3 and 2.4

![Chemical structures of (E)-2.3 and (E)-2.4]

The target compound 2.3 was prepared from the acid 2.46b as shown in Scheme 2.11. The amine salt 2.23 was coupled with the acid 2.46 using EDCI methodology to afford the α-hydroxyamide 2.47b in 60% yield. The TEMPO reagent was employed in the quantitative oxidation of the alcohol 2.47b to give the target compound 2.3 as a single diastereomer by TLC and $^{13}$C NMR. The optical rotation of 2.3 was measured to be $[\alpha]_D - 18^\circ (c 0.054$ acetonitrile).

**Scheme 2.11** Preparation of (E)- and (Z)- N-[4-(phenylazo)benzyl]-(3S)-3-[[N-Cbz-L-leucinyl]amino]-2-oxobenzenebutanamide 2.3

(i) 2.23, EDCI, HOBT, DIEA, DMF, CH$_2$Cl$_2$, 16 h, 60%; (ii) TEMPO, KBr, NaOCl, NaHCO$_3$, water, CH$_2$Cl$_2$, qu.

A problem was incurred in the attempted synthesis of 2.4 from the acid 2.46b using EDCI as the coupling agent. In the coupling reaction of acid 2.46b with 4-(phenylazo)aniline 2.48, mediated by EDCI, a mixture (2:1 by $^1$H NMR) of the α-hydroxyamide 2.49b and the side product 2.50 was isolated (Scheme 2.12). The dimeric
compound 2.50 was formed from a double esterification between two molecules of the starting material 2.46b. The most likely mechanism for the first esterification would be O-acylation by the activated carboxyl derivative of 2.46b onto a second molecule of 2.46b.24 The second esterification, being intramolecular, would be considerably more facile than the first. The dimer was prepared as a pure compound in a separate experiment, in which the acid 2.46b was treated with EDCI and HOBT in the absence of an amine nucleophile. The dimer 2.50 was obtained in 28% yield. When this reaction was repeated in the presence of base (DIEA, 1.1 equiv. relative to acid) 2.50 was isolated in 74% yield.

Scheme 2.12 EDCI-mediated coupling of 2.46b with 2.48
(i) EDCI, HOBT, DMF, CH2Cl2, 16 h, a mixture (2:1 by 1H NMR) of 2.49b/2.50.

The dimeric nature of 2.50 was proposed on the basis of high resolution mass spectral data that was consistent with the structural formula C48H57O10N4 (M+1)+. In addition, the 1H NMR of 2.50 had a resonance at 5.49 ppm that was shown to correspond with H2 by a 2D NMR COSY experiment. A key correlation was observed in the COSY experiment between H3 (4.66 ppm) and H2 (5.49 ppm). The chemical shift of H2 is consistent with a single proton attached to carbon substituted with an O-acyl group.25 A similar chemical shift was observed for H2 of the acetyl 2.45b (Section 2.5) at 5.53 ppm, whereas the H2 proton of the corresponding alcohol 2.45a (Section 2.5) resonated further upfield at 4.29 ppm. The two-fold axis of symmetry in the dimer 2.50 was supported by the presence of only twenty peaks in the 13C NMR spectrum. Furthermore, the optical rotation measurement of 2.50 in acetonitrile of [α]D -78° indicated that it was an optically active species.
An analogous side reaction did not arise in the synthesis of 2.3 (see Section 2.6), presumably because the amino group of 4-(phenylazo)benzylamine 2.22 is a much better nucleophile than that of 4-(phenylazo)aniline 2.48. The difference in the nucleophilicity of 2.22 and 2.48 was highlighted in the syntheses of compounds 2.1 and 2.2 (see Section 2.4). In the coupling reactions with the acid 2.39, 4-(phenylazo)benzylamine gave 64% product and 4-(phenylazo)aniline gave 46% product. This difference in nucleophilicity can be attributed to the difference in availability of the lone electrons of the amino group nitrogens.

\[
\begin{align*}
\text{(E)-2.48} & : \quad \text{NH}_2 \\
\text{(E)-2.22} & : \quad \text{NH}_2
\end{align*}
\]

In the reaction detailed in Scheme 2.12 it is probable that, because of the relatively poor nucleophilicity of 4-(phenylazo)aniline, the hydroxyl groups present in the starting material 2.46b were able to compete with the amino group for the activated carbonyl moiety of a second molecule of 2.46b. The related phenomenon of “overactivation” in peptide bond formation has been discussed by Bodanszky.24 In a peptide bond-forming reaction, side products can form if the activated derivative of the carboxyl component is too powerful to be selective in its reactions. For example, side products can result from the reaction of the “overactivated” acid derivative with primary or secondary hydroxyl groups. The relative rates of N-acylation and O-acylation are dependent on the nature of the protected amino acid, the activating moiety, the hydroxy group and on the reaction conditions.26 It has been observed that the rate of O-acylation of unprotected-serine containing peptides is enhanced by the presence of a base or HOBT.27 Also of note is the use of dicyclohexylcarbodiimide (DCC), which is closely related to EDCI in terms of reactivity and mechanism, as a effective dehydrating agent in the preparation of esters from carboxylic acids and alcohols.28

The products, 2.49b and 2.50, of the reaction outlined in Scheme 2.12 were not able to be separated chromatographically. Consequently, an alternative coupling
procedure that avoided the problem of $O$-acylation was sought. Esters of $N$-hydroxysuccinimide have been used as the activated species in amide formation from carboxylic acids.\textsuperscript{27} Although some activated esters have exhibited $O$-acylation in the presence of a base (imidazole),\textsuperscript{29} it is unlikely that an $N$-hydroxysuccinimide ester would lead to $O$-acylation considering that such intermediates are often purified by recrystallisation from alcohols.\textsuperscript{23b}

![Scheme 2.13](https://example.com/scheme213.png)

**Scheme 2.13** The synthesis of target compound 2.4

(i) $N$-hydroxysuccinimide, DCC, THF, 0 °C, 18 h; 2.48, 0 °C, 30 min then rt, 16 h, a mixture (5:6 by $^1$H NMR) of 2.49b/DCU; (ii) TEMPO, KBr, NaOCl, NaHCO$_3$, water, CH$_2$Cl$_2$, 0 °C, 20 min, rt, 39% over two steps.

As shown in **Scheme 2.13** the acid 2.46b was treated with $N$-hydroxysuccinimide and DCC to give a suspension of the $N$-hydroxysuccinimide ester of 2.46b and the reaction by-product $N,N'$-dicyclohexylurea (DCU). The suspension was filtered into a flask containing the amine 2.48 and the reaction mixture was stirred for 16 hours to afford a mixture (5:6 by $^1$H NMR) of 2.49b and DCU. There was no evidence of the $O$-acylation product 2.50 by $^1$H NMR. Because the components of this mixture were not able to be separated by chromatography, it was decided to further purify after the following synthetic step. The mixture of 2.49b and DCU was treated with TEMPO and the resulting mixture was separated effectively by flash chromatography on silica to give the $\alpha$-ketoamide 2.4 in 39% yield over two steps. Compound 2.4 was a single diastereomer by TLC and $^{13}$C NMR and gave an optical rotation of $[\alpha]_D - 16$ ° (c 0.038 acetonitrile).
2.7 Synthesis of a reported inhibitor of α-chymotrypsin 2.52

Compound 2.52 is a reported inhibitor of α-chymotrypsin.\textsuperscript{30} As part of the research described in this thesis, 2.52 was prepared to function as a standard for the α-chymotrypsin assay (Section 6.2). The synthesis (Scheme 2.14) proceeded from the cyanohydrin 2.14,\textsuperscript{4} which underwent a one-pot acidic hydrolysis and esterification in the presence of ethanol to form the α-hydroxyester 2.51 in 50% yield.\textsuperscript{5} The ester was oxidised with Dess-Martin periodinane to give 2.52 in 82% yield.

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\chemfig{\Ph\text{CbZ-Nly-CN\H\OH\2.14\Ph\text{CbZ-Nly-OEt\H\OH\2.51\Ph\text{CbZ-Nly-OEt\H\OH\2.52}}}}};
\node (i) at (-2.5,0) {i};
\node (ii) at (2.5,0) {ii};
\end{tikzpicture}
\end{center}

\textbf{Scheme 2.14} Synthesis of α-ketoester 2.52.

(i) HCl, EtOH, ether, 4 °C, 24 h, water, 4 °C, 3 d, 50%; (ii) periodinane, CH$_2$Cl$_2$, 20 h, 82%.

\textsuperscript{*} A sample of the cyanohydrin 2.14 was gifted by G. J. Foulds.
2.8 Summary of Chapter Two

Four compounds of the type A (2.1, 2.2, 2.3 and 2.4) were synthesised via the key intermediate 2.5. The synthesis of 2.5 was investigated by three routes and the most efficient of these routes gave the key intermediate via the cyanoketophosphorane 2.10 in three steps in 31% overall yield. Compounds 2.1 and 2.3 were synthesised from the key intermediate 2.23. The amine salt 2.23 and the corresponding free amine 2.22, syntheses for which had not been reported in the literature, were successfully prepared by separate routes.

Optically active samples of 2.1 and 2.2, which each comprise one amino acid, were readily synthesised (each three steps) from the key intermediate 2.5. For the preparation of the target compounds containing two amino acids, 2.3 and 2.4, the key intermediate 2.5b was extended by one amino acid (L-leucine) in three steps to give the acid 2.46b. From 2.46b, compound 2.3 was synthesised in two steps as a single diastereomer by $^{13}$C NMR. A dilactone side product 2.50 resulted from the attempted coupling of 2.46b with 4-(phenylazo)aniline using the coupling agent EDCI. The formation of this side product was avoided when the NHS ester of 2.46b was reacted with the amine to give 2.49b, which was oxidised to target compound 2.4.

The $\alpha$-ketoester 2.52 was prepared in two steps from the cyanohydrin 2.14 to function as a standard with known inhibition constant for the enzyme assay.
Chapter Two: Synthesis of compounds of the type A

2.9 References for Chapter Two


Chapter Two: Synthesis of compounds of the type A

22 (a) Peet, N. P.; Burkhart, J. P.; Angelastro, M. R.; Giroux, E. L.; Mehdi, S.; Bey, P.;
    Kolb, M.; Neises, B.; Schrîlîn, D. J. Med. Chem. 1990, 33, 394; (b) Ocain, T. D.;
23 (a) Kocienski, P. J. Protecting Groups; Thieme: Stuttgart, 1994; pp 195-199; (b)
    395-396.
    1990, 33, 11.
CHAPTER THREE

THE SYNTHESIS OF COMPOUNDS OF THE TYPE B
3.1 Introduction: Design of the Target Compounds

The type B compounds 3.1 to 3.3 are designed to be photoswitchable inhibitors of α-chymotrypsin (Figure 3.1). As discussed in Section 1.7, compounds of the type B comprise three main components, which read from the N-terminus; azobenzene (photoswitch), peptidyl (enzyme specificity), α-ketoamide (enzyme inhibition). Target compounds 3.1 to 3.3 are analogues of the reported α-chymotrypsin inhibitor 3.4 (Ki = 0.15 μM). The difference between the reported inhibitor and the target compounds is a simple change in amino protecting group from benzoyl to (phenylazo)benzoyl.

![Figure 3.1](image)

Figure 3.1 Target compounds of the type B (3.1 to 3.3) and a reported inhibitor of α-chymotrypsin (3.4).

The reason for the synthesis of the three isomers, 3.1 to 3.3, was to investigate the structure-activity relationships of azobenzene substitution of compounds of the type B. The substitution pattern of azobenzene is known to affect the composition of the PSS resulting from irradiation of the compound at a specific wavelength. In addition, the spatial characteristics of the ortho, meta and para substituted inhibitors are different and
this could lead to changes in active site specificity. Consequently, different levels of enzyme switching would be expected for each of these three target compounds.

Preliminary investigations into the water stability of compounds 3.1 and 3.2 (see Section 5.4 and Section 6.9 for a detailed explanation) revealed that the longer these compounds had been dissolved in water, the less active against α-chymotrypsin they became. The effect was accentuated in the presence of certain biological buffers. For instance a solution of the inhibitor 3.1 in acetonitrile incubated for two days reduced enzyme activity by 80%. A solution that was incubated in distilled water for two days only reduced enzyme activity by 55%. A solution that was incubated in water in the presence of HEPES buffer for two days gave only 15% loss of α-chymotrypsin activity.

Because of the poor water stability of compounds 3.1 and 3.2, other compounds of the type B were designed. A common feature of the compounds, which exhibited poor water stability, was the juxtaposition of the (phenylazo)benzoyl protecting group and the phenylalanine mimic. Target compounds 3.5 and 3.6 were designed with a spacer group to separate the azobenzene moiety from the phenylalanine residue containing the highly electrophilic ketone. The strategy for target compound 3.5 was to install a carbamate moiety into the molecule. The compound is analogous to the reported inhibitor of α-chymotrypsin 2.52. In the target compound 3.6 the azobenzene group was separated from the phenylalanine mimic by introducing a second amino acid, L-leucine, which corresponds to the P₂ subsite.

![Figure 3.2](image_url)  
**Figure 3.2** Target compounds of the Type B (3.4 and 3.3).
3.2 Synthesis of the target compounds 3.1 to 3.3

Most of the azobenzene derivatives prepared in the work described in this chapter were synthesised as predominantly the (E) isomer with traces of the (Z) isomer present. A solution of an azobenzene derivative reached the ambient light PSS (comprising (E) and (Z) isomers) after exposure to daylight for at least one day. NMR data was reported for the (E) isomers in the experimental section (Sections 8.3) and in selected cases this data was followed by information on the ambient light PSS composition. The NMR data for the (Z) isomers is reported in the experimental section on photoisomerisation (Section 8.5.3).

Peptidyl-α-ketoesters have been prepared by various routes. The most common is the oxidation of α-hydroxyesters, prepared from the hydrolysis of the corresponding cyanohydrin. The drawbacks of this route have been discussed in Section 2.2 with regard to peptidyl-α-ketoamide synthesis. Peptidyl-α-ketoesters have also been prepared by the oxidation of α,β-unsaturated carbonyl compounds, such as ethoxyvinyl ketones and acrylic acids. The Dakin-West procedure is a non-stereospecific preparation of peptidyl-α-ketoesters and involves the treatment of a carboxylic acid with ethyl oxalyl chloride. A recent innovation in the preparation of peptidyl-α-ketoesters is the oxidation of cyanoketophosphoranes by ozone in the presence of an alcohol.

Two routes to the target compounds were investigated. The first route, shown in Scheme 3.1, follows the synthetic route outlined by Wasserman. The formation of the cyanoketophosphorane 3.10 and its subsequent ozonolysis in the presence of methanol offered a convenient route to the target compound 3.1. The ester 2.36 (synthesised as described in Section 2.3) was hydrolysed in the presence of potassium hydroxide to afford 4-(phenylazo)benzoic acid 3.7 in 85% yield. The acid 3.7 was coupled with L-phenylalanine methyl ester to give 3.8 in 91% yield. Lithium hydroxide in methanol/water was used for the saponification of 3.8 to the acid 3.9 (quantitative). Unfortunately compound 3.10 was not able to be formed by reaction with 2.8 using the
methodology reported by Wassermann for the preparation of cyanoketophosphoranes.\textsuperscript{5,6} In this attempt, the acid 3.9 was activated with EDCI in the presence of the cyanophosphorane 2.8. A mixture of unidentified compounds resulted from this reaction, but there was no evidence by \textsuperscript{1}H NMR of starting material or the desired product. Consequently, investigations into this route were discontinued.

\[ \text{(E)-2.36} \quad R=\text{Et} \]
\[ \text{(E)-3.a} \quad R=\text{Me} \]
\[ \text{(E)-3.7} \quad R=\text{H} \]
\[ \text{(E)-3.9} \quad R=\text{H} \]
\[ \text{(E)-3.10} \]

\textbf{Scheme 3.1} Attempted synthesis of 3.1 from the cyanoketophosphorane 3.10

(i) KOH, EtOH, reflux, 4 h, 85%; (ii) L-phenylalanine methyl ester, EDCI, HOBT, DIEA, DMF, CH\textsubscript{2}Cl\textsubscript{2}, 16 h, 91%; (iii) LiOH, MeOH, water, 30 min, qu; (iv) 2.8 (PPh\textsubscript{3}=CHCN), EDCI, DMAP, CH\textsubscript{2}Cl\textsubscript{2}, 16 h, no product by \textsuperscript{1}H NMR.

The second route to the target compounds 3.1 to 3.3 took advantage of the availability of 2.5a, which was a key intermediate in the synthesis of 2.1 and 2.2 (as discussed in Section 2.2). For instance, the synthesis of target compound 3.1 involved the coupling of 4-(phenylazo)benzoic acid 3.7 with the amine salt derived from 2.5a. As shown in Scheme 3.2, the \textit{N}-CBZ protected amine 2.5a\textsuperscript{\dagger} was treated with HBr in acetic

\textsuperscript{\dagger} Absolute configuration of 2.5a assigned by comparison of \textsuperscript{1}H NMR and optical rotation data with literature compounds as discussed in Section 2.2
acid (33% v/v) to afford the hydrobromide salt 3.11a in 86% yield. The α-hydroxyester 3.12a was prepared as a single diastereomer by $^{13}$C NMR and TLC from the acid 3.7 and the amine salt 3.11a in excellent yield. The absolute stereochemistry of 3.12a, as shown in Scheme 3.2, was assigned on the basis of the known configuration of the starting material 2.5a. The target compound 3.1 was obtained from the TEMPO oxidation of 3.12a quantitatively. The optical rotation of 3.1 was measured to be $-\lbrack \alpha \rbrack_D ^{24} -52 \pm 5^\circ$ (c 0.29 acetonitrile).

![Scheme 3.2 Synthesis of target compound 3.1](image)

(i) HBr, AcOH, 20 min, 86%; (ii) EDCI, HOBT, DIEA, DMF, CH$_2$Cl$_2$, 16 h, 89%; (iii) TEMPO, KBr, NaOCl, NaHCO$_3$, water, CH$_2$Cl$_2$, qu.

The target compounds 3.2 and 3.3 were prepared by methods analogous to the route undertaken to prepare 3.1. Integral to these routes was the synthesis of (phenylazo)benzoic acids 3.15 and 3.18. A summary of the syntheses of these compounds is given in Table 3.1. The details of the synthesis of the para derivative 3.7, which were presented in Section 2.4, are repeated in Table 3.1 for comparison with the syntheses of the meta and ortho derivatives (3.15 and 3.18 respectively).
Table 3.1 Synthetic details for the preparation of the acids 3.7, 3.15 and 3.18.

The target compound 3.2 was then synthesised from the acid 3.15 as discussed above for 3.1. As shown in Scheme 3.3 the acid 3.15 was coupled with the amine salt 3.11a to give 3.19a in 39% yield. Compound 3.19a was obtained as a single

\[ \text{Product} \times \text{Reaction Type} \times \text{Appearance} \times \text{Melting/Boiling Point °C} \times \text{Yield} \]

<table>
<thead>
<tr>
<th>Product</th>
<th>Reaction Type</th>
<th>Appearance</th>
<th>Melting/Boiling Point °C</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.35</td>
<td>i, 24 h, reflux</td>
<td>cream solid</td>
<td>mp 84-86 (lit 92) (^7)</td>
<td>74%</td>
</tr>
<tr>
<td>2.36</td>
<td>ii, 4 h, reflux</td>
<td>orange solid</td>
<td>mp 76-82 (lit 86-87) (^8)</td>
<td>69%</td>
</tr>
<tr>
<td>3.7</td>
<td>iii, 4 h, reflux</td>
<td>orange solid</td>
<td>mp 247-249 (lit 249) (^7)</td>
<td>85%</td>
</tr>
<tr>
<td>3.13</td>
<td>i, 24 h, reflux</td>
<td>brown oil</td>
<td>bp 200-204 (lit 294) (^7)</td>
<td>88%</td>
</tr>
<tr>
<td>3.14</td>
<td>ii, 3 h, reflux</td>
<td>orange solid</td>
<td>mp 37-39 (lit 36) (^8)</td>
<td>72%</td>
</tr>
<tr>
<td>3.15</td>
<td>iii, 2 h, reflux</td>
<td>orange solid</td>
<td>mp 165-166 (lit 170-171) (^7)</td>
<td>76%</td>
</tr>
<tr>
<td>3.16</td>
<td>i, 24 h, reflux</td>
<td>clear oil</td>
<td>bp (<em>s) 100-110 (lit bp(</em>{15}) 145-147) (^7)</td>
<td>32%</td>
</tr>
<tr>
<td>3.17</td>
<td>ii, 24 h, 75 °C</td>
<td>red oil</td>
<td>(lit bp(_{12}) 206-210) (^7)</td>
<td>44%</td>
</tr>
<tr>
<td>3.18</td>
<td>iii, 2 h, reflux</td>
<td>brown solid</td>
<td>mp 82-83 (lit 95) (^7)</td>
<td>86%</td>
</tr>
</tbody>
</table>

\(^7\) The absolute configuration of 3.11a was assigned as (2R,3S) on the basis of the stereochemistry of its precursor 2.5b, which was in turn assigned by literature comparison (Section 2.2)
diastereomer by $^{13}$C NMR and TLC. The target compound 3.2 was afforded in excellent yield by the TEMPO oxidation of the $\alpha$-hydroxyester 3.19a. The optical rotation of 3.2 was $[\alpha]_D -53 \pm 2^o$ (c 0.67 acetonitrile).

\[
\begin{align*}
(\text{E})-3.19a \\
\text{3.11a} + \text{3.15} \xrightarrow{\text{i}} \text{(E)-3.19a} \quad \text{ii} \rightarrow \text{3.2}
\end{align*}
\]

**Scheme 3.3** Synthesis of target compound 3.2

(i) EDCI, HOBT, DIEA, DMF, CH$_2$Cl$_2$, 16 h, 39%; (ii) TEMPO, KBr, NaOCl, NaHCO$_3$, water, CH$_2$Cl$_2$, 95%.

The synthesis of target compound 3.3 was somewhat different from the previous two examples. The attempted coupling between the ortho-substituted benzoic acid 3.18 and the amine salt 3.11a using EDCI as a dehydrating agent gave a complex mixture comprising less than ten percent (by $^1$H NMR) of the coupled product 3.23a. An alternative coupling agent was sought. Examples of the activation of 2-(phenylazo)benzoic acid 3.18 in the literature are rare. Sunthankar et al. were successful in forming the acid chlorides from the $para$ and $meta$ substituted phenylazobenzoic acids, but were unsuccessful in forming the corresponding $ortho$ derivative. Instead of giving the desired product, treatment of 3.18 with phosphorus pentachloride gave the indazole derivative 3.20.

\[
\begin{align*}
\text{(E)-3.18} \xrightarrow{\text{PCl}_5} \text{3.20}
\end{align*}
\]

**Scheme 3.4** Attempted activation of 2-(phenylazo)benzoic acid 3.18.
In a successful case of the activation and subsequent amidation of \( 3.18 \), which was reported by Willner \textit{et al.}, the acid \( 3.18 \) was treated with one equivalent of dicyclohexylcarbodiimde (DCC) and one equivalent of \( N \)-hydroxysuccinimide (NHS) to form the activated ester \( 3.21 \).\(^{11}\) The reactive intermediate \( 3.21 \) was neither isolated nor characterised. The suspension was filtered to remove dicyclohexylurea and the supernatant was mixed with the enzyme, papain in basic solution. The free amines of the lysine residues of papain reacted with \( 3.21 \) to form species of the type \( 3.22 \). As discussed in \textbf{Section 1.5.2}, this reaction was an example of non-specific, covalent modification of an enzyme to give a photobiological switch.

\[ \text{OH} \quad \text{HN} \quad \text{DCC} \quad \text{NHS} \]
\[ \text{(E)-} 3.18 \quad \text{V} \quad \text{(E)-} 3.21 \quad \text{V} \quad \text{(E)-} 3.22 \]

\textbf{Scheme 3.5} Capping the lysine residues of papain with \( 3.18 \).\(^{11}\)

The DCC/NHS methodology was applied to the synthesis of target compound \( 3.3 \). As detailed in \textbf{Scheme 3.6}, the acid \( 3.18 \) was converted to the activated NHS ester \( 3.21 \), which was then reacted with the amine salt \( 3.11a \) in the presence of base. Flash chromatography of the resulting mixture afforded a compound that was identified by \( ^1H \) NMR and HRMS as the intermediate \( 3.21 \) (23\% yield). Further elution afforded the \( \alpha \)-hydroxyester \( 3.23a \) in 36\% yield. Compound \( 3.23a \) was a single diastereomer by \( ^{13}C \) NMR and TLC. The target compound \( 3.3 \) was prepared in 88\% yield by the subsequent oxidation of \( 3.23 \). An optical rotation measurement of \( [\alpha]_D = +16 \pm 1^\circ \) showed that \( 3.3 \) was optically active.
Scheme 3.6  Synthesis of target compound 3.3

(i) DCC, NHS, THF, 0 °C, 16 h; (ii) 3.11a, DIEA, THF, 4 h, 36% over two steps; (iii) TEMPO, KBr, NaOCl, NaHCO₃, water, CH₂Cl₂, 88%.

3.3 Synthesis of the target compound 3.5

Carbamates are often formed from the reaction between a chloroformate and a primary amine. This type of reaction is important in peptide synthesis because it is the basis for amino group protection by the Cbz protecting group (Figure 3.3). Benzylchloroformate 3.24 can be synthesised from the reaction of benzylalcohol with phosgene in the presence of pyridine, which acts as a nucleophilic catalyst in this reaction. Another commonly used amino protecting group is the Boc (t-butoxycarbonyl) group. Boc protected amino groups are prepared by the reaction of an amine with the anhydride 3.26 rather than the unstable chloroformate, tBuOOCOCI.

![Figure 3.3](image-url)  The protection of primary amines by the Cbz and Boc groups.
A one-pot synthesis of the carbamate moiety was investigated with a view to preparing the target compound 3.5. The work of Majer et al. in the formation of \( N,N' \)-unsymmetrically disubstituted ureas served as the template for this investigation.\(^{13}\) The ureas in this paper were synthesised in a one-pot reaction using triphosgene 3.28, a crystalline substitute for the gaseous and highly toxic phosgene 3.29.\(^{14}\) For example, the urea 3.32 in Scheme 3.7 was formed in 85% yield by the reaction of triphosgene with \( L \)-valine methyl ester 3.30 and the subsequent addition of the amine 3.31. This example is particularly important for the design of a route to target compound 3.5 because the amino acid 3.31 contains a secondary alcohol as is the case with the amine 3.11a. It appears that the presence of a secondary alcohol in one of the amines does not interfere with the desired reaction.

The route that was developed for the synthesis of target compound 3.5 is detailed in Scheme 3.7. In a one-pot reaction, the \( \alpha \)-hydroxyester 3.34 was formed from the alcohol 2.29 and the amine 3.11a (Steps i and ii). The alcohol 2.29 (1 equiv.), DIEA (1.2 equiv.) and triphosgene 3.28 (0.4 equiv.) were combined at 0 °C and then stirred at room temperature for thirty minutes. The proposed reaction intermediate is the chloroformate 3.33.\(^{12,14}\) A solution of the amine salt 3.11a (1 equiv.) and DIEA (2.2 equiv.) was added and the reaction mixture was stirred for a further thirty minutes. The crude product was purified by flash chromatography to return the alcohol 2.29 (23%
yield) and to afford the product 3.34 (34% yield) as a single diastereomer by $^{13}\text{C}$ NMR and TLC. The $\alpha$-hydroxyester 3.34 was oxidised with TEMPO to give the target compound 3.5 in quantitative yield. Compound 3.5 was optically active $\{[\alpha]_D -21 \pm 2^\circ \text{ (c 1.3 acetonitrile)}\}$.

Scheme 3.7 Synthesis of target compound 3.5
(i) 3.28, DIEA, CH$_2$Cl$_2$, 0 °C, 5 min; (ii) 3.11a, DIEA, CH$_2$Cl$_2$, 0 °C, 5 min, 41% by returned starting material over two steps; (iii) TEMPO, KBr, NaOCl, NaHCO$_3$, water, CH$_2$Cl$_2$, qu.

The return of the alcohol 2.29 from the reaction could mean that the intermediate 3.33 did not form quantitatively from the alcohol. A second possibility was that the intermediate was indeed formed, but the free amine from 3.11a did not react with the intermediate in full. In this case the intermediate would revert to the alcohol during aqueous workup. The former case is more likely because the reaction of triphosgene with an alcohol often requires a catalyst such as pyridine.$^{15}$

A potential problem in using pyridine as a catalyst for this reaction is that halogenation has been reported in the reaction between benzylic alcohols and triphosgene in the presence of excess pyridine.$^{16}$ For example benzyl alcohol in the presence of triphosgene (0.34 equiv.) and pyridine (2 equiv.) was converted to benzyl chloride (90%) in less than 15 min at room temperature. The researchers theorised that this reaction proceeded through the chloroformate 3.24.
In the reaction mixture of the preparation of carbamate 3.34 (reaction (ii) from Scheme 3.7), there was no evidence of 4-(phenylazo)benzylchloride by $^1$H NMR. In future attempts to improve the yield of 3.34, the reaction should be performed in the presence of a catalytic amount of pyridine. In a separate experiment the reaction time for the first half of the reaction should be extended from 30 min.

3.4 Synthesis of the target compound 3.6

\[ \text{(E)-3.7} \xrightarrow{i} \text{(E)-3.37a} \xrightarrow{iii} \text{(E)-3.6} \]

\[ \text{(E)-3.35 \text{ R=Me}} \]

\[ \text{(E)-3.36 \text{ R=H}} \]

\[ \text{(E)-3.37a} \]

Scheme 3.8 Synthesis of the target compound 3.6.

(i) L-leucine methyl ester hydrochloride, EDCI, HOBT, DIEA, DMF, CH$_2$Cl$_2$, 16 h, qu; (ii) LiOH, MeOH, water, 30 min, qu; (iii) 3.11a, EDCI, HOBT, DIEA, DMF, CH$_2$Cl$_2$, 16 h, qu; (iv) TEMPO, KBr, NaOCl, NaHCO$_3$, water, CH$_2$Cl$_2$, qu.
The proposed inhibitor **3.6** was synthesised as detailed in **Scheme 3.8**. The acid **3.7** was coupled with *L*-leucine methyl ester to give the amide **3.35** in excellent yield. Saponification of **3.35** yielded the acid **3.36**, which was then coupled with the amine salt **3.11a** to give the α-hydroxyester **3.37a** in excellent yield. Compound **3.37a** was obtained as a single diastereomer by \(^{13}\text{C}\) NMR. The target compound **3.6** was afforded in quantitative yield, as a single diastereomer by \(^{13}\text{C}\) NMR, from the oxidation of **3.37a**. The optical rotation of **3.6** was \([\alpha]_D^{-21} \pm 1^\circ\) (c 1.4 acetonitrile).

### 3.5 Summary of Chapter Three

Five optically active compounds of the type B were synthesised. The target compounds **3.1**, **3.2** and **3.3** were prepared from the corresponding (phenylazo)benzoic acids. The *para* isomer **3.1** was synthesised in the highest overall yield and the *ortho* isomer **3.3** in the lowest. In the synthesis of **3.3**, the DCC/NHS coupling reagents were found to successful for the reaction of an amine with 2-(phenylazo)benzoic acid. In the synthesis of **3.5**, a carbamate was prepared in one step from an alcohol and an amine by the use of triphosgene. The reaction was low yielding and returned some of the alcohol. Target compound **3.6** was prepared as a single diastereomer in good yield over twelve steps.

### 3.6 References for Chapter Three


CHAPTER FOUR

THE SYNTHESIS OF COMPOUNDS
OF THE TYPE C
4.1 Introduction: Design of the Target Compounds

The compounds 4.1-4.3 are compounds of the type C that were designed to be photoswitchable inhibitors of \( \alpha \)-chymotrypsin (Figure 3.1). As discussed in Section 1.7, compounds of the type C comprise three main components, which read from the N-terminus; azobenzene (photoswitch), \( \alpha \)-ketoamide (enzyme inhibition), peptidyl (enzyme specificity). Compound 4.1 is the exception, since it does not contain any component of intended enzyme specificity. As a result, the inhibition data for this compound will give an indication of the effectiveness of the basic type C design. It is possible, however, that one of the structural isomers of the azobenzene 4.1 aligns the unsubstituted phenyl ring into the \( S_1 \) subsite of \( \alpha \)-chymotrypsin, to improve the binding of that isomer. The \( S_1 \) subsite accommodates amino acid side chains with aromatic groups as discussed in Section 1.6.2.

![Figure 4.1](image)

Figure 4.1 Target compounds of the type B (4.1 to 4.3)

Compounds 4.2 and 4.3 contain amino acids, which were chosen to be specific for the \( S' \) subsites of \( \alpha \)-chymotrypsin. Because of the ambiguity in alignment resulting from the interaction of the active site with an \( \alpha \)-ketoamide instead of an amide, the amino acid on the carboxyl side of the \( \alpha \)-ketoamide may lie either in the \( S_{1}' \) or in the \( S_{2}' \) subsite. Consequently, an L-leucine residue, which is specific for the \( S_{2}' \) subsite of \( \alpha \)-
chymotrypsin, has been incorporated in target compound 4.2. The $S_1'$ subsite favours a basic amino acid side chain. Accordingly, target compound 4.3 contains an $L$-lysine residue. It is of note that the $S_1$ subsite is the primary specificity site of $\alpha$-chymotrypsin and any prospective substrate or inhibitor that does not take advantage of this strong interaction will tend to have poor activity. Therefore, high activity from the target compounds 4.1 to 4.3 will only arise from the alignment of the azobenzene group in the $S_1$ site.

4.2 Synthesis of the key intermediate 4.4

Most of the azobenzene derivatives prepared in the work described in this chapter were synthesised as predominantly the $(E)$ isomer with traces of the $(Z)$ isomer present. NMR data was reported for the $(E)$ isomers in the experimental section (Sections 8.4) and in selected cases this data was followed by information on the ambient light PSS composition. The NMR data for the $(Z)$ isomers is reported in the experimental section on photoisomerisation (Section 8.5.3).

![Scheme 4.1](image)

**Scheme 4.1** Retrosynthetic analysis of the synthesis of target compounds 4.2 and 4.3.

The $\alpha$-ketoamides 4.2 and 4.3 were synthesised from the corresponding $\alpha$-hydroxyacid 4.4 in a route analogous to the one discussed for the preparation of $\alpha$-ketoamides in Section 2.1. The key intermediate 4.4 (Scheme 4.1) was prepared by two routes, both of which gave mixtures of 4.4 with other compounds. However the target
compounds were able to be prepared from the crude samples of 4.4 by the amidation of 4.4 and subsequent purification by flash chromatography.

The first route to the key intermediate 4.4, shown in Scheme 4.2, follows the synthetic route outlined by Wasserman. The acid 3.7 was coupled to the cyanophosphorane 2.8 to give the cyanoketophosphorane 4.5 in 63% yield. The phosphorane was treated with ozone at -78°C in the presence of methanol to give the α-ketoester 4.6. This reaction (Step ii, Scheme 4.2) gave mixed results and highlighted one of the flaws of the cyanoketophosphorane methodology. A side product 4.7 was obtained from Step (ii) in varying amounts. In one reaction, no 4.7 was formed at all and in another reaction the products were obtained in the ratio 5:1 (4.6 / 4.7 by 1H NMR). There was no obvious difference in reaction conditions between these two cases. A mixture of 4.6 and 4.7 was used in the next step without purification in cases where 4.6 comprised at least 95% of the mixture by 1H NMR.

Scheme 4.2  Toward the key intermediate 4.4 via the cyanoketophosphorane 4.5

(i) 2.8, EDCI, DMAP, THF, CH₂Cl₂, 16 h, 63%; (ii) O₃, MeOH, CH₂Cl₂, -78°C, 15 min, a mixture of 4.6 and 4.7 (ratio varies).

The formation of side products analogous to 4.7 was observed in other reactions of cyanoketophosphoranes with ozone followed by the addition of a nucleophile. For example, the attempted, direct synthesis of target compound 4.2 from the corresponding
cyanoketophosphorane 4.5 led to the amide 4.8 instead of the desired α-ketoamide (Scheme 4.3). A similar result was discussed in Section 2.4, in which the amide 2.38 was formed from the cyanoketophosphorane 2.10. Further examples are presented in Scheme 4.3. In our hands, the conditions that Wasserman et al. reported for the preparation of α-keto acids,1 exclusively returned the original acids. For example 4.5 was treated with ozone in dichloromethane at -78 °C. The ozone was purged with nitrogen and a mixture (4:1) of THF and water was added. After workup the acid 3.7 was obtained in 41% yield. In a similar reaction the cyanoketophosphorane 2.10 gave the acid 2.9 in 51% yield rather than the desired α-ketoacid.

![Scheme 4.3](image)

Scheme 4.3 The cyanoketophosphorane methodology was not reliable and did not deliver the desired α-keto acid derivatives
(i) ozone, CH₂Cl₂, -78 °C, 10 min; N₂, -78 °C, 5 min; L-leucine methyl ester hydrochloride, DIEA, -78 °C, 1 h, 29%; (ii) and (iii) ozone, CH₂Cl₂, -78 °C, 10 min; N₂, -78 °C, 5 min; THF / water (4:1), -78 °C to rt, 5 h, 41% (3.7) and 51% (2.9)
The only efficacious ozonolysis reactions were those that had the nucleophile (i.e. methanol) present during the ozonolysis as co-solvent. The main difference between reactions that did not give an α-ketoacid (derivative) as the major product and the efficacious ozonolysis reactions was the presence or delayed addition of the nucleophile. It is reasonable to assume that the reactive intermediate was compromised under the reaction conditions of delayed nucleophile addition.

The remainder of the synthesis of key intermediate 4.4 is detailed in Scheme 4.4. The α-ketoester 4.6 (a mixture (>19:1) of 4.6/4.7 by $^1$H NMR) was reduced with sodium borohydride (0.5 equivalents) at 0 °C to give the α-hydroxyester 4.9 in excellent yield (a mixture (>19:1) of 4.9/4.7 by $^1$H NMR). The reaction was performed at low temperature because an over-reduced side product was formed at room temperature with 1.1 equivalents of reagent. The side product was a 1,2-diol resulting from the reduction of the ester to the corresponding primary alcohol.

![Scheme 4.4](image)

**Scheme 4.4** Synthesis of key intermediate 4.4 and side product 3.7
(i) NaBH$_4$ (0.5 equiv.), MeOH, 0 °C, qu.; (ii) LiOH, MeOH, water, 30 min, a mixture of 4.4 and 3.7 (ratio varies).

By some unknown mechanism, saponification of a mixture (>19:1 by $^1$H NMR) of 4.9/4.7 (Step ii, Scheme 4.4) produced the acid 3.7 as a side product in greater yield than would be expected based on the amount of 4.7 present. For example, a mixture (>19:1) of the methyl esters 4.9 and 4.7 (50 mg) was treated with lithium hydroxide to
give a mixture (3:1) of the desired \( \alpha \)-hydroxyacid 4.4 and the side product 3.7 (41 mg). The components of the mixture were not able to be separated by chromatographic means. The conversion of aryl-\( \alpha \)-hydroxyesters to aryl esters is not unprecedented. For instance, the oxidative decarboxylation of mandelic acid (\( \alpha \)-hydroxyphenylacetic acid) to benzoic acid derivatives has been reported in the literature.\(^2\) Mandelic acid was converted quantitatively to benzoic acid by the treatment of \( \text{NaBO}_3 \cdot 4\text{H}_2\text{O} \) in glacial acetic acid.

The synthesis of the key intermediate 4.4 was also approached from the cyanohydrins 4.11 and 4.12 (Scheme 4.5). The alcohol 2.29\(^i\) was oxidised with Dess Martin periodinane to give the aldehyde 4.10 in excellent yield. In the presence of potassium cyanide, zinc iodide and trimethylsilyl cyanide, the aldehyde 4.10 was converted to the corresponding cyanohydrin and its trimethylsilyl ether, which were isolated after flash chromatography in yields of 38% and 36% respectively.

\[ \text{Scheme 4.5} \quad \text{Synthesis of the cyanohydrin 4.11 and its TMS ether 4.12.} \]

(i) periodinane, \( \text{CH}_2\text{Cl}_2 \), 40 min, 98%; (ii) \( \text{KCN}, \text{ZnI}_2, \text{CH}_2\text{Cl}_2 \), 20 min, \( \text{TMSCN} \), 0 °C, 16 h, 38% (4.11), 36% (4.12).

Cyanohydrins can be hydrolysed in acidic conditions to give \( \alpha \)-hydroxyacids.\(^3\) In alkaline conditions, cyanohydrins are able to be hydrolysed, but the competing decomposition of the cyanohydrin to the aldehyde or ketone and cyanide is a major drawback.\(^4\) The hydrolysis of the cyanohydrin 4.11 was therefore attempted in acidic conditions. Treatment of 4.11 in dioxane with concentrated hydrochloric acid gave a

\(^{i}\) The synthesis of 2.29 is discussed in Section 2.4.
mixture of products. The composition of the mixture depended upon the reaction conditions as shown in Figure 4.2.

![Diagram showing the synthesis of compounds](image)

<table>
<thead>
<tr>
<th>Mass 4.11 (mg)</th>
<th>Reaction Temperature</th>
<th>Reaction Duration</th>
<th>Ratio (by $^1$H NMR)</th>
<th>Final Mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>rt</td>
<td>2 h</td>
<td>8 : 8 : 24 : 5</td>
<td>47</td>
</tr>
<tr>
<td>108</td>
<td>40 °C</td>
<td>24 h</td>
<td>n.o. : 8 : 3 : 6</td>
<td>56</td>
</tr>
<tr>
<td>100</td>
<td>55 °C</td>
<td>16 h</td>
<td>n.o.</td>
<td>n.o.</td>
</tr>
<tr>
<td>100</td>
<td>35 °C</td>
<td>3 d</td>
<td>n.o. : 8 : n.o : 3</td>
<td>82</td>
</tr>
<tr>
<td>100*</td>
<td>35 °C</td>
<td>3 d</td>
<td>n.o. : 4 : n.o : 1</td>
<td>82</td>
</tr>
</tbody>
</table>

**Figure 4.2** The acid hydrolysis of the cyanohydrin 4.11 afforded a mixture of compounds. (* solvent degassed, n.o. not observed)

(i) 0.2 M in dioxan/conc. HCl (1:1), temperature and duration as specified in table.

The amide 4.13 is the intermediate in the hydrolysis reaction and long reaction times (3 d) at moderate temperature (35 °C) were required to fully convert 4.13 to the desired acid 4.4. There was an upper limit on the reaction temperature, because after stirring for 16 hours at 55 °C no product was able to be isolated. The colour of the reaction mixture had changed from the characteristic orange of azobenzene derivatives to black. Formation of the side product 3.7 is not part of the normal mechanism of hydrolysis, nonetheless 3.7 was observed in all reactions, even after short reaction times at room temperature. It was thought that the side product 3.7 resulted from a mechanism involving oxidation, possibly with oxygen present in the solvent acting as the oxidant.
To test this theory the reaction was repeated under an atmosphere of nitrogen in solvent that had been degassed. The mixture isolated from this reaction contained less 3.7 relative to 4.4 than an analogous air-open reaction while still returning the same mass. Thus it appears that the impurity 3.7 does result from an oxidative process.

In summary, the key intermediate 4.4 was synthesised by two routes, but in both cases it was isolated as a mixture with the acid 3.7. The target compounds of the type B were synthesised from mixtures containing 4.4, 4.13 and 3.7. The details of these syntheses are given in Section 4.3.

4.3 Synthesis of the target compounds 4.2 and 4.3

![Scheme 4.6 Synthesis of the target compound 4.2](image)

(i) a mixture (6:1:1 by $^1$H NMR, cyanohydrin route) of 4.4, 4.13 and 3.7, L-leucine methyl ester, hydrochloride salt, EDCI, HOBT, DIEA, DMF, CH$_2$Cl$_2$, 16 h, 30% over last two steps; (ii) TEMPO, KBr, NaOCl, NaHCO$_3$, CH$_2$Cl$_2$, qu.

In the synthesis of target compound 4.2 (Scheme 4.6) a mixture (6:1:1 by $^1$H NMR) of 4.4, 4.13, 3.7 (54 mg, from the cyanohydrin route) was used for the coupling of the $\alpha$-hydroxyacid 4.4 with L-leucine methyl ester hydrochloride salt to give, after
chromatography, a mixture (1:1 by $^1$H NMR) of the diastereomers 4.14a and 4.14b (30 mg) in 30% yield over two steps. This mixture of compounds 4.14a and 4.14b was oxidised with TEMPO to give the 4.2 in quantitative yield. The target compound 4.2 was optically active $\{[\alpha]_D -30 \pm 3^\circ \ (c \ 0.215 \text{ acetonitrile})\}$.

In order to determine the geometry of the (E)-azobenzene moiety, a crystal of 4.2 was grown from chloroform. The single crystal analysis of 4.2 showed that the molecule crystallised in the chiral space group $P(2)$/$1$. A perspective drawing of 4.2 with atomic labelling is shown in Figure 4.3. The space group, $P(2)/1$ indicates that the crystal of 4.2 contained a single enantiomer. The molecule is depicted again in Figure 4.4 as a wireframe model. This figure emphasises the near planarity of the azobenzene group.

The C-N bond distances in (E)-4.2 (1.43 and 1.44 Å) are similar to the bond lengths of unsubstituted (E)-azobenzene (1.41 Å). These lengths are practically the same as a resonance-free C-N bond. The N=N distances of (E)-4.2 (1.26 Å) and (E)-azobenzene (1.23 Å) are comparable. The bond angles at the nitrogen atoms are smaller in (E)-4.2 (C1-N1-N2, 112 ° and N1-N2-C1', 115 °) than in (E)-azobenzene (121 °).
The synthesis of target compound 4.3 was more complex than the synthesis of 4.2 because of the need for protecting groups on the side chain of lysine. Two synthetic routes to 4.3, with different protecting groups (Cbz and Boc) for the amino group of
lysine, were investigated. The shorter route of the two employed N<sub>e</sub>-Cbz-L-lysine methyl ester hydrochloride salt, which was commercially available.

A mixture of the acids 4.4 and 3.7 (8:3 by ¹H NMR, from the cyanohydrin route) was reacted with EDCI and the methyl ester of N<sub>e</sub>-Cbz-L-lysine to afford a mixture (1:1 by ¹H NMR) of the α-hydroxyamides 4.15a and 4.15b (40% over the previous two steps) after column chromatography. The alcohols 4.15a and 4.15b were oxidised to the ketone 4.16 in excellent yield.

![Scheme 4.7](image)

**Scheme 4.7** Attempted synthesis of the target compound 4.3
(i) a mixture (8:3 by ¹H NMR, cyanohydrin route) of 4.4 and 3.7, N<sub>e</sub>-Cbz-L-lysine methyl ester, hydrochloride salt, EDCI, HOBT, DIEA, DMF, CH<sub>2</sub>Cl<sub>2</sub>, 16 h, 40% over last two steps; (ii) TEMPO, KBr, NaOCl, NaHCO<sub>3</sub>, water, CH<sub>2</sub>Cl<sub>2</sub>, 92%; (iii) various reagents, refer to text below.

Several reagents are available for the Cbz deprotection of amino groups. Catalytic hydrogenation would not be suitable for the deprotection of 4.16 because the conditions would reduce the ketone to the corresponding alcohol and possibly further to the alkane. Acidolysis of Cbz groups can be achieved with either protic (HBr) or Lewis acids (BBr<sub>3</sub> and TMSI). Treatment of 4.16 with HBr in acetic acid (30% v/v) at rt for twenty minutes gave a mixture of products and evidence of considerable methyl ester cleavage by ¹H NMR. Five equivalents of boron tribromide at −10 °C for one hour and
at rt for two hours led to not only the loss of the Cbz group from \(4.16\), but also complete loss of the methyl ester and decomposition of the azobenzene moiety. When \(4.16\) was treated with up to 25 equivalents of iodotrimethylsilane\(^*\) at rt for 3 h, the starting material \(4.16\) was returned.

\[
\begin{align*}
\text{CbzHN} & \quad \text{O} \quad \text{CbzHN} \quad \text{OMe} \\
\text{4.17} & \quad \text{+} \quad \text{NH}_2(C_6H_{12})_2 \\
& \quad \text{i} \quad \text{CbzHN} \quad \text{OMe} \\
& \quad \text{4.18} \\
& \quad \text{NHBOc} \\
3.7 & \quad + \quad 4.19 \\
& \quad \text{iii} \quad \text{N} \text{NN} \quad \text{OH} \quad \text{NBOc} \\
& \quad \text{4.20a} \\
& \quad \text{(E)-4.20b} \\
4.4 & \quad + \quad 4.19 \\
& \quad \text{ii} \quad \text{H}_3 \text{N} \quad \text{O} \quad \text{OMe} \\
& \quad \text{4.19} \\
& \quad \text{NHBOc} \\
& \quad \text{4.3} \\
& \quad \text{iv} \quad \text{N} \text{NN} \quad \text{O} \quad \text{COMe} \\
& \quad \text{(E)-4.21} \\
& \quad \text{NHBOc} \\& \quad \text{v} \quad \text{4.3}
\end{align*}
\]

**Scheme 4.8** Synthesis of the target compound \(4.3\)

(i) KHCO\(_3\), Mel, DMF, 4 h, 91%; (ii) Pd black, CHOOH, methanol, 10 min, qu; (iii) a mixture (6:1:1 by \(^1\)H NMR, cyanohydrin route) of \(4.4\), \(4.13\) and \(3.7\), EDCI, HOBT, DIEA, DMF, CH\(_2\)Cl\(_2\), 16 h, 8% over last two steps relative to cyanohydrin \(4.11\); (iv) TEMPO, KBr, NaOCl, NaHCO\(_3\), water, CH\(_2\)Cl\(_2\), 53%; (v) TMSI 5 equiv., CH\(_2\)Cl\(_2\), 30 min, 20%.

