THE DESIGN AND SYNTHESIS OF MECHANISM-BASED INHIBITORS OF SERINE PROTEASES

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To the family of friends on the outside who constantly berated me of the real world and whose support has been thankless and finally to my family Mum, Dad, Bo and the Cookie Marcus, and Steffi and Jesse:

'Res ipsa loquitur'

ABSTRACT

Serine proteases are involved in a number of physiological processes and have proved to be a valuable therapeutic target in the treatment of disease states resulting when the above processes move beyond homeostasis. The investigation of low molecular weight compounds as irreversible and reversible inhibitors of serine proteases has been fueled by the possibility of rational drug design and their use as mechanistic probes of enzyme action. As introduced in Chapter one particular attention has been focused on mechanism-based inactivators as these elicit clinically desirable specific, efficient and irreversible inhibition. The synthesis and assay of funtionalised imide mechanism-based inhibitors of the serine protease α-chymotrypsin is the subject of this thesis.

N-[(Sulfonyl)oxy]succinimides of type **1.41** (L = SO_2R') are known mechanism-based inhibitors of α-chymotrypsin operating via a Lossen rearrangement that unmasks an inactivating isocyanate species. Inhibitory activity has been found to be mediated by the nature of the R substituent and the R' substituent of the L group. Structure activity relationships were investigated by preparing a number of derivatives of type **1.41**. The design of the derivatives prepared focused on modulating the R' substituent to interact with extended binding sites of α-chymotrypsin, a strategy that would enhance inhibitory activity.

Chapter 2 describes the synthesis of **1.41** and **2.1**. Retrosynthetic analysis identified a route involving *N*-hydroxyimides to be favoured. A synthesis of **1.41** and **2.1** via this key intermediate required reaction between hydroxylamine and succinic and glutaric acid derivatives respectively. These derivatives were prepared using literature methods employing Guareschi, Michael and malonate ester reactions. A systematic study of the synthesis of succinic acids found the optimum route to involve the Stobbe condensation however a short synthesis employing amide base alkylation of succinimide was undertaken and this methodology may prove to be the ideal general route.

An aromatic series of derivatives, **1.41f-h** was therefore synthesised using the methodology above as were "dimeric" inhibitors **1.41m** and **n** capable of releasing two equivalents of inactivating species during inhibition. Succinimides with 3-C phenyl substituent rather than the benzyl substituent of the derivatives above, were prepared and a series of *N*-[(sulfonyl)oxy]glutarimides **2.1a**, **c-e** where the extent and type of substitution were varied were prepared. *N*-[(Acyl)oxy]imides **1.41r-o** and **2.1b** (L = C(O)R') may also inhibit α-chymotrypsin and these too were investigated.

Chapter 3 discusses the assay of inhibitory activity of compounds of type **1.41** and **2.1** against α-chymotrypsin. All the synthesised derivatives, excepting a series of

N[(acyl)oxy]succinimides **1.41o-r**, were found to be active to such a degree that all but one of the active compounds could not be assayed using sampling techniques. These potent inhibitors were then assayed using the progress curve method. Three compounds **1.41g** and **h** and **2.1c** were of such potency that the rate at which they inhibited α-chymotrypsin could not be measured even with the progress curve method. All three of these compounds possessed a benzyl substituent which was found to be a requiremnent for the exhibition of mechanism-based inhibition in the succinimide series. Compounds **1.41g** and **1.41h** owed their potency to being able to interact favourably with the S_n' subsites of α-chymotrypsin by containing aromatic substitution.

Chapter 4 discusses the use of Evan's oxazolidinone chemistry in the preparation of chiral succinates from which an enantiopure inhibitor of type **1.41** was prepared. Preliminary inhibition studies showed that **(*R*)-1.41f** was less active than its racemate indicating more activity resided in the (*S*)-enantiomer.

Chapter 5 discusses the design and synthesis of a potential novel imide mechanism-based inhibitor thought to act by unmasking a quinone imine methide in the active site of α-chymotrypsin. Although the compound released reactivity on hydrolysis it was not found to inhibit α-chymotrypsin significantly.

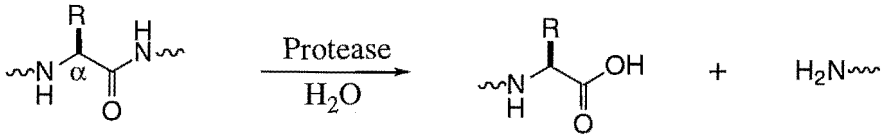
ABBREVIATIONS

α-CT	α-chymotrypsin
Ac	acetyl
AcCl	acetyl chloride
Ac_2O	acetic anhydride
Ala	alanine
Ar	aryl
Arg	arginine
Bn	Benzyl
t-Bu	tertiary butyl
n-BuLi	*n*-butyl lithium
t-BuOK	potassium tertiary butoxide
DCC	*N,N'*-dicyclohexylcarbodiimide
1,2-DCE	1,2-dichloroethane
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
EDAC	1-(3-dimethylaminopropyl)-3-ethyl carbodimide
Et	ethyl
Et_3N	triethylamine
EtOH	ethanol
h	hour(s)
His	histidine
HNE	human neutrophil elastase
HOBt	1-hydroxybenzotriazole
HRMS	high resolution mass spectrometry
LDA	lithium di-*iso*propylamide
Me	methyl
MeOH	methanol
mesyl	(methanesulfonyl)oxy
min	minute(s)
p-NA	*para*-nitroanilide
NMR	nuclear magnetic resonance
Ph	phenyl
Phe	phenylalanine
Pro	proline
i-PrNEt	Hunigs base
r^2	correlation coefficient
rt	room temperature
Ser	serine

CHAPTER 1

INTRODUCTION

Proteases constitute a large family of enzymes characterised by their function which is the catalysis of the hydrolytic cleavage of peptide bonds in proteins and polypeptides (**Scheme 1.1**).



peptide bond

Scheme 1.1. The protease catalysed hydrolysis of a peptide bond.

Proteases are involved in almost every aspect of life. The functions of proteases[1] include hydrolysis of proteins and polypeptides for digestive and nutritional purposes, release of peptide hormones and neuromodulators from inactive precursors, activation of enzymes, for example clotting factors, and termination of biological responses by degradation of the message-transmitting peptide.

Proteases have been classified into four classes each with their own distinct mechanism.[2,3] This classification is based on the most significant functional group associated with the protease activity. Hence the serine, aspartic, cysteine proteases derive their names from the most prominent catalytic amino acid residue in the active site whereas the metallo proteases are named according to the associated prosthetic metal ion. **Table 1.1** lists examples of proteases in each mechanistic category.

The following section of this introduction deals with the mechanism of action of serine proteases and the different approaches to inhibiting these enzymes.

Protease	Representative Enzymes	Normal Function
Serine	-thrombin, plasma kallikrein	-blood coagulation
	-trypsin, α-chymotrypsin, pancreatic elastase	-digestion
	-enterokinase plasmin, plasminogen activator	-fibrinolysis, destruction of blood clots
	-tissue kallikrein, post-protein cleaving enzyme	-hormone metabolism
	-elastase, cathepsin G, tryptases, mast cell chymases	-phagocytosis
	-acrosin	-fertilization
	-ATP dependent proteases (intracellular)	-protein turnover
Metallo	-angiotensin converting enzyme, aminopeptidases, renal dipeptidases	-blood pressure regulation
	-collagenase	-tissue remodelling
	-carboxypeptidase	-digestion
	-macrophage elastase	-peptide metabolism, blood pressure regulation
	-enkephalinases	-analgesic
Aspartic	-renin	-blood pressure regulation
	-HIV protease	-HIV replication
	-thermolysin, pepsin	-digestion
Cysteine	-cathepsins B, H, L, calcium activated neutral proteases	-protein turnover, bone resorbtion
	-papain	-digestion
	-picornavirus	-viral replication

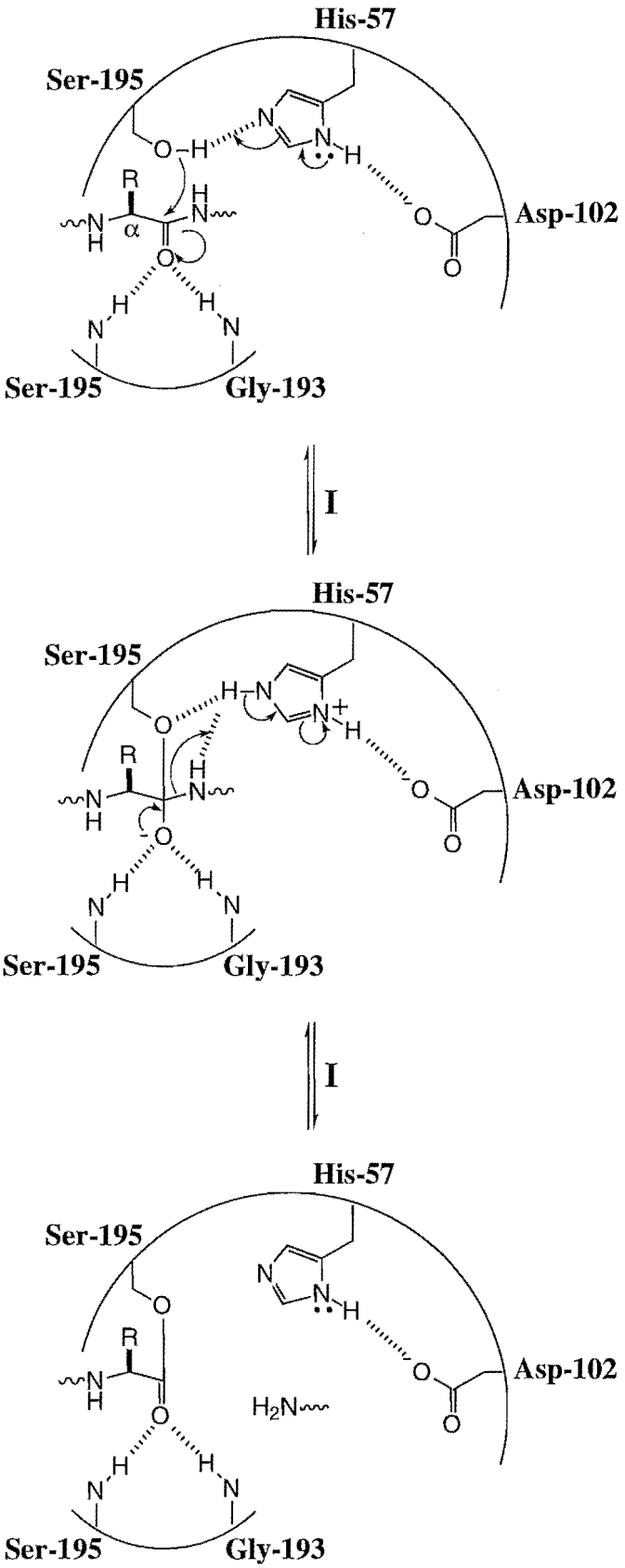
TABLE 1.1. Examples of Proteases, Subdivided into Mechanistic Categories

1.1 SERINE PROTEASES AND THEIR MECHANISM OF PEPTIDE HYDROLYSIS

More than 130 serine proteases have been classified to date.[4] Their different functions include digestion, processing of peptide prohormones, thrombolysis and fibrinolysis, fertilization and blastocyst implantation.[5,6] Even though the physiological functions of serine proteases are diverse, all employ a common catalytic mechanism. α-Chymotrypsin,[7,8] a serine protease from the mammalian pancreas involved in digestion, has been studied intensively and more information is available on its mode of catalysis than for almost any other enzyme.[9] Its active site has been well characterized by X-ray crystallography and intermediates of the hydrolysis reaction it catalyses are known. It is therefore representative of the mechanism of the whole class of serine proteases.

The proposed mechanism of peptide hydrolysis by α-chymotrypsin is illustrated in **Scheme 1.2**. The first step in the hydrolysis is the complementary approach of the enzyme and substrate that results in the non-covalent enzyme-substrate aggregate known as the Michaelis-Menten complex (**1.1**). The driving force for its formation depends on favourable binding interactions between the enzyme and substrate. The structure of serine proteases remains quite conserved but differences in key structural binding elements imparts specificity of action to a protease with regards to its substrate. Topographical studies of α-chymotrypsin, for example, have shown the presence of a hydrophobic pocket associated with the active-site which binds the amino acid α-C substituent on the carboxyl side of the peptide bond which is to be hydrolysed (the so-called scissile bond). Hence α-chymotrypsin selectively cleaves peptides on the carboxyl side of the amino acid residues; phenylalanine, tyrosine and tryptophan as these have aromatic side chains able to interact favourably with this pocket.

The driving force for hydrolysis is the catalytic triad consisting of Ser-195, His-57 and Asp-102 (the numbers 102, 57 and 195 denote the position of the amino acid residues Asp, His, and Ser, respectively in the polypeptide chain of the enzyme). His-57 and Asp-102 act as an electron sink and source in a charge relay system that enhances the nucleophilicity of Ser-195 (Step **I, Scheme 1.2**) and the incoming water molecule (Step **II, Scheme 1.2**). Catalysis is initiated by nucleophilic attack of the Ser-195 on the susceptible carbonyl carbon of the scissile peptide bond (depicted by the arrow) of the substrate to form a tetrahedral intermediate (**1.2**). The oxyanion of the tetrahedral adduct is stabilised by hydrogen-bonding to the backbone NH groups of Gly-193 and Ser-195 (see **1.2**) which incorporate a binding element referred to as the oxyanion hole. The proton on the serine hydroxyl group is ultimately transferred, via His-57, to the

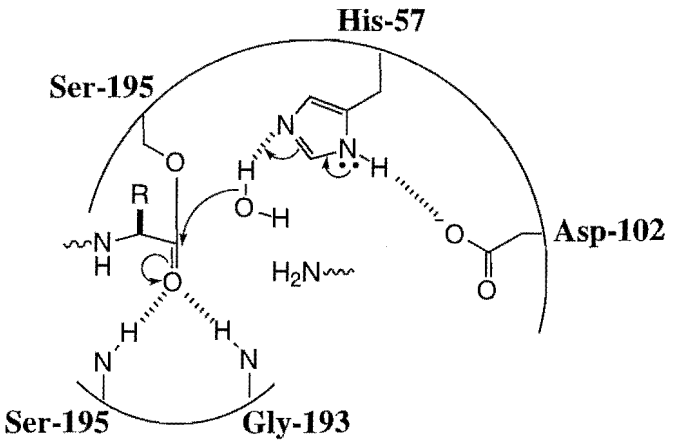


Michaelis Enzyme-substrate complex **1.1**

Tetrahedral Intermediate **1.2**

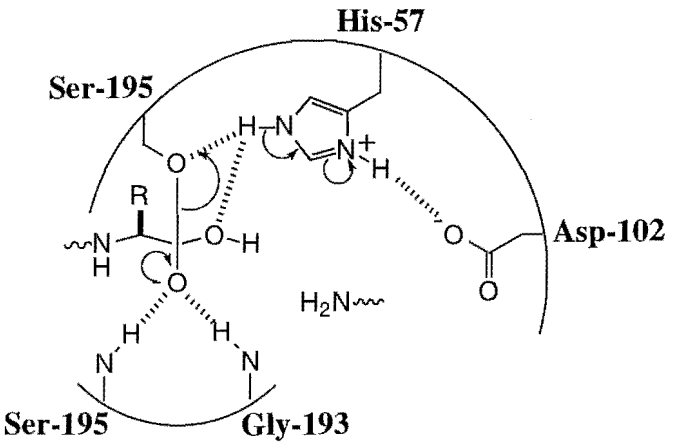
Acyl Enzyme **1.3a**

Scheme 1.2 Proposed mechanism of substrate hydrolysis by α-chymotrypsin



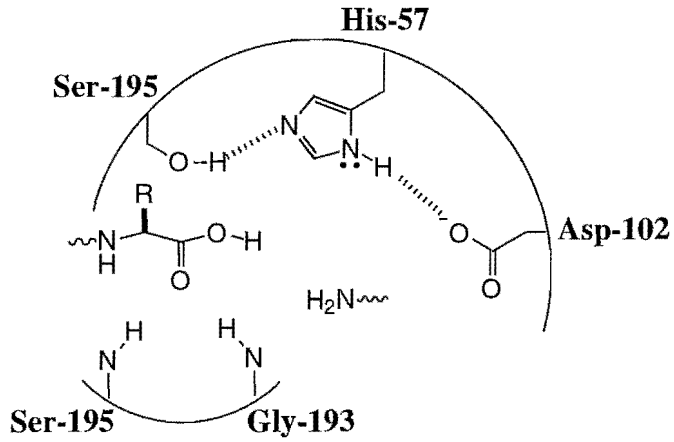
Acyl Enzyme **1.3b**

II



Tetrahedral Intermediate **1.4**

II



Products and Enzyme **1.5**

Scheme 1.2 (continued) Proposed mechanism of substrate hydrolysis by α-chymotrypsin

nitrogen atom of the eventual amine product, thereby facilitating collapse of the tetrahedral intermediate to the acyl-enzyme ester (**1.3a**) and release of the *C*-terminal peptide fragment. The acyl-enzyme **1.3b** is hydrolysed by the catalytic addition of water (Step **II**), facilitated by the charge relay system working in reverse via a second tetrahedral intermediate (**1.4**), to afford the *N*-terminal carboxylic acid fragment of the peptide, along with regenerated enzyme (**1.5**).

ENZYME-SUBSTRATE SPECIFICITY

The active site of proteases consists of two domains: a catalytic site where the covalent bond-forming and bond-breaking reactions take place, and an extended binding domain where non-covalent interactions occur between the enzyme and the amino acid residues of the substrate extending from either side of the scissile bond to be cleaved. It is these binding sites which impart specificity of action to a protease with regards to its substrate, as discussed above for α-chymotrypsin, thus enabling the diversity of the physiological function that exists within a protease class.[10]

The terminology of Schecter and Berger[11] has been widely adopted to describe the interactions between enzyme and substrate on binding. This nomenclature has been applied to identify all the relevant interactions between the amino acid side chains of a natural heptapeptide substrate and the specificity pockets of α-chymotrypsin (**Figure 1.1**). The amino acid residues of the substrate (or the inhibitor) are designated P_1, P_2, *etc*, numbering from the carbonyl end of the scissile amide bond in the (left-hand) direction of the amino terminal. The corresponding subsites of the enzyme are termed S_1, S_2, *etc*. The residues in the (right-hand) direction of the carboxy terminal from the scissile bond are designated P_1', P_2', *etc*, and the corresponding subsites S_1', S_2', *etc*.

Binding of the substrate occurs through non-covalent interactions such as hydrogen-bonds and hydrophobic forces. The most important of these interactions is that between the S_1 subsite of the enzyme and the P_1 residue of the substrate. This interaction is the primary determinant of substrate specificity, and consequently the point of cleavage. The specificities of a number of serine proteases is illustrated by their inhibition by a number of natural peptide aldehydes chymostatin (**1.6**),[12] elastatinal (**1.7**),[13] leupeptin (**1.8**).[14]

As discussed above, the size, shape and hydrophobic nature of the primary specificity pocket S_1 of chymotrypsin-like enzymes is such that these enzymes cleave peptides on the carboxyl side of aromatic residues: phenyalanine, tyrosine, tryptophan. The P_1 residue of chymostatin is phenylalanine and hence it is a potent inhibitor of α-chymotrypsin (K_i = 0.25μM).[15] Elastatinal **1.7** (P_1 is alanine) and leupeptin **1.8** (P_1 is

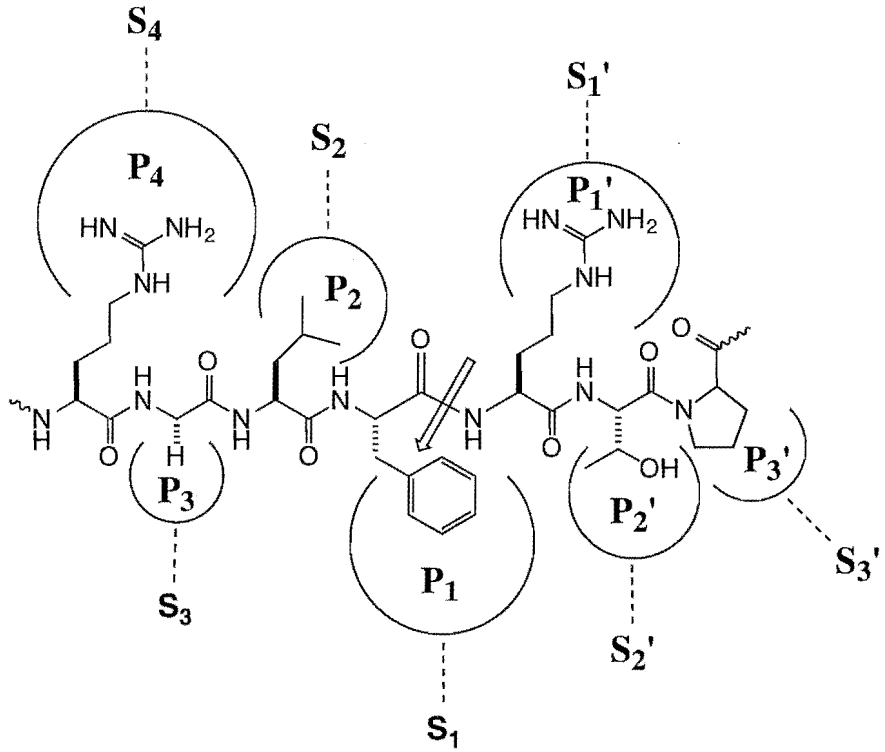


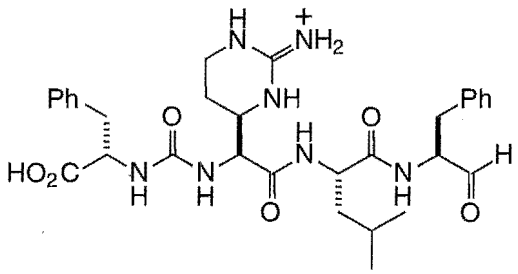
Figure 1.1 The interaction of an idealised heptapeptide substrate with a serine protease as described by Schecter and Berger terminology.

arginine) both lack a suitable bulky hydrophobic P_1 residue and are therefore poor inhibitors of α-chymotrypsin. The IC_{50} value for both leupeptin A **1.8a** and B **1.8b** is 1100μM[14] while elastatinal **1.7** does not inhibit α-chymotrypsin.[13a]

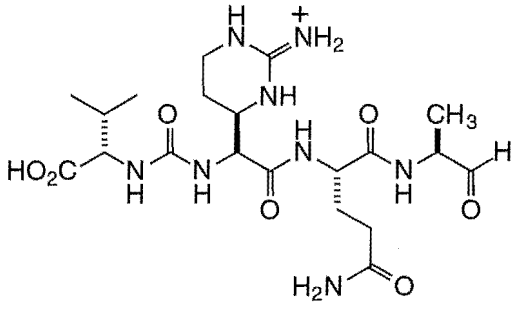
Elastases have shallow hydrophobic S_1 subsites which preferentially bind medium sized aliphatic amino acids: isoleucine, valine and alanine. These enzymes are inhibited by elastatinal **1.7** which has a P_1 alanine residue. The more potent inhibition of porcine pancreatic elastase by elastatinal **1.7** (K_i = 0.24μM)[15] is consistent with its smaller S_1 pocket[16] relative to human leukocyte elastase (K_i = 50-80μM for **1.7**)[15,17,18] which has a preference for valine at its P_1 position.[19] Since the S_1 subsite of these enzymes is quite small, enzyme substrate recognition is influenced, more so than other serine proteases, by interactions between secondary binding sites *e.g.* S_2-P_2 and S_2'-P_2' as well as the primary binding interaction S_1-P_1 hence these enzymes have relatively long extended active sites (S_5'-S_3).[20,21]

Trypsin has a negatively charged S_1 subsite due to the presence of the acidic amino acid Asp-189 in its active site and hence it prefers substrates with a complementary positive charge. Hence this enzyme is potently inhibited by leupeptin A **1.8a** (IC_{50} = 218μM)[15] and leupeptin B **1.8b** (IC_{50} = 211μM)[15] (P_1 for both is arginine). Elastatinal **1.7** and chymostatin **1.6** lack the required positively charged P_1 residue and are

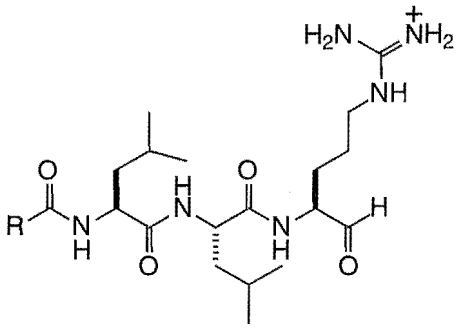
consequently poor inhibitors as exemplified by chymostatin **1.6** which does not inhibit trypsin.[12]



1.6



1.7



1.8

1.8a R = CH_3

1.8b R = $CH_3(CH_2)_2$

Figure 1.2 Natural peptide aldehyde reversible inhibitors of serine proteases

1.2 INHIBITION OF PROTEASE ENZYMES

The activity of proteases is strictly regulated by endogenous protease inhibitors.[3,22] However, a number of disease states, *e. g.* emphysema, inflammation, tumor metastasis, muscular dystrophy and hypertension, appear to be caused, to some extent, by excessive proteolytic activity brought on by abnormally low levels of the endogenous protease inhibitors.[5,23] Serine proteases, in particular, have been implicated in emphysema, adult respiratory distress syndrome, rheumatoid arthritis, pancreatitis, inflammation and digestive disorders.[5,24]

The involvement of serine proteases in a wide variety of physiological and pathological processes, coupled with their well studied mechanism of action, has made this class of enzyme an attractive target for the preparation of inhibitors. The rational design of drugs to specifically target key metabolic pathways under enzymatic control which have run amok, has been the main impetus for the development of enzyme inhibitors. These inhibition studies have also revealed information regarding substrate specificity and the catalytic mechanism of enzymes.

Active site directed competitive inhibitors are classically categorized as either reversible or irreversible.[25] There exists no definite time period for which an enzyme has to remain inactivated for the inhibitor to be classified as either reversible or irreversible. Therefore, there exists a continuum from inhibitors which form an enzyme-inactivator complex that is stable only for a short time before undergoing rapid decomposition to those inhibitors that form an enzyme-inactivator complex that is resistant to denaturation or even acid hydrolysis. A general distinction between the two exists in that reversible inhibitors closely resemble the normal substrate and act by competing with it to form a stable, non-covalent inhibitor-enzyme complex whereas irreversible inhibitors form a covalent or particularly strong inhibitor-enzyme association. Inactivation refers to an irreversible inhibition process.

Most enzyme inhibitor drugs are non-covalent, reversible inhibitors. Optimum activity of these requires maintenance of a high inhibitor concentration at the active site. Because of drug metabolism and excretion, repetitive administration of the drug is required. Inactivation has the distinct advantage over reversible inhibition in that a prolonged effect is achieved translating into smaller and fewer doses. Conversely reversible drugs are more manageable clinically in that if the enzyme is totally inactivated a potentially dangerous period of days[26] can pass before enzymic activity is regained through gene-encoded synthesis of new amounts of enzyme.

REVERSIBLE INHIBITION

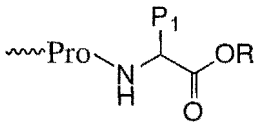
Peptides are a rational starting point in the design of inhibitors due to their high specificity and affinity for a target enzyme. A number of oligopeptides, for example **1.9**,[27] have been shown to inhibit human neutrophil elastase (HNE) by binding in the active site in such a manner that hydrolysis does not occur though the modes of action are uncertain.

$$Boc\text{-}(Val)_2\text{-}NH\text{-}(CH_2)_{11}CH_3$$

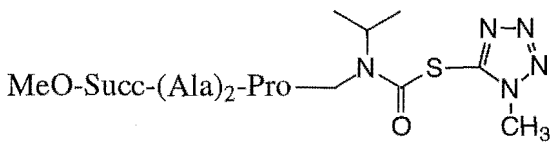
1.9 $K_i = 0.21\mu M$ / HNE[27]

Peptides, however, have poor pharmalogical profiles due to poor oral absorption and metabolic stability resulting in low *in vivo* potency. Peptidomimetics are small non-peptide ligands which retain the attractive potency and selectivity of the parent peptides whilst displaying more favourable pharmacological properties. In contrast to non-serine proteases, peptidomimetics have received little attention as inhibitors of serine proteases. This may, in part, be attributed to the fact that reactions with the active site serine provide an attractive inhibition strategy unavailable to other classes of enzyme.

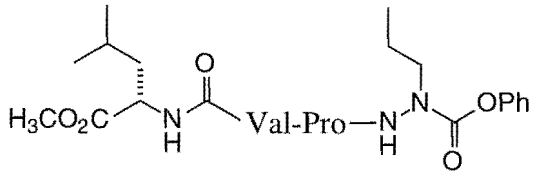
Peptidoisoteres, a class of peptidomimetic, are based on replacement of the amide bond with units that are functionally equivalent but due to the decreased peptide character are more stable. Two peptidoisoteres, carbamates (*e.g.* **1.11**)[27] and carbazates (*e.g.* **1.12**)[28] have been studied as inhibitors of human neutrophil elastase (HNE). Carbamates are obtained from a peptide ester substrate, represented by structure **1.10**, by reversal of the positions of the α-nitrogen and the α-carbon atoms (compare **1.11** with **1.10**) while replacement of the α-carbon with a nitrogen gives a carbazate (compare **1.12** with **1.10**).



1.10



1.11 $K_i = 4\mu M$ / HNE[28]



1.12 IC_{50} = 0.28μM / HNE[29]

Concurrent with the discovery of a number of natural peptide aldehydes (see **Figure 1.2**) as reversible inhibitors of serine proteases, synthetic aldehydes were reported as inhibitors of the cysteine protease papain.[30] Subsequently, peptide aldehydes have been developed as inhibitors of serine proteases. These react with Ser-195 to give a covalent tetrahedral adduct that mimics the the second tetrahedral intermediate (**1.4**) involved in serine protease catalysed peptide hydrolysis (**Scheme 1.2**). Optimization of the peptide backbones to match the target enzymes' natural substrates has resulted in very potent inhibitors as illustrated by the tetrapeptide **1.13**[31] whose carbobenzyloxy (Cbz) substituent mimics the desmosine residues in elastin the natural substrate of human leukocyte elastase.

While peptide aldehydes have been shown to be potent reversible inhibitors the aldehyde functionality is relatively unstable. An approach taken to improve stability and potency has been the replacement of the aldehydic proton with a group possessing greater electron withdrawing ability.[32] Examples of the resulting inhibitors are shown in **Table 1.2**.

Trifluoromethylketones,[33-5] such as (**1.15**),[36] have been found to be 10- to 100-fold more potent inhibitors of serine proteases than the corresponding aldehydes (compare **1.15** and **1.13**) and over 1000-fold more potent than the methyl ketones (**1.14**)[31] due to increased hydrogen-binding with the oxyanion hole leading to a more stable enzyme-inhibitor adduct.

Replacement of one of the fluorine atoms of **1.15** to give a difluoromethylketone decreases the electrophilicity of the scissile bond carbonyl and hence potency. However synthetic methodology has developed so that the hydrogen atom of a simple diflouoromethylketone can be replaced with an alkyl, keto, amide or ester group which have improved electronic properties. The replacement may also be tailored to interact with the S' subsites of the enzyme to give difluoromethylketone inhibitors which are more potent than the parent trifluoromethyl *e.g.* **1.16** is seven fold more active than **1.15**.

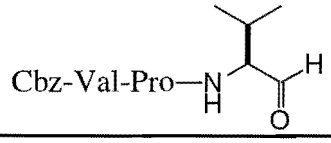
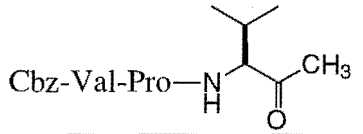
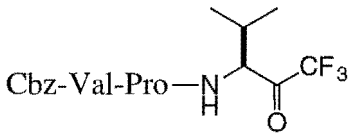
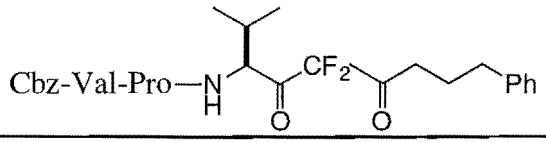
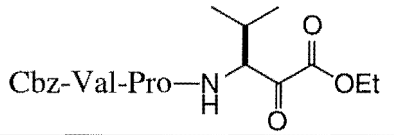
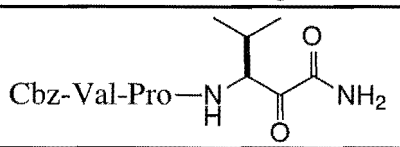
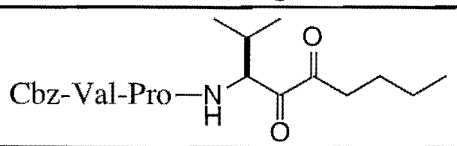
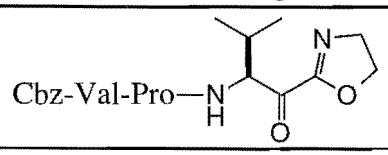
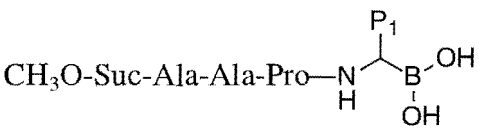
Compound number	Inhibitor	K_i (μM)/HNE
1.13		0.041[31]
1.14		8.0[31]
1.15		0.0016[36]
1.16		0.00023[37]
1.17		0.0006[38]
1.18		0.0018[37]
1.19		0.0016[37]
1.20		0.0006[31]

Table 1.2 Reversible inhibitors of human neutrophil elastase (HNE) based on peptide aldehydes

In addition to the trifluoromethyl and difluoromethyl groups discussed above, ester,[39-41] amide,[37] and keto[37,41,42] groups have been used to activate the carbonyl group in peptidyl aldehydes resulting in inhibitors **1.17-1.19** respectively. All three types are thought to operate with a mechanism which involves formation of a tetrahedral adduct with Ser-195. Peptidyl α-ketoheterocycles (*e. g.* **1.20**[31]) not only react with Ser-195 but the heterocyclic ring can also participate in hydrogen-bonding with His-57 enhancing potency.

Tailored peptides containing *C*-terminal α-amino- alkylboronic acid residues such as **1.21**[43] and **1.22**[44] have been shown to be some of the most potent reversible inhibitors

of serine proteases[45] including α-chymotrypsin (α-CT), α-lytic proteinase (ALP), thrombin and trypsin. These compounds contain a trigonal boron with a vacant 2p orbital which reacts with either Ser-195 (type 1) or His-57 (type 2). Type 1 boronic acids react with Ser-195 give a covalent tetrahedral adduct which mimics the second tetrahedral intermediate (**1.4**) involved in the hydrolysis of peptides (see **Scheme 1.2**) and are potent inhibitors. Type 2 acids however, lack the correct geometry to optimise binding with the enzyme's S_1 pocket and react with His-57 rather than Ser-195 and consequently are poor inhibitors.[43b]



1.21

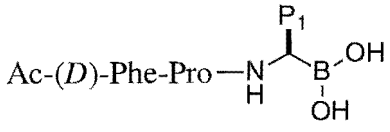
1.22a R = (*L*) $PhCH_2$

K_i = 0.54μM / ALP[43b]

K_i = 0.16nM / α-CT[43a]

1.22b R = *i*-Pr

K_i = 0.57nM / HLE[43a]



1.22

P_1 = (*L*)-Arg

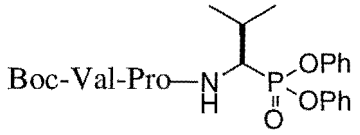
K_i = 0.04nM / Thrombin[44c]

K_i = 0.045nM / Trypsin[44c]

IRREVERSIBLE INHIBITION

A class of irreversible inhibitor includes reactive compounds referred to as affinity labeling agents.[25,46] These contain a reactive functional group, *e.g.* an α-halo ketone or isocyanate, and react directly with active site nucleophiles, generally via an S_N2 alkylation or acylation. These have been used extensively *in vitro* for mechanistic probing of enzyme active sites, however any potential clinical utility is undermined by their high and therefore indiscriminate reactivity within the biological environment, resulting in toxicity and side effects. Although clinically disadvantaged due to their high reactivity and associated poor stability, research has nonetheless been undertaken to develop these compounds as therapeutic agents by addressing these issues. In this regard, the incorporation of simple organofluorophosphonates[47] and organosulfonylfluorides[48] and isocyanates[49] into peptidomimetics has resulted in compounds with increased selectivity and stability such as **1.23**,[47d] whose inhibitory activity against human neutrophil elastase (HNE) and porcine pancreatic elastase (PPE) is shown below.

Although of lesser reactivity, esters react readily with serine proteases and consequently have undergone extensive development as substrates for kinetic assays. This provides an abundant pool of compounds suitable for the development of

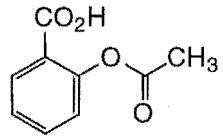


1.23

$k_{obs}/[I] = 27\ 000\ M^{-1}s^{-1}$ / HNE[47d]

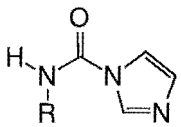
$k_{obs}/[I] = 11\ 000\ M^{-1}s^{-1}$ / PPE[47d]

therapeutic agents which can be realised by designing the esters such that they rapidly acylate the enzyme giving an inactive acyl-enzyme species which has a long lifetime if the deacylation reaction is slow. The most success has been achieved with aromatic pivaloates[50,51] however acetylsalicylic acid, aspirin, has obtained "cult" status as an inactivator of prostaglandin synthase and cure of the common headache.[52]



1.24

Stability may also be improved by masking the affinity label in a structure specific for a particular enzyme. Imidazole *N*-carboxamides (*e. g.* **1.25**)[53] have been modified by the attachment of enzyme recognition elements such as the *n*-hexyl group of **1.25a**[53b] and amino acid sequences (**1.25b**)[53c] to improve enzyme selectivity. Compounds **1.25** are examples of such "paracatalytic" inhibitors.[54] Their mechanism of inactivation involves deprotonation at nitrogen and subsequent elimination of imidazole to give an alkylisocyanate which inhibits the enzyme.



1.25

1.25a R = C_6H_{13}

1.25b R = amino acid sequence

The design of compounds that contain latent reactivity towards enzymes, as illustrated by the above example, has lead to the realisation of a second class of irreversible inhibition with important clinical applications known as mechanism-based inactivators[25] of which this thesis is concerned.

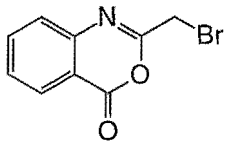
1.3 MECHANISM-BASED INACTIVATION

Mechanism-based inactivators[25] (also known as suicide inhibitors and k_{cat} inhibitors) are reasonably unreactive compounds which contain a latent reactive functionality. A mechanism-based inactivator is recognized by the target enzyme as a natural substrate. During catalysis, the latent reactive functionality in the inactivator is unmasked by the normal catalytic machinery of the target enzyme. The reactive group (generally an electrophilic site) becomes covalently bound to the enzyme (due to reaction with a nucleophilic residue in the enzyme). This renders the active site irreversibly blocked and the enzyme inactivated.

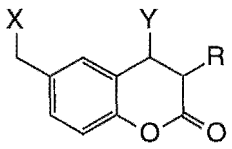
In contrast, to affinity labeling agents, mechanism-based inactivators are unreactive compounds and this is the key feature which makes them particularly amenable to the design of highly specific, low toxicity drugs. The potential for generating the reactive species exclusively within the active-site of the target enzyme imparts, in principle, a much higher degree of selectivity to mechanism-based inactivators than affinity labels. In the ideal case only the target enzyme would be capable of catalysing the appropriate conversion of the inactivator to the reactive species and only one inactivator molecule is needed per enzyme molecule for inactivation. This efficiency of inactivation is an important concept in drug design and is measured by the partition ratio.[54] In the ideal case case the partition ratio is equal to zero (no metabolites are formed per inactivation event). If the partition ratio is greater than zero the product(s) resulting from turnover may be hydrolysed to give a toxic product or react with other biomolecules to produce a toxic effect.