The target compound \(4.3\) was next approached from the \(N_\alpha\)-Boc protected lysine \(4.17\) (Scheme 4.8). The dicyclohexylamine salt \(4.17\) was converted to the corresponding methyl ester \(4.18\) (91%) by treatment with potassium bicarbonate and

\(^*\) Neat TMSI from a commercial source was used fresh.
methyl iodide. The Cbz protecting group of 4.18 was removed using catalytic transfer hydrogenation with formic acid as the hydrogen source.\textsuperscript{10} Compound 4.18 was treated with an equivalent mass of freshly prepared palladium black in formic acid (4.4\% v/v) in methanol to afford the formate salt 4.19 quantitatively. The $\alpha$-hydroxyamides 4.20\textsubscript{a} and 4.20\textsubscript{b} were prepared from the amine salt 4.19 and a mixture (6:1:1 by $^1$H NMR, obtained from the cyanohydrin 4.11) of 4.4, 4.13 and 3.7. The yield of the mixture (1:1 by $^1$H NMR) of diastereomers 4.20\textsubscript{a} and 4.20\textsubscript{b} over the previous two steps, with respect to the cyanohydrin 4.11, was a poor 8\%. The $\alpha$-ketoamide 4.21 was synthesised in 53\% yield by the oxidation of the alcohols 4.20\textsubscript{a} and 4.20\textsubscript{b}.

Boc protecting groups can be removed readily with iodotrimethylsilane.\textsuperscript{11} This reagent was chosen for the preparation of 4.3 from 4.21 since there were no anticipated side reactions with other functional groups in 4.21. The ketone moiety in 4.21 should be stable to TMSI, because TMSI is used to convert ketals into ketones.\textsuperscript{8} The methyl ester in 4.21 should also be stable to TMSI at rt, because in a reported Boc deprotection by TMSI, detectable amounts of ester cleavage by TMSI were only observed in the presence of excess reagent at elevated temperatures.\textsuperscript{9c} Therefore, compound 4.21 was reacted with TMSI (5 equiv., commercial source) for 30 min at rt to give the returned starting material 4.21 and the target compound 4.3 in isolated yields of 80\% and 20\% respectively. Compound 4.3 was optically active {[$\alpha$]$_D$ +8 ± 2 \degree (c 0.05 acetonitrile)}.

It is apparent that the commercially available TMSI was not sufficiently pure to effectively deblock a Boc group. Literature deprotections were complete in 6 minutes in the presence of 1.2 equivalents of TMSI.\textsuperscript{9c} In future experiments the reagent might be prepared immediately before use from TMSCl and magnesium iodide or from hexamethyldisiloxane, aluminium and iodine.\textsuperscript{12}
4.5 Summary of Chapter Four

The target compounds 4.1, 4.2 and 4.3 were successfully synthesised. The key intermediate to these compounds 4.4 was prepared by two routes, but in both cases 4.4 was prepared as an inseparable mixture of compounds. Compound 4.4 was further reacted as part of the crude mixture and the resulting product was purified in the following step. A crystal structure of (E)-4.2 confirmed that this compound was prepared as a single enantiomer. The yields for the syntheses of 4.2 and 4.3 were quite poor.
Chapter Four: Synthesis of compounds of the type C

4.6 References for Chapter Four


CHAPTER FIVE

ISOMERISATION AND HYDRATION STUDIES OF THE TARGET COMPOUNDS
5.1 Introduction

In this chapter the behaviour of the target compounds (type A: 2.1, 2.2, 2.3, 2.4; type B: 3.1, 3.2, 3.3, 3.5, 3.6; type C: 4.1, 4.2, 4.3) is discussed. Because of the complex functionality present in most of the target compounds, the behaviour of these compounds is quite complicated. The azobenzene moiety is reported to undergo \((E)/(Z)\) isomerisation. The activated ketone of \(\alpha\)-keto esters and amides is subject to hydration in the presence of water. Both of these phenomena were investigated in a representative example of each of the three types of target compound. Amino acids adjacent to the electrophilic ketone of an \(\alpha\)-ketoamide have been reported to epimerise, but there are discrepancies in the literature over the conditions for epimerisation. This issue was explored with the target compounds described in this thesis.

5.2 Hydration Studies of the Target Compounds

Peptidyl-\(\alpha\)-ketoamides and esters have been reported as inhibitors of a wide variety of enzymes, including serine\(^1\) and cysteine proteases,\(^2\) aspartyl proteases,\(^3\) aminopeptidases.\(^4\) There is general agreement in the literature that \(\alpha\)-ketoamides and esters are prone to hydration in the presence of water, however the mechanism of inhibition by these compounds can differ between different types of enzyme. In the case of metallo proteases and aspartyl proteases, experimental evidence strongly suggests that the inhibitor is present in the active site as the hydrate.\(^3b,4\) The gem-diol (Scheme 5.1) acts as a transition state mimic by imitating the tetrahedral intermediate formed between substrate and a catalytic water molecule. For cysteine and serine proteases, the mechanism of these inhibitors is more equivocal. The ease of hydration of the \(\alpha\)-ketoamides and esters suggests that a hydrate binds in the active site as a mimic of the tetrahedral intermediate formed between the substrate and a nucleophilic residue. A second theory is that the hydrate is not the active inhibitory species, rather it is the ketone that binds with the active site and undergoes nucleophilic attack by Ser-195 in the case of serine proteases.\(^1a,2b\) The resulting structure is a firmly bound, but reversible tetrahedral adduct. By this argument the “slow-binding” kinetics often observed with
electrophilic ketone inhibitors of serine and cysteine proteases, arises from the slow conversion of hydrated ketone to the active agent, free ketone.$^{1a,1c}$

![Scheme 5.1](image)

**Scheme 5.1** Hydration equilibria between ketones and gem-diols for peptidyl-α-ketoesters 5.1 and peptidyl-α-ketoamides 5.3.

Although the exact role of the hydrate in the mechanism of inhibition by peptidyl-α-ketoamides and esters is not well established, hydration equilibria are important in the inhibitory action of these compounds. Several groups have reported findings on gem-diol formation from α-ketoamides. Compound 5.5 was synthesised as an inhibitor of the cysteine protease, calpain.$^{2a}$ The formation of the hydrate was monitored by $^1$H NMR and the distinctive feature was the change in shift of the proton α to the ketone (β to the amide). In D$_2$O/DMSO-δ$_6$ (3:2) the α-ketoamide 5.5 rapidly reached an equilibrium state comprising ketone/gem-diol (4:5) by $^1$H NMR. The equilibrium state was defined as the state from which no further change in composition was observed and was reached within one hour. Compound 5.5 was also monitored by $^1$H NMR after dissolution in CD$_3$OD. Hemiketal formation was observed within 30 minutes at rt and the equilibrium composition, ketone/hemiketal (7:1), was reached within 47 hours.
Chapter Five: Isomerisation and Hydration Studies of the Target Compounds

Figure 5.1 Peptidyl-α-ketoamides for which hydration data are available.

Ocain and Rich synthesised the aminopeptidase inhibitor 5.6 and studied its hydration properties. Because of poor solubility of 5.6 in water, the compound was not able to be analysed by NMR in an aqueous system. The solubility limit for 5.6, D$_2$O/DMSO-$d_6$ (1:5), was found by adding D$_2$O to a solution of the ketone in deuterated DMSO. In this solvent system 5.6 equilibrated over an unspecified time period to approximately ketone/gem-diol (1:1) by $^1$H NMR. The gem-diol was further characterised by $^{12}$C NMR. In the mixture comprising ketone and gem-diol a new set of peaks arose; the characteristic resonance at 94.2 ppm corresponded to the carbon of the gem-diol adjacent to an amide carbonyl, which resonated at 172.2 ppm.

The reported behaviour of the α-ketoamide 5.7, synthesised by Slee et al. as an inhibitor of HIV protease, was different from the majority of cases recorded. In many cases peptidyl-α-ketoamides were able to be synthesised by the oxidation of α-hydroxyamides without loss of chirality of the carbon adjacent to the ketone. Reagents that were shown to retain the chirality of the P$_1$ residue included Dess Martin periodinane and TEMPO. However, when 5.7 was formed by the oxidation of the corresponding α-hydroxyamide with Dess Martin periodinane, the transformation led to the epimerisation of the β-carbon of the phenylalanine residue, giving a 3:1 mixture of diastereomers by $^1$H NMR. Furthermore the ratio of diastereomers changed from 3:1 to 1:1 on a change of solvent from deuterated chloroform to DMSO-$d_6$. Hydration studies of 5.7 were undertaken in D$_2$O/DMSO-$d_6$ (1:5). The $^{13}$C NMR results showed that the ketone moiety of the α-ketoamide remained unhydrated even after incubation in the mixed solvent for 24 hours. The authors doubted that hydration would occur in an aqueous medium, but suggested that hydration could occur in the active site of HIV protease (an aspartyl protease) as part of the normal catalytic mechanism.
The \( \alpha \)-ketoester \( 5.8 \) is an effective inhibitor of the aspartyl protease, renin.\(^3\) Peptidyl-\( \alpha \)-ketoesters are thought to bind in the active site of aspartyl proteases in the hydrated form. Strangely, in investigating the behaviour of \( 5.8 \), hemiketal formation was investigated instead of hydrate formation. The \( \alpha \)-ketoester \( 5.8 \) in CD\(_3\)OD was monitored over time by \( ^{13} \)C NMR. Initially only the ketone resonance at 194 ppm was observed. The resonance due to hemiketal formation (100 ppm) appeared gradually and intensified over time. It was concluded that hemiketal formation from \( 5.8 \) was a slow equilibrium process.

![Figure 5.2](image)

**Figure 5.2** The peptidyl-\( \alpha \)-ketoester \( 5.8 \) readily undergoes hydration.

The question to be explored is whether the target compounds in this thesis would exist as hydrates in water. From preliminary \( ^1 \)H NMR observations of the target compounds in \( d_3 \)-acetonitrile or \( d_6 \)-DMSO, the \( \alpha \)-ketoester \( 3.1 \) showed high propensity for hydration. Accordingly, this compound was used for the principal investigations into hydrate formation. Verification was sought on whether the impurity that arose from \( 3.1 \) in water-containing solvent was in fact the gem-diol and information was sought on the \( ^1 \)H and \( ^{13} \)C chemical shifts of the hydrate. Compound \( 3.1 \) belongs to the type B classification. Representative compounds of the types A and C were shown to undergo hydration by the NMR methodologies developed with \( 3.1 \).

Several experiments were undertaken to verify the processes that took place in a partially aqueous solution of \( 3.1 \). In the following experiment the purpose was to examine the reversible, water-dependent nature of hydration of the \( \alpha \)-ketoester and to show that hydration could occur over time in an solution of acetonitrile. A sample of \( 3.1 \)
in deuterchloroform was shown by $^1$H NMR to comprise exclusively $(E)$-3.1. The solvent was evaporated and the sample was dissolved in $d_1$-acetonitrile. After five minutes in $d_1$-acetonitrile, a new set of resonances were observed by $^1$H NMR. As discussed for compound 5.5 there was a distinctive signal that corresponded to the formation of gem-diol. The ratio of ketone $(E)$-3.1 to gem-diol $(E)$-5.9 was $> 19:1$. After 40 min in $d_1$-acetonitrile a $^1$H NMR spectrum indicated that the amount of hydrated compound in solution had increased and the additional process of $(E)/ (Z)$ isomerisation resulting from ambient lighting (as discussed in Section 2.3) had produced $(Z)$ isomers ($^1$H NMR spectrum is shown in Figure 5.4a). The solution comprised $(E)$-3.1 / $(Z)$-3.1 / $(E)$-5.9 / $(Z)$-5.9 in the ratio 64:13:18:5. After 26 h in solution still more hydrate had formed. The components $(E)$-3.1 / $(Z)$-3.1 / $(E)$-5.9 / $(Z)$-5.9 were present in the ratio 42:13:34:11 by $^1$H NMR.

![Diagram](image.png)

Figure 5.3 The species present in a sample of 3.1 exposed to light and water.

Water was then removed from the system by diluting the above sample with dichloromethane and drying over magnesium sulfate. The solvent was concentrated in vacuo and the sample was redissolved in deuterchloroform. By $^1$H NMR, the proportion of hydrate had decreased considerably: $(E)$-3.1 / $(Z)$-3.1 / $(E)$-5.9 were present in the ratio 81:13:6.
Figure 5.4a  $^1$H NMR of 3.1 and 5.9 after 40 min in $d_3$-acetonitrile.

Figure 5.4b  $^1$H NMR of 3.1 and 5.9 in $d_3$-acetonitrile/D$_2$O (1:1).
For a solution of 1 in $d_3$-acetonitrile/D$_2$O (2:1) the $^1$H NMR spectrum showed predominantly hydrate after 10 min, i.e. $(E)$-3.1 and $(Z)$-3.1 / $(E)$-5.9 and $(Z)$-5.9 were observed in the ratio 1:3. After 450 min in solution, the hydrate was present in the same ratio. It appears that the equilibrium composition was reached in less than 10 min. This result was different from the acetonitrile-only experiment, because in that experiment water was present as a limiting reagent whereas in the mixed solvent experiment water in available in excess. The mixed solvent results suggest that 5.9 is the exclusive species present in an aqueous medium. Of course, the low solubility of 3.1 in water would not allow for the NMR analysis of 3.1 in an aqueous medium.

The $^{13}$C NMR spectrum of 3.1 in deuterated chloroform showed resonances for the ketone at 191.5 ppm and the ester carbonyl at 160.7 ppm (Figure 5.5a). The same sample in $d_3$-acetonitrile/D$_2$O (2:1) gave a spectrum in which most of the peaks had doubled up but the peaks at 191.5 and 160.7 ppm were no longer observed (Figure 5.5b). New peaks were observed at 94.7 ppm (corresponding to gem-diol carbon of 5.9) and 171.9 ppm (corresponding to ester adjacent to gem-diol in 5.9).

Other compounds were tested for their propensity to undergo hydration. A compound of the type A, 2.4, was dissolved with heating in $d_6$-DMSO/D$_2$O (9:1) to give a mixture of ketone and gem-diol (3:1) by $^1$H NMR. In a sample of 2.4 in neat DMSO at rt and at 60 °C hydrate was barely present. The ratio of ketone and gem-diol was >19:1 in both cases. A representative compound of the type C, 4.2, was dissolved in $d_3$-acetonitrile/D$_2$O (3:1), but the extent of hydrate formation could not be quantified by $^1$H NMR. The shifts of the resonances of protons proximal to the ketone/gem-diol did not change noticeably and only a broadening of signals was observed. The $^{13}$C NMR spectrum, however, gave evidence for the hydration of the ketone 4.2 (gem-diol 111.5 ppm).
Figure 5.5a  $^{13}$C NMR spectrum of target compound 3.1 in deuterochloroform

Figure 5.5b  $^{13}$C NMR spectrum of a solution of 3.1 in $d_3$-acetonitrile/D$_2$O (2:1)
In conclusion, 3.1 exhibited behaviour in solutions containing water that was consistent with hydrate formation. The gem-diol moiety of 5.9 was observed by $^1$H NMR and $^{13}$C NMR. It is probable that a solution of 3.1 in water would be completely hydrated, as suggested for literature examples of $\alpha$-ketoesters and $\alpha$-ketoamides.\textsuperscript{2,3,4} Evidence of hydrate formation was gathered for compounds of the type A (2.4) and compounds of the type C (4.2). It appears that these types are less prone to hydration than compounds of the type B (3.1).

### 5.3 Isomerisation studies of the target compounds

Aromatic azo compounds are interconvertible between the (E) and (Z) isomers. There are considerable differences between the isomers in terms of shape, polarity and stability. These differences make azobenzene photoisomerisation a good process for photobiological switching (as discussed in Section 1.424). (E)-Azobenzene (commonly named trans) is planar across the whole molecule, whereas the phenyl groups of the (Z) isomer (or cis isomer) are twisted.\textsuperscript{5} (Z)-Azobenzene is planar across the azo moiety (C-N=N-C), but the aryl groups are twisted out of the plane of the azo moiety by 53° because of steric hindrance between the ortho hydrogen atoms.\textsuperscript{6} The spatial dissimilarity of the isomers leads to a substantial difference in dipole moments of 3.0 D.\textsuperscript{7} The (E) isomer is more stable than the (Z) isomer by 50 kJmol\textsuperscript{-1}. The less stable (Z)-azobenzene can thermally reconvert to (E), but there is an activation barrier of 96 kJmol\textsuperscript{-1} in solution or 130 kJmol\textsuperscript{-1} in the solid so the corresponding thermal half life of the (Z) isomer is approximately one day at rt.\textsuperscript{5}

The UV spectra of (E)-azobenzenes\textsuperscript{4} have two diagnostic absorption bands (a characteristic spectrum is shown in Figure 5.6).\textsuperscript{11} An intense band arising from a $\pi \rightarrow \pi^*$ transition occurs at approximately 340 nm. The specific wavelength of this band depends on the substitution of the phenyl rings of the azo compound. A less intense band, from a $n \rightarrow \pi^*$ transition occurs at approximately 440 nm. The wavelength of this transition varies little between different azobenzene derivatives,

\textsuperscript{4} Excluding azobenzenes substituted in ortho or para positions with hydroxyl or amino groups.
because the transition is determined by the local symmetry of the azo group rather than the molecular symmetry. Although the \( n \rightarrow \pi^* \) transition in \((E)\) azobenzene derivatives is forbidden by selection rules, there is sufficient vibrational coupling with the \( \pi \rightarrow \pi^* \) transition for the transition to occur. For \((Z)\) azobenzene derivatives (Figure 5.6) the \( n \rightarrow \pi^* \) transition is symmetry allowed and consequently the band is more intense for the \((Z)\) isomer than the corresponding band for the \((E)\) isomer: for \((E)\)-azobenzene \( \varepsilon_{449} = 405 \text{ M}^{-1}\text{cm}^{-1} \), for \((Z)\)-azobenzene \( \varepsilon_{449} = 1250 \text{ M}^{-1}\text{cm}^{-1} \). \(^{11}\)

![UV spectra of \((E)-3.5\) and \((Z)-3.5\) obtained from HPLC chromatography](image)

**Figure 5.6** UV spectra of \((E)-3.5\) and \((Z)-3.5\) obtained from HPLC chromatography

Photostationary state mixtures of azobenzenes arise from irradiations at all wavelengths, because at all wavelengths there is absorbance overlap for the \((E)\) and \((Z)\) isomers.\(^{7}\) The composition of the PSS is determined by the processes represented in the energy profile (Figure 5.7). Irradiation of the material at a specific wavelength leads to the excitation of isomer \(A\) to the high-energy state \(A^*\) that decays to the excited transition state \(P^*\). In turn \(P^*\) decays to the transition state \(P\), which can then relax to either \(A\) or \(B\). The probability of \(A\) formation from \(P\) is \(\alpha\) and the probability of \(B\) formation from \(P\) is \((1-\alpha)\). Because isomer \(B\) absorbs at the wavelength of irradiation, then \(B\) will also be excited and relax to the transition state \(P\). As a result the
composition of the isomers $A$ and $B$ is determined by the steady state equilibrium expressed in **Equation 5.1**, where $\frac{e_B}{e_A}$ is the ratio of molar absorptivity coefficients.

\[
\frac{[A]}{[B]} = \frac{\frac{e_B}{e_A}}{\alpha} \cdot \frac{\alpha}{1-\alpha}
\]

**Equation 5.1**

Furthermore, the energy levels of isomers $A$ and $B$ are not equal as shown in **Figure 5.7**. Because $B$ is of higher energy than $A$, the activation energy for the thermal back-isomerisation of $B$ to $A$ is much lower than for the forward process. As a result thermal back-isomerisation is often associated with the primary photoisomerisation process. In the case of substituted azobenzenes, the thermal \((Z) \rightarrow (E)\) isomerisation for compounds in solution has activation energies in the range 88 to 100 kJmol$^{-1}$, which correspond to thermal half-lives in the order of 2 to 20 hours at rt.

![Diagram](image)

**Figure 5.7** These diagrams represent the concurrent photochemical (left) and thermal (right) isomerisation processes that occur during the photoisomerisation of a photochromic material between states A and B. The common transition state of these processes is P and * denotes an excited state. Diagram modified from Willner and Willner.\(^7\)

Applying the discussion above to the preparation of isomer-enriched PSS mixture of azobenzenes, a specific wavelength for photoisomerisation can be selected by observing the differences between the UV spectra of the \((E)\) and \((Z)\) isomers of an azobenzene derivative (**Figure 5.6**). The photostationary state composition afforded by

\(^7\) Excluding azobenzenes substituted in ortho or para positions with hydroxyl or amino groups.
irradiation of a compound at a specific wavelength is proportional to the ratio of $\varepsilon(E)$ to $\varepsilon(Z)$ at that wavelength.\(^9\) For example, an $(E)$ rich PSS is obtained by irradiating the sample at a wavelength for which the $(Z)$ isomer absorbs more strongly than the $(E)$ isomer. Over time the same PSS composition is arrived at regardless of the initial isomeric composition, sample concentration or the light intensity.\(^10\)

![Figure 5.8 Rotation and inversion pathways for azobenzene isomerisation.](image)

Two mechanisms have been proposed for the isomerisation of azobenzene (Figure 5.8).\(^11\) These pathways involve twisted transition states obtained by the rotation or inversion of a phenyl substituent. As discussed above, following photoexcitation of either the $(E)$ or $(Z)$ double bond structure to a common excited state, the excited state decays to the twisted transition state.\(^7\) The transition state of the inversion pathway has sp-hybridisation at the N-atom proximal to the twisted phenyl substituent and is thought to be associated with the $^1(n, \pi^*)$ state. The rotation pathway, on the other hand, has a transition state with N,N-single bond behaviour that allows for bond rotation. The rotation mechanism is associated with the deactivation of the $^1(\pi, \pi^*)$ state. The
mechanism of isomerisation is not yet fully understood, but the models are becoming more refined as new experiment and theory continues to yield information.

In the isomerisation studies described in this section the aim was to create PSS mixtures rich in either the (E) or (Z) isomer. Sample solutions were irradiated at specific wavelengths and the resulting PSS compositions were measured by $^1$H NMR. Typically a (E) rich PSS is afforded by irradiation at wavelengths over 400 nm and a (Z) rich PSS is afforded by irradiation with wavelengths in the vicinity of 360 nm. PSS compositions tend to be reported in the literature when there was an intended application for the PSS mixture, for instance in photobiological switching or photoregulated supramolecular chemistry. General photoisomerisation studies reported quantum yields rather than PSS compositions. Furthermore, the bulk of work done to date with azobenzenes used azo dyes, azobenzenes substituted with hydroxyl, carboxyl, amino or sulfonic acids, and these compounds do not exhibit the same behaviour as the target compounds in this thesis. Consequently few examples of PSS composition of compounds directly analogous to the target compounds have been reported. Some reported results are presented below for comparison with the PSS composition results of the target compounds.

The first examples of reported azobenzene isomerisation are 4,4'-dialkyl substituted azobenzenes 5.10 and 5.11 (Figure 5.9), which provide a suitable comparison with the 4-alkyl substituted target compounds 2.1, 2.3 and 3.5. The disubstituted azobenzene 5.10 was an anomalous example of (Z) isomer enrichment. A solution of 5.10 was irradiated with UV light to give quantitative conversion to the (Z) isomer by $^1$H NMR ($\lambda=366$ nm, 5 min, 4W UV lamp, c 0.05 M). Usually irradiation of an azobenzene with UV light would give a PSS comprising (E)/(Z) from 1:1 to 1:9. For example the PSS for the reference compound 5.11 at $\lambda=366$ nm was (E)/(Z) = 4:1. The reason for the unusually high yield of (Z)-5.10 from the photoisomerisation was postulated to be due to stabilisation of this isomer by intramolecular hydrogen bonding between the cyclic peptides.
Irradiation with visible light afforded a PSS comprising (E)/(Z) = 6:1 by $^1$H NMR ($\lambda>$400 nm, 20 min, 15 W fluorescent lamp, $c$ 0.05 M). A sample of (E)-5.10 in chloroform in daylight contained small amounts of (Z)-5.10: the (E)/(Z) ratio was 9:1 by $^1$H NMR. Note that this composition is similar to the composition arrived at from irradiation of 5.10 with visible light. The rates of thermal isomerisation, (Z) $\rightarrow$ (E), were measured at rt for both (Z)-5.10 and the reference compound (Z)-5.11. The peptidic compound (Z)-5.10 isomerised in the dark 7.5 times more slowly than (Z)-5.11. Once again the slower rate for (Z)-5.10 was attributed to the stabilisation from intramolecular hydrogen bonding. The thermal isomerisation rate for (Z)-5.11 at 293 K was $2.2 \times 10^{-6}$ s$^{-1}$.

![Figure 5.9](image_url) 4,4'-Disubstituted azobenzenes 5.10 and 5.11.

A second example of azobenzene isomerisation involves an azobenzene substituted with the nitrogen of an amide group, 5.12. This substitution pattern is analogous to the target compounds 2.2 and 2.3. The moiety shown in Figure 5.10 was one unit of a photochromic polymer. Irradiation of the polymeric gel 5.12 with UV light ($330>\lambda>370$ nm) gave a PSS comprising (E)-5.12 / (Z)-5.12 = 1:1. The mixture of isomers reverted exclusively to (E)-5.12 on irradiation with visible light ($\lambda>$400 nm).

![Figure 5.10](image_url) PSS compositions have been reported for azobenzenes 5.12 and 5.13.
The reference did not specify the means for measuring the PSS composition, nor the UV or visible light sources, nor the length of the irradiation period. There was no mention of precautions taken to exclude ambient light from the azo-containing gel.

The 4-carboxyamino substituted azobenzene 5.13, which was synthesised as an $\alpha$-chymotrypsin inhibitor, has similar structural features to the target compounds 3.1 and 3.6. A solution of 5.13 was irradiated with UV light to give a PSS comprising $\text{(E)}$-5.13 / $\text{(Z)}$-5.13 = 1:5 by HPLC (330 < $\lambda$ < 370 nm, 150 W Xe arc lamp). Irradiation with visible light gave a solution of exclusively $\text{(E)}$-5.13 ($\lambda$ > 400 nm, 150 W Xe arc lamp). There was no discussion of the effect of ambient light on the PSS composition of solutions of 5.13 nor was there any mention of precautions taken to exclude ambient light from the sample.

The target compounds described in this thesis were irradiated with light from a 200W high pressure mercury arc lamp. The light was filtered to allow passage of wavelengths between 330 and 370 nm for $\text{(Z)}$ isomer enrichment and wavelengths over 400 nm for $\text{(E)}$ enrichment. For the purposes of PSS composition measurement, solutions of the target compounds in $d_3$-acetonitrile or $d_6$-DMSO (20-40 mM) were irradiated in quartz NMR tubes for 60 min. The PSS compositions were measured by $^1$H NMR immediately after irradiation of the solution. The results of the PSS measurements are presented in groups according to the target compound type.

The results from the irradiation of the target compounds of the type A are presented in Table 5.1. It can be seen that the PSS compositions from irradiation with visible light ($\lambda$ > 400 nm) and ambient light are equal for each compound within the error resulting from NMR integral measurements ($\pm 5\%$). The $^1$H NMR spectra indicated that there was no hydrate formation for these compounds during isomerisation. The target compound that has the best enrichment of $\text{(Z)}$ with UV light and $\text{(E)}$ with visible light is 2.1. The behaviour of the target compounds does not compare well with the analogous literature compounds 5.10 to 5.12.
Table 5.1  Photostationary state compositions of compounds of the type A and analogous literature compounds: n.a. data not available, * \( \lambda = 366 \text{ nm} \), ** means of PSS composition measurement not specified.
Chapter Five: Isomerisation and Hydration Studies of the Target Compounds

The results for the isomerisation of compounds of the type B are presented in Table 5.2 and are repeated in graphical format for compounds 3.1, 3.2 and 3.3 in Chart 5.1. Each of the target compounds underwent hydrate formation during isomerisation. The hydrates were formed either over a period of days in solution or as a result of irradiation. Presumably, sample heating during irradiation led to an increased proportion of hydrate. Compound 3.5 appears to be the most resistant to hydrate formation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PSS composition ( (E\text{-keto})/(E\text{-hydrate})/(Z\text{-keto})/(Z\text{-hydrate}) ) by (^1)H NMR at the specified wavelengths</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>20:7:47:26</td>
</tr>
<tr>
<td>3.2</td>
<td>40:6:47:7</td>
</tr>
<tr>
<td>3.3</td>
<td>15:10:45:30</td>
</tr>
<tr>
<td>3.5</td>
<td>16:6:56:22 75:2:23(\text{n.o.})</td>
</tr>
<tr>
<td>5.13</td>
<td>1:5(^*) exclusively ((E)) (\text{n.a.})</td>
</tr>
</tbody>
</table>

Table 5.2 Photostationary state compositions of compounds of the type B in \(d_3\)-acetonitrile and a literature compound 5.13 that is analogous in substitution pattern to target compounds 3.1 and 3.6: n.o. not observed, n.a. data not available, \(^*\) \((E)/(Z)\)
The isomerisation data can be evaluated more easily by observing the histogram (Chart 5.1), which emphasises by colour-coding the difference between the \((E)\) isomers and the \((Z)\) isomers. It is clear from the histogram that, although the proportion of hydrate varied considerably, the ratio of \((E)\) to \((Z)\) in the PSS from visible light was equal to the ratio in the PSS for ambient light. The exception to this rule was the ortho substituted azobenzene 3.3, for which the ambient PSS comprised 10% more \((E)\)-keto plus \((E)\)-gem diol relative to the PSS arising from irradiation with visible light.

**Chart 5.1** Photostationary state compositions of 3.1, 3.2 and 3.3 in \(d_3\)-acetonitrile.
The data from the isomerisation studies of compounds of the type C in $d_3$-acetonitrile is presented in Table 5.3. In general, these compounds have given poor results for the PSS compositions at the wavelength ranges measured. There are low levels of (Z)-isomer enrichment resulting from irradiation with UV light. There was no evidence by $^1$H NMR of hydration during the photoisomerisation.

![Chemical structures]

### Table 5.3 Photostationary state compositions of compounds of the type C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PSS composition (E)/(Z) by $^1$H NMR at the specified wavelengths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>330&gt;λ&gt;370 nm</td>
</tr>
<tr>
<td>4.1</td>
<td>7:3</td>
</tr>
<tr>
<td>4.2</td>
<td>1:1</td>
</tr>
<tr>
<td>4.3</td>
<td>1:2</td>
</tr>
</tbody>
</table>

In summary, the isomerisation properties of azobenzene derivatives can be grouped according to the type of azobenzene substituent. For *para* derivatives the most efficient switching was observed for azobenzenes substituted with methylene groups (such as 2.1 and 3.5 with the exception of 2.3) or N-amide groups (such as 2.2 and 2.4). Intermediate results were observed for azobenzenes substituted with C-amide groups (such as 3.1 and 3.6), which switched from an ambient light PSS of 25% (Z) isomer to a UV light PSS of 75% (Z) isomer. Poor results were observed for azobenzenes substituted with a *para* ketone (such as 4.1, 4.2 and 4.3), for which the UV light PSS ranged from 30% to 66% (Z) isomer.
The concentration of the target compounds required for isomerisation studies by $^1$H NMR spectroscopy was considerably higher (up to 1000-fold) than the concentration of these compounds used for the enzyme assay. The assumption was made that the PSS compositions measured at NMR concentration would be the same as the PSS compositions at the assay concentration. This assumption was supported by the reported finding that samples of different concentration that were irradiated with the same wavelength reached the same PSS concentration. However, the time taken to reach equilibrium was concentration dependent. This concentration-based time difference was emphasised by low light intensities.

In order to test this assumption, solutions of target compound 3.5, at two different concentrations, were analysed using reverse phase HPLC. A solution of 3.5 (4.1 mM) in acetonitrile that had reached the ambient light PSS was eluted with acetonitrile on a reverse phase C18 HPLC column and detected by absorbance at 190 nm (Figure 5.11).

![HPLC trace of 3.5 (4.1 mM) detected by absorbance at 190 nm](image)

**Figure 5.11** HPLC trace of 3.5 (4.1 mM) detected by absorbance at 190 nm

Two distinct peaks were observed (2.9 and 3.3 min) and each of these peaks were forerun by shoulders, which had the same UV characteristics as the adjacent major peak. The major peaks were characterised by their UV profiles. The characterisation was consistent with the anticipated order of elution with respect to the isomer polarities. The (Z) isomer is considerably more polar than the (E) isomer and as such would elute more quickly on a reverse phase column. (Z)-3.5 eluted at 2.9 min and (E)-3.5 eluted at
3.3 min. The shoulders on these peaks were assigned as the corresponding hydrates on the basis of their UV profiles and retention times. It was known from a $^1$H NMR of the sample that 3.5 was partially hydrated. The absorbance ratio of (E)-ketone and (E)-gem diol to (E)-ketone and (E)-gem diol at 190 nm was 72:28. This ratio did not necessarily reflect the composition of the solution because the extinction coefficients of the isomers at 190 nm were not known. The solution was diluted 20-fold to give a concentration of 0.21 mM. The absorbance ratio of (E)-ketone and (E)-gem diol to (E)-ketone and (E)-gem diol at 190 nm was 70:30. The ratios for the two solutions of different concentration are equal within experimental error. Thus the dilution of a solution of an azobenzene derivative would not be expected to affect the isomer composition.

5.4 Racemisation studies of $\alpha$-ketoester 3.1

There are conflicting reports that discuss the conditions for the epimerisation of peptidyl-$\alpha$-ketoamides and peptidyl-$\alpha$-ketooesters. For instance, Harbeson et al. found that peptidyl-$\alpha$-ketoamides epimerised on silica gel, whereas Wasserman and co-workers purified peptidyl-$\alpha$-ketoamides by flash chromatography on silica gel to afford single diastereomers. Harbeson et al. investigated the instability of their compounds further by monitoring solutions of 5.5 with varying pH and buffer concentration. It was found that acidic solutions (pH3) and low buffer concentration retarded epimerisation. All solutions at pH7 and pH10 had epimerised completely after 15 days.

It was claimed that the peptidyl-$\alpha$-ketoester 5.8 (Figure 5.2) slowly epimerised on standing. By $^{13}$C NMR the single diastereomer of 5.8 had epimerised completely within one week at rt. As discussed in Section 5.2, the $\alpha$-ketoamide 5.7 was reported to epimerise on changing solvent from chloroform to methanol or DMSO. A final condition that was reported to cause epimerisation of peptidyl-$\alpha$-ketoamides and peptidyl-$\alpha$-ketooesters was the method of synthesis, for example the Swern oxidation of a chiral $\alpha$-hydroxyester was reported to lead to mixtures of enantiomers.
The racemisation of target compound 3.1 was investigated after it was discovered that this compound lost activity against \(\alpha\)-chymotrypsin over time while in buffered aqueous solution (for a more detailed discussion refer to Section 6.8). Several experiments were undertaken to establish whether this loss of activity could have been due to racemisation. The stability of this compound on silica was investigated. A sample of 3.1 with an ORD measurement of \(-52 \pm 5^\circ\) \((c\ 0.3,\ \text{acetonitrile})\) was passed down a silica column to give a sample with a rotation of \(-57 \pm 5^\circ\) \((c\ 0.2,\ \text{acetonitrile})\). A similar result was observed for another \(\alpha\)-ketoester 3.5, which had an initial rotation of \(-20 \pm 2^\circ\) \((c\ 1.3,\ \text{acetonitrile})\). After column chromatography the rotation of a solution of 3.5 was \(-18 \pm 2^\circ\) \((c\ 1.3,\ \text{acetonitrile})\). It was evident that the peptidyl-\(\alpha\)-ketoesters did not racemise on silica under these conditions.

The pH-dependence of the stability of 3.1 was investigated by \(^1\text{H}\) NMR studies in \(d_3\)-acetonitrile/\(D_2O\) (2:1) (see Chart 5.2 for results). One solution was prepared with \(D_2O\) that had been acidified to pH 3. The aqueous component of the second solution was buffered to pH 7.8 with HEPES buffer. The components of the buffered \(D_2O\) were at the same concentration as the enzyme assay buffer solution, i.e. HEPES 0.1 M, calcium chloride 0.02 M, Triton X-100 0.05% w/v. The integral corresponding to the proton adjacent to the electrophilic ketone (\(\beta\)-proton) was monitored over time. There were actually four species in solution, due to isomerisation and hydrate formation as discussed in Section 5.2, and the integral of the \(\beta\)-proton was measured as the sum of the integrals of these four species. A reduction in the value of this integral sum relative to the integral of one aromatic proton would indicate that the proton was exchanging with deuterium from the solvent. This exchange would imply that the stereocentre was scrambling and that 3.1 was undergoing racemisation.

The results of this investigation are presented in Chart 5.2 for which the proportion of \(\beta\)-proton relative to one aromatic proton remaining by \(^1\text{H}\) NMR is plotted against time. The solution containing 3.1 at pH 3 did not display any measurable deuterium exchange over thirty hours (crimson line in Chart 5.2). For the solution of 3.1 at pH 7.8 in the presence of HEPES buffer approximately one third of the \(\beta\)-proton had experienced deuterium exchange after 30 h. This result is consistent with the
finding of Harbeson et al that α-ketoamides epimerised in general base conditions and this rate of epimerisation was increased by high concentrations of buffer. Epimerisation was not observed in slightly acidic solutions of the α-ketoamide.\(^{2a}\)

![Proportion of proton remaining vs. time](chart.png)

**Chart 5.2** The pH-dependence of the racemisation of 3.1 over time.

Preliminary isomerisation studies of 3.1 were undertaken without a water filter between the sample and the light source. After a sample of 3.1 in \(d_3\)-acetonitrile was irradiated for one hour with a wavelength filter (330<\(\lambda<370\) nm) the \(^1\)H NMR showed that the integral for the β-proton was considerably reduced. When the experiment was repeated with a water filter (10 mm thickness) there was no evidence of β-proton loss by \(^1\)H NMR. It was assumed that the cause of the increased rate of racemisation on irradiation was the sample heating due to infrared absorption by water in the sample. A similar effect was observed with the target compounds 3.2 and 3.3, which have similar structure to 3.1. The other target compounds (type A, type C) did not show any sign of racemisation resulting from irradiation.

The compromised sample of 3.1 was examined further in order to quantify the extent of deuteration. By \(^1\)H NMR the proportion of the original value of the integral of the β-proton that remained after irradiation was 23 ± 10%. The optical rotation of the sample was \(-20 ± 3^\circ\) (c 0.2, acetonitrile). Comparing the ORD value of the racemised sample with that of the initial solution, \(-57 ± 5^\circ\) (c 0.2, acetonitrile), led to a result of 32 ± 8% non-deuterated β-proton. The final estimation of the extent of deuteration was
made by a mass spectrometry isotopic incorporation calculation. This technique compares the height of the peaks for a non-deuterated sample with the peak heights of the deuterated sample. This technique gave a figure of 24% singly deuterated fragment and 76% non-deuterated fragment for the fragment ion $M^+ = 328$. This figure of 76% non-deuterated $\beta$-proton is considerably higher than the results from ORD and NMR, and should be considered the most accurate of the three results because of the errors associated with the ORD and NMR measurements.

From the research on the racemisation of 3.1 a number of conclusions can be drawn. Flash chromatography on silica is a reliable method for the purification of peptidyl-$\alpha$-ketoamides and peptidyl-$\alpha$-ketoesters as advocated by Wasserman.\textsuperscript{15b} The target compounds 3.1, 3.2 and 3.3 were most prone to hydration and appear at this stage to be more susceptible to racemisation upon irradiation with UV light in the absence of a water filter and in an aqueous solution containing HEPES buffer at pH7.8 than other target compounds. Because of the racemisation of 3.1 that occurred in conditions approximating the conditions of the enzyme assay, the activity of 3.1 against $\alpha$-chymotrypsin would be expected to change on incubation in HEPES buffer at pH 7.8.
Chapter Five: Isomerisation and Hydration Studies of the Target Compounds

5.5 Summary of Chapter Five

The hydration, photoisomerisation and racemisation of the target compounds were investigated. Hydration studies of 3.1 showed that this compound was readily hydrated in solutions containing water. The gem-diol moiety of 5.9 (hydrate of 3.1) was observed by $^1$H NMR and $^{13}$C NMR. It is probable that a solution of 3.1 in water would be completely hydrated, as suggested for literature examples of $\alpha$-ketoesters and $\alpha$-ketoamides. Evidence of hydrate formation was gathered for compounds of the type A (2.4) and compounds of the type C (4.2). It appears that these types are less prone to hydration than compounds of the type B (3.1). The photoisomerisation of the target compounds was studied and trends were observed according the substitution of the azobenzene moiety. For example, the most efficient switching was observed for azobenzenes substituted in the para position with methylene groups and poor results were observed for azobenzenes substituted with a para ketone. Racemisation studies showed that 3.1 was prone to deuteration in a HEPES buffer solution at pH 7.8 and upon irradiation with UV light in the absence of a water filter.

5.6 References for Chapter Five


CHAPTER SIX

ENZYME INHIBITION STUDIES
OF THE TARGET COMPOUNDS
6.1 Introduction

Each of the target compounds was assayed against α-chymotrypsin. The active compounds were converted to the ambient light PSS and the UV light PSS and the level of α-chymotrypsin inhibition was measured for each PSS. The aims of this study were to measure the extent of inhibition by the compounds, to determine the type of inhibition and to quantify the difference in activity between the (E) and (Z) isomers. In the next section the enzyme kinetics and the methods for analysis of the data are discussed. In following sections the activity results and the mode of inhibition for the target compounds are grouped by the compound type.

6.2 Michaelis-Menten kinetics

![Figure 6.1 Initial rate dependence on substrate concentration](image)

A typical plot of the initial rate of an enzyme-catalysed reaction versus substrate concentration (Figure 6.1) shows saturation behaviour. The term “saturation” means
that the initial rate of the reaction approaches a limiting rate, $V_{\text{max}}$, at high substrate concentration. The limiting rate cannot be achieved in practice, regardless of the substrate concentration, because it is the theoretical optimum operating rate of the enzyme that could only be reached at infinite substrate concentration. A model for the observed kinetic behaviour of an enzyme-catalysed reaction is shown in Equation 6.1. In this model, the reversible binding of substrate (S) with the enzyme (E) gives the enzyme-substrate complex (ES), which undergoes conversion to give product (P) and regenerated enzyme.

\[
\begin{align*}
E + S & \xrightarrow{k_1} ES \quad \xrightarrow{k_2} E + P
\end{align*}
\]  

Equation 6.1

If it is assumed that the concentration of the enzyme-substrate complex is static throughout the course of the reaction (except of course at the very start of the reaction), the resulting kinetic equations lead to the Michaelis-Menten equation (Equation 6.2, for a derivation see Appendix 1). The Michaelis-Menten equation is the fundamental equation of enzyme kinetics. The important variables in this equation are the limiting rate ($V_{\text{max}}$) and the Michaelis-Menten constant ($K_M$). The limiting rate is equal to the product of the total enzyme concentration ($[E]_T = [E] + [ES]$) and the rate constant $k_2$, which is known as the catalytic constant. Because of the contribution of the catalytic constant to the limiting rate, the limiting rate is a measure of the catalytic activity of the enzyme. The Michaelis-Menten constant quantifies the binding efficiency of the substrate with the enzyme since its value specifies the relative concentrations of free enzyme, free substrate and enzyme-substrate complex. $K_M$ is not actually an equilibrium constant since it defines the concentrations under steady-state conditions rather than at equilibrium.
Chapter Six: Enzyme inhibition studies of the target compounds

\[ V_0 = \frac{V_{\text{max}}[S]}{K_M + [S]} \]

where \[ K_M = \frac{k_1 + k_2}{k_1} \] \hspace{1cm} \text{Equation 6.2}

and \[ V_{\text{max}} = [E]_T k_2 \]

\[ \frac{1}{V_0} = \frac{1}{V_{\text{max}}} + \left( \frac{K_M}{V_{\text{max}}} \right) \left( \frac{1}{[S]} \right) \]

\hspace{1cm} \text{Equation 6.3}

The values that are sought in investigations of enzyme systems are the limiting rate and the Michaelis-Menten constant. By graphical means, the values are obtained from linearised versions of the substrate curve. The most common linear plot of the Michaelis-Menten equation is the double reciprocal (Lineweaver-Burk) plot.\(^1\) From a reformulation (Equation 6.3) of the Michaelis-Menten equation, it is evident that when \( 1/V_0 \) is plotted against \( 1/[S] \), the kinetic parameters, \( K_M \) and \( V_{\text{max}} \), are obtained from the axis intercept values (as shown in Figure 6.2a). The double reciprocal plot, in spite of its popularity and its consequent ease of interpretation, is flawed in that experimental errors in small values of \( V_0 \) and \([S]\) are exaggerated. From the three possible ways to plot the Michaelis-Menten equation as a straight line, the double reciprocal plot gives the least accurate values for the kinetic parameters. The alternative plots are \([S]/V_0\) against \([S]\) and \( V_0 \) against \( V_0/[S] \) (the Eadie-Hofstee plot).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.2.png}
\caption{a. The double reciprocal plot \hspace{1cm} b. A modified direct linear plot}
\end{figure}
An alternative type of graphical analysis to the linear plots is the modified direct linear plot, which is shown in Figure 6.2b, and comes from the Michaelis-Menten equation reformulation Equation 6.4. For the purposes of the direct linear plot $1/V_0$ and $[S]/V_0$ are treated as constant and are the y- and x-intercepts respectively for each of a series of lines. Each point on a line is a $(1/V_{\text{max}}, K_M/V_{\text{max}})$ data pair that is consistent with the observation $1/V_0$ and $[S]/V_0$. The points of intersection of these lines provide estimates for $1/V_{\text{max}}$ and $K_M/V_{\text{max}}$. For n lines, or n observations at different substrate concentrations, at best $\sum_{1}^{n} (n-1)$ estimates will be given and it is common practise to use the median value for the best estimate.

$$\frac{1}{V_{\text{max}}} = \frac{1}{V_0} - \left( \frac{1}{[S]} \right) \left( \frac{K_M}{V_{\text{max}}} \right)$$

Equation 6.4

The observed effect that the presence of inhibitor has on the initial rate of an enzyme falls mainly into one of the four categories: competitive, uncompetitive, mixed and noncompetitive inhibition. The categories are assigned on the basis of observed initial rate data and do not necessitate a particular mechanism. There is, however, a mechanism for each category that deals with the simplest possible scenario consistent with the observed kinetics. Full details on the mechanisms and corresponding plots are given in Appendix 2. In this section, the expected observations, mechanisms and graphical results for competitive inhibition only are given. This is because the peptidyl-\(\alpha\)-ketoesters and peptidyl-\(\alpha\)-ketoamides that have been reported in the literature as inhibitors of serine and cysteine proteases have exclusively given competitive inhibition.\(^2\)

$$E \quad S \overset{k_1}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} E + P$$

Equation 6.5

The characteristic feature of competitive inhibition is an increase in $K_M$ and no change in $V_{\text{max}}$ as the inhibitor concentration increases. One mechanism devised to explain these observations (Equation 6.5) is that inhibitor binding to the active site is in
competition with substrate binding. The corollary is that increasing the substrate concentration will act to “wash out” the inhibitor from the active site. A competitive inhibitor decreases the apparent affinity of the substrate for the enzyme giving a decreased $K_M$, but does not affect the reactivity of the ES complex once formed, so $V_{\text{max}}$ is unchanged.

$$V_i = \frac{V_{\text{max}}[S]}{K_M(1 + [I]/K_i) + [S]} \quad \text{Equation 6.6}$$

$$\frac{1}{V_i} = \frac{1}{V_{\text{max}}} + \left( \frac{K_M}{V_{\text{max}}} \right) \left( \frac{1}{[S]} \right) \left( 1 + \frac{[I]}{K_i} \right) \quad \text{Equation 6.7}$$

The rate equation for a competitive inhibitor (Equation 6.6) is derived from the simple model given in Equation 6.5. The double-reciprocal reformulation (Equation 6.7) of the rate equation is directly analogous to the case in the absence of inhibitor (Equation 6.3), however the observed limiting rate ($V_{\text{max}}^{\text{app}}$) and Michaelis-Menten constant ($K_M^{\text{app}}$) are dependent on the inhibitor concentration i.e. $K_M^{\text{app}} = K_M(1+[I]/K_i)$ and $V_{\text{max}}^{\text{app}} = V_{\text{max}}(1+[I]/K_i)$. A double reciprocal plot for a series of substrate, rate data sets at $n$ different inhibitor concentrations yields $n$ lines, which intersect the $1/V_i$ axis at the same point and intersect the $1/[S]$ axis at different points. Inputting a fitted data point from a line on the plot into Equation 6.7 gives an estimate of $K_i$ for that line. Of course, the same reservations discussed above on the poor accuracy of the double-reciprocal plot hold for kinetic parameters derived from this type of analysis.

An alternative set of plots which yield inhibition constants and are diagnostic for inhibition type (in tandem) are the Dixon and modified-Dixon plots. These plots avoid at least some of the inaccuracy incurred by the double reciprocal plot. The Dixon plot has $1/V_i$ on the ordinate axis, which gives some degree of inaccuracy. The ordinate axis of the modified Dixon, on the other hand, has $[S]/V_i$, the variance of which does not vary greatly with $V_i$ if $V_i$ is distributed uniformly. The typical Dixon and modified Dixon plots for a competitive inhibitor are given in Figure 6.3 and the plots for other inhibition types are detailed in Appendix 2. From Equation 6.7 at any value of $[I]$ the
value of $1/V_i$ varies with $[S]$ unless $(1 + K_i) = 0$. Therefore a Dixon plot ($1/V_i$ against $[I]$, Figure 6.3a) and at $n$ substrate concentrations gives $n$ lines which intersect at points for which $[I] = K_i$ holds true. Therefore, each intersection provides an estimate for the inhibition constant. The corresponding modified Dixon plot ($[S]/V_i$ against $[I]$) for a competitive inhibitor (Figure 6.3b) has a set of parallel lines.

**Figure 6.3**  
*a.* The Dixon plot of a competitive inhibitor  
*b.* The modified Dixon plot of a competitive inhibitor
6.3 Assay design, assay calibration and the peculiarities of acetonitrile in the assay medium

Scheme 6.1 The cleavage of succ-Ala-Ala-Pro-Phe-p-NA by α-chymotrypsin

The activity of α-chymotrypsin was measured by monitoring the formation of p-nitroaniline from the enzyme-catalysed hydrolysis of succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Scheme 6.1). The absorbance of p-nitroaniline was measured at $\lambda = 404$ nm for five minutes from the time of mixing to obtain a value for the initial rate. The assay procedure was developed from the technique described by Geiger.\textsuperscript{4} Two major alterations were made to this technique. The buffer was changed from TRIS to HEPES in accordance with the procedure developed for peptidyl-α-ketoesters by Angelastro et al.\textsuperscript{5} The order of solution addition to the assay cuvette was also changed. In the technique of Geiger, enzyme and inhibitor were pre-incubated before the addition of substrate. This order of addition to the cuvette was changed so that true competition between the substrate and inhibitor for the enzyme was being measured. In the altered
assay substrate and inhibitor were incubated and the assay was instigated by the addition of enzyme.

A plot of absorbance against time (slope equals rate of reaction) gave a straight line over the entire time course in almost all cases and the slope was used as the initial rate measurement. The exceptions were the assays with very low substrate concentrations, for which the initial rate was determined from the slope of the initial straight line period of the curve. The initial rate values were processed by double reciprocal and direct linear graphical analyses.

The kinetic parameters, $K_M$ and $V_{max}$, were determined from rate measurements at six substrate concentrations. The parameters were calculated by double reciprocal plot and a modified direct linear plot. As can be seen in Table 6.1a, the results from these methods are in good agreement. The value denoted $R^2$ is a measure of the goodness of fit for the line in the double reciprocal plot. A value of unity indicates a data set with an ideal fit to the model. The double reciprocal plot for the determination of $K_M$ and $V_{max}$ is presented in Figure 6.4.

<table>
<thead>
<tr>
<th>Double reciprocal</th>
<th>Direct linear</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$ (µM)</td>
<td>$V_{max}$ (µmol s$^{-1}$mg$^{-1}$)</td>
</tr>
<tr>
<td>48</td>
<td>1200</td>
</tr>
</tbody>
</table>

Table 6.1a Kinetic parameters for the assay
Figure 6.4 Double reciprocal plot for $V_{\text{max}}$ and $K_M$ determination in the absence of acetonitrile.

The peptidyl-$\alpha$-ketoester 2.52 was reported by Angelastro et al. as an inhibitor of $\alpha$-chymotrypsin ($K_i = 0.13 \, \mu\text{M}$).\(^5\) Angelastro et al. determined the inhibition constant with rate measurements at two inhibitor concentrations. The data was inputted into Equation 6.7 to give two approximations for the inhibition constant, the average of which was used as the inhibition constant.

![Chemical Structure of Compound 2.52]
Target compound 2.52 was synthesised (as discussed in Section 2.7) as a standard for the calibration of the enzyme assay relative to the reported data. The compound was analysed for bioactivity by the same method as Angelastro et al., except that the inhibitor was stored as a stock acetonitrile solution rather than a stock buffer solution. It was discovered that upon storage in buffer (HEPES) solution 2.52 became less active against α-chymotrypsin (see Table 6.2) whereas 2.52 in acetonitrile retained its activity against α-chymotrypsin for several weeks. The calculated inhibition constant of 2.52 (K_i = 0.17 μM) was comparable with the literature value. This result implies that the kinetic parameters calculated from this assay for each of the target compounds are able to be compared meaningfully, in magnitude at least, with the values reported by Angelastro et al..

<table>
<thead>
<tr>
<th>Time in solution</th>
<th>Solvent</th>
<th>[S] µM</th>
<th>[I] µM</th>
<th>V_i</th>
<th>V_i/V_o</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>acetonitrile</td>
<td>0.48</td>
<td>0.6</td>
<td>810</td>
<td>0.58</td>
</tr>
<tr>
<td>24 h</td>
<td>acetonitrile</td>
<td>0.48</td>
<td>0.6</td>
<td>840</td>
<td>0.61</td>
</tr>
<tr>
<td>5 min</td>
<td>water (HEPES)</td>
<td>0.48</td>
<td>1.7</td>
<td>560</td>
<td>0.41</td>
</tr>
<tr>
<td>40 min</td>
<td>water (HEPES)</td>
<td>0.48</td>
<td>1.7</td>
<td>680</td>
<td>0.48</td>
</tr>
<tr>
<td>24 h</td>
<td>water (HEPES)</td>
<td>0.48</td>
<td>1.7</td>
<td>1020</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Table 6.2 The activity of 2.52 against α-chymotrypsin after incubation in acetonitrile or HEPES buffer (0.1 M): Units for V_i: μmols⁻¹mg⁻¹.

As described for 2.52, each target compound was stored in an organic solvent during testing, since a solution of an inhibitor in water was expected to lose activity against α-chymotrypsin over time. Consequently, the assays were run with increasing acetonitrile concentration as inhibitor concentration increased. At the completion of the enzyme assays for the target compounds, it was found that the presence of even a small proportion of acetonitrile increased the rate of substrate turnover relative to an entirely aqueous system. This activation of α-chymotrypsin was unexpected and somewhat peculiar. A discussion of enzyme activation, in which activation kinetics are developed, is given by Cornish-Bowden.⁶
In order to establish a measure of the effect of acetonitrile on the enzyme activity, the kinetic parameters, $K_M$ and $V_{\text{max}}$, were measured at acetonitrile concentrations of 1.9% and 5.7% v/v. As shown in Table 6.1b both $K_M$ and $V_{\text{max}}$ increase as the proportion of acetonitrile in the assay medium increases. The effect of the changing $K_M$ and $V_{\text{max}}$ with increasing inhibitor concentration was minimised in the calculations of the inhibition constant from the double reciprocal plots because the $K_i$ calculation at each inhibitor concentration required the input of $K_M$, $V_{\text{max}}$ and $V_0$, each of which were acetonitrile-concentration dependent. However the Dixon and modified Dixon plots did not allow an adjustment for this aberration and as a consequence returned less reliable results. In future kinetic investigations on related compounds, the concentration of acetonitrile in the assay should be kept constant at the concentration required for the highest inhibitor concentration.

<table>
<thead>
<tr>
<th>MeCN (v/v)</th>
<th>Double reciprocal</th>
<th>Direct linear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (µM)</td>
<td>$V_{\text{max}}$ (µmols$^{-1}$mg$^{-1}$)</td>
</tr>
<tr>
<td>0.0%</td>
<td>48</td>
<td>1200</td>
</tr>
<tr>
<td>1.9%</td>
<td>96</td>
<td>1400</td>
</tr>
<tr>
<td>5.7%</td>
<td>91</td>
<td>1700</td>
</tr>
</tbody>
</table>

Table 6.1b Kinetic parameters for the $\alpha$-chymotrypsin assay in the presence of varying amounts of acetonitrile as determined by double reciprocal and direct linear analyses.
6.4 A representative data set: Full kinetic data for 3.2

For each of the target compounds described in this thesis, preliminary assays against α-chymotrypsin were performed in order to check for enzyme inhibition. For the successful inhibitors large data sets (5 × [S], 5 × [I]) were obtained in duplicate for the PSS composition resulting from ambient light. These data sets were fitted to the Michaelis-Menten model with double-reciprocal, direct linear, Dixon and modified Dixon plots. From these analyses the type of inhibition was determined and the kinetic parameters $K_{M,\text{app}}, V_{\text{max,app}}$ and $K_i$ were calculated.

Since the compositions of the ambient light and visible light PSS were comparable (see Section 5.3), the inhibition of α-chymotrypsin by the ambient light PSS was used as an approximation for the inhibition of the enzyme by the visible light PSS. This approximation was tested for each of the active inhibitors by cross-checking the extent of inhibition at two inhibitor concentrations for the ambient light PSS with the results for the visible light PSS.

The data sets for the UV light PSS (4 × [S], 3 × [I]) were smaller than the data sets obtained for the ambient light PSS. Such a data set allowed reliable analyses with double reciprocal and direct linear analyses only. A large data set is required for the determination of inhibition type using Dixon and modified Dixon plots. The smaller sets were used for the UV light PSS in order to save time and resources, considering that the type of inhibition was already determined for each mixture of isomers, albeit at different ratios.
Figure 6.4  Double reciprocal plot for the ambient light PSS of 3.2

Figure 6.5  A representative direct linear plot for which [3.2] = 0.45 μM
Chapter Six: Enzyme inhibition studies of the target compounds

**Figure 6.6** Dixon plot for the ambient light PSS of 3.2

**Figure 6.7** Modified Dixon plot for the ambient light PSS of 3.2
The plots for the ambient light PSS of 3.2 are presented in Figures 6.4–6.7. The type of inhibition for this mixture of isomers is clearly competitive, because of the structure of the Dixon and modified Dixon plots. The lines of the Dixon plot (Figure 6.6) all intersect in the fourth quadrant, giving an estimate for the inhibition constant and the lines of the corresponding modified Dixon plot (Figure 6.7) are parallel. The kinetic parameters for the inhibition of \( \alpha \)-chymotrypsin by the ambient light PSS of 3.2 are given in Table 6.2. Both the direct linear and double reciprocal results show that \( K_M^{\text{app}} \) increases and \( V_{\text{max}}^{\text{app}} \) remains the same as inhibitor concentration increases, which is indicative of competitive inhibition.

<table>
<thead>
<tr>
<th>[I]</th>
<th>MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
<th>Dixon</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu M )</td>
<td>v/v</td>
<td>( K_M^{\text{app}} ) mM</td>
<td>( V_{\text{max}}^{\text{app}} )</td>
<td>( V_{\text{max}}^{\text{app}} )</td>
</tr>
<tr>
<td>0.11</td>
<td>0.5%</td>
<td>0.13</td>
<td>1100</td>
<td>0.13</td>
</tr>
<tr>
<td>0.22</td>
<td>1.0%</td>
<td>0.22</td>
<td>1100</td>
<td>0.22</td>
</tr>
<tr>
<td>0.34</td>
<td>1.4%</td>
<td>0.31</td>
<td>1200</td>
<td>0.25</td>
</tr>
<tr>
<td>0.45</td>
<td>1.9%</td>
<td>0.39</td>
<td>1200</td>
<td>0.41</td>
</tr>
<tr>
<td>0.56</td>
<td>2.4%</td>
<td>0.39</td>
<td>1100</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Table 6.2 The kinetic parameters for the inhibition of \( \alpha \)-chymotrypsin by the ambient light PSS of 3.2. Units for \( V_{\text{max}}^{\text{app}} \): \( \mu \text{mols}^{-1} \text{mg}^{-1} \)

The inhibition constant from the Dixon plot (\( K_i = 0.12 \) \( \mu M \)) is higher than the inhibition constants from the double reciprocal plot (mean \( K_i = 0.08 \) \( \mu M \)). A difference in the results from these two analytical methods was expected because only the double reciprocal analysis could take into account the fact that \( K_M \) and \( V_{\text{max}} \) increased as the acetonitrile concentration increased. A second point about these analytical methods is that both the Dixon plot and the double reciprocal plot would exaggerate errors in the rate measurements. The conclusion to be drawn is that the double-reciprocal analysis, although flawed, probably provides the best data in this situation. The activity of the
ambient light PSS of 3.2 by the double reciprocal plot (mean $K_i = 0.08 \ \mu M$) is comparable to the activity of the inhibitor standard 2.52 ($K_i = 0.17 \ \mu M$).

The results from the UV light PSS of 3.2 are tabulated below (Table 6.3). The trend for $K_M$ and $V_{max}$ as inhibitor concentration increases corresponds with competitive inhibition as above, although the values for $K_M$ at $[I] = 0.45 \ \mu M$ (i.e. 2.13 & 2.18 mM) and the values for $V_{max}$ at $[I] = 0.45 \ \mu M$ (i.e. 3400 & 3400 \mu mols^{-1}mg^{-1}$) are higher than expected for the trend. The inhibition constant from the double reciprocal plot (mean $K_i = 0.04 \ \mu M$) is two-fold lower than the inhibition constant from the double reciprocal analysis for the ambient light PSS, therefore the (Z) isomer is a more active inhibitor than the (E) isomer. The inhibition results for 3.2 combined with the photoisomerisation data for 3.2 are given in Section 6.5 with the other compounds of the type B.

<table>
<thead>
<tr>
<th>[I] MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
</tr>
</thead>
<tbody>
<tr>
<td>[\mu M] v/v $K_M^{\text{app}}$ [mM] $V_{max}^{\text{app}}$ [mmols$^{-1}$mg$^{-1}$]</td>
<td>$K_M^{\text{app}}$ [mM] $V_{max}^{\text{app}}$ [mmols$^{-1}$mg$^{-1}$]</td>
<td>$R^2$</td>
</tr>
<tr>
<td>0.11 0.5% 0.26 1300</td>
<td>0.24 1300</td>
<td>0.96</td>
</tr>
<tr>
<td>0.22 1.0% 0.43 1400</td>
<td>0.43 1400</td>
<td>0.97</td>
</tr>
<tr>
<td>0.45 1.9% 2.13 3400</td>
<td>2.18 3400</td>
<td>0.99</td>
</tr>
</tbody>
</table>

**Table 6.3** The kinetic parameters for the inhibition of $\alpha$-chymotrypsin by the UV light PSS of 3.2. Units for $V_{max}^{\text{app}}$: \mu mols$^{-1}$mg$^{-1}$.

<table>
<thead>
<tr>
<th>[S] [mM]</th>
<th>[I] [\mu M]</th>
<th>$V_i$ (visible light PSS)</th>
<th>$V_i$ (ambient light PSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>0.22</td>
<td>470</td>
<td>480</td>
</tr>
<tr>
<td>0.20</td>
<td>0.45</td>
<td>340</td>
<td>350</td>
</tr>
</tbody>
</table>

**Table 6.4** The rates of substrate turnover by $\alpha$-chymotrypsin inhibited with the visible light PSS of 3.2 and the ambient light PSS of 3.2.

A sample of 3.2 at the visible light PSS was assayed against $\alpha$-chymotrypsin to test the assumption that the visible light PSS and ambient light PSS have the same effect.
on the enzyme activity. As shown in Table 6.4 both of the photostationary states give the same level of inhibition. Therefore, the inhibition data from the ambient light PSS of 3.2 is a good approximation for the inhibition data from the visible light PSS of 3.2. Similar results were obtained for each of the target compounds, except of course compound 3.3, for which the UV light and ambient light photostationary states do not correlate.

6.5 Inhibition of α-chymotrypsin by compounds of the type A

![Chemical structures](E)-2.1 and (E)-2.2

The kinetic data for the ambient light PSS of 2.1 is presented in Table 6.5. Both the direct linear and double reciprocal plots showed the trend of increasing apparent $K_M$ and stable apparent $V_{max}$ with increasing inhibitor concentration, which is indicative of competitive inhibition. Note that the direct linear data point at $[I] = 37 \mu M$ (i.e. $K_{M}^{app} = 0.10 \text{ mM}$, $V_{max}^{app} = 800 \text{ \mu mol s}^{-1} \text{mg}^{-1}$) does not fit the trend. There was no reliable information available from the Dixon or modified Dixon plots, presumably because these plots cannot take account for the fact that $K_M$ and $V_{max}$ increase as the organic solvent concentration increases. Using the full data set the Dixon plot returned a negative value for the best estimate of $K_i$, however, when the outlying data set for $[S] = 0.24 \text{ mM}$ was disregarded, more consistent values for the inhibition parameters were returned ($K_i = 5.5 \mu M$, $V_{max}^{app} = 1100 \text{ \mu mol s}^{-1} \text{mg}^{-1}$).

The UV light PSS of 2.1 gave the values shown in Table 6.6 when assayed against α-chymotrypsin. These results were poor with low line-fit values and low apparent $K_M$ and $V_{max}$ values. The inhibition constant for the UV light PSS of 2.1 (mean $K_i = 4.2 \mu M$) was more than twofold lower than the inhibition constant for the ambient
light PSS (mean $K_i = 4.2 \mu M$), which indicates that the (Z) isomer is more active against the enzyme than the (E) isomer.

![Table 6.5](image)

**Table 6.5** The kinetic parameters for the inhibition of $\alpha$-chymotrypsin by the ambient light PSS of 2.1. Units for $V_{\text{max}}^{\text{app}}$: $\mu$mol s$^{-1}$ mg$^{-1}$

![Table 6.6](image)

**Table 6.6** The kinetic parameters for the inhibition of $\alpha$-chymotrypsin by the UV light PSS of 2.1. Units for $V_{\text{max}}^{\text{app}}$: $\mu$mol s$^{-1}$ mg$^{-1}$.

When the ambient light PSS of 2.2 was tested against $\alpha$-chymotrypsin, $V_{\text{max}}^{\text{app}}$ was found to rise dramatically as a function of inhibitor concentration (**Table 6.7**). This behaviour is not consistent with competitive inhibition. From the four inhibition types discussed in Appendix 1 the only type that allows for increasing $V_{\text{max}}^{\text{app}}$ with increasing inhibitor concentration is mixed inhibition. However, the modified Dixon plot for the ambient light PSS of 2.2 is not consistent with mixed inhibition. Interestingly, the results for the UV light PSS of 2.2 strongly supported competitive kinetics; i.e. $V_{\text{max}}$ stayed constant as inhibitor concentration increased and the modified Dixon plot gave
parallel lines (Figure 6.8). The results from 2.2 suggest that two different PSS compositions of the same isomers can have different inhibition constants and different mechanisms.

<table>
<thead>
<tr>
<th>[I]</th>
<th>DMSO v/v</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
<th>Dixon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[M]</td>
<td>[K_M] (app) mM</td>
<td>[V_max] (app)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.0%</td>
<td>0.3</td>
<td>1500</td>
<td>0.3</td>
</tr>
<tr>
<td>18</td>
<td>1.4%</td>
<td>0.7</td>
<td>2400</td>
<td>0.6</td>
</tr>
<tr>
<td>24</td>
<td>1.9%</td>
<td>1.0</td>
<td>2800</td>
<td>1.3</td>
</tr>
<tr>
<td>30</td>
<td>2.4%</td>
<td>3.6</td>
<td>7500</td>
<td>3.2</td>
</tr>
<tr>
<td>36</td>
<td>2.9%</td>
<td>4.9</td>
<td>9600</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 6.7 The kinetic parameters for the inhibition of α-chymotrypsin by the ambient light PSS of 2.2. Units for $V_{\text{max}}^{\text{app}}$: μmol s$^{-1}$ mg$^{-1}$.
Chapter Six: Enzyme inhibition studies of the target compounds

Table 6.8  The kinetic parameters for the inhibition of α-chymotrypsin by the UV light PSS of 2.2. Units for $V_{\text{max}}^{\text{app}}$: μmols$^{-1}$mg$^{-1}$.

<table>
<thead>
<tr>
<th>[I]</th>
<th>DMSO</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>v/v</td>
<td>$K_M^{\text{app}}$ mM</td>
<td>$V_{\text{max}}^{\text{app}}$</td>
</tr>
<tr>
<td>12</td>
<td>1.0%</td>
<td>0.16</td>
<td>920</td>
</tr>
<tr>
<td>18</td>
<td>1.4%</td>
<td>0.19</td>
<td>870</td>
</tr>
<tr>
<td>24</td>
<td>1.9%</td>
<td>0.26</td>
<td>880</td>
</tr>
</tbody>
</table>

The kinetic results for the ambient light PSS of 2.3 (Table 6.10) show typical behaviour for competitive inhibition, with the exception of the [I] = 12.2 μM dataset. The Dixon graph has a closely sited set of intersections, giving an inhibition constant ($K_i = 6.1$ μM) that is comparable in magnitude to the value derived from the double reciprocal plot ($K_i = 2.7$ μM). The UV light PSS of 2.3 (Table 6.9) gave a lower value for the inhibition constant by the double reciprocal plot ($K_i = 1.3$ μM), which indicates that the (Z) isomer is a stronger inhibitor of α-chymotrypsin than the (E) isomer.
Chapter Six: Enzyme inhibition studies of the target compounds

<table>
<thead>
<tr>
<th>[I]</th>
<th>MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v/v</td>
<td>$K_M^{app}$ mM</td>
<td>$V_{max}^{app}$ mM</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5%</td>
<td>0.12</td>
<td>1000</td>
</tr>
<tr>
<td>4.1</td>
<td>1.0%</td>
<td>0.73</td>
<td>2400</td>
</tr>
<tr>
<td>8.1</td>
<td>1.9%</td>
<td>0.40</td>
<td>1400</td>
</tr>
</tbody>
</table>

Table 6.9 The kinetic parameters for the inhibition of $\alpha$-chymotrypsin by the UV light PSS of 2.3. Units for $V_{max}^{app}$: µmols$^{-1}$mg$^{-1}$.

<table>
<thead>
<tr>
<th>[I]</th>
<th>MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
<th>Dixon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v/v</td>
<td>$K_M^{app}$ mM</td>
<td>$V_{max}^{app}$ mM</td>
<td>$V_{max}^{app}$ mM</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5%</td>
<td>0.13</td>
<td>1300</td>
<td>0.08</td>
</tr>
<tr>
<td>4.1</td>
<td>1.0%</td>
<td>0.10</td>
<td>1000</td>
<td>0.09</td>
</tr>
<tr>
<td>8.1</td>
<td>1.9%</td>
<td>0.16</td>
<td>1000</td>
<td>0.12</td>
</tr>
<tr>
<td>12.2</td>
<td>2.9%</td>
<td>0.37</td>
<td>1300</td>
<td>0.11</td>
</tr>
<tr>
<td>16.2</td>
<td>3.8%</td>
<td>0.19</td>
<td>900</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Table 6.10 The kinetic parameters for the inhibition of $\alpha$-chymotrypsin by the ambient light PSS of 2.3. Units for $V_{max}^{app}$: µmols$^{-1}$mg$^{-1}$

Preliminary assays on the ambient light PSS of 2.4 showed that the compound was markedly less active than the other compounds of the type A and that there would not be sufficient compound available to undergo extensive testing. A crude calculation of the inhibition constant, as used by Angelastro et al., put the inhibition constant (assuming competitive inhibition) in the range of 50 µM to 150 µM. Therefore 2.4 would be approximately 100-fold less active than its analogue 2.3. One would expect about a 5-fold difference in activity between 2.3 and 2.4, corresponding to the difference in activity between 2.1 and 2.2. It must be concluded that another factor has caused the sizeable drop in activity from 2.3 to 2.4. Compound 2.4 was purified by flash chromatography on silica whereas 2.3 was synthesised without the need for subsequent
purification. It is possible that the silica epimerised the stereogenic carbon in the phenylalanine residue of 2.4 leading to a mixture of diastereomers with less activity than the single $L,L$ diastereomer that was synthesised. Such an epimerisation occurred during silica chromatography of peptidyl-$\alpha$-ketoamides prepared by Harbeson et al.$^{2c}$ However, this theory was discounted by the $^{13}$C NMR spectrum of 2.4 after silica chromatography, which showed a single diastereomer.
6.6 Inhibition of α-chymotrypsin by compounds of the type B

The full dataset for the ambient light PSS of 3.1 (as given in Table 6.11) provided a good fit to the Michaelis-Menten kinetics of a competitive inhibitor. The double reciprocal and Dixon plots gave excellent coincidence for the inhibition constant ($K_i = 0.22 \mu M$). The double reciprocal plot for the UV light PSS of 3.1 (results given in Table 6.12) had very good line fit values and gave an average inhibition constant of 0.13 μM. The UV light PSS was therefore 2-fold more active against α-chymotrypsin than the ambient light PSS.

<table>
<thead>
<tr>
<th>[I]</th>
<th>MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
<th>Dixon</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>v/v</td>
<td>$K_M^{app}$ mM</td>
<td>$V_{max}^{app}$</td>
<td>$K_M^{app}$ mM</td>
</tr>
<tr>
<td>0.14</td>
<td>1.0%</td>
<td>0.10</td>
<td>1200</td>
<td>0.10</td>
</tr>
<tr>
<td>0.28</td>
<td>1.9%</td>
<td>0.13</td>
<td>1100</td>
<td>0.14</td>
</tr>
<tr>
<td>0.43</td>
<td>2.9%</td>
<td>0.25</td>
<td>1500</td>
<td>0.26</td>
</tr>
<tr>
<td>0.57</td>
<td>3.8%</td>
<td>0.21</td>
<td>1200</td>
<td>0.24</td>
</tr>
<tr>
<td>0.71</td>
<td>4.8%</td>
<td>0.31</td>
<td>1500</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Table 6.11 The kinetic parameters for the inhibition of α-chymotrypsin by the ambient light PSS of 3.1. Units for $V_{max}^{app}$: μmols^{-1}mg^{-1}
Chapter Six: Enzyme inhibition studies of the target compounds

<table>
<thead>
<tr>
<th>[I]</th>
<th>MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>v/v</td>
<td>K_{M_{app}} mM</td>
</tr>
<tr>
<td>0.14</td>
<td>1.0%</td>
<td>0.15</td>
<td>1200</td>
</tr>
<tr>
<td>0.28</td>
<td>1.9%</td>
<td>0.13</td>
<td>1000</td>
</tr>
<tr>
<td>0.43</td>
<td>2.9%</td>
<td>0.17</td>
<td>1100</td>
</tr>
</tbody>
</table>

Table 6.12 The kinetic parameters for the inhibition of α-chymotrypsin by the UV light PSS of 3.1. Units for \( V_{max_{app}} \): μmols\(^{-1}\)mg\(^{-1}\).

<table>
<thead>
<tr>
<th>[I]</th>
<th>MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
<th>Dixon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>v/v</td>
<td>K_{M_{app}} mM</td>
<td>( V_{max_{app}} )</td>
</tr>
<tr>
<td>0.9</td>
<td>1.0%</td>
<td>0.13</td>
<td>1300</td>
<td>0.12</td>
</tr>
<tr>
<td>1.7</td>
<td>1.9%</td>
<td>0.18</td>
<td>1300</td>
<td>0.12</td>
</tr>
<tr>
<td>2.6</td>
<td>2.9%</td>
<td>0.17</td>
<td>1200</td>
<td>0.15</td>
</tr>
<tr>
<td>3.5</td>
<td>3.8%</td>
<td>0.22</td>
<td>1300</td>
<td>0.20</td>
</tr>
<tr>
<td>4.4</td>
<td>4.8%</td>
<td>0.30</td>
<td>1500</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table 6.13 The kinetic parameters for the inhibition of α-chymotrypsin by the ambient light PSS of 3.3. Units for \( V_{max_{app}} \): μmols\(^{-1}\)mg\(^{-1}\).

<table>
<thead>
<tr>
<th>[I]</th>
<th>MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>v/v</td>
<td>K_{M_{app}} mM</td>
</tr>
<tr>
<td>0.9</td>
<td>1.0%</td>
<td>0.6</td>
<td>2200</td>
</tr>
<tr>
<td>1.7</td>
<td>1.9%</td>
<td>0.5</td>
<td>1600</td>
</tr>
<tr>
<td>3.5</td>
<td>3.8%</td>
<td>1.1</td>
<td>2200</td>
</tr>
</tbody>
</table>

Table 6.14 The kinetic parameters for the inhibition of α-chymotrypsin by the UV light PSS of 3.3. Units for \( V_{max_{app}} \): μmols\(^{-1}\)mg\(^{-1}\).

The ortho substituted azobenzene derivative 3.3 proved to have an inhibition profile (refer to Tables 6.13 and 6.14) that was comparable to both 3.1 (refer to Tables...
6.11 and 6.12) and 3.2 (refer to Tables 6.7 and 6.8). The data for 3.3 fitted the competitive inhibition model and it was found that the (Z)-rich PSS of 3.3 ($K_i$ (mean double reciprocal) = 0.36 μM) was more active against α-chymotrypsin than the (E)-rich PSS of 3.3 ($K_i$ (mean double reciprocal) = 1.2 μM).

The assay results for the ambient PSS for 3.5 show a smooth trend of increasing apparent $K_M$ and static apparent $V_{max}$ with increasing inhibitor concentration by the direct linear and double reciprocal methods. The modified Dixon also supports a competitive mechanism for this mixture of isomers. A large data set was obtained for the UV light PSS of 3.5 in order that reliable Dixon plots could be analysed for both PSS compositions.

<table>
<thead>
<tr>
<th>[I] MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
<th>Dixon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v/v</td>
<td>$K_{M_{app}}$ mM</td>
<td>$V_{max_{app}}$</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0%</td>
<td>0.19</td>
<td>1000</td>
</tr>
<tr>
<td>3.0</td>
<td>1.4%</td>
<td>0.39</td>
<td>1300</td>
</tr>
<tr>
<td>4.1</td>
<td>1.9%</td>
<td>0.37</td>
<td>1100</td>
</tr>
<tr>
<td>5.2</td>
<td>2.4%</td>
<td>0.59</td>
<td>1500</td>
</tr>
<tr>
<td>6.2</td>
<td>2.9%</td>
<td>0.63</td>
<td>1500</td>
</tr>
</tbody>
</table>

Table 6.15 The kinetic parameters for the inhibition of α-chymotrypsin by the ambient light PSS of 3.5. Units for $V_{max_{app}}$: μmols⁻¹mg⁻¹

The trend in inhibition constants from the Dixon plots did not coincide with the inhibition constants from the double reciprocal plots. The Dixon plots had the ambient
light PSS more active than the UV light PSS and the double reciprocal analyses gave the opposite trend. Because the UV light photostationary states were the more active for the other inhibitors tested in this work, it is likely that the values from the double reciprocal plot were more reliable for the 3.5 data sets. Errors in the Dixon plot would likely have arisen from the $K_M$ and $V_{max}$ dependency on acetonitrile concentration.

<table>
<thead>
<tr>
<th>[I]</th>
<th>MeCN</th>
<th>Direct linear</th>
<th>Double Reciprocal</th>
<th>Dixon</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>v/v</td>
<td>$K_M^{app}$ mM</td>
<td>$V_{max}^{app}$</td>
<td>$K_M^{app}$ mM</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0%</td>
<td>0.7</td>
<td>1300</td>
<td>0.78</td>
</tr>
<tr>
<td>3.0</td>
<td>1.4%</td>
<td>1.1</td>
<td>1400</td>
<td>0.99</td>
</tr>
<tr>
<td>4.1</td>
<td>1.9%</td>
<td>1.7</td>
<td>1800</td>
<td>2.1</td>
</tr>
<tr>
<td>5.2</td>
<td>2.4%</td>
<td>1.2</td>
<td>1400</td>
<td>1.3</td>
</tr>
<tr>
<td>6.2</td>
<td>2.9%</td>
<td>0.77</td>
<td>900</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 6.16 The kinetic parameters for the inhibition of α-chymotrypsin by the UV light PSS of 3.5. Units for $V_{max}^{app}$: μmol s⁻¹ mg⁻¹.

When the ambient light PSS of 3.6 was assayed against α-chymotrypsin interesting kinetic behaviour was observed (as in Figure 6.9). It was found that when inhibitor was preincubated with enzyme and the assay was instigated by the addition of substrate there was a slow initial rate, $V_B$ (Case B, Figure 6.9). The rate increased over time and stabilised to a constant rate, $V_S$ after about five minutes. When the enzyme was added to a mixture of inhibitor and substrate the reaction began with a high initial rate, $V_A$ that tended downward over time reaching a steady rate $V_S$ after about five minutes (Case A, Figure 6.9). In both cases the final rate $V_S$ was the same if the same concentrations were used (a dataset is given in Table 6.17).
Chapter Six: Enzyme inhibition studies of the target compounds

Figure 6.9 Kinetic characteristics of a slow, tight binder

Table 6.17 The initial (approximate) and final reaction rates for the inhibition of α-chymotrypsin by the ambient light PSS of 3.6 at the conditions specified in the table. Units for rate: μmol·l⁻¹·mg⁻¹

<table>
<thead>
<tr>
<th>Conditions: [I] = 0.82 μM, [S] = 0.2 mM, [E] = 6.7 nM</th>
<th>Approximation to initial rate (0-100 s)</th>
<th>Final rate (300-600 s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>950</td>
<td>950</td>
</tr>
<tr>
<td>Inhibitor &amp; no pre-incubation</td>
<td>490</td>
<td>170</td>
</tr>
<tr>
<td>Inhibitor with pre-incubation</td>
<td>90</td>
<td>170</td>
</tr>
</tbody>
</table>

The kinetic behaviour described above fits with the behaviour of a "tight-binding" inhibitor. "Tight-binding" inhibition is the delayed onset of inhibition due to slow formation of the enzyme-inhibitor complex. Therefore the high initial rate $V_A$ is equal to the uninhibited initial rate $V_0$ for Case A in Figure 6.9 because at $t = 0$ no enzyme-inhibitor complex has been formed. The reduction in rate over time is explained by the slow formation of enzyme-inhibitor complex to give a final steady state velocity.
In Case B of Figure 6.9 the pre-incubation of enzyme and inhibitor gives sufficient time for the tightly-bound enzyme-inhibitor complex to form and the initial rate results from the residual amount of unbound free enzyme. The rate increases over time as substrate competes with inhibitor for the enzyme active site to reach the steady state rate $V_s$ for the system. "Tight-binding" inhibition has been reported for a number of different electrophilic carbonyl inhibitors of serine proteases such as peptidyl aldehydes, peptidyl fluoromethylketones, and peptidyl $\alpha$-ketoesters. It was theorised that the "tight-binding" inhibition of electrophilic carbonyl inhibitors resulted from a pre-binding equilibrium involving dehydration of the hydrated electrophilic carbonyl moiety. There is considerable evidence from the TFMK inhibitors to support this theory.

Cha investigated the kinetics of "tight-binding" inhibitors and devised kinetic models for the determination of the kinetic parameters of the inhibited reaction. Cha wrote that the inhibition constant for a "tight-binding" inhibitor can be determined from the initial rates of enzyme pre-incubated with inhibitor by the following method. The double reciprocal plot of these measurements should give a pattern that resembles non-competitive inhibition (i.e. a set of lines intersecting at the $1/[S]$ axis as discussed in Appendix 2). A plot of the $1/V_i$ intercepts against $[I]$ then gives a hyperbola that intercepts the $[I]$ axis at $[E] - K_i$. A set of such hyperbola at different values of $[E]$ allows the plotting of $[I]$-intercept versus $[E]$ to obtain a value for the inhibition constant.

In an attempt to apply the analysis described by Cha to the ambient light PSS of 3.6, the initial rates of 3.6 that had been pre-incubated with enzyme were measured. As can be seen in Figure 6.10, the double reciprocal plot resulting from the pre-incubation rate measurements of 3.6 does not at all resemble the pattern for non-competitive inhibition as required by Cha. A reason for this could have been that the rate of change of the reaction rate at the beginning of the reaction was high and the initial rate could not be directly measured due to a necessary mixing time of 20 seconds so only a poor estimate of the true initial rate was obtained. Several different techniques were employed to reduce the mixing time but each proved unsuccessful. As a result the
inhibition constant was not determined for the ambient light PSS of 3.6. In future, a method of extrapolation could be applied to this problem to give a better estimate of the initial rate.\(^\text{10}\)

![Figure 6.10](image)

Figure 6.10  A double reciprocal plot of the pre-incubated ambient light PSS of 3.2

6.7  **Inhibition of \(\alpha\)-chymotrypsin by compounds of the type C**

\[ \text{(E)-4.1} \]

\[ \text{(E)-4.2} \]

\[ \text{(E)-4.3} \]

The target compounds 4.1, 4.2 and 4.3 were found to be inactive at a micromolar level against \(\alpha\)-chymotrypsin. There was no loss of enzyme activity when 4.1 was
present at 260 µM or when 4.2 was present at 90 µM. Compound 4.3 had no effect on the enzyme activity at a concentration of 30 µM.

6.8 Inhibition constants of the target compounds at UV and ambient PSS compositions

In this section the isomerisation data from Section 5.3 and the inhibition data from Sections 6.4 and 6.5 are brought together in a simplified format for easy comparison. The most obvious trends from the inhibition studies were that the (Z) isomer for each inhibitor was more active than the (E) isomer and that the order of activity for the three types of compound synthesised, at the same (E)/(Z) ratio, from the most active to the least active was: B>A>C (compare Table 6.18 with Table 6.19).

Further trends have been examined by considering the data within a compound type (Tables 6.18 and 6.19). The PSS compositions were taken from the $^1$H NMR measurements in Section 5.3. The composition descriptions were simplified by categorising the moieties present in the mixture as either (E) or (Z) regardless of hydration. This measure is valid if it is assumed that the compounds are completely hydrated in an aqueous medium and that there is no change in (E)/Z ratio during hydration. The results from the hydration studies in Section 5.2 and numerous literature examples supported this assumption. The ambient light PSS has been taken as an approximation for the visible light PSS in all cases except for 3.5 (as discussed in Section 5.3). The change in inhibition constant between the ambient light PSS and the UV light PSS was denoted $\Delta K_i$ in the tables and were calculated by difference and by quotient (rounded to the nearest integer).

Table 6.18 contains the details for the compounds of the type A. The most active compound of this series is 2.3, which includes two amino acid residues (L-Leu-L-Phe). The less active inhibitors 2.1 and 2.2 have just one amino acid residue (L-Phe). A comparison of the inhibition constants for the UV light PSS of these three compounds shows that 2.1 and 2.2 have approximately the same inhibition constant (4.2 µM and 3.9 µM respectively) whereas 2.3 is at least three-fold more active (1.3 µM). Thus the UV
Chapter Six: Enzyme inhibition studies of the target compounds

PSS of 2.3 was more active than the single amino acid inhibitors in spite of the fact that the UV PSS of 2.3 comprised comparatively less active (Z) isomer (68%) than the UV PSS of either 2.1 (87%) or 2.2 (81%). In terms of switching efficiency 2.1 has the largest change between PSS ratios (from 20% to 87% of (Z) isomer) and the largest change in inhibition constant (>2 fold).

![Chemical structures](image)

<table>
<thead>
<tr>
<th></th>
<th>PSS (E):(Z)</th>
<th>Percent (Z)</th>
<th>K_i (µM)</th>
<th>ΔK_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 ambient</td>
<td>4:1</td>
<td>20 ± 5</td>
<td>9.6</td>
<td>5.4 µM</td>
</tr>
<tr>
<td>2.1 UV</td>
<td>1:7</td>
<td>87 ± 5</td>
<td>4.2</td>
<td>2-fold</td>
</tr>
<tr>
<td>2.2 ambient</td>
<td>7:3</td>
<td>29 ± 5</td>
<td>4.7</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>2.2 UV</td>
<td>1:4</td>
<td>81 ± 5</td>
<td>3.9</td>
<td>no change</td>
</tr>
<tr>
<td>2.3 ambient</td>
<td>3:1</td>
<td>27 ± 5</td>
<td>2.7</td>
<td>1.4 µM</td>
</tr>
<tr>
<td>2.3 UV</td>
<td>3:7</td>
<td>68 ± 5</td>
<td>1.3</td>
<td>2-fold</td>
</tr>
</tbody>
</table>

**Table 6.18** The inhibition constants for the inhibition of α-chymotrypsin by the UV and ambient light PSS of compounds of the type A.

The results for the compounds of the type B in **Table 6.19** give an indication of the structure-activity relationship for azobenzene substitution. The *meta* isomer 3.2 was the most active, followed by the *para* isomer 3.1 with the *ortho* isomer 3.3 being the least active. The range from the most active to the least active across these isomers with respect to the ambient light PSS was 15-fold. The isomer that showed the best switching
ability with respect to \( \alpha \)-chymotrypsin was 3.3, for which there was a more than 3-fold difference in inhibition constant between the ambient and visible light PSS.

![Chemical structures of compounds](image)

<table>
<thead>
<tr>
<th></th>
<th>PSS ((E):(Z))</th>
<th>Percent ((Z))</th>
<th>(K_i) ((\mu)M)</th>
<th>(\Delta K_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 ambient</td>
<td>3:1</td>
<td>26 ± 5</td>
<td>0.24</td>
<td>0.11 (\mu)M</td>
</tr>
<tr>
<td>3.1 UV</td>
<td>1:3</td>
<td>73 ± 5</td>
<td>0.13</td>
<td>2-fold</td>
</tr>
<tr>
<td>3.2 ambient</td>
<td>3:1</td>
<td>28 ± 5</td>
<td>0.081</td>
<td>0.039 (\mu)M</td>
</tr>
<tr>
<td>3.2 UV</td>
<td>4:5</td>
<td>54 ± 5</td>
<td>0.042</td>
<td>2-fold</td>
</tr>
<tr>
<td>3.3 ambient*</td>
<td>7:3</td>
<td>32 ± 5</td>
<td>1.2</td>
<td>0.84 (\mu)M</td>
</tr>
<tr>
<td>3.3 UV</td>
<td>1:3</td>
<td>75 ± 5</td>
<td>0.36</td>
<td>3-fold</td>
</tr>
<tr>
<td>3.5 ambient</td>
<td>4:1</td>
<td>18 ± 5</td>
<td>0.77</td>
<td>0.48 (\mu)M</td>
</tr>
<tr>
<td>3.5 UV</td>
<td>1:4</td>
<td>78 ± 5</td>
<td>0.29</td>
<td>3-fold</td>
</tr>
</tbody>
</table>

**Table 6.19** The inhibition constants for the inhibition of \( \alpha \)-chymotrypsin by the UV and ambient light PSS of compounds of the type B. * The ambient light PSS of 3.3 does not provide a good approximation for the visible light PSS of 3.3.
The effect of changing the amide $N$-protecting group functionality of 3.1 to an carbamate $N$-protecting group in 3.5 was to improve the switching efficiency in terms of isomer ratio and consequently in terms of change in inhibition constant relative to 3.1. However the carbamate 3.5 was approximately three-fold less active against $\alpha$-chymotrypsin relative to the ambient light PSS than the amide 3.1.

6.9 **Stability of inhibitors in the assay medium: towards an *in situ* reversible assay**

In an *in situ* reversible assay a solution of the photobiological switch and the enzyme is irradiated with UV light. To an aliquot of this solution the substrate is added and the enzyme activity measured. The solution is then irradiated with visible light and another aliquot removed for activity measurement. The whole cycle is repeated a number of times in order to determine the reversibility of the enzyme-photoswitch system. An *in situ* reversible assay was reported for azobenzene-containing inhibitors of $\alpha$-chymotrypsin over three cycles.\(^{12}\)

A requirement for a successful *in situ* assay is that the inhibitor does not lose activity because of the storage conditions. It was observed, however, in the assays of some of the target compounds that the activity of the compounds decreased over time when the compounds were stored in buffer solutions. Experiments were undertaken to investigate this effect by storing the inhibitors in the absence of buffer or in different buffers. In the first experiment, 3.1 was stored in Milli-Q purified water and in HEPES buffer (0.1 M) at rt. Aliquots were taken at times ranging from 5 min to 6 days for assay against $\alpha$-chymotrypsin. The rate of substrate turnover was monitored over 10 min. The results (Figure 6.11) show that the loss of inhibitor activity was considerably faster in the presence of HEPES buffer. Even after 5 min incubation the inhibitor that was dissolved in Milli-Q water was noticeably more active (20% enzyme activity remaining) than the inhibitor that was dissolved in HEPES solution (28% enzyme activity remaining). From this experiment it was determined that the inhibitor slowly became less active when it was stored in water and that HEPES buffer accelerated rate of inhibitor deactivation. The increased rate of loss of activity in the HEPES buffer could
either be due to the presence of HEPES or the change in pH (the Milli-Q water was at pH 4.0 and the HEPES solution was at pH 7.8).

**Figure 6.11** Inhibitor 3.1 loses activity against α-chymotrypsin over time.

**Figure 6.12** Inhibitor 2.4 is most stable in the presence of either water or phosphate buffer
A second experiment provided a comparison of the rates of inhibitor deactivation in different buffers. Three biological buffers (HEPES, TRIS, phosphate) that are commonly used for the analysis of α-chymotrypsin were chosen. Compound 2.4 was incubated in either Milli-Q water, HEPES (0.1 M, pH 7.8), TRIS (0.1 M, pH 7.8) or phosphate buffer (0.1 M HPO₄⁻, pH 7.3) and aliquots from each of these solutions were assayed against α-chymotrypsin at different times. The assay was performed in HEPES buffer (0.1 M), CaCl₂ (0.02 M) and Triton X-100 (0.05% w/v). A problem was caused by the precipitation of Ca₃(PO₄)₂ in the assays from inhibitor/phosphate buffer solutions. The rate of reaction was still able to be measured in spite of the cloudiness of the assay solution, but another set of assays were taken to check the validity of the results. The second set of assays for the inhibitor/phosphate buffer solution that were run in phosphate buffer (0.1 M), KCl (0.03 M) and Triton X-100 (0.05% w/v) gave comparable results to the assays that were run in HEPES buffer in the presence of Ca²⁺ (see Section 8.6.5 for experimental results).

The results for the buffer experiment (Table 6.12) show that inhibitor that was incubated in water retained its activity most effectively and there was no noticeable loss of activity after 48 h. Both the TRIS and HEPES buffers led to a complete loss of inhibition by 2.4 within 24 h, but 2.4 deactivated more slowly in the presence of phosphate buffer. There was still 50% inhibition of the enzyme remaining after 24 h incubation in phosphate buffer. The results from this experiment suggest that the two factors that could contribute to the loss of activity of the inhibitors while in solution are the type of buffer chosen and the pH of the solution.

There is a need for buffering of the enzyme/photoswitch solution in an in situ reversible assay, because enzyme activity is dependent on pH. There is evidence, however, that the inhibitors described in this thesis become less active over time in the presence of certain buffers (especially HEPES and TRIS). From the three buffers tested the phosphate buffer would the buffer of choice for an in situ reversible assay.
6.10 Summary of Chapter Six

The Michaelis-Menten constant and the limiting rate were measured and were found to be dependent on the acetonitrile concentration. This dependency affected the ability of the Dixon and modified Dixon plots to give useful values for the inhibition constants and, consequently, the inhibition constants from the double reciprocal analyses were quoted as the more reliable. In future experiments of this nature, the acetonitrile concentration should be kept constant throughout the entire data accumulation period for a compound. The level of \( \alpha \)-chymotrypsin inhibition by reference compound 2.52 was measured \((K_i = 0.17 \, \mu M)\) to be comparable to the literature value for this compound \((K_i = 0.13 \, \mu M)\). For all of the target compounds the \((Z)\) isomer was more active against \( \alpha \)-chymotrypsin than the \((E)\) isomer. As expected, the type of inhibition for all of the compounds fitted most closely to a competitive model of inhibition. The main exception was the ambient light PSS of 2.2, for which the limiting rate increased markedly with increased inhibitor concentration according to the Lineweaver-Burk and direct linear plots. Such behaviour points to mixed inhibition. Interestingly the UV light PSS of 2.2 exhibited textbook competitive inhibition by the modified Dixon plot. The results for 2.2 suggest that \((E)-2.2\) and \((Z)-2.2\) inhibit \( \alpha \)-chymotrypsin by different mechanisms.

The order of activity for the three types of compound synthesised from the most active to the least active was: B > A > C. For compounds of the type A, the dipeptide 2.3 was the most active, but 2.1 was the most efficient in terms of switching of the PSS ratio and the inhibition constant. For compounds of the type B, the meta isomer 3.2 was the most active however the ortho isomer was the most efficient in switching \( \alpha \)-chymotrypsin. Compounds of the type C did not show activity against \( \alpha \)-chymotrypsin at millimolar concentration.

A study into the stability of the target compounds in various media showed that 3.1 lost activity against \( \alpha \)-chymotrypsin slowly over time in water at pH 4. In a solution of HEPES at pH 7.8, compound 3.1 lost activity more quickly. Compound 2.4 retained
activity against α-chymotrypsin when stored in water, but became less active in the presence of buffer (HEPES, TRIS, phosphate). From the three buffers tested the phosphate buffer would be the buffer of choice for an in situ reversible assay of 2.4.

6.11 References for Chapter Six

CHAPTER SEVEN

SUMMARY AND FUTURE WORK
Three series of photobiological switches were synthesised and tested against α-chymotrypsin. Each of the photobiological switches comprised an azobenzene group, an α-ketocarbonyl moiety and a peptidic region (with the exception of 4.1). Changes in the order of assembly of these three components gave rise to compounds of the types A, B and C (refer to Section 1.7 for a description).