The design of mechanism-based inactivators for a target enzyme requires a knowledge of the enzyme mechanism and structure. As discussed earlier, the mechanism of serine protease catalyzed peptide hydrolysis has been extensively studied and consequently a large number of mechanism-based inactivators have been based on electrophilic heterocycles which are capable of being attacked by the nucleophilic active site serine hydroxyl of the serine protease. The resulting ring-opened acyl enzyme intermediate reacts further to unmask a species that ultimately inactivates the enzyme. Herein provides a brief review of heterocyclic mechanism-based inactivators of serine proteases illustrating design features that have been employed to increase target enzyme specificity and more favourable inactivator pharmacodynamics and kinetics.

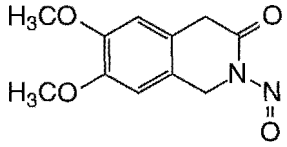
BENZOXAZINONES, COUMARINS, *N*-NITROSOAMINES



1.26



1.27



1.28

1.27a R, Y = H, X = Br

$k_{obs}/[I] = 170\ M^{-1}s^{-1}$ / α-CT[58b]

1.27b R, Y, X = Br

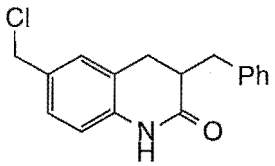
$k_{obs}/[I] = 3\ 000\ M^{-1}s^{-1}$ / α-CT[58b]

1.27c R = $PhCH_2$, Y = H, X = Cl

$k_{obs}/[I] = >20\ 000\ M^{-1}s^{-1}$ / α-CT[58b]

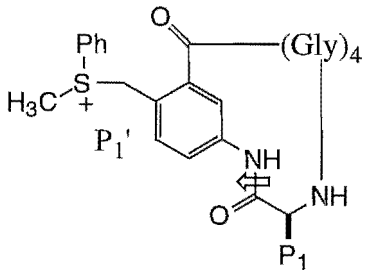
The earliest enzyme-activated inhibitors studied were halomethylbenzoxazinones (**1.26**)[56] and *N*-nitrosoamines (**1.28**)[57] and which were found to inhibit α-chymotrypsin. The first "true" mechanism-based inhibitors reported were the halomethyl dihydrocoumarins (**1.27**) which were observed to inactivate a number of serine proteases particularly α-chymotrypsin (α-CT),[58] as shown above, and human neutrophil elastase,[59] porcine pancreatic elastase,[60] and trypsin[60] but less effectively. The inactivation results from unmasking of a quinone methide which alkylates the imidazole side chain of His-57. Substitution at the 3- and 4-positions permits interaction with the S_1 subsite increasing inhibitory potency and efficiency as observed by comparison of the kinetic constants of inhibition for **1.27b** and **1.27c** against **1.27a**.

The related functionalized 3,4-dihydroquinolinone **1.29** was found to be inactive probably because the *cis*-lactam ring is not opened by the enzyme.[61] However Wakselman *et al*[62] have shown that the macrocyclic analogs represented by struture **1.30** are mechanism-based inhibitors of serine proteases since the scissile amide bond has the necessary *trans* configuration. These macrocyclic peptides contain a latent quinone imine methide species that resides between P_1-P_1' mimics of which can be tailored to impart selective action against a particular target enzyme.



1.29

inactive[61]

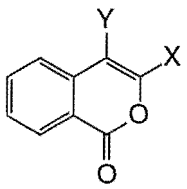


1.30

P_1 = (*L*)-Arg

$k_{obs}/[I]$ = 2 300 $M^{-1}s^{-1}$ / urokinase[62c]

ISOCOUMARINS



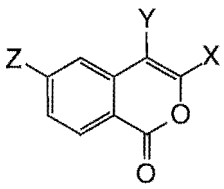
1.31

1.31a X = Cl, Y = H

$k_{obs}/[I]$ = 3 900 $M^{-1}s^{-1}$ / HNE[64]

1.31b X, Y = Cl

$k_{obs}/[I]$ = 8 900 $M^{-1}s^{-1}$ / HNE[65]



1.32

1.32a X = OCH_3, Y = H, Z= NO_2

Alternate substrate[68]

1.32b X = OCH_3, Y = Cl, Z = NH_2

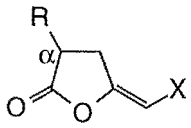
$k_{obs}/[I]$ = 10 000 $M^{-1}s^{-1}$ / HNE[68]

Isocoumarin derivatives **1.31** and **1.32** have been found to be mechanism-based inhibitors of serine proteases particularly of human neutrophil elastase (HNE).[63] Introduction of a second chlorine atom in the 4-position of the first generation inhibitor **1.31a**[64] gives **1.31b**[65] which is more potent due to increased electrophilicity of the carbonyl group. Electrospray mass spectrometry studies of the inhibition of porcine pancreatic elastase by **1.31b**[66] and proton inventories[67] suggests the mechanism of action involves an unmasked active site tethered acid chloride which, unless hydrolysed, forms a salt linkage with His-57 to inactivate enzyme.

While 3-alkoxy-isocoumarins such as **1.32a** are only alternate substrate inhibitors lacking reactivity on ring-opening,[68,69] introduction of a chlorine atom at the 4-position gave inactivators, *e. g.* **1.32b**, of human leukocyte elastase.[67,68] The mechanism of inactivation was found by X-ray analysis of a porcine pancreatic elastase-**1.32b** complex[69,70] to differ from that of the simple isocoumarins **1.31** in that an unmasked quinone imine methide irreversibly alkylates His-57. The specificity of **1.32** can be tailored by modifying the 3-alkoxy substituent such that **1.32b** and **1.32c** were found to

be more reactive towards human neutrophil elastase and α-chymotrypsin respectively.[67,68]

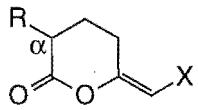
HALOENOL LACTONES



1.33

1.33a R = Ph, X = Br

$k_{inact}/[I]$ = 23 $M^{-1}s^{-1}$ / α-CT[72]



1.34

1.34a R = Ph, X = Br

$k_{inact}/[I]$ = 73 000 $M^{-1}s^{-1}$ / α-CT[72]

1.34b R = *i*-Pr, X = Br

(*E*)- $k_{inact}/[I]$ = 14 500 $M^{-1}s^{-1}$ / HNE[77]

(*Z*)- $k_{inact}/[I]$ = 37 500 $M^{-1}s^{-1}$ / HNE[77]

1.34c R = Ph, X = I

$k_{inact}/[I]$ = 130 000 $M^{-1}s^{-1}$ / α-CT[72]

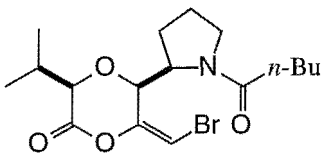
1.34d R = naphthyl, X = I

$k_{inact}/[I]$ = 292 000 $M^{-1}s^{-1}$ / α-CT[72]

Both mono α-substituted 5- and 6-membered haloenol lactones (**1.33** and **1.34**) have been found to be mechanism-based inhibitors of serine proteases.[71,72] These compounds were first proposed by Rando[73] however it was a decade later that an efficient synthetic route was developed.[74] The mechanism of action involves ring-opening followed by a tautomeric shift to reveal an α-halo ketone[75] which, as indicated by reactivation studies and molecular mechanics analysis, irreversibly alkylates the His-57 imidazole.[75,76]

The potency and efficiency of inactivation is related to the lactone ring size, geometry, halogen and substitution. Six membered haloenol lactones are more effective than five membered rings (compare **1.33a** and **1.34a**) which may result from the fact that the reactive α-halo ketone is on a longer tether and has better access to His-57. The olefin geometry has a relatively moderate effect with (*Z*)-enols being more potent than the corresponding (*E*)-enols [compare **1.34b** (*E*) and (*Z*)] as does the halogen with iodo enol lactones being more potent than bromo enol lactones (compare **1.34a** and **c**). Judicious modification of the α-substituent can impart selectivity of action. Haloenol lactones with aryl α-substitutents (**1.34a**, **c**, and **d**) target specifically α-chymotrypsin[72] while those with an α-isopropyl substituent (**1.34b**) are selective for human neutrophil elastase (HNE).[77] The most potent inhibitor in the monosubstituted series, **1.34d**, is

also very efficient with a partition ratio equal to 1.7. As a strategy for increasing specificity haloenol lactones have been incorporated into psuedo dipeptides.[78,79] The *trans* peptidyl haloenol lactone **1.35**,[79] which contains a proline-valine peptide sequence typical of elastase substrates, has been found to be an effective inactivator of α-chymotrypsin (α-CT) and human neutrophil elastase (HNE) with specificity superior to other mono substituted haloenol lactones.

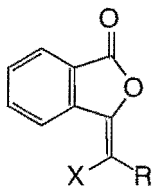


1.35

$k_{obs}/[I] = 8\ 000\ M^{-1}s^{-1}$ / α-CT[79]

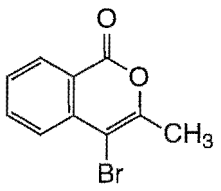
$k_{obs}/[I] = 1100\ M^{-1}s^{-1}$ / HNE[79]

The "benzo" lactones represented by structures **1.36-8** were proposed to act by unmasking a latent α-halo ketones as postulated for the halo enol lactones. The isobenzofuranone derivative **1.36** and the isocoumarin **1.37** are not effective substrates of serine proteases and hence the latent reactivity cannot be released. Although isobenzofuranones of type **1.38** are potent inhibitors, the observation of biphasic inactivation kinetics and the results of reactivation studies indicate that due to their poor stability the benzopyran-1,4-dione is responsible for inactivation via a non mechanism-based acylation process.[80]



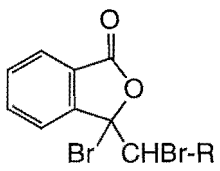
1.36

Inactive[80]



1.37

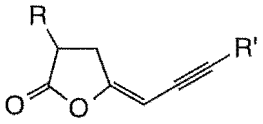
Inactive[80]



1.38

Acylating agent[80]

YNENOL LACTONES



1.39

1.39a R, R' = H

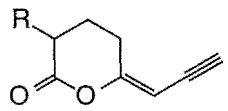
inactive[82]

1.39b R = $PhCH_2$, R' = H

$k_{inact}/[I]$ = 7 600 $M^{-1}s^{-1}$ / HNE[82]

1.39c R = $PhCH_2$, R' = CH_3

$k_{inact}/[I]$ = 2.4 $M^{-1}s^{-1}$ / HNE[65]



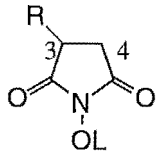
1.40

1.40a R = $PhCH_2$

$k_{inact}/[I]$ = 28 000 $M^{-1}s^{-1}$ / HNE[82]

Five and six membered ynenol lactones represented by structures **1.39** and **1.40** respectively have been found to be mechanism based inhibitors similar to haloenol lactones of serine proteases.[81,82] The chemical competence of the mechanism of action has been examined[83] and involves unmasking of a reactive allene ketone which irreversibly alkylates the enzyme although the site remains unknown. Five and six membered and (*E*) and (*Z*) ynenol lactones are similarly equipotent. Substitution at the α-position is required for inhibition (compare **1.39b** and **1.39a**) however substitution of the acetylene reduces the rate of inactivation or eliminates it completely (compare **1.39c** and **1.39b**). This is thought to be because of decreased rate of conversion to the reactive allenone and/or active site geometries restricting alkylation.

SUCCINIMIDES



1.41

1.41a R = *i*-Pr, L = SO_2CH_3

$k_{obs}/[I] = 3\ 817\ M^{-1}s^{-1}$ / HLE[87a]

1.41b R = *i*-Pr, L = SO_2*trans*-styryl

$k_{obs}/[I] = >100\ 000\ M^{-1}s^{-1}$/ HLE[87a]

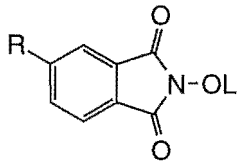
1.41c R = *i*-Bu, L = $PO(OCH_2Ph)$

$k_{obs}/[I] = 6\ 180\ M^{-1}s^{-1}$ / HLE[89]

Acylating agent[89]

The series of succinimide inhibitors represented by structure **1.41** are unique in that the reactive intermediate is generated through molecular rearrangement.[84,85] Proton NMR studies support a mechanism which proceeds with reaction of Ser-195 to give a ring-opened *N*-sulfonyloxycarboxamide which undergoes a Lossen rearrangement[86] to generate a reactive isocyanate if the *N*-substituent is a good leaving group. The reactive isocyanate then acylates His-57 to form a doubly bound inactive enzyme-inhibitor adduct. SAR study of **1.41** undertaken by Groutas *et al*[87] has shown selectivity can be obtained by modification of the C-3 substituent that is proposed to interact with the S_1 binding site of the enzyme. Introduction of a *trans* styryl group as the *N*-substituent resulted in a dramatic increase in inhibitory activity due to proposed interaction with the extended S_n' binding sites (compare **1.41a** and **1.41b**).

N-Phosphoryloxyimides are also known to undergo a Lossen rearrangement on treatment with nucleophiles[88] and hence compounds of type **1.41c** were also examined as mechanism-based inhibitors. However ^{31}P NMR studies indicated these compounds were only potent acylating agents.[89] *N*-(Sulfonyloxyphthalimides) **1.42** have also been found to inactivate serine proteases with a mechanism established by flourescence spectroscopy to involve the Lossen rearrangement.[90,91]

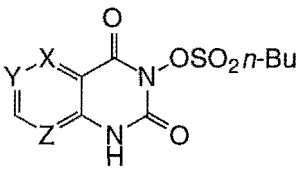


1.42

1.42a L = SO_2CH_3, R = NH_2

$k_{obs}/[I]$ = 110 000 $M^{-1}s^{-1}$ / HLE[91]

Other heterocyclic compounds, *e.g.* **1.43-46**, which contain the masked isocyanate have been investigated as potential mechanism-based inhibitors but have been found to be less potent than the succinimide based derivatives **1.41**.[92-95] Of particular note is the reversal of the trend in activity observed with the succinimide series in the dihydrouracil inhibitors **1.44**.[93] Here incorporation of a *trans*-styryl group results in less potent inhibition thought to reflect the increased steric demands of these six membered inhibitors.



1.43

1.43a X, Y, Z = C

$k_{obs}/[I]$ = 180 $M^{-1}s^{-1}$ / HLE[92]

1.43b X = N, Y, Z = C

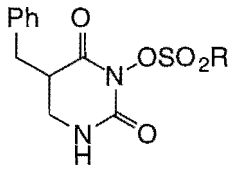
$k_{obs}/[I]$ = 64 $M^{-1}s^{-1}$ / HLE[92]

1.43c Y = N, X, Z = C

$k_{obs}/[I]$ = 58 $M^{-1}s^{-1}$ / HLE[92]

1.43d X, Z =N, Y = C

$k_{obs}/[I]$ = 20 $M^{-1}s^{-1}$ / HLE[92]



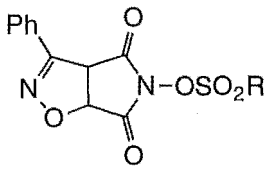
1.44

1.44a R = CH_3

$k_{obs}/[I]$ = 3310 $M^{-1}s^{-1}$ / HLE[93]

1.44b R = *trans*-styryl

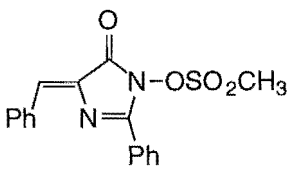
$k_{obs}/[I]$ = 480 $M^{-1}s^{-1}$ / HLE[93]



1.45

R = *trans*-styryl

Alternate substrate[94]

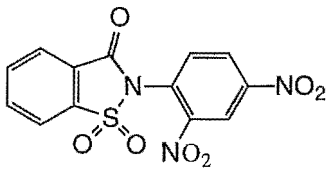


1.46

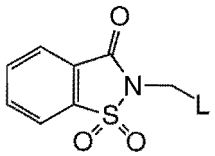
Acylating agent[95]

SACCHARIN

Mechanism-based inhibition of serine proteases by saccharin derivatives was first reported by Hlasta *et al*[96] based on the premise that incorporation of a leaving group into known acylating agents,[97] such as **1.47**, would permit further inactivation.[98] Groutas *et al*[99] arrived at the design of inhbitors of type **1.48** on consideration of a possible mechanism of inactivation based on the known chemistry of saccharin and its derivatives towards nucleophiles.

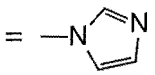


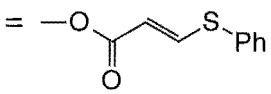
1.47



1.48

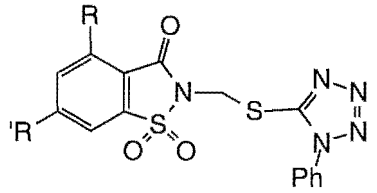
L = halogen





= (*L*)-Leu-*N*-Cbz

The potency of these compounds depends on the electron withdrawing properties of the leaving group and modification of the saccharin nucleus to act as an enzyme recognition element. Preliminary SAR studies undertaken by Hlasta *et al*[99] identified 1-phenyl mercapto tetrazole as a favourable leaving group while computer modelled "docking" studies of substituted saccharins with human leukocyte elastase predicted that substituents at the 4-position would interact with the S_1 specificity pockets of serine proteases resulting in the design of compound **1.49**.[103] Judicious choice of the 4-substituent may therefore enhance potency as was observed on incorporation of a 4-*iso*-propyl substituent (compare **1.49a** and **1.49b**). The kinetic constant K_i^* serves as a measure of inhibitory activity for the following saccharin derivatives. A low value corresponds to potent inhibition. All values quoted are for inhibition of human leukocyte elastase.



1.49

1.49a R = H

$K_i^* = 15nM$, $t_{1/2} < 5min$[107]

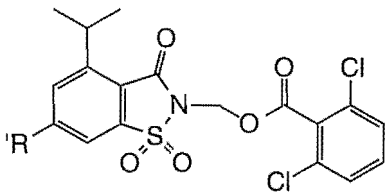
1.49b R = *i*-Pr

$K_i^* = 0.3nM$, $t_{1/2} = 45min$[107]

1.49c R = *i*-Pr, R' = OCH_3

$K_i^* = 0.27nM$, $t_{1/2} = 260min$[107]

Further SAR study included replacement of the mercapto tetrazole with aryl ethers[101] and phosphate esters,[102] a novel class of leaving group. Based on the results of Smith *et al*[103] with related compounds, aryl carboxylates were examined as leaving groups resulting in the selection of 2,6-dichlorobenzoate (as in **1.50**) based on its ability to confer superior *in vitro* activity and stability.[104]



1.50

1.50a R' = H

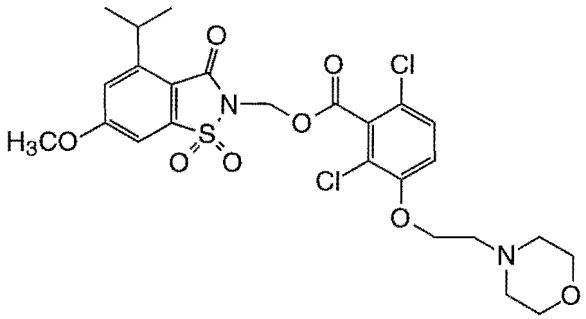
$K_i^* = 0.03nM$, $t_{1/2} = 30min$[107]

1.50b R' = OCH_3

$K_i^* = 0.023nM$, $t_{1/2} = 140min$[107]

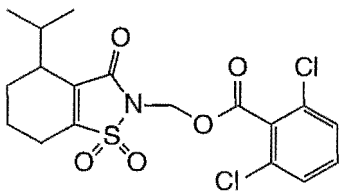
Clinical development of these saccharin derivatives has met the same hurdles experienced with other mechanism-based inhibitors of serine proteases in that *in vivo* activity by oral administration has been difficult to achieve because of poor hydrolytic and metabolic stability.[105] A probable cause of the low stability is the reactivity of compounds **1.48-50** towards esterases.[106] It has been hypothesised that these preferentially attack the carbonyl of the saccharin nucleus and hence increasing the electron density and/or the steric hindrance around this carbonyl would reduce esterase

cleavage and therefore improve stability.[107] Introduction of an alkoxy group at the 6-position (**1.49c** and **1.50b**) lead to a dramatic increase in stability, due to combined electronic and steric effects, and potency (K_i^* = 0.08nM) due to hydrogen bonding between the C-6 alkoxy group and Val-216 of human leukocyte elastase.[108] However, these compounds are very lipophilic with poor aqueous solubility and hence have poor *in vivo* activity. To improve bioavailability, compounds such as **1.51** with aqueous solubilizing substituents were prepared and though a decrease in stability was observed, **1.51** displays both potent *in vitro* and *in vivo* activity.[109]



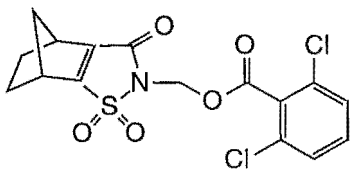
1.51 K_i^* = 0.013nM, $t_{1/2}$ = 36min[109]

Compounds with a modified saccharin skeleton have also been examined for inhibitory activity against human leukocyte elastase. A class of inhibitors based on a tetrahydrosaccharin heterocycle was shown to have different SAR than the corresponding saccharin inhibitors with increased inhibitory activity being predicted by modelling and observed with less sterically demanding C-4 substituents. Compound **1.52** was the most potent inhibitor in the series displaying similar activity to the bicyclo derivative **1.53**.[110]



1.52

K_i^* = 0.03nM[110]

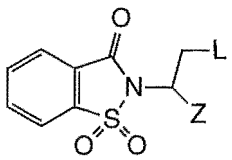


1.53

K_i^* = 0.8nM[110]

The biochemical rationale underlying the design of compound **1.54**, a potential mechanism-based inhibitor of human leukocyte cathepsin G (Cath G),[111] is based on the Gabriel-Colman rearrangement of either phthalimido- or saccharino-acetic esters or ketones.[112] Preliminary kinetic studies support the proposed mechanism which involves Ser-195 induced ring-opening of the heterocycle and a prototropic shift of the resulting imide anion to a carbanion. Subsequent elimination of the leaving group (L) gives a

reactive imine that undergoes a Michael reaction with His-57 to give doubly bound inactive enzyme inhibitor. Compounds **1.55** and **1.56** have been found to be mechanism-based inhibitors of the same ilk.[113,114]



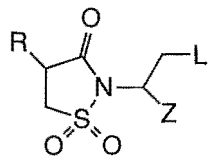
1.54

L = leaving group

Z = activating group

1.54a L = F, Z = SO_2Ph

$k_{obs}/[I] = 60\ M^{-1}s^{-1}$ / Cath G[111]



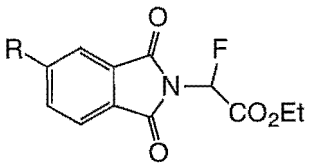
1.55

L = leaving group

Z = activating group

1.55a L = F, Z = SO_2Ph

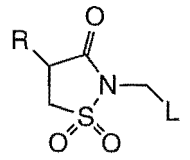
$k_{obs}/[I] = 420\ M^{-1}s^{-1}$ / HLE[113]



1.56

$k_{obs}/[I] = 100\ M^{-1}s^{-1}$ / HLE[114]

Compounds of type **1.58**, related to the saccharin example, have been found to be mechanism-based inhibitors of serine proteases.[115,116] The mechanism of action of these derivatives involves Ser-195 induced ring-opening of the heterocycle to give a reactive electrophilic species that irreversibly alkylates the enzyme. In addition to increasing the inhibitory activity by tailoring the R substituent and leaving group L to interact with the S_1 and $S_{n'}$ subsites respectively of the enzyme the design of L has considered the pathophysiology of disease states resulting from protease/anti-protease imbalance.



1.57

1.57a R = $PhCH_2$, L = ibuprofen

$k_{obs}/[I] = 45\ M^{-1}s^{-1}$ / HLE[115]

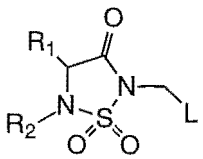
1.57b R = *i*-Pr

L = $OPO(OCH_2Ph)_2$

$k_{inact}/K_I = 95\ 000\ M^{-1}s^{-1}$ / HLE[116]

Connective tissue diseases such as emphysema are associated with elevated levels of oxidants and are inflammatory in nature.[117] Incorporation of an anti-inflammatory agent such as ibuprofen for release into the surrounding milieu during the inhibition process provides a second therapeutic effect. However obtention of this "dual function" comes at a cost in potency as seen by comparison of **1.57a**[115] and **1.57b**.[116]

Based on the knowledge of the chemistry of the compounds represented by structure **1.58**, these were proposed to be mechanism-based inhibitors of serine proteases.[118]



1.58

1.58a $R_1 = PhCH_2$, $R_2 = PhCH_2$

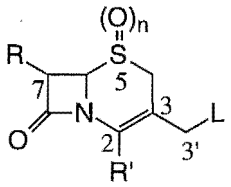
L = $OPO(OCH_2Ph)_2$

$k_{inact}/[I] = 6\ 000\ 000\ M^{-1}s^{-1}$ / HLE[116]

A noteworthy feature of the design process of these compounds was the use of available X-ray crystal structures of human leukocyte elastase and inhibitor complexes to model the binding of **1.58** in the active site. These studies suggested that the heterocyclic ring may act as a highly effective peptidomimetic suitable for appending peptidyl and nonpeptidyl recognition elements allowing the optimization of binding interactions with the S_1 subsite (through modification of R_1) and the $S_{2\text{-}n}$ subsites (modification of R_2). The choice of a good leaving group, (L = halogen, SO_2R, O_2CR, *N*-protected amino acids, phosphate esters),[116,118,119] directly increases potency but L may also be choosen such that interaction with the S_n' subsites can be achieved enhancing activity.

The optimisation of R_1, R_2 and L has lead to some of the most potent inhibitors of serine proteases for example compound **1.58a**[116] which mimics the preferred P_1 residue (valine) ($R_1 = CH(CH_3)_2$) and P_n and P_n' bulky residues of human leukocyte elastase [$R_2 = PhCH_2$, $L = OP(O)(OCH_2Ph)$]. Due to the dense functionalisation present in **1.58** this class of inhibitor could be considered to be general in nature as different serine proteases can be targeted by judicious modification of R_1, R_2 and L. The high stability and efficiency (the partition ratio of compound **1.58a** was found to be *ca* zero) of these inhibitors enhances their suitability as potential *in vivo* modifiers.

CEPHALOSPORINS

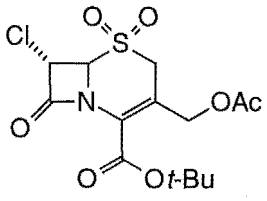


1.59

Inhibitors containing a β-lactam nucleus are probably the most intensively studied mechanism-based inhibitors of serine proteases and are unique in that they have been used clinically for disease modification. The rationale underlying their design was based on elucidation of their mode of action as antibiotics which inhibited bacterial serine proteases[120] and β-lactamases.[121] The first reported inhibitors were cephalosporins represented by structure **1.59**.[122] These form a tetrahedral intermediate on reaction with Ser-195 which collapses with loss of the C-3' leaving group to form a reactive imine tethered in the active site which unless hydrolysed, slowly but irreversibly alkylates His-57 to give one of three possible doubly bound inactive enzyme-inhibitor complexes depending on the nature of the C-7 substituent.[123-126]

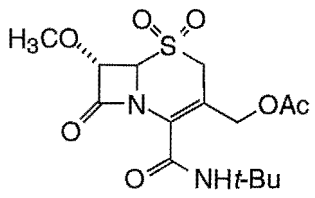
SAR study by the Merck group[124,127-130] indicated activity was optimised by modification of the substitution at the 2-, and 7-positions: the C-2 substituent is thought to allow interaction with the S_n' subsites while the C-7 substituent activates the lactam ring towards nucleophilic attack and if it has the correct orientation (α-isomer) is believed to bind in the enzyme's S_1 specificity pocket. The oxidation state of the C-5 sulfur atom is also important with sulfones being more active than sulfoxides or sulfides. Variations of the leaving group at the C-3' position did not significantly alter activity. Whereas **1.60** was found to be the most potent *in vitro* inhibitor **1.61** was more potent *in vivo* when administered intratracheally. The increased *in vivo* potency was thought to be due to the C-2 amide imparting aqueous stability and hence optimisation of both stability and potency lead to the synthesis of **1.62**. This optimum compound still

lacked systemic activity due to poor stability in blood and a remedy was sought in the investigation of simple moncyclic β-lactams.[131]



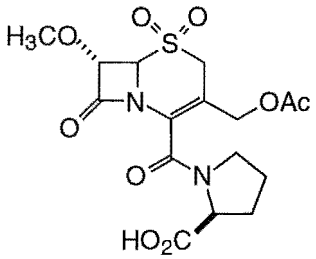
1.60

$k_{obs}/[I]$ = 161 000 $M^{-1}s^{-1}$ / HLE[128]



1.61

$k_{obs}/[I]$ = 2 200 $M^{-1}s^{-1}$ / HLE[130]

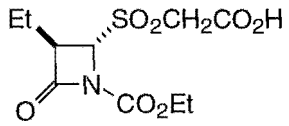


1.62

$k_{obs}/[I]$ = 3 800 $M^{-1}s^{-1}$ / HLE[130]

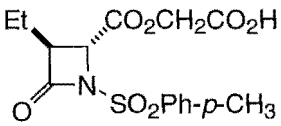
MONOCYCLIC β-LACTAMS

Two classes of azetidinones have been developed as mechanism based inhibitors of serine proteases such as elastase[132] and thrombin.[133] Human cytomegalovirus protease[134] has also been targeted for potential treatment of herpes and inhibition studies coupled with X-ray crystallography have lead to the discovery that this enzyme has a novel mechanism of hydrolysis which involves a catalytic triad of Ser-132, His-63 and His-157.[135] The first class (I) represented by structure **1.63**[136] has an electron withdrawing group on the nitrogen and leaving group at the C-4 position: in the second class (II) (**1.64**)[136] the nature of the substituents is reversed. The binding and mechanism of action were proposed[131,137] to be the same as that described above for the cephalosporin derived inhibitors excepting that class II azetidinones only form a singly bound enzyme-inhibitor adduct which is functionally irreversible.[138] The C-3 substituent occupies the S_1 subsite of the target enzyme while that of the nitrogen atom interacts with the S_n' subsites.[136]



1.63

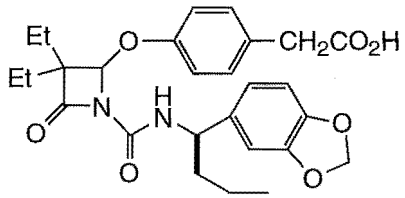
$k_{obs}/[I] = 5\ 615\ M^{-1}s^{-1}$ / HNE[136]



1.64

$k_{obs}/[I] = 5\ 615\ M^{-1}s^{-1}$ / HNE[136]

Structure-activity optimisation has lead to some of the most potent inactivators of human neutrophil elastase reported. Azetidinone **1.65**[139] is only 2.5 times less reactive than the natural human neutrophil elastase inhibitor α_1-proteinase inhibitor (k_{inact}/K_I = 2 200 000 $M^{-1}s^{-1}$)[140] and possesses improved stability relating to the *N*-carbamoyl group and the dialkyl substitution at C-3.[139,141] Azetidinones related to **1.65** are among the few compounds reported to be orally active human neutrophil elastase inhibitors.[142,143]



1.65

$k_{obs}/[I] = 867\ 000\ M^{-1}s^{-1}$ / HNE[139]

This thesis is concerned with the study of substituted succinimide and glutarimides which are potential mechanism-based inhibitors of the serine protease α-chymotrypsin. Through the employment of general synthetic routes to these compounds it is a goal to prepare a number of derivatives so that the relation between the extent and type of functionalisation and substitution and inhibitory activity can be determined on assay against α-chymotrypsin.

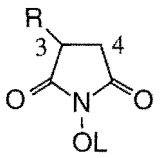
CHAPTER 2

SUBSTITUTED *N*-[(ACYL AND ALKYL ANF ARYL SULFONYL)OXY]IMIDES

2.1 SYNTHETIC STRATEGY

A cornerstone of contemporary organic synthesis is the principle of so-called retrosynthetic analysis. The application of retrosynthetic analysis involves the disconnection of larger target molecules into smaller molecules the chemist can recognise as freely available starting materials either from a commercial or synthetic source. There may exist several ways to disconnect a molecule on paper providing a "tree" of alternatives for its synthesis. The route chosen is a product of logical reasoning, based on the chemist's knowledge of reactivity and chemical reactions, and intuition.

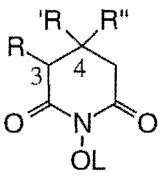
The potential mechanism-based inhibitors of serine proteases, **1.41**, **2.1**, and **2.2**, contain two common structural elements: an imide skeleton essential for enzyme recognition and a reactive moiety essential for activity. Due to this similarity it could be perceived that each inhibitor be prepared using slight modifications of one central synthetic strategy using similar pools of precursors. In order to design such a strategy retrosynthetic analysis of **1.41** was undertaken, being representative of the other target inhibitors **2.1** and **2.2**.



1.41

L = SO_2R'

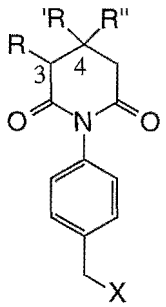
= C(O)R'



2.1

L = SO_2R'''

= C(O)R'''



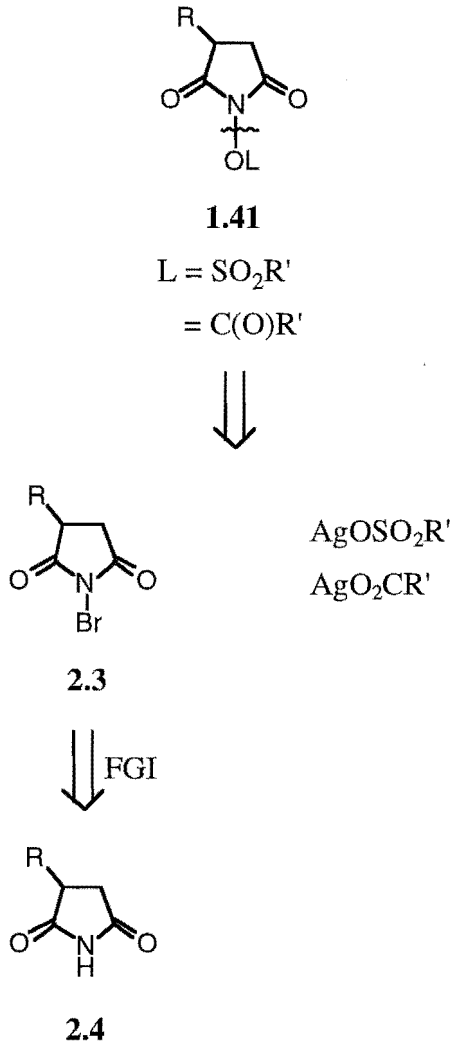
2.2

X = leaving group

The aim of the following discussion is to present briefly those disconnections that exemplify the points and features that were considered in a desire to cultivate an

attractive and aesthetic general synthetic strategy. When considering the preliminary disconnections of a target molecule a commonly employed strategy is to disect the molecule in half. This often cultivates a shorter, more convergent synthesis than that which is obtained from a retrosynthetic analysis that whittles away at the molecule's skeleton. Following this principle, the two following retrosynthetic analyses were examined to the exclusion of others.

FIRST RETROSYNTHETIC ANALYSIS OF 1.41



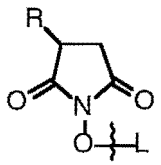
Scheme 2.1 First retrosynthetic analysis of **1.41**.

The arbitrary first disconnection that appeared favourable was to cleave the bond between the nitrogen of the succinimide ring and the oxygen adjacent to the reactive group L (as shown by the wavy line in **Scheme 2.1**). This would lead to the silver alkylsulfonate and alkylcarboxylate salts and 2-substituted-*N*-bromosuccinimides **2.3** as reagents. The retrosynthetic analysis continues with the functional group interconversion (FGI) of **2.3** to the 2-substituted succinimides **2.4**. These are readily

prepared from either the respective succinic acid or if the substituent is an alkyl group, from succinimide and the appropriate alkylhalide.

SECOND RETROSYNTHETIC ANALYSIS OF 1.41

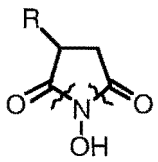
The second strategy proposed for the synthesis of the target inhibitors is depicted by the retrosynthetic analysis shown in **Scheme 2.2**. This proceeds by cleaving the bond between the oxygen and the reactive moiety L which gives the 3-substituted-*N*-hydroxysuccinimides **2.5**, and alkylsulfonic and alkylcarboxylic acid derivatives as reagents. Disconnection of **2.5** proceeds by cleavage of the imide bonds, as indicated by the wavy lines, to give hydroxylamine and 2-substituted succinic acid derivatives **2.6** and **2.7** as reagents.



1.41

L = SO_2R'

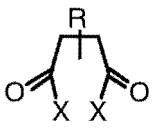
= C(O)R'

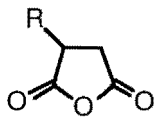


$R'SO_2X$

R'C(O)X

2.5





H_2NOH

2.6

2.6a X = Cl

2.6b X = OH

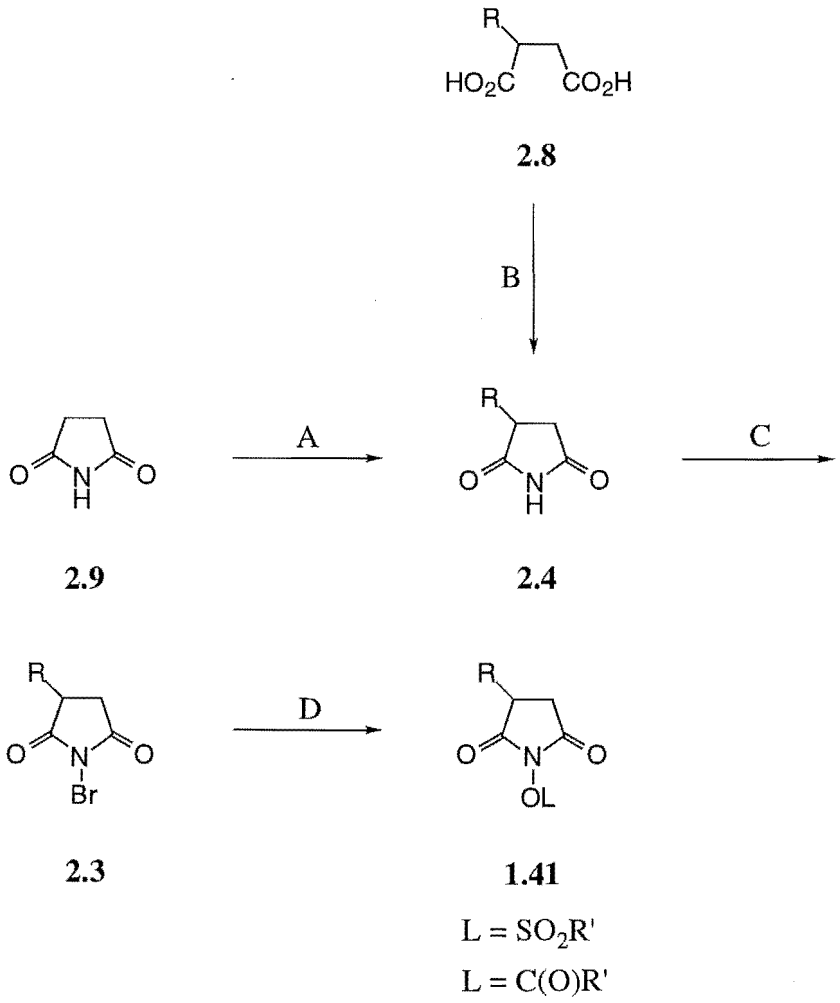
2.6c X = OR'

2.7

Scheme 2.2 Second retrosynthetic analysis of target inhibitor **1.41**

The above retrosynthetic analyses proposed two possible forward syntheses of the target inhibitor. The chemical competency of each of these syntheses was then investigated to identify the most favourable route to the target inhibitor **1.41**. Of aid in this investigation was the substantial literature describing the synthesis of succinic and phthalic acid derivatives, however there existed little documentation, of which was disjointed, as to the synthesis of substituted succinate derivatives. An aim of this thesis, therefore, was to make a systematic study of the preparation of these compounds.