Four compounds of the type A were synthesised. Each of the target compounds showed switching action with 2.1 and 2.4 giving the best results in the photoisomerisation studies. Compounds of the type A were shown to undergo hydration in semi-aqueous media. The dipeptide 2.3 was more active against α-chymotrypsin than the single residue compounds 2.1 and 2.2. Although there was not sufficient compound available to fully test the second dipeptide 2.4, it appeared to be considerably less active against α-chymotrypsin than all of the compounds of the type A. For 2.1, 2.2 and 2.3 the (Z) isomer was more active than the (E) isomer. Consequently the (Z) rich PSS was the "off" state for the bioswitch and the (E) rich PSS was the "on" state.

Five compounds of the type B were synthesised. Compounds 3.1, 3.2 and 3.3 allowed for structure-activity relationship analysis of the regioisomeric substitution of the azobenzene group. These compounds were particularly prone to hydration and racemisation. Compound 3.1, for example, became hydrated in a solution of acetonitrile and became deuterated in a semi-aqueous solution containing HEPES at pH 7.8. This compound was also shown to lose activity against α-chymotrypsin after incubation in solution different biological buffers. Compounds of the type B were shown, however, to be stable to flash chromatography on silica. The isomerisation results did not change markedly across this series of compounds, with the notable exceptions being the comparatively poor (Z)-enriched PSS of 3.2 and the fact the ambient light PSS of 3.3 did not provide a good approximation for the visible light PSS of 3.3. Compound 3.2 was the most active compound against α-chymotrypsin prepared in this research and compound 3.3 gave the best switching of α-chymotrypsin. Compound 3.6 showed behaviour that was consistent with a "slow-tight binder". Unfortunately the method of Cha1 was not able to be applied to the inhibitor because the initial rate of the enzyme-
catalysed reaction in the presence of 3.6 could not be measured reliably. For 3.1, 3.2, 3.3 and 3.5 the (Z) isomer was more active than the (E) isomer.

The compounds of the type C were designed to make use of the S' specificity of α-chymotrypsin. These three compounds showed poor photoisomerisation with the UV light PSS ranging from 30% to 66% (Z) isomer. None of the compounds of the type C were active against α-chymotrypsin at the millimolar level.

\[
\begin{align*}
\text{(E)-1.34} \\
\end{align*}
\]

The enzyme switching ability of the inhibitors (typically 2-3 fold) was entirely consistent with a reported example of an azobenzene bioswitch 1.34 that targeted α-chymotrypsin. Furthermore, the aim of improving on the activity of 1.34 ((E) rich PSS, \(K_i = 11 \mu\text{M}\)) by incorporating peptidyl groups into the inhibitors was achieved. The switching ability of a photoswitch would be considerably better with a switch that gave a pure, single-isomer state on irradiation rather than the photostationary states reached by the photoisomerisation of the azobenzene group. An ideal photoswitch would have no “on”-component present at all in the “off”-state. In spite of this drawback, the azobenzene group did prove to be synthetically versatile and readily formed. The isomers were sufficiently stable to allow multiple assays to be run without any change in the composition of the mixture. Importantly, the moderate inhibition of α-chymotrypsin shows that the azobenzene group could be accommodated in the active site of the enzyme. This is a reflection that azobenzene has similar size and structure to existing protecting groups that were used in inhibitors of α-chymotrypsin. An obvious future extension of this work is the search for a photochromic group that does not form photostationary states, but rather fully photoconverts from one state to the other. The challenge would be the choice of a group that fulfils the other criteria of a photobiological switch as well as azobenzene does.
Potential compounds for investigation that build directly on the work done in this thesis on the azobenzenes include multiply substituted azobenzenes and multiaza compounds. A structure-activity relationship study on substitution of the distal azobenzene phenyl ring of the inhibitors may provide novel inhibitors with better switching performance. Another possibility is extension in the π direction of the active inhibitor 3.2 by replacing the methyl ester for L-lysine methyl ester (compound 7.1). The chemistry for the synthesis of such a compound has been covered in the work described in this thesis.

Because of solvent problems with the kinetic analysis of the target compounds, the double reciprocal plot proved to be more reliable than the Dixon and modified Dixon plots. It was found that the addition of acetonitrile to the assay changed the $K_M$ and $V_{max}$ for the assay. The use of the double reciprocal plot to calculate the inhibition constant enables corrections to be made for these changing kinetic parameters. The Dixon plots are based on the assumption that $V_{max}$ and $K_M$ are static for all data points. Normally the Dixon plots inherently have less error than the double reciprocal, but in this case, the double reciprocal appeared to be giving more consistent results. The reason why acetonitrile was present in the assay was that the inhibitor needed to be in an organic solution. Some of the inhibitors were shown to become less active against α-chymotrypsin after incubation in water (HEPES, pH 7.8). In future experiments, the assays could be run with a constant concentration of acetonitrile. Alternatively, the inhibitors could be stored in mildly acidic buffered water as follows on from the work in Section 5.4.
7.2 References for Chapter Seven


CHAPTER EIGHT

EXPERIMENTAL
8.1 General Methods and Experimental Procedures

**Compound Numbering**

The compounds described in this thesis were numbered for the purposes of NMR characterisation as follows:

Azobenzene derivatives (singly substituted) Fmoc protecting group

![Compound Numbering Diagram]

**NMR data reporting for azobenzene derivatives**

Most of the azobenzene derivatives prepared in the work described in this thesis were synthesised as predominantly the (E) isomer with traces of the (Z) isomer present. A solution of an azobenzene derivative reached the ambient light PSS (comprising (E) and (Z) isomers) after exposure to daylight for at least one day. NMR data was reported for the (E) isomers in the experimental section (synthetic, Sections 8.2 to 8.4) and in selected cases this data was followed by information on the ambient light PSS composition. The NMR data for the (Z) isomers is reported in the experimental section (photoisomerisation, Section 8.5.3).

**Elemental Analysis**

Elemental analyses were performed for carbon, nitrogen and hydrogen at the University of Otago microanalytical laboratory.

**Flash Chromatography**

Flash chromatography was performed on Merck silica 60 following the guidelines given by Still et al. All eluting solvents were distilled before use.
HPLC Chromatography

Analytical HPLC was performed on a Shimadzu VP system, which included a Shimadzu LC-10AC VP liquid chromatograph coupled to a SIL-10A VP autoinjector, a CTO-10A VP column oven set to 40 °C, a SPD-M10A VP diode array detector and a RID-10A VP refractive index detector. This system was controlled by a Shimadzu CLASS-VP (Version 5.02) software. A Shimadzu degasser was used for the degassing of solvents with helium. The instrument was equipped with a Brownlee Labs reverse phase C18 column (dimensions 220 mm (L) x 4.6 mm (ID), 5 μm particle size). Acetonitrile (BDH HiperSolv™ “Far UV” grade) was used at a solvent flow rate of 1 mL/min.

Infrared Spectroscopy

IR spectra were obtained using a Shimadzu 8201PC series FTIR. Spectra were run either in chloroform solution, neat or as a nujol mull.

Mass Spectrometry

Mass spectrometry was performed on a Kratos MS80 Mass Spectrometer operating at 4 kV. Various ionisation techniques were used including Electron Impact (EI) at 70 eV, Fast Atom Bombardment (FAB) with an Ion Tech ZNIFN ion gun using xenon as the reagent gas, operating at 8 kV and 2 mA with NOBA (m-nitrobenzyl alcohol) as the matrix or Electrospray (ES) with a Micromass LCT probe at 150 °C operating at 3200 V and an acetonitrile/water (1:1) carrier from a source at 80 °C.

Melting Points

Melting points were taken on an Electrothermal apparatus and are uncorrected.

Nuclear Magnetic Resonance

Proton-detected NMR spectra were obtained on a Varian Unity 300 spectrometer operating at 300 MHz. Carbon-detected NMR spectra were obtained on a Varian XL300 spectrometer operating at 75 MHz. Spectra were obtained at 23 °C unless specified. Chemical shifts were reported in parts per million (ppm) on the δ scale and were referenced to the appropriate solvent peaks: CDCl₃ referenced to (CH₃)₄Si at
$\delta_H 0.00$ ppm ($^1$H) and CDCl$_3$ at $\delta_C 77.0$ ppm ($^{13}$C); CD$_3$OD referenced to CHD$_2$OD at $\delta_H 3.30$ ppm ($^1$H) and CD$_3$OD at $\delta_C 49.3$ ppm ($^{13}$C); acetone-$d_6$ referenced to (CD$_3$)(CHCD$_2$)CO at $\delta_H 2.17$ ppm ($^1$H) and (CD$_3$)$_2$CO at $\delta_C 29.2$ ppm ($^{13}$C); acetonitrile-$d_3$ referenced to CHD$_2$CN at $\delta_H 2.00$ ppm ($^1$H) and CD$_3$CN at $\delta_C 1.3$ ppm ($^{13}$C); DMSO-$d_6$ referenced to (CD$_3$)(CHD$_2$)SO at $\delta_H 2.50$ ppm ($^1$H) and (CD$_3$)$_2$SO at $\delta_C 39.6$ ppm ($^{13}$C). $^1$H NMR spectra were obtained using an acquisition time ($A_t$) of 2 s. $^{13}$C NMR were obtained with an $A_t$ of 0.878 s and a delay ($D_1$) typically of 1 s. HSMQC experiments were obtained with $A_t$ =0.2 s, $^1J_{CH} = 145$ Hz and $D_1$ was set for individual experiments when setting the null value. HMQC experiments with the pulsed field gradient system were run with $A_t =0.137$ s, $^1J_{CH} = 140$ Hz and $D_1 = 1.0$ s. HMBC experiments were obtained with $A_t =0.21$ s (or 0.137 s with the pulsed field gradient system), $^1J_{CH} = 140$ Hz, $^3J_{CH} = 8.3$ Hz and a relaxation delay of 0.3 s.

**Optical Rotary Dispersion**

Optical rotation measurements were performed either on a JASCO J-20C recording spectropolarimeter with a 10 mm path length or a Perkin Elmer polarimeter Model 341 with a 100 mm path length. Measurements were taken at rt in acetonitrile at $\lambda = 589$ nm. $[\alpha]_D$ values are given in units of $^o$ mL/g dm and the sample concentration given in units of g/100 mL.

**Thin Layer Chromatography**

Analytical TLC was conducted on aluminium-backed Merck-Kieselgel KG60F$_{254}$ plates. Visualisation was by ultraviolet light or potassium permanganate solution or iodine.

**Ultraviolet Spectroscopy**

UV spectra were obtained using a Hewlett Packard 8452A diode array spectrophotometer. Spectra were either run in a solution of methanol, acetonitrile or chloroform.
Reagents and Solvents

Unless otherwise indicated all experiments were performed in oven-dried glassware under an atmosphere of nitrogen. Solvents and reagents used in reactions were purified according to well-established procedures. Tetrahydrofuran and ether were distilled from sodium benzophenone ketal immediately prior to use. Dichloromethane, chloroform, triethylamine were distilled from calcium hydride immediately prior to use. Methanol and ethanol were distilled from iodine-magnesium turnings and were stored under nitrogen over 4 Å molecular sieves. Petroleum ether describes a mixture of hexanes in the bp range 50-70 °C.

General Procedure A1: Coupling of acid and amine salt via EDCI methodology.3

To a solution of the acid (1.0 equiv.) and the amine salt (1.1 equiv.) in 1:1 dimethylformamide/dichloromethane (ca. 0.1 M) at rt under nitrogen was added EDCI (1.3 equiv.) and HOBT (1.5 equiv.). The reaction mixture was stirred for 5 min and DIEA (1.1 equiv.) was added. The reaction mixture was stirred 16-20 h. The solution was diluted with either dichloromethane or ethyl acetate, washed with 1 N HCl, saturated aqueous NaHCO₃, brine, dried over MgSO₄ and concentrated in vacuo. The residue was purified by flash chromatography.

General Procedure A2: Coupling of acid and free amine via EDCI methodology.3a

To a solution of the acid (1.0 equiv.) and the free amine (1.1 equiv.) in dichloromethane (ca. 0.1 M) at rt under nitrogen was added EDCI (1.5 equiv.) and HOBT (2.0 equiv.). The reaction mixture was stirred 16-20 h. The solution was diluted with dichloromethane and washed with 3 M NaCl. The aqueous phase was back-extracted with dichloromethane. The combined organic washings were dried over MgSO₄ and concentrated in vacuo. The residue was purified by flash chromatography.

General Procedure B1: Oxidation of alcohol using Dess-Martin periodinane.1b,4

To a stirred solution of Dess-Martin periodinane (1.0 equiv.) in dichloromethane (20-50 mM) was added dropwise a solution of the alcohol (1.0 equiv.) in dichloromethane (20-50 mM). The solution was stirred at rt under nitrogen for 0.5-3.0 h. When the reaction was complete, as determined by thin layer chromatography, the
reaction mixture was diluted with either ethyl acetate or dichloromethane. The solution was washed with 10% Na$_2$S$_2$O$_3$ in saturated NaHCO$_3$, brine, dried over MgSO$_4$ and concentrated in vacuo. The residue was purified when necessary by flash chromatography.

**General Procedure B2: Oxidation of alcohol using TEMPO.**

To TEMPO (ca. 0.1 equiv.), KBr (ca. 0.2 equiv.) and water (ca. 0.01 equiv.) was added a solution of the alcohol (1.0 equiv.) in dichloromethane (ca. 0.1 M). The reaction mixture was stirred at 0 °C. A buffered bleach solution was prepared by the addition of NaHCO$_3$ (300 mg, 3.6 mmol) to a 5.25% sodium hypochlorite solution (9.7 mL commercial bleach, 15.3 mL distilled water) and the resulting mixture was stirred until all of the solid was dissolved. The pH of the bleach solution was checked to be within 8.6-9.5 (the required range for HOCl distribution between both phases). The buffered bleach solution (one part) was added dropwise to the above reaction mixture (two parts) at 0 °C. After rapid stirring for 30 min the reaction mixture was diluted with ethyl acetate and the organics were separated. The organics were then washed with 0.5 N HCl, saturated aqueous NaHCO$_3$, brine, dried over MgSO$_4$ and concentrated in vacuo. The residue was purified by flash chromatography when necessary.

**General Procedure C1: Saponification using potassium hydroxide.**

To a solution of the ester (1.0 equiv.) in 1:4 water/ethanol (ca. 0.1 M) potassium hydroxide (2.0 equiv.) was added. After the solution was heated at reflux for 90 min, the solvent was evaporated to dryness. The residue was dissolved in water and the resulting solution was washed with ether. The aqueous phase was cooled in an ice-bath and acidified by the addition of 10 % v/v H$_2$SO$_4$ (acid by litmus). The aqueous phase was extracted with either ether or ethyl acetate. The combined organic washings were washed with brine, dried over MgSO$_4$ and concentrated in vacuo to give the acid.

**General Procedure C2: Saponification using lithium hydroxide.**

To a solution of the ester in methanol (0.05-0.1 M, 1 part) at 0 °C was added a solution of 0.25 M lithium hydroxide (1.5 parts) in 2:1 methanol/water. After the reaction mixture was stirred for 0.5-4 h, sufficient 1N HCl was added to acidify the
solution (pH 5 by Universal Indicator) and the methanol was evaporated in vacuo. Water was added to the residue and the organics were extracted with ethyl acetate. The combined organic extracts were washed with water, brine, dried over MgSO₄ and concentrated in vacuo to give the acid.

**General Procedure D: Ethyl ester preparation.**

A saturated solution of HCl in ethanol (100 mL) was poured onto the acid (ca. 10 g) and the reaction mixture was set to reflux. After 24 h, the solution was poured into an equal volume of water and cooled to 10 °C by the addition of ice. Solid Na₂CO₃ was added until the supernatant was basic to litmus. The resulting precipitate was filtered, washed with water and dried under vacuum in the presence of P₂O₅ to afford the ester.

**General Procedure E: Formation of substituted azobenzenes by the condensation of amines with nitroso compounds.**

A solution of nitrosobenzene (1.0 equiv.) and the amine (1.0 equiv.) in glacial acetic acid (ca. 0.25 M) was stirred at 100 °C for 4 h. On cooling, the solution was diluted with water (1.5 parts) and extracted with dichloromethane. To the organic washings water was added (0.25 parts) and then solid Na₂CO₃ was added until effervescence ceased. The organic phase was separated, washed with water, dried over MgSO₄ and concentrated in vacuo. The black residue was purified on an alumina (Grade H) column eluting with petroleum ether-dichloromethane (1:1). The crude product was further purified by flash chromatography.

**General Procedure F: Formation of α-hydroxyesters by ozonolysis.**

Ozone was administered, at a rate of 1-2 bubbles per second, to a stirred solution of the ketophosphorane in (7:3) dichloromethane/methanol (ca. 0.1 M) at −78 °C for 15 min. The solution was flushed with nitrogen for 10 min, then allowed to warm to rt. The solution was evaporated to dryness and the resulting residue was purified by flash chromatography.
8.2 Experimental Work Described in Chapter Two

8.2.2 Synthesis of the key intermediates \((2R,3S)\)- and \((2S,3S)-3-[[\text{benzyloxy}][\text{carbonyl}][\text{amino}]-2\text{-hydroxybenzenebutanoic acid methyl ester 2.5a, 2.5b}}

\[
\begin{align*}
\text{2.5a} & \quad \text{2.5b} \\
\end{align*}
\]

8.2.2.1 Synthesis of 2.5 from the cyanoketophosphorane 2.10

\[
\begin{align*}
\text{Cl} & \quad \text{CN} \quad \rightarrow \quad \text{PPh}_3 \quad \text{Cl}^- \quad \rightarrow \quad \text{PPh}_3 \quad \text{CN} \\
\text{2.6} & \quad \text{2.7} & \quad \text{2.8} \\
\end{align*}
\]

**Cyanomethylenetriphenylphosphorane 2.8**

A solution of chloroacetonitrile \text{2.6} (5.00 g, 66.1 mmol) and triphenylphosphine (13.0 g, 50.0 mmol) in nitromethane (75 mL) was refluxed for 5 h. The reaction mixture was allowed to cool over 2 h, during which time the product had precipitated. The solid was collected on a scintillated funnel, washed with cold nitromethane (2 x 20 mL) and air-dried to give \text{2.7} (12.4 g, 73%) as small white crystals:

mp 275–278 °C, (literature mp 278-279 °C); IR (film) 2918, 2848, 2635, 1458, 1436, 1377, 1112 cm\(^{-1}\).

Compound \text{2.7} (1.61 g, 5.3 mmol) was dissolved in water (20 mL) and the solution was cooled to 0 °C. Aqueous 10% NaOH (2 mL) was added and the resulting solid was promptly filtered, washing with water (2 x 10 mL) and ether (2 x 10 mL). The
solid was dried under vacuum in the presence of P₂O₅ to give 2.8 (1.39 g, 97%) as a white solid:

mp 194-200 °C, (literature mp 195-196 °C); IR (film) 2924, 2856, 1710, 1460, 1441, 1377, 1190 cm⁻¹.

(4S)-4-[[benzyloxy]carbonyl]amino]-3-oxo-2-triphenylphosphoranylidene benzenepentanenitrile 2.10

To a mixture of Cbz-L-phenylalanine, 2.9 (0.40 g, 1.3 mmol) in dichloromethane (14 mL) at 0 °C were added EDCI (0.27 g, 1.4 mmol) and DMAP (0.016 g, 0.13 mmol), followed by the dropwise addition of a solution of cyanophosphorane 2.8 (0.81 g, 2.7 mmol) in dichloromethane (6 mL). The reaction mixture was allowed to warm to rt and was stirred under nitrogen for 20 h. The reaction mixture was poured over 1:1 dichloromethane/water (15 mL) and the phases were separated. The aqueous phase was back-extracted with dichloromethane (2 x 5 mL) and the combined organic extracts were washed with brine (30 mL), dried over MgSO₄ and concentrated in vacuo. The resulting residue was purified by flash chromatography, eluting with dichloromethane-ethyl acetate (4:1), to give 2.10 (0.61 g, 78%) as a white solid:

[MS (FAB): 583 (M+H)⁺, 539, 475, 328, 302, 262, 183, 154, 136; HRMS (FAB) calcd for C₃₇H₃₂O₃N₂P (M+H)⁺ 583.2150, found 583.2145; mp 101-103 °C; Rf 0.34 (1:1 ethyl acetate-petroleum ether); IR (film) 3420, 3009, 1716, 1587, 1498, 1438, 1359, 1236, 1180, 1053 cm⁻¹;]

¹H NMR (CDCl₃) δ 7.61-7.66 (3H, m, PPh₃), 7.45-7.60 (12H, m, PPh₃), 7.30 (5H, m, Ar), 7.21 (5H, m, Ar), 5.53 (1H, d, J = 7.8 Hz, NH), 5.19 (1H, m, CH), 5.06 (2H, s, CbzCH₂), 3.35 (1H, dd, J = 4.8, 13.5 Hz, CHCH₄Ph), 3.08 (1H, dd, J = 6.8, 13.5 Hz, CHCH₃Ph);
Chapter Eight: Experimental

$^{13}$C NMR (CDCl$_3$) $\delta$ 192.8 (CO), 155.4 (CbzCO), 136.8, 133.6, 133.4, 133.2, 129.6, 129.2, 129.0, 128.3, 128.0, 127.7 & 126.4 (ArC & ArCH), 122.4 (d, $J_{PCq}$ = 92.8 Hz, CN), 120.9 (d, $J_{PCa}$ = 16 Hz, P-C$_q$), 66.2 (CbzCH$_2$), 57.2 (d, $J_{PCq}$ = 9 Hz, CH), 47.8 (d, $J_{PCa}$ = 125 Hz, P=C), 38.7 (CHCH$_2$).

\((3S)-3-[(\text{benzyloxy})\text{carbonyl}]\text{amino}-2\text{-oxobenzenebutanoic acid methyl ester} \ 2.11\)

\[
\text{Cbz} \quad \begin{array}{c}
\text{Ph} \\
\text{CN}
\end{array} \quad \text{PPh$_3$} \quad \begin{array}{c}
\text{Cbz} \\
\text{OMe}
\end{array}
\]

Compound \(2.10\) (538 mg, 0.949 mmol) was treated with ozone in the presence of methanol as described in Method F. The resulting crude product was purified by flash chromatography, eluting with petroleum ether-ethyl acetate (3:2), to give \(2.11\) (249 mg, 77%) as a white solid:

[MS (FAB): 342 (M+1)$^+$, 298, 254, 210, 154, 136, 107, 91; HRMS (FAB) caled for C$_{19}$H$_{20}$O$_5$N (M+1)$^+$ 342.134, found 342.133]; mp 64-73 \(^\circ\)C; \(R_F\) 0.37 (2:3 ethyl acetate-petroleum ether); IR (film) 3431, 2950, 1736, 1720, 1506, 1456, 1338 cm$^{-1}$;

$^1$H NMR (CDCl$_3$) $\delta$ 7.20-7.38 (10H, m, Ar), 7.09 (1H, d, $J$ = 7.5 Hz, NH), 5.27 (1H, m, CH), 5.07 (2H, s, CbzCH$_2$), 3.85 (3H, s, OCH$_3$), 3.23 (1H, m, CHCH$_2$Ph), 3.05 (1H, m, CHCH$_2$Ph);

$^{13}$C NMR (CDCl$_3$) $\delta$ 191.8 (CO), 160.7 (COOMe), 155.5 (CbzCO), 136.0 (CbzC), 134.7 (ArC), 129.3, 128.7, 128.6, 128.5, 128.2 & 127.3 (ArCH), 64.1 (CbzCH$_2$), 58.1 (CH), 53.2 (CH$_3$), 37.1 (CHCH$_2$).
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(2R,3S)- and (2S,3S)-3-[(benzyloxy)carbonylamino]-2-hydroxybenzenbutanoic acid methyl ester 2.5a, 2.5b

To a solution of the α-ketoester 2.11 (3.00 g, 8.8 mmol) in methanol (50 mL) was added sodium borohydride (0.36 g, 9.7 mmol). The reaction mixture was stirred at rt for 20 min before quenching with saturated NH₄Cl (50 mL). The organics were evaporated in vacuo and the resulting aqueous residue was extracted with ethyl acetate (3 x 50 mL). The combined organic washings were washed with brine (100 mL), dried over MgSO₄ and concentrated in vacuo to afford a cream solid. Flash chromatography eluting with petroleum ether-ethyl acetate (5:1) afforded 2.5a (0.826 g, 27%) as a white solid:

[MS (FAB): 344 (M+1)+, 300, 254, 210, 154, 136, 107, 91; HRMS (FAB) calcd for C₁₉H₂₂O₅N (M+1)⁺ 344.1500, found 344.1512];

Rf 0.40 (2:3 ethyl acetate-petroleum ether); [α]D - 60 ± 5° (c 1.1 dichloromethane), lit. [α]D - 45 ± 5° (c 3.2 dichloromethane)

1H NMR (CDCl₃) δ 7.20-7.40 (10H, m, Ar), 5.10 (1H, d, J = 9.8 Hz, NH), 5.04 (2H, s, CbzCH₂), 4.33 (1H, ddd, J = 9.8, 9.8, 9.8 Hz, CHCHOH), 4.08 (1H, br s, CHCHOH), 3.70 (3H, s, CH₃), 3.20 (1H, d, J = 3.0 Hz, CHCHOH), 2.80 (2H, m, CH₂Ph).

Further elution with petroleum ether-ethyl acetate (5:3) afforded 2.5b (0.749 g, 24%) as a white solid:

Rf 0.30 (2:3 ethyl acetate-petroleum ether); [α]D - 13 ± 1° (c 0.69 methanol), lit. [α]D - 5° (c 2.0 methanol)

1H NMR (CDCl₃) δ 7.20-7.40 (10H, m, Ar), 5.22 (1H, d, J = 9.3 Hz, NH), 5.04 (2H, s, CbzCH₂), 4.38 (1H, m, CHCHOH), 4.35 (1H, s, CHCHOH), 3.56 (3H, s, CH₃), 2.80 (2H, m, CH₂Ph).
8.2.2.2 Synthesis of 2.5 from the cyanohydrin 2.14

(2R,3S)- and (2S,3S)-3-[[([benzyloxy]carbonyl]amino]-2-hydroxybenzenebutanoic acid methyl ester 2.5a, 2.5b

To a saturated solution of HCl in (3:1) ether/methanol (60 mL) at 0 °C was added the cyanohydrin 2.14 (1.14 g, 3.67 mmol). The solution was stirred at 4 °C for 24 h, before cold water (13 mL) was added dropwise and the reaction mixture was stirred a further 48 h at 4 °C. The organics were evaporated in vacuo and the resulting mixture was extracted with dichloromethane (2 x 25 mL). The combined organic extracts were washed with water (40 mL), brine (40 mL), dried over MgSO4 and concentrated in vacuo to afford a mixture of the methyl esters 2.5a and 2.5b. Purification by flash chromatography, eluting with petroleum ether-ethyl acetate (3:2), gave 2.5a (0.62 g, 26%) as a white solid:
Rf 0.36 (2:3 ethyl acetate-petroleum ether);
1H NMR (CDCl3) δ 7.20 (10H, m, Ar), 5.05 (3H, m, CbzCH2 & NH), 4.35 (1H, m, CHCHOH), 4.08 (1H, d, J = 2.5 Hz, CHCHOH), 3.70 (3H, s, OCH3), 3.15 (1H, d, J = 4.2 Hz, CHCHOH), 2.95 (2H, m, CHCH2Ph).
Further elution gave 2.5b (0.32 g, 13%) as a white solid:
Rf 0.24 (2:3 ethyl acetate-petroleum ether);
1H NMR (CDCl3) δ 7.15-7.40 (10H, m, Ar), 5.05-5.15 (3H, m, CbzCH2 & NH), 4.35 (2H, m, CHCHOH), 3.58 (3H, s, OCH3), 3.20 (1H, br s, CHCHOH), 2.80 (2H, m, CHCH2Ph).

1 A sample of the cyanohydrin 2.14 was gifted by G. J. Foulds.
8.2.2.3 Synthesis of 2.5 from homophenylalanine 2.21

\((3S)-3-\text{[(benzyloxy)carbonyl]amino}-2\text{-oxo-4-phenydiazobutane}\) 2.20\(^{11}\)

![Structure of 2.20](image)

To 2.9 (13.1 g, 43.8 mmol) in THF/ether (1:1, 200 mL) stirring at -10 °C were added triethylamine (6.08 mL, 43.6 mmol) and then ethyl chloroformate (4.19 mL, 43.8 mmol) over 5 minutes. The solution was stirred at -5 °C for 30 minutes. An ethereal diazomethane solution was prepared from Dizald® (32.1 g, 150 mmol) in ether (190 mL) and KOH (9.0 g) in ether/water/carbitol (1:1:3, 80 mL) by the method of Hudlicky.\(^{11a}\) The solution of diazomethane was transferred with caution to the flask containing the solution of mixed anhydride. The reaction mixture was stirred at 0 °C for 1 h and then refrigerated for 3 days. At rt the unstoppered reaction vessel was stirred for 90 min to vent excess diazomethane. The reaction mixture was filtered and the filtrate was washed with cold saturated NaHCO\(_3\) (2 x 150 mL), brine (150 mL), dried over MgSO\(_4\) and concentrated \textit{in vacuo} to give crude product as a pale yellow solid. The solid was dissolved in benzene (100 mL) and the benzene was evaporated \textit{in vacuo}. To the solid, petroleum ether (100 mL) was added and the suspension was filtered. The solid was washed with petroleum ether (3 x 40 mL). The solid was recrystallised from benzene/petroleum ether (2:3, 130 mL) to afford 2.20 (11.2 g, 79%) as a pale yellow solid:

mp 85–86 °C, (literature mp 81–82.5 °C)\(^{11b}\); \(R_F\) 0.30 (1:4 ethyl acetate-petroleum ether);

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.16-7.36 (10H, m, Ar), 5.39 (1H, br s, NH), 5.20 (1H, s, CHN\(_2\)), 5.08 (2H, s, CbzCH\(_2\)), 4.48 (1H, m, CH/CH\(_2\)), 3.04 (2H, d, \(J = 6.3\) Hz, CHCH\(_2\)Ph).
(3S)-3-[[benzyloxy]carbonyl]amino]benzenebutanoic acid methyl ester 2.21

To a solution of the diazoketone 2.20 (7.88 g, 24.4 mmol) and methanol (1.4 mL) in THF (250 mL) at 0 °C was added a solution of silver benzoate (0.69 g, 3.0 mmol) in triethylamine (10.5 mL, 34.0 mmol). The reaction mixture was allowed to warm to rt and the reaction was determined to be complete by thin layer chromatography after 90 min. The solvent was evaporated in vacuo and the resulting residue was taken up with ethyl acetate (200 mL). The solution was washed with 10% HCl (2 x 100 mL), saturated NaHCO₃ (100 mL), brine (100 mL), dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography, eluting with ethyl acetate-petroleum ether (1:9) to afford a pale yellow solid. The solid was recrystallised from ethyl acetate-petroleum ether (1:4, 25 mL) to afford 2.21 (5.08 g, 64%) as a white solid:

mp 54–55 °C; R_F 0.17 (1:9 ethyl acetate-petroleum ether);

^1^H NMR (CDCl₃) δ 7.16 - 7.38 (10H, m, Ar), 5.30 (1H, d, J = 7.3 Hz, NH), 5.07 (2H, s, CbzCH₂), 4.23 (1H, m, CH), 3.67 (3H, s, CH₃), 2.94 (1H, m, CHCH₂Ph), 2.84 (1H, m, CHCH₂Ph), 2.51 (2H, m, CHCH₂COOMe);

^13^C NMR (CDCl₃) δ 171.2 (COOMe), 155.6 (CbzCO), 137.4 & 136.5 (ArC), 129.3, 128.6, 128.5, 128.1, 128.0, 126.7 (ArCH), 66.6 (CbzCH₂), 51.7 (CH₃), 49.3 (CH₂CO₂Me), 40.2 (CH), 37.3 (CHCH₂Ph).

Davis reagent: (±)-trans-3-phenyl-2-(phenylsulfonyl)oxaziridine 2.16

PhSO₂NH₂ + Ph → PhSO₂⁺N=PhH → PhSO₂⁺N=PhH
Into a two-necked round-bottomed flask equipped with a Dean-Stark water separator and a condenser were placed Amberlyst-15 ion exchange resin (0.4 g), benzenesulphonamide (31.4 g, 0.20 mmol), 4Å powdered molecular sieves (30 g), freshly distilled benzaldehyde (15.0 mL, 0.15 mmol) and toluene (330 mL). The reaction mixture was stirred and heated at reflux under nitrogen. At regular intervals water was removed from the Dean-Stark trap. When water separation ceased (20 h), the mixture was filtered and the resulting residue was washed with toluene (3 x 30 mL). The solvent was evaporated in vacuo and the resulting solid was powdered using a glass rod. Petroleum ether (100 mL) was added and the mixture was filtered. The solid was washed with petroleum ether (3 x 50 mL) and air dried to afford 2.16a (32.4 g, 88%) as a pale yellow powder:

mp 79-80 °C, (literature mp 78-80 °C)\textsuperscript{14};

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 9.06 (1H, s, NCH), 7.92–8.03 (4H, m, Ar), 7.46–7.65 (6H, m, Ar).

To a stirred mixture of 2.16a (10.0 g, 41 mmol) in toluene (400 mL) and K\(_2\)CO\(_3\) (47.5 g, 47 mmol) in water (250 mL) was added a solution of Oxone™ (30.0 g, 49 mmol) in water (220 mL) from a dropping funnel over a 15 min period. The reaction mixture was stirred for a further 30 min and the layers were separated. The aqueous phase was back-extracted with toluene (100 mL) and the combined organic phases were washed with 10% Na\(_2\)SO\(_3\) (150 mL), dried over MgSO\(_4\) and concentrated in vacuo. The resulting white solid was triturated with petroleum ether (40 mL), filtered and washed with petroleum ether (2 x 40 mL). The product was recrystallised by dissolving in ethyl acetate (50 mL) at rt, adding petroleum ether (40 mL) then storing at 4 °C overnight to afford 2.16 (4.5 g, 42%) as a white solid:

mp 91-92 °C, (literature mp 94-95 °C)\textsuperscript{14};

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.05 (2H, d, J = 4.9 Hz, Ar), 7.60–7.75 (3H, M, Ar), 7.40–7.47 (5H, m, Ar), 5.49 (1H, s, CH).
Attempted preparation of \((2R,3S)-\) and \((2S,3S)-3-\{(benzyloxy)carbonyl\}amino\}\)-2-hydroxybenzenebutanoic acid methyl ester \(2.5a, 2.5b\) via Davis reagent\(^1\)

To a solution of KHMDS (2 mL, 0.5 M solution in toluene) in THF (3 mL) at 
\(-78^\circ C\) was added dropwise a solution of \(2.21\) (56 mg, 0.17 mmol) in THF (6 mL). The resulting pale yellow solution was stirred until the acetone/dry ice bath reached \(-25^\circ C\) (60 min), then was returned to a \(-78^\circ C\) bath and \(2.16\) (134 mg, 0.51 mmol) was added in one portion. The reaction mixture was stirred at \(-70^\circ C\) for 3 h. The reaction was quenched with saturated \(\text{Na}_2\text{SO}_3\) (1.5 mL) followed by saturated \(\text{NH}_4\text{Cl}\) (1.5 mL). The phases were separated and the aqueous phase was extracted with THF (5 mL). The combined organic phases were washed with 10\% \(\text{HCl}\)/brine (1:1, 10 mL), 2\% \(\text{Na}_2\text{CO}_3\) (10 mL), brine (10 mL), dried with \(\text{MgSO}_4\) and concentrated \textit{in vacuo}. Analysis of the resulting mixture (183 mg) by \(^1\text{H}\) NMR showed exclusively \(2.21\) and \(2.16\).

Attempted preparation of \((2R,3S)-\) and \((2S,3S)-3-\{(benzyloxy)carbonyl\}amino\}\)-2-hydroxybenzenebutanoic acid methyl ester \(2.5a, 2.5b\) via MoOPH\(^{15a}\)

A solid-addition arm containing MoOPH (286 mg, 0.52 mmol) was fitted to a two-necked round-bottomed flask and the system was evacuated and flushed with nitrogen. A solution of KHMDS (4 mL, 0.5 M solution in toluene) in THF (2 mL) was placed into the flask via syringe. The solution was stirred at \(-78^\circ C\) and a solution of \(2.21\) (112 mg, 0.34 mmol) in THF (4 mL) was added dropwise. The solution was stirred at \(-78^\circ C\) for 15 min, then transferred to a \(\text{CCl}_4\)/dry ice bath and stirred at \(-25^\circ C\) for 20 min. The reaction mixture was cooled back to \(-78^\circ C\) and the MoOPHi was added in one portion. The reaction mixture was stirred at \(-70^\circ C\) for 2 h. The reaction was quenched with saturated \(\text{Na}_2\text{SO}_3\) (1.5 mL), followed by saturated \(\text{NH}_4\text{Cl}\) (1.5 mL). The phases were separated and the aqueous phase was extracted with THF (5 mL). The combined
organic phases were washed with 10% HCl/brine (1:1, 10 mL), 2% Na₂CO₃ (10 mL), brine (10 mL), dried with MgSO₄ and concentrated in vacuo. Analysis of the resulting mixture (85 mg) by ¹H NMR showed 2.21 and 2.5 (4:1 by ¹H NMR).

8.2.3 Synthesis of (E)- and (Z)-4-(phenylazo)benzylamine 2.22 and (E)- and (Z)-4-(phenylazo)benzylamine hydrochloride salt 2.23

4-amino-N-FMOC-benzylamine 2.27

To a suspension of 2.25 (500 mg, 2.9 mmol) in dichloromethane (30 mL) under nitrogen was added 9-fluorenylmethyl chloroformate (686 mg, 2.9 mmol) and DIEA (2.32 mL, 13.3 mmol). The reaction mixture was stirred at rt for 2 h and quenched with 5% HCl (30 mL). The phases were separated and the organic phase was washed with water (30 mL), dried over MgSO₄ and concentrated in vacuo. The resulting solid was recrystallised from ethyl acetate (3 mL) to afford 2.26 (819 mg, 83%) as small yellow crystals:

[MS (EI): 374 (M⁺), 196, 178, 166, 165, 132, 89; HRMS (FAB) calcd for C₂₂H₁₈N₂O₄ (M⁺) 374.1266, found 374.1268]; mp 155-156 °C; IR (CDCl₃) 3452, 3068, 1724, 1608, 1522, 1348 em⁻¹; ¹H NMR (CDCl₃) δ 8.16 (2H, d, J = 8.3 Hz, ArH3), 7.78 (2H, d, J = 7.3 Hz, FmocH1), 7.59 (2H, d, J = 7.3 Hz, FmocH4), 7.26-7.44 (6H, m, ArH2 & FmocH2&3), 5.27 (1H, br s, NH), 4.54 (2H, d, J = 6.5 Hz, FmocCH2), 4.44 (2H, d, J = 5.5 Hz, NHCH₂), 4.21 (1H, t, J = 6.5 Hz, CH); ¹³C NMR (CDCl₃) δ 156.4 (CO), 146.0 (ArC), 143.7 (FmocC1a), 141.4 (FmocC4a), 127.9 (ArCH), 127.7 (FmocC3), 127.4 (ArC), 127.1 (FmocC2), 124.9 (FmocC4), 123.9 (ArCH), 120.0 (FmocC1), 66.7 (FmocCH₂), 47.3 (CH), 44.3 (NHCH₂).
To a solution of 2.26 (820 mg, 2.2 mmol) in ethyl acetate (150 mL) was added platinum dioxide (10 mg). The reaction vessel was evacuated and flushed with hydrogen four times, then stirred for 16 h under an atmosphere of hydrogen. The reaction mixture was filtered through a Celite pad and the solvent was removed in vacuo to give 2.27 (530 mg, 70%): [MS (EI): 344 (M⁺), 196, 178, 164, 106, 77; HRMS (FAB) calcd for C₂₂H₂₀N₂O₂ (M⁺) 344.1525, found 344.15141; mp 147-147.5 °C; IR (CDCl₃) 3448, 3068, 1717, 1624, 1517, 1450, 1223 cm⁻¹; 

¹H NMR (CDCl₃) δ 7.67 (2H, d, J = 7.5 Hz, FmocH₁), 7.51 (2H, d, J = 7.5 Hz, FmocH₄), 7.31 (2H, t, J = 7.5 Hz, FmocArH), 7.21 (2H, t, J = 7.5 Hz, FmocArH), 6.98 (2H, d, J = 8.0 Hz, ArH₂), 6.55 (2H, d, J = 8.0 Hz, ArH₃), 4.98 (1H, br s, NH), 4.35 (2H, d, J = 7.0 Hz, FmocCH₂), 4.15 (3H, m, CH & NHCH₂); 

¹³C NMR (CDCl₃) δ 156.5 (CO), 145.9 (ArC), 143.9 (FmocCl₁), 141.3 (FmocC₄a), 128.9 (ArCH), 128.1 (ArC), 127.6 (FmocC₃), 127.0 (FmocC₂), 125.0 (FmocC₄), 119.9 (FmocCl), 115.1 (ArCH), 66.5 (FmocCH₂), 47.2 (CH), 44.7 (NHCH₂).

(E)- and (Z)-N-FMOC-4-(phenylazo)benzylamine 2.28₁⁶

A solution of 2.27 (770 mg, 2.2 mmol) and nitrosobenzene, 2.33 (480 mg, 4.5 mmol) in glacial acetic acid (30 mL) was stirred at rt for 48 h. The reaction was diluted with water (100 mL) and the organics were extracted with dichloromethane (2 x 80 mL). To the organic washings was added water (80 mL) and solid Na₂CO₃ was added until effervescence ceased. The layers were separated and the organic phase was washed with water (2 x 80 mL), dried over MgSO₄ and concentrated in vacuo. The resulting residue was purified by flash chromatography, eluting with eluting with ethyl acetate-petroleum ether (1:2) to give the compound 2.28 (490 mg, 50%) as an orange solid:
Chapter Eight: Experimental

[MS (FAB): 434 (M+1)$^+$, 289, 255, 179, 154, 136, 107, 77; HRMS (FAB) calcd for C$_{21}$H$_{15}$O$_2$N$_3$ (M+1)$^+$ 434.1869, found 434.1884]; mp 164.5-165.5 °C; $R_f$(E) 0.32 $R_f$(Z) 0.19 (1:2 ethyl acetate-petroleum ether); IR (film) 1793, 1718, 1512, 1466, 1450, 1381 cm$^{-1}$;

$^1$H NMR (CDCl$_3$) $\delta$ 7.87-7.93 (4H, m, H$_2$, H$_2'$), 7.77 (2H, d, J = 7.5 Hz, FmocH1), 7.60 (2H, d, J = 7.0 Hz, FmocH4), 7.47-7.55 (3H, m, H$_3'$ & H$_4'$), 7.23-7.43 (6H, m, H$_3$, FmocH2 & FmocH3), 5.14 (1H, br s, NH), 4.50 (2H, d, J = 6.6 Hz, FmocCH$_2$) 4.46 (2H, d, J = 8.0 Hz, NHCH$_2$), 4.23 (1H, t, J = 6.5 Hz, FmocCH);

$^{13}$C NMR (CDCl$_3$) $\delta$ 156.5 (CO), 152.6 (C1'), 152.0 (C1), 143.8 (FmocC1a), 141.4 (C4), 141.3 (FmocC4a), 131.0 (C4'), 129.1 (C3'), 128.0 (C3), 127.7 (FmocC3), 127.0 (FmocC2), 124.9 (FmocC4), 123.2 (C2), 122.8 (C2'), 120.0 (FmocC1), 66.7 (FmocCH$_2$), 47.3 (CH), 44.7 (NHCH$_2$).

When the above $^1$H NMR sample of (E)-2.28 was left on the bench for at least 1 day, a PSS comprising (E)-2.28 / (Z)-2.28 (4:1 by $^1$H NMR) resulted.

$^1$H NMR (CDCl$_3$, selected data for the mixture) $\delta$ 6.83 (4H, (Z)-2.28, m, H2 & H2'), 5.02 (1H, (Z)-2.28, br s, NH), 4.29 (2H, (Z)-2.28, m, NHCH$_2$).

(E)- and (Z)-4-(phenylazo)benzylamine 2.22$^1$

![Diagram]

To a solution of 2.28 (20 mg, 46 $\mu$mol) in THF (1.8 mL) was added a solution of TBAF (1.0 M, 200 $\mu$L) in THF and the reaction mixture was stirred 5 min at rt. Methanol (180 $\mu$L, 460 $\mu$mol) was added and the reaction mixture was loaded onto a silica column. The column was eluted with dichloromethane (50 mL) and then with ethanol-dichloromethane (1:9) to afford 2.22 (9 mg, 90%) as an orange solid:

[MS (EI): 211 (M$^+$), 134, 105, 89, 77; HRMS (EI) calcd for C$_{13}$H$_{13}$N$_3$ (M$^+$) 211.111, found 211.112]; mp 101-104 °C; IR (CH$_3$CN) 3626, 3541, 3381, 1631, 1301 cm$^{-1}$;
\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.91 (4H, m, H2, H2'), 7.45-7.55 (5H, m, H3, H3' & H4'), 3.96 (2H, s, CH\(_2\));
\(^1\)C NMR (CDCl\(_3\)) \(\delta\) 152.6 (C1'), 151.6 (C1), 146.3 (C4), 130.8 (C4'), 129.0 (C3'), 127.7 (C3), 123.1 & 122.8 (C2 & C2'), 46.2 (CH\(_2\)).

(E)- and (Z)-N-phthalimido-4-(phenylazo)benzylamine 2.35\(^\text{18}\)

\[
\text{(E)-2.29} \xrightarrow{\text{HOC}} \text{(E)-2.30}
\]

A solution of DEAD (1.12 mL, 7.1 mmol) in THF (50 mL) was added dropwise to a stirred solution of the alcohol 2.29 (1.50 g, 7.1 mmol), triphenylphosphine (1.85 g, 7.1 mmol) and phthalimide (1.04 g, 7.1 mmol) in THF (125 mL) at rt. The reaction mixture was stirred for 20 h under N\(_2\) and the solvent was concentrated \textit{in vacuo}. The residue was purified by flash chromatography, eluting with petroleum ether-dichloromethane (1:9) to give the compound 2.30 (1.97 g, 82%) as an orange-red solid: [Anal. calcd for C\(_{21}\)H\(_{13}\)O\(_2\)N\(_3\), C 73.89, H 4.43, N 12.31; found C 73.63, H 4.31, N 12.43; MS (EI): 341 (M\(^+\)), 285, 236, 149, 105, 77; HRMS (EI) calcd for C\(_{21}\)H\(_{13}\)O\(_2\)N\(_3\) (M\(^+\)) 341.1164, found 341.1167]; mp 205–207 °C; R\(_f\) 0.57 (1:2 ethyl acetate-petroleum ether); IR (film) 3050, 1771, 1717, 1470, 1394, 1348 cm\(^{-1}\);

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.88 (6H, m, H2, H2' & PthH2), 7.73 (2H, dd, J = 5.5, 3.0 Hz, PthH3), 7.55 (2H, d, J = 8.5 Hz, H3), 7.50 (3H, m, H3' & H4'), 4.92 (2H, s, CH\(_2\));

\(^1\)C NMR (CDCl\(_3\)) \(\delta\) 168.0 (PthCO), 152.6 (C1'), 152.1 (C1), 139.1 (C4), 134.1 (PthC2), 132.1 (PthC1), 131.1 (C4'), 129.3 (C3), 129.0 (C3'), 123.4 (PthC3), 123.2 (C2), 122.8 (C2'), 41.3 (CH\(_2\)).

When the above \(^1\)H NMR sample of (E)-2.30 was left on the bench for at least 1 day, a PSS comprising (E)-2.30 / (Z)-2.30 (4:1 by \(^1\)H NMR) resulted.

\(^1\)H NMR (CDCl\(_3\), selected data for the mixture) \(\delta\) 7.20-7.30 (5H, (Z)-2.30, m, H3, H3' & H4'), 6.82 (4H, (Z)-2.30, m, H2 & H2'), 4.77 (2H, (Z)-2.30, s, CH\(_2\)).
(E)- and (Z)-4-(phenylazo)benzylamine hydrochloride salt 2.23

Procedure A:

Compound 2.30 (70 mg, 0.21 mmol) was stirred in THF (3 mL). An aqueous 40% solution of methylamine (3 mL) was added and the solution was stirred for 4 h. The solvent was removed in vacuo and the resulting mixture was purified by flash chromatography, eluting with dichloromethane-ethanol (9:1) to give a compound tentatively assigned as 2.31 (14 mg, 18%) as an orange solid:

\[ \text{MS (EI): 372 (M^+), 341, 236, 211, 161, 105, 89, 77; HRMS (EI) calcd for C}_{22}\text{H}_{20}\text{N}_{4}\text{O}_{2} (M^+) 372.159, \text{found 372.157;}} \]

\[ ^1\text{H NMR (CDCl}_3) \delta 7.90 (4\text{H, m, H}_2 & \text{H}_2'), 7.45-7.57 (9\text{H, m, PthH, H}_3' & \text{H}_4'), 6.60 (1\text{H, m, NHCH}_3), 4.67 (2\text{H, d, } J = 6.0 \text{ Hz, CH}_2), 3.74 (1\text{H, t, } J = 6.0 \text{ Hz, NHCH}_2), 2.90 (3\text{H, d, } J = 5.0 \text{ Hz, CH}_3). \]

Further elution gave 2.22 (28 mg, 62%) as an orange solid.