FIRST FORWARD SYNTHESIS OF 1.41



Scheme 2.3 A, 1) $NaNH_2$, 1h, -78 °C 2) $PhCH_2Br$, 1h, -78 °C
B, urea, 3h *ca* 200 °C
C, HOBr or Br_2
D, $AgOSO_2R'$ or AgO_2CR', CH_3CN, rt

The key 2-substituted succinimides **2.4** (Scheme **2.3**) were prepared in two ways. In the case where the R substituent of **2.4** is alkyl, it may be obtained by $NaNH_2$ mediated alkylation of succinimide.[144] For example, 2-benzylsuccinimide **2.4** ($R=PhCH_2$) was prepared in 12% yield using this methodology which is discussed later in this chapter.

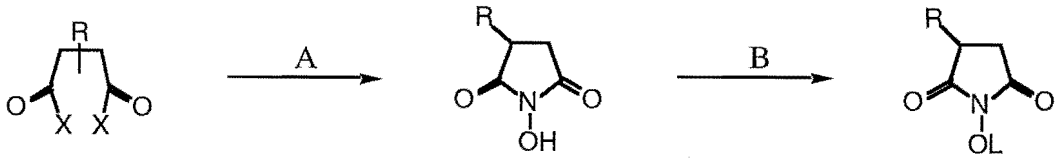
When this reaction was not applicable, as in the case of aromatic succinimides, *e.g.* **2.4** (R = Ph), the 2-substituted succinimides were otherwise obtained by heating a mixture of the corresponding succinic acid **2.8** with two equivalents of urea for 3h at *ca* 200 °C (Step B, **Scheme 2.3**).[145] Both 2-phenylsuccinimide **2.4** (R=Ph) and 2-benzylsuccinimide **2.4** (R=$PhCH_2$) were obtained in 65% yield in this manner.

The remainder of this synthetic route (Steps C - D, **Scheme 2.3**) was examined using succinimide **2.9** as a model substrate. Due to the availability of *N*-bromosuccinimide the bromination reaction of Step C (**Scheme 2.3**) was not immediately investigated, however, it was anticipated that 2-substituted *N*-bromosuccinimides **2.3** could be prepared following well established literature procedures.[146]

The reaction of silver tosylate salts with alkylhalides to give alkyltosylates has been documented[147] but to our knowledge this methodology has not been expanded to include a study of the reaction of halo-imides and silver sulfonate or acetate salts as a means of preparing compounds of type **1.41**. Initial studies using commercially available *N*-bromosuccinimide (NBS) and silver (I) tosylate (prepared from reaction between sodium tosylate and silver nitrate)[148] failed and hence the preparation of the target inhibitors **1.41** using the route displayed in **Scheme 2.1** was abandoned.

SECOND FORWARD SYNTHESIS OF 1.41

The synthesis of the target inhibitor **1.41** was carried out according to the route shown in **Scheme 2.4**.



2.6

2.6a X = Cl

2.6b X = OH

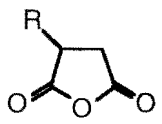
2.6c X = OR'

2.5

1.41

L = SO_2R'

L = C(O)R'



2.7

Scheme 2.4 A, H_2NOH B, $R'SO_2X$ or R'C(O)X

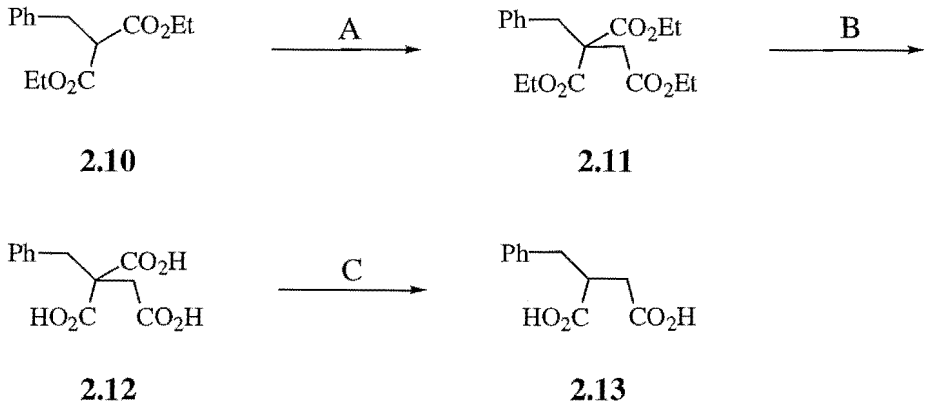
The intermediate 3-substituted *N*-hydroxysuccinimides **2.5** were prepared from 2-substituted succinic acid derivatives **2.6** and **2.7** by reaction with hydroxylamine. It is possible that on reaction of **2.6** and **2.7** with hydroxylamine (Step A, **Scheme 2.4**) mono- and di-acylation could occur at either the oxygen or nitrogen atom to give a mixture of products.[149] Jencks, however, has shown that acylation of the amino group of hydroxylamine does not usually require protection of the hydroxyl group.[150] However, as will be discussed, it proved more practical to utilise an *O*-protected form of hydroxylamine - *O*-benzyloxyamine - in many cases. The choice of whether to use hydroxylamine or *O*-benzyloxyamine was found to depend on the type of 2-substituted succinic acid derivative used. Hence reaction of **2.6** or **2.7** with either hydroxylamine or *O*-benzyloxyamine gave 3-substituted *N*-hydroxysuccinimides **2.5**. Base mediated coupling of these with alkylsulfonic and alkylcarboxylic acid derivatives (in the presence of an acylation catalyst) (Step B of **Scheme 2.4**) then gave the desired compounds **1.41**.

A detailed discussion of the preparation of the succinimide inhibitors **1.41** according to Scheme **2.4** follows, beginning with the synthesis of 2-substituted succinic acids which were prepared using three methods.

2.2 THE SYNTHESIS OF 2-SUBSTITUTED SUCCINIC ACIDS

METHOD A: MALONATE ESTER ROUTE

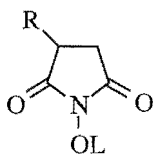
Monosubstituted succinic acids have been traditionally prepared using malonate ester condensation reactions of the type shown in **Scheme 2.5**.[151] A range of 2-substituted succinic acids can be obtained employing this methodology using different malonic esters as starting materials.



2.10 **2.11** **2.12** **2.13**

Scheme 2.5 A ethyl bromoacetate, NaOEt / EtOH, reflux 14.25h
B KOH / EtOH, reflux 4h
C 2.25h at 160-170 °C

Our initial goals were aimed at obtaining a good supply of 2-benzylsuccinic acid as the corresponding inhibitors of type **1.41** (R = $PhCH_2$, L = SO_2R') have been found to be more specific for our target enzyme α-chymotrypsin (α-CT) than those with other substitution (compare the inhibitory activities of **1.41a** and **1.41d** towards α-chymotrypsin which are shown below).[84]



1.41

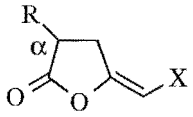
1.41a R = *i*- Pr, L = SO_2CH_3

$k_{obs}/[I]$ = 1 050 $M^{-1}s^{-1}$ / α-CT[84]

1.41d R = $PhCH_2$, L = SO_2CH_3

$k_{obs}/[I]$ = 9 000 $M^{-1}s^{-1}$ / α-CT[84]

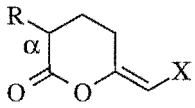
Halo enol lactones **1.33a** and **1.34a** with an α-phenyl substituent have been found to be potent inhibitors of α-chymotrypsin (α-CT),[72] hence it was proposed that inhibitors of type **1.41** (R = Ph) may similarly be potential inhibitors.



1.33

1.33a R = Ph, X = Br

$k_{inact}/[I]$ = 23 $M^{-1}s^{-1}$ / α-CT[72]



1.34

1.34a R = Ph, X = Br

$k_{inact}/[I]$ = 73 000 $M^{-1}s^{-1}$ / α-CT[72]

The commercial availability of 2-phenylsuccinic acid provided a quick entry point into the synthesis of the corresponding inhibitors **1.41** (R = Ph, L = C(O)R', SO_2R') while 2-benzylsuccinic acid was prepared by a number of routes as detailed in this section.

The initial preparation of 2-benzylsuccinic acid **2.13** was carried out as depicted in **Scheme 2.5** following the directions of Cohen and Milovanovic.[152] Diethyl benzylmalonate **2.10**# was alkylated with ethyl bromoacetate in a reaction mediated by sodium ethoxide. Despite the long reaction course (14.25h) and attempts to remove unreacted starting material during work-up, the isolated product still contained unreacted starting material: the ratio of starting material to product was found to be 4:5 by ^1H NMR analysis. This mixture was submitted to the subsequent hydrolysis (Step B, **Scheme 2.5**) to effect the synthesis of the triacid **2.12**, however, a crude solid was isolated containing a substantial amount of benzyl malonic acid impurity which was due to hydrolysis of the diethyl benzylmalonate **2.10** that was present in the starting material. Decarboxylation (Step C, **Scheme 2.5**) was carried out by heating this crude solid for 2.25h at 160-170 °C. The desired 2-benzylsuccinic acid **2.13** was then obtained in an overall yield of 17% by what was a lengthy and messy work-up procedure (see experimental section for details).

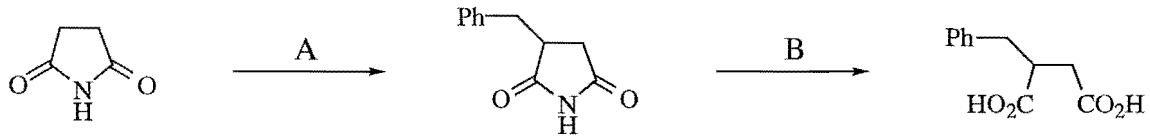
METHOD B: AMIDE BASE ALKYLATION OF SUCCINIMIDE

A more general synthesis of 2-substituted succinic acids could involve first the construction of the essential basic succinic skeleton which is then modified by incorporation of the R substituent at a late stage. Taking into account the relatively poor yielding reactions of the above synthesis and difficulties with purification it was deemed advantageous, considering our desire to obtain a good supply of 2-

We utilised commercial diethyl benzylmalonate. In our hands the literature procedure for preparing this compound[153] resulted in difficult to separate mixtures.

benzylsuccinic acid, that cleaner and more direct preparations of 2-alkylsuccinic acids be examined.

Bryant and Hauser[144] reported the potassium amide mediated benzylation of succinimide **2.9** to give 2-benzylsuccinimide **2.14** from which 2-benzylsuccinic acid **2.13** can be obtained by acid hydrolysis (**Scheme 2.6**). The preparation of **2.14** was carried out in the reported low yield of 29% due to competing reactions. A substantial amount of higher C-alkylated product and a quantity of stilbene (generated from the self-condensation of benzyl chloride - a reaction which is quite facile under these conditions) were also obtained.



2.9 **2.14** **2.13**

Scheme 2.6 A, 1) KNH_2 or $NaNH_2$, $NH_{3(l)}$ 2) $PhCH_2X$ (X = Br, Cl), $NH_{3(l)}$
B, 6N HCl, reflux 1d

There is literature precedent that the yield of the key reaction (Step A) of **Scheme 2.6** may be optimised by changing the reaction conditions.[144,154] The yield of the mono-alkylated product appears to be dependent on the reaction temperature. Bryant and Hauser isolated only higher C-alkylated products when the preparation of **2.14** was carried out at higher temperatures in refluxing Et_2O or toluene. Description of the apparatus used for the above authors' preparation of **2.14** in 29% yield indicated the reaction was carried out in refluxing ammonia (-33 °C) hence a higher yield (>29%) of monoalkylated product may be realised if lower temperatures are used *i.e.* the reaction is carried out at <-33 °C.

Wolfe and Rodgers[154b] report improved yields for the benzylation of disodio glutarimide when potassium amide (65%) is replaced with sodium amide (80%). Greater success was also observed when a higher molar equivalency of amide base and longer alkylation periods were employed. The replacement of benzyl chloride with benzyl bromide may also result in a higher yielding reaction. This advantage may be offset in that the bromide may activate self condensation, producing greater amounts of stilbene, relative to the chloride.

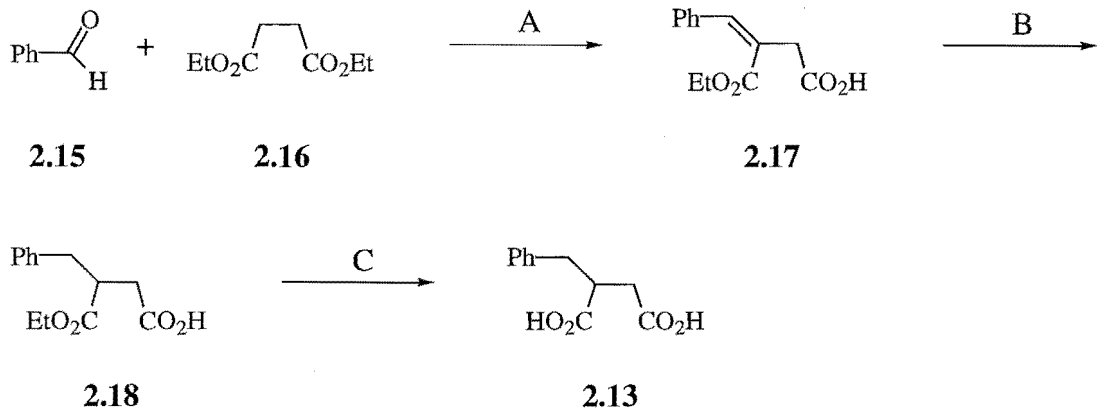
In consideration of the above the following procedure for the preparation of 2-benzylsuccinimide was used. Succinimide **2.9** was added to a solution of sodium amide[155] in liquid ammonia to give disodio succinimide which was benzylated on

addition of a solution of benzylbromide in ether to give pure 2-benzylsuccinimide **2.14** in 12% yield. Acid hydrolysis of **2.14** with 6N HCl over a day period then gave 2-benzylsuccinic acid **2.13** in quantatative yield. Although the yield of the monoalkylated product was low, the reaction provides a direct and short two step synthesis of 2-alkylsuccinic acids. This route is also versatile in that it allows the preparation of a number of different 2-alkylsuccinic acids by simply substituting the alkylating agent used.

METHOD C: STOBBE CONDENSATION ROUTE

The Stobbe condensation[156] has been employed as a general method for the preparation of 2-substituted succinic acid derivatives.[157] Groutas *et al*[87a] have reported the preparation of 2-alkylsuccinic acids by the route shown (**Scheme 2.7**) using a potassium *t*-butoxide (*t*-BuOK) mediated Stobbe condensation between the appropriate aldehyde and diethyl succinate followed by reductive hydrogenation and hydrolysis. Subsequently the preparation of 2-benzylsuccinic acid was undertaken using this route.

Sodium ethoxide was used to mediate the condensation between benzaldehyde (**2.15**) and diethyl succinate (**2.16**) (Step A, **Scheme 2.7**) to give **2.17**. Reductive hydrogenation of **2.17** gave the corresponding saturated 2-benzylsuccinic acid monoethyl ester (**2.18**) as a yellow oil (60%). Hydrolysis of this ester was attempted a number of times by treatment with ethanolic NaOH as prescribed by literature procedures[152,157d] however this was unsuccessful. Hence more vigorous reaction conditions were employed and 2-benzylsuccinic acid **2.13** was successfully obtained in 82% yield (overall yield 37%) by refluxing **2.18** in ethanolic KOH for 5h.



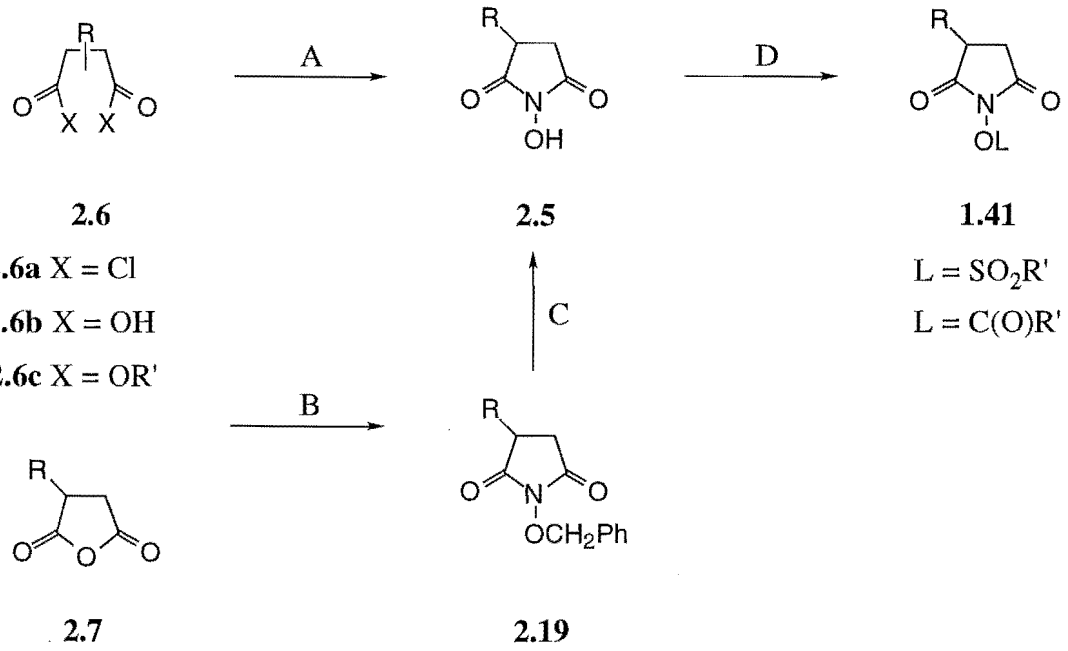
Scheme 2.7 A NaOEt / EtOH, reflux 12h
B. H_2 / 10% Pd-C / EtOAc 19h
C. KOH / EtOH, reflux 5h

SUMMARY

The preparation of 2-substituted succinic acids by **Method C** (**Scheme 2.7**) as represented by the synthesis of 2-benzylsuccinic acid **2.13** gave the best overall yield (37% over three steps). The malonate ester route **Method A** (**Scheme 2.5**) gave **2.13** in an overall yield of 16% over three steps while **2.13** was prepared in an overall yield of 12% in two steps using **Method B** (**Scheme 2.6**). On this basis, the method of choice for the preparation of 2-substituted succinic acids is **Method C**. However the short two step synthesis of **Method B** and its general nature make it more attractive excepting the low yielding alkylation reaction (Step B, **Scheme 2.6**). The chemistry and practice of this reaction was not as established as those in **Method C**, however there is literature precedent to suggest that optimisation of this reaction is possible and once undertaken **Method B** could provide the ideal route to the desired 2-alkylsuccinic acids.

2.3 THE SYNTHESIS OF 3-SUBSTITUTED-*N*-HYDROXYSUCCINIMIDES

The next step towards the preparation of the desired inhibitors **1.41** is the synthesis of the intermediate 3-substituted-*N*-hydroxysuccinimides **2.5** from the corresponding 2-substituted succinic acid derivatives **2.6** and **2.7** (**Scheme 2.8**) by reaction with either hydroxylamine (conditions A) or *O*-benzyloxyamine (conditions C). The following discussion presents an investigation of the preparations of 3-alkyl-*N*-hydroxysuccinimides from 2-alkylsuccinic acid derivatives in order of increasing utility.



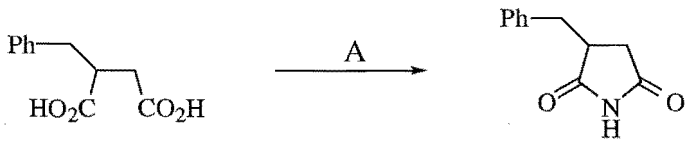
Scheme 2.8 A H_2NOH
B H_2NOCH_2Ph
C H_2, Pd catalyst
D $R'SO_2X$ or R'C(O)X

DIESTER

The use of dicarboxylic acid diesters of the type **2.6c** in reactions with *O*-benzyloxyamine favours formation of monoacylated *N*-benzyloxyhydroxamic acids rather than the desired *N*-benzyloxyimides. *N*-Hydroxyphthalimide, however, has been obtained in a one step synthesis by acidification of the salt obtained from reaction of diethyl phthalate with hydroxylamine.[158] Reaction of diethyl 2-benzylsuccinate with an ethanolic hydroxylamine solution failed, however, to give the proposed salt and attempts to obtain 3-benzyl-*N*-hydroxysuccinimide **2.5** (R = $PhCH_2$) using this methodology was abandoned.

DIACID

It is possible to prepare imides from the diacid *e.g.* **2.6b** and amine constituents under relatively harsh dehydrating conditions. For example 2-benzylsuccinimide **2.14** was prepared by heating a mixture of 2-benzylsuccinic acid **2.13** and 2 equivalents of urea for 3h at 170-200 °C (**Scheme 2.9**). However, it was thought unlikely that a desirable preparation of 3-substituted *N*-hydroxysuccinnimides **2.5** could be realised by the reaction between the appropriate 2-alkylsuccinic acid and hydroxylamine hydrochloride despite the obvious attraction of the short one step synthesis. The observation that hydroxylamines form explosive mixtures at elevated temperatures also adds caution to such a synthesis.[149]



2.13 **2.14**

Scheme 2.9 A, urea, 3h at *ca* 170-200 °C

A milder preparation of 3-alkyl-*N*-hydroxysuccinimides requires the use of activated acid derivatives such as acid chlorides, *e.g.* **2.6c**, anhydrides *e.g.* **2.7** or by activating the diacid **2.6b** *in situ*. A preparation of substituted *N*-benzyloxyglutarimides has been reported by the EDAC/HOBt mediated condensation of *O*-benzyloxyamine and the respective substituted glutaric acid.[159] This methodology was successfully applied by us to the preparation of a 4,4-dialkyl-*N*-benzyloxyglutarimide (see **Section 2.7** of this chapter) with the corresponding *N*-hydroxyglutarimide being obtained by the removal of the *O*-benzyl protecting group by catalytic hydrogenation. The preparation of 3-benzyl-*N*-benzyloxysuccinimide was attempted using this methodology but was unsuccessful.

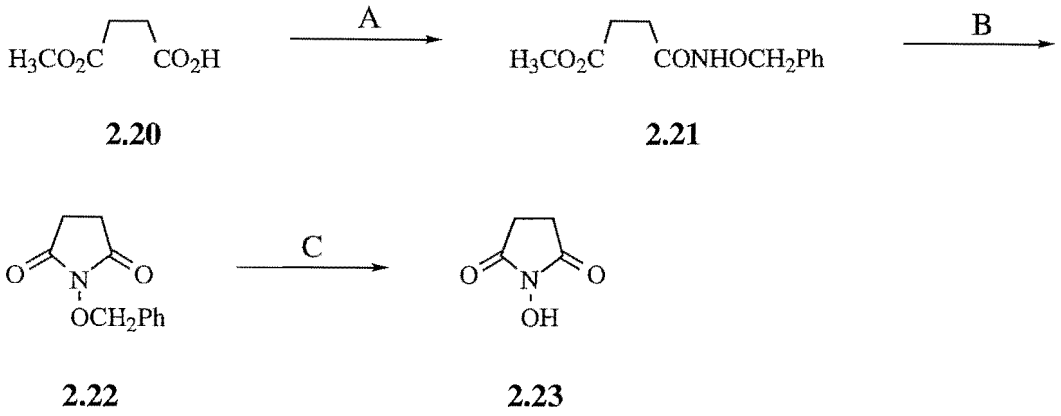
DIACID CHLORIDE

N-Hydroxyphthalimide has been prepared from reaction between the respective diacid chloride and hydroxylamine.[160] A route to 3-alkyl-*N*-hydroxysuccinimides based on this reaction first requires a reliable method for the preparation of substituted succinic diacid chlorides. In general, acid chlorides are obtained by the treatment of the parent acid with chlorinating agents for example succinyl dichloride has been obtained by reaction of succinic acid and PCl_5.[161] The preparation of 2-benzylsuccinyl chloride was attempted by heating the acid with three molar equivalents of $SOCl_2$ however the isolated white solid was identified by NMR, melting point and mass spectrometry to be

the respective anhydride. A milder preparation was employed using oxalyl chloride and DMF at room temperature but the anhydride was again obtained. Hence, in light of these perhaps not suprising results, it was not possible to design a synthesis of 3-substituted *N*-hydroxysuccinimides from diacid chlorides as their preparation favoured activated ring closure to give the corresponding anhydride.

MONO ETHYL ESTER DERIVATIVES

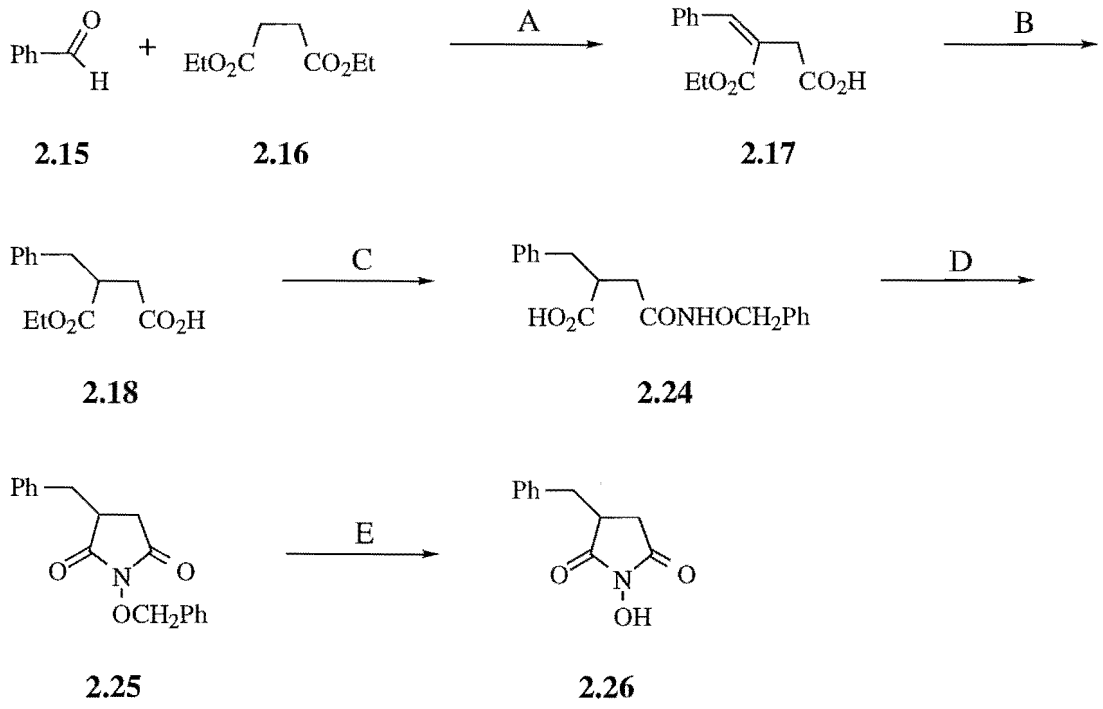
The preparation of 3-alkyl-*N*-hydroxysuccinimides from 2-alkylsuccinic acid mono ethyl ester derivatives was also investigated on the basis of study of the functionalisation of the succinimide ring undertaken previously within the University of Canterbury Department of Chemistry[162] it was observed that methyl hydrogen succinate **2.20** undergoes coupling to *O*-benzyloxyamine under standard DCC/HOBt conditions to give methyl *N*-benzyloxysuccinamate **2.21**. Treatment of **2.21** with either Et_3N or *iso*-propylamine gave *N*-benzyloxysuccinmide **2.22** which on catalytic hydrogenation gave **2.23**.



2.20 **2.21** **2.22** **2.23**

Scheme 2.10 A, $HCl.H_2NOCH_2Ph$, Et_3N, DCC, HOBt, CH_2Cl_2, overnight 0-5 °C-rt
B, Et_3N, CH_2Cl_2, rt 2h
C, H_2, 10% Pd-C, THF, 3h

Consequently possible preparations of substituted *N*-hydroxysuccinimides were investigated by applying this methodology to substituted succinic acid mono ethyl esters. Of the two esters available, ethyl 3-benzylidenesuccinate **2.17** and ethyl 3-benzylsuccinate **2.18**, obtained in the course of preparing 2-benzylsuccinic acid using Stobbe condensation chemistry (see **Scheme 2.7**) a route to 3-benzyl-*N*-hydroxysuccinimide **2.26** was investigated from **2.18**. The synthesis of 3-benzyl-*N*-hydroxysuccinimide **2.26** from **2.18** in five steps is shown below **Scheme 2.11**.



Scheme 2.11 A, NaOEt, EtOH, reflux 12h
B, H_2, 10% Pd-C, EtOAc, 19h
C, $HCl.H_2NOCH_2Ph$, Et_3N, DCC, HOBt, CH_2Cl_2, overnight 0-5 °C-rt
D, *p*-TsOH, DCE, reflux 13h
E, H_2, 10% Pd-C, THF, overnight

The mono ethyl ester **2.18** was prepared as discussed previously by reductive hydrogenation of the Stobbe condensation product **2.17** in 46% yield (Steps A and B of **Scheme 2.11**). Coupling of *O*-benzyloxyamine hydrochloride to **2.18** using standard DCC / HOBt conditions gave a 2:1 mixture, by ^1H NMR, of the ring opened *N*-benzyloxy hydroxamic acid derivative **2.24** and the ring closed *N*-benzyloxysuccinimide **2.25**.

To complete conversion to the ring closed product **2.25** a solution of a sample of the isolated mixture of **2.24** and **2.25** in CH_2Cl_2 was treated with triethylamine employing the conditions used for the cyclisation of the model compound methyl *N*-benzyloxysuccinamate **2.21** discussed above (**Scheme 2.10**). Proton NMR analysis of the isolated product indicated no further cyclisation had taken place implying that harsher conditions were required.

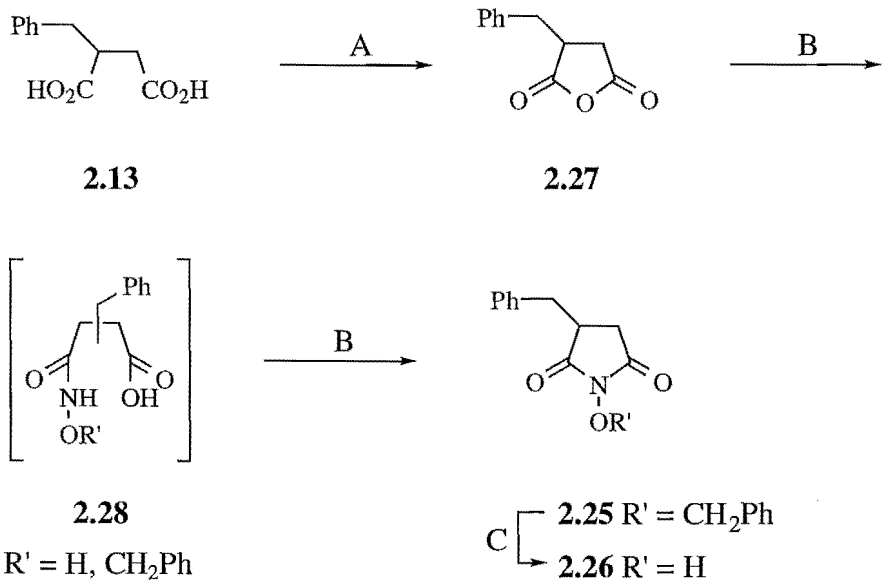
To this end a neat synthetic sample of the mixture obtained from the coupling of **2.18** and *O*-benzyloxyamine hydrochloride was heated for 2h at 160 °C. The isolated product was found by ^1H NMR to contain a 2:3 mixture of ring-opened **2.24** and ring-closed **2.25** implying that some further cyclisation had ocurred. The harsh conditions used

were deemed to be destructive considering that incomplete cyclisation took place and therefore a milder alternative was sought.

Park *et al*[163] have prepared a series of *N*-CBz-α-aminoglutarimides and succinimides from the *N*-protected glutamic and aspartic acids respectively. The ultimate step in the four step synthesis they employed involved cyclisation of methyl *N*-Cbz-α-aminoglutaramates and succinanamates by refluxing with 0.5 equivalent of *p*-TsOH in
toluene for 8h in 65-82% yields. Consequently we tried these cyclisation conditions only to find that the reaction mixture decomposed. However, on changing the solvent from toluene to 1,2-dichloroethane (DCE) and refluxing for 13h we isolated the desired ring-closed product **2.25** in 85% yield over the two steps (C and D of **Scheme 2.11**). The desired 3-benzyl-*N*-hydroxysuccinimide **2.26** was then obtained by hydrogenolysis of **2.25** in 72% yield (Step E , **Scheme 2.11**).

ANHYDRIDE

The synthesis of 3-alkyl*N*-hydroxysuccinimides was carried out via an anhydride using modifications of procedures adopted by Groutas *et al.*[85,87]



Scheme 2.12 A, Ac_2O, reflux
B, H_2NOR' (R' = H or CH_2Ph), toluene or xylene, reflux
C, H_2, 10% Pd-C, 1:1 MeOH/EtOH, EtOAc, or THF

2-Benzylsuccinic acid **2.13**, prepared as discussed earlier in this chapter, was refluxed with excess acetic anhydride to give the anhydride **2.27** in 90% yields.

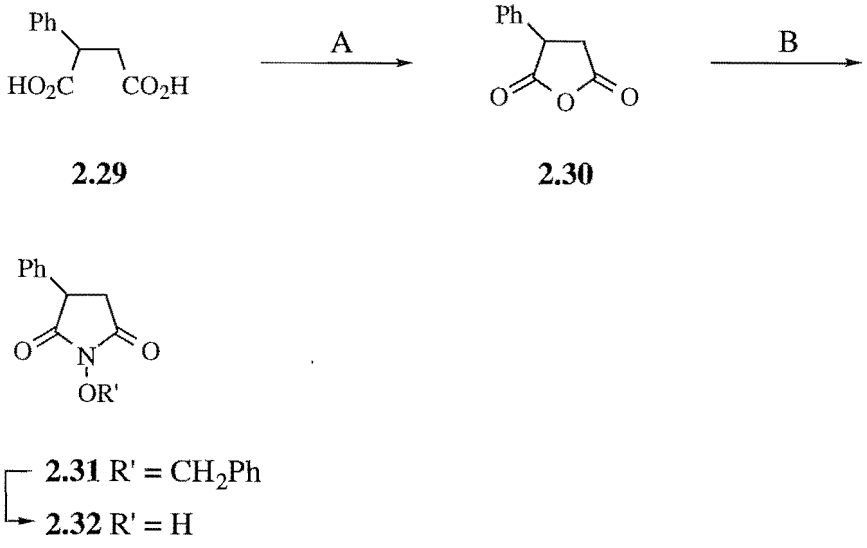
However, it was found that the reaction of 2-benzylsuccinic anhydride **2.27** with *O*-benzyloxyamine was low yielding using the conditions described by Groutas *et al.*[85]

A review of the literature preparations[149,164] of the model compounds *N*-benzyloxysuccinimide and *N*-benzyloxyphthalimide suggested that this reaction proceeds by initial attack of *O*-benzyloxyamine with concomitant ring-opening to give the intermediate which then undergoes ring closure with elimination of water to give the *N*-benzyloxyimide. Depending on the reaction conditions (reaction time, temperature) employed, either the ring opened intermediate or ring closed product can be obtained. For example, *N*-benzyloxysuccinimide has been prepared from *O*-benzyloxyamine and succinic anhydride in refluxing benzene with a reaction time of 0.5h but *N*-benzyloxysuccinamic acid was obtained employing a reaction time equal only to the time required to mix the reagents.[164]

To ensure formation of the desired *N*-hydroxysuccinimides we sought to employ longer reaction times than those used in literature preparations. Additional improvements on the existing literature procedures were sought by employing azeotropic removal of water from the reaction mixture to drive formation of the ring-closed *N*-benzyloxyimide and substituting the higher boiling xylenes (bp[165] 137-144 ^{o}C) for toluene (bp[165] 111 ^{o}C). On increasing the reaction time and employing the azeotropic removal of water during the reaction we observed an increase in yield for the preparation of 3-benzyl-*N*-benzyloxysuccinimide (78% *cf* 60%). On substituting xylenes for toulene there was no observed improvement in yield, however, the work-up of these reactions which involved evaporation of the reaction solvent proved more difficult.

The original conditions employed by Groutas[84] for the hydrogenolysis of **2.25** used a 1:1 MeOH/EtOH solvent system, desirable because of the high solubility of H_2 in both MeOH and EtOH. Considering that both are nucleophilic it could be possible that formation of ring-opened derivatives of type **2.28** could occur during the hydrogenation reaction. Hence the work-up procedure includes evaporation of the solvent and then reflux of a toluene solution of the resulting residue with azeotropic removal of water to promote formation of the ring closed product. Substitution of 1:1 MeOH/EtOH (61% yield) with the non-nucleophilic solvent THF (72%) resulted in higher yields and simplified work-up procedures.

3-Phenyl-*N*-hydroxysuccinimide was also prepared using the reactions described above (**Scheme 2.13**).



2.29 **2.30**

Scheme 2.13 A, AcCl, reflux
B, H_2NOR' (R' = H, CH_2Ph)
C, H_2, 10% Pd-C, THF

Commercially available 2-phenylsuccinic acid (**2.29**) was refluxed with an excess of acetyl chloride to give 2-phenylsuccinic anhydride (**2.30**) in 69% yield after recrystallisation from toluene/petroleum ether.[166] Addition of *O*-benzyloxyamine to a refluxing solution of **2.30** in toluene followed by reflux with azeotropic removal of water for 7 hours gave 3-phenyl-*N*-benzyloxysuccinimide (**2.31**) in 70% yield. Hydrogenolysis of **2.31** in THF with 10% Pd-C for 4.5 hours gave 3-phenyl*N*-hydroxysuccinimide (**2.32**) with 52% mass recovery after radial chromatography. Despite this purification, inspection of the ^1H NMR spectrum still showed impurities.

Similar results have been observed on the hydrogenolysis of *O*-benzyl hydroxamates similar to **2.31**.[167] Despite using different types of palladium catalyst Pd-C (5%, 10% as above, 20%), Pd black, 10% Pd-$SrCO_3$[164] and varying the reaction pressure (10-50psi) the desired products were not obtained cleanly the corresponding amide being a major impurity. The catalyst 5% Pd/$BaSO_4$ proved to be more effective in the hydrogenation of the described *O*-benzyl hydroxamates to the desired hydroxamic acids and hence prompted its use to effect a clean synthesis of **2.32**.

Hydrogenolysis at 50psi of 3-phenyl-*N*-benzyloxysuccinimide **2.31** in MeOH with 5% Pd/$BaSO_4$ for 8 hours gave 3-phenyl-*N*-hydroxysuccinimide **2.32** in 37% yield (17% overall) and good purity after chromatography. Although a greatly reduced yield was observed with this catalyst, the ^1H NMR spectrum of the crude product showed less impurities than that of the reaction using 10% Pd-C as catalyst.

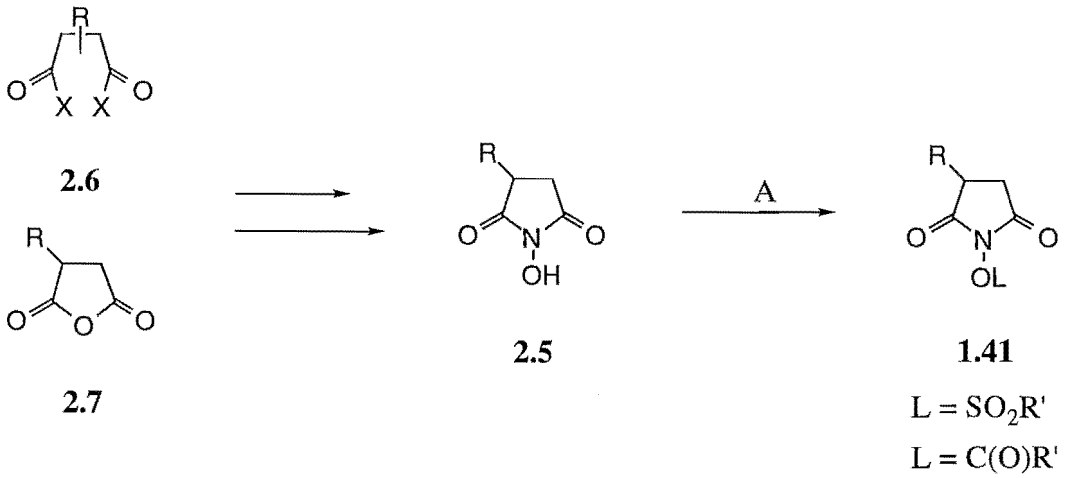
The *O*-protected form of hydroxylamine, *O*-benzyloxyamine, has been preferred in the above preparations of 2-substituted *N*-hydroxysuccinimides to reduce the risk of side reactions. Jencks[150] has shown that acylation of the amino group of hydroxylamine does not usually require protection of the hydroxyl group and hence shorter syntheses of substituted *N*-hydroxysuccinimides may be perceived from reaction of the anhydrides and hydroxylamine.

The model compounds *N*-hydroxysuccinimide and *N*-hydroxyphthalimide have been obtained by reaction of the respective anhydride with hydroxylamine.[168,169] *N*-Hydroxyphthalimide has been obtained by simply heating a mixture of hydroxylamine hydrochloride, phthalic anhydride and Na_2CO_3 in H_2O for one hour.[169] These reaction conditions have also been used by Groutas in the preparation of 3-alkylthio-*N*-hydroxysuccinimides.[87f]

Both 3-benzyl- and 3-phenyl-*N*-hydroxysuccinimide (**2.26** and **2.32**) were prepared in this manner. Hence **2.26** was prepared in 51% yield by refluxing a solution of the 2-benzylsuccinic anhydride **2.27**, hydroxylamine hydrochloride (2 equiv.) and anhydrous potassium carbonate (1 equiv.) for two hours and **2.32** by refluxing a solution of **2.30**, hydroxylamine hydrochloride and anhydrous sodium carbonate for five hours (82% yield).

2.4 THE DESIGN AND SYNTHESIS OF 3-SUBSTITUTED *N*-[(ACYL AND ALKYL AND ARYL SULFONYL) OXY]SUCCINIMIDES

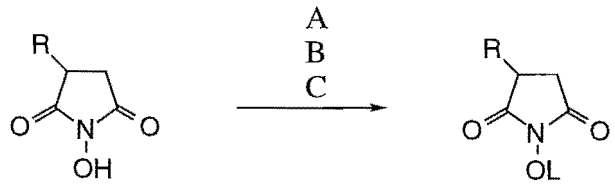
The ultimate step in the synthesis of the target inhibitors **1.41** was the coupling of alkylcarboxylic and alkylsulfonic acid derivatives to the 3-substituted *N*-hydroxysuccinimides **2.5** (**Scheme 2.14**).



Scheme 2.14 A, $R'SO_2X$ or R'C(O)X

The object of this thesis was to examine the effect the L group of **1.41** had on inhibitory activity. The design of these derivatives is discussed later but aspects included the incorporation of favourable substitution capable of harnessing the binding energy available through interaction with the S_n and S_n' subsites of α-chymotrypsin and increasing the number of equivalents of reactive species released during inhibition.

The preparation of the *N*-[(sulfonyl)oxy]succinimide derivatives **1.41** (L = SO_2R') in **Table 2.1** was undertaken according to literature procedures for *N*-hydroxyphthalimide or succinimide substrates,[85,87,91,170] which prescribe the treatment of **2.5** with base followed by the alkylcarboxylic and alkylsulfonic acid derivative. A variety of bases have been used for this reaction including aqueous sodium bicarbonate and hydroxide, and pyridine (two equivalents have been normally employed) or triethylamine if organic solvents (benzene, dichloromethane, chloroform, toluene) have been used. Different reaction times and temperatures have also been used with a continuum ranging from 20min at 0-5 °C followed by the same period at room temperature to room temperature overnight to ultimately 40 °C overnight.



2.26 R = $PhCH_2$ **1.41**

2.32 R = Ph L = SO_2R'

Conditions A Sulfonylation at 0-5 °C

B Sulfonylation at rt

C Sulfonylation at 50 °C

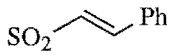
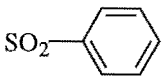
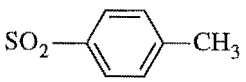
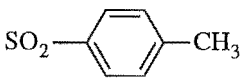
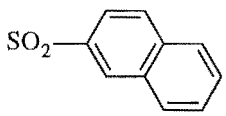
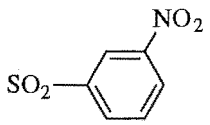
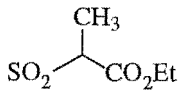
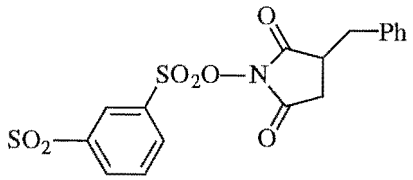
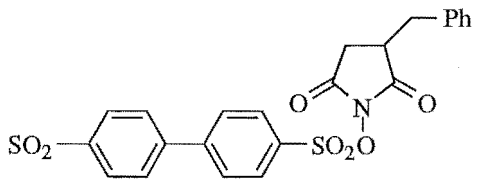
Compound number	R	L	Conditions
1.41d	$PhCH_2$	SO_2CH_3	B
e	Ph	SO_2CH_3	A
f	$PhCH_2$		A
g	$PhCH_2$		B
h	$PhCH_2$		A
i	Ph		A
j	$PhCH_2$		A
k	$PhCH_2$		A
l	$PhCH_2$		C
m	$PhCH_2$		A
n	$PhCH_2$		C

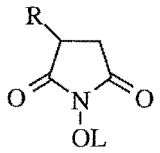
Table 2.1 *N*-[(sulfonyl)oxy]succinimide inhibitors of type **1.41**

To effect the sulfonylation of **2.5** three distinct procedures were employed. The mildest (**Method A**) involved treating an organic solution of **2.5** with either two equivalents of pyridine or a slight excess of triethylamine followed by the appropriate sulfonyl chloride for 0.5h at 0-5 °C and then allowing the reaction mixture to rise to room temperature normally overnight. A second procedure (**Method B**) was a slight modification of the first with the reaction being carried out at room temperature. Dichloromethane was generally used as the reaction solvent except when solubility problems were encountered requiring a third procedure (**Method C**) in which toluene replaced dichloromethane and the reaction was carried out at an elevated temperature of 40-50 °C.

When **2.26** was the substrate there was no noticeable difference in the nearly quantatative yields observed between reactions mediated with pyridine or triethylamine however when the latter was employed triethylamine hydrochloride deposited from the reaction mixture requiring a filtration step in the work-up procedure. However treatment of a solution of 3-phenyl-*N*-hydroxysuccinimide **2.32** in toluene with pyridine followed by methanesulfonyl chloride using the conditions of **Method B** (24 hours at room temperature) failed to give the desired compound **1.41e** (R = Ph, L = SO_2CH_3) in anything greater than *ca* 5-10% by ^1H NMR. Similar results were obtained using the same reagents and employing the conditions of **Method C** with the temperature raised to 50 °C for two hours. These results prompted a change in base. Employing **Method A**, a solution of **2.32** in dichloromethane was treated with Hunigs base (*N,N*-diisopropylethylamine) followed by methanesulfonyl chloride which gave **1.41e** in 98% yield and high purity.

The Mitsunobu reaction[172] was also investigated as a method for the esterification of compounds of this type, using *N*-hydroxysuccinimide as a model. A solution of *N*-hydroxysuccinimide, triphenylphosphine, diethylazodicarboxylate and methanesulfonic acid was stirred at room temperature for 24 hours. Although similar compounds have been prepared in this way, *N*-[(methanesulfonyl)oxy]succinimide was not obtained.[173]

It has been found that the potency of the inhibitors **1.41** can be enhanced by judicious choice of the R' substituent of the reactive moiety L. Substitution of the R' group with improved electron withdrawing capabilites relative to the simple analogues **1.41d** and **1.41e** will result in increased inhibitory activity. In addition the R' substituent may be modified to allow interaction with the secondary S_n' subsites of the serine protease improving enzyme-inhibitor recognition and hence inhibitory activity. For example Groutas *et al*[87a] observed a dramatic ten-fold increase enhancement of activity against human leukocyte elastase on replacement of R' methyl substituent (**1.41a**) with a *trans*-styryl group (**1.41b**).



1.41

1.41a R = *i*- Pr, L = SO_2CH_3

$k_{obs}/[I] = 3\ 817\ M^{-1}s^{-1}$ / HLE[87a]

1.41b R = *i*-Pr, L = SO_2*trans*styryl

$k_{obs}/[I] > 100\ 000\ M^{-1}s^{-1}$ / HLE[87a]

This result prompted the synthesis of derivatives of type **1.41** with R' aromatic substituents that may utilise the potential increased binding energy available by interaction with the S_n' subsites (for a definition see **Section 1.1**, Chapter 1). Hence benzene sulfonyl chloride was coupled to **2.26** and *p*-toluenesulfonylchloride coupled to both **2.26** and **2.32** to give inhibitors **1.41g-i** respectively. The more sterically demanding 2-napthalenesulfonyl chloride was coupled to **2.26** to give an inhibitor **1.41j** which extended further into the S_n' subsites. To study the increased electron withdrawing effects within this aromatic series 3-nitrobenzenesulfonyl chloride, obtained from the available sodium sulfonate by reaction with $PCl_5/POCl_3$,[174] was coupled to **2.26** to give **1.41k**.

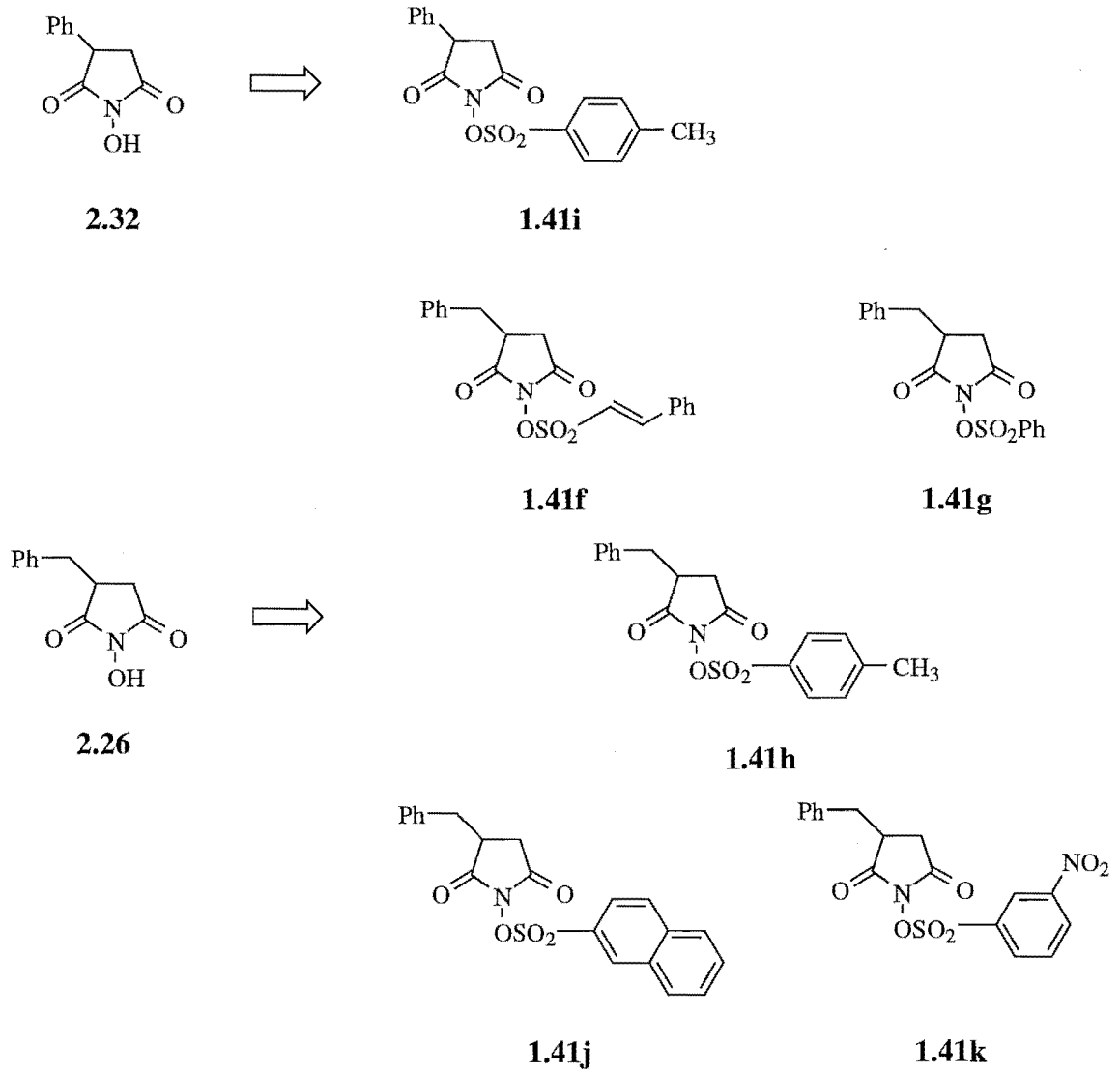
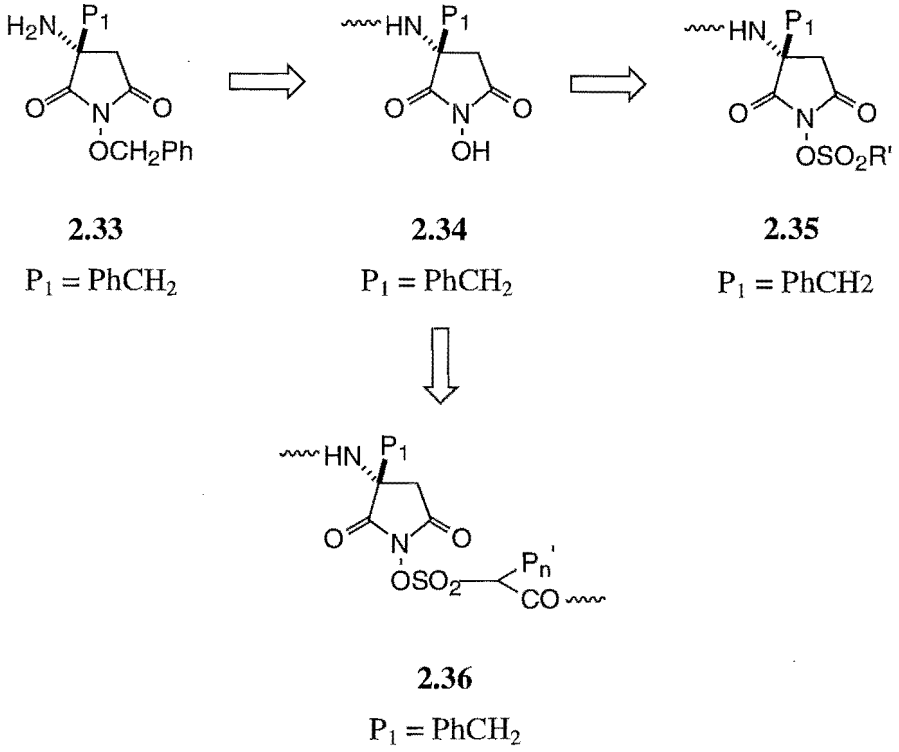


Figure 2.1 Aromatic series of succinimide inhibitors **1.41**

The stereoselective synthesis of the 3-amino-3-alkyl-*N*-benzyloxysuccinimide **2.33** permitted the preparation via the *N*-hydroxysuccinimide intermediate **2.34** of imide containing psuedo peptides **2.35**,[175] which were found to be inhibitors of serine proteases with extension in the *N* direction.[176] An aim was to expand this work completed within the University of Canterbury Department of Chemistry to the incorporation of an inhibitor of type **1.41** into a complete psuedo peptide sequence **2.36**.



2.33
$P_1 = PhCH_2$

2.34
$P_1 = PhCH_2$

2.35
$P_1 = PhCH2$

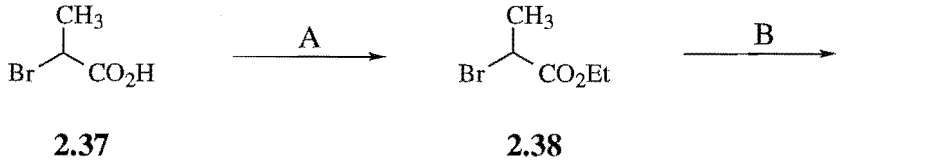
2.36
$P_1 = PhCH_2$

Figure 2.2 Synthetic Strategy for preparation of "extended" inhibitors

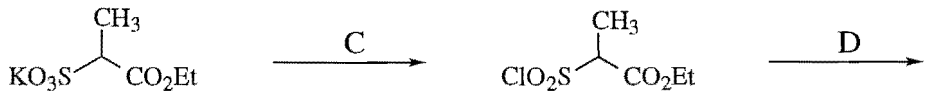
The strategy employed for the preparation of "extended" inhibitors of type **2.36** involved the synthesis of an alkylsulfonyl chloride with substitution similar to that of a natural amino acid hence containing structural features able to improve enzyme-inhibitor recognition and the functionality to permit further extension by the proposed coupling of other amino acids. Coupling of such a designed sulfonyl chloride to the peptidic *N*-hydroxysuccinimide **2.34** using the reaction conditions described above would give a peptidyl inhibitor of type **2.36**. Compound **1.411** was targeted as an initial step towards this goal.

The synthesis of the sulfonyl chloride **2.40**[176] designed as a P_n' alanine mimic and the subsequent coupling reaction to give the corresponding inhibitor **1.411** extending in the *C*-direction is shown in **Scheme 2.15**.

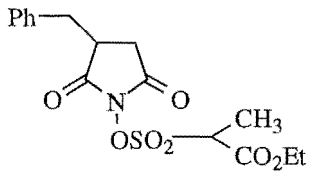
Commercially available 2-bromo-propionic acid (**2.37**), the starting point of the above scheme, was esterified with $SOCl_2$ and EtOH to give ethyl 2-bromopropionate (**2.38**) in 30% yield. The potassium sulfonate salt **2.39** was obtained from the Strecker[178] reaction between an ethanolic solution of **2.38** and aqueous K_2SO_3. Activation with PCl_5 and $POCl_3$ gave the sulfonyl chloride **2.40** in 11% yield which on pyridine mediated coupling to **2.26** gave **1.411** in 28% yield after purification by flash SiO_2 chromatography.



2.37 **2.38**



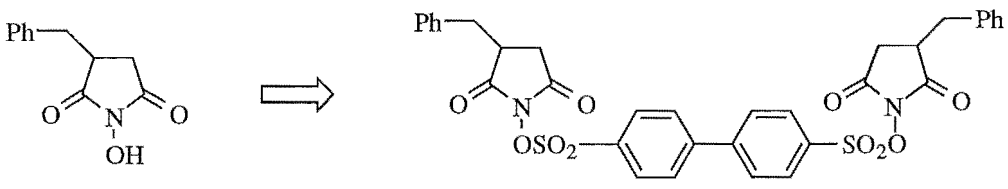
2.39 **2.40**



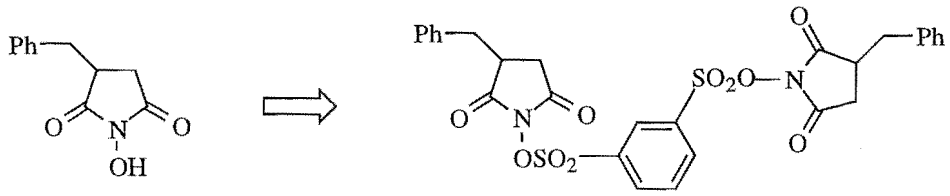
1.41l

Scheme 2.15 A, 1) $SOCl_2$, reflux 2) EtOH, 0-5 °C-rt
B, K_2SO_3, 12% v/v EtOH, reflux
C, PCl_5, $POCl_3$, overnight 70 °C
D, **2.26**, pyridine, CH_2Cl_2, 18h 50 °C

Inhibitors such as **1.41l** interact with α-chymotrypsin in the manner that one molecule of **1.41l** yields one molecule of inactivating species. Increased inactivation may be realised if on interaction with α-chymotrypsin an inhibitor yielded two equivalents of inactivating species. Of mechanistic interest, therefore, was the activity of the two so-called 'dimeric' inhibitors **1.41m** and **1.41n** prepared from the coupling of the commercially available disulfonyl chlorides and **2.26**. It is proposed that the stoichiometry of inactivation will be different from the monomeric inhibitors, *e.g.* **1.41d**, resulting in two molecules of enzyme being inhibited for one molecule of inhibitor resulting in a more rapid inactivation.



2.26 **1.41m**

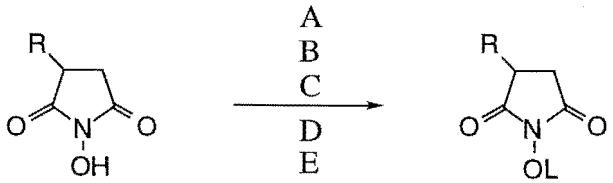


2.26 **1.41n**

The biochemical rationale underlying the design of *N*-sulfonyloxy succinimide mechanism based inhibitors discussed above was based on the knowledge that these derivatives are known to undergo spontaneous Lossen rearrangements on reaction with nucleophiles to produce aliphatic isocyanates. These are known affinity label inactivators of serine proteases reacting with His-57. The Lossen rearrangement was first observed in amide systems[88] and has also been observed in *N*-[(acyl)oxy]phthalimide derivatives[179] and hence we proposed that *N*-acyloxy imides **1.41** (R = Ph, $PhCH_2$, L = C(O)R') may be potential mechanism based inhibitors operating with the same mechanism of action as their sulfonylated counterparts **1.41** (R = Ph, $PhCH_2$, L = SO_2R').

Inhibitors of type **1.41** (R = Ph, $PhCH_2$, L = C(O)R') , see Table **2.2**, were prepared by the base mediated coupling of either acetic anhdride with *N,N*-dimethyl-4-aminopyridine (DMAP) as a reaction catalyst, or acetyl chloride to **2.26** and **2.32** to give the acetyl derivatives **1.41o** and **1.41p**. Reaction of cinnamic acid with **2.26** mediated by triethylamine and catalysed by DMAP gave the proposed "dual" inhibitor **1.41q** which in addition to inhibiting α-chymotrypsin will release cinnamic acid, a known anti-oxidant, to combat the high level of free radicals associated with the disease states resulting from unregulated α-chymotrypsin activity. To enhance inhibitory activity through interaction with the enzyme's S_n' subsites the extended inhibitor **1.41r** was prepared by DCC/HOBt mediated coupling of **2.26** to *N*-benzyloxycarbonyl-(L)-phenylalanine (N-CBz-(L)-Phe).

The syntheses of **1.41o-r** by these methods gave crude products which required further purification either by acid and base washing and crystallisation or chromatography. Hence a cleaner acylation procedure was sought. Procopiou *et al*[180] have published results describing the use of trimethylsilyl trifluoromethanesulfonate (TMSOTf) as a catalyst in what are fast and efficient acylations of a variety of alcohols. Applying this methodology initially to the synthesis of **1.41o** a faster and cleaner reaction in higher yield was observed requiring less equivalents of acylating agent and catalyst. Specifically, reaction of **2.26** with Ac_2O (1.5 equiv.) and TMSOTf (0.02 equiv.) gave **1.41o** as a white crystalline solid in 86% yield, whereas under the original conditions, reaction of **2.28** and Ac_2O (2 equiv.), Et_3N (1.1 equiv.) and DMAP (0.1



2.26 R = $PhCH_2$ **1.41**

2.32 R = Ph L = C(O)R'

Conditions A Et_3N or *i*-PrNEt and DMAP catalysed acylation
B Et_3N or *i*-PrNEt acylation
C TMSOTf acetylation
D TMSOTf acylation with *in situ* mixed anhydride
E DCC, HOBt mediated acid coupling

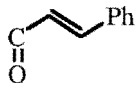
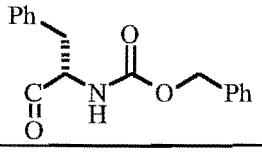
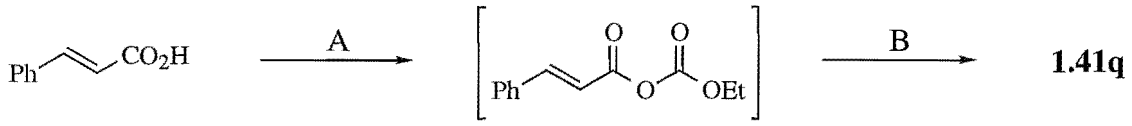
Compound number	R	L	Conditions
1.41o	$PhCH_2$	Ac	A, C
p	Ph	Ac	B
q	$PhCH_2$		A, D
r	$PhCH_2$		E

Table 2.2 Inhibitors of type **1.41** (R = Ph, $PhCH_2$, L = C(O)R')

equiv.) gave **1.41o** as a brown residue in 79% yield after purification by acid and base washing.

Procopiou's method requires the use of anhydrides as acylating agents. The synthesis of **1.41q** and **1.41r** would therefore require a supply of *trans*-cinnamic acid anhydride and *N*-Cbz-(*L*)-phe acid anhydride both of which are not commercially available and difficult to prepare. The use of mixed anhydrides generated *in situ* as acylating agents was investigated as a solution to this problem hence the synthesis of **1.41p** was attempted using this methodology (**Scheme 2.16**).



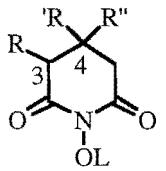
2.41

Scheme 2.16 A *N*-methyl morpholine, ClC(O)OEt, 0 °C
B **2.26**, TMSOTf, overnight, 0-5 °C-rt

Trans -cinnamic acid (2 equiv.) was treated with ethyl chloroformate (2 equiv.) and *N* -methyl morpholine (2 equiv.) at 0 °C to generate the mixed ahydride **2.41**. Reaction of this with **2.26** and TMSOTf (0.02 equiv.) gave a brown residue that was found to contain **2.41** and the desired product **1.41q** in a 1:1 ratio, by ^{1}H NMR. It was proposed a cleaner reaction may be observed if the equivalency of the mixed anhydride and therefore that of *trans*-cinnamic acid and *N*-methyl morpholine used be lowered. Subsequently the reaction was repeated with only 1.05 equivalents of acid and base and pure **1.41q** as a white solid in 86% yield.

2.7 DESIGN AND SYNTHESIS OF SUBSTITUTED *N*-[(ACETYL AND METHANESULFONYL)OXY] GLUTARIMIDES

In order to study the effect ring size has on inhibitory activity a number of previously unknown substituted *N*-[(acyl and alkylsulfonyl)oxy]glutarimides represented by structure **2.1** were prepared which are represented in **Table 2.3**.



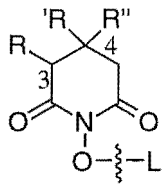
2.1

Compound number	R	R'	R"	L
2.1a	Ph	H	H	SO_2CH_3
2.1b	Ph	H	H	Ac
2.1c	$PhCH_2$	H	H	SO_2CH_3
2.1d	H	Ph	H	SO_2CH_3
2.1e	H	R', R" = cyclohexyl		SO_2CH_3
2.1f	H	CH_3	CH_3	SO_2CH_3

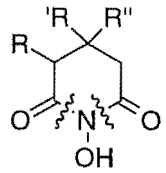
Table 4.1 Potential inhibitors of type **2.1**

The α-C substituents of the related succinimide inhibitors **1.41** are proposed to interact with the S_1 subsite of α-chymotrypsin hence the potent and specific inhibition of this enzyme by derivatives of type **1.41** with an aromatic α-C substituent as illustrated by the inhibitory activities of **1.41d** against human leukocyte elastase and α-chymotrypsin. It was proposed that by judicious choice of the extent and type of substitution present on the glutarimide ring that similar increased activity could be observed.

The retrosynthetic analysis of **2.1** (**Scheme 2.17**) proceeds in the same manner as the second retrosynthetic analysis described for **1.41** (see **Scheme 2.2, Section 2.1**). Disconnection of the bond between the oxygen and that of the reactive moiety (L) gives substituted *N*-hydroxyglutarimides **2.42** and alkylsulfonic and alkylcarboxylic acid derivatives. The preferred syntheses of 2-substituted *N*-hydroxysuccimides, the key intermediates in the synthesis of derivatives of type **1.41**, involved either the anhydride



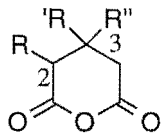
2.1



$L = SO_2R'''$

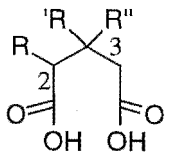
$= C(O)R'''$

2.42



2.43

FGI



2.44

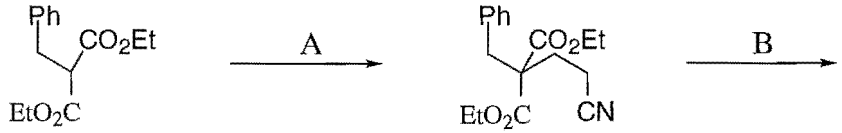
Scheme 2.17 Retrosynthetic analysis of glutarimide inhibitors **2.1**

or mono ethyl ester. The latter was prepared using the Stobbe condensation of diethyl succinate and appropriate aldehyde. Diethyl glutarate cannot be used as a substrate in this reaction[157c] and hence an efficient route to *N*-hydroxyglutarimides can only be realised through the anhydride. Hence disconnection of **2.42** gives the the glutaric acid anhydride **2.43** and hydroxylamine. The anhydride **2.43** is derived from the corresponding glutaric acid **2.44**.

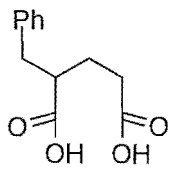
The substitution present on the glutarimide ring is directly obtained from that present in the glutaric acid starting materials. The prepartion of potent inhibitors therefore required a supply of aromatic glutaric acids. The aromatic 2- and 3-phenylglutaric acids

are commercially available and hence provided a quick entry point into the synthesis of desired inhibitors **2.1** (R, R', R" is aromatic). Of particular interest was the inhibitory activity of **2.1c** (R = $PhCH_2$, R', R" = H) which possesses the α-benzyl substitutent found to impart higher inhibitory activity towards α-chymotrypsin in the succinimide series (compare **1.41a** and **1.41d** above).

The required synthesis of 2-benzyl-glutaric acid **2.44b** was carried out employing Michael reaction chemistry[181] following literature procedures for the preparation of 2-alkyl glutaric acids.[182]



2.10 **2.45**



2.44b

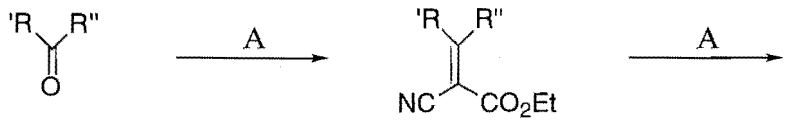
Scheme 2.17 A, acrylonitrile, 30% KOH, MeOH, *t*-BuOH, 4.5h, rt
B, 68% HBr, 1d, reflux

A Michael reaction between diethyl benzylmalonate **2.10** and acrylonitrile gave the malonate eser **2.45** in 50% yield after distillation at reduced pressure. Acid hydrolysis of **2.47** then followed to give 2-benzylglutaric acid **2.44b** in 72% yield.

As part of research being undertaken into anti-epilepsy agents within the University of Canterbury Chemistry Department methodology for the preparation 3,3-disubstituted glutaric acids **2.44** was being developed. To extend the scope of the SAR study of the glutarimide inhibitor series 4,4-disubstituted inhibitors **2.1** (R = H, R', R" = alkyl) were prepared from the corresponding 3,3-disubstituted glutaric acids.

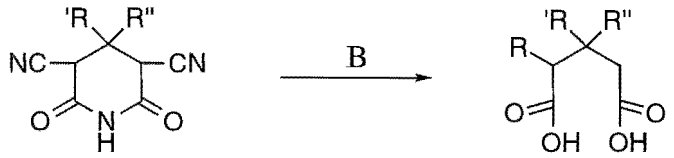
Of the synthetic methods available for the synthesis of 3,3 disubstituted glutaric acid derivatives the Guareschi route (**Scheme 2.18**) is the most favoured where applicable. The formation of the key "Guareschi" imide intermediate **2.48** of **Scheme 2.18** is believed to occur in two steps at least. The starting ketone represented by structure **2.46** undergoes a Knoevenagel condensation with ethyl 2-cyanoacetate, which was used in our synthesis, catalysed by the liquid ammonia solvent to give the α-β unsaturated

compound **2.47**. This undergoes a Michael reaction with 2-cyanoacetamide resulting from solvolysis of ethyl cyanoacetate and concomitant cyclisation to give the Guareschi imide 4,4-dialkyl-3,5-dicyanoglutarimide **2.48**. Simple acid hydrolysis of **2.48** gives the desired 3,3-disubstituted glutaric acids.



2.46 **2.47**

2.46a R', R" = CH_3



2.48 **2.44**

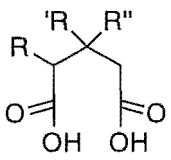
2.48a R', R" = CH_3 **2.44e** R = H, R', R" = CH_3

Scheme 2.18 Guareschi route to 3,3-disubstituted glutaric acids

The Guareschi route has been applied to a number of substrates however the key Guareschi imide intermediate has not been observed in a number of examples.[185] This occurs when the starting materials do not suit the chemistry of the Knoevenagel condensation or if suitable give derivatives of type **2.47** which are unsuitable for the following Michael reaction and ring closure. Hence the scope of the reaction is limited to ketones which are not sterically hindered and less certainly those that have an enolizable α-proton.[186] Acetone fits this criteria and, therefore, it was proposed to use this Guareschi imide methodology in the preparation of 3,3-dimethyl glutaric acid **2.44e**. Cyclohexylglutaric acid **2.44d** was also prepared in this manner and the corresponding Guareschi imide was a generous gift from Dr. Rob McKeown.

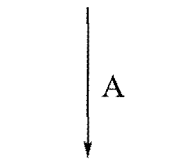
Subsequently the synthesis of 3,3-dimethyl glutaric acid **2.44e** was carried out using the Guareschi route. The Guareschi imide **2.48a** was prepared in 42% by acidification of the ammonium salt that was deposited on storing a solution of ethylcyanoacetate and acetone **2.46a** in ethanol saturated with ammonia for three days at *ca* -5 °C. Subsequent sulfuric acid hydrolysis of **2.48a** gave the desired 3,3-dimethylglutaric acid **2.44e**.

The preparation of the desired substituted *N*-[(alkylsulfonyl)oxy]glutarimide inhibitors **2.2** from glutaric acid precursors was then carried out according to **Scheme 2.19**

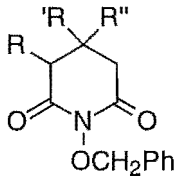


2.44

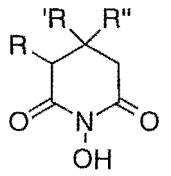
2.44a R = Ph, R', R"= H
2.44b R = $PhCH_2$, R', R" = H
2.44c R,R" = H, R' = Ph,
2.44d R=H, R', R" = cyclohexyl
2.44e R= H, R', R" = CH_3



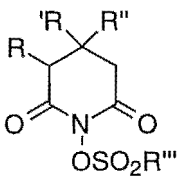
2.43a-e



2.49a-d



2.42a-e



2.1a-e

Scheme 2.19 A Ac_2O, reflux
B H_2NOCH_2Ph, toluene, xylenes, reflux
C H_2, 10% Pd-C, THF
D base, R'"SO_2Cl, R'"C(O)Cl

The variously substituted glutaric acids whether obtained from a commercial source or synthesised as described above were treated with either acetyl chloride (in the case of **2.44a**) or acetic anhydride (for **2.44b-e**) under refluxing conditions to give the respective anhydrides in high yields ranging from 84% for the preparation of 2-phenylglutaric anhydride **2.43a** to 91% for cylcohexylglutaric anhydride **2.43d**.

The anhydrides **2.43** were then refluxed with *O*-benzyloxyamine to give the *N*-protected glutarimides **2.49** in typically 80% yield. Hydrogenolysis of these gave the *N*-hydroxyglutarimides **2.42** in yields of 70% which were then treated with the appropriate alkyl sulfonylchloride or acid chloride to give the desired inhibitors. As observed with the succinimide series of compounds this reaction proceeded in nearly quantatative yield.

Shorter routes to the desired inhibitors were realised by the reaction of hydroxylamine directly with the anhydride **2.43**. Both 4,4-dimethyl-*N*-hydroxyglutarimide **2.42e** and 3-phenyl-*N*-hydroxyglutarimide were prepared in 50% yield in this manner. A short route to **2.42e** was also investigated by coupling *O*-benzyloxyamine directly to the acid **2.44e** using EDAC however the low yield of this reaction (12%) indicated this route was of poor preparative value considering the high yields of the reactions of **Scheme 2.19**.

2.6 SUMMARY

A brief retrosynthetic analysis of the target inhibitor **1.41** suggested two general syntheses. The chemical competency of these was examined and a route involving 3-substituted N-hydroxysuccinimides as key intermediates was identified to be the most desirable to the demise of a route proceeding via 2-substituted succinimides. This route involved the reaction of succinimides with silver carboxylate and sulfonate salts as a means of preparing inhibitors of type **1.41** but this reaction was unsuccessful.

The synthesis of 3-substituted *N*-hydroxysuccinimides **2.8** was carried out by reaction of activated 2-substituted succinic acid derivatives and either hydroxylamine or a protected form of this *O*-benzyloxyamine. The synthesis of 3-benzyl-*N*-hydroxysuccinimide was a key goal as inhibitors derived from this display increased specificity for α-chymotrypsin. Consequently some effort was directed towards investigating routes to this key intermediate from activated 2-benzylsuccinates requiring a good synthetic supply of 2-benzylsuccinic acid. Three methods for the preparation of the representative 2-benzylsuccinc acid were therefore compared. A route employing the Stobbe condensation between benzaldehyde and diethylsuccinate gave the highest overall yield and was preferred for the ease of its well established reactions. However a short (two step) and general synthesis which involved the sodium amide mediated benzylation of succinimide as a key reaction may provide the ideal route to 2-alkylsuccinic acids with further optimisation.

The failed preparation of 3-benzyl-*N*-hydroxysuccinimide from the reaction of 2-benzylsuccinic acid activated *in situ* by treatment with the coupling reagent EDAC with *O*-benzyloxyamine prompted the use of more activated derivatives. Of those derivatives successful syntheses of 3-benzyl-*N*-hydroxysuccinimide were carried oput from the anhydride and 2-benzylsuccinic acid mono ethyl ester in six and five steps respectively with *O*-benzyloxyamine. The reaction of hydroxylamine and the anhydride proved to be a successful preparation of 3-benzyl-*N*-hydroxysuccinimide in five steps whereas the use of hydroxylamine in reaction with the mono ethyl ester failed.Of the two successful five step syntheses a greater overall yield was observed with that route involving the mono ethyl ester (30%) than that involving the anhydride (18%) and in conclusion this synthesis was found to be the preferred preparation of 3-benzyl-*N*-hydroxysuccinimide.