Procedure B:

Compound 2.30 (152 mg, 0.45 mmol) was stirred in THF (3 mL) at 60 °C. An aqueous 40% solution of methylamine (3 mL) was added and the solution was stirred for 5 min. Concentrated HCl (3 mL) was added dropwise over 5 min causing two layers to form. The flask was cooled on ice and the resulting precipitate was collected by filtration and washed with 1N HCl (2 x 5 mL) to afford 2.23 (89 mg, 80%) as an orange solid:
[MS (FAB): 212 (M\(^+\)), 195, 154, 136, 107, 89, 77; HRMS (FAB) calcd for C\(_{13}\)H\(_{14}\)N\(_3\) (M\(^+\)) 212.1187, found 212.1186; mp 241–243 °C; IR (film) 3283, 2955, 2924, 1460, 1377 cm\(^{-1}\); ¹H NMR (CD\(_3\)OD) \(\delta\) 7.98 (4H, m, H2, H2'), 7.65 (2H, d, J = 8.3 Hz, H3), 7.55 (3H, m, H3' & H4'), 4.22 (2H, s, CH\(_2\)); ¹³C NMR (CD\(_3\)OD) \(\delta\) 154.5 & 154.2 (C1 & C1'), 137.6 (C4), 133.0 (C4'), 131.3 & 130.7 (C3 & C3'), 124.7 & 124.2 (C2 & C2'), 44.2 (CH\(_2\)).

**Nitrosobenzene 2.33\(^{20}\)**

\[
\begin{array}{c}
\text{2.32}
\end{array}
\quad \rightarrow
\begin{array}{c}
\text{2.33}
\end{array}
\]

In a beaker 2.32 (25.5 mL, 250 mmol) and NH\(_4\)Cl (15 g, 280 mmol) were stirred vigorously in water (500 mL). Zinc dust (37.5 g, 516 mmol) was added portionwise over 10 min at a rate such that the temperature of the reaction mixture reached 45 °C. The reaction mixture was stirred a further 15 min, then filtered to remove zinc oxide. The solid was washed with hot water (3 x 100 mL). The combined yellow filtrate and washings were cooled to 0 °C quickly by the addition of ice. Ice was added to concentrated H\(_2\)SO\(_4\) (75 mL) until the solution was at 0 °C and without delay this solution was added to the filtrate, followed by a solution of Na\(_2\)CrO\(_7\) (17 g, 57 mmol) in ice-water (60 mL). The resulting brown precipitate was filtered and washed with water (2 x 50 mL). The impure solid was steam distilled to afford 2.33 (11.2 g, 42%) as a lime green solid:

[MS (EI): 107 (M\(^+\)), 77, 51]; mp 63–67 °C, (literature mp 66–67 °C)\(^{20}\); IR (CHCl\(_3\)) 3068, 1506, 1466, 1443, 1312, 1178, 1117 cm\(^{-1}\); ¹H NMR (CDCl\(_3\)) \(\delta\) 7.90 (2H, dt, J = 7.0, 1.5 Hz, H2), 7.70 (1H, tt, J = 7.0, 1.5 Hz, H4), 7.62 (2H, dt, J = 7.0, 1.5 Hz, H3).
4-aminobenzoic acid ethyl ester 2.35

\[
\begin{align*}
&\text{H}_2\text{N} \quad \text{O} \\
&\text{2.34} \quad \rightarrow \quad \text{H}_2\text{N} \quad \text{O} \\
&\text{Et}
\end{align*}
\]

Compound 2.34 (11.3 g, 82 mmol) was treated with a saturated solution of HCl in ethanol as described in Method D to afford the ester 2.35 (10.1 g, 74%) as a cream solid:

mp 84–86 °C, (literature mp 92 °C)\(^{21}\);

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.95 (2H, m, H3), 6.67 (2H, m, H2), 4.32 (2H, q, J = 7.0 Hz, CH\(_2\)CH\(_3\)), 1.37 (3H, t, J = 7.0 Hz, CH\(_2\)CH\(_3\)).

\((E)-\) and \((Z)-4-(phenylazo)benzoic acid ethyl ester 2.36

\[
\begin{align*}
&\text{NO} \\
&\text{2.33} \quad + \quad \text{H}_2\text{N} \quad \text{O} \\
&\text{2.35} \quad \rightarrow \quad \text{N} \quad \text{N} \\
&\text{Et} \quad \text{Et}
\end{align*}
\]

Compounds 2.33 (6.7 g, 63 mmol) and 2.35 (10.4 g, 63 mmol) were condensed by reflux in glacial acetic acid as described in Method E. The crude product formed was purified by flash chromatography, eluting with petroleum ether-dichloromethane (3:2), to give the ester 2.36 (11.0 g, 69%) as an orange solid:

[MS (EI): 254 (M\(^+\)), 207, 174, 143, 105, 77; HRMS (EI) calcd for C\(_{15}\)H\(_{14}\)O\(_2\)N\(_2\) (M\(^+\)) 254.1055, found 254.1061]; mp 76–82 °C, (literature mp 86-87 °C)\(^{21}\); R\(_F\) 0.51 (1:4 ethyl acetate-petroleum ether); IR (film) 2953, 2856, 1720, 1461, 1377, 1272 cm\(^{-1}\);

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.24 (2H, d, J = 8.5 Hz, H3), 7.98 (4H, m, H2 & H2'), 7.55 (3H, m, H3' & H4'), 4.45 (2H, q, J = 7.0 Hz, CH\(_2\)CH\(_3\)), 1.46 (3H, t, J = 7.0 Hz, CH\(_2\)CH\(_3\));

\(^13\)C NMR (CDCl\(_3\)) \(\delta\) 164.9 (CO), 155.0 (C1), 152.5 (C1'), 132.1 (C4), 131.6 (C4'), 130.5 (C3), 129.1 (C3'), 123.1 (C2), 122.5 (C2'), 61.2 (CH\(_2\)CH\(_3\)), 14.3 (CH\(_2\)CH\(_3\)).
(E)- and (Z)-4-(phenylazo)benzylalcohol 2.29

![Chemical Structure](image)

To a solution of the ester 2.36 (5.49g, 21.6 mmol) in ether (200 mL) under nitrogen at rt, lithium aluminium hydride (1.64 g, 43.2 mmol) was added and the reaction mixture was stirred for 20 min. The suspension was diluted with saturated NH₄Cl (150 mL) and the phases were separated. The aqueous phase was extracted with ethyl acetate (2 x 50 mL). The combined organics were washed with water (150 mL), brine (150 mL), dried (MgSO₄) and concentrated in vacuo to afford the alcohol 2.29 (4.54 g, 99%) as an orange solid:

[MS (EI): 212 (M⁺), 135, 107, 77, 51; HRMS (EI) calcd for C₁₁H₁₀N₂ (M⁺) 212.09496, found 212.09492; mp 141–145 °C, (literature mp 141.5 °C); R_f 0.34 (1:2 ethyl acetate-petroleum ether); IR (CHCl₃) 3616, 3062, 2932, 1709, 1609, 1583, 1520, 1504, 1489, 1420, 1371, 1308 cm⁻¹;]

[^1]H NMR (CDCl₃) δ 7.90 (4H, m, H2 & H2'), 7.55 (5H, m, H3, H3' & H4'), 4.77 (2H, s, CH₂), 1.71 (1H, br s, OH);

[^13]C NMR (CDCl₃) δ 152.6 (C1), 152.1 (C1'), 143.8 (C4), 131.0 (C4'), 129.1 (C3'), 127.4 (C3), 123.1 & 122.9 (C2 & C2'), 64.9 (CH₂).

When the above[^1]H NMR sample of (E)-2.29 was left on the bench for at least 1 day, a PSS comprising (E)-2.29 / (Z)-2.29 (4:1 by[^1]H NMR) resulted.

[^1]H NMR (CDCl₃, selected data for the mixture) δ 6.84 (4H, (Z)-2.29, m, H2 & H2'), 4.63 (2H, (Z)-2.29, s, CH₂).
8.2.4 Synthesis of the peptidyl-α-ketoamides 2.1 and 2.2

\[
\begin{align*}
(E)-2.1 & : \quad \text{Cbz} \quad \text{Ph} \quad \text{N} \quad \text{N} \\
(E)-2.2 & : \quad \text{Cbz} \quad \text{Ph} \quad \text{N} \quad \text{N}
\end{align*}
\]

Attempted preparation of \(N\)-benzyl-(3S)-3-[[(benzyloxy)carbonyl]amino]-2-oxobenzenebutanamide 2.37

\[
\begin{align*}
\text{2.10} & \quad \text{Cbz} \quad \text{Ph} \quad \text{N} \quad \text{N} \\
\text{2.37} & : \quad \text{Cbz} \quad \text{Ph} \quad \text{N} \quad \text{N} \\
\text{2.38} & : \quad \text{Cbz} \quad \text{Ph} \quad \text{N} \quad \text{N}
\end{align*}
\]

To a solution of 2.10 (75 mg, 0.129 mmol) in dichloromethane (6 mL) at -78 °C was administered ozone at a rate of approximately 1 bubble per second for 10 min. Nitrogen was administered to the solution for 5 min and then benzylamine (30 μL, 0.284 mmol) was added. The reaction mixture was stirred at -78 °C for 90 min, then allowed to warm to rt and concentrated \textit{in vacuo}. The desired product 2.37 was not apparent by \(^1\text{H} \text{NMR}\) in the complex mixture. There was evidence for the presence of amide 2.38 by \(^1\text{H} \text{NMR}\) and HRMS.
(2R,3S)- and (2S,3S)-3-[[benzyloxy]carbonyl]amino]-2-hydroxybenzenebutanoic acid 2.39a, 2.39b

A mixture (1:1 by \(^1\)H NMR) of the \(\alpha\)-hydroxyesters 2.5a and 2.5b (482 mg, 1.40 mmol) was treated with lithium hydroxide as described in Method C2 to afford a mixture (1:1 by \(^1\)H NMR) of the \(\alpha\)-hydroxyacids 2.39a and 2.39b (459 mg, 99%) as a cream solid:

[MS (FAB): 352 (M+Na)\(^+\), 330, 286, 154, 136; HRMS (FAB) calcd for C\(_{18}\)H\(_{19}\)O\(_3\)NNa (M+Na)\(^+\) 352.1161, found 352.1156];

\(^1\)H NMR (CD\(_3\)OD, selected data for the mixture) \(\delta\) 7.15-7.35 (each 1OH, m, Ar, isomers a and b), 4.95 (each 3H, m, NH & CbzCH\(_2\), isomers a and b), 4.25 & 4.10 (each 2H, m, CHCHOH, isomers a and b), 2.8-3.0 (each 2H, m, CHCH\(_2\), isomers a and b);

\(^13\)C NMR (CD\(_3\)OD, selected data for the mixture) \(\delta\) 176.5, 175.9, 158.6, 158.5, 140.0, 139.8, 138.6, 130.7, 130.6, 129.7, 129.6, 129.6, 129.1, 129.1, 128.9, 128.8, 127.8, 127.6, 74.3, 72.1, 67.6, 67.5, 57.1, 57.0, 39.3, 36.6.

(E)- and (Z)-N-[4-(phenylazo)benzyl]-(2R,3S)- and (2S,3S)-3-[[benzyloxy]carbonyl]amino]-2-hydroxybenzenebutanamide 2.40a, 2.40b

A mixture (1:1 by \(^1\)H NMR) of the acids 2.39a and 2.39b (100 mg, 0.30 mmol) was coupled to the amine salt, 2.23 (83 mg, 0.33 mmol) as described in Method A1. The products were purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:9) to give a mixture (2:3 by \(^1\)H NMR) of diastereomers A and B of
2.40 (97 mg, 61%) as a pale orange solid. A sample (30 mg) of the original mixture was recrystallised from ethanol to give a 4:1 mixture by ¹H NMR of diastereomers A and B (7 mg):

Data from original mixture:

[MS (FAB): 523 (M+1)⁺, 307, 210, 195, 154, 136; HRMS (FAB) calcd for C₃₁H₃₁O₄N₄ (M+1)⁺ 523.235, found 523.236]; Rf 0.16, 0.09 (1:4 ethyl acetate-petroleum ether); IR (CHCl₃) 3687, 3427, 1776, 1672, 1602, 1517, 1456 cm⁻¹;

Diastereomer A (data from the recrystallised sample):

mp 199–200 °C;

¹H NMR (DMSO) δ 8.60 (1H, t, J = 5.8 Hz, NHCH₂Ar), 7.82-7.88 (4H, m, H₂ & H₂'), 7.50-7.62 (5H, m, H₃, H₃' & H₄'), 7.12-7.30 (10H, m, ArH), 6.03 (1H, d, J = 5.8 Hz, CbzNH), 4.92 (2H, ABₜ, CbzCH₂), 4.40 (2H, d, J = 5.4 Hz, NHCH₂Ar), 4.31 (2H, m, CHCHOH), 2.60 & 2.70 (2H, m, CH₂Ph);

¹³C NMR (DMSO) δ 171.7 (CHOHCONH), 155.2 (CbzCO), 151.6 (C₁'), 150.5 (C₁), 143.3 (C₄), 138.9 & 136.9 (ArC), 131.1 (C₄'), 129.1 (C₃), 128.7 & 128.0 (ArCH), 127.9 (C₃'), 127.9, 127.7, 127.0 & 125.6 (ArCH), 122.2 (C₂), 122.2 (C₂'), 73.3 (CHOH), 64.6 (CbzCH₂), 55.2 (CHCHOH), 41.4 (NHCH₂Ar), 33.7 (CH₂Ph).

Diastereomer B (data from the original mixture):

¹H NMR (DMSO) δ 8.48 (1H, t, J = 5.9 Hz, NHCH₂Ar), 7.76-7.85 (4H, m, H₂ & H₂'), 7.56-7.60 (3H, m, H₃' & H₄'), 7.43 (2H, d, J = 8.3 Hz, H₃), 7.12-7.30 (10H, m, ArH), 5.97 (1H, d, J = 6.8 Hz, CbzNH), 4.92 (2H, m, CbzCH₂), 4.40 (2H, m, NHCH₂Ar), 4.31 (1H, m, CHCHOH), 3.94 (1H, m, CHCHOH), 2.84 (1H, dd, J = 6.8, 13.7 Hz, CHCH₃Ph), 2.70 (1H, m, CHCH₃Ph);

¹³C NMR (DMSO) δ 172.0 (CHOHCONH), 155.2 (CbzCO), 151.6 (C₁'), 150.5 (C₁), 143.3 (C₄), 138.4 & 136.8 (ArC), 131.1 (C₄'), 129.1 (C₃), 128.7 & 128.0 (ArCH), 127.9 (C₃'), 127.9, 127.7, 127.0 & 125.6 (ArCH), 122.2 (C₂), 122.2 (C₂'), 71.3 (CHOH), 64.9 (CbzCH₂), 55.1 (CHCHOH), 41.4 (NHCH₂Ar), 37.1 (CH₂Ph).
(E)- and (Z)- N-[4-(phenylazo)benzyl]-(3S)-3-[(benzyloxy)carbonyl]amino]-2-oxobenzenebutanamide 2.1: DMPO

A mixture (2:3 by $^1$H NMR) of compounds 2.40a and 2.40b (30 mg, 57 µmol) was oxidised with Dess-Martin periodinane as described in Method B1 to afford 2.1 (30 mg, qu) as an orange solid. An analytical sample was obtained by recrystallisation from ethanol:

[Anal. calcd for C$_{31}$H$_{28}$O$_4$N$_4$, C 71.39, H 5.41, N 10.74; found C 71.17, H 5.65, N 10.70; MS (FAB): 521 (M+1)$^+$, 460, 387, 307, 289, 195, 154, 136; HRMS (FAB) calcd for C$_{31}$H$_{29}$O$_4$N$_4$ (M+1)$^+$ 521.219, found 521.220]; mp 178.5-179 °C; R$_f$ 0.55 (1:4 ethyl acetate-dichloromethane); IR (CHCl$_3$) 3412, 1714, 1693, 1620, 1585, 1506, 1330 cm$^{-1}$; $[\alpha]_D$ +5 ± 5° (c 0.10 dichloromethane);

$^1$H NMR (DMSO) $\delta$ 9.45 (1H, t, J = 6.4 Hz, NHCH$_2$Ar), 7.87 (4H, m, H2 & H2'), 7.60 (3H, m, H3' & H4'), 7.48 (2H, d, J = 8.3 Hz, H3), 7.28-7.36 (11H, m, CbzNH & ArH), 5.06 (1H, m, CH), 4.96 (2H, AB$_q$, CbzCH$_2$), 4.45 (2H, d, J = 5.8 Hz, NHCH$_2$Ar), 3.14 (1H, dd, J = 3.4, 14.2 Hz, CHCH$_2$Ph), 2.73 (1H, dd, J = 10.7, 13.7 Hz, CHCH$_2$Ph);

$^{13}$C NMR (DMSO) $\delta$ 197.0 (COCONH), 161.1 (COCONH), 156.0 (CbzCO), 152.0 (C1'), 151.1 (C1), 142.3 (C4), 137.7 & 136.9 (ArC), 131.6 (C4'), 129.6 (C3), 129.1 (ArCH), 128.4 (C3'), 127.9, 127.7, 126.6 (ArCH), 122.7 & 122.2 (C2 & C2'), 65.6 (CbzCH$_2$), 57.5 (CH), 42.0 (NHCH$_2$Ar), 35.0 (CH$_2$Ph).

(E)- and (Z)- N-[4-(phenylazo)benzyl]-(3S)-3-[(benzyloxy)carbonyl]amino]-2-oxobenzenebutanamide 2.1: TEMPO

A mixture of compounds 2.40a and 2.40b (14 mg, 27 µmol) was oxidised with TEMPO as described in Method B2 to afford 2.1 (14 mg, qu) as an orange solid:
[\alpha]_D +4 \pm 2 ^\circ (c 0.23 \text{ acetonitrile});

$^1$H NMR (DMSO) δ 9.46 (1H), 7.87 (4H), 7.60 (3H), 7.48 (2H), 7.28-7.36 (11H), 5.08 (1H), 4.97 (2H), 4.45 (2H), 3.13 (1H), 2.72 (1H).

(E)- and (Z)- N-[4-(phenylazo)phenyl]-(2R,3S)- and (2S,3S)-3-[(benzyloxy)carbonyl]amino]-2-hydroxybenzenebutanamide 2.41a, 2.41b

A mixture (1:1 by $^1$H NMR) of the acids 2.39a and 2.39b (91 mg, 0.28 mmol) was coupled to 4-(phenylazo)aniline (60 mg, 0.31 mmol) as described in Method A2. The products were purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:9) to give a mixture (1:1 by $^1$H NMR) of the diastereomers A and B of 2.41 (65 mg, 46%) as a pale orange solid:

Data from the mixture of diastereomers:

[MS (FAB): 509 (M+1)$^+$, 307, 289, 217, 195, 155, 136; HRMS (FAB) calcd for C$_{30}$H$_{29}$O$_4$N$_4$ (M+1)$^+$ 509.2188, found 509.2185]; mp 205-207 °C; R$_f$ 0.28, 0.16 (2:3 ethyl acetate-petroleum ether); IR (CHCl$_3$) 3688, 3435, 2928, 1696, 1602, 1

Diastereomer A:

$^1$H NMR (DMSO, data from the mixture) δ 10.04 (1H, s, NHAr), 7.87 (6H, m, H2, H2' & H3), 7.53-7.62 (3H, m, H3' & H4'), 7.17-7.35 (10H, m, ArH), 6.07 (1H, d, J = 6.3 Hz, CbzNH), 4.90 (2H, m, CbzCH$_2$), 4.15 (1H, m, CHCHOH), 4.09 (1H, d, J = 2.9 Hz, CHCHOH), 2.93 (1H, m, CHCH$_2$Ph), 2.77 (1H, m, CHCH$_2$Ph);

$^{13}$C NMR (DMSO, data from the mixture) δ 171.6 (CHOHCONH), 155.7 (CbzCO), 152.1 (C1'), 147.9 (C1), 141.6 (C4), 138.8 & 137.3 (ArC), 131.2 (C4'), 129.6 (C3'), 129.4, 129.1, 128.3, 127.6, 127.3, & 126.2 (ArCH), 123.6 (C2'), 122.5 (C2'), 120.2 (C3), 72.5 (CHOH), 65.0 (CbzCH$_2$), 56.2 (CHCHOH), 34.9 (CH$_2$Ph);
Diastereomer B:

$^1$H NMR (DMSO, data from the mixture) $\delta$ 10.09 (1H, s, NHAr), 7.98 (2H, d, J = 9.3 Hz, H3), 7.87 (4H, m, H2 & H2'), 7.53-7.62 (3H, m, H3' & H4'), 7.17-7.35 (10H, m, ArH), 6.33 (1H, d, J = 5.4 Hz, CbzNH), 4.90 (2H, m, CbzCH$_2$), 4.15 (2H, m, CHCHOH) 2.77 (2H, m, CH$_2$Ph);

$^{13}$C NMR (DMSO, data from the mixture) $\delta$ 171.6 (CHOHCONH), 155.7 (CbzCO), 152.1 (C1'), 147.9 (C1), 141.6 (C4), 139.0 & 137.3 (ArC), 131.2 (C4'), 129.6 (C3'), 129.4, 129.1, 128.3, 127.6, 127.3, & 126.2 (ArCH), 123.6 (C2'), 122.5 (C2'), 120.3 (C3), 74.3 (CHOH), 65.0 (CbzCH$_2$), 55.6 (CHCHOH), 37.3 (CH$_2$Ph).

(E)- and (Z)- $N$-[4-(phenylazo)phenyl]-[(3S)-3-[[benzyloxy]carbonyl]amino]-2-oxobenzenebutanamide 2.2: DMPO

A mixture (1:1 by $^1$H NMR) of compounds 2.41a and 2.41b (14 mg, 28 µmol) was treated with Dess Martin periodinane as described in Method B1 to afford 2.2 (14 mg, qu) as an orange solid:

[MS (FAB): 507 (M+)$^+$, 307, 289, 210, 154, 136; HRMS (FAB) calcd for C$_{30}$H$_{27}$O$_4$N$_4$ (M+)$^+$ 507.2032, found 507.2027]; mp 161-165 ºC; R$_f$ 0.19 (1:9 ethyl acetate-dichloromethane); IR (CHCl$_3$) 3433, 3381, 1718, 1699, 1531, 1506, 1354 cm$^{-1}$;

$^1$H NMR (DMSO) $\delta$ 11.01 (1H, s, NHAr), 8.07 (2H, m, H3), 7.86-7.99 (4H, m, H2 & H2'), 7.59 (3H, m, H3' & H4'), 7.23-7.32 (10H, m, ArH), 5.18 (1H, m, CH), 4.99 (2H, m, CbzCH$_2$), 3.22 (1H, dd, J = 3.3, 13.8 Hz, CHCH$_2$Ph), 2.79 (1H, dd, J = 10.5, 13.3 Hz, CHCH$_2$Ph);

$^{13}$C NMR (DMSO) $\delta$ 196.1 (COCONH), 159.2 (COCONH), 155.7 (CbzCO), 151.7 (C1'), 148.1 (C1), 140.1 (C4), 137.3 & 136.5 (ArC), 131.0 (C4'), 129.1 (C3), 128.8, 128.0, 127.5, 127.2, 126.2 (ArCH), 123.1 (C2'), 122.1 (C2), 120.6 (C3), 65.2 (CbzCH$_2$), 56.8 (CH), 34.7 (CH$_2$Ph).
(E)- and (Z)- \( N-[4-(phenylazo)phenyl]-(3S)-3-[[\text{benzyloxy}carbonyl]amino]-2-oxobenzenebutanamide \): TEMPO

A mixture (1:1 by \(^1\text{H} \text{NMR}\)) of compounds \(2.41a\) and \(2.41b\) (10 mg, 20 \(\mu\)mol) was oxidised with TEMPO as described in Method B2 to afford \(2.2\) (10 mg, qu) as an orange-brown solid:

\[ [\alpha]_D^0 +9 \pm 3^\circ (c 0.39 \text{ acetonitrile}) ; \]

\(^1\text{H} \text{NMR} \) (DMSO): \(8 1.03\) (1H), \(8.07\) (2H), \(7.88-8.00\) (4H), \(7.57\) (3H), \(7.23-7.32\) (10H), \(5.18\) (1H), \(4.98\) (2H), \(3.20\) (1H), \(2.78\) (1H).

8.2.5 Synthesis of the key intermediate \(2.46b\)

\[
\text{CbzLeu} \quad \begin{array}{c}
\text{Ph} \\
\text{OH} \\
\text{N}\end{array}
\]
\(2.46b\)

\((2S,3S)-3\text{-amino-2-hydroxybenzenebutanoic acid, methyl ester, hydrobromide} \) \(2.42b\) and \((2S,3S)-2\text{-acetoxy-3-amino} \)benzenebutanoic acid, methyl ester, hydrobromide \(2.43b\) \(^{23}\)

\[
\begin{array}{c}
\text{Cbz} \\
\text{H} \\
\text{N} \\
\text{O} \\
\text{Me} \\
\text{Ph}
\end{array}
\rightarrow
\begin{array}{c}
\text{Ph} \\
\text{O} \\
\text{Me}
\end{array}
\]
\(2.5b\)

\(2.42b\) \(R = H\)

\(2.43b\) \(R = \text{Ac}\)

Compound \(2.5b\) (500 mg, 1.47 mmol) was dissolved in a solution of HBr in acetic acid (33%, 1.0 ml) and the resulting mixture was stirred at rt for 20 minutes. The addition of ether (2 mL) caused the amine salt to precipitate. The solvent was drawn off and the resulting mass was retained. To the ethereal solution, petroleum ether (4 mL) was added and further product precipitated. The solvent was drawn off and the two batches
of solid were combined and dried to afford a mixture (397 mg, 12:1 by $^1$H NMR) of the amine salts 2.42b (86%) and 2.43b (6%) as a brown tar.

Data from the mixture:

**MS (FAB):** 252 (M$,^+$, 2.43b), 219, 210 (M$,^+$, 2.42b), 176, 154, 137, 136, 120.

Data from the mixture for compound 2.42b:

[HRMS (FAB) calcd for C$_{11}$H$_{16}$O$_3$N (M$^+$) 210.1130, found 210.1126];

$^1$H NMR (D$_2$O) $\delta$ 7.19-7.31 (5H, m, ArH), 4.51 (1H, d, J = 2.5 Hz, CHCHOH), 4.03 (1H, m, CHCHOH), 3.38 (3H, s, CH$_3$), 2.89 (2H, m, CH$_2$Ph).

Selected data from the mixture for compound 2.43b:

[HRMS (FAB) calcd for C$_{13}$H$_{18}$O$_4$N (M$^+$) 252.1236, found 252.1238];

$^1$H NMR (D$_2$O) $\delta$ 7.19-7.31 (5H, m, ArH), 5.41 (1H, d, J = 1.7 Hz, CHCHOAc), 4.25 (1H, m, CHCHOAc), 3.41 (3H, s, COOCH$_3$), 3.00 (2H, m, CH$_2$Ph), 2.18 (3H, s, OCOCH$_3$).

(2S,3S)-3-[[N-Cbz-L-leucinyl]amino]-2-hydroxybenzenebutanoic acid, methyl ester 2.44b and (2S,3S)-2-acetoxy-3-[[N-Cbz-L-leucinyl]amino]benzenebutanoic acid, methyl ester 2.45b

To a mixture of the amine salts 2.42b and 2.43b (64 mg, 9:1 by $^1$H NMR, 0.22 mmol) EDCI (47 mg, 0.25 mmol) and HOBT (32 mg, 0.24 mmol) were added. A solution (1.0 M) of N-Cbz-L-leucine in N-methylpyrrolidinone (240 $\mu$L, 0.24 mmol), DMF (250 $\mu$L) and DIEA (43 $\mu$L, 0.25 mmol) were then added and the reaction mixture was stirred overnight at rt under nitrogen. The reaction mixture was diluted with ethyl acetate (4 mL) and washed with 10% HCl (3 x 2 mL), saturated NaHCO$_3$ (3 x 2 mL), brine (2 mL), dried over MgSO$_4$ and concentrated in vacuo to afford a mixture (9:1 by $^1$H NMR) of the esters 2.44b and 2.45b (69 mg, 69%) as a white solid.

Data from the mixture:
[MS (FAB): 499 (M+1)^+, 2.45b], 457 (M+1)^+, 2.44b], 414, 307, 210, 154, 136]; mp 138-144 °C; IR (CHCl_3) 3688, 3416, 2961, 1734, 1676, 1601, 1500, 1440 cm\(^{-1}\).

Data from the mixture for compound 2.44b:

[HRMS (FAB) calcd for C_{11}H_{16}O_3N (M+1)^+ 457.2339, found 457.2334];

\(^1H\) NMR (CDCl_3) \(\delta\) 7.14-7.35 (10H, m, ArH), 6.51 (1H, d, \(J = 8.8\) Hz, PheNH), 5.09 (3H, m, CbzCH_2 & CbzNH), 4.51 (1H, ddd, \(J = 2.9, 7.3, 16.1\) Hz, CHCHOH), 4.29 (1H, br s, CHCHOH), 4.11 (1H, m, NHCHCO), 3.59 (3H, s, OCH_3), 2.79 (2H, m, PheCH_2), 1.61 (3H, m, LeuCH & LeuCH_A), 1.39 (1H, m, LeuCH_B), 0.89 (6H, m, 2 x LeuCH_3);

\(^13\)C NMR (CDCl_3) \(\delta\) 172.8 (COOMe), 172.0 (NHCHCO), 156.2 (CbzCO), 136.7 (PhC=O), 136.0 (CbzC=O), 129.3, 128.5, 128.3, 128.1, 126.7 (ArCH), 72.0 (CHO), 67.2 (CbzCH_2), 53.7 (NHCHCO), 53.0 (CHCHOH), 52.5 (COOCH_3), 41.0 (LeuCH_3), 35.4 (PheCH_2), 24.6 (LeuCH), 22.8 (Leu(CH_3)_A), 21.9 (Leu(CH_3)_B).

Selected data from the mixture for compound 2.45b:

\(^1H\) NMR (CDCl_3) \(\delta\) 6.42 (1H, m, PheNH), 5.53 (1H, s, CHOAc), 3.63 (3H, s, COOCH_3), 2.16 (3H, s, OCOCH_3).

(2S,3S)-3-[N-Cbz-L-leucinyl]amino-2-hydroxybenzenebutanoic acid 2.46b

A mixture of the esters 2.44b and 2.45b (60 mg, 0.13 mmol, 9:1 by \(^1H\) NMR) was treated with lithium hydroxide as described in Method C2 to afford the acid 2.46b (58 mg, qu) as a white solid;

[MS (FAB): 465 (M+Na)^+, 443, 399, 286, 176, 153, 136]; HRMS (FAB) calcd for C_{24}H_{30}O_6N_2Na (M+Na)^+ 465.2002, found 465.1996]; mp 131-134 °C; IR (CHCl_3) 3433, 2963, 1720, 1660, 1510, 1456, 1340, 1217 cm\(^{-1}\);

\(^1H\) NMR (CD_3OD) \(\delta\) 7.82 (1H, d, \(J = 9.3\) Hz, CbzNH), 7.33 (5H, m, ArH), 7.10-7.19 (5H, m, ArH), 5.07 (2H, m, CbzCH_2), 4.49 (1H, m, CHCHOH), 4.17 (1H, d, \(J = 4.4\) Hz, CHCHOH), 4.07 (1H, m, NHCHCO), 2.80 (2H, m, PheCH_2), 1.58 (1H, m, LeuCH), 1.33 (2H, m, LeuCH_2), 0.88 (6H, m, 2 x LeuCH_3);
\[ 1^3\text{C NMR (CD}_3\text{OD}) \delta 175.8 & 175.2 (\text{CHCONH} & \text{COOH}), 158.6 (\text{CbzCO}), 139.6 (\text{PheArC}), 138.4 (\text{CbzArC}), 130.7, 129.8, 129.6, 129.3, 129.2, 127.6 (\text{ArCH}), 73.9 (\text{CHOH}), 68.0 (\text{CbzCH}_2), 55.4 (\text{NHCHCO}), 54.8 (\text{CHCHOH}), 42.5 (\text{LeuCH}_2), 36.4 (\text{PheCH}_2), 26.1 (\text{LeuCH}), 23.6 (\text{Leu(CH}_3)_A), 22.2 (\text{Leu(CH}_3)_B). \]

8.2.6 Synthesis of the peptidyl-\(\alpha\)-ketoamides 2.3 and 2.4

(E)- and (Z)- \(N\)-(4-(phenylazo)benzyl)-(2S,3S)-3-[[N-Cbz-L-leucinyl]amino]-2-hydroxybenzenebutanamide 2.47b

The acid 2.46b (50 mg, 0.11 mmol) was coupled to the amine 2.23 (31 mg, 0.12 mmol) as described in Method A1. The products were purified by flash chromatography, eluting with ethyl acetate-dichloromethane (2:3) to give 2.47b (43 mg, 60%) as an orange-brown solid:

[MS (FAB): 636 (M+1)^+, 592, 460, 397, 307, 289, 195, 154, 136; HRMS (FAB) calcd for C\(_{37}\)H\(_{42}\)O\(_8\)N\(_5\) (M+1)^+ 636.3186, found 636.3190]; mp 204-208 °C; IR (CHCl\(_3\)) 3427, 3325, 2961, 1716, 1672, 1510, 1439, 1217 cm\(^{-1}\);

\[^1\text{H NMR (DMSO)} \delta 8.59 (1\text{H, t, J = 5.9 Hz, NHCH}_2), 7.86 (4\text{H, m, H}_2 \text{ & H}_2'), 7.70 (1\text{H, d, J = 9.3 Hz, PheNH}), 7.59 (3\text{H, m, H}_3' \text{ & H}_4'), 7.51 (2\text{H, d, J = 8.3 Hz, H}_3), 7.23-7.35 (5\text{H, m, ArH}), 7.09-7.19 (5\text{H, m, ArH}), 6.05 (1\text{H, d, J = 4.9 Hz, CbzNH}), 5.02 (2\text{H, AB}_\text{q}, \text{CbzCH}_2), 4.38 (3\text{H, m, CHCHOH & NHCH}_2), 3.98 (2\text{H, m, NHCHCO &}}
CH(OH), 2.68 (1H, m, PheCH\textsubscript{A}), 2.57 (1H, m, PheCH\textsubscript{B}), 1.47 (1H, m, LeuCH), 1.28 (2H, m, LeuCH\textsubscript{2}), 0.81 (6H, m, 2 x LeuCH\textsubscript{3});

\(^{13}\)C NMR (DMSO) \(\delta\) 172.1 (CHOHCONH), 171.8 (NHCHCO), 155.9 (CbzCO), 152.0 (C1'), 151.0 (C1), 143.6 (C4), 138.9 (PheArC), 137.1 (CbzArC), 131.6 (C4'), 129.6 (C3'), 129.2, 128.5 (ArCH), 128.3 (C3), 128.0, 127.9, 127.8, 126.0 (ArCH), 122.7 & 122.6 (C2 & C2'), 73.6 (CHOH), 65.5 (CbzCH\textsubscript{2}), 53.4 (NHCHCO), 53.1 (CHCHOH), 41.8 (NHCH\textsubscript{2}), 41.0 (LeuCH\textsubscript{2}), 34.3 (PheCH\textsubscript{2}), 24.2 (LeuCH), 23.1 (Leu(CH\textsubscript{3})\textsubscript{A}), 21.6 (Leu(CH\textsubscript{3})\textsubscript{B}).

When the above \(^{1}\)H NMR sample of (E)-2.47\textsubscript{b} was left on the bench for at least 1 day, a PSS comprising (E)-2.47\textsubscript{b} / (Z)-2.47\textsubscript{b} (4:1 by \(^{1}\)H NMR) resulted.

\(^{1}\)H NMR (DMSO, selected data for the mixture) \(\delta\) 8.48 (1H, (Z)-2.47\textsubscript{b}, t, NHCH\textsubscript{2}), 6.79 (4H, (Z)-2.47\textsubscript{b}, m, H2 & H2').

(E)- and (Z)- \(N-(4-(\text{phenylazo})\text{benzyl})-(3S)-3-[[\text{N-Cbz-L-leuciny}]\text{amino}]-2-\text{oxo benzenebutanamide 2.3}

\[
2.47\text{b} \quad \text{CbzLeu} \quad \text{Ph} \quad \text{N} = \quad \text{N} \quad \text{(E)-2.3}
\]

Compound 2.47\textsubscript{b} (12 mg, 20 \(\mu\)mol) was oxidised with TEMPO as described in Method B2 to afford 2.3 (12 mg, qu) as an orange solid:

[MS (FAB): 634 (M+1)\textsuperscript{+}, 477, 395, 307, 289, 195, 154, 136; HRMS (FAB) calcld for C\textsubscript{37}H\textsubscript{40}O\textsubscript{5}N\textsubscript{5} (M+1)\textsuperscript{+} 634.3029, found 634.3025]; mp 165-172 °C; R\textsubscript{f} 0.65 (1:4 ethyl acetate-dichloromethane); IR (CHCl\textsubscript{3}) 3683, 3620, 3413, 3013, 2976, 1710, 1692, 1518, 1421, 1203 cm\textsuperscript{-1}; [\(\alpha\)]\textsubscript{D} = -18 ° (c 0.54 acetonitrile);

\(^{1}\)H NMR (CD\textsubscript{3}CN) \(\delta\) 7.97 (4H, m, H2 & H2'), 7.65 (3H, m, H3' & H4'), 7.57 (2H, d, J = 14.2 Hz, H3), 7.20-7.41 (1IH, m, ArH & PheNH), 5.89 (1H, d, J = 7.8 Hz, CbzNH), 5.38 (1H, m, CHCOCO), 5.11 (2H, AB, CbzCH\textsubscript{2}), 4.57 (2H, d, J = 7.0 Hz, NHCH\textsubscript{2}).
4.12 (1H, m, CbzNHCH), 3.28 (1H, m, PheCH₂), 3.01 (1H, m, PheCH₃), 1.65 (1H, m, LeuCH), 1.45 (2H, m, LeuCH₂), 0.93 (6H, m, 2 x LeuCH₃);

¹³C NMR (CD₃CN) δ 195.2 (COCONH), 172.0 (COCONH), 159.2 (LeuCO), 156.1 (CbzCO), 152.5 & 152.2 (C1 & C1'), 139.7 (C4), 136.0 (CbzArC), 135.5 (PheArC), 131.1 (C4'), 129.3, 129.1, 128.6, 128.5, 128.4, 128.2, 127.9, 127.1 (ArCH, C3 & C3'), 123.3 (C2), 122.8 (C2'), 67.1 (CbzCH₂), 55.7 (CHCOCO), 53.2 (CbzNHCH), 43.0 (NHCH₂), 41.0 (LeuCH₂), 37.1 (PheCH₂), 24.6 (LeuCH), 22.8 (Leu(CH₃)ₐ), 21.9 (Leu(CH₃)ₐ).

(E)- and (Z)- N-[4-(phenylazo)benzyl]- (2S,3S)-3-[[N-Cbz-L-leucinyl]amino]-2-hydroxybenzenebutanamide 2.49b: EDCI

The acid 2.46b (88 mg, 0.20 mmol) was coupled to the amine 2.48 (44 mg, 0.22 mmol) as described in Method A2. The products were purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:4) to give an inseparable mixture (2:1 by ¹H NMR) of 2.49b and 2.50 (44 mg) as an orange solid:

Data from the mixture:
[MS (FAB): 849 ((M+1)+, 2.50), 620 ((M+1)+, 2.49b), 550, 455, 395, 308, 293, 210, 195, 154, 136; HRMS (FAB) caled for C₃₆H₃₈O₅N₅ ((M+1)+, 2.49b) 620.287, found 620.288]; Rₚ 0.21 (1:4 ethyl acetate-dichloromethane); IR (CHCl₃) 3710, 3645, 1703, 1607, 1531, 1504, 1466 cm⁻¹;

Compound 2.49b:
¹H NMR (DMSO, data from the mixture) δ 10.05 (1H, s, NHAr), 7.86-7.96 (6H, m, H₂, H₂' & H₃), 7.74 (1H, d, J = 8.8 Hz, PheNH), 7.59 (3H, m, H₃' & H₄'), 7.16-7.35 (10H, m, ArH), 6.23 (1H, d, J = 6.4 Hz, CbzNH), 5.02 (2H, ABq, CbzCH₂), 4.38 (1H, m,
CHCHOH), 3.98-4.09 (2H, m, NHCHCO & CHOH), 2.83 (2H, m, PheCH2), 1.15-1.48 (3H, m, LeuCH & LeuCH2), 0.73 (6H, m, 2 x LeuCH3);

$^{13}$C NMR (DMSO, data from the mixture) δ 171.5 & 171.1 (CHOHCONH & NHCHCO), 155.5 (CbzCO), 151.7 (C1'), 147.5 (C1), 141.3 (C4), 138.1 & 136.8 (ArC), 130.8 (C4'), 129.1, 129.0, 128.7, 128.0, 127.7, 127.4, 125.6 (C3' & ArCH), 123.1 (C2'), 122.0 (C2), 119.8 (C3), 73.5 (CHOH), 65.1 (CbzCH2), 53.0 & 52.7 (NHCHCO & CHCHOH), 40.6 (LeuCH2), 34.8 (PheCH2), 23.7 (LeuCH), 22.6 (Leu(CH3)A), 21.2 (Leu(CH3)B).

Lactone dimer of 2.46b

To a solution of the acid 2.46b (14 mg, 32 μmol) in DMF (1 mL) was added EDCI (8 mg, 41 μmol), HOBT (7 mg, 47 μmol), dichloromethane (1 mL) and DIEA (6 μL, 35 μmol). The reaction mixture was stirred overnight at rt before being diluted with dichloromethane (10 mL), washed with 10% HCl (10 mL), brine (10 mL), dried over MgSO4 and concentrated in vacuo to afford 2.50 (10 mg, 74%) as a white solid:

[MS (ES): 871 (M+Na)$^+$, 849 (M+1)$^+$, 647, 556, 537, 464, 316, 289, 275, 249; HRMS (ES) calcd for C48Hs70lON4 (M+ $^+$) 849.408, found 849.405]; Rf 0.19 (1:4 ethyl acetate-dichloromethane); IR (CHCl3) 3628, 3354, 1767, 1722, 1683, 1228 cm$^{-1}$; [α]$^D$ - 78 (c 0.082 acetonitrile);

$^1$H NMR (DMSO) δ 8.28 (2H, d, J = 8.2 Hz, 2 x PheNH), 7.16-7.35 (22H, m, ArH & 2 x CbzNH), 5.49 (2H, s, 2 x CHOR), 5.01 (4H, s, 2 x CbzCH2), 4.66 (2H, m, 2 x CHCHOR), 4.05 (2H, m, 2 x NHCHCO), 2.81 (4H, m, 2 x PheCH2), 1.49 (2H, m, 2 x LeuCH), 1.36 (2H, m, 2 x LeuCHA), 1.27 (2H, m, 2 x LeuCHB), 0.81 (12H, m, 4 x LeuCH3);

$^{13}$C NMR (CD3Cl) δ 172.1 (COCOR), 165.7 (LeuCO), 155.4 (CbzCO), 137.6 (PheArC), 136.7 (CbzArC), 128.7, 128.1, 128.0, 127.6, 127.5, 126.1 (ArCH), 77.6
(COR), 65.2 (CbzCH₂), 52.8 (COCHNH), 49.9 (NHCHCOR), 40.7 (LeuCH₂), 33.8 (PheCH₂), 23.9 (LeuCH), 22.6 (Leu(CH₃)ₐ), 21.3 (Leu(CH₃)ₙ).

(E)- and (Z)- N-(4-(phenylazo)benzyl)-(2S,3S)-3-[[N-Cbz-L-leuciny]amino]-2-oxo benzenebutanamide 2.49: NHS

\[
\begin{align*}
\text{2.46b} \rightarrow \text{CbzLeu} \rightarrow \text{2.4} \\
\text{(E)-2.49b}
\end{align*}
\]

A solution of DCC (11 mg, 54 µmol) in THF (0.25 mL) at 0 °C was added to a cold solution of the acid 2.46b (24 mg, 54 µmol) and N-hydroxysuccinimide (6 mg, 54 µmol) in THF (0.70 mL). The reaction mixture was stirred at 0 °C for 2 h then stored in a freezer overnight. The resulting mixture was filtered through Celite, washing with cold THF (1 mL). To the amine 2.48 (53 mg, 270 µmol) was added the solution of N-hydroxysuccinimide ester and the reaction mixture was stirred at 0 °C for 30 min and at rt overnight. The solution was diluted with dichloromethane (10 mL), washed with saturated NH₄Cl (10 mL), brine (10 mL), dried over MgSO₄ and concentrated in vacuo. The residue was purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:4) to give an inseparable mixture (5:6 by ¹H NMR) of 2.49b (data as detailed above) and DCU (36 mg) as an orange solid: Rf 0.18 (1:4 ethyl acetate-dichloromethane).

A sample of the preceding mixture (5:6 by ¹H NMR) of 2.49b and DCU (20 mg) was oxidised with TEMPO as described in General Method B2. The resulting mixture was purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:19) to give afford 2.4 (7 mg, 39% over two steps) as an orange solid: [MS (FAB): 620 (M+1)⁺, 550, 395, 308, 293, 289, 259, 210, 175, 154, 137; HRMS (FAB) calcd for C₃₆H₃₈O₅N₅ (M+1)⁺ 620.287, found 620.288]; Rf 0.53 (1:9 ethyl acetate-dichloromethane); IR (CHCl₃) 3684, 3620, 3431, 3010, 2976, 1720, 1697, 1602, 1521, 1477, 1421, 1200 cm⁻¹; [α]D - 16 ° (c 0.038 acetonitrile);
\(^1\)H NMR (CDCl\(_3\)) 8.86 (1H, s, NHAr), 7.94 (4H, m, H2 & H2'), 7.81 (2H, m, H3), 7.53 (3H, m, H3' & H4'), 7.15-7.35 (10H, m, ArH), 6.78 (1H, m, PheNH), 5.59 (1H, m, CHCOCO), 5.03-5.13 (3H, m, CbzCH\(_2\) & CbzNH), 4.19 (1H, m, CbzNHCH), 3.13 (1H, dd, J = 5.4, 13.7 Hz, PheCH\(_A\)), 3.13 (1H, dd, J = 7.6, 14.5 Hz, PheCH\(_B\)), 1.40-1.62 (3H, m, LeuCH & LeuCH\(_2\)), 0.88 (6H, m, 2 x LeuCH\(_3\));

\(^13\)C NMR (DMSO) 195.5 (COCONH), 172.6 (COCONH), 159.5 (LeuCO), 155.6 (CbzCO), 151.7 (C1'), 148.0 (C1), 140.4 (C4), 137.2 & 137.0 (ArC), 131.1 (C4'), 129.2, 129.0, 128.9, 128.1, 127.6, 127.4, 126.3 (ArCH & C3'), 123.2 (C2), 122.2 (C2'), 120.6 (C3), 65.1 (CbzCH\(_2\)), 55.0 (CHCOCO), 52.4 (CbzNHCH), 40.5 (LeuCH\(_2\)), 35.0 (PheCH\(_2\)), 23.8 (LeuCH), 22.6 (Leu(CH\(_3\))\(_A\)), 21.3 (Leu(CH\(_3\))\(_B\)).

The \(^1\)H NMR spectrum of 2.4 in \(d_6\)-DMSO showed doubling of the amide protons:

\(^1\)H NMR (DMSO, selected data from the mixture) 8.95 (0.4H, s, NHAr, minor), 10.90 (0.6H, s, NHAr, major), 8.62 (0.4H, d, J = 6.8 Hz, NH, minor), 8.56 (0.6H, d, J = 6.4 Hz, NH, major), 7.81 (2H, d, J = 8.8 Hz, H3, both), 7.88 (4H, m, H2 & H2', both), 7.70 (0.4H, d, J = 8.8 Hz, NH, minor), 7.58 (3.6H, m, H3' & H4', both, NH, major), 7.23-7.38 (10H, m, ArH, both), 5.19 (1H, m, CHCOCO, both), 5.02 (2H, AB\(_q\), CbzCH\(_2\), both), 4.09 (1H, m, CbzNHCH, both), 3.30 (1H, m, PheCH\(_A\), both), 2.94 (1H, m, PheCH\(_B\), both), 1.55 (1H, m, LeuCH\(_A\), both), 1.35 (2H, m, LeuCH\(_2\), both), 1.26 (1H, m, LeuCH\(_B\), both), 0.79 (6H, m, 2 x LeuCH\(_3\), both).