To complete the synthesis of the target inhibitors **1.41** (R = Ph, $PhCH_2$, L = SO_2R') various sulfonyl chlorides were coupled to the 3-substituted *N*-hydroxysuccinimides **2.28** and **2.34** using modifications of literature procedures. The nature of the sulfonyl chloride used affects the potency of the resulting inhibitors and hence is an important design element. Literature SAR results indicated that bulky sulfonyl chlorides with

good electron withdrawing properties would furnish the most potent inhibitors in this series and subsequently an aromatic series was studied. To add to a conceived strategy of enhancing inhibitory activity by incorporating the inhibitors of type **1.41** into a complete psuedo peptide sequence a tailored sulfonyl chloride was synthesised permitting the preparation of an inhibitor extending in the *C*-direction. Two examples of so-called "dimeric" inhibitors were prepared to study the effects of stoichiometry on inhibition.

N-Acyloxyimides are also known to undergo a Lossen rearrangement when treated with nucleophiles hence these were proposed to be potential mechanism-based inhibitors of the same ilk as *N*-sulfonyloxyimides.[88] The acylation of **2.28** and **2.34** using Hunigs base or triethylamine with DMAP as a catalyst gave crude products. An improved procedure employed the use of TMSOTf as a catalyst for acetylations which was expanded to include acylations using mixed anhydride methodology. Acyl chlorides and anhydrides were chosen so that a number of the the resulting inhibitors possessed the same substitution as the related *N*-sulfonyloxyimides to provide a direct comparison in activity. An extended inhibitor of type **1.41** (R = $PhCH_2$, L = C(O)R') was prepared by the coupling of *N*-protected phenylalanine to **2.28** .

To study the effect of ring size on inhibitory activity six membered glutarimide inhbitors were prepared using the synthetic methodology developed above for the succinimide series. The favoured route for the succinimides using the Stobbe condensation was not applicable to the glutarimides series as this reaction cannot tolerate glutarimide substrates and therefore the method employed proceeded through the anhydride.

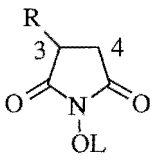
It was also desirable to examine what effect the type and extent of substitution had on inhibitory activity which required a supply of both mono and disubstituted alkyl and aromatic glutaric acids. The synthesis of α-benzylglutaric acid was effected through Michael reaction chemistry and a number of dialkyl glutaric acids were prepared using the Guareschi route. Glutarimide inhibitors were then prepared in good yield from the respective acids through a four step synthetic sequence proceeding through the anhydride, *N*-protected glutarimide and *N*-hydroxyglutarimide.

CHAPTER 3

α-CHYMOTRYPSIN INHIBITION ASSAYS

3.1 MECHANISM FOR INACTIVATION OF α-CHYMOTRYPSIN BY *N*-[(ACYL AND ALKYL AND ARYL SULFONYL) OXY]IMIDES

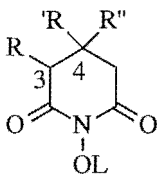
The mechanism of inactivation of α-chymotrypsin by the substituted *N*-[(acyl and sulfonyl)oxy]imides (**1.41** and **2.1**) is proposed to be the same as that identified by Groutas *et al*[85] for the succinimide inhibitor **1.41d**. This mechanism is depicted in **Scheme 3.1**.



1.41

L = SO_2R'

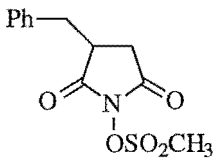
= C(O)R'



2.1

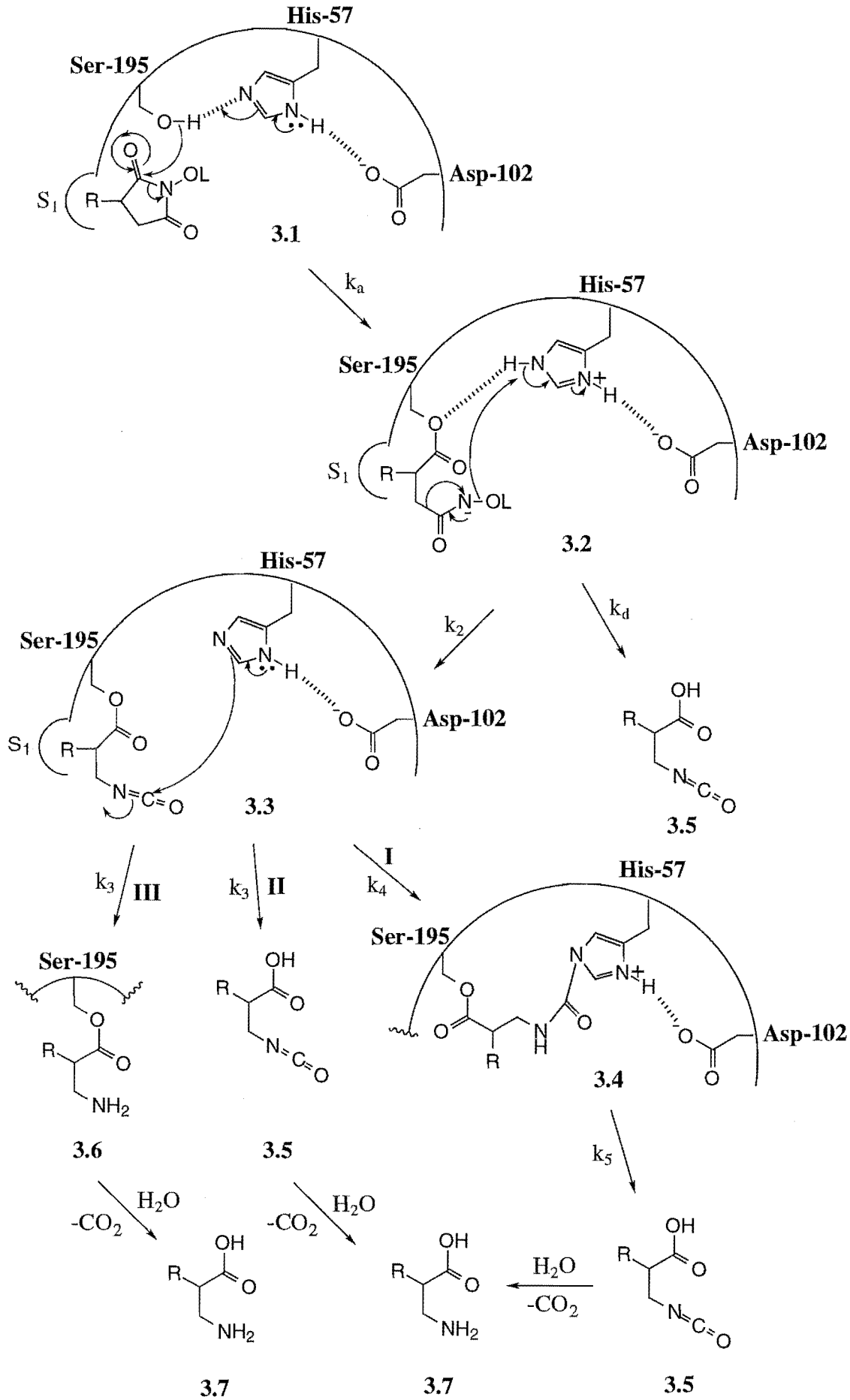
L = SO_2R'''

= C(O)R'''



1.41d

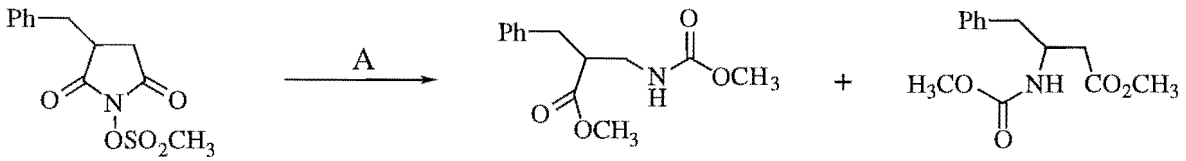
Accommodation of the inhibitor in the active-site of the enzyme through interactions between the R substituent and the primary specificity site S_1 of the enzyme to give the enzyme-inhibitor Michaelis complex (**3.1**), is followed by acylation of the active site serine (Ser-195) to yield **3.2**. Lossen rearrangement of **3.2** then generates an enzyme bound isocyanate **3.3** which competes with deacylation which gives the free isocyanate **3.5**. The enzyme bound isocyanate **3.3** then reacts rapidly with a nucleophile present in the active site. In particular, acylation of the imidazole ring of the active site amino acid His-57 (Pathway **I**) results in formation of **3.4**, the likely final inactive enzyme species. Hydrolysis of the Ser-195 ester linkage of **3.3** (Pathway **II**) results in the release of the reactive free isocyanate **3.5** from the enzyme in a "turnover" event. Hydrolysis of the isocyanate **3.3** (Pathway **III**) gives the acyl enzyme **3.6**. If stable, **3.6** may inactivate the enzyme during its lifetime which depends on the rate of hydrolysis to the amine **3.7**. It is believed that the acyl enzyme **3.6** is short lived and hence pathway **III** will be acting predominantly as a "turnover" pathway. The urea linkage of the inactive enzyme-inhibitor adduct **3.4** is also susceptible to hydrolysis, by analogy with related imidazole *N*-carboxamides,[53] leading to formation of **3.5**.



Scheme 3.1 Proposed mechanism of inhibition of α-chymotrypsin by **1.41** and **2.1**

The reactive enzyme bound isocyanate **3.3** reacts in three ways (Pathways **I-III**) all of which ultimately lead to the release of free isocyanate **3.5** from the enzyme. This undergoes rapid hydrolysis with concomitant decarboxylation to form the highlighted amine **3.7**. Aliphatic isocyanates, such as **3.5**, have been shown to be act as inactivators of serine proteases, including α-chymotrypsin,[49] and hence it is thought that the isocyanate **3.5** or the amine **3.7** may play a part in the inactivation process in concert with the enzyme bound isocyanate **3.3**.

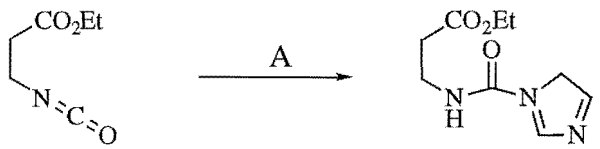
Indirect evidence supporting the proposed mechanism (**Scheme 3.1**) has been obtained.[84,87e] Reaction of equivalent amounts of the inhibitor **1.41d** and sodium methoxide in methanol resulted in formation of a mixture of two isomeric Lossen rearrangement products **3.8** and **3.9** (**Scheme 3.2**).



1.41d **3.8** **3.9**

Scheme 3.2 A, $NaOCH_3$, CH_3OH, 1h, rt.

The isocyanate **3.10** has also been observed to react with imidazole to give the imidazole *N*-carboxamide **3.11** in quantitative yield.



3.10 **3.11**

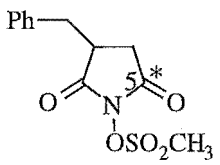
Scheme 3.3 A, imidazole, 1h, room temperature

The urea linkage of imidazole-*N*-carboximides such as **3.4** is labile to nucleophiles, and hence treatment of inactivated enzyme with appropriate nucleophilic reagents should result in reactivation of the enzyme. Reactivation of inactivated enzyme has been observed in experiments undertaken with hydroxylamine and dithiothreitol, results which support formation of **3.4**.

Isocyanates **3.3** and **3.5**, are both reactive towards nucleophiles. If an external nucleophile is present during the inactivation process it will scavenge both **3.3** and **3.5**, and if these are involved in the inactivation process, the enzyme will be protected.

Experimentally a lower rate of inactivation will be observed for an inhibition experiment carried out in the presence of an external nucleophile than the control experiment carried out in its absence. This result has been observed in these types of experiments performed by Groutas *et al*,[84] and supports the existence of isocyanates **3.3** and **3.5** in the proposed mechanism (**Scheme 3.1**).

Direct evidence[85] in support of the proposed mechanism of inactivation has been obtained from a high resolution ^{13}C NMR experiment in D_2O (7.5% DMSO-d_6) using ^{13}C labelled (C-5) inhibitor **3.12**. Incubation of an equivalent amount of **3.12** with α-chymotrypsin led to the appearance of resonances at 176 and 126ppm in the ^{13}C NMR spectrum. These resonances are consistent with the formation of the imidazole-*N*-carboximide **3.4** due to pathway **I** and the free isocyanate **3.5** presumably resulting from pathway **II** of **Scheme 3.1**.



3.12

The elucidation of the mechanism of inactivation of **Scheme 3.1** provides evidence that inhibition of α-chymotrypsin by **1.41d** involves the catalysis by the enzyme. This is one criterium of a set of criteria that have been developed to establish the occurrence of mechanism-based inhibition. Important criteria include:

Time-dependent loss of activity: for mechanism-based inactivation enzyme activity should decrease over time. Ideally, psuedo first-order kinetics is observed.

Saturation: the rate of the inactivation is dependent on the extent of formation of the Michaelis enzyme-inhibitor complex. A point will be reached when all the enzyme is saturated with inhibitor and the addition of further amounts of inhibitor will not affect the rate of inactivation. Therefore there will exist a maximum rate of inactivation for a mechanism-based inhibitor.

Irreversibility: mechanism-based inactivation is irreversible in nature; in general the inactive enzyme-inhibitor adduct contains a functionally covalent linkage and, unlike a reversible inhibitor, no enzyme activity will be recovered on gel filtration or dialysis.

Inactivation Occurs Prior to Release of the Activated Species: one of the features of mechanism-based inhibition is that it is specific. This specificity of action will be lost if the active species escapes and then returns to inactivate the enzyme as is the case for

affinity labels or a so-called "paracatalytic" inhibitors. Mechanism-based inactivation will show no "lag" phase and will not be affected by the presence of nucleophiles.

Partition Ratio: this concept is unique to mechanism-based inhbition. This term introduced by Walsh,[54] describes the ratio of the number of latent inhibitor molecules converted and released as product relative to each turnover leading to enzyme inactivation. The partition ratio, therefore is a measure of the efficiency of the inhibition.

Frequently in practice, as in this thesis, only a few of the criteria developed for mechanism-based inactivation are investigated when a potential mechanism-based inactivator is studied. However, to unambiguously charaterize an inactivator as being mechanism-based the criteria above should be satisfied. The first two criteria define any covalent or tightly bound, slow binding, non-covalent competitive inhibitor which would be analysed in a different manner than a mechanism-based inhibitor. The last three criteria, in combination, are definitive for a mechanism-based inhibitor. It is the object of this chapter to discuss the assay of *N*-[(acyl and sulfonyl)oxy]imides **1.41** and **2.1** using kinetic models developed for mechanism-based inactivation. The question then arises are we justified in using these models?

A preliminary answer to this question and a preview of the activity of compounds **1.41** and **2.1** can be provided by the results Groutas *et al*[84,87a] obtained in carrying out experiments prescribed to establish mechanism-based inhibition for **1.41d**. This compound has been found to inhibit α-chymotrypsin in a time dependent manner with the observation of pseudo first-order kinetics over 15 minutes. Experiments carried out at increasing inhibitor concentration point to the a maximum rate of inactivation. The urea linkage of the inactive enzyme (see **Scheme 3.1**, structure **3.4**) is susceptible to hydrolysis ($t_{1/2}$ = 1h) and therefore inhibitors of type **1.41** and **2.1** will only be acting as irreversible inhibitors over a short time.

Although the addition of the nucleophile dithiothreitol results in a lower rate of inhibition it is believed that inactivation does occur before the reactive species escapes the active site as there is no "lag" phase for inhibition. This result may be explained by dithiothreitol entering the active site and reacting with the enzyme bound isocyanate (**Scheme 3.1**, structure **3.3**).

The partition ratio for **1.41d** will depend on the reactivity of the enzyme bound isocyanate, its rate of hydrolysis and hence diffusion from the active site, and the proximity of the imidazole ring for carbamate bond formation. No partition ratio analysis has been undertaken for **1.41d**, however ^{13}C NMR and reactivation studies

carried out by Groutas *et al*[84,85] suggest that the rate of hydrolysis of the enzyme bound isocyanate **3.3** and inactive enzyme **3.4** are significant. It is therefore proposed that the partition ratios for inhibitors of type **1.41** and **2.1** will be far from the ideal value of zero.

The predicted high partition ratios for **1.41** and **2.1** will have practical implications for the inhibition assays as depletion of the inhibitor during assay will be significant and problematic. It appears therefore that the *N*-[(acyl and sulfonyl)oxy]imide inhibitors **1.41** and **2.1** will only be acting as true mechanism-based inactivators at high concentrations and over a short period of time after which deviation away from psuedo first-order kinetics is expected. There is also evidence to suggest that a species other than the initial compound **1.41d**, most probably the amine **3.7**, contribute to the inactivation, although not initially.

5.2 INACTIVATION KINETICS

The evaluation of the inhibitory activity of a compound is routinely carried out using two methods. The first procedure commonly referred to as the "incubation method" or "pre-incubation method"[71,72,79,187-9] as it name suggests involves incubating an assay mixture containing the enzyme and an excess of inhibitor. Periodically, aliquots are removed from this assay mixture and reacted with a "monitoring" substrate. The rate of hydrolysis of the substrate is a measure of the enzyme activity of this sample. A chromogenic substrate is used so that its rate of hydrolysis can be followed spectrophotometrically by noting the proportional rate of increase in absorbance of its hydrolysis product. The assay mixture contains only enzyme and inhibitor and hence kinetic analysis involves consideration of only those two species.

The incubation method can be used only to assay relatively slow inhibitors because sampling techniques are employed. Faster or more potent inhibitors cannot be adequately examined using this method hence a second assay procedure capable of measuring rapid inactivation is required. The procedure commonly employed is the progress curve method.[190-192] To an assay mixture containing "monitoring" substrate and inhibitor an aliquot of enzyme is added, and the progress of the reaction followed by the rate of hydrolysis of the substrate.

Practically, the incubation method of assay is more demanding. Several inhibition and control experiments have to be undertaken to afford the rate measurements that on simple analysis give the inhibitory activity of the compound being assayed. For the incubation method the bimolecular constant $k_{obs}/[I]$ is used exclusively as the index for inhibitory activity. As discussed in the following section, for slow inhibitors $k_{obs}/[I]$ serves as an approximation for the ratio k_{inact}/K_I which is the ideal constant for quantifying inhibition. For potent inhibitors this ratio is high, for weaker inhibitors it is lower.

For those fast inhibitors able to be analysed using the incubation method $k_{obs}/[I]$ approximates k_{inact}/K_I less well but k_{obs}/K_I is still used as an index of potency, faster inhibitors again displaying a higher value. A rigorous value for k_{inact}/K_I can be obtained using the incubation method but requires that experiments be carried out at several different inhibitor concentrations (five are recommended)[25] which adds to the already high experimental demand for this method.

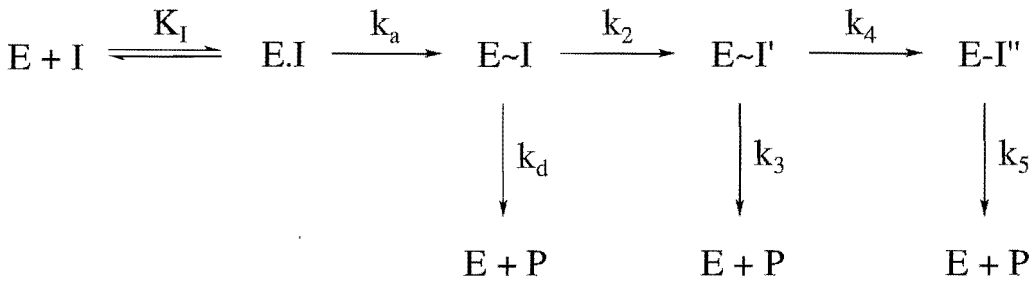
The progress curve method requires only one inhibition experiment for evaluation of $k_{obs}/[I]$ but the data analysis is more complicated and time consuming. The expression for $k_{obs}/[I]$ for the progress curve method is different from that for the incubation

method having a different dependence on the inhibitor concentration and including substrate terms and hence the two are not compared. The ratio k_{inact}/K_I is exclusively reported and can be determined rigorously using the progress curve method. Again experiments are run at different inhibitor concentrations but the fact that the progress curve method is less labour intensive facilitates these operations. Values for k_{inact}/K_I can be compared between the two methods and a good agreement is normally observed.[62c]

The assay mixture for the incubation method contains enzyme and inhibitor whereas for the progress method the assay mixture contains enzyme, inhibitor, and substrate and hence a more complicated kinetic analysis will be required. The kinetic models used in the analysis of inhibitors using these two methods is discussed below.

INCUBATION METHOD KINETICS

The corresponding kinetic scheme for the interaction between enzyme and inhibitor for the proposed mechanism of inactivation of α-chymotrypsin by **1.41** and **2.1** is shown below (**Scheme 3.4**).



Scheme 3.4. Kinetic scheme for mechanism-based inhibitors of type **1.41** and **2.1**

In this scheme E is the free enzyme; I is the *N*-acyl or sulfonyloxy imide inhibitor **1.41** or **2.4**; E.I the Michaelis complex for the enzyme and inhibitor (structure **3.1** of **Scheme 3.1**); E~I is the acyl enzyme (**3.2**); and E~I' is the reactive isocyanate (**3.3**) tethered at the active site; E-I'' is the inactivated enzyme with the inhibitor covalently attached (**3.4**); and P represents the free isocyanate (**3.5**) and the amine (**3.7**) which are the products resulting from hydrolysis of the inhibitor.

The inhibitor initially interacts with the enzyme in the same way as a substrate to give an enzyme-inhibitor Michaelis complex E.I. The binding of enzyme and inhibitor is described by K_I the dissociation constant for the Michaelis complex. Acyl enzyme formation (k_a) proceeds as with a normal substrate, however, it is accompanied by the release of the latent reactivity *i.e.* the Lossen rearrangement (k_2) to the enzyme bound isocyanate. Deacylation of the acyl enzyme (k_d) may also occur via a so-called

"turnover" pathway which does not result in enzyme inactivation. Acylation of the imidazole ring of His-57 by the enzyme bound isocyanate (k_4) leads to inactivated enzyme (E-I''). This competes with other "turnover" hydrolysis pathway (k_3). The rate constant for hydrolysis of the inactivated enzyme species (k_5) determines the "irreversibility" of the inactivator.

Jung and Metcalfe[187b] have developed a kinetic model for mechanism-based inhibitors extending the work undertaken by Kitz and Wilson[187a] for the kinetic analysis of active-site directed irreversible inhibitors. Groutas *et al*[84,85,87] have analysed inhibitors of type **1.41** (L = SO_2R') such as **1.41d**, using the model of Jung and Metcalfe and hence this model was adopted for the initial inhibition studies of this thesis.

Since the progress of the overall inhibition reaction is monitored by the decrease in total active enzyme (*i.e.* accumulation of E-I''), E.I, E~I, and E~I' are not observed independently and hence **Scheme 5.2** can be simplified to give the kinetic scheme shown below (**Scheme 3.5**).

The assumption made in this approximation is that the inhibition reaction is carried out with an excess of inhibitor so that the concentration of the Michaelis complex is constant throughout the assay. If the inhibition experiment is designed so that these ideal conditions are maintained then psuedo first-order kinetics will be observed.

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E^*I \xrightarrow{k_{inact}} E\text{-}I''$$

Scheme 3.5 Simplified incubation method kinetic scheme for mechanism-based inhbitors **1.41** and **2.1**

In this scheme E*I represents the kinetic composite of E.I, E~I, and E~I' and k_{inact} is the rate constant for the inactivation process. The rate constant k_{inact} is a mixture of the microscopic rate constants k_2, k_3, k_4 and k_5 of **Scheme 3.2**. Waley[193] has applied a steady-state hypothesis to the kinetic scheme for a general mechanism-based inactivator which includes all the terms considered in **Scheme 3.4** excepting k_5 as this is zero for inactivation. The expression obtained for k_{inact} is shown below in equation (3.1).

$$k_{inact} = \frac{k_2 k_4}{k_2 + k_3 + k_4} \qquad (3.1)$$

From equation (3.1) it is observed that the magnitude of k_{inact} is proportional to k_2, the rate constant for the Lossen rearrangement to the inactivating species; and k_4, the

rate constant for the reaction between the inactivating species and His-57 to form inactive enzyme. The magnitude of k_{inact} is inversely proportional to k_3, the rate constant for the turnover pathways which result in no net inactivation.

Typically k_2 is found to be the rate determining step in the inhibition process, in this case equation (3.1) reduces to:

$$k_{inact} = \frac{k_2 k_4}{k_3 + k_4} \qquad (3.2)$$

From equation (3.2) the magnitude of k_{inact} will be proportional directly to k_3 and k_4 (but in a more complicated manner) but inversely proportional to k_3.

The ratio k_{inact}/K_I provides a measure of inhibitory activity. A potent inhibitor will have a high k_{inact}/K_I value whereas a weaker inhibitor will have a lower value. Hence a potent inhibitor will have a low K_I, demonstrating that it has high binding affinity for α-chymotrypsin and a high k_{inact} demonstrating that the Lossen rearrangement and reaction of the resulting isocyanate with His-57 is rapid, but deacylation and hydrolysis of the isocyanate and inactive enzyme are slow.

The kinetic scheme above (**Scheme 3.5**) can be analysed by the method of Kitz and Wilson.[187] Provided the enzyme-inhibitor solution is extensively diluted before assay, and that the inhibitor concentration is at such an excess over enzyme that it remains constant during the course of the assay it can be shown that:

$$\ln\frac{[\varepsilon]}{[E_o]} = -k_{obs}.t \qquad (3.3)$$

where

$$k_{obs} = \frac{k_{inact}}{1 + \frac{K_I}{[I]}} \qquad (3.4)$$

Experimentally, the quantity [ε] is measured by the rate of hydrolysis of the monitoring substrate in the presence of inhibitor, v_t. The quantity $[E_o]$ is measured by the rate of hydrolysis of monitoring substrate in the absence of inhibitor, v_o, such that equation (3.3) can also be expressed as:

$$\ln\frac{v_t}{v_o} = -k_{obs}.t \qquad (3.5)$$

Therefore, a plot of $\ln\frac{v_t}{v_o}$, which serves as a measure of enzyme activity, versus time should give a straight line with a slope of $-k_{obs}$.

Looking at equation (3.4):

$$k_{obs} = \frac{k_{inact}}{1 + \frac{K_I}{[I]}} \qquad (3.6)$$

If $[I] << K_I$, for a poor inhibitor, then:

$$k_{obs} \approx \frac{k_{inact} \cdot [I]}{K_I}$$

$$\therefore \frac{k_{obs}}{[I]} \approx \frac{k_{inact}}{K_I} \qquad (3.7)$$

Thus, $\frac{k_{obs}}{[I]}$ can be an approximation for the second order rate constant $\left(\frac{k_{inact}}{K_I}\right)$

The inhibitory constant k_{inact}/K_I can be obtained in a more rigorous manner using the method of Kitz and Wilson.[187a] The experimental quantity k_{obs} is obtained for a number of inhibition experiments where the excess of inhibitor over enzyme is varied. The half-lives for inhibition, ($t_{1/2}$), for these experiments are then simply calculated using equation (3.8) which is derived from equation (3.5):

$$t_{1/2} = \frac{\ln 2}{k_{obs}} \qquad (3.8)$$

If the $t_{1/2}$ values for inhibition are plotted against the reciprocal of the inhibitor concentrations, according to equation (3.9), obtained from substitution of equation (3.8) into equation (3.6) , two possibilities can exist (**Figure 3.1**).

$$t_{1/2} = \frac{\ln 2}{k_{inact}} + \frac{\ln 2 K_I}{k_{inact}} \qquad (3.9)$$

Of the plots displayed in **Figure 3.1** Graph **A** depicts the case when enzyme saturation is observed while Graph **B** depicts the situation when saturating conditions do not exist. The inhibitory constant k_{inact}/K_I can be only obtained from the gradient and y-intercept of Graph **A** as shown, demonstrating the importance of maintaining saturating inhibitor concentrations during the course of the enzyme assay.

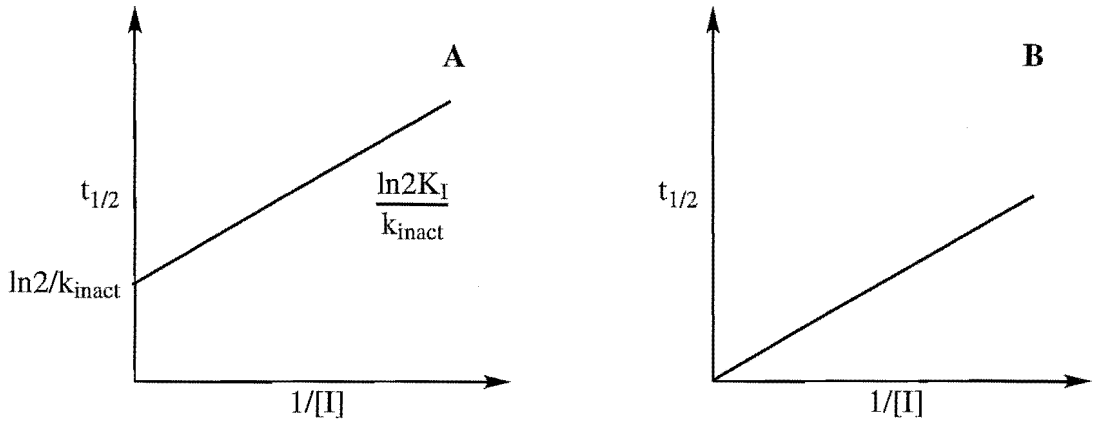


Figure 3.1 Kitz and Wilson plot for an inhibitor exhibiting saturation (**A**) or (**B**) not exhibiting saturation

To determine k_{inact}/K_I using Graph **A** requires first the calculation of $t_{1/2}$ from k_{obs} as described above and then of the gradient and y-intercepts. A modified Kitz and Wilson plot of $[I]/k_{obs}$ versus [I] (**Figure 3.2**) according to equation (3.10) , derived from equation (3.6), also gives a straight line with positive slope and postive y-intercept. A value for k_{inact}/K_I can be obtained directly from the y-intercept of this graph and hence more simply than a determination using Graph **A** of **Figure 3.1** and without the required $t_{1/2}$ calculations.

$$\frac{[I]}{k_{obs}} = \frac{K_I}{k_{inact}} + \frac{[I]}{k_{inact}} \qquad (3.10)$$

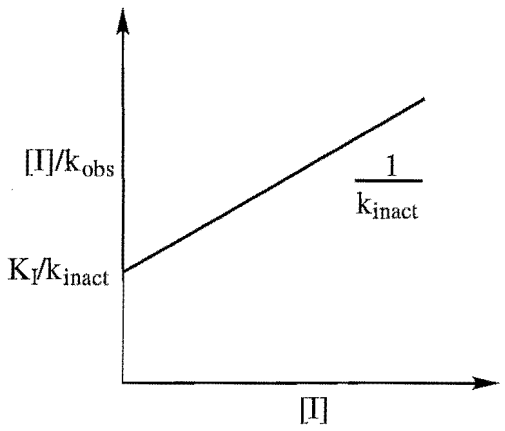
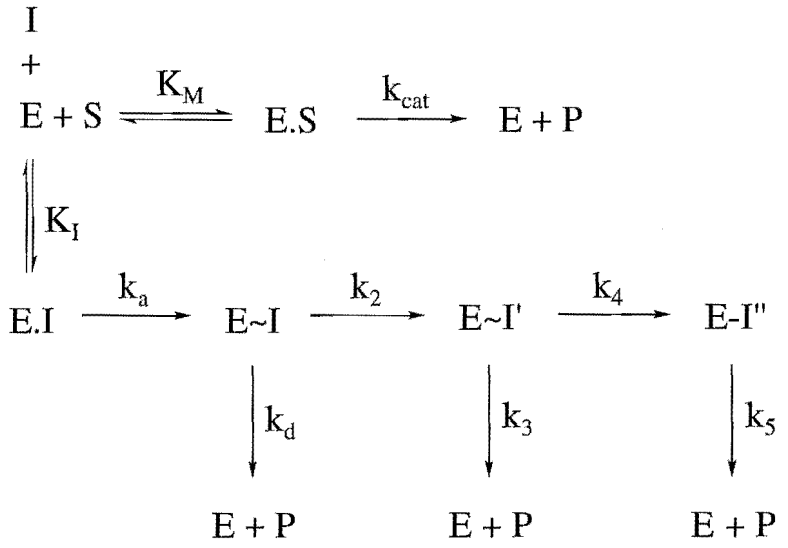


Figure 3.2 Plot of $[I]/k_{obs}$ versus [I] to determine k_{inact}/K_I

PROGRESS CURVE KINETICS

The kinetic scheme for the interaction of enzyme, inhibitor and substrate for the proposed mechanism of inactivation of α-chymotrypsin by **1.41** and **2.1** is shown below.



Scheme 3.6 Progress curve method kinetic scheme for mechanism-based inhibitors of type **1.41** and **2.1**

This kinetic scheme can be analysed according to the method of Tsou.[190] Provided that the rate of the enzyme-substrate reaction is faster than the enzyme-inhibitor reaction and the inhibitor and substrate concentrations remain constant during the course of the assay it can be shown that there will be an exponential release of product with time according to equation (3.11).

$$[P] = \frac{v}{k_{obs}}(1 - e^{-k_{obs}t}) \qquad (3.11)$$

At $t = \infty$, from equation (3.11):

$$[P_\infty] = \frac{v}{k_{obs}} \qquad (3.12)$$

where k_{obs} is the rate constant for the observed inhibition and v the rate of the enzyme-substrate reaction in the absence of inhibitor which are given by:

$$k_{obs} = \frac{k_{inact} + [I_o]}{[I_o] + K_I[1 + \frac{[S_o]}{K_M}]} \qquad (3.13)$$

$$v = \frac{V_m}{1 + \frac{K_M}{[S_o]}[1 + \frac{[I_o]}{K_I}]}$$

$$\frac{k_{obs}}{[I]} = \frac{k_{inact}}{K_I[1 + \frac{[S_o]}{K_M}] + [I_o]} \qquad (3.14)$$

To determine the kinetic constants for irreversible inhibition, $k_{obs}/[I]$ and k_{inact}/K_I, using the progress curve kinetic method, a suitable irreversible curve of type **A** (**Figure 3.3**) which is described by equation (3.11) must be obtained. Therefore an important experimental task when using the progress curve method is the design of inhibition conditions so that this irrevesible curve is obtained. This involves changing inhibitor and substrate concentrations, however, the change must be such that the inhibitor and substrate are present at saturating levels. Once a suitable curve is obtained analysis can be carried out as follows.

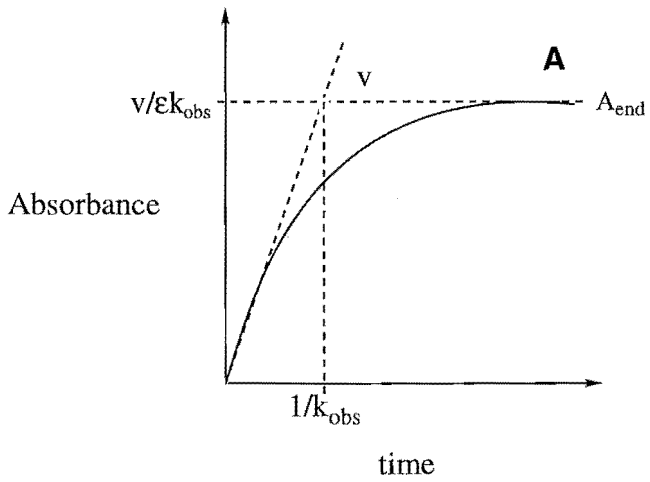


Figure 3.3 Irreversible Curve **A**

From equations (3.11) and (3.12) it can be simply shown that:

$$\ln([P_\infty] - [P_t]) = \ln[P_\infty] - k_{obs}.t \qquad (3.15)$$

The quantities $[P_t]$ and $[P_\infty]$ can be interchanged with the corresponding absorbances, A_t and A_{end}, which can be directly read off the absorbance versus time curves obtained experimentally (see **Figure 3.3**). Therefore equation (3.15) can also be expressed as:

$$\ln(A_{end} - A_t) = \ln(A_{end}/\varepsilon) - k_{obs}.t \qquad (3.16)$$

Hence k_{obs} can be obtained from the slope of the initial portion of the logarithmic plot $\ln(A_{end} - A_t)$ versus time using linear regression analysis. Alternatively k_{obs} can be determined by fitting experimental absorbance versus time data to equation (5.9) using

non-linear regression analysis with initial parameter values obtained from the extrapolations shown in **Figure 3.3** as suggested by Walsh and Morrison.[191]

$$A = \frac{v}{k_{obs}}(1 - e^{-k_{obs}t}) + A_o \qquad (3.17)$$

Determination of the inhibition constant k_{inact}/K_I is carried out by evaluating $[I]/k_{obs}$ at different inhibitor concentrations at saturating conditions. A Tsou plot of $[I]/k_{obs}$ versus [I] according to equation (3.17) should give a straight line for a mechanism-based inactivator with positive slope and y-intercept as shown in **Figure 3.4** which can be determined by linear regression analysis. Since K_M[194] and $[S_o]$ are known k_{inact}/K_I can be simply calculated.

$$\frac{[I]}{k_{obs}} = \frac{K_I[1 + \frac{[S_o]}{K_M}]}{k_{inact}} + \frac{[I]}{k_{inact}} \qquad (3.18)$$

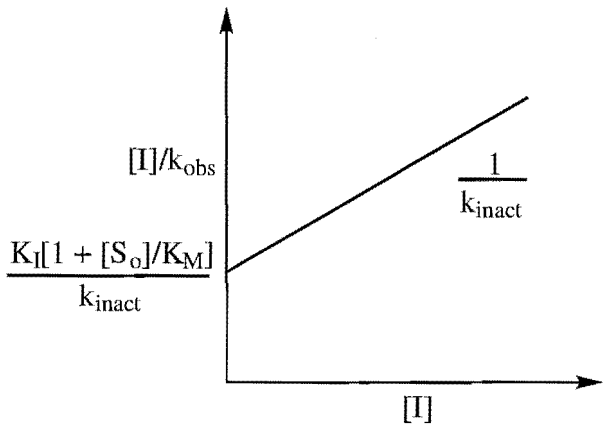


Figure 3.4 Tsou plot used to determine k_{inact}/K_I.

Earlier the differences between the incubation and progress curve methods were discussed. The question arose as to whether results obtained for compounds which are poor inhibitors, assayed using the incubation method can be compared with those obtained for compounds, which are potent inhibitors, assayed using progress curve techniques. The following section compares the kinetic models for the two methods to provide an answer to this question.

The quantity $k_{obs}/[I]$ is used routinely as an index of inhibitory activity, with more potent inhibitors displaying a higher value than those compounds which are less potent. The equations for $k_{obs}/[I]$ derived for the incubation method (3.6) and (3.7) and progress curve method (3.14) are shown respectively below.

$$\frac{k_{obs}}{[I]} = \frac{k_{inact}}{1 + \frac{K_I}{[I]}} \qquad (3.6)$$

$$\frac{k_{obs}}{[I]} = \frac{k_{inact}}{K_I} \qquad (3.7)$$

$$\frac{k_{obs}}{[I]} = \frac{k_{inact}}{K_I[1 + \frac{[S_o]}{K_M}] + [I_o]} \qquad (3.14)$$

It can be observed that both the equations are not independent of the inhibitor concentration and cannot therefore serve as an index of inhibitory activity unless inhibitors are compared at the same inhibitor concentration in which case $k_{obs}/[I]$ values can only be compared if obtained using the same assay technique; no valid comparison can be made between the two methods. As discussed above, if a poor inhibitor is being assayed using the incubation method $k_{obs}/[I]$ serves as an approximation for k_{inact}/K_I, however this is not the case for compounds assayed using progress curve kinetics.

The equations for the experimental quantity $[I]/k_{obs}$ for the incubation (3.10) and progress curve method (3.18) are shown respectively below.

$$\frac{[I]}{k_{obs}} = \frac{K_I}{k_{inact}} + \frac{[I]}{k_{inact}} \qquad (3.10)$$

$$\frac{[I]}{k_{obs}} = \frac{K_I[1 + \frac{[S_o]}{K_M}]}{k_{inact}} + \frac{[I]}{k_{inact}} \qquad (3.18)$$

Observation of these equations shows that they are of the same form and demonstrate the same dependence with the inhibitor concentration [I]. Hence it is by considering the term $[I]/k_{obs}$ that the two methods converge; plots according to these equations will show the same trends (**Figure 3.5**) and as discussed can be manipulated to determine a value for k_{inact}/K_I which is independent of which method is used.

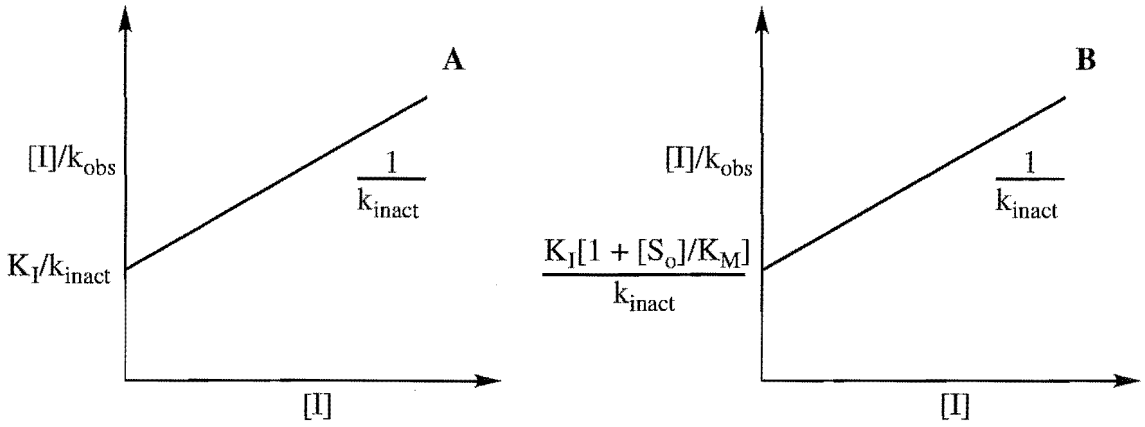


Figure 3.5 Analytical graphs for incubation method (**A**) and progress curve method (**B**)

3.3 EXPERIMENTAL PROCEDURES

GENERAL

Kinetic assays were performed using a Hewlett-Packard 8451A single beam diode array spectrophotometer and 1cm path length quartz cuvettes incubated in a customised heating block fitted to the spectrophotometer. α-Chymotrypsin (bovine pancreatic; three times crystallised and free of autolysis products and low molecular contaminants) and *N*-Succ-(Ala)$_2$-Pro-Phe-*p*-nitroanilide were obtained from Sigma Chemical company. *N*-Benzoyl-L-tyrosine ethyl ester (BTEE)[195] was purchased from Aldrich. All kinetic studies were performed at 25°C.

The following solutions were prepared fresh for assay:

0.1M KH_2PO4/K_2HPO_4 potassium phosphate buffer, pH 7.2. Prepared from a 0.1M solution of KH_2PO_4 in distilled H_2O and adjusting the pH of this solution with 5M NaOH as monitored by a meter previously calibrated with sodium phthalate buffer, pH 4.01.

α-Chymotrypsin: Stock solution of 20μM (4.3mg in 10mL 0.1M phosphate buffer). Bovine pancreatic α-chymotrypsin has M_r = 21 600 Da.[196] The stock solution was diluted 5-fold in phosphate buffer before use, to give a concentration of 4μM.

N-Succ-(Ala)$_2$-Pro-Phe-*p*-nitroanilide: Stock solution 120μM in phosphate buffer. This required slight warming for dissolution.

BTEE: Stock solution 120μM in 1:1:2 MeOH/H_2O/phosphate buffer.

Inhibitor solutions: Stock solutions of 5mM or 10mM in CH_3CN were prepared and were diluted when required by the assay.

INCUBATION ASSAY PROCEDURE

Typically, a solution of α-chymotrypsin in 0.1M (potassium) phosphate buffer (1mL, 4μM, final concentration 3.922μM) was incubated at 25^{0}C with various concentrations of inhibitor (three-, five-, ten- or 25-fold excess over enzyme) added as a volume [xμL of a 5mM or more dilute stock MeCN inhibitor solution plus a volume (20-x)μL of acetonitrile (MeCN)]. Aliquots (25μL) were withdrawn at typically 1, 3, 5, 7, 9, 11, 15, 20 and 30min intervals (though assays were carried out at longer time intervals) and added to quartz cuvettes containing a solution of either BTEE in 1:1:2 MeOH/H_2O/buffer (1mL, 120μM) for compounds **1.41d-q** and **2.1b** and **2.d-f**; or *N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide in buffer (1mL, 120μM) for compounds **1.41e** and **h** and **2.1f** and **2.1e** held in a heating block thermostated at 25°C.

Reaction progress was then followed immediately by recording the increase of absorbance at 256nm (BTEE) or 410nm (*N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide) for 80s relative to a "blank" cuvette containing BTEE (1mL, 120μM in 1:1:2 MeOH/H_2O/buffer, final concentration 117μM) or *N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (1mL, 120μM in buffer) and buffer (25μL).

The absorbance versus time data obtained were linear plots with slope equal to the rate of reaction, (in the presence of inhibitor), v_t. This was calculated by linear regressional analysis of data over 20-70s. The rate of reaction (in the absence of inhibitor) v_o was determined in the same manner but enzyme was incubated with 20μL MeCN. V_o was observed to remain constant over the 30min period of incubation and hence an average value was used in subsequent calulations. The ratio v_o/v_t is a measure of residual enzyme activity.[187a]

PROGRESS CURVE PROCEDURE

Typically into a 1mL quartz cuvette 958μL of the stock solution of BTEE (final concentration 117μM) and a solution of inhibitor in acetonitrile were combined. The inhibitor was added as a volume x mL of a 15mM or more dilute stock inhibitor solution in acetonitrile plus a volume (25-x)μL of acetonitrile. The final concentrations of inhibitor ranged from 0.189μM (three-fold excess over enzyme) to 18.25μM (250-fold excess over enzyme). This solution was incubated for five minutes at 25 °C after which 17μL of the buffered enzyme stock solution (final concentration 0.073μM) was added and the reaction progress was then followed immediately by recording the change

in absorbance at λ = 256nm over a time interval not greater than 30min. The change in absorbance was measured relative to a "blank" cuvette containing 958μL of BTEE stock solution, 25μL acetonitrile and 17μL phosphate buffer.

A control experiment in the absence of inhibitor was also routinely carried out in the same manner excepting 958μL of the stock BTEE and 25μL of acetonitrile only were incubated before addition of the enzyme. The reaction was monitored as above.

The time delay involved from addition (at t = 0) of the 17μL aliquot of the buffered enzyme stock solution to the incubated inhibitor-substrate solution in an open cuvette, removal of the cuvette from the water bath, placement of the cuvette's plastic cap, agitation of the cuvette to mix the assay solution and placement of the cuvette into the incubated cell of the spectrophotometer and finally employment of the latter took approximately 15s in our experience. This mixing time was noted for each inhibition experiment and subsequently was entered into the analysis of absorbance data.

Three types of absorbance versus time curve were generally obtained and are shown in **Figure 3.6**. Graph **A** depicts a progress curve typical of irreversible inhibition and can be analysed using the Tsou logarithmic plot of $\ln(A_{end}-A_t)$ versus time, or by non-linear regression analysis to obtain a value for the observed inhibiton rate constant k_{obs}.

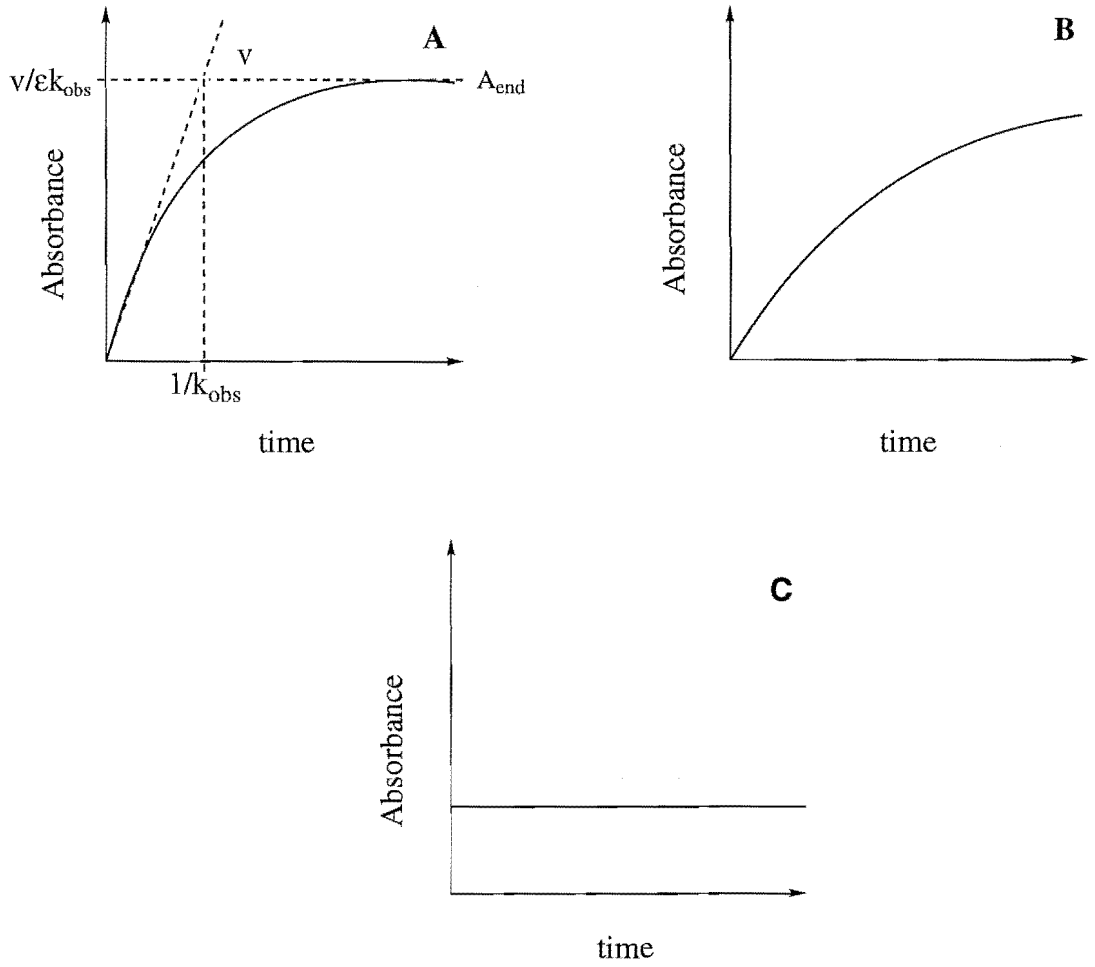


Figure 3.6 Idealised experimental progress curves

Graph **B** depicts a progress curve observed typically for reversible inhibition but was observed for the *N*-acyl and sulfonyloxy imide inhibitors **1.41** and **2.1** studied. This result suggests that the experiment was run at a low inhibitor concentration and this was depleted during the course of the inhibition experiment. This was considered to be a likely event for **1.41** and **2.1** because their partition ratios are predicted to be high. When this result was obtained the inhibition experiment was repeated at a higher inhibitor concentrations to obtain saturating conditions such that an irreversible curve of type **A** was observed.

Graph **C** depicts a case where a high excess of inhibitor was used having the effect that the enzyme was totally inactivated within the mixing period. In reality a straight line was not obtained but rather a noisy absorbance trace with a positive slope indicating that with time the inhibitor was being depleted and enzyme activity returning. When this result was obtained the inhibition experiment was repeated at a lower inhibitor concentration in an effort to obtain a curve of type **A**.

The major task therefore when using the progress curve assay method is finding suitable inhibitor concentrations that give curves of type **A** suitable for analysis of k_{obs}. Several inhibition experiments were carried out to determine k_{obs} at different inhibitor concentrations and the resulting data analysed using a Tsou plot of $[I]/k_{obs}$ versus [I] from which k_{inact}/K_I can be obtained.

5.4 ASSAY RESULTS

Each inhibitor of type **1.41** and **2.1** shown in **Tables 3.1** and **3.2** respectively was assayed using the incubation method detailed above to quickly determine the activity-time profile. Subsequent experiments depended on the potency and type of inhibition observed in these preliminary experiments. In general five results were obtained from the incubation assay.

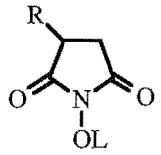
a) The compounds were inactive.

b) The inhibitors inactivated α-chymotrypsin in a time-dependent manner at such a rate that a number of data points could be obtained for analysis. The inhibition constants k_{obs} and $k_{obs}/[I]$ were then determined as described above.

c) A rapid loss in enzymic activity was observed followed by a rapid regain of this activity. It was proposed that these compounds were acting as poor alternate substrate inhibitors. No further analysis was undertaken.

d) The rate of inactivation was too fast to be determined using the incubation assay. These potent inhibitors were assayed using progress curve kinetic methods.

e) The compounds were of such potency that they could not be measured accurately using the progress curve method at an inhibitor concentration of ten-fold excess over enzyme.



1.41

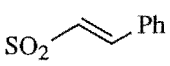
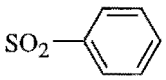
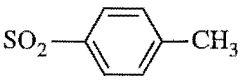
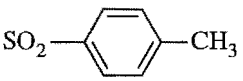
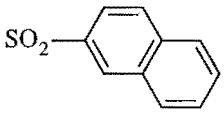
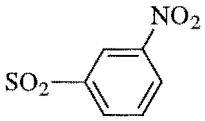
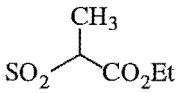
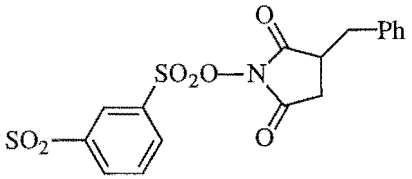
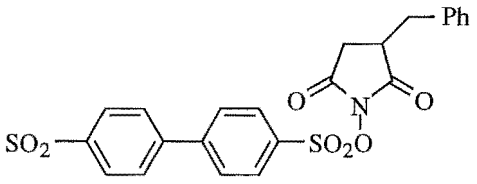
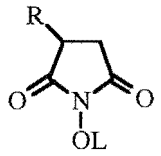
Compound number	R	L
1.41d	$PhCH_2$	SO_2CH_3
e	Ph	SO_2CH_3
f	$PhCH_2$	
g	$PhCH_2$	
h	$PhCH_2$	
i	Ph	
j	$PhCH_2$	
k	$PhCH_2$	
l	$PhCH_2$	
m	$PhCH_2$	
n	$PhCH_2$	

Table 3.1 *N*-[(sulfonyl)oxy]imide inhibitors of type **1.41**



1.41

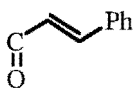
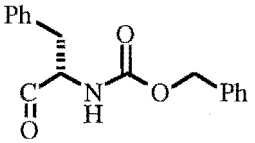
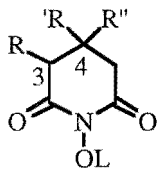
Compound number	R	L
o	$PhCH_2$	Ac
p	Ph	Ac
q	$PhCH_2$	
r	$PhCH_2$	

Table 3.2 *N*-[(acyl)oxy]imide inhibitors of type **1.41**



2.1

Compound number	R	R'	R''	L
2.1a	Ph	H	H	SO_2CH_3
2.1b	Ph	H	H	Ac
2.1c	$PhCH_2$	H	H	SO_2CH_3
2.1d	H	Ph	H	SO_2CH_3
2.1e	H	R', R'' = cyclohexyl		SO_2CH_3
2.1f	H	CH_3	CH_3	SO_2CH_3

Table 3.3 *N*-[(acyl and alkylsulfonyl)oxy]glutarimide inhibitors of type **2.1**

INACTIVE COMPOUNDS

When no appreciable net loss of enzyme activity was monitored over the time course of the experiment the compounds being assayed were considered to be inactive. In these cases the compound concentration was increased up to 50-fold excess over enzyme in an attempt to increase the rate of inactivation so that it could be measured, however, still no inhibitory activity was observed. **Figure 3.7** depicts the results obtained on assay of **1.41o** which is representative of the *N*-[(acyl)oxy]succinimides **1.41o-r** which were found to be inactive.

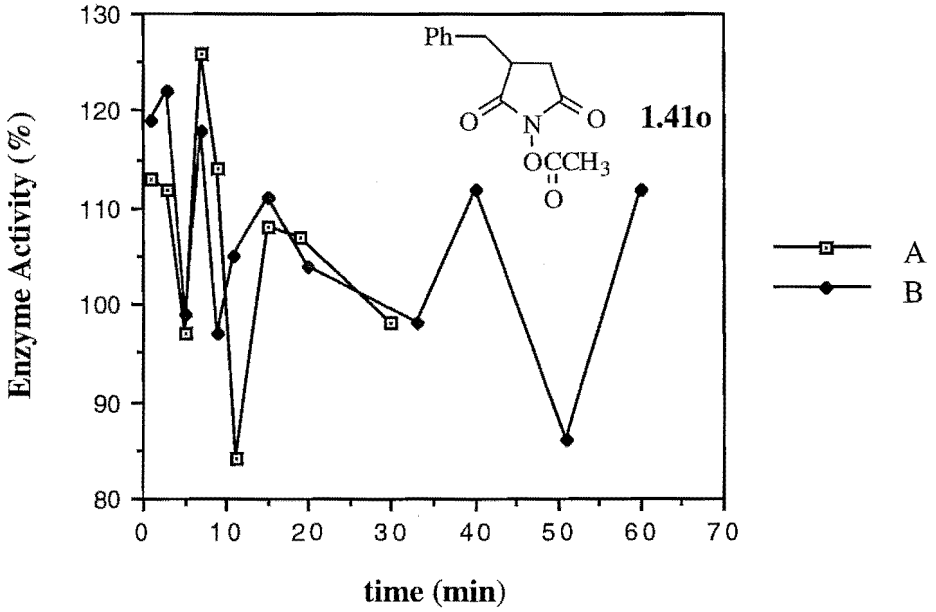


Figure 3.7 Assay of inhibitory activity of compound **1.41** against α-chymotrypsin. α-Chymotrypsin (4μM) was incubated with (A, 4μM; B, 100μM) and enzyme activity assayed using either *N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (A, 117μM) or BTEE (B, 117μM).

The oscillating nature of the graph suggests that there is a period of inhibition followed by a temporary regain in activity. This trend could be explained in the terms that several inactivating species are present in the assay mixture each of which only inhibit the enzyme for a transient period before being metabolised by the enzyme. These results are more likely to be explained, however, by the error associated with the sampling measurements used in the incubation method. Although the individual rate measurements are precise (± 1-2% error observed) they are difficult to reproduce accurately; hence erroneous results can give the impression of enzyme inhibition or activation. Therefore experiments need to be run in duplicate or triplicate for true activity versus time profiles to become evident.

TIME DEPENDENT INHIBITORS

Once it was shown by the appropriate logarithmic plot that inhibition was psuedo-first order, work was undertaken to optimise the inhibitor concentration so that an accurate value of k_{obs} was obtained. In practice this required finding the highest inhibitor concentration that gave linearity over as many data points over the time course of the assay. The analysis of the inhibition of α-chymotrypsin by compound **2.1f** whose activity versus time profile (**Figure 3.8**) serves as a representative example.

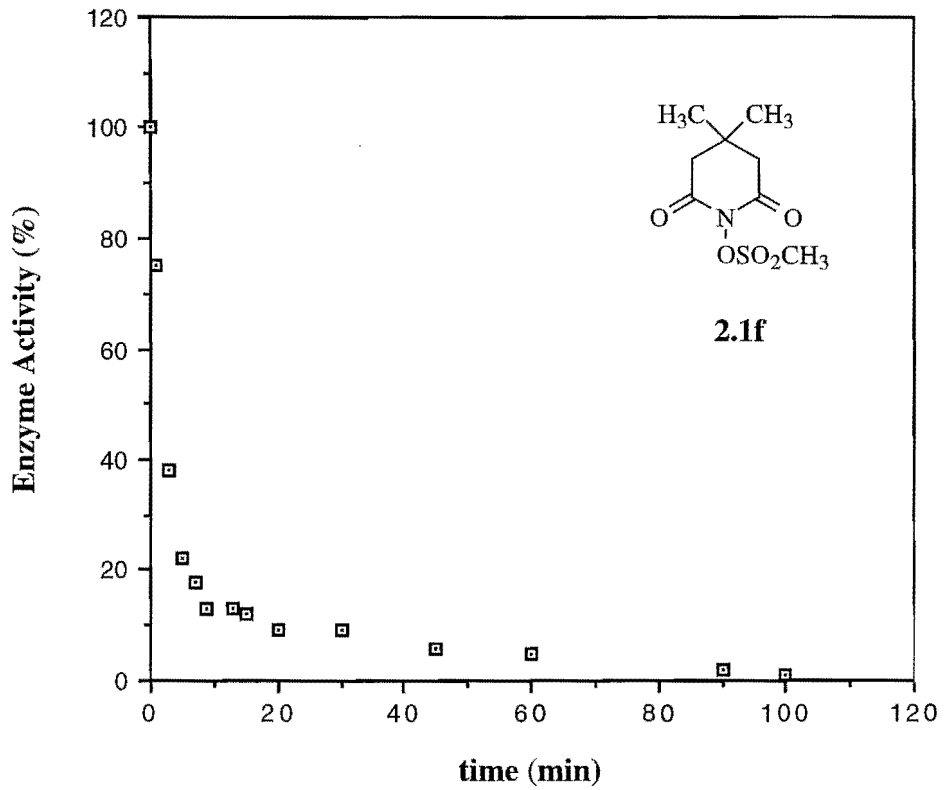


Figure 3.8 Time-dependent inactivation of α-chymotrypsin by **2.1f**. α-Chymotrypsin (4μM) was incubated with **2.1f** (100μM) and enzyme activity assayed using *N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (117μM).

Figures 3.9 and **3.10** display logarithmic plots obtained from the activity versus time data of **Figure 3.8** firstly over the full time course of the assay (100 minutes) and then over the initial nine minutes.

Observation of the logarithmic plot displayed in **Figure 3.9** indicates there is deviation away from the psuedo first-order kinetics with time. The logarithmic plot displays three phases. Initially psuedo-first order kinetics is observed. There is no "lag phase" indicating immediate inhibition of α-chymotrypsin is due to the *N*-

mesylglutarimide rather than a metabolite. This initial inhibition phase is followed by an upward deviation away from psuedo-first-order kinetics *i.e.* the rate of inactivation decreases with time. This may be accounted for by depletion of inhibitor due to turnover. The final phase depicts a downward deviation indicating a second period of inactivation of the enzyme at a slower rate than that observed initially. These observations are consistent with possibly a hydrolysis product of the *N*-mesylglutarimide being a potential inhibitor of α-chymotrypsin. Over the time course of the assay this species has increased beyond a threshold concentration such that has returned to inactivate the enzyme.

Given that there is deviation from psuedo-first order kinetics as the time course of the assay increases linear regression analysis of data to determine k_{obs} was carried out only over the initial portion of the logarithmic plot of **Figure 3.9** as shown in **Figure 3.10**. The initial data points are more dependable as indicated by comparison of the correlation coefficients of the regressions carried out for the plots of **Figures 3.9** (r^2 = 0.827) and **3.10** (r^2 = 0.965). Analysis of the data in **Figure 3.10** gives a value of 39 $M^{-1}s^{-1}$ for $k_{obs}/[I]$ for derivative **2.1f** when *N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide was used as the incubation assay substrate.

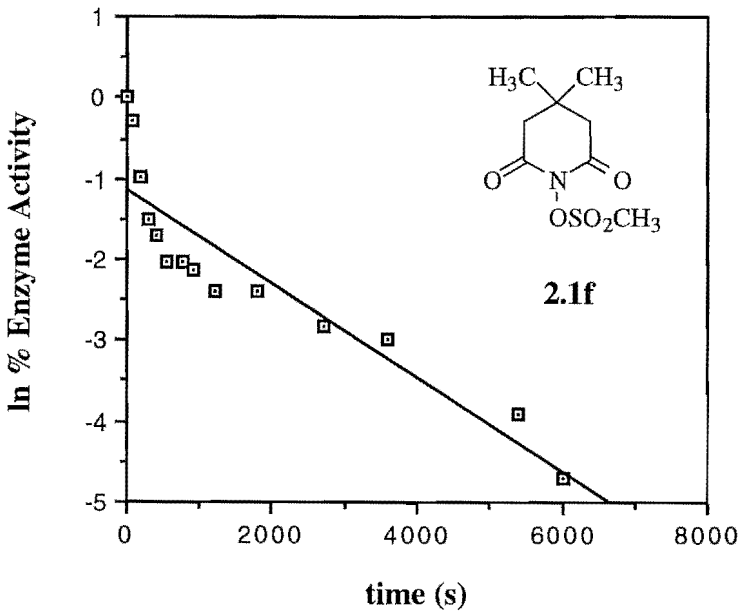


Figure 3.9 Logarithmic plot of enzyme activity versus time over 100 minutes for the data displayed in **Figure 3.8**.

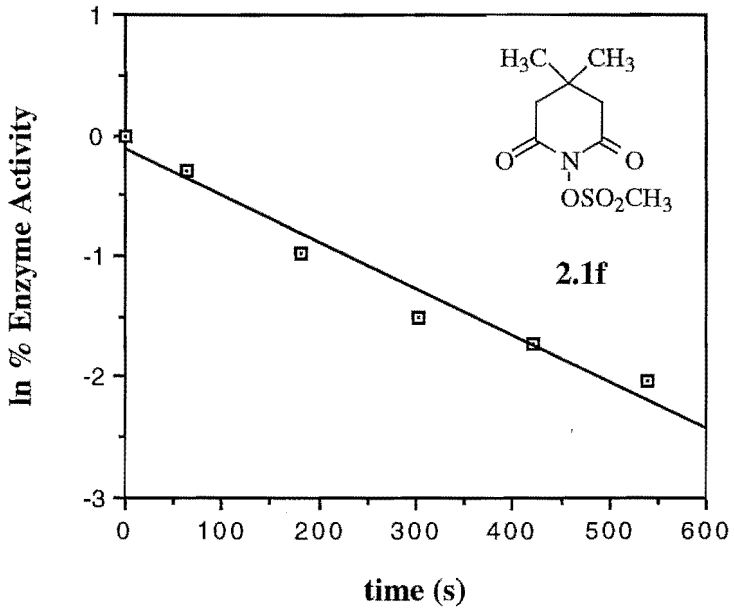


Figure 3.10 Logarithmic plot of enzyme activity versus time over nine minutes for inhibition data displayed in **Figure 3.8**.

The inhibition of α-chymotrypsin by **2.1f** was then studied at different inhibitor concentrations at 15-, 20-, 25-, 30-fold excess over enzyme with the ultimate aim of determining a rigorous value for k_{inact}/K_I for this inhibitor. The trend of the k_{obs} values with inhibitor concentration is shown below (see **Figure 3.11**).

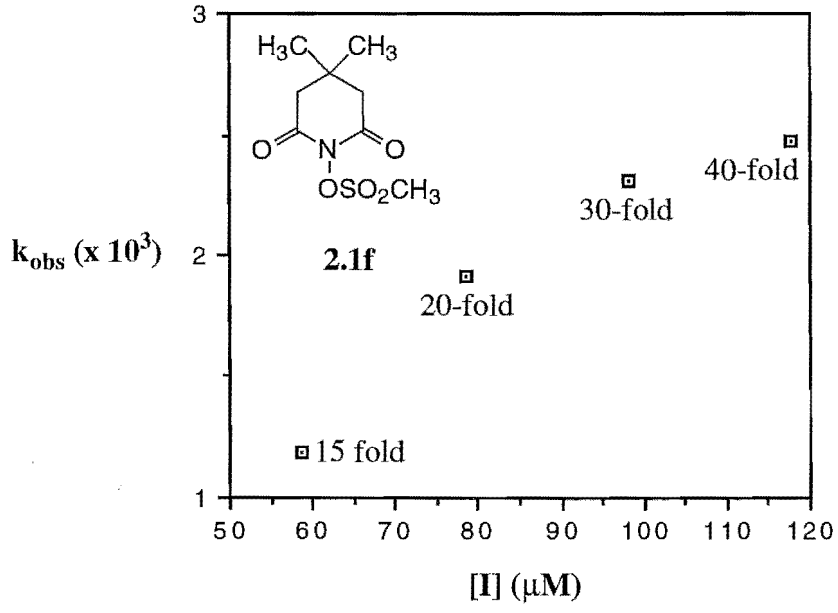


Figure 3.11 Plot of k_{obs} versus $[I_o]$ for compound **2.1f**

As observed from **Figure 3.11** the value for k_{obs} is increasing with inhibitor concentration but the graph is characterized by an asymptote indicating there is a maximum value for k_{obs}. This results confirms that saturating kinetics are being observed for the inhibition of α-chymotrypsin by compound **2.1f** which is one of the features that identifies true mechanism-based inactivation (see **Section 3.1** for a discussion).

The analysis of this data using the Kitz and Wilson plot described previously (**Section 3.2**) for determination of k_{inact}/K_I is shown below (**Figure 3.12**).

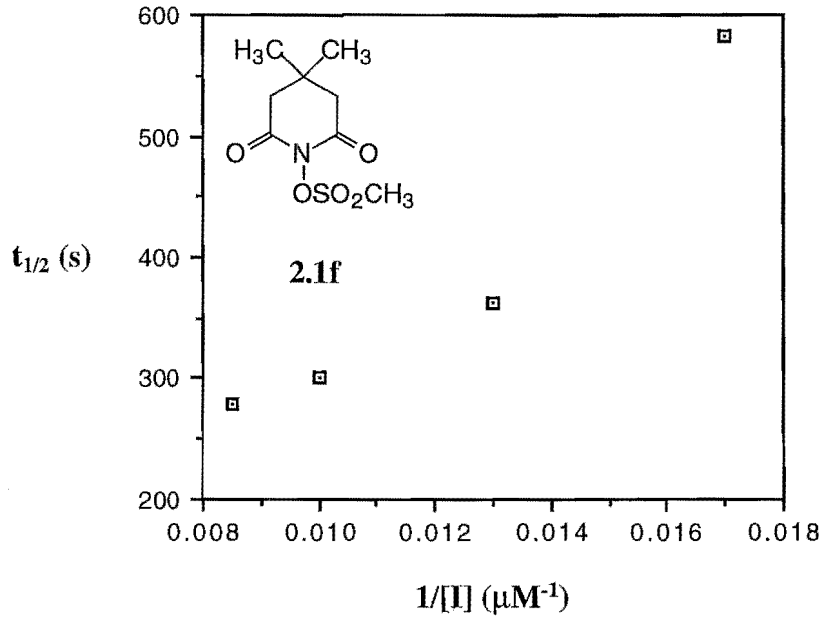


Figure 3.12 Kitz and Wilson plot for inhibition of α-chymotrypsin by **2.1f**

The above plot is of the type obtained when non-saturating conditions are prevalent. Linear regression analysis of this plot gives the y-intercept as negative and hence a value for k_{inact}/K_I cannot be obtained. This result may be explained in the terms that the k_{inact} for the compound is high, however, analysis by the incubation method has shown this compound to be a poor inhibitor which would normally possess a relatively low k_{inact} value. The error for each individual calculation of $t_{1/2}$ at each of the four inhibitor concentrations is high (± 15%) and will contribute to the non-saturating shape of the graph. However the above graph is distinguished by a sharp rise in $t_{1/2}$ at a lower inhibitor concentration and therefore it could be considered that analysis at this inhibitor concentration ($[I_0]$ = 59mM, 15-fold excess over enzyme) has been undertaken in non-saturating conditions.

A Kitz and Wilson plot of the first three data points of **Figure 3.12** which were deemed to be obtained from inhibition experments carried out at saturating inhibitor concentrations is shown below (**Figure 3.13**).

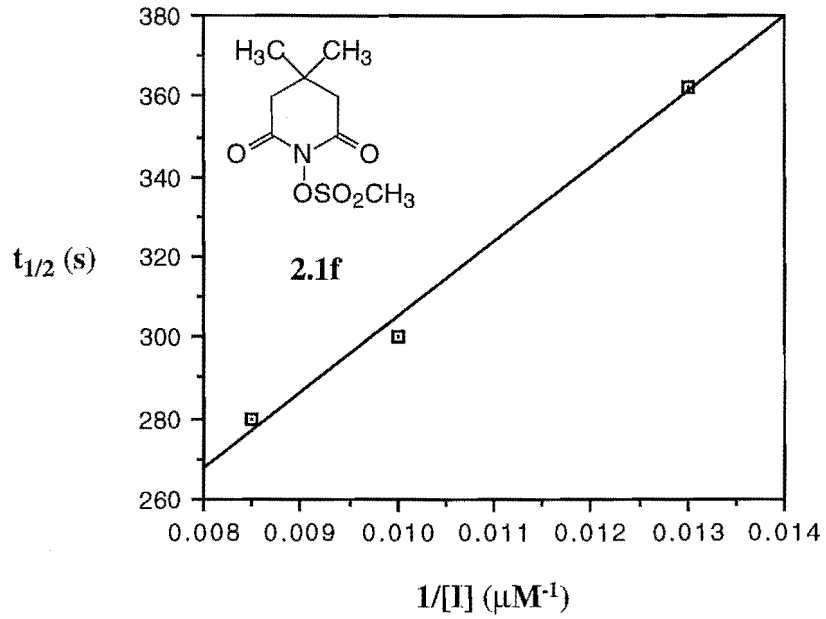


Figure 3.13 Kitz and Wilson plot for inhibition of α-chymotrypsin by **2.1f** at saturating conditions

The above plot is of the type obtained at saturating conditions permitting analysis of k_{inact}/K_I which was determined to be 37 $M^{-1}s^{-1}$ (k_{inact} = 0.006 s^{-1}, K_I = 156 μM). A plot of $[I]/k_{obs}$ versus [I] also gave a value of 37 $M^{-1}s^{-1}$.

This highlights the caution required when using $k_{obs}/[I]$ values as approximations for k_{inact}/K_I. For a poor inhibitor, the $k_{obs}/[I]$ values calculated from inhibition experiments where the inhibitor concentration is less than the K_I serve as an approximation for k_{inact}/K_I. The data points of **Figure 3.13** were obtained from inhibition experiments carried out at inhibitor concentrations 20-fold (78μM), 25-fold (98μM), and 30-fold (117μM) excess over enzyme. The $k_{obs}/[I]$ values calculated from these experiments were 24 $M^{-1}s^{-1}$, 24 $M^{-1}s^{-1}$ and 21 $M^{-1}s^{-1}$ respectively. These inhibitor concentrations are lower than the K_I calculated for **2.1f** (156μM) but the $k_{obs}/[I]$ values poorly approximate k_{inact}/K_I.

In a series of other experiments a determination of k_{inact}/K_I was undertaken using the progress curve method. A plot of $[I]/k_{obs}$ versus [I] gave a plot typical of non-saturating conditions hence k_{inact}/K_I was unable to be calculated. However a value for k_{inact} (0.003s^{-1}) was obtained from the slope of this graph and is of the same magnitude

of that value obtained from the incubation method above (k_{inact} = 0.006s^{-1}). On this basis there appears to be a good agreement between the two methods for compound **2.1f**.

ALTERNATE SUBSTRATE INHIBITORS

Alternate substrate inhibitors[71,80,97(a),197] function as substrates of the target enzyme; however instead of chemically reacting with the enzyme, the alternate substrate becomes bound so tightly to the active site that further access by natural substrate molecules is prevented.

Compounds **1.41** and **2.1** may act as alternate substrate inhibitors if on formation of the acyl enzyme the Lossen rearrangement is unable to occur. In this case the enzyme would remain inhibited for the lifetime of the acyl enzyme species. The goal therefore in the design of alternate substrate inhibitors is to prolong the life span of the acyl enzyme by hindering the deacylation process using strategies based on either electronic or steric reasoning.[189] If the deacylation process is rapid then the inhibitors will merely be acting as substrates of the enzyme.

Compounds **1.41e** and **1.41h** displayed activity time profiles consistent with alternate substrate inhibitors that only transiently inhibit the enzyme. As the results obtained on assay of compound **1.41e** (**Figure 3.14**) show there is initial rapid inhibition of the enzyme the rate of inhibition increasing for increased inhibitor concentration (compare graphs A and B). The enzyme then remains inhibited for a period of time dependent on the initial concentration of inhibitor ($[I_o]$) used in the experiment. When $[I_o]$ was ten-fold excess over enzyme this period was *ca* 7minutes but increased to ten minutes when $[I_o]$ was increased to 25-fold excess over enzyme. During this period the inactive enzyme is actively being hydrolysed but there still remains enough inhibitor to elicit inactivation until a point where this is depleted and enzyme activity is regained.

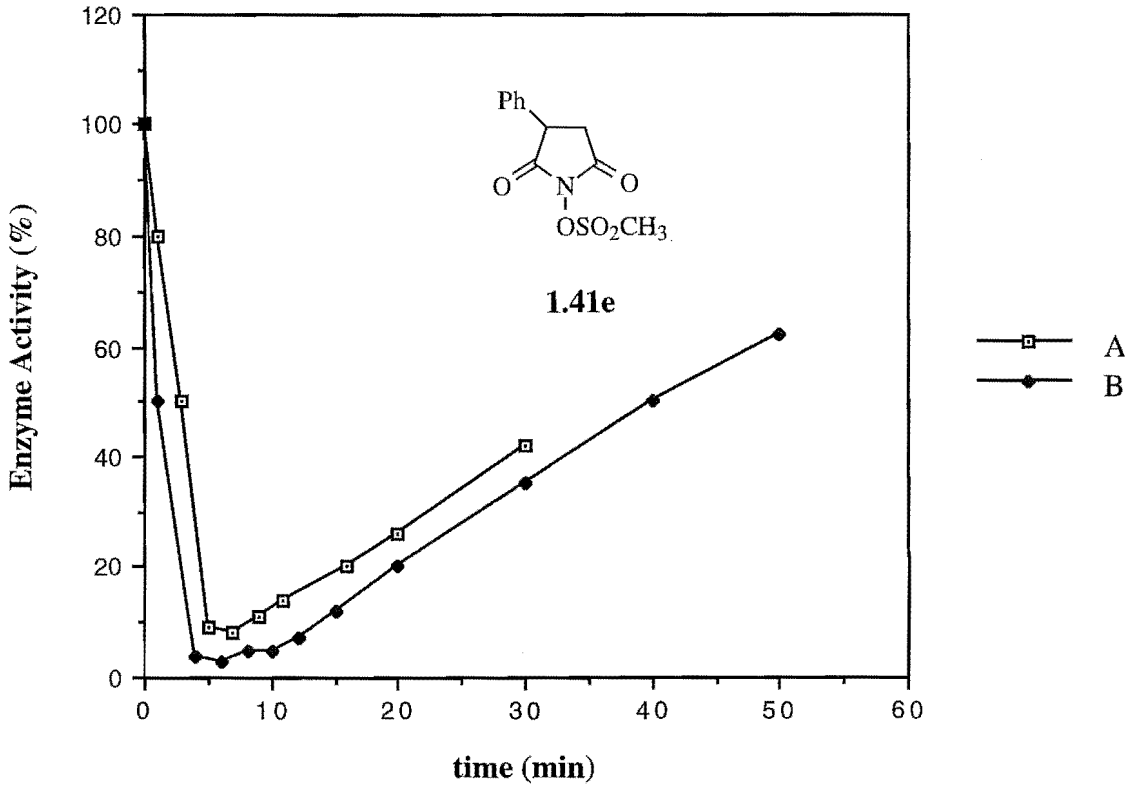


Figure 3.14 Assay of inhibitory activity of compound **1.41e** against α-chymotrypsin. α-Chymotrypsin (4μM) was incubated with **1.41e** (A, 40μM; B, 100μM) in 0.1M phosphate buffer, pH = 7.2, and 2.5% CH_3CN. Aliquots (25μL) were removed periodically and assayed for activity for enzymic activity using *N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (A, B 117μM) according to the incubation assay procedure discussed previously.