\((3S)-3-[[\text{benzyloxy}carbonyl]\text{amino}]\text{-2-oxobenzenebutanoic acid ethyl ester} 2.52\)

\[
\begin{align*}
\text{Cbz} & \quad \text{Ph} \\
\text{OH} & \quad \text{CN} \\
2.14 & \quad \longrightarrow & \quad \text{Cbz} & \quad \text{Ph} \\
\text{OH} & \quad \text{OEt} \\
2.51 & \quad \longrightarrow & \quad \text{Cbz} & \quad \text{Ph} \\
\text{NH} & \quad \text{OEt} \\
2.52 &
\end{align*}
\]

To a saturated solution of HCl in (3:1) ether/ethanol (40 mL) at 0 °C was added a mixture (1:1 by \(^1\)H NMR) of the cyanohydrins 2.14\(\alpha\) and 2.14\(\beta\) (0.326 g, 1.05 mmol). The solution was stirred at 4 °C for 24 h, before cold water (10 mL) was added dropwise and the reaction mixture was stirred a further 3 days at 4 °C. The organics
were evaporated in vacuo and the resulting mixture was extracted with ethyl acetate (2 x 25 mL). The combined organic extracts were washed with water (40 mL), brine (40 mL), dried over MgSO₄ and concentrated in vacuo. Purification by flash chromatography, eluting with dichloromethane, gave a mixture (1:1 by ¹H NMR) of the ethyl esters 2.51a and 2.51b (0.186 g, 50%) as a white solid:

[MS (EI): 254 (M-CHOHCOOEtv), 222 (M-BnOCO)⁺, 210, 158, 107, 91; HRMS (EI) calcd for C₁₆H₁₆O₂N (M-CHOHCOOEtv) 254.118, found 254.121, calcd for C₁₂H₁₆O₃N (M-BnOCO)⁺ 222.1131, found 222.1139]; Rf 0.32 (1:9 ethyl acetate-dichloromethane);
IR (CDCl₃) 3672, 3524, 3435, 3067, 3032, 2984, 2939, 1725, 1605, 1506, 1456, 1406 cm⁻¹;
¹H NMR (CDCl₃, selected data for the mixture) δ 7.18-7.38 (each 10H, m, Ar, isomers a and b), 5.12 & 5.18 (each 1H, d, NH, isomers a and b), 5.03 & 5.04 (each 2H, s, CbzCH₂, isomers a and b), 4.30-4.42 (each 1H, m, CHCHOH, isomers a and b) (one isomer 1H, m, CHCHOH), 4.05-4.21 (each 1H, m, CH₂CH₃, isomers a and b) & (one isomer 1H, m, CHCHOH), 2.92 (each 1H, m, CHH₄Ph, isomers a and b), 2.80 (each 1H, m, CHH₄Ph, isomers a and b), 1.21 (each 3H, m, CH₂CH₃, isomers a and b);

A mixture (1:1 by ¹H NMR) of the α-hydroxy-esters 2.51a and 2.51b (91 mg, 0.25 mmol) was oxidised with Dess Martin periodinane as described in Method B1 to afford the α-keto-ester 2.52 (74 mg, 82%) as a yellow oil:

[MS (EI): 355 (M⁺), 335, 293, 254, 210, 176, 149, 91, 77; HRMS (EI) calcd for C₂₀H₂₁O₅N (M⁺) 355.14197, found 355.14162; Rf 0.61 (dichloromethane); IR (CDCl₃) 3512, 3433, 2927, 1728, 1585, 1498, 1456, 1339, 1261, 1223 cm⁻¹; [α]D - 20 ± 5 ° (c 0.076 ethanol);
¹H NMR (CDCl₃) δ 7.08-7.34 (10H, m, Ar), 5.30 (2H, m, CH & NH), 5.08 (2H, s, CbzCH₂), 4.31 (2H, q, J = 6.9 Hz, CH₂CH₃), 3.23 (1H, dd, J = 4.9, 14.1 Hz, CHCH₄Ph), 3.06 (1H, dd, J = 4.9, 13.7 Hz, CHCH₄Ph), 1.35 (3H, t, J = 6.8 Hz, CH₂CH₃);
8.3 Experimental Work Described in Chapter Three

8.3.2 Synthesis of the peptidyl-\(\alpha\)-ketoesters 3.1 to 3.3

\[ (E)-3.1 \]

\[ (E)-3.2 \]

\[ (E)-3.3 \]

8.3.2.1 Attempted synthesis of 3.1 via the cyanoketophosphorane 3.10

\[ (E)-\text{and}(Z)-4-(\text{phenylazo})\text{benzoic acid }3.7^7 \]

\[ (E)-2.36 \rightarrow (E)-3.7 \]

The ester 2.36 (508 mg, 2.0 mmol) was treated with potassium hydroxide as described in General Procedure C1 to afford 3.7 (382 mg, 85%) as a shiny orange solid: mp 247-249 °C, (literature mp 249 °C) \(^{21}\),
\[^1\]H NMR (DMSO) $\delta$ 8.29 (2H, d, $J = 10.5$ Hz, H3), 8.08 (4H, m, H2 & H2'), 7.76 (3H, m, H3' & H4').

(E)- and (Z)-(2S)-2-[[4-(phenylazo)benzene|carbonyl|amino]benzenepropanoic acid 3.9

The acid 3.7 (100 mg, 0.44 mmol) was coupled to L-phenylalanine methyl ester (105 mg, 0.49 mmol) according to General Procedure A1. The crude product was purified by flash chromatography, eluting with dichloromethane to give the compound 3.8 (156 mg, 91%) as an orange solid:

[MS (EI): 387 (M'), 355, 225, 209, 143, 104, 77, 51; HRMS (EI) calcd for C_{23}H_{21}O_{3}N_{3} (M') 387.15829, found 387.15836]; mp 142-143 °C; $R_f$ (E) 0.60 $R_f$ (Z) 0.37 (1:9 ethyl acetate-dichloromethane); IR (CHCl$_3$) 3439, 1741, 1662, 1518, 1485, 1361, 1234 cm$^{-1}$;

\[^1\]H NMR (CDCl$_3$) $\delta$ 7.95 (4H, m, H2, H2'), 7.87 (2H, m, H3), 7.52 (3H, m, H3' & H4'), 7.29 (3H, m, ArH), 7.15 (2H, m, ArH), 6.73 (1H, d, $J = 7.8$ Hz, NH), 5.12 (1H, m, CH), 3.78 (3H, s, CH$_3$), 3.28 (2H, m, CH$_2$);

\[^{13}\]C NMR (CDCl$_3$) $\delta$ 171.9 (PheCO), 166.1 (COAr), 154.4 (C1), 152.5 (C1'), 135.7 (ArC), 135.5 (C4), 131.6 (C4'), 129.3, 128.1, 128.6, 128.0, 127.2 (C3, C3' & ArCH), 123.0 & 122.9 (C2 & C2'), 53.6 (CH$_3$), 52.4 (CH), 37.8 (CH$_2$).

The ester 3.8 (100 mg, 0.26 mmol) was treated with lithium hydroxide solution as described in General Procedure C2 to afford 3.9 (95 mg, qu) as an orange solid:

[MS (FAB): 374 (M'$^+$), 307, 289, 209, 154, 137; HRMS (FAB) calcd for C_{23}H_{21}O_{3}N_{3} (M'$^+$) 374.1505, found 374.1506]; mp 180-183 °C;

\[^1\]H NMR (DMSO) $\delta$ 8.94 (1H, d, $J = 7.8$ Hz, NH), 8.02 (2H, d, $J = 8.3$ Hz, H3), 7.95 (4H, m, H2, H2'), 7.61 (3H, m, H3' & H4'), 7.21-7.35 (5H, m, ArH), 4.66 (1H, m, CH), 3.23 (1H, dd, $J = 4.4, 13.7$, CH$_A$), 3.10 (1H, dd, $J = 10.8, 13.7$, CH$_B$);
\(^{13}\text{C} \text{ NMR} (\text{DMSO} \ \delta \ 173.2 \ (\text{PheCO}), 165.7 \ (\text{COAr}), 153.5 \ (\text{C1}), 152.0 \ (\text{C1'}), 138.2 \ (\text{ArC}), 136.1 \ (\text{C4}), 132.2 \ (\text{C4'}), 129.7, 129.2, 128.8, 128.3, 126.5 \ (\text{C3}, \text{C3'} \ & \text{ArCH}), 122.9 \ & 122.5 \ (\text{C2} \ & \text{C2'}), 54.4 \ (\text{CH}), 36.4 \ (\text{CH}_2)\). \\

**Attempted preparation of (E)- and (Z)-(4S)-3-oxo-4-[4-(phenylazo)benzene carbonyl]amino]-2-(triphenylphosphoranylidene)benzenepentanenitrile acid 3.10**

\[ \text{3.9} \rightarrow X \rightarrow \text{3.10} \]

To a solution of the acid 3.9 (24 mg, 64 \text{\mu mol}) in dichloromethane (2 mL) at 0°C were added EDCI (13 mg, 67 \text{\mu mol}) and DMAP (1 mg, 6 \text{\mu mol}), followed by the dropwise addition of the phosphorane 2.8 (39 mg, 67 \text{\mu mol}) in dichloromethane. After stirring overnight at rt the reaction mixture was diluted with dichloromethane (5 mL) and washed with water (5 mL). The water phase was back-extracted with dichloromethane (2 x 5 mL) and the combined organics were washed with brine (10 mL), dried over MgSO\(_4\) and concentrated \textit{in vacuo} to afford a complex mixture that contained neither starting material nor desired product by \(^1\text{H} \text{NMR} \).

**8.3.2.2 \textit{Synthesis of 3.1 via the peptidyl-\alpha-hydroxyester 3.12a}**

\((2R,3S)-3\text{-amino-2-hydroxybenzenebutanoic acid, methyl ester, hydrobromide 3.11a}^{23}\)

\[ \text{2.5a} \rightarrow \text{3.11a} \]
Compound 2.5a (795 mg, 2.31 mmol) was dissolved in a solution of HBr in acetic acid (33%, 1.0 ml) and the resulting mixture was stirred at rt for 20 minutes. The addition of ether (2 mL) caused the amine salt to precipitate. The mixture was kept at 0 °C for 30 min and then filtered and washed with ether (3 x 2 mL) to give 3.11a (574 mg, 86%) as a white solid:

[MS (FAB): 210 (M⁺), 196, 176, 154, 137, 136, 120]; mp 160-161 °C;

¹H NMR (D₂O) δ 7.22-7.35 (5H, m, ArH), 4.28 (1H, d, J = 3.4 Hz, CHCHOH), 3.87 (1H, m, CHCHOH), 3.64 (3H, s, CH₃), 2.99 (2H, m, CH₃Ph);

¹³C NMR (D₂O) δ 172.9 (CO), 134.8 (ArC), 129.4, 129.2, 127.7 (ArCH), 68.5 (CHOH), 54.2 (CHCHOH), 53.1 (CH₃), 35.1 (CH₂).

(E)− and (Z)-(2R,3S)-2-hydroxy-3-[(4-(phenylazo)benzene]carbonyl]amino] benzenebutanoic acid methyl ester 3.12a

\[\text{3.7} + \text{3.11a} \rightarrow \text{(E)-3.12a}\]

The acid 3.7 (71 mg, 0.31 mmol) was coupled to the amine salt 3.11a (100 mg, 0.34 mmol) according to General Procedure A1 to afford 3.12a (127 mg, 89%) as an orange solid:

[Anal. calcd for C₂₄H₂₃O₄N₃, C 69.05, H 5.55, N 10.06; found C 68.80, H 5.66, N 10.24; MS (EI): 417 (M⁺), 328, 308, 209, 105, 77; HRMS (EI) calcd for C₂₄H₂₃O₄N₃ (M⁺) 417.1689, found 417.1693]; mp 176 °C; Rf 0.43 (1:9 ethyl acetate-dichloromethane); IR (CHCl₃) 1734, 1666, 1514, 1483, 1382, 1290, 1265 cm⁻¹;

¹H NMR (CDCl₃) δ 7.95 (4H, m, H₂, H₂'), 7.82 (2H, d, J = 8.3 Hz, H₃), 7.54 (3H, m, H₃' & H₄'), 7.24-7.36 (5H, m, ArH), 6.44 (1H, d, J = 9.1 Hz, NH), 4.82 (1H, m, CH), 4.22 (1H, d, J = 2 Hz, CHOH), 3.77 (3H, s, CH₃), 3.31 (1H, d, J = 4 Hz, CHOH), 3.05 (2H, m, CH₂);

¹³C NMR (CDCl₃) δ 174.3 (ArCO), 166.5 (COOMe), 154.4 (C1), 152.5 (C1'), 137.2 (ArC), 135.9 (C4), 131.6 (C4'), 129.4 (ArCH), 129.2 (C3'), 128.7 (ArCH), 127.9 (C3),
126.9 (ArCH), 123.1 & 122.9 (C2 & C2'), 70.1 (CHOH), 53.4 (CH), 53.1 (CH3), 38.0 (CH2).

When the above $^1$H NMR sample of (E)-3.12a was left on the bench for at least 1 day, a PSS comprising (E)-3.12a / (Z)-3.12a (5:1 by $^1$H NMR) resulted. Rf 0.17 (1:9 ethyl acetate-dichloromethane);

$^1$H NMR (CDCl$_3$, selected data for the mixture) δ 6.88 (4H, (Z)-3.12a, m, H2 & H2'), 6.37 (1H, (Z)-3.12a, d, NH), 4.75 (1H, (Z)-3.12a, m, CH), 4.19 (1H, (Z)-3.12a, d, CHOH), 3.73 (3H, (Z)-3.12a, s, CH3), 3.29 (1H, (Z)-3.12a, d, CHOH).

(E)- and (Z)-(3S)-2-oxo-3-|4-(phenylazo)benzene|carbonyl|amino|benzene butanoic acid methyl ester 3.1

The alcohol 3.12a (43 mg, 0.10 mmol) was oxidised with TEMPO as described in General Procedure B2 to give 3.1 (43 mg, qu) as an orange solid:

[MS (El): 415 (M$^+$), 356, 328, 209, 105, 77; HRMS (El) calcd for C$_{24}$H$_{21}$O$_4$N$_3$ (M$^+$) 415.1532, found 415.1539]; mp 138-139 °C; Rf 0.61 (1:9 ethyl acetate-dichloromethane); IR (CHCl$_3$) 1733, 1664, 1514, 1483, 1383, 1286, 1096 cm$^{-1}$; [α]$_D$ -52 ± 5 (c 0.29 acetonitrile);

$^1$H NMR (CDCl$_3$) δ 7.98 (4H, m, H2, H2'), 7.84 (2H, m, H3), 7.55 (3H, m, H3' & H4'), 7.17-7.30 (5H, m, ArH), 6.67 (1H, d, J = 4.9 Hz, NH), 5.65 (1H, m, CH), 3.91 (3H, s, CH3), 3.41 (1H, dd, J = 5.9, 13.7 Hz, CHA), 3.25 (1H, dd, J = 6.6, 13.7 Hz, CHB);

$^{13}$C NMR (CDCl$_3$) δ 191.5 (COCOOMe), 166.3 (COAr), 160.7 (COOMe), 154.6 (C1), 152.5 (C1'), 134.9 & 134.9 (ArC & C4), 131.6 (C4'), 129.4, 129.2, 128.9, 128.0, 127.5 (C3, C3' & ArCH), 123.1 & 123.0 (C2 & C2'), 57.1 (CH), 53.3 (CH3), 36.9 (CH2).
8.3.2.3 Synthesis of 3.2 and 3.3 via the acids 3.15 and 3.18 respectively

3-aminobenzoic acid ethyl ester 3.13

\[ \text{H}_2\text{N} \quad \text{O} \rightarrow \quad \text{H}_2\text{N} \quad \text{O} \quad \text{Et} \]

\[
\begin{align*}
\text{3.13} \\
\text{3-aminobenzoic acid ethyl ester 3.13}
\end{align*}
\]

The compound, 3-aminobenzoic acid (14.8 g, 107 mmol) was treated with a saturated solution of HCl in ethanol as described in General Method D to afford the ester 3.13 (15.7 g, 88%) as a brown oil:

[MS (EI): 165 (M+), 137, 120, 92, 65; HRMS (EI) calcd for C_{9}H_{10}O_{2}N (M+) 165.0790, found 165.0791];

\[ ^{1}\text{H NMR} \text{(CDCl}_3) \delta 7.43 \text{(1H, d, J = 7.5 Hz, H6)}, 7.35 \text{(1H, s, H2)}, 7.21 \text{(1H, t, J = 7.8 Hz, H5)}, 6.85 \text{(1H, d, J = 7.8 Hz, H5)}, 4.35 \text{(2H, q, J = 7.2 Hz, CH}_2\text{CH}_3\text{)}, 3.78 \text{(2H, br s, NH}_2\text{)}, 1.38 \text{(3H, t, J = 7.2 Hz, CH}_2\text{CH}_3\text{)}; \]

\[ ^{13}\text{C NMR} \text{(CDCl}_3) \delta 166.7 \text{(CO)}, 146.5 \text{(C3)}, 131.3 \text{(C1)}, 129.0 \text{(C5)}, 119.4 \& 119.2 \text{(C4 \& C6)}, 115.5 \text{(C2)}, 60.7 \text{(CH}_2\text{)}, 14.1 \text{(CH}_3\text{)}; \]

\( (E)\)- and (Z)-3-(phenylazo)benzoic acid ethyl ester 3.14

\[
\begin{align*}
\text{2.33} + \quad \text{3.13} \rightarrow \quad \text{(E)-3.14} \\
\text{N=N} \quad \text{O} \quad \text{Et} \quad \text{O} \quad \text{Et} \quad \text{N=N} \quad \text{O} \quad \text{Et} \quad \text{O} \\
\text{2.33} \quad \text{3.13} \quad \text{(E)-3.14}
\end{align*}
\]

Compounds 2.33 (8.0 g, 74 mmol) and 3.13 (12.2 g, 74 mmol) were condensed by reflux in glacial acetic acid as described in Method E. The crude product formed was purified by flash chromatography, eluting with petroleum ether-ethyl acetate (9:1), to give the ester 3.14 (13.6 g, 72%) as an orange solid:
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[MS (EI): 254 (M+), 209, 149, 105, 77; HRMS (EI) calcd for C_{15}H_{14}O_{2}N_{2} (M+) 254.1055, found 254.1061]; mp 37-39 °C, (literature mp 36 °C)\textsuperscript{22}; R\text{f} 0.72 (1:4 ethyl acetate-petroleum ether); IR (film) 3480, 2965, 1720, 1360, 1340 cm\textsuperscript{-1};

\textsuperscript{1}H NMR (CDCl\textsubscript{3}) \(\delta\) 8.57 (1H, d, J = 1.5 Hz, H2), 8.16 (1H, d, J = 7.8 Hz, H4), 8.10 (1H, d, J = 7.8 Hz, H6), 7.95 (2H, d, J = 7.5 Hz, H2'), 7.59 (1H, t, J = 7.8 Hz, H5), 7.54 (3H, m, H3' & H4'), 4.43 (2H, q, J = 7.2 Hz, CH\textsubscript{2}CH\textsubscript{3}), 1.44 (3H, t, J = 7.2 Hz, CH\textsubscript{2}CH\textsubscript{3});

\textsuperscript{13}C NMR (CDCl\textsubscript{3}) \(\delta\) 166.0 (CO), 152.5 (C1), 152.4 (C1'), 131.6 & 131.6 (C3 & C4), 131.3 (C4'), 129.1 & 129.1 (C5 & C3'), 126.5 (C6), 124.2 (C2), 123.0 (C2'), 61.2 (CH\textsubscript{2}CH\textsubscript{3}), 14.3 (CH\textsubscript{2}CH\textsubscript{3}).

(E)- and (Z)-3-(phenylazo)benzoic acid 3.15

\[
\begin{align*}
3.14 & \rightarrow \text{(E)-3.15} \\
& \begin{array}{c}
\text{N=}
\end{array} \\
& \text{OH}
\end{align*}
\]

The ester 3.14 (2.7 g, 11 mmol) was treated with potassium hydroxide as described in General Procedure C1 to afford 3.15 (1.8 mg, 76%) as an orange solid: mp 165-166 °C, (literature mp 165-166 °C,\textsuperscript{7} 170-171°C\textsuperscript{21});

\textsuperscript{1}H NMR (CDCl\textsubscript{3}) \(\delta\) 8.66 (1H, s, H2), 8.23 (1H, d, J = 7.8 Hz, H4), 8.17 (1H, d, J = 7.8 Hz, H6), 7.97 (2H, d, J = 6.3 Hz, H2'), 7.65 (1H, t, J = 7.8 Hz, H5), 7.55 (3H, m, H3' & H4');

\textsuperscript{13}C NMR (CDCl\textsubscript{3}) \(\delta\) 171.1 (CO), 152.7 (C1), 152.4 (C1'), 132.2 (C4), 131.5 (C4'), 130.4 (C3), 129.3 (C5), 129.2 (C3'), 127.6 (C6), 124.7 (C2), 123.1 (C2').
(E)- and (Z)-(2R,3S)-2-hydroxy-3-[[3-(phenylazo)benzene]carbonyl]amino] benzenebutanoic acid methyl ester 3.19a

The acid 3.15 (37 mg, 0.16 mmol) was coupled to the amine salt 3.11a (52 mg, 0.18 mmol) according to General Procedure A1. The crude product was purified by flash chromatography, eluting with eluting with ethyl acetate-dichloromethane (1:4) to afford 3.19a (26 mg, 39%) as an orange solid:

$[\text{MS (EI)}: 417 (M^+), 328, 308, 209, 105, 77; \text{HRMS (EI) calcd for } C_{24}H_{23}O_4N_3 (M^+) 417.1689, \text{ found } 417.1691; \text{ RF } 0.29 (1:9 \text{ ethyl acetate-dichloromethane); IR (CHCl}_3) 3435, 3031, 2956, 1734, 1668, 1585 \text{ cm}^{-1};$

$^1H \text{ NMR (CDCl}_3) \delta 8.18 (1H, s, H2), 8.02 (1H, d, J = 7.8 \text{ Hz, H6}), 7.92 (2H, d, J = 8.1 \text{ Hz, H2'}), 7.82 (1H, d, J = 7.5 \text{ Hz, H4}), 7.53 (4H, m, H5, H3' & H4'), 7.24-7.35 (5H, m, ArH), 6.68 (1H, m, NH), 4.86 (1H, m, CHCHOH), 4.24 (1H, m, CHO), 3.75 (3H, s, CH$_3$), 3.09 (2H, m, CH$_2$);

$^{13}C \text{ NMR (CDCl}_3) \delta 174.2 (\text{COOMe}, 166.5 (\text{ArCO}), 152.5 & 152.4 (\text{C1 & C1'}), 137.2 (\text{ArC}), 135.2 (\text{C3}), 131.4 (\text{C4'}, 129.4 & 129.1 (\text{C3', C5, C4 & ArCH}), 128.7, 126.8 (\text{ArCH}), 125.6 (\text{C6}), 123.0 (\text{C2}), 121.2 (\text{C2'}), 70.1 (\text{CHO}), 53.4 & 53.0 (\text{CHCHOH & CH$_3$}), 37.9 (\text{CH$_2$}).$
The alcohol \textbf{3.19a} (27 mg, 65 \mu mol) was oxidised with TEMPO as described in General Procedure B2 to give \textbf{3.2} (26 mg, 95\%) as an orange solid:

[MS (EI): 415 (M\(^{+}\)), 356, 328, 209, 105, 77; HRMS (EI) calcd for C\(_{24}\)H\(_{21}\)O\(_4\)N\(_3\) (M\(^{+}\)) 415.1532, found 415.1531; IR (CHCl\(_3\)) 3433, 2974, 1734, 1666, 1508, 1475 cm\(^{-1}\); \([\alpha]_D\) -53 \pm 2 ° (c 0.67 acetonitrile);

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.21 (1H, s, H\(_2\)), 8.07 (1H, d, J = 7.8 Hz, H\(_6\)), 7.94 (2H, d, J = 6.5 Hz, H\(_2'\)), 7.84 (1H, d, J = 7.5 Hz, H\(_4\)), 7.56 (4H, m, H\(_5\), H\(_3'\) & H\(_4'\)), 7.17-7.33 (5H, m, ArH), 6.76 (1H, d, J = 6.7 Hz, NH), 5.65 (1H, m, CH), 3.90 (3H, s, CH\(_3\)), 3.41 (1H, dd, J = 6.0, 14.0 Hz, CH\(_A\)), 3.25 (1H, dd, J = 6.5, 14.0 Hz, CH\(_B\))

\(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 191.5 (COCOOME), 166.4 (ArCO), 160.7 (COOME), 152.5 & 152.3 (C1 & C1'), 134.9 (ArC), 134.3 (C3), 131.5 (C4'), 129.6, 129.4, 129.2 (C3', C5, C4 & ArCH), 128.8, 127.5 (ArCH), 126.4 (C6), 123.0 (C2), 121.0 (C2'), 57.1 (CH\(_3\)), 53.3 (CHCO), 36.8 (CH\(_2\))

\textbf{2–aminobenzoic acid ethyl ester 3.16}

\[
\begin{array}{c}
\text{NH}_2 \\
\text{O}
\end{array} & \longrightarrow & \\
\begin{array}{c}
\text{NH}_2 \\
\text{OEt}
\end{array}
\]

\textbf{3.16}

The compound, 2–aminobenzoic acid (15.2 g, 111 mmol) was treated with a saturated solution of HCl in ethanol as described in General Method D. The crude product was distilled under vacuum to afford the ester \textbf{3.16} (6.0 g, 32\%) as a colourless oil:

bp\(_3\) 100-102 °C, (literature bp\(_{15}\) 145-147 °C)\(^{21}\)

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.87 (1H, d, J = 8.3 Hz, H\(_6\)), 7.25 (1H, t, J = 8.5 Hz, H\(_5\)), 6.63 (2H, m, H\(_3\) & H\(_4\)), 5.69 (2H, br s, NH\(_2\)), 4.31 (2H, q, J = 7.3 Hz, CH\(_2\)CH\(_3\)), 1.38 (3H, t, J = 7.3 Hz, CH\(_2\)CH\(_3\)).
(E)- and (Z)-2-(phenylazo)benzoic acid 3.18

Compounds 2.33 (1.50 g, 14 mmol) and 3.16 (2.2 g, 13 mmol) were condensed in glacial acetic acid as described in Method E. The crude product formed was purified by flash chromatography, eluting with dichloromethane-ethyl acetate (4:1), to give the ester 3.17 (1.5 g, 44%) as a red oil:

\[ \text{MS (EI): 254 (M^+), 209, 165, 152, 121, 105, 77; HRMS (EI) caled for C}_{13}\text{H}_{14}\text{O}_{2}\text{N}_{2} \text{ (M^+) 254.1055, found 254.1063}; \ R_F \ 0.14 (2:3 \text{ dichloromethane-petroleum ether); IR (CHCl}_3 \text{) 3026, 1716, 1596, 1475, 1367, 1296, 1234 cm}^{-1}; \]

\(^1\text{H NMR (CDCl}_3 \text{) } \delta \ 7.92 (2H, d, J = 8.3 \text{ Hz, } H_2'), 7.83 (1H, d, J = 8.8 \text{ Hz, } H_3), 7.45-7.59 (6H, m, H4, H5, H6, H3' & H4'); 4.37 (2H, q, J = 7.3 \text{ Hz, } CH_2CH_3), 1.30 (3H, t, J = 7.2 \text{ Hz, } CH_2CH_3);

\(^13\text{C NMR (CDCl}_3 \text{) } \delta \ 167.4 \text{ (CO), 152.5 (C1'), 151.9 (C1), 131.7 (C4), 131.3 (C4'), 129.6 & 129.6 (C3' & C5), 129.0 (C3), 128.5 (C2), 123.1 (C2'), 118.6 (C6), 61.3 (CH}_2\text{CH}_3), 14.2 (CH}_2\text{CH}_3).\]

The ester 3.17 (405 mg, 1.6 mmol) was treated with potassium hydroxide as described in General Procedure C1 to afford 3.18 (304 mg, 86%) as a brown solid:

mp 82-83 °C, (literature mp 95 °C).\(^{21}\);

\(^1\text{H NMR (DMSO) } \delta \ 7.86 (2H, m, H2'), 7.80 (1H, dd, J = 1.5, 7.8 \text{ Hz, } H_3), 7.54-7.72 (6H, m, H4, H5, H6, H3' & H4');

\(^13\text{C NMR (DMSO) } \delta \ 168.3 \text{ (CO), 152.2 (C1'), 151.0 (C1), 131.9 & 131.8 (C4 & C4'), 130.5 (C5), 129.6 (C3'), 129.3 (C3), 129.1 (C2), 122.9 (C2'), 118.1 (C6).}
Attempted preparation of \((E)\)- and \((Z)\)-(2R,3S)-2-hydroxy-3-[[2-(phenylazo)benzene]carbonyl]amino]benzenebutanoic acid methyl ester 3.23a (EDCI)

The acid 3.18 (14 mg, 63 \(\mu\)mol) and the amine salt 3.11a (20 mg, 68 \(\mu\)mol) were treated according to General Procedure A1 to give a complex mixture (19 mg) comprising approximately 10% of 3.23a by \(^1\)H NMR.

\((E)\)- and \((Z)\)-(2R,3S)-2-hydroxy-3-[[2-(phenylazo)benzene]carbonyl]amino]benzenebutanoic acid methyl ester 3.23a (DCC/NHS)

A solution of DCC (77 mg, 0.40 mmol) in THF (1.5 mL) at 0 °C was added to a cold solution of acid 3.18 (90 mg, 0.40 mmol) and \(N\)-hydroxysuccinimide (46 mg, 0.40 mmol) in THF (3 mL). The reaction mixture was stirred 2 h at 0 °C then stored at 4 °C overnight. The reaction mixture was filtered, washing with cold THF (2 x 1 mL). To the solution was added 3.11a (166 mg, 0.77 mmol) and DIEA (76 \(\mu\)L, 0.44 mmol) and the reaction mixture was stirred 1 h at 0 °C and 4 h at rt. The reaction mixture was diluted with dichloromethane (20 mL), washed with saturated \(NH_4\)Cl (20 mL), brine (20 mL), dried over \(MgSO_4\) and concentrated in vacuo. The crude product was purified by flash chromatography, eluting with dichloromethane, to afford an orange solid tentatively assigned as 3.21 (30 mg, 23%):
[HRMS (ES) calcd for C_{17}H_{13}O_{4}N_{3}K (M+K)^+ 362.054, found 362.055]; R_{f} 0.56 (1:9 ethyl acetate-dichloromethane);

^{1}H NMR (CDCl_{3}) \delta 8.06 (3H, m, H3 & H2'), 7.75 (2H, m, H4 & H6), 7.48-7.59 (4H, m, H5, H3' & H4'), 2.86 (4H, s, 2 x CH_{2}).

Further elution with dichloromethane-ethyl acetate (19:1) gave 3.23a (62 mg, 37%) as an orange solid:

[MS (EI): 417 (M^+), 308, 209, 152, 105, 91, 77; HRMS (EI) calcd for C_{24}H_{23}O_{4}N_{3} (M^+) 417.1689, found 417.1692]; mp 127-130 °C; R_{f} 0.27 (1:9 ethyl acetate-dichloromethane); IR (CHCl_{3}) 3622, 3533, 3310, 1743, 1656 cm^{-1};

^{1}H NMR (CDCl_{3}) \delta 9.02 (1H, d, J = 9.0 Hz, NH), 8.43 (1H, m, H3), 7.97 (2H, d, J = 8.1 Hz, H2'), 7.76 (1H, m, H6), 7.54 (5H, m, H4, H5, H3' & H4'), 7.17-7.34 (5H, m, ArH), 4.90 (1H, m, CHCHOH), 4.22 (1H, s, CHO), 3.68 (3H, s, CH_{3}), 3.35 (1H, br s, OH), 3.04 (2H, m, CH_{2});

^{13}C NMR (CDCl_{3}) \delta 174.2 (COOMe), 165.3 (ArCO), 152.3 (C1'), 149.6 (C1), 137.5 (ArC), 132.2 (C4'), 131.8 (C4), 131.5 (C3), 131.3 (C5), 130.2 (C2), 129.3 & 129.3 (ArCH & C3'), 128.5, 126.6 (ArCH), 123.4 (C2'), 115.8 (C6), 70.0 (CHOH), 53.9 (CHCHOH), 52.8 (CH_{3}), 38.1 (CH_{2}).

When the above ^{1}H NMR sample of (E)-3.23a was left on the bench for at least 1 day, a PSS comprising (E)-3.23a / (Z)-3.23a (4:1 by ^{1}H NMR) resulted.

R_{f} 0.13 (1:9 ethyl acetate-dichloromethane);

^{1}H NMR (CDCl_{3}, selected data for the mixture) \delta 8.43 (1H, (Z)-3.23a, m, H3), 6.85 (2H, (Z)-3.23a, dd, J = 1.5, 7.0 Hz, H2'), 6.05 (1H, (Z)-3.23a, d, J = 8.5 Hz, H6), 4.44 (1H, s, (Z)-3.23a, CHO), 3.71 (3H, (Z)-3.23a, s, CH_{3})

^{13}C NMR (CDCl_{3}, selected data for the mixture) \delta 137.3 ((Z)-3.23a, ArC), 120.8 ((Z)-3.23a, C2'), 116.7 ((Z)-3.23a, C6), 70.4 ((Z)-3.23a, CHOH).
(E)- and (Z)-(3S)-2-oxo-3-[[2-(phenylazo)benzene]carbonyl]amino]benzene butanoic acid methyl ester 3.3

The alcohol 3.23a (24 mg, 57 µmol) was oxidised with TEMPO as described in General Procedure B2 to give 3.3 (21 mg, 88%) as an orange solid:

\[
\text{[MS (EI): 415 (M^+), 356, 310, 235, 209, 152, 91, 77; HRMS (EI) calcd for C}_{24}\text{H}_{21}\text{O}_{4}\text{N}_{3} (M^+) 415.1532, found 415.1524]; IR (CHCl}_3) 3622, 2541, 1739, 1635, 1049 \text{ cm}^{-1}; [\alpha]_D^0 +16 \pm 1 ^\circ \text{ (c 0.94 acetonitrile);}
\]

\[\^1\text{H NMR (CDCl}_3) \delta 9.36 (1\text{H, d, } J = 5.5 \text{ Hz, NH}), 8.41 \text{ (1H, m, H3), 7.78 (1H, m, H6), 7.44-7.66 (7H, m, H4, H5, H2', H3' & H4'), 6.95-7.09 (5H, m, ArH), 5.77 (1H, m, CH), 3.87 (3H, s, CH}_3), 3.37 (1\text{H, dd, } J = 5.9, 14.2 \text{ Hz, CH}_A), 3.23 (1\text{H, dd, } J = 6.4, 14.2 \text{ Hz, CH}_B);\]

\[\^13\text{C NMR (CDCl}_3) \delta 191.4 \text{ (COCOOMe), 165.2 (ArCO), 160.8 (COOMe), 152.2 & 149.8 (C1 & C1'), 135.1 (ArC), 132.2, 132.1, 131.6 & 131.3 (C3, C4, C5 & C4'), 130.6 (C2), 129.3 & 129.2 (C3', ArCH), 128.3 & 127.0 (ArCH), 123.3 (C2'), 115.9 (C6), 57.6 (CH), 53.1 (CH}_3), 36.8 (CH}_2).\]

8.3.3 Synthesis of the peptidyl-\(\alpha\)-ketoester 3.5
(E)- and (Z)-(2R,3S)-2-hydroxy-3-[[4-(phenylazo)benzyloxy]carbonyl[amino]benzenebutanoic acid methyl ester 3.34a

2.29 + 3.11a → (E)-3.34a

To a solution of triphosgene (42 mg, 0.14 mmol) in dichloromethane (0.5 mL) at 0 °C was added dropwise over 5 min to a solution of the alcohol 2.29 (80 mg, 0.38 mmol) and DIEA (72 µL, 0.41 mmol) in dichloromethane (4 mL). The reaction mixture was stirred at 0 °C for 5 min and at rt for 30 min. A solution of the amine 3.11a (100 mg, 0.34 mmol) and DIEA (132 µL, 0.76 mmol) in dichloromethane (1 mL) was added in two portions over 1 min and the reaction mixture was stirred at rt for 30 min. The solvent was evaporated in vacuo and the resulting residue was taken up in ethyl acetate (20 mL). The organics were washed with 10% K2HSO4 (2 x 10 mL), 5% NaHCO3 (2 x 10 mL), brine (10 mL), dried over MgSO4 and concentrated in vacuo. The residue was purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:19), to afford the starting material 2.29 (18 mg, 23%). Further elution with dichloromethane-ethyl acetate (19:1) gave 3.34a (53 mg, 34%) as an orange solid:

[MS (FAB): 448 (M+1)⁺, 404, 195, 154, 137; HRMS (FAB) calcd for C23H26O5N3 (M+1)⁺ 448.1874, found 448.1873; RF 0.42 (1:9 ethyl acetate-dichloromethane); IR (CHCl3) 3699, 3651, 1733, 1604, 1504 cm⁻¹;]

1H NMR (CDCl3) δ 7.92 (4H, m, H2 & H2'), 7.41-7.55 (5H, m, H3, H3' & H4'), 7.24-7.34 (5H, m, ArH), 5.12 (3H, m, CH2O & NH), 4.36 (1H, m, CHCHOH), 4.12 (1H, m, CHOH), 3.72 (3H, s, CH3), 3.21 (1H, s, OH), 2.97 (2H, m, PheCH2);

13C NMR (CDCl3) δ 174.1 (COOMe), 155.6 (CONH), 152.6 & 152.2 (C1 & C1'), 139.3 (C4), 137.2 (ArC), 131.1 (C4'), 129.4, 129.1, 128.6, 128.3, 126.8 (C3, C3' & ArCH), 122.9 & 122.8 (C2 & C2'), 70.1 (CHOH), 66.1 (CH2O), 54.7 (CHCHOH), 52.9 (CH3), 38.3 (PheCH2).
(E)- and (Z)-(3S)-2-oxo-3-[[4-(phenylazo)benzyl]oxy]carbonyl][amino]benzene butanoic acid methyl ester 3.5

The alcohol 3.34a (28 mg, 65 μmol) was oxidised with TEMPO according to General Procedure A1 to afford 3.5 (28 mg, qu) as a brown solid:

\[ \text{[MS (EI) 445 (M^+), 314, 233, 195, 107, 77; HRMS (EI) calcd for } \text{C}_{25}\text{H}_{24}\text{O}_{3}\text{N}_{3} \text{ (M^+)} 445.164, \text{ found 445.165] ; IR (CHCl}_3 \text{) 3622, 3355, 1735, 1604, 1253, 1157 cm}^{-1} \text{; } \alpha_d - 21 \pm 2 ^\circ \text{ (c 1.3 acetonitrile) } \]

\[ \text{H NMR (CD}_3\text{CN) } \delta 7.95 (4H, m, H}_2 \text{ & H}_2' \text{), } 7.62 (3H, m, H}_3 \text{ & H}_4' \text{), } 7.51 (2H, d, J = 8.3 Hz, H}_3 \text{), 7.26-7.41 (5H, m, ArH), 6.26 (1H, d, J = 7.4 Hz, NH), 5.15 (3H, m, CH}_2\text{O & CH), 3.86 (3H, s, OCH}_3 \text{), 3.30 (1H, dd, J = 4.9, 14.5 Hz, PheCH}_A \text{), 2.93 (1H, dd, J = 9.5, 14.2 Hz, PheCH}_B \text{); } \]

\[ \text{C NMR (CD}_3\text{CN) } \delta 192.8 (\text{COCOOMe}), 161.6 (\text{COOMe}), 156.2 (\text{CONH}), 152.8 & 152.4 (\text{C}_1 \text{ & C}_1' \text{), 140.6 (C}_4 \text{), 137.0 (ArC), 131.7 (C}_4' \text{), 129.7, 129.7, 128.9, 128.7 & 127.3 (ArCH, C}_3 \text{ & C}_3' \text{), 123.1 & 123.0 (C}_2' \text{ & C}_2 \text{), 66.1 (CH}_2\text{O), 59.1 (CH), 53.1 (CH}_3 \text{), 35.8 (PheCH}_2 \text{). } \]

8.3.4 Synthesis of the peptidyl-\(\alpha\)-ketoester 3.6
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(E)- and (Z)-N-[[4-(phenylazo)benzene]carbonyl]-L-leucine 3.36

The acid 3.7 (100 mg, 0.39 mmol) was coupled with L-leucine methyl ester, hydrochloride salt (78 mg, 0.51 mmol) according to General Procedure A1 to give 3.35 (137 mg, qu) as an orange solid:

[MS (EI) 353 (M)+, 297, 265, 209, 104, 77; HRMS (EI) calcd for C20H23O3N3 (M)+ 353.174, found 353.175]; mp 94-95 °C; IR (CHCl3) 3689, 3438, 2959, 1741, 1664, 1518, 1438, 1274, 1209, 1155 cm⁻¹;

1H NMR (CDCl3) δ 7.94 (6H, m, H2, H3 & H2'), 7.51 (3H, m, H3' & H4'), 6.80 (1H, d, J = 8.3 Hz, NH), 4.90 (1H, m, NHCH), 3.79 (3H, s, CH3), 1.76 (3H, m, LeuCH & LeuCH2), 1.00 (6H, t, J = 5.9 Hz, 2 x LeuCH3);

13C NMR (CDCl3) δ 173.7 (COOMe), 166.4 (ArCO), 154.3 (C1), 152.4 (C1'), 135.5 (C4), 131.5 (C4'), 129.0 (C3'), 128.0 (C3), 123.0 & 122.9 (C2 & C2'), 52.4 (CH3), 51.2 (NHCH), 41.7 (LeuCH2), 25.0 (LeuCH), 22.8 & 22.0 (LeuCH3).

The ester 3.35 (30 mg, 84 µmol) was treated with lithium hydroxide according to General Procedure C2 to give 3.36 (30 mg, qu) as an orange solid:

[MS (EI) 339 (M+), 321, 283, 209, 104, 77; HRMS (EI) calcd for C19H21O3N3 (M+) 339.1583, found 339.1588]; mp 115-115.5 °C; IR (film) 3705, 1728, 1660, 1604 cm⁻¹;

1H NMR (CD3OD) δ 8.71 (1H, d, J = 7.8 Hz, NH), 8.01 (2H, d, J = 7.7 Hz, H3), 7.91 (4H, m, H2 & H2'), 7.51 (3H, m, H3' & H4'), 4.72 (1H, m, NHCH), 1.78 (3H, m, LeuCH & LeuCH2), 0.99 (6H, t, J = 5.3 Hz, 2 x LeuCH3);
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$^{13}$C NMR (CD$_3$OD) $\delta$ 176.4 (COOMe), 169.8 (ArCO), 155.8 (C1), 154.1 (C1'), 137.5 (C4), 133.1 (C4'), 130.6 & 129.9 (C3 & C3'), 124.3 & 124.0 (C2 & C2'), 53.0 (NHCH), 41.6 (LeuCH$_2$), 26.5 (LeuCH), 23.7 & 22.1 (2 x LeuCH$_3$).

(E)- and (Z)-(2R,3S)-2-hydroxy-3-[[4-(phenylazo)benzene]carbonyl]-L-leucinyl amino]benzenebutanoic acid methyl ester 3.37a

The acid 3.36 (30 mg, 88 $\mu$mol) was coupled to the amine salt 3.11a (28 mg, 97 $\mu$mol) according to General Procedure A1 to afford 3.37a (45 mg, qu) as an orange solid:

[MS (FAB) 531 (M+1)$^+$, 457, 387, 209, 182, 136, 120, 105; HRMS (FAB) calcd for C$_{30}$H$_{35}$O$_5$N$_4$ (M+1$^+$) 531.2608, found 531.2600; IR (film) 3699, 2962, 1735, 1655, 1604, 1514, 1265 em$^{-1}$; IH NMR (CDCl$_3$) $\delta$ 7.95 (6H, m, H$_2$, H$_3$ & H$_2'$), 7.56 (3H, m, H$_3'$ & H$_4'$), 7.13-7.29 (5H, m, ArH), 6.50 (2H, m, 2 x NH), 4.59 (2H, m, NHCHCO & CHCHOH), 4.15 (1H, s, CHOH), 3.74 (3H, s, OCH$_3$), 3.32 (1H, d, J = 3.9 Hz, CHOH), 2.95 (2H, m, PheCH$_2$), 1.59 (3H, m, LeuCH & LeuCH$_2$), 0.97 (6H, m, 2 x LeuCH$_3$); 13C NMR (CDCl$_3$) $\delta$ 173.8 (COOMe), 172.0 (CHCO), 166.7 (ArCO), 154.3 (C1), 152.4 (C1'), 137.3 (ArC), 135.3 (C4), 131.5 (C4'), 129.3 (ArCH), 129.1 (C3'), 128.8, 128.4, (C3, ArCH), 126.5 (ArCH), 123.0 & 122.7 (C2 & C2'), 70.6 (CHOH), 53.4 (NHCHCHOH), 52.5 & 52.4 (NHCHCO & OCH$_3$), 41.2 (LeuCH$_2$), 37.7 (PheCH$_2$), 24.8 (LeuCH), 22.6 & 22.5 (2 x LeuCH$_3$).

When the above $^1$H NMR sample of (E)-3.37a was left on the bench for at least 1 day, a PSS comprising (E)-3.37a / (Z)-3.37a (3:1 by $^1$H NMR) resulted.

$^1$H NMR (CDCl$_3$, selected data for the mixture) $\delta$ 6.87 (2H, (Z)-3.37a, H2 & H2'), 3.66 (3H, (Z)-3.37a, s, CH$_3$), 0.89 (6H, (Z)-3.37a, t, J = 5.8 Hz, 2 x LeuCH$_3$);
$^{13}$C NMR (CDCl$_3$, selected data for the mixture) δ 173.5 ((Z)-3.37a, COOMe), 166.8 ((Z)-3.37a, ArCO), 137.4 ((Z)-3.37a, ArC), 135.5 ((Z)-3.37a, C4), 120.1 & 120.6 ((Z)-3.37a, C2 & C2'), 70.8 ((Z)-3.37a, CHOH), 41.0 ((Z)-3.37a, (LeuCH$_2$), 24.7 ((Z)-3.37a, (LeuCH).

(E)- and (Z)-(3S)-2-oxo-3-[[[4-(phenylazo)benzene]carbonyl]-L-leuciny] amino benzenebutanoic acid methyl ester 3.6

\[
\text{\includegraphics[width=0.5\textwidth]{structure.png}}
\]

The alcohol 3.37a (26 mg, 49 μmol) was oxidised with TEMPO according to General Procedure A1 to afford 3.6 (26 mg, qu) as an orange solid:

[MS (FAB) 529 (M+1)$^+$, 322, 289, 154, 136; HRMS (FAB) calcd for C$_{30}$H$_{33}$O$_5$N$_4$ (M+1)$^+$ 529.245, found 529.246]; IR (CHCl$_3$) 3695, 3429, 2968, 1733, 1660, 1604, 1514, 1485, 1375, 1249 cm$^{-1}$; [α]$_D$ = −21 ± 1° (c 1.4 acetonitrile)

$^1$H NMR (CDCl$_3$) δ 7.88-7.96 (6H, m, H2, H3 & H2'), 7.53 (3H, m, H3' & H4'), 7.13-7.26 (5H, m, ArH), 6.72 (1H, d, J = 6.3 Hz, PheNH), 6.50 (1H, d, J = 6.8 Hz, LeuNH), 5.37 (1H, m, CHCOCO), 4.75 (1H, m, CHCONH), 3.82 (3H, t, OCH$_3$), 3.26 (1H, m, PhCH$_2$), 3.02 (1H, m, PhCH$_2$), 1.63 (3H, m, LeuCH & LeuCH$_2$), 0.89 (6H, m, 2 x LeuCH$_3$);

$^{13}$C NMR (CD$_3$CN) δ 192.2 (COOCOMe), 173.0 (CHCONH), 166.8 (ArCO), 161.8 (COOMe), 154.5 (C1), 152.9 (C1'), 137.0 (C4), 136.3 (ArC), 132.3 (C4'), 129.9, 129.8, 129.2 & 128.9 (ArCH, C3 & C3'), 127.2 (ArCH), 123.3 (C2'), 122.9 (C2), 57.5 (CHCOCO), 53.2 (OCH$_3$), 52.6 (CHCONH), 40.7 (LeuCH$_2$), 35.7 (PheCH$_2$), 25.0 (LeuCH), 22.7 & 21.4 (2 x LeuCH$_3$).
8.4 Experimental Work Described in Chapter Four

8.4.2 Synthesis of the key intermediate $(E)$- and $(Z)$-$(2RS)$-2-hydroxy-2-$[4$-$(phenylazo)$]benzene$|$acetic acid 4.4

8.4.2.1 Route 1 to 4.4: via the cyanophosphorane 4.5

$(E)$- and $(Z)$-2-oxo-2-$[4$-$(phenylazo)$]benzene$|$acetic acid methyl ester 4.6

To a solution of 3.7 (150 mg, 0.66 mmol) in THF (7 mL) at 0°C were added EDCI (133 g, 0.70 mmol) and DMAP (8 mg, 0.066 mmol). A solution of the cyanophosphorane 2.8 (400 mg, 1.33 mmol) in dichloromethane (4 mL) was added dropwise. After stirring at rt for 16 h, the reaction mixture was poured into dichloromethane (15 mL) and water (15 mL). The organic layer was removed and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The combined organic washings were washed with brine (40 mL), dried over MgSO$_4$ and concentrated in vacuo. The residue was purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:19) to give the compound 4.5 (213 g, 63%) as an orange solid: [MS (EI): 509 (M$^+$), 403, 308, 277, 231, 185, 126, 105, 77; HRMS (EI) calcd for C$_{33}$H$_{24}$ON$_3$P (M$^+$) 509.1657, found 509.1651]; mp 221-223°C; R$_F$ 0.21 (2:3 ethyl
acetate-petroleum ether); IR (CHCl₃) 3067, 2255, 2175, 1583, 1545, 1485, 1438, 1342, 1213 cm⁻¹;
¹H NMR (CDCl₃) δ 8.15 (2H, d, J = 8.5 Hz, H3), 7.94 (4H, m, H2 & H2'), 7.60-7.80 (9H, m, H3', H4' & PPh₃H2), 7.40-7.55 (9H, m, PPh₃H3 & PPh₃H4);
¹³C NMR (CDCl₃) δ 189.0 (CO), 153.6 (C1), 152.5 (C1'), 141.0 (C4), 133.6 & 133.5 (PPh₃C2 & PPh₃C3), 133.0 (PPh₃C4), 131.1 (C4'), 129.2 & 129.0 (C3 & C3'), 123.7 (PPh₃Cl), 122.9 (C2'), 122.4 (C2), 120.2 (d, JPC₈ = 57 Hz, PPh₃CCN), 49.3 (d, JPC₆ = 126 Hz, PPh₃CCN).

When the above ¹H NMR sample of (E)-4.5 was left on the bench for at least 1 day, a PSS comprising (E)-4.5 / (Z)-4.5 (6:1 by ¹H NMR) resulted.
¹H NMR (CDCl₃, selected data for the mixture) δ 6.89 (4H, (Z)-4.5, m, H2 & H2')

Compound 4.5 (100 mg, 0.20 mmol) was treated with ozone in the presence of methanol as described in Method F. The crude product was purified by flash chromatography, eluting with ethyl acetate-petroleum ether (1:2) to give a mixture (>19:1 by ¹H NMR) of 4.6 and 4.7 (37 mg, 70%) as a shiny orange solid: [MS (El): 268 (M⁺), 209, 149, 104, 77; HRMS (El) calcd for C₁₅H₁₂O₃N₂ (M⁺) 268.08479, found 268.08480]; mp 85-86 °C; Rₚ 0.54 (3:7 ethyl acetate-petroleum ether); IR (CHCl₃) 3072, 2957, 1740, 1688, 1597, 1487, 1446, 1410, 1323, 1205 cm⁻¹; Data from the mixture for 4.6:
¹H NMR (CDCl₃) δ 8.19 (2H, d, J = 9.0 Hz, H3), 8.00 (4H, m, H2 & H2'), 7.56 (3H, m, H3' & H4'), 4.01 (3H, s, CH₃);
¹³C NMR (CDCl₃) δ 184.6 (ArCO), 163.1 (COOMe), 155.4 (C1), 152.0 (C1'), 133.1 (C4), 131.6 (C4'), 130.7 (C3), 128.7 (C3'), 122.7 & 122.5 (C2 & C2'), 52.4 (CH₃).
Data from the mixture for 4.7:
¹H NMR (CDCl₃) δ 3.96 (3H, s, CH₃).
(E)- and (Z)-(2RS)-2-hydroxy-2-[4-(phenylazo)benzene]acetic acid methyl ester 4.9

To a stirred solution of the mixture described above (>19:1) of α-ketoester 4.6 and ester 4.7 (50 mg, 0.19 mmol) in methanol (5 mL) at 0 °C was added sodium borohydride (4 mg, 0.09 mmol). The reaction mixture was stirred 10 min at 0 °C, before quenching with saturated NH₄Cl (10 mL). The phases were separated and the aqueous phase was extracted with ethyl acetate (2 x 10 mL). The combined organic washings were washed with brine (15 mL), dried over MgSO₄ and concentrated in vacuo to give a mixture (>19:1 by ¹H NMR) of 4.9 and 4.7 (50 mg, qu) as an orange solid:

[MS (EI): 270 (M⁺), 211, 165, 147, 105, 77; HRMS (EI) calcd for C₁₅H₁₄O₃N₂ (M⁺) 270.1004, found 270.1005]; mp 77-78 °C; Rₚ 0.71 (3:2 ethyl acetate-petroleum ether); IR (CHCl₃) 3528, 3065, 2957, 1732, 1487, 1440, 1261, 1186 cm⁻¹;

Data from the mixture for 4.9:
¹H NMR (CDCl₃) δ 7.93 (4H, m, H2 & H2'), 7.45-7.60 (5H, m, H3, H3' & H4'), 5.28 (1H, s, CHO), 3.77 (3H, s, CH₃);
¹³C NMR (CDCl₃) δ 173.6 (COO), 152.5 & 152.5 (C1 & C1'), 140.8 (C4), 131.1 (C4'), 129.0 (C3'), 127.3 (C3), 123.0 & 122.8 (C2 & C2'), 72.5 (CHOH), 53.1 (CH₃).

Data from the mixture for 4.7:
¹H NMR (CDCl₃) δ 8.21 (2H, d, H3), 3.96 (3H, s, CH₃).
(E)- and (Z)-(2RS)-2-hydroxy-2-[4-(phenylazo)benzene]acetic acid 4.4

\[
\begin{align*}
4.9 & \rightarrow & (E)-4.4 \\
+ & & \rightarrow \\
4.7 & \rightarrow & (E)-3.7
\end{align*}
\]

The mixture from above (>19:1 by \(^1\)H NMR) of 4.9 and 4.7 (50 mg, 0.18 mmol) was treated as described in Method C2 to give a mixture (3:1 by \(^1\)H NMR) of 4.4 and 3.7 (41 mg) as an orange solid:

[MS (EI): 256 (M\(^+\), 4.4), 226 (M\(^+\), 3.7), 210, 152, 105, 77, 45; HRMS (EI) calcd for C\(_{14}\)H\(_{12}\)O\(_3\)N\(_2\) (M\(^+\)) 256.0848, found 256.0843; IR (CHCl\(_3\)) 3528, 3065, 2957, 1732, 1487, 1440, 1186 cm\(^{-1}\);

Data from the mixture for 4.4:

\(^1\)H NMR (CD\(_3\)OD) \(\delta\) 7.89 (4H, m, H2 & H2'), 7.66 (2H, d, J = 8.3 Hz, H3), 7.50-7.60 (3H, m, H3' & H4'), 5.25 (1H, s, CHOH);

\(^1\)C NMR (CD\(_3\)OD) \(\delta\) 176.1 (CO), 154.2 & 153.9 (C1 & C1'), 144.3 (C4), 132.6 (C4'), 130.5 (C3'), 129.0 (C3), 124.1 & 124.1 (C2 & C2'), 74.1 (CHOH).

Data from the mixture for 3.7:

\(^1\)H NMR (CD\(_3\)OD) \(\delta\) 8.19 (2H, d, H3).

8.4.2.2 Route 2 to 4.4: via the cyanohydrins 4.11

(E)- and (Z)-4-(phenylazo)benzaldehyde 4.10
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The alcohol 2.29 (50 mg, 0.24 mmol) was oxidised with Dess Martin periodinane as described in Method B1 to give the aldehyde 4.10 (49 mg, 98%) as an orange–red solid:

[Anal. calcd for C13H10O2, C 74.27, H 4.79, N 13.32; found C 74.10, H 4.70, N 13.16; MS (EI): 210 (M⁺), 152, 105, 77; HRMS (EI) calcd for C13H10O2 (M⁺) 210.07931, found 210.07931]; mp 117.5-119 °C, (literature mp 121-122 °C)\(^{21}\); Rf 0.78 (dichloromethane); IR (CHCl₃) 2928, 1705, 1601, 1581, 1487, 1296, 1201 cm\(^{-1}\);

\(^1\)H NMR (CDCl₃) δ 10.10 (1H, s, CHO), 8.04 (4H, s, H2 & H3), 7.97 (2H, m, H2'), 7.55 (3H, m, H3' & H4');

\(^13\)C NMR (CDCl₃) δ 191.6 (CO), 155.9 (C1), 152.5 (C1'), 137.4 (C4), 131.4 (C4'), 130.7 (C3), 129.2 (C3'), 123.3 & 123.2 (C2 & C2').

\((E)-\) and \((Z)-(2RS)-2\text{-hydroxy}-2\text{-[4-(phenylazo)benzene]ethanenitrile 4.11 and (E)-}\) and \((Z)-(2RS)-2\text{-hydroxy}-2\text{-[4-(phenylazo)benzene]ethanenitrile trimethylsilylether 4.12}^{25}\)

\[
\begin{align*}
4.10 & \rightarrow \quad \text{(E)-4.11} \quad + \quad \text{(E)-4.12} \\
\end{align*}
\]

To the aldehyde 4.10 (1.01 g, 4.8 mmol) in dichloromethane (10 mL), was added potassium cyanide (0.31 g, 4.8 mmol) and zinc iodide (1.52 g, 4.8 mmol) and the reaction mixture was stirred under nitrogen at rt for 20 min. The solution was cooled to 0 °C and a solution of trimethylsilylcyanide (1.59 mL, 11.9 mmol) in dichloromethane (10 mL) at 0 °C was added in one portion. The reaction was stirred at 0 °C for 20 h, followed by quenching with the dropwise addition of concentrated HCl (1 mL). The solution was diluted with water (50 mL) and the phases were separated. The aqueous phase was extracted with dichloromethane (2 x 25 mL) and the combined organic washings were dried over MgSO₄ and concentrated \textit{in vacuo} to afford a mixture of the compounds 4.11 and 4.12. Purification by flash chromatography, eluting with dichloromethane gave 4.12 (0.54 g, 36%) as an orange solid:
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[MS (FAB): 310 (M+H)+, 283, 220, 204, 154, 136; HRMS (FAB) calcd for C17H20ON3Si (M+H)+ 310.1371, found 310.1376; mp 112-114 °C; Rf 0.78 (dichloromethane); IR (CHCl3) 3072, 2960, 1585, 1487, 1446, 1413, 1304, 1258 cm⁻¹; ¹H NMR (CDCl3) δ 7.96 (4H, m, H2 & H2'), 7.63 (2H, d, J = 8.4 Hz, H3), 7.52 (3H, m, H3' & H4'), 5.58 (1H, s, CHOTMS), 0.27 (9H, s, TMS); ¹³C NMR (CDCl3) δ 153.0 & 152.5 (Cl & Cl'), 137.8 (C4), 131.4 (C4'), 129.2 (C3'), 127.1 (C3), 123.3 & 123.2 (C2 & C2'), 118.8 (CN), 63.3 (CHOTMS), -0.3 (TMS).

Further elution gave 4.11 (0.43 g, 38%) as an orange solid:

[MS (FAB): 238 (M+H)+, 211, 154, 136; HRMS (FAB) calcd for C14H11ON3 (M+H)+ 238.0981, found 238.0982; Rf 0.15 (dichloromethane); IR (CHCl3) 3649, 3062, 2738, 2638, 1585, 1487, 1446, 1412, 1304 cm⁻¹; ¹H NMR (CDCl3) δ 7.98 (4H, m, H2 & H2'), 7.69 (2H, d, J = 8.7 Hz, H3), 7.54 (3H, m, H3' & H4'), 5.64 (1H, s, CHOH), 2.95 (1H, br s, CHO); ¹³C NMR (CDCl3) δ 153.2 & 152.4 (Cl & Cl'), 137.4 (C4), 131.6 (C4'), 129.2 (C3'), 127.5 (C3), 123.5 & 123.0 (C2 & C2'), 118.4 (CN), 63.3 (CHO).

Representative examples of the preparation of (E)- and (Z)-(2RS)-2-hydroxy-2-(4-phenylazo)benzene|acetic acid 4.4 as a mixture with (E)- and (Z)-4-(phenylazo)benzoic acid 3.7 and/or (E)- and (Z)-(2RS)-2-hydroxy-2-[4-(phenylazo)benzene|ethanamide 4.13

A mixture of dioxan (2 mL) and concentrated HCl was degassed under vacuum in the presence of a nitrogen bleed for 10 min, before the cyanohydrin 4.11 (100 mg, 0.42 mmol) was added from a solid addition arm. The reaction mixture was stirred
under nitrogen for 3 d at 35 °C. Water (4 mL) was added dropwise with the reaction vessel in an ice-salt bath and the resulting suspension was extracted with ethyl acetate (2 x 10 mL). The organic washings were dried over MgSO₄ and concentrated in vacuo to afford a mixture (3:1 by ¹H NMR) of the compounds 4.4 and 3.7 (82 mg), which was used without further purification.

A solution of 4.11 (141 mg, 0.59 mmol) in dioxan (4 mL) and concentrated HCl (3 mL) was stirred at 40 °C for 24 h. The reaction was quenched by the addition of water (10 mL) and the resulting suspension was extracted with ethyl acetate (3 x 10 mL). The organic washings were dried over MgSO₄ and concentrated in vacuo to afford a mixture (7:1:2 by ¹H NMR) of the compounds 4.4, 4.13 and 3.7 (106 mg), which was used without further purification.

**8.4.3 Synthesis of the target compounds 4.2 and 4.3**

\[(E)-\text{ and } (Z)-(2S,5RS)-3-\text{aza-2-(2-methylpropyl)-4,5-dioxo-5-[4-(phenylazo)benzene]pentanoic acid methyl ester 4.2}\]

A mixture (6:1:1 by ¹H NMR, from the cyanohydrin 4.11 route) of the compounds 4.4, 4.13 and 3.7 (54 mg) and L-leucine methyl ester, hydrochloride salt (38 mg, 0.21 mmol) were treated as described in Method A1. The crude product was purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:4) to
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give a mixture (1:1 by \(^1\)H NMR) of the diastereomers \(\mathbf{4.14a}\) and \(\mathbf{4.14b}\) (30 mg, 30% over two steps from \(\mathbf{4.11}\)) as an orange solid:

[MS (EI): 383 (M\(^+\)), 351, 246, 212, 143, 105, 77; HRMS (EI) calcd for \(\text{C}_{21}\text{H}_{25}\text{N}_{3}\text{O}_{4}\) (M\(^+\)) 383.1845, found 383.1843]; \(R_f\) 0.54, 0.36 (1:4 ethyl acetate-dichloromethane);

Data from the mixture for diastereomer \(\Lambda\):

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.93 (4H, m, H2 & H2'), 7.46-7.60 (5H, m, H3, H3' & H4'), 6.64 (1H, d, J = 8.5 Hz, NH), 5.16 (1H, s, CHOH), 4.63 (1H, m, NHCH), 3.72 (3H, s, OCH\(_3\)), 1.46-1.68 (3H, m, LeuCH\(_2\) & LeuCH), 0.88 (6H, m, 2 x LeuCH\(_3\));

\(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 172.9 & 171.4 (CONH & COOMe), 152.7 & 152.5 (C1 & C1'), 141.9 (C4), 131.2 (C4'), 129.0 (C3'), 127.5 (C3), 123.3 & 122.9 (C2 & C2'), 73.8 (CHOH), 52.4 (OCH\(_3\)), 50.8 (NHCH), 41.4 (LeuCH\(_2\)), 24.8 (LeuCH), 22.7 & 21.8 (2 x LeuCH\(_3\)).

Data from the mixture for diastereomer \(\Lambda\):

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.93 (4H, m, H2 & H2'), 7.46-7.60 (5H, m, H3, H3' & H4'), 6.64 (1H, d, J = 8.5 Hz, NH), 5.16 (1H, s, CHOH), 4.63 (1H, m, NHCH), 3.72 (3H, s, OCH\(_3\)), 1.46-1.68 (3H, m, LeuCH\(_2\) & LeuCH), 0.88 (6H, m, 2 x LeuCH\(_3\));

\(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 172.9 & 171.4 (CONH & COOMe), 152.7 & 152.5 (C1 & C1'), 141.9 (C4), 131.2 (C4'), 129.0 (C3'), 127.5 (C3), 123.3 & 122.9 (C2 & C2'), 73.8 (CHOH), 52.4 (OCH\(_3\)), 50.8 (NHCH), 41.4 (LeuCH\(_2\)), 24.8 (LeuCH), 22.7 & 21.8 (2 x LeuCH\(_3\)).

A mixture (1:1 by \(^1\)H NMR) of the compounds \(\mathbf{4.14a}\) and \(\mathbf{4.14b}\) (30 mg, 0.080 mmol) was oxidised with TEMPO as described in Method B1. The crude product was purified by flash chromatography, eluting with ethyl acetate-dichloromethane (2:3) to give \(\mathbf{4.2}\) (30 mg, 75%) as an orange solid:

[MS (EI): 381 (M\(^+\)), 209, 182, 104, 77; HRMS (EI) calcd for \(\text{C}_{21}\text{H}_{23}\text{N}_{3}\text{O}_{4}\) (M\(^+\)) 381.1689, found 381.1688]; mp 77-78 °C; \(R_f\) 0.34 (2:3 ethyl acetate-dichloromethane);

IR (CHCl\(_3\)) 3402, 2960, 1743, 1672, 1597, 1517, 1438, 1276, 1199 cm\(^{-1}\); [\(\alpha\)]\(_D\) -30 ± 3 ° (c 0.215 acetonitrile);

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.42 (2H, d, J = 8.8 Hz, H3), 7.87 (4H, m, H2 & H2'), 7.44 (4H, m, NH, H3' & H4'), 4.60-4.70 (1H, m, NHCH), 3.70 (3H, s, OCH\(_3\)), 1.60-1.75 (3H, m, LeuCH\(_2\) & LeuCH), 0.90 (6H, m, 2 x LeuCH\(_3\));
\[^{13}\text{C} \text{NMR (CDCl}_3\text{)} \delta 186.1 (\text{CO}), 172.3 (\text{COOMe}), 161.2 (\text{CONH}), 155.5 (\text{C1}), 152.5 (\text{C1'}) , 134.5 (\text{C4}), 132.4 (\text{C3}), 131.9 (\text{C4'}), 129.1 (\text{C3'}), 123.2 (\text{C2}), 122.6 (\text{C2'}), 52.5 (\text{NHCH}), 50.8 (\text{OCH}_3), 41.3 (\text{LeuCH}_2), 24.9 (\text{LeuCH}), 22.7 \& 21.8 (2 \times \text{LeuCH}_3)\].

Crystallographic structure determination for compound 4.2 by X-ray analysis

C$_{21}$H$_{23}$N$_3$O$_4$, M 381.42, mp 77-78 °C, crystal dimensions 0.70 x 0.15 x 0.03 mm, monoclinic, \(a = 10.1885(3) \, \text{Å}, b = 5.0764(2) \, \text{Å}, c = 19.2977(5) \, \text{Å}, V = 982.90(5) \, \text{Å}^3\), space group P(2)/1, \(Z = 2\), \(F(000) = 404\), \(D_{\text{calc}} = 1.289 \, \text{mg/m}^3\), absorption coefficient 0.090 mm$^{-1}$, \(\theta\) range for data collection 2.45 to 21.50 °, index ranges \(-10 \leq h \leq 10\), \(-2 \leq k \leq 5\), \(19 \leq l \leq 19\), data/restraints/parameters 1739/1/256, goodness of fit on \(F^2\) was 1.165, final \(R\) indices \([I \geq 2\sigma(I)]\) \(R_1 = 0.0705\), \(wR_2 = 0.1816\), \(R\) indices (all data) \(R_1 = 0.0816\), \(wR_2 = 0.1932\), largest peak difference and hole 0.251 and --0.233 e. Å$^{-3}$.

A unique data set was measured at 163(2) K within \(2\theta_{\text{max}} = 43\) ° limit (\(\omega\) scans). Of the 5431 reflections obtained, 1739 were unique \((R_{\text{int}} = 0.1359)\) and were used in the full-matrix least squares refinement.\(^{26}\) The structure was solved by direct methods.\(^{27}\) Hydrogen atoms were fixed in idealised positions. All non-hydrogen atoms were refined with anisotropic atomic displacement parameters. Neutral scattering factors and anomalous dispersion corrections for non-hydrogen atoms were taken from Ibers and Hamilton.\(^{28}\)

\((E)\)- and \((Z)\)-(2S,5RS)-3-aza-2-[4-[[\text{benzyloxy}][\text{carbonyl}][\text{amino}][\text{butyl}]]-5-hydroxy-4-oxo-5-[4-(\text{phenylazo})\text{benzene}][\text{pentanoic acid methyl ester 4.15a} \text{ and 4.15b}}

\[\text{\begin{center}
\includegraphics[width=0.3\textwidth]{image.png}
\end{center}}\]

A mixture (8:3 by \(^{1}H\) NMR, from the cyanohydrin 4.11 route) of the compounds 4.4 and 3.7 (82 mg) and \(N_c\)-Cbz-L-lysine methyl ester, hydrochloride salt (87 mg, 0.26 mmol) were treated as described in Method A1. The crude product was purified by flash
chromatography, eluting with ethyl acetate-dichloromethane (1:4) to give a mixture (1:1 by $^1$H NMR) of the diastereomers 4.15a and 4.15b (91 mg, 40% over two steps from 4.11) as an orange solid:

[HRMS (ES) calcd for C$_{29}$H$_{32}$N$_4$O$_6$Na (M+Na)$^+$ 555.222, found 555.223]; R$_F$ 0.14, 0.11

(1:4 ethyl acetate-dichloromethane);

$^1$H NMR (d$_6$-benzene, data from the mixture) δ 8.09-8.21 (each 4H, m, H$_2$ & H$_2'$, isomers a and b), 7.82 (each 2H, m, H$_3$, isomers a and b), 7.41 (each 3H, m, H$_3'$ & H$_4'$, isomers a and b), 7.16-7.30 (each 5H, m, ArH, isomers a and b), 5.28 $\pm$ 4.0Hz & 5.24$\pm$4.9Hz (each 1H, 2 x d, CHOH, isomers a and b), 5.17 (each 2H, m, CbzCH$_2$, isomers a and b), 4.75 (each 1H, m, NHCH$_2$, isomers a and b), 4.54 & 4.41 (each 1H, 2 x t, NHCH$_3$, isomers a and b), 4.50$\pm$3.9Hz & 4.22$\pm$4.5Hz (each 1H, 2 x d, OH, isomers a and b), 3.35 & 3.31 (each 3H, s, OCH$_3$, isomers a and b), 2.90 (each 2H, m, Lys$_8$CH$_2$, isomers a and b), 1.76 & 1.47 (each 2H, m, Lys$_8$CH$_2$, isomers a and b), 1.14 (each 4H, m, Lys$_8$CH$_2$ & Lys$_8$CH$_2$, isomers a and b);

$^{13}$C NMR (CDCl$_3$, data from the mixture) δ 172.5, 172.3, 172.0 (CHOHCONH & COOMe, isomers a and b), 156.6, 156.5 (CbzCO, isomers a and b), 152.4, 152.4, 152.4 (C1 & C1', isomers a and b), 142.0 (C4, isomers a and b), 136.4, 136.3 (ArC, isomers a and b), 131.0 (C4', isomers a and b), 129.0, 128.7, 128.4, 128.4, 128.1, 127.9, 127.4, 127.2, 127.0 (C3, C3', ArCH, isomers a and b), 122.9, 122.9, 122.8 (C2 & C2', isomers a and b), 73.7, 73.6 (CHOH, isomers a and b), 66.6, 66.5 (CbzCH$_2$, isomers a and b), 52.4, 52.4 (OCH$_3$, isomers a and b), 51.8, 51.7 (NHCH, isomers a and b), 40.4 (Lys$_8$CH$_2$, isomers a and b), 31.6 (Lys$_8$CH$_2$, isomers a and b), 29.2 (Lys$_8$CH$_2$, isomers a and b), 22.2, 22.0 (Lys$_8$CH$_2$, isomers a and b).

(E)- and (Z)-(2S)-3-aza-2-[4-[(benzyl oxy)carbonyl]amino]butyl]-4,5-dioxo-5-[4-( phenylazo)benzene]pentanoic acid methyl ester 4.16
A mixture (1:1 by $^1$H NMR) of the compounds 4.15a and 4.15b (26 mg, 48 μmol) was oxidised with TEMPO according to General Procedure B2 to afford 4.16 (24 mg, 92%) as an orange solid:

[HRMS (ES) calcd for C$_{29}$H$_{30}$N$_4$O$_6$Na (M+Na)$^+ 553.2063$, found 553.2062]; mp 104-105 ºC; R$_F$ 0.20 (1:19 ethyl acetate-dichloromethane); IR (CHCl$_3$) 3618, 3382, 1743, 1720, 1674, 1597, 1242, 1211, 1141 cm$^{-1}$;

$^1$H NMR (CDCl$_3$) $\delta$ 8.50 (2H, d, J= 8.8 Hz, H3), 7.95 (4H, m, H2 & H2’), 7.70 (1H, d, J = 8.7 Hz, NHCH), 7.52 (3H, m, H3' & H4’), 7.33 (5H, m, ArCH), 5.08 (2H, s, CbzCH$_2$), 4.91 (1H, m, Lys$_n$NH), 4.68 (1H, m, NHCH), 3.78 (3H, s, OCH$_3$), 3.20 (2H, m, Lys$_E$CH$_2$), 2.03 & 1.86 (2H, m, Lys$_g$CH$_2$) 1.56 (2H, m, Lys$_g$CH$_2$), 1.44 (2H, m, Lys$_x$CH$_2$);

$^{13}$C NMR (CDCl$_3$) $\delta$ 186.1 (COCONH), 171.7 (COOMe), 161.4 (COCONH), 156.5 (CbzCO), 155.5 (C1), 152.5, (C1’), 136.5 (ArC), 134.4 (C4), 132.3 (C3), 131.9 (C4’), 129.1, 128.4, 128.4, 128.0 (C3’, ArCH), 123.2, (C2), 122.6 (C2’), 66.6 (CbzCH$_2$), 52.6, 52.1 (OCH$_3$ & NHCH), 40.4 (Lys$_g$CH$_2$), 31.6 (Lys$_g$CH$_2$), 29.4 (Lys$_g$CH$_2$), 22.3 (Lys$_x$CH$_2$).

**Attempted preparation of (E)- and (Z)-(2S)-2-[4-aminobutyl]-3-aza-4,5-dioxo-5-[4-(phenylazo)benzene]pentanoic acid methyl ester 4.3**

A. To 4.16 (8 mg, 0.015 mmol) was added a solution (33% v/v) of HBr in acetic acid (400 μL) and the reaction mixture was stirred 10 min at rt. The reaction mixture was diluted with ether and from the resulting precipitate the supernatant was drawn. By $^1$H NMR the residue contained a mixture of compounds. Not only had the Cbz
group been removed, but also most of the methyl ester was hydrolysed and the azobenzene had undergone decomposition.

B. To a solution of 4.16 (9 mg, 0.03 mmol) in dichloromethane (0.75 mL) at -10 °C was added a solution (1.0 M) of BBr₃ in dichloromethane (150 μL, 0.15 mmol). The reaction mixture was stirred at -10 °C for 1 h and at rt for 2 h. The reaction was quenched by the dropwise addition of water (1 mL) and the layers were separated. The organics were extracted with water (3 x 1 mL) and the combined aqueous extracts were concentrated in vacuo to give a complex mixture, by ¹H NMR. The Cbz group had been removed and most of the methyl ester was hydrolysed and the azobenzene had undergone decomposition.

C. To a solution of 4.16 (10 mg, 0.019 mmol) in dichloromethane (1 mL) was added a solution of TMSI (0.4 M, 1.25 mL, 0.5 mmol, commercial source) in dichloromethane. The reaction mixture was stirred 3 h at rt then it was quenched with methanol (40 μL, 1.0 mmol) and the solvent was removed in vacuo. The resulting residue was partitioned over 1N HCl (1 mL) and ether (1 mL) and the aqueous layer was basified by the dropwise addition of saturated NaHCO₃ and extracted with ethyl acetate (2 x 1.5 mL). The combined organic washings were dried over MgSO₄ and concentrated in vacuo to give no compound. The ether layer was dried over MgSO₄ and concentrated in vacuo to return starting material 4.21 (8 mg) as an orange solid.

\[ \text{Ns-Boc-L-lysine methyl ester, formate salt 4.19} \]

\[
\text{CbzHN} \quad \text{O} \quad \text{O} \quad \text{CbzHN} \quad \text{O} \quad \text{OMe} \quad \text{CbzHN} \quad \text{O} \quad \text{OMe} \\
\text{NH}_{2}(\text{C}_6\text{H}_{12})_2 \quad \text{OH} \quad \text{H}_3\text{N} \quad \text{OH} \\
4.17 \quad 4.18 \quad 4.19
\]

To a suspension of 4.17 (100 mg, 0.18 mmol) in DMF (1.0 mL) were added KHCO₃ (18 mg, 0.18 mmol) and methyl iodide (20 μL, 0.29 mmol) and the reaction mixture was stirred 4 h at rt. The reaction mixture was quenched with water (3 mL) and the organics were extracted with ethyl acetate (3 x 2 mL). The combined organic
extracts were washed with water (2 x 2 mL), 5% aqueous \( \text{Na}_2\text{SO}_3 \) (2 mL), brine (2 mL), dried over \( \text{MgSO}_4 \) and concentrated \textit{in vacuo}. The crude product was purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:9) to give \textbf{4.18} (64 mg, 91%) as a white semisolid:

\[ \text{[MS (FAB): 395 (M+1)}^+\text{, 339, 295, 232, 182, 154; HRMS (FAB) calcd for C}_{20}\text{H}_{31}\text{N}_2\text{O}_6\ (\text{M}^+)^+\text{ 395.218, found 395.219}; \text{ R}_f\text{ 0.68 (1:4 ethyl acetate-dichloromethane); IR (CHCl}_3\text{) 3448, 2938, 1721, 1711, 1507, 1246, 1211 cm}^{-1}; \]

\( ^1\text{H NMR (CDCl}_3\text{) } \delta \text{ 7.36 (5H, m, ArCH), 5.41 (1H, d, J = 6.8 Hz, NHCH), 5.11 (2H, s, CbzCH}_2\text{), 4.58 (1H, m, Lys}_n\text{NH}), 4.36 (1H, m, NHCH), 3.72 (3H, s, OCH}_3\text{), 3.10 (2H, m, Lys}_n\text{CH}_2\text{), 1.83 & 1.69 (2H, m, Lys}_o\text{CH}_2\text{), 1.34-1.49 (13H, m, Lys}_x\text{CH}_2\text{, Lys}_o\text{CH}_2 \& 3 \times \text{BocCH}_3\text{);} \]

\( ^{13}\text{C NMR (CDCl}_3\text{) } \delta \text{ 172.8 (COOMe), 156.0, 155.9 (CbzCO & BocCO), 136.2 (ArC), 128.4, 128.1, 128.1 (ArCH), 79.1 (BocC}_n\text{), 66.9 (CbzCH}_2\text{), 53.6 (NHCH), 52.3 (OCH}_3\text{), 39.9 (Lys}_n\text{CH}_2\text{), 32.1 (Lys}_p\text{CH}_2\text{), 29.5 (Lys}_o\text{CH}_2\text{), 28.3 (3 \times \text{BocCH}_3\text{), 22.3 (Lys}_x\text{CH}_2\text{).} \]

To a rapidly stirred suspension of palladium black (25 mg, freshly prepared) in 4% formic acid in methanol (1 mL) was added a solution of \textbf{4.18} (23 mg, 58 \( \mu \)mol) in 4% formic acid in methanol (0.5 mL). After 10 min the reaction mixture was filtered through Celite, washing with methanol (1.5 mL) and water (1.5 mL). The solvent was evaporated \textit{in vacuo} to give \textbf{4.19} (19 mg, qu) as a colourless solid:

\[ \text{[MS (FAB): 261 (M^-), 205, 154, 136; HRMS (FAB) calcd for C}_{12}\text{H}_{24}\text{N}_2\text{O}_4\ (\text{M}^-)\text{ 261.1814, found 261.1806}; \text{ IR (CHCl}_3\text{) 3961, 2938, 1743, 1684, 1601, 1507, 1367, 1247, 1171 cm}^{-1}; \]

\( ^1\text{H NMR (D}_2\text{O) } \delta \text{ 8.23 (s, CHOO^-), 4.02 (1H, t, J = 6.3 Hz, CH), 3.72 (3H, s, OCH}_3\text{), 2.94 (2H, m, Lys}_n\text{CH}_2\text{), 1.82 (2H, m, Lys}_p\text{CH}_2\text{), 1.29-1.41 (13H, m, Lys}_x\text{CH}_2\text{, Lys}_o\text{CH}_2 \& 3 \times \text{BocCH}_3\text{);} \]

\( ^{13}\text{C NMR (CDCl}_3\text{) } \delta \text{ 172.4 (CHOO^-), 170.4 (COOMe), 159.9 (BocCO), 82.4 (BocC}_n\text{), 55.2 (OCH}_3\text{), 54.4 (NHCH), 41.0 (Lys}_n\text{CH}_2\text{), 31.1 (Lys}_p\text{CH}_2\text{), 30.1 (Lys}_o\text{CH}_2\text{), 29.4 (3 \times \text{BocCH}_3\text{), 23.1 (Lys}_x\text{CH}_2\text{).} \]
(E)- and (Z)-(2S,5RS)-3-aza-2-[4-[[ tert-butylox y] carbonyl] amino]butyl]-5-hydroxy-4-oxo-5-[4-(phenylazo)benzene] pentanoic acid methyl ester 4.20a, 4.20b

A mixture (6:1:1 by $^1$H NMR, from the cyanohydrin 4.11) of the compounds 4.4, 4.13 and 3.7 (71 mg) and 4.19 (79 mg, 0.26 mmol) were treated as described in Method A1. The crude product was purified by flash chromatography, eluting with ethyl acetate-dichloromethane (1:4) to give a mixture (1:1 by $^1$H NMR) of the diastereomers 4.20a and 4.20b (16 mg, 8% over last two steps from 4.11) as an orange solid:

[MS (FAB): 499, (M+1)$^+$, 399, 289, 233, 189, 154, 137; HRMS (FAB) calcd for C$_{26}$H$_{33}$N$_4$O$_6$ (M+1)$^+$ 499.256, found 499.255; R$_f$ 0.10 (1:4 ethyl acetate-dichloromethane);

$^1$H NMR (CDCl$_3$) $\delta$ 7.91 (each 4H, m, H$_2$ & H$_2'$, isomers a and b), 7.62 (each 2H, m, H$_3$, isomers a and b), 7.50 (each 3H, m, H$_3'$ & H$_4'$, isomers a and b), 6.61 (each 1H, NHCH$_2$, isomers a and b), 5.23 & 5.22 (each 1H, 2 x s, CHO$_2$, isomers a and b), 4.56-4.73 (each 2H, m, NHCH & NHCH$_2$, isomers a and b), 3.75 & 3.72 (each 3H, s, OCH$_3$, isomers a and b), 3.04 (each 2H, m, Lys$_3$CH$_2$, isomers a and b), 1.71 & 1.87 (each 2H, m, Lys$_9$CH$_2$, isomers a and b), 1.26-1.50 (each 13H, m, Lys$_5$CH$_2$, Lys$_8$CH$_2$ & 3 x BocCH$_3$, isomers a and b);

$^{13}$C NMR (CDCl$_3$, data from the mixture) $\delta$ 172.5, 172.4, 171.9 (CHOHCONH & COOMe, isomers a and b), 156.2 (CbzCO, isomers a and b), 152.5, 152.5, 152.4 (C1 & C1', isomers a and b), 142.2, 142.1 (C4, isomers a and b), 131.0 (C4', isomers a and b), 129.0, 127.5, 127.3 (C3, C3', isomers a and b), 123.0, 122.8 (C2 & C2', isomers a and b), 79.2 (BocC$_3$), 73.8, 73.7 (CHOH, isomers a and b), 52.5, 52.4, 51.9, 50.7 (OCH$_3$ & NHCH, isomers a and b), 40.0, 39.0 (Lys$_6$CH$_2$, isomers a and b), 31.8 & 31.6 (Lys$_9$CH$_2$,
isomers a and b), 29.6, 29.5 (Lys\textsubscript{6}CH\textsubscript{2}, isomers a and b), 28.4 (3 \times \text{BocCH}\textsubscript{3}, isomers a and b), 22.4, 22.3 (Lys\textsubscript{2}CH\textsubscript{2}, isomers a and b).

(E)- and (Z)-(2S)-3-aza-2-[4-[[\text{tert}-butyloxy]carbonyl]amino]butyl]-4,5-dioxo-5-[4-(phenylazo)benzene]pentanoic acid methyl ester 4.21

\[
\begin{align*}
4.20a & \rightarrow 4.20b \\
\text{(E)-}4.21 & \quad \text{NH} \quad \text{O} \\
\end{align*}
\]

A mixture (1:1 by \textsuperscript{1}H NMR) of the compounds 4.20\textit{a} and 4.20\textit{b} (15 mg, 30 \textmu mol) was oxidised with TEMPO according to General Procedure B2. The crude product was purified by flash chromatography eluting with ethyl acetate-dichloromethane (1:9) to afford 4.21 (8 mg, 53\%) as an orange solid:

[HRMS (ES) calcd for C\textsubscript{26}H\textsubscript{32}N\textsubscript{4}O\textsubscript{6}Na (M+Na)\textsuperscript{+} 519.222, found 519.223]; IR (CHCl\textsubscript{3}) 3625, 3541, 1747, 1674, 1631, 1597, 1162 cm\textsuperscript{-1};

\textsuperscript{1}H NMR (CDCl\textsubscript{3}) \delta 8.51 (2H, d, J = 8.8 Hz, H3), 7.97 (4H, m, H2 & H2'), 7.63 (1H, d, J = 7.8 Hz, NH\textsubscript{CH}), 7.54 (3H, m, H3' & H4'), 4.68 (1H, m, NH\textsubscript{CH}'), 4.57 (1H, m, Lys\textsubscript{6}NH), 3.80 (3H, s, OCH\textsubscript{3}), 3.13 (2H, m, Lys\textsubscript{6}CH\textsubscript{2}), 2.10 & 1.85 (2H, m, Lys\textsubscript{6}CH\textsubscript{2}), 1.54 (2H, m, Lys\textsubscript{6}CH\textsubscript{2}), 1.43 (11H, m, Lys\textsubscript{2}CH\textsubscript{2} \& 3 x BocCH\textsubscript{3});

\textsuperscript{13}C NMR (CDCl\textsubscript{3}) \delta 186.1 (COCONH), 171.7 (COOMe), 161.3 (COCONH), 156.0 (BocCO), 155.6 (C1), 152.5, (C1'), 134.5 (C4), 132.4 (C3), 131.9 (C4'), 129.2 (C3'), 122.6 & 123.2 (C2 & C2'), 79.3 (Boc\textsubscript{C}\textsubscript{6}), 52.6 & 52.2 (CH & OCH\textsubscript{3}), 40.1 (Lys\textsubscript{6}CH\textsubscript{2}), 31.9 (Lys\textsubscript{6}CH\textsubscript{2}), 29.6 (Lys\textsubscript{6}CH\textsubscript{2}), 28.4 (3 x BocCH\textsubscript{3}), 22.5 (Lys\textsubscript{2}CH\textsubscript{2}).
(E)- and (Z)-(2S)- 3-aza-2-[4-aminobutyl]-4,5-dioxo-5-[4-(phenylazo)benzene]
pentanoic acid methyl ester 4.3

To a solution of 4.21 (5 mg, 0.010 mmol) in dichloromethane (1 mL) was added
a solution of TMSI (0.4 M, 125 µL, 0.05 mmol, commercial source) in
dichloromethane. The reaction mixture was stirred 20 h at rt then it was quenched with
methanol (10 µL, 0.25 mmol) and the solvent was removed in vacuo. The resulting
residue was partitioned over IN HCl (1 mL) and ether (1 mL) and the aqueous layer
was basified by the dropwise addition of saturated NaHCO₃ (basic to litmus) and
extracted with ethyl acetate (2 x 1.5 mL). The combined organic washings were dried
over MgSO₄ and concentrated in vacuo to give 4.3 (1mg, 20%) as a brown solid:

[HRMS (ES) calcd for C₂₁H₂₅N₄O₄ (M+1)⁺ 397.188, found 397.189]; [α]D +8 ± 2 ° (c 0.05 acetonitrile);

¹H NMR (CDCl₃) δ 8.37 (2H, d, J= 8.8 Hz, H3), 7.80-8.00 (6H, m, NH₂, H2 & H2'),
7.51 (3H, m, H3' & H4'), 4.65 (1H, m, NHCH), 3.77 (3H, s, OCH₃), 3.12 (2H, m,
Lys₆CH₂), 1.80-1.96 (2H, m, Lys₇CH₂), 1.53 (2H, m, Lys₈CH₂), 1.42 (2H, m, Lys₉CH₂).
The ether layer was dried over MgSO₄ and concentrated in vacuo to return 4.21 (4 mg,
80%) as an orange solid.
8.5 Experimental Work Described in Chapter Five

8.5.2 Hydration studies of 3.1

Experiment 8.5.2.1 The hydration equilibrium for 3.1

\[ (E)-3.1 \]

\[ (Z)-3.1 \]

\[ (E)-5.9 \]

\[ (Z)-5.9 \]

A \(^1\)H NMR spectrum of 3.1 was run in dry CDCl\(_3\):

\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.98 (4H, m, H2, H2'), 7.84 (2H, m, H3), 7.55 (3H, m, H3' & H4'), 7.17-7.30 (5H, m, ArH), 6.67 (1H, d, J = 4.9 Hz, NH), 5.65 (1H, m, CH), 3.91 (3H, s, CH\(_3\)), 3.41 (1H, dd, J = 5.9, 13.7 Hz, CH\(_A\)), 3.25 (1H, dd, J = 6.6, 13.7 Hz, CH\(_B\)).

The solvent was evaporated and the sample was dissolved in dry \(d_3\)-acetonitrile that was open to the atmosphere. After 5 min, 40 min, 26 h further \(^1\)H NMR spectra were run. Selected data for (E)-3.1 from the mixture:

\(^1\)H NMR (CD\(_3\)CN) \(\delta\) 7.92-8.08 (6H, m, H2, H2', H3), 7.63 (3H, m, H3' & H4'), 7.27-7.39 (5H, m, ArH), 5.32 (1H, dd, J = 5.4, 9.3 Hz, CH), 3.85 (3H, s, CH\(_3\)), 3.39 (1H, dd, J = 5.4, 14.2 Hz, CH\(_A\)), 3.13 (1H, dd, J = 9.3, 14.2, CH\(_B\)).
Selected data for (Z)-3.1 from the mixture:

\(^1\)H NMR (CD\(_3\)CN) \(\delta \) 7.92-8.08 (2H, m, H3), 7.63 (3H, m, H3' & H4'), 7.27-7.39 (5H, m, ArH), 6.89 (4H, m, H2, H2'), 5.24 (1H, m, CH), 3.81 (3H, s, CH\(_3\)).

Selected data for (E)-5.9 from the mixture:

\(^1\)H NMR (CD\(_3\)CN) \(\delta \) 7.92-8.08 (6H, m, H2, H2', H3), 7.84 (2H, d, \(J = 8.8\) Hz, NH), 7.63 (3H, m, H3' & H4'), 7.27-7.39 (5H, m, ArH), 4.79 (1H, dd, \(J = 3.4, 11.7\) Hz, CH), 3.78 (3H, s, CH\(_3\)), 3.35 (1H, m, CH\(_A\)), 3.10 (1H, m, CH\(_B\)).

Selected data for (Z)-5.9 from the mixture:

\(^1\)H NMR (CD\(_3\)CN) \(\delta \) 7.92-8.08 (2H, m, H3), 7.63 (3H, m, H3' & H4'), 7.27-7.39 (5H, m, ArH), 6.89 (4H, m, H2, H2'), 4.63 (1H, m, CH), 3.74 (3H, s, CH\(_3\)).

Water was then removed from the system by diluting the sample with dichloromethane and drying over MgSO\(_4\). The solvent was concentrated in vacuo and the sample was redissolved in dry CDCl\(_3\). The solution was examined by \(^1\)H NMR. The integral ratios for this experiment are given in Table 8.1.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Time (min)</th>
<th>(E)-3.1</th>
<th>(Z)-3.1</th>
<th>(E)-5.9</th>
<th>(Z)-5.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDCl(_3) (dry)</td>
<td>5</td>
<td>95</td>
<td>&lt;5</td>
<td>n.o.</td>
<td>n.o.</td>
</tr>
<tr>
<td>CD(_3)CN</td>
<td>5</td>
<td>80</td>
<td>20</td>
<td>&lt;5</td>
<td>n.o.</td>
</tr>
<tr>
<td>CD(_3)CN</td>
<td>40</td>
<td>64</td>
<td>13</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>CD(_3)CN</td>
<td>1560</td>
<td>42</td>
<td>13</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>CDCl(_3) (dry)</td>
<td>5</td>
<td>81</td>
<td>13</td>
<td>6</td>
<td>n.o.</td>
</tr>
</tbody>
</table>

**Table 8.1** Percentage by \(^1\)H NMR integral of 3.1 and 5.9 over time in CDCl\(_3\) and CD\(_3\)CN solution (n.o. not observed).
**Experiment 8.5.2.2** $^1$H NMR of 3.1 in part aqueous solvent

<table>
<thead>
<tr>
<th>CD$_3$CN/D$_2$O</th>
<th>Time (min)</th>
<th>(E)-3.1</th>
<th>(Z)-3.1</th>
<th>(E)-5.9</th>
<th>(Z)-5.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:1</td>
<td>10</td>
<td>21</td>
<td>4</td>
<td>73</td>
<td>12</td>
</tr>
<tr>
<td>2:1</td>
<td>450</td>
<td>21</td>
<td>5</td>
<td>70</td>
<td>14</td>
</tr>
</tbody>
</table>

**Table 8.2** Percentage by $^1$H NMR integral of 3.1 and 5.9 over time in mixed CD$_3$CN/D$_2$O solution.

**Experiment 8.5.2.3** $^{13}$C NMR of 3.1 in mixed solvent (2:1) CD$_3$CN/D$_2$O

Selected data from the mixture:

$^{13}$C NMR (CD$_3$CN/D$_2$O 2:1) \( \delta \) 171.8 (COOMe), 167.4 (CONH), 153.8 (C1), 152.2 (C1'), 138.3 & 136.6 (C4 & ArC), 131.8 (C4'), 94.7 (COCOOMe5), 56.8 (CH), 52.5 (CH$_3$), 33.8.

**Experiment 8.5.2.4** Hydration in other target compounds

A solution of 2.4 in 9:1 $d_6$-DMSO /D$_2$O yielded a 3:1 mixture of ketone-2.4/hydrate-2.4.

Selected data from the $^1$H NMR spectrum:

$^1$H NMR ($d_6$-DMSO/D$_2$O 9:1) \( \delta \) 5.17 (1H, m, CHCOCO, ketone), 4.37 (1H, m CHC(OH)$_2$, hydrate), 0.73 (6H, m, 2 x LeuCH$_3$, ketone), 0.58 (6H, m, 2 x LeuCH$_3$, hydrate).

Selected data from the mixture resulting from 4.2 in 3:1 CD$_3$CN/D$_2$O:

$^{13}$C NMR (CD$_3$CN/D$_2$O 3:1) \( \delta \) 189.5 (COCONH, ketone), 111.5 (C(OH)$_2$, hydrate).
8.5.3 Isomerisation studies of the target compounds

Experiment 8.5.3.1 The isomerisation of target compounds

Isomer-enriched photostationary states for the compounds described in this thesis were obtained by irradiation of a solution of the compound with filtered light from a 200W high pressure mercury arc lamp. The light was filtered with an Oriel 59810 filter to allow passage of UV light (330<\(\lambda\)<370 nm) for (Z) isomer enrichment. For (E) enrichment the light was filtered with an Oriel 59494 filter (\(\lambda\)>400 nm) to allow the transmittance of visible light. The glass filter was held in a cylinder 500 mm from the light source and the sample was held a further 80 mm past the filter. Between the sample and the glass filter there was a water filter of 10 mm thickness to reduce sample heating by infrared radiation. For the purposes of PSS composition measurement, solutions of the target compounds in \(d_3\)-acetonitrile or \(d_6\)-DMSO (20-40 mM) were irradiated in quartz NMR tubes for 60 min. The PSS compositions were measured by \(^1\)H NMR immediately after irradiation of the solution. The solution was protected from ambient light during transferral from the irradiation setup to the NMR spectrometer. The isomer ratios were calculated where possible by comparison of the integrals of the methyl peaks. Otherwise, the value of the integral of one (Z)-proton was determined from the distinctive H2 and H2\(^\prime\) protons that resonate at 6.8-6.9 ppm. The value of the integral of one (E) plus one (Z) proton was obtained from the total aromatic integral. From these two values the proportion of (Z) was determined. The (Z) isomer \(^1\)H NMR details are given below and the PSS ratios are presented in Section 5.3.

(Z)-2.1: Selected data from the mixture

\(^1\)H NMR (DMSO) \(\delta\) 9.41 (1H, t, \(J = 6.3\) Hz, NHCH\(_2\)Ar), 7.22-7.45 (15H, m, ArH, H3, H3\(^\prime\) & H4\(^\prime\)), 6.92 (4H, m, H2 & H2\(^\prime\)), 5.16 (1H, m, CH), 5.05 (2H, AB\(_q\), CbzCH\(_2\)), 4.38 (2H, d, \(J = 5.9\) Hz, NHCH\(_2\)Ar), 3.19 (1H, dd, \(J = 3.9, 14.1\) Hz, CHCH\(_3\)Ph), 2.79 (1H, dd, \(J = 10.3, 14.1\) Hz, CHCH\(_3\)Ph).
(Z)-2.2: Selected data from the mixture
$^1$H NMR (DMSO) $\delta$ 10.88 (1H, s, NHAr), 7.84 (2H, d, J = 8.8 Hz, H3), 7.28-7.46 (13H, m, ArH, H3' & H4'), 6.97 (4H, m, H2 & H2'), 5.23 (1H, m, CH), 5.06 (2H, AB$_q$, CbzCH$_2$), 3.26 (1H, dd, J = 3.4, 13.7 Hz, CHCH$_{Ph}$), 2.83 (1H, dd, J = 10.3, 14.1 Hz, CHCH$_{Ph}$).