POTENT INHIBITORS

The rate of inhibition of α-chymotrypsin by compounds **1.41d**, **1.41f**, **1.41g**, **1.41h**, **1.41j**, **1.41k**, **1.41m**, **1.41n**, **2.1a**, **2.1c**, **2.1d**, **2.1e** was too fast to be determined using the incubation assay indicating that these were potent inhibitors. To obtain a measurable rate the inhibitor concentration was reduced but even at the lower inhibitor concentrations employed some inhibitors still inactivated α-chymotrypsin at a rate too rapid to be measured by the incubation assay. With other inhibitors an increased number of assay points was able to be obtained however substantial deviation from psuedo first order kinetics was observed.

In another attempt to determine a value for the inhibitory activity of these potent inhibitors using the incubation method the sampling periods were changed for analysis.

In a typical incubation experiment aliquots of the enzyme-inhibitor assay mixture were withdrawn at 1, 3, 5, 7, 9 *etc.* minute intervals. To obtain a greater number of data points for the initial period of inhibition aliquots were withdrawn at smaller time intervals requiring that the duration of the data set collection for the calculation of v_t, be decreased from 80s to 50s. The inhibitory activity of **1.41d** was assayed in this manner (**Figure 3.15**) and the subsequent logarithmic plot is shown below (**Figure 3.16**).

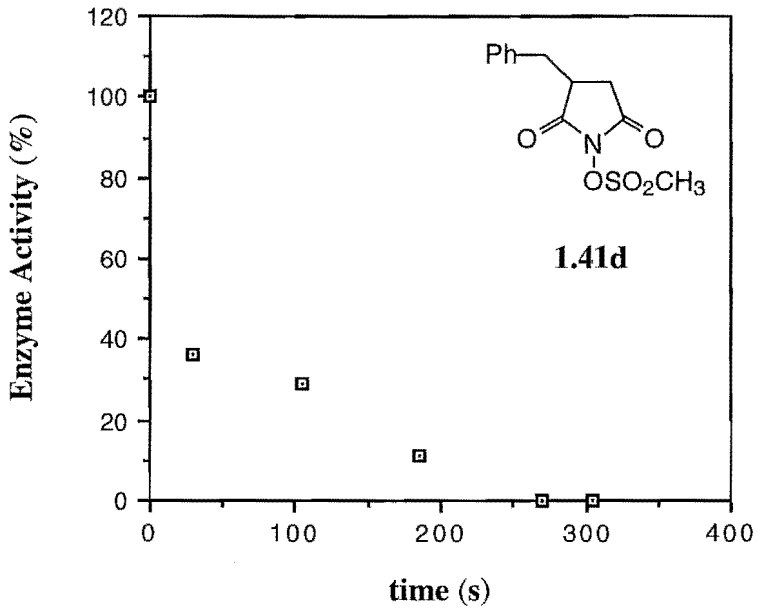


Figure 3.15 Assay of inhibitory activity of compound **1.41d** against α-chymotrypsin. Incubation of α-chymotrypsin (4mM) with **1.41d** (21mM, 5-fold excess over enzyme) in 0.1M phosphate buffer, pH = 7.2, with 2.5% CH_3CN. Aliquots were withdrawn at t = 0.5, 1.75, 3min intervals before the enzyme was totally inactivated.

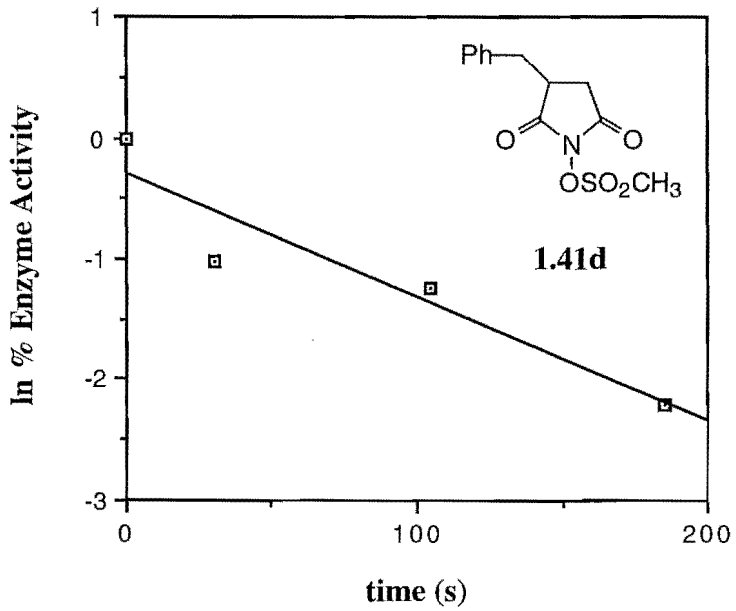


Figure 3.16 Logarithmic plot of enzyme activity versus time over 200 seconds for the data displayed in **Figure 3.15**.

A logarithmic plot with negative slope is obtained but as alluded above there is substantial deviation away from psuedo first-order kinetics (r^2 = 0.886). Therefore $k_{obs}/[I]$ (502 $M^{-1}s^{-1}$) will serve as a poor approximation of k_{inact}/K_I, but none the less is a useful figure as it serves as a lower limit for k_{inact}/K_I. Comparison with the value obtained for the glutarimide inhibitor **2.1f** (k_{inact}/K_I = 37 $M^{-1}s^{-1}$, see previous section) indicates that **1.41d** is at least 13-fold more potent than **2.1f**. Both **1.41d** and **2.1f** contain a mesylate leaving group hence the difference in inhibitory activity is related to the different ring size and substitution possessed by these compounds.

The activity versus time profile of the inhibition of α-chymotrypsin by compound **2.1d** (**Figure 3.17**) serves to illustrate the activity displayed by compounds that were found to be potent inhibitors over an extended period of time.

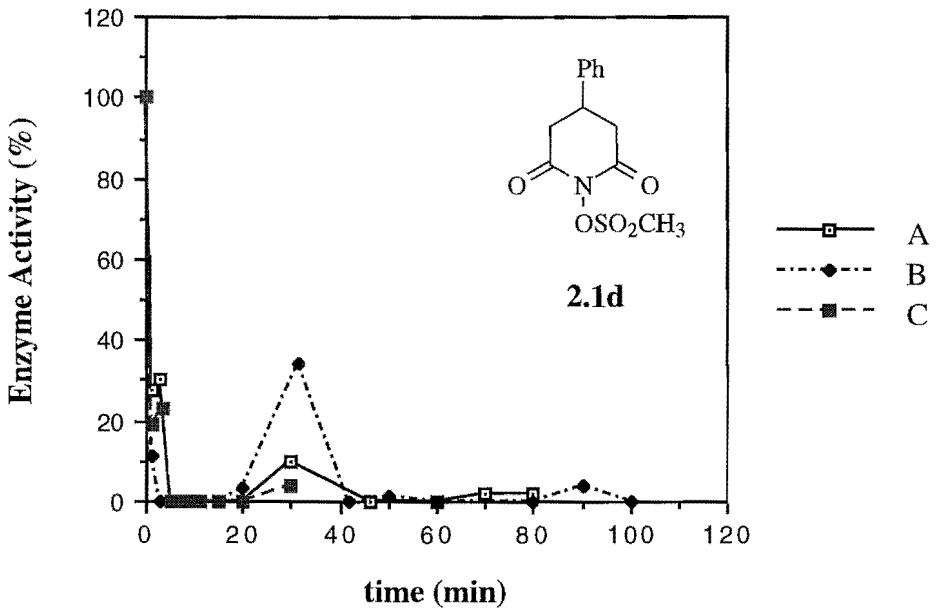


Figure 3.17 Assay of inhibitory activity of compound **2.1d** against α-chymotrypsin. Incubation of α-chymotrypsin (4μM) with **2.1d** (A, 10μM, 3-fold excess over enzyme; B, C, 20μM, 5-fold excess over enzyme) in 0.1M phosphate buffer, pH = 7.2 with 2.5% CH_3CN. Aliquots (25μL) were withdrawn periodically and assayed for enzymic activity using BTEE employing the incubation method.

Incubation assay of compound **2.1d** carried out at an inhibitor concentration five-fold excess over enzyme (data sets B and C of **Figure 3.17**) resulted in a rapid loss of

enzyme activity followed by a partial regain in enzyme activity (*ca* t = 20-30min) then a second rapid and ireversible loss of activity. A subsequent experiment was carried out at a lower inhibitor concentration (three-fold excess over enzyme) (**Figure 3.17**, Data Set A) in an attempt to lower the rate of inactivation and hence obtain data suitable for analysis. Less rapid inactivation was observed (<1% activity remaining after seven minutes) followed by a smaller recovery of enzymic activity compared to experiments B and C. After this partial regain in enzymic activity a second irreversible loss was also observed.

These results are similar to those obtained for the assay of compound **2.1f** (see **Figures 3.8-3.10**) and support an inhibition scenario where initial inactivation of α-chymotrypsin is due to the unreactive imides **1.41** and **2.1** acting as "true" mechanism-based inactivators. Hydrolysis of the inactive enzyme and inhibitor results in a temporary regain in enzyme activity followed by a second but less rapid phase of inactivation thought to be due to a hydrolysis product of the imides. These rates of hydrolysis appear to be greater for more potent inhibitors. For example, there is a smaller recovery of enzyme activity for the inhibition of α-chymotrypsin by **2.1d** at three-fold excess over enzyme than at five-fold excess (compare experiment A with B and C of **Figure 3.17**). To whit there was only a slight recovery of enzyme activity observed on assay of compound **2.1f** (see **Figures 3.8** and **3.9**).

Assay of **1.41d** using the incubation method gave results indicating that the rate of inactivation of α-chymoptrypsin by this compound was too rapid to measured by this technique. The inhibitory activity was then evaluated using progress curve kinetics. Groutas *et al*[84] have previously assayed **1.41d** using this technique obtaining a value for $k_{obs}/[I]$ of 9 000 $M^{-1}s^{-1}$. This experiment was repeated and the results are shown below.

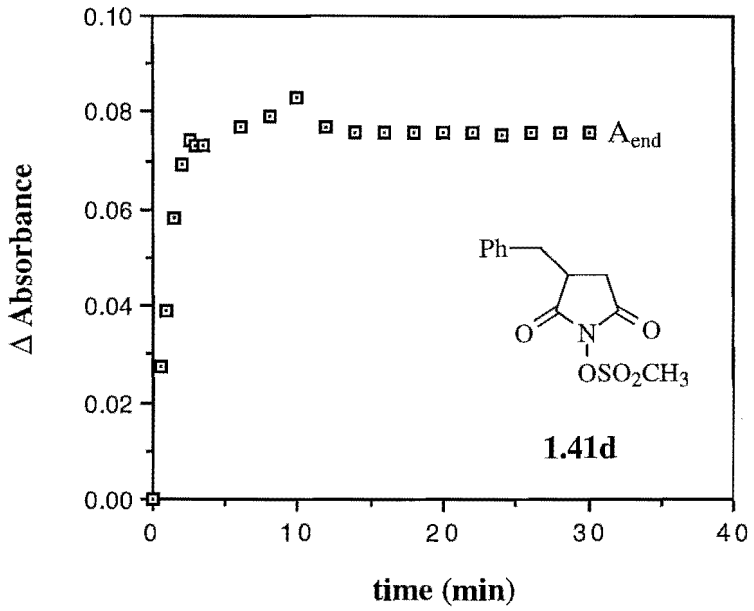


Figure 3.18 Progress curve assay of inhibitory activity of α-chymotrypsin of compound **1.41d** against α-chymotrypsin. α-Chymotrypsin (0.073μM) was added to an incubated solution of **1.41d** (0.189μM; 3-fold excess over enzyme) and BTEE (117μM). Reaction progress was monitored by change in absorbance at λ = 256nm.

Analysis of this curve according to the graphical method of Tsou is shown below (**Figure 3.19**).

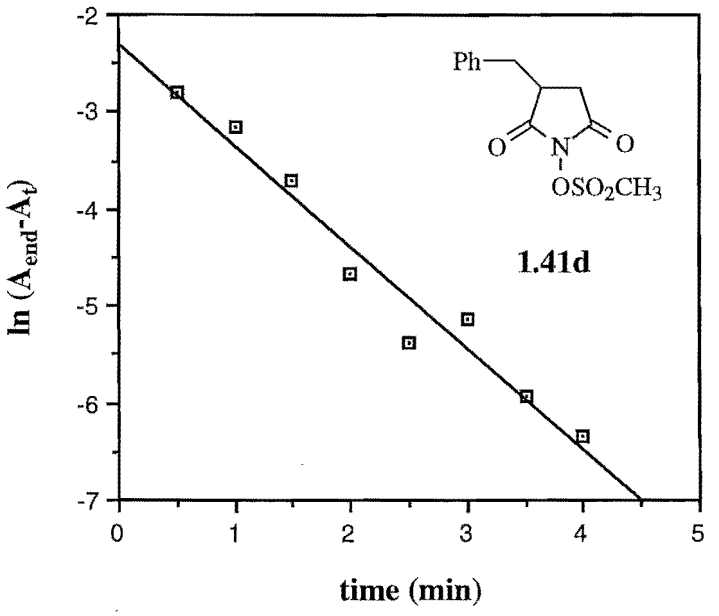


Figure 3.19 Initial portion of Tsou plot obtained for absorbance curve (see **Figure 3.18**)

There is a good correlation between the data and the kinetic model ($r^2 = 0.962$) and a value for $k_{obs}/[I]$ of 64 497 $M^{-1}s^{-1}$ ± 10% was obtained. The ± 10% error in this measurement, which is typical of progress curve measurements, was determined by addition of the error evaluated by the regression process in the calculation of k_{obs} (± 5%) and that in the experimental measurement of the inhibitor concentration, which incorporated weighing of the inhibitor to prepare the stock solution and subsequent dilution of this as required by each inhibition experiment.

It appears therefore that the progress curve measurements are more accurate than those obtained with the incubation method where errors of up to ± 15% were calculated for determination of k_{obs} which reflects the greater number of experimental measurements that need to made to determine k_{obs} when using the incubation method. However results were obtained for identical progress curve inhibition experiments that were far beyond within 10% of each other even though the same buffer, enzyme, inhibitor and substrate solutions had been used. It is considered that the extent of mixing and the time taken to achieve this are of critical importance in obtaining accurate measurements. Even though a mixing time of not more than 20 seconds was strictly adhered to the extent of mixing is a hard variable to control. To minimise the error in measurements experiments were repeated so that two values for k_{obs} within 10% of each other were obtained.

The fact that the logarithmic plots conceal alot of error in progress curve measurements contributes to the difference in the value we obtained for the inhibition of α-chymotrypsin by **1.41d** ($k_{obs}/[I] = 64\ 497 M^{-1}s^{-1}$) to that obtained by Groutas *et al*[84] ($k_{obs}/[I] = 9\ 000\ M^{-1}s^{-1}$).

These values differ by an order in magnitude and illustrate the care that needs to be taken when comparing inhibition results between laboratories.

Of the experimental details offered by Groutas *et al*[84] in publishing their $k_{obs}/[I]$ value the only difference between the experimental conditions we employed is the use of DMSO as opposed to CH_3CN however the volume of these solvents used in the assay is small and is considered to have a negligible effect compared to other assay variables. These include inhibitor purity and the freshness of the solutions used in the assay; the loss of enzyme activity of a stock solution of enzyme in phosphate buffer was noted and stock solutions of BTEE should be prepared daily.[195b] The above experiments have also been run at low inhibitor concentration of 3-fold excess over enzyme and hence there will be deviation away from the kinetic model used; this will also contribute to the discrepancy between the two results.

As stressed earlier it is important that inhibition experiments are carried out at saturating inhibitor and substrate concentrations for the steady-state assumption of the kinetic model to remain valid. It is quoted that less than ten percent of the substrate should undergo hydrolysis during the assay so that the steady-state for enzyme-substrate reaction of an inhibition experiment does not differ from that of the control experiment.[198] The extent of hydrolysis can be calculated directly from the absorbance versus time curve using the Beer-Lambert Law. Calculation for the above curve (**Figure 3.18**) indicates that 70% of BTEE has undergone hydrolysis.

An experiment run at this low inhibitor concentration could be considered to be overstretching the steady-state assumption of the kinetic model even though other researchers have reported assay conditions with similar low inhibitor excesses. It has been shown that an inhibitor with a low partition ratio can be analysed at low inhibitor excess with little deviation away from psuedo first-order kinetics.[25] There is evidence to suggest, however, that significant "turnover" pathways for *N*-acyl and sulfonyloxy imide inhibitors **1.41** and **2.1** exist and hence their partition ratios will be high.

For accurate measurement of $k_{obs}/[I]$ using the progress curve method high concentrations of inhbitor will have to be used to achieve the all important saturating conditions. This was observed in the inhibition of α-chymotrypsin by compound **2.1f** where a Kitz and Wilson plot to test for saturating conditions gave a result indicative of non-saturating conditions when low inhibitor concentrations were employed (see **Figure 3.12**).

The results of the inhibition of α-chymotrypsin by **1.41d** at higher concentrations is shown below (**Figure 3.20**).

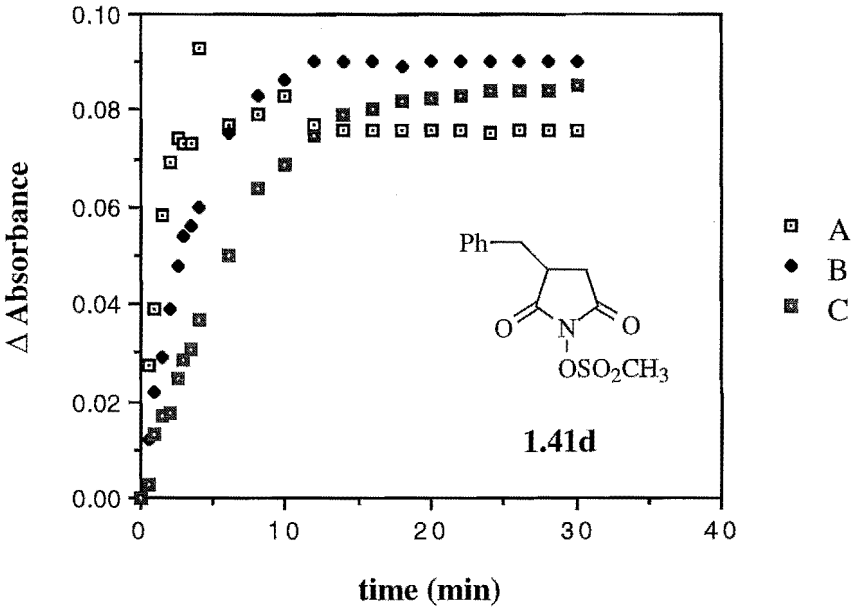


Figure 3.20 Progress curves for inhibition of α-chymotrypsin by **1.41d**. α-Chymotrypsin (0.073μM) was added to an incubated solution of **1.41d** (A, 0.189μM, 3-fold excess over enzyme; B, 0.365mM, 5-fold excess; C, 0.949μM, 13-fold excess) and BTEE (117μM). Reaction progress was monitored by change in absorbance at λ = 256nm.

From **Figure 3.20** it can be observed that curves B and C fall over more sharply than does curve A. Hence the rate of inhibition of α-chymotrypsin is more rapid at higher concentrations. Analysis of curve B gives $k_{obs}/[I]$ = 17 790 $M^{-1}s^{-1}$ (r^2 = 0.989) and $k_{obs}/[I]$ = 3 562 $M^{-1}s^{-1}$ for curve C (r^2 = 0.993) and as alluded the correlation between data and model is better for higher inhibitor concentrations. Suprisingly the A_{end} values are higher for these curves than that of curve A suggesting the extent of substrate hydrolysis is greater. Curve C has the appearance of a curve associated normally with reversible inhibition. These results suggest that the inhibitor is being depleted significantly during the course of the experiment making the maintainence of saturating conditions difficult for this inhibitor. This is confirmed by the Tsou plot of $[I]/k_{obs}$ versus [I] for **1.41d** (**Figure 3.21**).

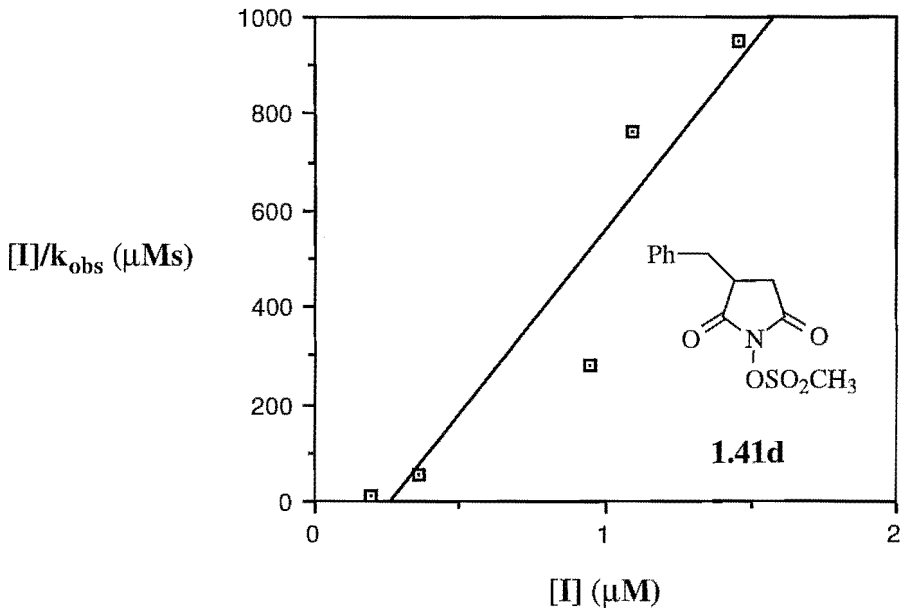


Figure 3.21 Tsou plot of $[I]/k_{obs}$ versus [I] for **1.41d**

The above plot is of the type obtained when non-saturating conditions are prevalent characterized by a poor correlation ($r^2 = 0.886$). The y-intercept is negative and hence a value for k_{inact}/K_I can not be obtained however a value for k_{inact} ($0.0013s^{-1}$) was obtained from the slope which provides valuable information regarding the inhibition process.

The graph above however is distinguished by a sharp rise in $[I]/k_{obs}$ at higher inhibitor concentrations and therefore it could be considered that these have been obtained at the necessary saturating conditions required for analysis. A plot of these two data points cut the positive y-axis and subsequently an approximate value of k_{inact}/K_I was found to be 4 653 $M^{-1}s^{-1}$. Comparison with the k_{inact}/K_I value obtained for the glutarimide inhibitor **2.1f** (k_{inact}/K_I = 37 $M^{-1}s^{-1}$) indicates that **1.41d** is 100-fold more potent. Comparison of the k_{inact} and K_I values obtained for these inhibitors provides the valuable insight that **1.41d** owes its increased potency to greater binding affinity for α-chymotrypsin.

To allow an accurate k_{inact}/K_I analysis a number of experiments have to be carried out at high inhibitor concentration however this was found to be excruciatingly frustrating to achieve as illustrated by the progress curve analysis of compound **1.41g**.

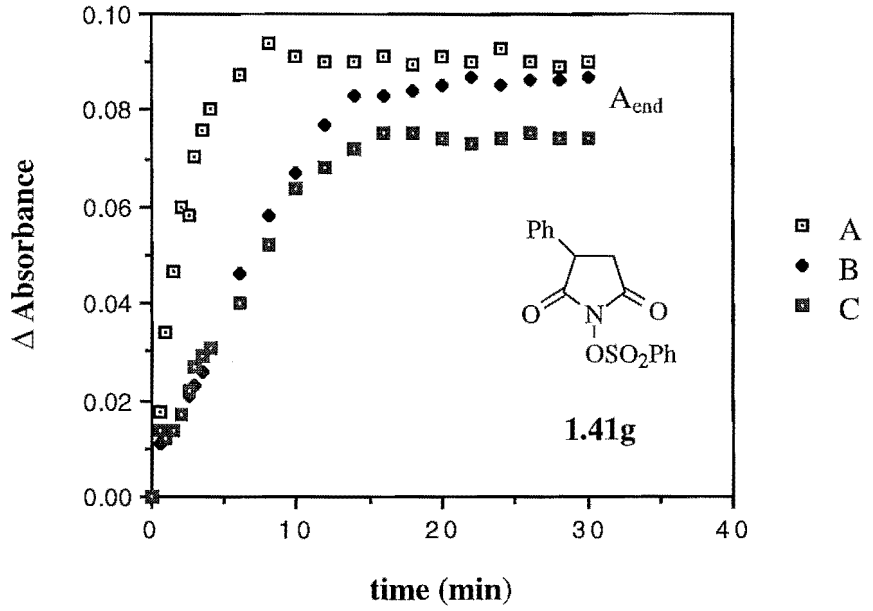


Figure 3.22 Progress curves for inhibition of α-chymotrypsin by **1.41g**. α-Chymotrypsin (0.073μM) was added to an incubated solution of **1.41g** (A, 0.189μM, $[I_o]/[E_o]$ = 3; B, 0.365mM, $[I_o]/[E_o]$ = 5; C, 0.55mM, $[I_o]/[E_o]$ = 8) and BTEE (117μM). Reaction progress was monitored by change in absorbance at λ = 256nm.

Figure 3.22 displays the progress curves obtained for inhibition of α-chymotrypsin by **1.41g** obtained at three different inhibitor concentrations. As the inhibitor concentration increases the curves fall over more steeply as seen in the trend from curve A to curve C. The initial rates of reaction are also observed to decrease reflecting increased rate of inhibition and the A_{end} value is lower hence less substrate has been hydrolysed. Therefore saturating conditions are more likely to be prevalent in the inhibition experiment C than A or B.

This is confirmed by the Tsou plot of $[I]/k_{obs}$ versus [I] for **1.41g** which has a negative y-intercept. As observed in the Tsou plot for **1.41d** (**Figure 3.21**) there is a sharp rise from a data point considered to be obtained in non-saturating conditions to data points obtained at saturating conditions. A plot of $[I]/k_{obs}$ versus [I] for these two points (not shown) also cuts the negative y-axis.

To determine k_{inact}/K_I for compound **1.41g** it was therefore necessary that experiments be carried out at higher inhibitor concentrations. However, in an experiment carried out at an inhibitor concentration ten-fold excess over enzyme the enzyme was found to be totally inactivated within the mixing period as characterized by a flat absorbance versus time graph (see graph **C** of **Figure 3.6**). It was not possible,

therefore, to obtain a value of k_{inact}/K_I for this compound as the rate of inhibition at saturating inhibitor concentrations was too rapid to be measured.

These results indicate that the progress curve method is inadequate for measuring the inhibition of α-chymotrypsin by **1.41f**. The potent activity displayed by **1.41f** is such that only the activity at low inhibitor concentrations can be measured however these measurements will be inaccurate and deviate away from the pseudo first-order kinetics of the model. An accurate measurement of the rate of inhibition of α-chymotrypsin by compound **1.41g** in order to determine k_{inact}/K_I would require the use of stopped-flow methods.

The results described above still, however, provide a qualitative description of structure and activity relation when compared to those obtained for other inhibitors at the same concentration. For example, the inhibition experiment carried out for **1.41d** at an inhibitor concentration ten-fold excess over enzyme resulted in a normal progress curve (see progress curve C of **Figure 3.20**) whereas, as above, **1.41g** totally inactivated α-chymotrypsin at this concentration. Therefore the inhibition of α-chymotrypsin by **1.41g** is more rapid than that of **1.41d**.

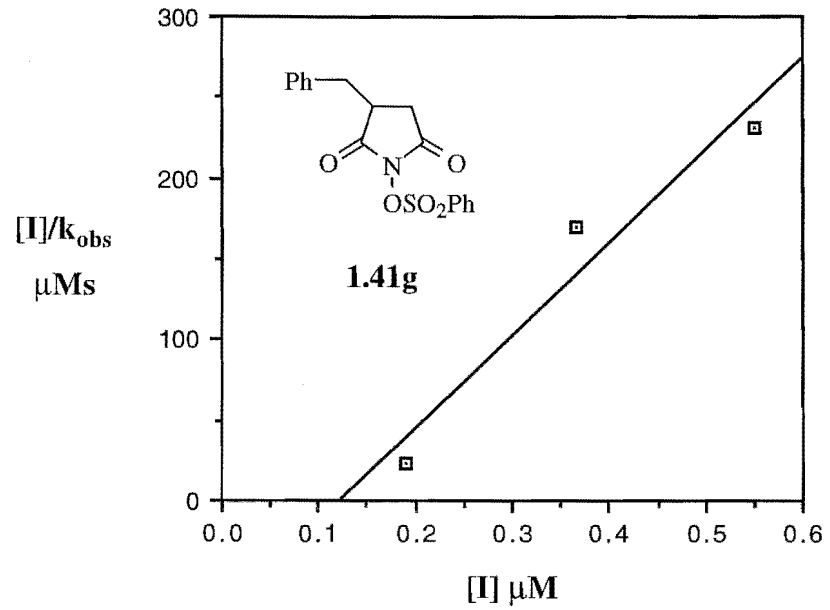


Figure 3.23 Tsou plot of $[I]/k_{obs}$ versus [I] for **1.41g**

For an accurate measurement of k_{inact}/K_I we require k_{obs} values at saturating inhibitor concentrations. Accuracy will be improved if a greater number of inhibition concentrations are used. A larger data set has been obtained for the glutarimide inhibitor **2.1g**, with [I]/kobs values being obtained from inhibitor concentrations ranging from

0.189μM (three-fold excess over enzyme) to 18.25μM (250-fold excess over enzyme). The Kitz and Wilson plot for **2.1e** is shown below (**Figure3.17**).

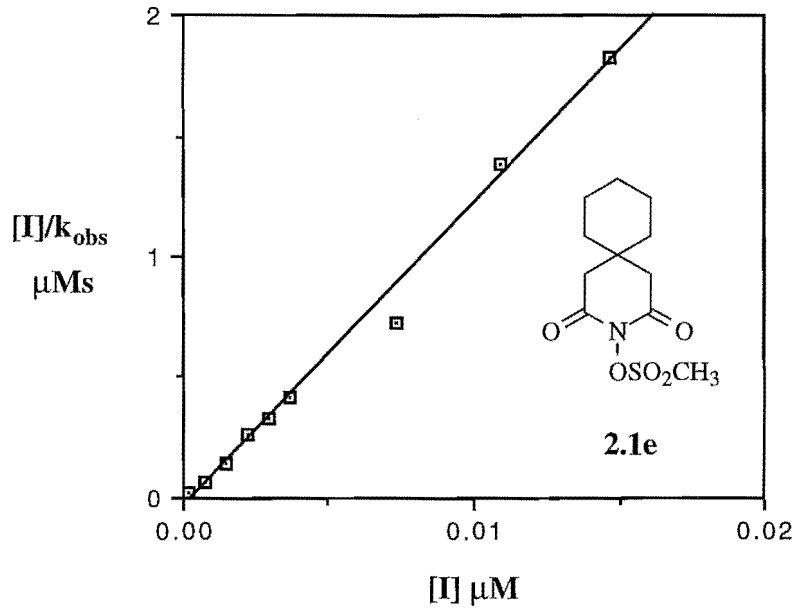


Figure 3.24 Tsou plot of [I]/k_{obs} versus [I] for **2.1e**

Again a straight line with negative slope is obtained from which a value for k_{inact} ($0.009s^{-1}$) can be obtained however the y-intercept is not positive, which does not permit analysis of k_{inact}/K_I.

The results discussed for the succinimide inhibitors **1.41d** and **1.41f** and the glutarimide inhibitor **2.1g** were representative for the other potent inhibitors of type **1.41** and **2.1**. Due to the inability to calculate k_{inact}/K_I which was to be used as the measure of inhibitory activity providing the basis for structure activity comparison other means have to be used.

As discussed earlier $k_{obs}/[I]$ is conventionally used when comparing the potencies of inhibitors. A high $k_{obs}/[I]$ value for a compound indicates that it is a more potent inhibitor than a compound with a lower $k_{obs}/[I]$ value. The constant $k_{obs}/[I]$ is used routinely to compare compounds assayed using the incubation method however this term is not independent of inhibitor concentration for potent inhibitors assayed using the progress curve method; hence for a valid comparison to be made experiments for each inhibitor will have to be run at the same inhibitor concentration (an inhibitor concentration of ten-fold exces over enzyme was chosen for this study).

In noting the trends in $k_{obs}/[I]$ values for compounds assayed using the progress curve method we found that, suprisingly, those compounds which were determined to be more potent inhibitors by the incubation method possessed lower $k_{obs}/[I]$ values than those determined to be weaker. For example, compound **1.41d** could not be analysed accurately using the incubation method because it inactivated α-chymotrypsin rapidly within four minutes at an inhibitor concentration five-fold excess over enzyme. By decreasing the time intervals used in the sampling a lower limit for $k_{obs}/[I]$ was able to be calculated ($k_{obs}/[I] \approx k_{inact}/K_I$ = 502 $M^{-1}s^{-1}$) indicating that **1.41d** was at least 13-fold more potent than the glutarimide **2.1f** (k_{inact}/K_I = 37 $M^{-1}s^{-1}$). Compound **2.1f** only inactivated α-chymotrypsin within 100minutes at an inhibitor concentration 25-fold excess over enzyme. On progress curve analysis the converse trend in the $k_{obs}/[I]$ values was observed; **1.41d** (3 562 $M^{-1}s^{-1}$) versus **2.1f** (7 888 $M^{-1}s^{-1}$).

This trend is also observed in comparing the inhibitory activities of compounds **1.41d** and **2.1e**. Compound **2.1e**, like **1.41d**, could not be assayed accurately using the incubation method because it rapidly inactivated α-chymotrypsin. Assay at a low inhibitor concentration 3-fold excess over enzyme served to provide a lower limit for k_{inact}/K_I for the inhibition of α-chymotrypsin by **2.1e** ($k_{obs}/[I] \approx k_{inact}/K_I$ = 403 $M^{-1}s^{-1}$) hence **1.41d** ($k_{obs}/[I] \approx k_{inact}/K_I$ =502 $M^{-1}s^{-1}$) is more potent.

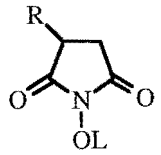
Progress curve assay of these compounds confirmed this result. At a concentration of twenty-fold excess over enzyme compound **1.41d** totally inactivated α-chymotrypsin within the mixing time. In comparison, at a concentration of **2.1e** 250-fold excess over enzyme α-chymotrypsin remained active. Based on these results **1.41d** is more potent than **2.1e** but this compound has a lower $k_{obs}/[I]$ value **1.41d** ($k_{obs}/[I]$ = 3 562 $M^{-1}s^{-1}$) versus **2.1e** ($k_{obs}/[I]$ = 14 321 $M^{-1}s^{-1}$).

A preliminary explanation for the reversal in trends for $k_{obs}/[I]$ may lie in consideration of the inhibitor concentration dependence for $k_{obs}/[I]$. For the mechanism-based inhibitors of type **1.41** and **2.1** studied $[I]/k_{obs}$ is observed to increase with inhibitor concentration. For $k_{obs}/[I]$ the converse will be observed *i.e.* $k_{obs}/[I]$ decreases at increased inhibitor concentration. A tentative hypothesis predicts a lower $k_{obs}/[I]$ value for a more potent inhibitor if for inhibition experiments run at the same inhibitor concentration the more potent inhibitor is acting as the weak inhibitor but at a higher concentration.

In the absence of a theoretical basis for an explanation as to why more potent inhibitors possess lower $k_{obs}/[I]$ values when assayed using progress curve kinetics this method for comparison has its basis in the empirical observations described for the representative inhibitors above.

In the following structure-activity discussion the activities of inhibitors have beencompared on the basis of $k_{obs}/[I]$ values obtained from inhibition experiments run at a ten-fold excess of inhibitor over enzyme where there exists no more rigorous information regarding the inhibition process. Hence compounds possessing a low $k_{obs}/[I]$ when assayed employing the progress curve are considered to be more potent inhibitors than compounds with high values.

3.5 STRUCTURE-ACTIVITY DISCUSSION



1.41

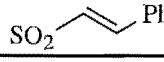
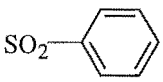
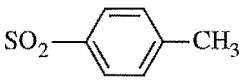
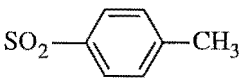
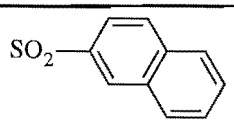
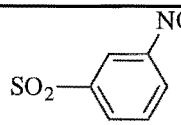
Compound number	R	L	k_{inact} (s^{-1})	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)
1.41d	$PhCH_2$	SO_2CH_3	0.0013	3 562[B]
e	Ph	SO_2CH_3	A	Alternate Substrate
f	$PhCH_2$		A	6 471[B]
g	$PhCH_2$		A	C
h	$PhCH_2$		A	C
i	Ph		A	Alternate Substrate
j	$PhCH_2$		0.0004[D]	826[B]
k	$PhCH_2$		0.003[D]	6 427[B]

TABLE 3.3 Inhibition of α-chymotrypsin by inhibitors of type **1.41**

A Unable to be calculated because alternate substrate inhibitor or inactivated α-chymotrypsin too rapidly at $[I_o/E_o] > 10$ for $k_{obs}/[I]$ values to be determined.

B Value obtained from progress curve analysis $[I_o/E_o] = 10$; $[E_o] = 0.073\mu M$; $[S] = 117\mu M$. The lower the value the more potent the inhibitor.

C Inhibition too rapid to be measured by progress curve method at $[I_o/E_o] = 10$; $[E_o] = 0.073\mu M$; $[S] = 117\mu M$.

D Value obtained from slope of Tsou plot of $[I]/k_{obs}$ versus [I] by linear regression analysis

In comparison to the "reference" inhibitor **1.41d** it can be seen that the incorporation of an aromatic substitutent can result in an increase in inhibitory activity. On the basis that both **1.41d** and **1.41f** totally inactivated α-chymotrypsin at an inhibitor excess 20-fold over enzyme these compounds are essentially equipotent however a dramatic increase in activity was observed with compounds **1.41g** and **1.41h**. Both inhibited α-chymotrypsin at a rate too fast to be measured at an inhibitor concentration ten-fold excess over enzyme suggesting that these have successfully harnessed the binding energy available in interacting with the S_n' subsites of α-chymotrypsin. Compound **1.41j** was very potent with a $k_{obs}/[I]$ value of 826 $M^{-1}s^{-1}$ (a low value indicates potent inhibition) but less so than **1.41g** and **h**. This perhaps suggests that its larger bulk dissuades favourable binding which corroborates with the result obtained with **1.41f**, also a bulkier inhibitor, which showed no enhancement in inhibitory activity relative to **1.41d**.

Compound **1.41k** was designed with the aim of enhancing the activity of **1.41g** as the increased electron withdrawing properties of the nitro group would be thought to increase inhibitory activity in two ways. The rate of the Lossen rearrangement has been determined to be related directly to the strength of the acid that is released during formation of the isocyanate.[86] It is considered that *m*-nitrobenzene sulfonic acid which is released during the inhibition of α-chymotrypsin by **1.41k** is a stronger acid than benzene sulfonic acid released during the inhibition cycle of **1.41g** due to the inductive effect of the nitro group. A more rapid Lossen rearrangement leads to an increase in k_{inact} resulting in more potent inhibition. The increased electron withdrawing ability of the nitro group is also thought to have a secondary influence on the potency of inhibitors by increasing the electrophilicity of the scissile carbonyl of the succinimide ring. This would activate formation of the acyl-enzyme again increasing k_{inact}.