(Z)-2.3: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 7.18-7.41 (15H, m, ArH, H3, H3' & H4'), 6.88 (4H, m, H12 & H2'), 5.87 (1H, d, J = 7.8 Hz, CbzNH), 5.38 (1H, m, CHCOCO), 5.11 (2H, AB$_q$, CbzCH$_2$), 4.40 (2H, m, NHCH$_2$), 4.12 (1H, m, CbzNHCH), 3.28 (1H, m, PheCH$_A$), 3.01 (1H, m, PheCH$_B$), 1.65 (1H, m, LeuCH), 1.45 (2H, m, LeuCH$_2$), 0.93 (6H, m, 2 x LeuCH$_3$).

(Z)-2.4: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 9.15 (1H, S, NHAr), 7.66 (2H, m, H3), 7.25-7.47 (13H, m, ArH, H3' & H4'), 6.91 (4H, m, H2 & H2'), 6.78 (1H, d, J = 7.8 Hz, PheNH), 5.37 (1H, m, CHCOCO), 5.09 (2H, m, CbzCH$_2$), 4.10 (1H, m, CbzNHCH), 3.29 (1H, dd, J = 4.4, 13.7 Hz, PheCH$_A$), 3.00 (1H, m, PheCH$_B$), 1.40-1.62 (3H, m, LeuCH & LeuCH$_2$), 0.90 (6H, m, 2 x LeuCH$_3$).

(Z)-3.1: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 7.92-8.08 (2H, m, H3), 7.63 (3H, m, H3' & H4'), 7.27-7.39 (5H, m, ArH), 6.89 (4H, m, H2, H2'), 5.24 (1H, dd, J = 5.4, 9.3, CH), 3.81 (3H, s, CH$_3$).

(Z)-3.2: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 6.86 (2H, d, J = 8.3 Hz, H2'), 5.25 (1H, m, CH), 3.77 (3H, s, CH$_3$).

(Z)-3.3: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 8.77 (1H, d, J = 6.4 Hz, NH), 6.98 (2H, d, J = 8.8 Hz, H2'), 6.24 (1H, m, H6), 5.34 (1H, m, CH), 3.87 (3H, s, CH$_3$).
(Z)-3.5: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 7.23-7.37 (10H, m, ArH, H3, H3' & H4'), 6.87 (4H, m, H2 & H2'), 6.14 (1H, m, NH), 4.98 (2H, m, CH$_2$O), 4.84 (1H, m, CH), 3.85 (3H, s, OCH$_3$), 3.28 (1H, m, PheCH$_A$), 2.91 (1H, m, PheCH$_B$).

(Z)-3.6: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 7.77 (2H, d, $J = 7.4$ Hz, H3), 7.17-7.36 (8H, m, Ar, H3' & H4'), 6.93 (4H, m, H2 & H2'), 5.03 (1H, m, CHCOCO), 4.48 (1H, m, CHCONH), 3.80 (3H, s, OCH$_3$), 3.26 (1H, m, PheCH$_A$), 2.94 (1H, m, PheCH$_B$), 1.63 (3H, m, LeuCH & LeuCH$_2$), 0.95 (6H, m, 2 x LeuCH$_3$).

(Z)-4.1: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 7.97 (2H, d, $J = 8.7$ Hz, H3), 7.25-7.38 (3H, m, H3' & H4'), 6.83 & 7.03 (4H, m, H2 & H2'), 3.97 (3H, s, CH$_3$).

(Z)-4.2: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 8.10 (2H, m, H3), 7.22-7.37 (3H, m, H3' & H4'), 6.91-7.02 (4H, m, H2 & H2'), 4.59 (1H, m, CH), 3.76 (3H, s, CH$_3$), 1.75 (3H, m, LeuCH & LeuCH$_2$), 0.99 (6H, m, 2 x LeuCH$_3$).

(Z)-4.3: Selected data from the mixture
$^1$H NMR (CD$_3$CN) $\delta$ 8.12 (2H, m, H3), 7.26-7.37 (3H, m, H3' & H4'), 6.93 & 7.01 (4H, m, H2 & H2'), 4.54 (1H, m, CH), 3.78 (3H, s, CH$_3$), 3.00 (2H, m, Lys$_6$CH$_2$).

Experiment 8.5.3.2  HPLC measurement of isomer ratios of an azobenzene at different concentrations

Two solutions of 3.5 (4.1 mM and 0.21 mM) in acetonitrile that had reached the ambient light PSS (1 d under fluorescent lighting) were eluted with acetonitrile on a reverse phase C18 HPLC column and the peaks were detected by their absorbance at $\lambda = 190$ nm. For each solution two distinct peaks were observed (2.9 and 3.3 min) and
each of these peaks were forerun by shoulders which had the same UV characteristics as the major peak. The retention times and peak areas are given in Table 8.3

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Retention time (min)</th>
<th>Relative Area Percent</th>
<th>Retention time (min)</th>
<th>Relative Area Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>2.9</td>
<td>28</td>
<td>3.3</td>
<td>72</td>
</tr>
<tr>
<td>0.21</td>
<td>2.9</td>
<td>30</td>
<td>3.3</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 8.3 HPLC trace details for the elution of solutions of 3.5

8.5.4 Racemisation studies of 3.1 and 3.5

Experiment 8.5.4.1 Stability of peptidyl-\(\alpha\)-ketoesters on silica

The optical rotations of pure samples of 3.1 and 3.5 that had not been chromatographed were measured. The samples were run through a silica column and the optical rotations were measured once again. The results are given in Table 8.4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>[(\alpha)]D before column</th>
<th>[(\alpha)]D after column</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>(-52 \pm 5^\circ ) (c 0.3, acetonitrile)</td>
<td>(-57 \pm 5^\circ ) (c 0.2, acetonitrile)</td>
</tr>
<tr>
<td>3.5</td>
<td>(-20 \pm 2^\circ ) (c 1.3, acetonitrile)</td>
<td>(-18 \pm 2^\circ ) (c 1.3, acetonitrile)</td>
</tr>
</tbody>
</table>

Table 8.4 Optical rotations for peptidyl-\(\alpha\)-ketoesters before and after flash column chromatography.

Experiment 8.5.4.2 pH dependence of the racemisation of 3.1

An NMR solution of 3.1 was prepared in (2:1) \(d_3\)-acetonitrile/acidified D\(_2\)O. The D\(_2\)O had been acidified to pH 3 by Universal Indicator by the addition of HCl. An NMR solution of 3.1 was prepared in (2:1) \(d_3\)-acetonitrile/buffered D\(_2\)O. The buffered D\(_2\)O solution at pH 7.8 comprised HEPES buffer (0.1 M), CaCl\(_2\) (0.02 M) and Triton X-100 (0.05% w/v), which is analogous to the enzyme assay buffer solution. The two solutions
were monitored for racemisation over a period of 30 h by $^1$H NMR. The extent of racemisation of these solutions was measured by comparing the value for the integral of the proton adjacent to the electrophilic ketone ($\beta$-proton) with the integral of one aromatic proton. There were four species present in the solution \{(E)-3.1, (Z)-3.1, (E)-5.9, (Z)-5.9\} and the integral of the $\beta$-proton was measured as a sum of the integrals of the four species. The results for both solutions are given in Table 8.5.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Proportion of $\beta$ proton remaining by $^1$H NMR (all ± 0.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acidic D$_2$O (pH3)</td>
</tr>
<tr>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>0.75</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 8.5  Time dependency of value of $\beta$ proton relative to an aromatic proton.

Experiment 8.5.4.3  Measuring the extent of deuteration of a sample of 3.1

A sample of 3.1 in $d_3$-acetonitrile was irradiated for one hour by a high pressure mercury arc lamp with a wavelength filter (330$<\lambda<$370 nm) as specified in Experiment 8.5.3.1, however there was no water filter between the light source and the sample. A $^1$H NMR was taken of the sample and the extent of racemisation of the sample was measured by comparing the value for the integral of the $\beta$-proton with the integral of one aromatic proton. There were four species present in the solution \{(E)-3.1, (Z)-3.1, (E)-5.9, (Z)-5.9\} and the integral of the $\beta$-proton was measured as a sum of the integrals of the four species. The ratio of integrals was $\beta$-proton/aromatic proton = 1:3.3.

Irradiated sample (contains S and R): \[[\alpha]_D = 20 \pm 3^\circ (c 0.2, \text{acetonitrile})\]
Original sample (exclusively S): \[[\alpha]_D = 57 \pm 3^\circ (c 0.2, \text{acetonitrile})\]
Calculation of deuterium incorporation: \[\text{if } \chi_S = y, \text{ then } \chi_R = 1 - y\]
\[-55x_S + 55x_R = -20\]
\[-55y + 55(1-y) = -20\]

\[y = 0.68\]
\[3/20 + 3/55 = 1/5\]

therefore \(x_S = 0.68\), then \(x_R = 0.32\)

because all R is deuterated and if the amount of R deuterated = amount of S deuterated

then \(x_S\) (non-deuterated) = 0.36 ± 0.08

Mass spectra (intensities relative to cleavage ion at 328 given in brackets)

Irradiated sample:
MS (EI): 325 (1.5), 326 (8.9), 327 (9.8), 328 (100.0), 329 (57.3), 330 (15.2), 331 (2.2)

Original sample:
MS (EI): 325 (1.3), 326 (6.8), 327 (7.7), 328 (100.0), 329 (27.4), 330 (4.2), 331 (0.4)

Isotopic label incorporation calculation programme gave the following results:

<table>
<thead>
<tr>
<th>Number of labels</th>
<th>Percentage of label</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>75.8</td>
</tr>
<tr>
<td>1</td>
<td>24.2</td>
</tr>
</tbody>
</table>
8.6 Experimental Work Described in Chapter Six

8.6.3 Setup of the enzyme assay

8.6.3.1 Equipment used for enzyme assay

Absorbance measurements were made on a Hewlett Packard 8452A diode array spectrophotometer, which was controlled by Hewlett Packard 89532A UV-VIS operating software. The plastic cuvettes (1.5 mL) were held during the assays in a custom-made thermostatted cell block that was set at 25.0 ± 0.2 °C. Buffer, enzyme and substrate solutions were stored during use in a water bath thermoregulated at 25 °C by a Techne Tempette TE-8A water bath thermostat. The solutions were transferred by use of Eppendorf micropipettes (100, 200, 1000 µL). All buffer solutions were pH tested at 25 °C with a custom-made digital pH meter that had been referenced with borax solution (0.01 M) to pH 9.18. All stock inhibitor solutions were prepared in acetonitrile (BDH HiperSolv™ “Far UV” grade).

8.6.3.2 Solutions for enzyme assay

A. Buffer (HEPES) solution

HEPES 0.1 M, pH 7.8; Ca\(^{2+}\) 0.02 M; Triton X-100, 0.05% (w/v)

HEPES (N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid), Sigma®, 11.9 g, 0.05 mol), CaCl\(_2\)·6H\(_2\)O (analytical grade, BDH, 2.2 g, 0.01 mol) and Triton X-100 (Aldrich, 0.25 g) were dissolved in Milli-Q ionised water (400 mL) in a 500 mL volumetric flask. Sufficient 1M NaOH solution was added (analytical grade, approx. 40 mL) to reach pH 7.8 and the solution was made up to 500 mL with more Milli-Q water.
B. Substrate solution

\[ \text{\textit{N-Succinyl-(Ala)}_2\text{-Pro-Phe-4-nitroanilide 3.4 mM; HEPES 0.1 M, pH 7.8; Ca}^{2+} 0.02 \text{ M}} \]

HEPES (11.9 g, 0.05 mol) and CaCl\(_2\cdot6\text{H}_2\text{O}\) (2.2 g, 0.01 mol) were dissolved in Milli-Q ionised water (400 mL) in a 500 mL volumetric flask. Sufficient 1M NaOH solution was added (approx. 40 mL) to reach pH 7.8 and the solution was made up to 500 mL with more Milli-Q water. \textit{N-Succinyl-(Ala)}\(_2\)-Pro-Phe-4-nitroanilide (Sigma®, 21 mg, 34 \(\mu\)mol) was dissolved in the solution described above (10 mL) by sonication for 10 min in cold water. The solution was stored for up to two weeks below 0 °C. The concentration of the solution was determined at the start of each day from its UV spectrum (\(\varepsilon_{315} = 14 \text{ 000 Lmol}^{-1}\text{cm}^{-1}\)).

C. Enzyme solution

\[ \text{\textit{\alpha-chymotrypsin 240 nM; HCl 2.5 mM; Triton X-100, 0.05\% (w/v)}} \]

A stock solution of \textit{\alpha-chymotrypsin} was prepared from \textit{\alpha-chymotrypsin} (3.03 mg, 120 nmol, Sigma®, Type II: from bovine pancreas 3 x crystallised from 4 x crystallised chymotrypsinogen) and Milli-Q water (1 mL). To a 100 mL volumetric flask was added stock solution (200 \(\mu\)L, 24 nmol), Triton X-100 (50 mg, 0.05\% w/v) and conc. HCl (analytical grade, 10 \(\mu\)L, 1.2 mM) and Milli-Q water (about 50 mL). The flask was shaken at length and the solution was made to 100 mL with Milli-Q water. The enzyme solutions were prepared fresh each day. The enzyme concentration was standardised each day by fitting to the Michaelis-Menten plot shown in Figure 6.4.

8.6.3.3 Determination of the Michaelis-Menten constant and the limiting rate

The assay procedure was developed from the technique described by Geiger, except that HEPES buffer was used instead of TRIS buffer and that the order of addition of enzyme and substrate was inverted. To a plastic cuvette (1.5 mL) was added the buffer solution ((1.02-x-y) mL), substrate solution (x mL, x in the range 0.02 to 0.15 mL) and acetonitrile (y mL, y either 0.00, 0.02, 0.06 mL). The solution was mixed by inversion of the cuvette and then the assay medium was incubated at 25 °C for five minutes. Enzyme solution (0.03 mL) was added, then the solution was homogenised by
inversion and the absorbance of the product (4-nitroaniline, $e_{405} = 10,200 \text{ Lmol}^{-1}\text{cm}^{-1}$) was measured at $\lambda = 404 \text{ nm}$ for five minutes. A plot of absorbance against time (slope equals rate of reaction) gave a straight line over the entire time course in almost all cases and the slope was used as the initial rate measurement. The exceptions were the assays with very low substrate concentrations, for which the initial rate was determined from the slope of the initial straight line period of the curve. Each measurement was made in duplicate. The raw data is given in Table 8.6

<table>
<thead>
<tr>
<th>0.0% v/v acetonitrile</th>
<th>1.9% v/v acetonitrile</th>
<th>5.7% v/v acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S] (mM)</td>
<td>$V_0$ (µmol s$^{-1}$mg$^{-1}$)</td>
<td>[S] (mM)</td>
</tr>
<tr>
<td>0.480</td>
<td>1090</td>
<td>1120</td>
</tr>
<tr>
<td>0.384</td>
<td>1030</td>
<td>980</td>
</tr>
<tr>
<td>0.288</td>
<td>960</td>
<td>990</td>
</tr>
<tr>
<td>0.192</td>
<td>920</td>
<td>900</td>
</tr>
<tr>
<td>0.096</td>
<td>790</td>
<td>800</td>
</tr>
<tr>
<td>0.048</td>
<td>570</td>
<td>540</td>
</tr>
</tbody>
</table>

Table 8.6 Kinetic data for $K_M$ and $V_{max}$ determination.

8.6.3.4 Calibration of the enzyme assay with a reported inhibitor

The assay procedure was performed as described by Angelastro et al. To a plastic cuvette (1.5 mL) was added the buffer solution ((0.87-$\gamma$) mL), substrate solution (0.15 mL) and enzyme solution (0.03 mL). The solutions were mixed by inversion of the cuvette and the assay medium was incubated at 25 °C for five minutes. A solution of 2.52 in acetonitrile (60.2 µmol L$^{-1}$, $\gamma$ mL, $\gamma$ either 0.01 or 0.03 mL) was added, then the solution was homogenised by inversion and the absorbance of the 4-nitroaniline was measured at $\lambda = 404 \text{ nm}$ for five minutes. A plot of absorbance against time (slope equals rate of reaction) gave a straight line over the entire time course in all cases and the slope was used as the initial rate measurement. Each measurement was made in duplicate. The raw data is given in Table 8.7
Table 8.7 Inhibition of α-chymotrypsin by 2.52

<table>
<thead>
<tr>
<th>% v/v MeCN</th>
<th>[I] μM</th>
<th>[S] μM</th>
<th>( V_i ) (μmol s⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.6</td>
<td>0.48</td>
<td>700</td>
</tr>
<tr>
<td>2.8</td>
<td>1.7</td>
<td>0.48</td>
<td>570</td>
</tr>
</tbody>
</table>

The assay procedure described above was repeated for assays of 2.52 (60.2 μmol L⁻¹) that had been stored in either acetonitrile or HEPES (solution A). Measurements were taken at 5 min, 40 min (HEPES only) and 24 h after the preparation of the inhibitor solutions (results in Table 8.8).

Table 8.8 The activity of 2.52 against α-chymotrypsin after incubation in acetonitrile or HEPES buffer (0.1 M)

<table>
<thead>
<tr>
<th>Time in solution</th>
<th>Solvent</th>
<th>[S] μM</th>
<th>[I] μM</th>
<th>( V_i ) (μmol s⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>acetonitrile</td>
<td>0.48</td>
<td>0.6</td>
<td>810</td>
</tr>
<tr>
<td>24 h</td>
<td>acetonitrile</td>
<td>0.48</td>
<td>0.6</td>
<td>840</td>
</tr>
<tr>
<td>5 min</td>
<td>water (HEPES)</td>
<td>0.48</td>
<td>1.7</td>
<td>560</td>
</tr>
<tr>
<td>40 min</td>
<td>water (HEPES)</td>
<td>0.48</td>
<td>1.7</td>
<td>680</td>
</tr>
<tr>
<td>24 h</td>
<td>water (HEPES)</td>
<td>0.48</td>
<td>1.7</td>
<td>1020</td>
</tr>
</tbody>
</table>

8.6.4 Enzyme assay of the target compounds

To a plastic cuvette (1.5 mL) was added the buffer solution ((1.02-x-y) mL), substrate solution (x mL, x in the range 0.02 to 0.15 mL) and a solution of the inhibitor in acetonitrile (y mL, y in the range 0.005 to 0.060 mL). The solutions were mixed by inversion of the cuvette and then the assay medium was incubated at 25 °C for five minutes. Enzyme solution (0.03 mL) was added, then the solution was homogenised by inversion and the absorbance of the product, 4-nitroaniline, was measured at \( \lambda = 404 \) nm for five minutes. A plot of absorbance against time (slope equals rate of reaction) gave a straight line over the entire time course in almost all cases and the slope was
used as the initial rate measurement. The exceptions are discussed by case. Each measurement was made in duplicate.

For each of the target compounds described in this thesis preliminary assays against \(\alpha\)-chymotrypsin were performed in order to check for enzyme inhibition. If the compounds proved to be inhibitors of the enzyme the following study was undertaken. A solution of the compound in acetonitrile was put under fluorescent lighting for at least one day to give the ambient light PSS. The solution was then assayed at five substrate concentrations and at five inhibitor concentrations (large data set). The inhibitor solution was irradiated with UV light for 60 min to give the UV light PSS as described in Section 8.5.3.1. During the assays this solution was shielded from light and the cuvettes were covered during the incubation period of the assay. This solution was assayed at four substrate concentrations and at three inhibitor concentrations (small data set). The inhibitor solution was irradiated with visible light for 60 min to give the visible light PSS as described in Section 8.5.3.1. During the assays this solution was shielded from light and the cuvettes were covered during the incubation period of the assay. In order to test the assumption that the ambient and visible light photostationary states have the same effect on \(\alpha\)-chymotrypsin, the visible light PSS was assayed against the enzyme at two inhibitor concentrations.

Both large and small data sets were fitted to the Michaelis-Menten model with double-reciprocal and direct linear plots.\(^{34}\) From these analyses the kinetic parameters \(K_M^{\text{app}}\), \(V_{\text{max}}^{\text{app}}\) and \(K_i\) were calculated. The large data sets were sufficiently comprehensive to apply the data to Dixon and modified Dixon plots in order to further ascertain the type of inhibition.\(^{34b}\) The analyses were performed in Microsoft Excel\(^\text{TM}\) by regression analysis, axis intercept formulae and line intersection formulae. In this section the raw data is presented, while the apparent Michaelis-Menten constants, the apparent limiting rates and the inhibition constants are given in Sections 6.4, 6.5 and 6.6.
Chapter Eight: Experimental

The target compounds 4.1, 4.2 and 4.3 were found to be inactive against α-chymotrypsin at the following concentrations:

- 4.1 at 260 μM
- 4.2 at 90 μM
- 4.3 at 30 μM
<table>
<thead>
<tr>
<th>[I] μM</th>
<th>9</th>
<th>20</th>
<th>28</th>
<th>37</th>
<th>47</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.096</td>
<td>470</td>
<td>380</td>
<td>310</td>
<td>260</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>390</td>
<td>330</td>
<td>290</td>
<td>260</td>
</tr>
<tr>
<td>0.144</td>
<td>690</td>
<td>560</td>
<td>460</td>
<td>400</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>570</td>
<td>490</td>
<td>450</td>
<td>380</td>
</tr>
<tr>
<td>0.192</td>
<td>730</td>
<td>660</td>
<td>620</td>
<td>520</td>
<td>490</td>
</tr>
<tr>
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**Table 8.9** Initial rates (μmols·mg⁻¹) for 2.1 ambient light PSS

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<tr>
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<tr>
<td></td>
<td>590</td>
<td>460</td>
<td>280</td>
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**Table 8.10** Initial rates (μmols·mg⁻¹) for 2.1 UV light PSS

<table>
<thead>
<tr>
<th>[S] mM</th>
<th>[I] μM</th>
<th>$V_i$ (ambient light PSS)</th>
<th>$V_i$ (visible light PSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.144</td>
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**Table 8.11** Initial rates (μmols·mg⁻¹) for 2.1 ambient and visible light PSS
### Table 8.12 Initial rates ($\mu$mol$\cdot$m$^{-1}$) for 2.2 ambient light PSS

<table>
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<td>310</td>
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<td>520</td>
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### Table 8.13 Initial rates ($\mu$mol$\cdot$m$^{-1}$) for 2.2 UV light PSS

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<th>24</th>
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<td>340</td>
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<tr>
<td>0.192</td>
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<td>460</td>
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<tr>
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<td>500</td>
<td>440</td>
<td>390</td>
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<td>0.256</td>
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<tr>
<td></td>
<td>560</td>
<td>480</td>
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### Table 8.14 Initial rates ($\mu$mol$\cdot$m$^{-1}$) for 2.2 ambient and visible light PSS

<table>
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<th>[S] mM</th>
<th>[I] $\mu$M</th>
<th>$V_i$ (ambient light PSS)</th>
<th>$V_i$ (visible light PSS)</th>
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<tr>
<td>0.192</td>
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Table 8.14 Initial rates ($\mu$mol$\cdot$m$^{-1}$) for 2.2 ambient and visible light PSS
### Table 8.15 Initial rates (μmol s⁻¹ mg⁻¹) for 2.3 ambient light PSS

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<th>8.1</th>
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<td>410</td>
<td>400</td>
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<tr>
<td>[S] mM</td>
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<td>600</td>
<td>540</td>
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<td></td>
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<td>490</td>
<td>440</td>
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### Table 8.16 Initial rates (μmol s⁻¹ mg⁻¹) for 2.3 UV light PSS

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<th>8.1</th>
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<td>310</td>
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<td>490</td>
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<td>340</td>
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<td>460</td>
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</tr>
<tr>
<td></td>
<td>520</td>
<td>420</td>
<td>380</td>
</tr>
<tr>
<td>[S] mM</td>
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<td>520</td>
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<td>450</td>
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<tr>
<td></td>
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<td>560</td>
<td>480</td>
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### Table 8.17 Initial rates (μmol s⁻¹ mg⁻¹) for 2.4 ambient light PSS

<table>
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<tr>
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<th>[I] μM</th>
<th>V_i (ambient light PSS)</th>
<th>% v/v MeCN</th>
<th>V_0</th>
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<td>0.192</td>
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Table 8.16 Initial rates (μmol s⁻¹ mg⁻¹) for 2.3 UV light PSS
## Table 8.18 Initial rates (µmols⁻¹mg⁻¹) for 3.1 ambient light PSS

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<td>570</td>
<td>470</td>
<td>450</td>
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<tr>
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<td>560</td>
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Table 8.19 Initial rates (µmols⁻¹mg⁻¹) for 3.1 UV light PSS

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<th>$V_i$ (visible light PSS)</th>
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<td>490</td>
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<td>0.14</td>
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Table 8.20 Initial rates (µmols⁻¹mg⁻¹) for 3.1 ambient and visible light PSS
Table 8.21 Initial rates (\(\mu\text{mols}^\text{1} \text{mg}^{-1}\)) for 3.2 ambient light PSS

<table>
<thead>
<tr>
<th>[I] (\mu\text{M})</th>
<th>0.11</th>
<th>0.22</th>
<th>0.34</th>
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<th>0.56</th>
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<td>390</td>
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<td>0.384</td>
<td>820</td>
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<td>670</td>
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<td>860</td>
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Table 8.22 Initial rates (\(\mu\text{mols}^\text{1} \text{mg}^{-1}\)) for 3.2 UV light PSS

<table>
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<tr>
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<th>0.45</th>
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<tbody>
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<td>850</td>
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Table 8.23 Initial rates (\(\mu\text{mols}^\text{1} \text{mg}^{-1}\)) for 3.2 ambient and visible light PSS

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<th>(V_i) (ambient light PSS)</th>
<th>(V_i) (visible light PSS)</th>
</tr>
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### Table 8.24
Initial rates (μmol·mg⁻¹) for 3.3 ambient light PSS

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### Table 8.25
Initial rates (μmol·mg⁻¹) for 3.3 UV light PSS

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### Table 8.26 Initial rates (μmols⁻¹mg⁻¹) for 3.5 ambient light PSS

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### Table 8.27 Initial rates (μmols⁻¹mg⁻¹) for 3.5 UV light PSS

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<td>350</td>
<td>310</td>
<td>280</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>0.336</td>
<td>410</td>
<td>320</td>
<td>290</td>
<td>280</td>
<td>280</td>
</tr>
<tr>
<td>0.384</td>
<td>410</td>
<td>330</td>
<td>280</td>
<td>300</td>
<td>270</td>
</tr>
<tr>
<td>0.432</td>
<td>450</td>
<td>370</td>
<td>320</td>
<td>330</td>
<td>300</td>
</tr>
<tr>
<td>0.480</td>
<td>460</td>
<td>360</td>
<td>350</td>
<td>350</td>
<td>310</td>
</tr>
<tr>
<td>0.532</td>
<td>490</td>
<td>420</td>
<td>360</td>
<td>350</td>
<td>330</td>
</tr>
<tr>
<td>0.632</td>
<td>450</td>
<td>400</td>
<td>370</td>
<td>360</td>
<td>330</td>
</tr>
</tbody>
</table>
In an attempt to apply the analysis described by Cha to the ambient light PSS of 3.6 the initial rates of 3.6 that had been pre-incubated with enzyme were measured. To a plastic cuvette (1.5 mL) were added the buffer solution \{(1.02-x-y) mL, 0.1 M HEPES\}, enzyme solution \{0.03 mL, 2.4 \mu M \alpha\text{-chymotrypsin}\} and a solution 3.6 in acetonitrile \{43 \mu M, y mL, y either 0.005, 0.010, 0.020, 0.030 mL\}. The solutions were mixed by inversion of the cuvette and then the assay medium was incubated at 25 °C for five minutes. Substrate solution \{x mL, x in the range 0.04 to 0.10 mL\} was added, then the solution was homogenised by inversion and the absorbance of the product, 4-nitroaniline, was measured at \(\lambda = 404\) nm from exactly 20 seconds after the addition of substrate. Measurements were taken over 50 seconds and the slope of the absorbance versus time plot was calculated by a zero order calculation. Measurements were made in duplicate.

<table>
<thead>
<tr>
<th>[S] mM</th>
<th>[I] \mu M</th>
<th>(V_i) (ambient light PSS)</th>
<th>(V_i) (visible light PSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24</td>
<td>4.1</td>
<td>470</td>
<td>470</td>
</tr>
<tr>
<td>0.336</td>
<td>6.2</td>
<td>520</td>
<td>520</td>
</tr>
</tbody>
</table>

**Table 8.28** Initial rates (\(\mu\)mols\(^{-1}\)mg\(^{-1}\)) for 3.5 ambient and visible light PSS

<table>
<thead>
<tr>
<th>[I] \mu M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>0.128</td>
</tr>
<tr>
<td>0.192</td>
</tr>
<tr>
<td>0.256</td>
</tr>
<tr>
<td>0.320</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[S] mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.128</td>
</tr>
<tr>
<td>0.192</td>
</tr>
<tr>
<td>0.256</td>
</tr>
<tr>
<td>0.320</td>
</tr>
</tbody>
</table>

**Table 8.29** Initial rates (\(\mu\)mols\(^{-1}\)mg\(^{-1}\)) for pre-incubated 3.6 UV light PSS
8.6.5 Stability of inhibitors in different buffer solutions

8.6.5.1 Stability of 3.1 in HEPES and Milli-Q water

From a stock solution of 3.1 in acetonitrile (4.7 mM) were prepared solutions (0.094 mM) of 3.1 in Milli-Q water and in HEPES buffer (Solution A from Section 8.6.4). The inhibitor solutions were tested against α-chymotrypsin (as described in Section 8.6.4 with [S] = 0.48 mM, [I] = 2.7 μM) at 5 min, 20 min, 1 h, 1 d (HEPES only), 2 d, 3 d (Milli-Q only), 6 d (HEPES only). The initial rate in the absence of inhibitor for [S] = 0.48 mM is \( V_0 = 1060 \, \mu\text{mol}s^{-1}\cdot\text{mg}^{-1} \).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>( V_i ) (3.1, Milli-Q, ( \mu\text{mol}s^{-1}\cdot\text{mg}^{-1} ))</th>
<th>( V_i ) (3.1, HEPES, ( \mu\text{mol}s^{-1}\cdot\text{mg}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>210</td>
<td>290</td>
</tr>
<tr>
<td>0.33</td>
<td>190</td>
<td>300</td>
</tr>
<tr>
<td>1</td>
<td>190</td>
<td>320</td>
</tr>
<tr>
<td>24</td>
<td>N.A.</td>
<td>790</td>
</tr>
<tr>
<td>48</td>
<td>480</td>
<td>890</td>
</tr>
<tr>
<td>72</td>
<td>670</td>
<td>N.A.</td>
</tr>
<tr>
<td>144</td>
<td>N.A.</td>
<td>1060</td>
</tr>
</tbody>
</table>

Table 8.29 Initial rates for ambient light PSS 3.1 incubated in water or HEPES.

8.6.5.2 Buffer solutions for 2.4 enzyme assay

A. HEPES solution

HEPES 0.1 M, pH 7.8; \( \text{Ca}^{2+} \) 0.02 M; Triton X-100, 0.05% (w/v)
HEPES (11.9 g, 0.05 mol), \( \text{CaCl}_2\cdot6\text{H}_2\text{O} \) (2.2 g, 0.01 mol) and Triton X-100 (0.25 g) were dissolved in Milli-Q ionised water (400 mL) in a 500 mL volumetric flask. Sufficient 1M NaOH solution was added (approx. 40 mL) to reach pH 7.8 and the solution was made up to 500 mL with more Milli-Q water.
TRIS solution

TRIS 0.1 M, pH 7.8, Ca$^{2+}$ 0.02 M; Triton X-100, 0.05% (w/v)

TRIS (tris(hydroxymethyl)aminoethane, 1.21 g, 0.01 mol), CaCl$_2$.6H$_2$O (0.44 g, 0.002 mol) and Triton X-100 (0.05 g) were dissolved in Milli-Q ionised water (75 mL) in a 100 mL volumetric flask. Sufficient 1M HCl solution was added (approx. 40 mL) to reach pH 7.8 and the solution was made up to 100 mL with more Milli-Q water.

Phosphate solution

K$_2$HPO$_4$ 0.1 M, pH 7.3, K$^+$ 0.03 M; Triton X-100, 0.05% (w/v)

K$_2$HPO$_4$ (1.36 g, 0.01 mol), KCl (0.22 g, 0.002 mol) and Triton X-100 (0.05 g) were dissolved in Milli-Q ionised water (75 mL) in a 100 mL volumetric flask. Sufficient 1M NaOH solution was added (approx. 40 mL) to reach pH 7.8 and the solution was made up to 100 mL with more Milli-Q water.

8.6.5.3 Stability of 2.4 in different buffer solutions

From a stock solution of 2.4 in acetonitrile (4.5 mM) were prepared solutions (0.28 mM) of 2.4 in Milli-Q water, and in HEPES, TRIS and phosphate buffers (PHOS). The inhibitor solutions were tested against α-chymotrypsin (as described in Section 8.6.4 with [S] = 0.48 mM, [I] = 8.1) at 5 min, 25 min, 80 min, 160 min (Milli-Q and HEPES only), 27 h. The initial rate in the absence of inhibitor for [S] = 0.48 mM is $V_0 = 1060$ μmols$^{-1}$mg$^{-1}$.

The initial rates under the heading - $V_1$ (PHOS)* - were from assays run in phosphate buffer rather than HEPES buffer and were obtained in the following manner: to a plastic cuvette (1.5 mL) was added the phosphate buffer solution ((1.02-x-y) mL), substrate solution (0.15 mL) and a solution of 2.4 in phosphate buffer (0.03 mL, 0.28 mM). The solutions were mixed by inversion of the cuvette and the assay medium was incubated at 25 °C for five minutes. Enzyme solution (0.03 mL) was added, then the solution was homogenised by inversion and the absorbance of the solution was measured at $\lambda = 404$ nm for five minutes. A plot of absorbance against time (slope
equals rate of reaction) gave a straight line over the entire time course and the slope was used as the initial rate measurement. Each measurement was made in duplicate.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>( V_1 ) (Milli-Q)</th>
<th>( V_1 ) (HEPES)</th>
<th>( V_1 ) (TRIS)</th>
<th>( V_1 ) (PHOS)</th>
<th>( V_1 ) (PHOS)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>210</td>
<td>180</td>
<td>220</td>
<td>280</td>
<td>190</td>
</tr>
<tr>
<td>0.42</td>
<td>220</td>
<td>270</td>
<td>270</td>
<td>160</td>
<td>170</td>
</tr>
<tr>
<td>1.33</td>
<td>210</td>
<td>290</td>
<td>260</td>
<td>230</td>
<td>150</td>
</tr>
<tr>
<td>3.75</td>
<td>130</td>
<td>450</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>27</td>
<td>220</td>
<td>940</td>
<td>930</td>
<td>450</td>
<td>390</td>
</tr>
</tbody>
</table>

**Table 8.30**  Initial rates (\( \mu \text{mols}^{-1} \text{mg}^{-1} \)) for ambient light PSS 2.4 incubated in water or one of three buffers (* enzyme assays were run in phosphate buffer).
8.7 References for Chapter Eight

Chapter Eight: Experimental


*Beilsteins Handbuch der organischen Chemie*; Springer-Verlag: Berlin.


Sheldrick, G. M. *SHELXL 93*, University of Göttingen.


APPENDIX
Appendix One

Derivation of the Michaelis-Menten equation for the one-intermediate case

Equation A.1 models an enzyme-catalysed reaction, in which enzyme E binds with substrate S to form the enzyme-substrate complex ES. This step is not necessarily an equilibrium, but it is reversible. The ES reacts to give the product P and the regenerated enzyme. It is assumed that [ES] does not change during the reaction, except of course at the very start of the reaction.

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P
\]

**Equation A.1**

- \([E]_T = \text{total concentration of enzyme} = [E] + [ES]\)
- \(V_0 = \text{rate of reaction} = k_2[ES] \text{ because the conversion of ES to } E + P \text{ is the rate determining step}\)
- \(V_{\text{max}} = \text{limiting rate} = k_2[E]_T \text{ because at saturation } V_0 = V_{\text{max}} \text{ and } [E] = 0 \text{ thus } [E]_T = [ES]\)
- \(K_M = \text{Michaelis-Menten constant} = \left(\frac{k_{-1} + k_2}{k_1}\right)\)

At steady state: the rate of formation of ES = the rate of destruction of ES

- rate of formation of ES = \(k_1[S][E]_T - [ES])
- rate of destruction of ES = \(k_{-1}[ES] + k_2[ES]\)

thus \(k_1[S][E]_T - [ES]) = k_{-1}[ES] + k_2[ES]\)

\(k_1[S][E]_T - k_1[ES][S] = k_{-1}[ES] + k_2[ES]\)

\([ES](k_1[S] + k_{-1} + k_2) = k_1[S][E]_T\)

\([ES] = k_1[S][E]_T / (k_1[S] + k_{-1} + k_2)\)

\([ES] = [S][E]_T / ([S] + (k_{-1} + k_2) / k_1)\)

\(V_0 = k_2[ES] = k_2[S][E]_T / ([S] + (k_{-1} + k_2) / k_1)\)

\(V_0 = V_{\text{max}}[S] / ([S] + K_M) \text{ Equation A.2}\)
The Michaelis-Menten equation for the two-intermediate case

Equation A.3 represents the mechanism for catalysis by \( \alpha \)-chymotrypsin, for which there are two intermediates: i.e. the enzyme-substrate complex (ES) and the acyl enzyme adduct (ES').

\[
\begin{align*}
E + S & \xrightleftharpoons[k_1]{k_{-1}} ES \\
& \xrightarrow[k_2]{k_3} ES' \\
& \xrightarrow{k_3} E + P
\end{align*}
\]

Equation A.3

The rate equation for Equation A.3 is given below (Equation A.4) and was derived by a method analogous to that used to obtain Equation A.2. The rate equation for the two intermediate system is of the same form as Equation A.2, but the kinetic constants \((V_{\text{max}}', \text{ and } K_{M}')\)\(^2\) are considerably more complex.

\[
V_0 = \frac{V_{\text{max}}'[S]}{([S] + K_{M}')}
\]

where \(V_{\text{max}}' = \frac{k_2 k_3 [E]}{k_2 + k_2 + k_3}\)

and \(K_{M}' = \frac{k_1 k_2 + k_1 k_3 + k_2 k_3}{k_1 (k_2 + k_2 + k_3)}\)

Equation A.4

Derivation of the double reciprocal (Lineweaver-Burk) plot:

\[
\begin{align*}
V_0 &= \frac{V_{\text{max}}[S]}{([S] + K_M)} \\
\frac{1}{V_0} &= \frac{[S]}{(V_{\text{max}}[S]) + K_M / (V_{\text{max}}[S])} \\
\frac{1}{V_0} &= 1 / V_{\text{max}} + (K_M / V_{\text{max}})(1 / [S])
\end{align*}
\]

Equation A.5

A plot of \(1 / V_0\) versus \(1 / [S]\) gives a straight line with

- \((1 / V_0)\) intercept \(= 1 / V_{\text{max}}\)
- \((1 / [S])\) intercept \(= -1 / K_M\)

\(^2\)In the body of this thesis, the limiting rate is referred to as \(V_{\text{max}}\) and the Michaelis-Menten constant as \(K_M\) regardless of mechanism.
Derivation of the modified direct linear plot:

\[ V_0 = \frac{V_{\text{max}}[S]}{([S] + K_M)} \]
\[ \frac{1}{V_0} = \frac{1}{V_{\text{max}}} + \left(\frac{K_M}{V_{\text{max}}}\right)\left(1 / [S]\right) \]
\[ \frac{1}{V_{\text{max}}} = \frac{1}{V_0} - \left(\frac{K_M}{V_{\text{max}}}\right)\left(1 / [S]\right) \]

Equation A.6

\[
\begin{align*}
\text{if} & \quad \frac{1}{V_{\text{max}}} & = & \quad 0 \\
\text{then} & \quad \frac{1}{V_0} - \left(\frac{K_M}{V_{\text{max}}}\right)\left(1 / [S]\right) & = & \quad 0 \\
\text{and consequently} & \quad \frac{K_M}{V_{\text{max}}} & = & \quad \frac{[S]}{V_0} \\
\text{if} & \quad \frac{K_M}{V_{\text{max}}} & = & \quad 0 \\
\text{then} & \quad \frac{1}{V_{\text{max}}} & = & \quad \frac{1}{V_0}
\end{align*}
\]

For the purposes of the modified direct linear plot \(1/V_0\) and \([S]/V_0\) are treated as constant and are the y- and x-intercepts respectively for each of a series of lines. Hence the equations above show that a line on a \(1/V_{\text{max}}\) versus \(K_M/V_{\text{max}}\) plot has its the axis intercepts at \(1/V_0\) and \([S]/V_0\) respectively. Each point on the line is a \((1/V_{\text{max}}, K_M/V_{\text{max}})\) data pair that is consistent with the observation \(1/V_0\) and \([S]/V_0\). The points of intersection of these lines provide estimates for \(K_M/V_{\text{max}}\) and \(1/V_{\text{max}}\) and consequently \(K_M\) and \(V_{\text{max}}\). For \(n\) lines, or \(n\) observations at different substrate concentrations, at best \(\sum_{i=1}^{n} (n-1)\) estimates will be given and it is common practise to use the median value for the best estimate.
Appendix Two

Inhibition Kinetics

The way that an inhibitor affects the initial rate of an enzyme-catalysed reaction gives clues to the type of interaction between the inhibitor and the enzyme. The pattern of change of the initial rate can be described as belonging predominantly to one of the following categories: competitive, uncompetitive, mixed and noncompetitive inhibition. The categories are assigned purely on the basis on observed initial rate data and do not necessitate a particular mechanism. There is, however, a mechanism for each category that deals with the simplest possible scenario consistent with the observed kinetics. In this section the simple mechanisms are given for each of the four inhibition types along with details on how kinetic parameters are determined for each type of inhibition.

Notation as per Cornish-Bowden (1974).

$K_i$ = the dissociation constant of the EI complex

$K_i'$ = the dissociation constant of the ESI complex

A. Competitive Inhibition

\[
E + S \overset{k_1}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} E + P \quad \text{Equation A.7}
\]

The characteristic feature of competitive inhibition is an increase in $K_M$ and no change in $V_{\text{max}}$ as the inhibitor concentration increases. One mechanism employed to explain these observations (Equation A.7) is that inhibitor binding to the active site to give the enzyme-inhibitor complex (EI) is in competition with substrate binding that gives the enzyme–substrate complex (ES). The corollary is that increasing the substrate concentration will act to “wash out” the inhibitor from the active site. A competitive inhibitor decreases the apparent affinity of the substrate for the enzyme (reflected in an
increase in $K_M$), but does not affect the reactivity of the ES complex once formed (i.e. no change in $V_{max}$). By applying the derivation used in Appendix 1 the rate equation corresponding to Equation A.7 is:

$$V_0 = V_{max}[S] / ([S] + K_M (1 + [I] / K_i))$$

Double reciprocal: A series of lines intersecting at the $1/[V_0]$-axis; $V_{max}^{app}$ is the $1/[V_0]$ intercept and $K_M^{app}$ is the $1/[S]$ intercept for each line.

Dixon: $K_i$ is given as the $[I]$-axis value for the median of the line intersections in the fourth quadrant

Modified Dixon: A series of parallel lines

B. Uncompetitive Inhibition

$$E + S \xrightleftharpoons[k_1]{k_2} E + P$$

Equation A.8

Uncompetitive inhibition, which is manifested in decreased $V_{max}$ and decreased $K_M$ with increasing inhibitor concentration, is the polar opposite to competitive inhibition. The simplest, however unlikely, mechanism for this observed behaviour is that the enzyme-substrate complex binds reversibly and unproductively with the inhibitor (as shown in Equation A.8). The mechanism is unlikely because an inhibitor that binds ES would be expected to have affinity for the free enzyme as well. The rate equation corresponding to Equation A.8 is:

$$V_0 = V_{max}[S] / ([S] (1 + [I] / K_i') + K_M)$$

Double reciprocal: A series of parallel lines; $V_{max}^{app}$ is the $1/[V_0]$ intercept and $K_M^{app}$ is the $1/[S]$ intercept for each line.

Dixon plot: A series of parallel lines

Modified Dixon: $K_i'$ is given as the $[I]$-axis value for the median of the line intersections in the fourth quadrant
C. **Mixed Inhibition**

Mixed inhibition can be viewed as the general case for which competitive and uncompetitive inhibition are the extremes (Equation A.9). Alternatively, it can be seen as comprising contributions from both competitive and uncompetitive inhibition. The kinetics are manifested in both $V_{\text{max}}$ and $K_M$ which may either stay the same or change with increasing inhibitor concentration. The rate equation corresponding to Equation A.9 is:

$$V_0 = \frac{V_{\text{max}}[S]}{/[I] (1 + [I] / K_i) + K_M (1 + [I] / K_i)}$$

Double reciprocal: A series of lines with no predefined pattern; $V_{\text{max}}^{\text{app}}$ is the $1/[V_0]$ intercept and $K_M^{\text{app}}$ is the $1/[S]$ intercept for each line.

Dixon plot: $K_i$ is given as the $[I]$-axis value for the median of the line intersections in the fourth quadrant.

Modified Dixon: $-K_i'$ is given as the $[I]$-axis value for the median of the line intersections in the third quadrant.

D. **Noncompetitive Inhibition**

Noncompetitive inhibition is characterised by a decrease in $V_{\text{max}}$ and no change in $K_M$ with increasing inhibitor concentration. A mechanism for noncompetitive inhibition is the binding of the inhibitor at an alternative site to the active site, leading to a decrease in catalytic activity but no change in substrate binding. The inhibitor would bind equally well to the enzyme or the enzyme substrate complex (as shown in
Equation A.10). This mechanism has been discredited as a category for real enzymes by some enzymologists who believe that binding at a second site must affect the substrate binding at the active site.

\[
\begin{align*}
E + S & \underset{k_1}{\overset{k_i}{\rightleftharpoons}} ES \quad \overset{k_2}{\longrightarrow} E + P \\
I + S \quad \overset{k_i}{\longrightarrow} I \\
K_i & \quad K_i' \\
E & \quad E + I \\
K_i & \quad K_i' \\
\end{align*}
\]

Regardless of whether any enzyme would fit to noncompetitive inhibition, the classification is useful in a theoretical sense. For instance the method of Cha for the analysis of slow-tight binders makes use of the concept of noncompetitive inhibition as discussed in Section 6.6. The rate equation corresponding to Equation A.10 is:

\[
V_0 = V_{\text{max}}[S] / (([S] + K_M)(1 + [I] / K_i))
\]

Double reciprocal: A series of lines intersecting at the 1/[S]-axis; \(V_{\text{max}}\) is the 1/[\(V_0\)] intercept and \(K_M\) is the 1/[S] intercept for each line.

Dixon and Modified Dixon plots:

-\(K_i\) is equal to -\(K_i'\) and is given as the median [I]-axis intercept for the series of lines in both the Dixon and modified Dixon plots.

The Dixon and Modified Dixon plots

\[
1/V_i = (K_M / ([S]V_{\text{max}}))(1 + [I] / K_i) + (1 / V_{\text{max}})(1 + [I] / K_i')
\]

The Dixon plot is based on Equation A.11, which is derived from the mixed inhibition (general) case. If \(1/V_i\) is plotted against [I] an estimation of \(K_i\) (if relevant) is given.

\[
[S]/V_i = (K_M / V_{\text{max}})(1 + [I] / K_i) + ([S] / V_{\text{max}})(1 + [I] / K_i')
\]
The modified Dixon plot is based on Equation A.12, which is derived from the mixed inhibition (general) case. If \([S]/V_i\) is plotted against \([I]\) an estimation of \(K_i'\) (if relevant) is given.

**Figure A.1**  

a. The Dixon plot of a competitive inhibitor  
b. The modified Dixon plot of a competitive inhibitor

**Figure A.2**  

a. The Dixon plot of an uncompetitive inhibitor  
b. The modified Dixon plot of an uncompetitive inhibitor
Figure A.3  

a. The Dixon plot of a mixed inhibitor  
b. The modified Dixon plot of a mixed inhibitor

Figure A.4  

a. The Dixon plot of a noncompetitive inhibitor  
b. The modified Dixon plot of a noncompetitive inhibitor

Units used in the kinetic plots

*Double reciprocal plot:*

1/$V_0$ axis in mg.s.$\mu$mol$^{-1}$ from rate in $\mu$mol.s$^{-1}$.mg$^{-1}$, that is the number of micromoles of substrate converted per second per milligram of enzyme.

1/[$S$] axis in L.mol$^{-1}$
Modified direct linear plot:
$1/V_{\text{max}}$ axis in mg.s.$\mu$mol$^{-1}$ as above for the $1/V_0$ axis of the double reciprocal.
$[K_M]/V_{\text{max}}$ axis in mol.s.mg.L$^{-1}$.$\mu$mol$^{-1}$

Dixon plot:
$1/V_0$ axis in mg.s.$\mu$mol$^{-1}$
$[I]$ axis in mol.L$^{-1}$

Modified Dixon plot:
$[S]/V_0$ axis in mol.s.mg.L$^{-1}$.$\mu$mol$^{-1}$
$[I]$ axis in mol.L$^{-1}$

Major sources for Appendices One and Two