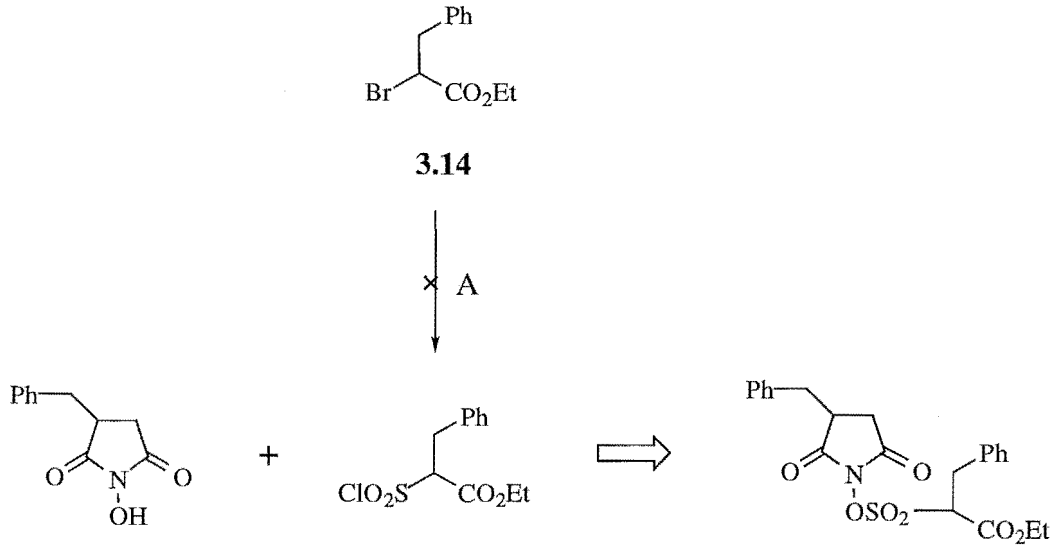
Despite the prediction of increased inhibitory activity the converse was observed, with **1.41k** found to be less potent than **1.41g** but comparable inactivity to **1.41f**. It must be noted that the increased electron withdrawing effect of the nitro group in **1.41k** may also increase the rate of deacylation so that it competes with the Lossen rearrangement such that k_{inact} may remain constant for **1.41k** and **1.41g**. A definitive comparison of the k_{inact} values for **1.41g** and **1.41k** was not possible however as the inhibition of α-chymotrypsin by **1.41g** was too rapid to measure. However a comparison of the k_{inact} values of compounds **1.41j** (k_{inact} = 0.0004 s^{-1}), which is thought to have similar electronic properties to **1.41g**, and **1.41k** (k_{inact} = 0.003 s^{-1}) does indicate there is a favourable ten-fold increase in k_{inact}. Despite this ten-fold increase compound **1.41g** is more potent, hence it is quite likely that compound **1.41k** lacks the topology to interact with α-chymotrypsin to the same extent as does **1.41g** hence the lower inhibitory activity.

Compound **1.41i** in comparison to compound **1.41k** has an electron donating methyl group on the phenyl ring. This compound was found to have comparable activity to **1.41g** hence the methyl group has no effect on inhibitory activity *i.e.* the electron donating and steric effects of this group are minimal.

Compounds **1.41e** and **1.41i**, both of which have a phenyl substituent (R) rather than the benzyl substituent of the compounds discussed above, were found only to be transient alternate substrate inhibitors of α-chymotrypsin. This result may be explained in the terms that the Lossen rearrangement to form the inactivating isocyanate has not occurred or if it has, the binding of these compounds to α-chymotrypsin has been such that the resulting isocyanate does not have the correct geometry or proximity to react with His-57. The Lossen rearrangement has been found to not only depend upon the strength of the departing acid as discussed above but also on the electron releasing power of the substitution present in the resultant isocyanate.[86] On this basis it may be considered that the benzyl substituent is more favourable in promoting the Lossen rearrangement and binds more effectively with the S_1 pocket of α-chymotrypsin. The results obtained with compounds **1.41e** and **1.41i** indicate that a benzyl substituent is required if a compound is to exhibit mechanism-based irreversible inhibition.

The increased activity of the aromatic compounds **1.41g** and **1.41h** relative to the simple derivative **1.41d** is almost certainly due to a favourable hydrophobic interaction between the phenyl ring contained within the fomer with either Phe-81 or Phe-192 which are located in the S_n' subsites of α-chymotrypsin.[199] To harness this available binding energy and in an attempt to further increase enzyme-inhibitor recognition compound **1.41l** (**Table 3.4**) was prepared which incorporated the design of the L group as an amino acid mimic. Initial design plans focused on the synthesis of the sulfonyl chloride **3.15**, a phenylalanine mimic, that on coupling to **2.25** would give compound **3.13** that possesses an aromatic substituent incorporated into an amino acid like substituent. An attempted synthesis of **3.13** using the Strecker reaction with the phenylalanine derivative **3.14** however was unsuccessful presumably due to the steric crowding of the α-centre of **3.14** a conclusion which is supported by documented low yields of the Strecker reaction when using secondary alkyl halides.[173]

As shown below in **Table 3.4** **1.41k**, which lacks the desired aromatic substitution, was found only to be a moderate inhibitor of α-chymotrypsin.



Scheme 3.7 A, 1) K_2SO_3, $EtOH/H_2O$, reflux 2) PCl_5, $POCl_3$, 70 °C

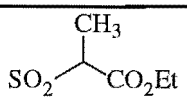
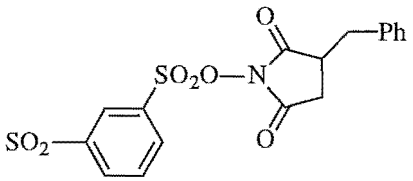
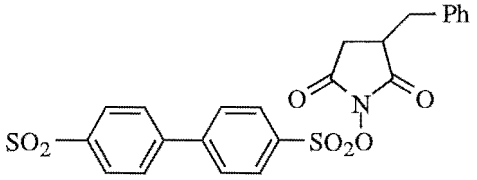
Compound number	R	L	k_{inact} (s^{-1})	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)
1.41l	$PhCH_2$		A	B
m	$PhCH_2$		0.001[C]	1 609[D]
n	$PhCH_2$		0.012[C]	12 589[D]

Table 3.4 Inhibition of α-chymotrypsin by derivatives of type **1.41**

A Unable to be calculated

B Value ($k_{obs}/[I]$ = 278 $M^{-1}s^{-1}$) obtained from incubation analysis using *N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide [I_o/E_o] = 10; [E_o] = 0.073μM; [S] = 117μM. Note that this value cannot be compared to those obtained for **1.41m** and **n**.

C Determined from slope of a plot of $[I]/k_{obs}$ versus [I]

D Value obtained from progress curve analysis [I_o/E_o] = 10; [E_o] = 0.073μM; [S] = 117μM. The lower the value the more potent the inhibitor.

The "dimeric" compounds **1.41m** and **1.41n** were designed to release a species capable of inhibiting α-chymotrypsin providing a second cycle of inhibition. It was thought that compound **1.41m** might be more potent than the related monomeric compound **1.41f**. Although these compounds showed reasonable activity, the opposite trend was observed suggesting that the second inactivating species is ineffectual, probably because it escapes from the enzyme active site.

If a second species derived from the initial compounds **1.41m** and **n** was involved in the inhibition process these compounds would display complicated multiphasic kinetic behaviour relative to the monomeric compounds, however, in the preliminary studies undertaken this was not observed. Further development of these types of inhibitors may include incorporation of structural features, *i.e.* macrocycles, to prevent the release of the second inactivating species from the active site.

Of interest is the difference in the k_{inact} values for the "dimeric" inhibitors **1.41m** and **1.41n**. There is a ten-fold increase in k_{inact} between the *meta* substituted compound **1.41m** and the *para* substituted compound **1.41n** however the former is the more potent inhibitor (as indicated by the lower $k_{obs}/[I]$ value; a lower value indicates more potent inhibition. This result suggests that **1.41n** binds less favourably to α-chymotrypsin than **1.41m**, perhaps due to its increased steric demands, resulting in poorer inhibition. This is consistent with the trend observed with the monomeric compounds where compounds **1.41f** and **1.41j** were found to be less potent than **1.41g** and **h**. In the same manner the "extended" dimeric compound **1.41n** was found to be two-fold less potent than **1.41l** on comparison of $k_{obs}/[I]$ values.

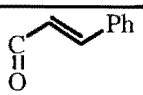
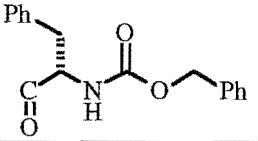
Compound number	R	L	Result
1.41o	$PhCH_2$	Ac	Inactive
p	Ph	Ac	Inactive
q	$PhCH_2$		Inactive
r	$PhCH_2$		Inactive

Table 3.5 Inhibition results for *N*-[(acyl)oxy]succinimides

All the *N*-[(acyl)oxy]succinimides **1.41o-r** displayed in **Table 3.5** were found to be inactive suggesting that an acyl leaving group is unfavourable for inhibition. To improve the activity of **1.41o** compound **1.41q** was prepared which has a better leaving

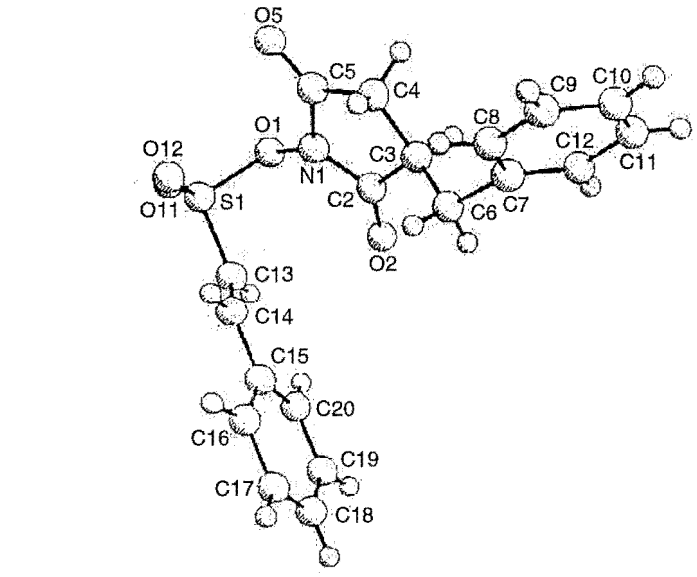
group as evident by comparison of the pKa values of the respective acids (pKa acetic acid 4.74, pKa *trans*-cinnamic acid 4.44), however this compound too was inactive. An attempt was also made to impart activity to this series by increasing enzyme-inhibitor recognition and to this end compound **1.41r** which contains *N*-Cbz (*L*)-phenylalanine as a leaving group was prepared however this too was found to be inactive.

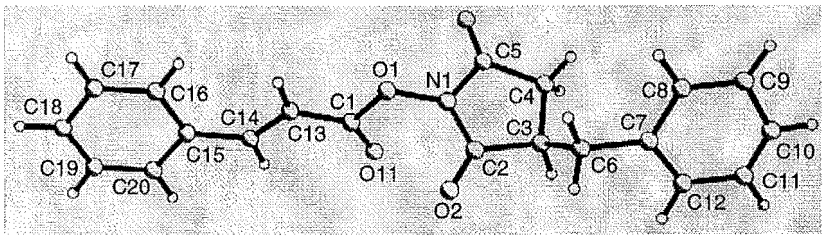
The inactivity of the *N*-[(acyl)oxy]succinimides of type **1.41o-r** appears to be due to the lack of a suitable leaving group but may also be rationalised in terms of their ability to mimic a natural peptide substrate. The crystal structures of compounds **1.41o-q** were determined during the course of this thesis and comparison to a crystal struture of an active inhibitor may prove valuable in determining if *N*-[(acyl)oxy]succinimides possess the favourable binding properties that are required for inhibition. This proposed analysis is only approximate as the crystal structures which are obtained in the solid state give no indication of the conformations acquired by compounds in the active site.

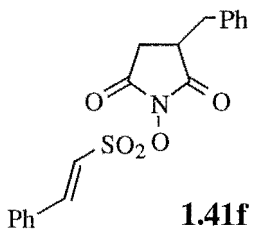
The crystal structure of **1.41f**, whose inhibitory activity against both α-chymotrypsin and human leukocyte elastase is discussed above, has been solved,[87g] and is pictured in **Figure 3.18** in contrast to a topological picture obtained for **1.41q** from data (see **Appendix**) provided by the refinement for this compound. A comparison of **1.41f** with **1.41q** is thought to be representative of the other *N*-[(acyl)oxy]succinimides **1.41o, p**, and **r** as **1.41q** and **1.4f** both contain a *trans*-styryl group hence the differences in conformations will be illustrated more clearly.

The first point to note on comparison of the inactive *N*-[(acyl)oxy]succinimide **1.41q** and the active *N*-[(sulfonyl)oxy]succinimide **1.41f** (**Figure3.18**) is that **1.41q** lacks the typical horseshoe or H shape of **1.41f** which is commonly observed in the crystal structures of dipeptides containing (*L*)-phenylalanine. This characteristic conformation results from the two amino acid side chains extending from the backbone of the dipeptide and running approximately parallel.[200-203] This geometry displayed by the natural substrates of serine proteases is reflected in the complementary shape of the active sites of these enzymes.[199,204]

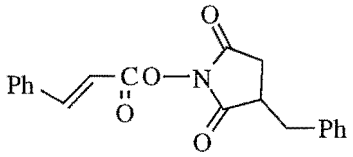
It therefore appears that the sulfonyl(oxy) segment of potent inhbitors not only imparts favourable electronic properties for inhibition but has a role in setting the geometry of the inhibitor such that it mimics a natural substrate. The carbonyl group lacks this geometry and imposes a linear and flat conformation to the *N*-[(acyl)oxy]imides which is unfavourable for binding to serine proteases.







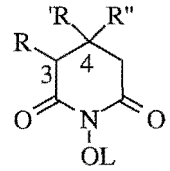
1.41f



1.41q

Figure 3.18 Comparison of crystal structure for **1.41f** (above) against that for **1.41q** (below)

The results obtained on assay of the six-membered glutarimide compounds **2.1a-f** against α-chymotrypsin are presented in **Table 3.6**. These compounds provided a valuable insight into the importance ring size has with regards to structure-activity relation.



2.1

L = SO_2R'''

= $C(O)R'''$

Compound number	R	R'	R''	L	k_{inact} (s^{-1})	k_{inact}/K_I ($M^{-1}s^{-1}$)	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)
2.1a	Ph	H	H	SO_2CH_3	0.011	100 000[A]	7 784[B]
2.1b	Ph	H	H	Ac	C	C	15 845
2.1c	$PhCH_2$	H	H	SO_2CH_3	C	C	D
2.1d	H	Ph	H	SO_2CH_3	C	C	452[B]
2.1e	H	cyclohexyl		SO_2CH_3	0.009	C	14 321[B]
2.1f	H	CH_3	CH_3	SO_2CH_3	0.006[E]	37[E]	F

Table 3.6 Inhibition of α-chymotrypsin by derivatives of type **2.1**

A Values determined from Tsou plot of $[I]/k_{obs}$ versus [I]

B Value obtained from progress curve analysis $[I_o/E_o] = 10$; $[E_o] = 0.073\mu M$; [S] = 117μM. The lower the value the more potent the inhibitor.

C Unable to be calculated due to inactivation of α-chymotrypsin being too rapid at $[I_o/E_o] = 10$ or Tsou plot of $[I]/k_{obs}$ versus [I] having a negative y-intercept.

D Inhibition too rapid to be measured by progress curve method at $[I_o/E_o] = 10$; $[E_o] = 0.073mM$; [S] = 117μM.

E Values obtained from Kitz and Wilson plot of $t_{1/2}$ versus 1/[I]

F Value ($k_{obs}/[I] = 20\ M^{-1}s^{-1}$) obtained from incubation method analysis $[I_o/E_o]$ = 25; $[E_o] = 3.922\mu M$; [S] = 117μM. Note that this value cannot be compared with those obtained using the progress curve method.

The *N*-mesyl glutarimide **2.1f** was found to be a poor inhibitor (k_{inact}/K_I = 37 $M^{-1}s^{-1}$) when assayed using the incubation method. However, the glutarimide **2.1e** which possesses more suitable substitution to interact with the hydrophobic S_1 pocket was found to be a more potent inhibitor. This compound was unable to be assayed accurately using the incubation method although a lower limit for k_{inact}/K_I was calculated to be 408 $M^{-1}s^{-1}$ hence **2.1e** is at least 11-fold more potent than **2.1f**. An analysis using the progress curve method gave a $k_{obs}/[I]$ value of 14 321 $M^{-1}s^{-1}$ hence **2.1f** is less active than the five-membered succinimide compounds (see **Table 3.3**). The difference in the observed potencies of these compounds lies in consideration of their binding affinity for α-chymotrypsin since their k_{inact} values were found to be of the same magnitude; K_I for the more potent **2.1e** must therefore be lower than the value for **2.1f** (K_I = 156μM) calculated from a Kitz and Wilson plot of $t_{1/2}$ versus 1/[I].

The introduction of an aromatic substituent onto the ring resulted in a dramatic 2 000 fold increase in inhibitory activity as shown by comparison of k_{inact}/K_I values for **2.1f** (k_{inact}/K_I = 37 $M^{-1}s^{-1}$) and **2.1a** (k_{inact}/K_I = 100 000 $M^{-1}s^{-1}$) which has a favourable α-phenyl substituent. The k_{inact} value for **2.1a** was only slightly higher than that for **2.1f** hence the dramatic increase in activity is due to increased binding affinity as confirmed by the comparison of the K_I values for **2.1a** (0.1μM) and **2.1f** (156μM).

Although no k_{inact}/K_I analysis was made for **2.1d** comparison of the $k_{obs}/[I]$ values obtained for **2.1a** ($k_{obs}/[I]$ = 7 784 $M^{-1}s^{-1}$) and **2.1d** ($k_{obs}/[I]$ = 452 $M^{-1}s^{-1}$) indicate that the placement of the phenyl substituent at the β-position leads to an increase in inhibitory activity (note that studies have shown that a lower value corresponds to more potent inhibition). This may suggest that **2.1d** may be able to bind in a different manner in the active-site projecting both the phenyl substituent and a portion of the glutarimide ring into the S_1 specificity pocket such that this compound can mimick the P_1 phenylalanine residue of the natural substrate while retaining the correct conformation to react with Ser-195.

As observed in the succinimide series incorporation of an α-benzyl substituent onto the glutarimide ring afforded the most potent inhibition observed in the glutarimide series. Compound **2.1c** totally inactivated α-chymotrypsin at a concentration 10-fold excess over enzyme within the mixing period and is therefore of comparable activity to the succinimides **1.41g** and **h**.

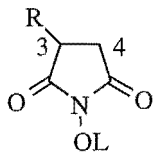
As mentioned previously these potent compounds would require the use of stopped flow methods to determine k_{inact}/K_I. However an indication of their relative potencies can be obtained by comparison of the k_{inact} values obtained for the succinimide and glutarimide series. With the exception of the "dimeric" inhibitor **1.41n** whose k_{inact} is

of the same magnitude the k_{inact} values of the glutarimide inhibitors are an order of magnitude higher.

Assuming that compounds **1.41g** and **h**, and **2.1c** have comparable binding affinity for α-chymotrypsin it appears that **2.1c** may be the more potent inhibitor due to the higher k_{inact} value observed in the glutarimide series. This trend has been reported in the comparison of five and six membered haloenol lactones discussed previously (**Section 1.3**, Chapter 1) where the higher inhibitory activity of the six membered derivatives was rationalised in the terms that the α-halo ketone reactive species unmasked was on a longer tether and therefore closer to His-57 promoting formation of the inactive enzyme.

Suprisingly, the *N*-acetylglutarimide **2.1b** showed inhibitory activity against α-chymotrypsin. If the mechanism of inactivation of α-chymotrypsin by **2.1b** involves the Lossen rearrangement the question arises as to why the related *N*[(acyl)oxy]succinimides did not exhibit similar activity. The differences in activity between the *N*-acyloxy glutarimide and succinimide derivatives could be explained in the terms that the glutarimide binds more favourably to α-chymotrypsin and in such a way that allows a mode of inhibition to proceed which is unavailable to the succinimides.

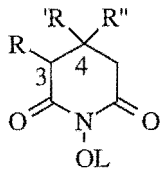
In summary, we assayed the inhibitory activity of the series of five- and six-membered imides represented by structures **1.41** and **2.1** against α-chymotrypsin using both the incubation method, for slower inhibitors, and the progress curve method for faster more potent inhibitors.



1.41

L = SO_2R'

= C(O)R'



2.1

L = SO_2R'''

= C(O)R'''

These results suggest that for potent inhibition to be observed the ring substitution must be aromatic or hydrophobic in nature; alkyl type substitution resulted in poor inhibition. A benzyl substituent was found to be optimum for activity being equivalent to the side chain of phenyalanine the preferred P_1 residue of natural substrates of α-chymotrypsin.

No activity was observed in the succinimide compounds **1.41o-r**, probably due to the fact that an acyl group is not a sufficiently good leaving group. Suprisingly, activity was observed in the glutarimide example **2.1b** suggesting that the six-membered ring has favourable binding properties.

An increase in inhibitory activity in the succinmide series was observed when the R substituent of the leaving group (OSO_2R) was aromatic. It is thought that this R group may be interacting favourably with the S_n' subsites of α-chymotrypsin and their increased activity owing to increased binding energy. However not all aromatic type inhibitors of type **1.41** displayed a favourable enhancement in activity; those derivatives with bulkier subtituents *e.g.* **1.41f** and **1.41i** displayed slightly decreased potency indicating there are steric limitations to the extent of binding with the S_n' subsites.

The glutarimides **2.1a**, **e** and **f** were analysed to have higher k_{inact} values than the succinimides **1.41**. This is thought to result from an increase in the rate of reaction of the reactive isocyanate formed in the Lossen rearrangement with His-57. This is explained in the terms that the isocyanate is on a longer tether and hence closer to His-57 for glutarimides. Due to the glutarimide compounds possessing higher k_{inact} values it is thought that appropriately substituted derivatives, having high binding affinity for α-chymotrypsin, will display the optimum activity of the inhibitors whose assay has been discussed in this chapter. The greater number of substitution sites on the glutarimide ring will also make these compounds more amenable to the development of imides as potent and selective mechanism-based inactivators of serine proteases.

CHAPTER 4

ENANTIOPURE *N*-[(ALKYLSULFONYL)OXY] SUCCINIMIDES AND INHIBITION

4.1 CHIRALITY AND BIODISCRIMINATION

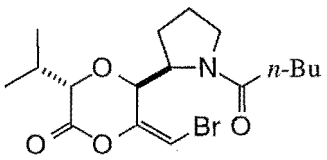
Chirality is a property of molecules which defines important aspects of our biochemistry and interaction with the environment. It also determines the biological activity of molecules finding wider everyday use as pharmaceuticals, agrochemicals, and foodstuffs.

Biological activity arises from the interaction of a compound with a biomolecule such as an enzyme or a receptor. These biomolecules are constructed from amino acids and carbohydrates - nature's own chiral building blocks - and hence are chiral themselves. If the compound is chiral then the type and magnitude of the interaction, and therefore the biological response, is related to its stereochemistry. Thus the stereoisomers of an organic compound are likely to interact differently with its biomolecule.[205,206]

This biodiscrimination is manifest throughout nature,[207,208] and has long been recognised in taste and odour perception[209-211] and other physiological responses. Perhaps the most striking example is the stereoselective action of the enzymes and receptors responsible for the chemical reactions that instill life. The enzymes of our body, with few exceptions, metabolize only the (*L*)-enantiomers of amino acids and the (*D*)-enantiomers of carbohydrates and receptors act on the spatial elements these stereochemistries dictate.

TYPES OF BIODISCRIMINATION

In general, there are four different ways in which stereoisomers act on their target chiral biomolecule.[212] Of these four, only three are observed when studying a single enzyme system *in vitro* as in this thesis. In one situation the biological activity resides totally with one stereoisomer while another is devoid of activity: for example the (*S,S*)-peptidyl haloenol lactone **1.35** is active against α-chymotrypsin (α-CT) and human neutrophil elastase (HNE) while the (*S,R*)-peptidyl haloenol lactone **4.1** is inactive at the same concentration.[79]

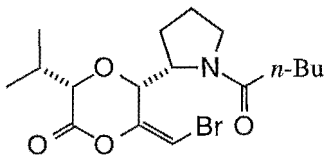


(*S,S*)

1.35

$k_{obs}/[I] = 8\ 000\ M^{-1}s^{-1}$/α-CT[79]

$k_{obs}/[I] = 1100\ M^{-1}s^{-1}$/HNE[79]

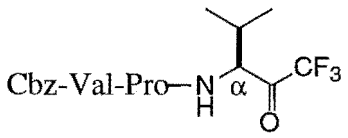


(*S,R*)

4.1

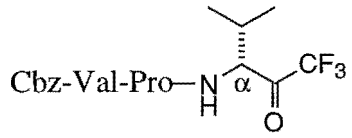
inactive

Alternatively, the stereisomers may both be active but differ in potency. This case is illustrated by the reversible inhibition of human neutrophil elastase (HNE) by peptidyl trifluoromethylketones (see **Section 1.3**, Chapter 1 for a discussion). Compound **1.15**[36] has the correct (*S*)-configuration at the α-position to mimick the natural peptide and hence is 100-fold more active than the epimer **4.2**[36] which has an unfavourable non-natural (*R*)-configuration, and two-fold more active than the racemate **4.3**.



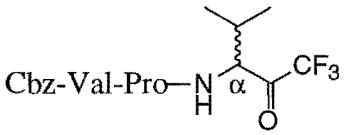
1.15

K_i = 0.0008 μM / HNE[36]



4.2

K_i = 0.015 μM / HNE[36]

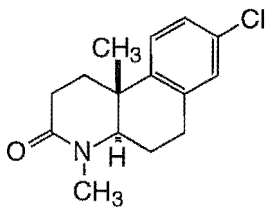


4.3

K_i = 0.0016 μM / HNE[192]

Discussion of the biodiscrimination displayed in the above two situations has lent itself to the nomenclature proposed by Lehmann *et al.*[213] The more biologically active stereoisomer has been named the eutomer; the corresponding less potent or inactive isomer is then called the distomer. The ratio of the activities (eutomer:distomer), the so-called eudismic ratio, is a measure of the degree of stereoselectivity of the biological activity. The eudismic ratio for the above example ($K_{i\mathbf{1.15}}$:$K_{i\mathbf{4.3}}$) is *ca* 200 which is a value typical of chiral medicinal agents.[214]

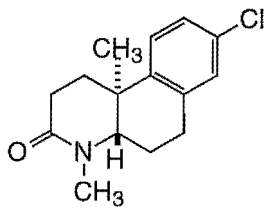
The remaining biodiscrimination type observed is relatively rare. In this situation both stereoisomers may have similar or equal activities as illustrated by the inhibition of Type 1 human steroid 5α-reductase by **4.4** and **4.5**.[215]



4.4

$K_{i,app}$ = 9nM

Type 1 Steroid 5α-reductase

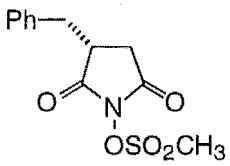


4.5

$K_{i,app}$ = 10nM

Type 1 Steroid 5α-reductase

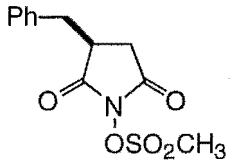
The stereoselective inhibition of serine proteases by mechanism-based inhibitors of type **1.41** has been undertaken by Groutas *et al.*[87e] The results for the inhibition of human leukocyte elastase by (-)-(***R***)- and (+)-(***S***)-**1.41d** are shown below.



(-)-(***R***)

1.41d

k_2/K_I = 330 $M^{-1}s^{-1}$ / HLE[87e]



(+)-(***S***)

1.41d

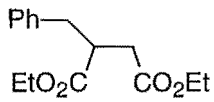
k_2/K_I = 1500 $M^{-1}s^{-1}$ / HLE[87e]

The eudismic ratio for the above example is equal to five suggesting there is low stereoselectivity for the inhibition which has been rationalised in the terms that both enantiomers of **1.41d** can display the correct spatial orientation to be accomodated in the active site.

Human leukocyte elastase has a long and shallow active site and its substrate specificity at S_1 has been found to be regulated by substrate length.[216] The preferred P_1 residue is valine however the hydrophobic S_1 pocket of human leukocyte elastase will also accomodate residues such as alanine and phenylalanine with short substrates. With extended substrates the specficity demands increase and only those substrates with valine as the P_1 residue are accomodated, in the same manner the stereoselectivity of inhibition will increase a commonly observed observation that is known as Pfeiffer's rule.[217] The low stereoselectivity of inhibition observed does not mean that human leukocyte elastase acts with low stereospecificity; it is thought that with longer inhibitors the difference in rates of inhibition for stereoisomers will be larger and a higher eudismic ratio observed.

Groutas *et al*[87e] obtained similar results to those above for human leukocyte elastase when investigating α-chymotrypsin *i.e.* (-)-(***R***)- and (+)-(***S***)-**1.41d** were equipotent.

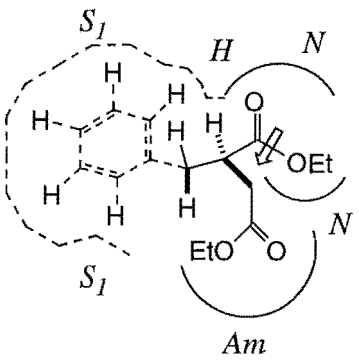
This enzyme does not display the broad substrate specificity of human leukocyte elastase and invoking Pfeiffer's rule it may have been expected that the two enantiomers would display different levels of activity. The stereoselective action of α-chymotrypsin has been explained in terms of binding requirements to specific structural motifs in a model of the active site proposed by Cohen and Milovanovic in a study of the absolute steric course of hydrolysis of a number of esters of substituted succinic and propionic acids.[152] **Figure 4.1** depicts the binding of diethyl 2-benzylsuccinate (**4.6**) in the active site proposed by these authors.



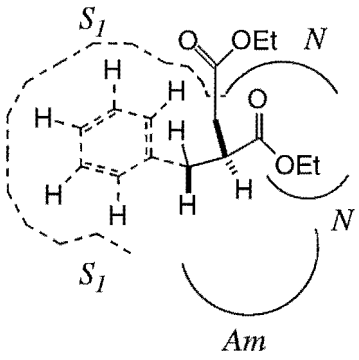
4.6

⇩ **α-chymotrypsin**

α-chymotrypsin active site model



(*R*)-4.6 accomodated



(*S*)-4.6 no fit

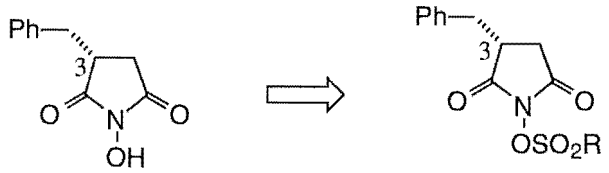
Figure 4.1 The binding of diethyl 2-benzylsuccinate **4.6** in Cohen and Milovanovic's active site model for α-chymotrypsin

From **Figure 4.1** only **(*R*)-4.6** is hydrolysed by α-chymotrypsin as it is the enantiomer that has the correct spatial orientation to bind properly within the active site. For this enantiomer, the phenyl ring is juxtaposed so that it can interact favourably with the S_1 hydrohobic binding pocket while the scissile bond is aligned correctly in the hydrolytic site, *N*. The α-carbethoxymethylene group is in the correct position to permit favourable hydrogen bonding with the acylamino binding pocket *Am*. The α-H of **(*R*)-4.6** points toward the interior of the active site at a domain H of restricted volume, contiguous with *Ar*.

This binding domain H can only accomodate structural moieties of small size; substitution of a substantially larger group decreases reactivity greatly. Hence (***S***)**-4.6** if it is to interact correctly with the S_1 hydrophobic pocket places the large α-carbethoxymethylene group at *H* which explains the poor reactivity of (***S***)**-4.6** in conjuction with the loss of the favourable hydrogen bonding iteraction with the acyl amino binding site.

The stereospecificity of action is lost if the α-carbethoxymethylene group is replaced with a group of smaller size and this has been observed with α-chloro and hydroxy propionic acid esters[218] and cyclised substrates.[219] That the enantiopure inhibitors (-)-(***R***)- and (+)-(***S***)-**1.41d** exhibit the same potency may be rationalised in the terms that the *N*-mesylate is sufficiently small that both enantiomers can be accomodated in the active site.

A goal of this thesis was to explore the stereoselective inhibition of α-chymotrypsin by derivatives of type **1.41**, as shown below, where the alkyl sulfonyl group was larger than a mesylate so that a difference in the activites betwen the two enantiomers may be observed and that the stereoselectivity of the interaction with the S_n' subsites of the enzyme could be examined.



(3*R*) **(3*R*)**

2.26 **1.41**

A method for obtaining enantiopure derivatives of type **1.41** therefore was required as a step towards accomplishing this goal. *N*-Hydroxysuccinimides, such as **2.26**, are key precursors of **1.41** therefore we required an enantioselective synthesis of these derivatives.

4.2 METHODS FOR OBTAINING CHIRAL SUCCINATES

Biodiscrimination has had implications for the agrochemical and foodstuffs industries: chiral herbicides and plant growth factors display biodiscrimination, and large industries have developed for the stereoselective synthesis of (*L*)-amino acids as food supplements and (*D*)-glucoses as nonnutritive sweeteners.[220,221] Chiral medicinal agents have been known to exhibit biodiscrimination as long as 80 years ago[222] and this phenomenon abounds in the medical field[223] and hence has held the greatest consequence for the pharmaceutical industry.

Industry's requirement for chiral pharmaceuticals, agrochemicals and foodstuffs has fueled research into the synthesis of enantiopure compounds and subsequently the analysis of the enantiomer compositions of molecules. This has had a huge "wash-over" effect for academic pursuit as there exist several ways of obtaining chiral, non-racemic compounds essential for the study of nature's stereoselective processes, whether that be the investigation of the stereospecificity of an enzyme's catalytic action, or mimicking her biosynthetic prowess by threading the stereochemical labyrinth of her structurally diverse and complicated molecules through asymmetric synthesis.[224]

There exist a number of methods for the preparation of chiral succinates and these are discussed briefly in turn.

CLASSICAL RESOLUTION

Traditionally, optically pure compounds have been obtained by classical resolution techniques at a favourable stage during the synthesis of their racemates. The use of enantiomerically pure starting materials and enzymes in synthesis and of the latter in kinetic resolutions and the advent of stereoselective synthesis has reduced, though not totally, the need for classical resolution. A synthesis purporting all of these design principles can still be subject to rabid racemization requiring resolution of intermediates and products.

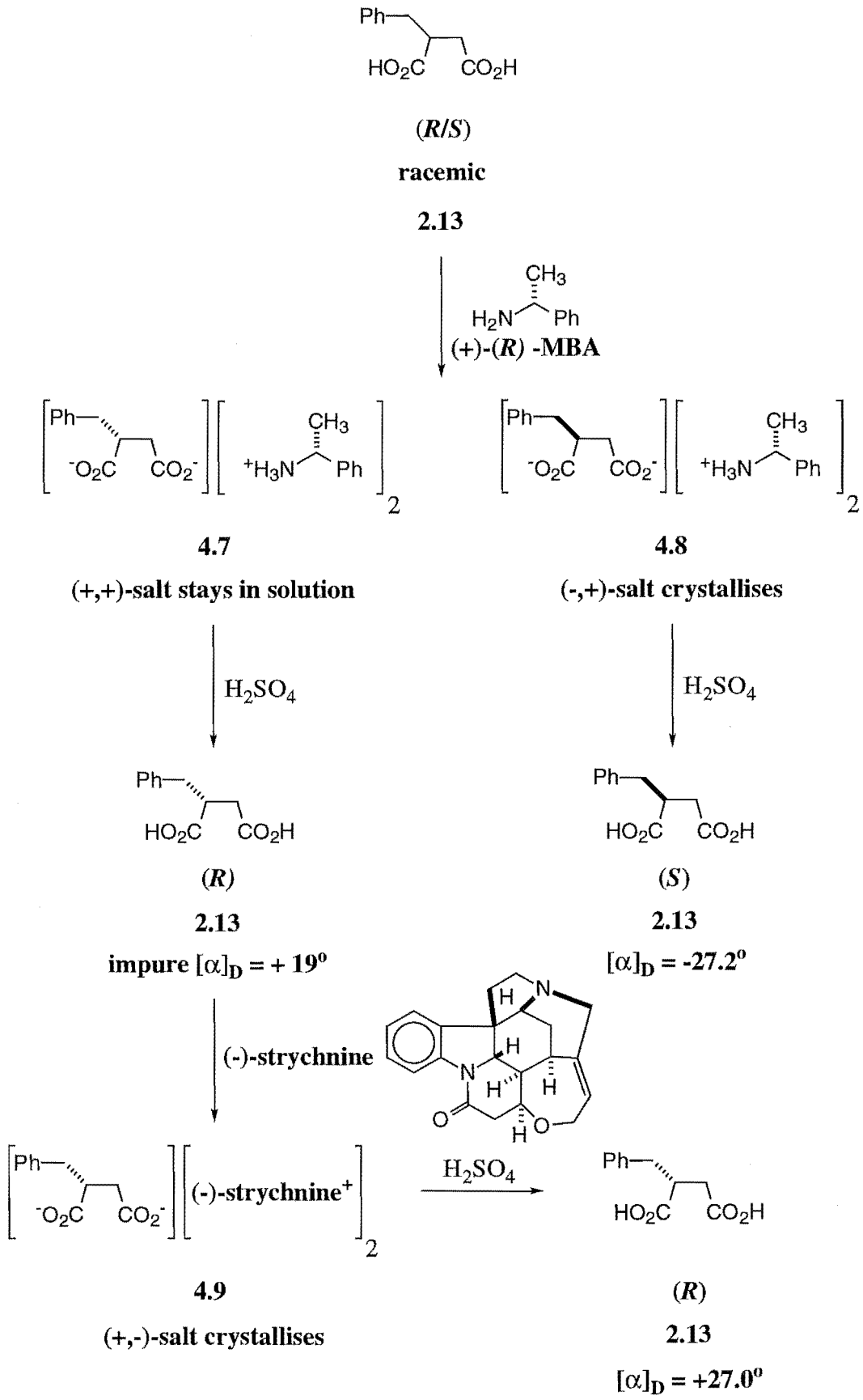
Earlier in this thesis (**Section 2.1**, Chapter 2) 2-substituted succinic acids were identified as intermediates in the synthesis of 3-substituted *N*-hydroxysuccinimides key precursors in the preparation of the target inhibitors **1.41**. These acids also represent a favourable target for classical resolution. The resolution of 2-substituted succinic acids in particular 2-benzylsuccinic acid **2.13** by the optically active amine (+)-(*R*)-methybenzylamine (MBA) followed by the alkaloid strychnine (**Scheme 4.1**) has been carried out by Fredga.[225] Succinic acid mono esters featured in one synthesis of 2-

substituted succinic acids employing Stobbe condensation methodology (**Method C**, **Scheme 2.5**, Chapter 2) and these also present themselves as targets for classical resolution. The resolution of methyl 2-isopropylsuccinate by both enantiomers of methylbenzylamine has been achieved.[226]

A resolution procedure may prove wasteful if the undesirable enantiomer cannot be used in a different application or recycled. The above resolution procedures are economical in this sense as both enantiomers of the target inhibitors **1.41** provide valuable information regarding the stereospecifics of inhibition of α-chymotrypsin. Classical resolution therefore is a competitive route to desired enantiopure inhibitors **1.41** considering the resolving agents employed, strychnine and methylbenzylamine, are both commercially available.

Classical resolution, however, involves numerous recrystallisations each with inherent loss of material, and is lengthy (in terms of time). For example, the resolution of racemic 2-benzylsuccinic acid **2.13** (**Figure 4.1**) as described in the literature,[225] was undertaken using pure **2.13**, requiring repeated recrystallisation of the material obtained from synthesis (in our experience four recrystallisations from water were required).

The racemic acid **2.13** is treated with two equivalents of (+)-(*R*)-methylbenzylamine to give the diastereoisomeric salts **4.7** and **4.8**. The required separation of these is trivial as **4.8** crystallises from the reaction solution, and **4.7** remains in the mother liquor. Repeated recrystallisation of salt **4.8** from H_2O completes the resolution of (-)-(*S*)-2-benzylsuccinic acid **2.13** ($[\alpha]_D$ = -27.2°) and this is obtained by decomposition of the purified salt **4.8** with dilute H_2SO_4 and extractive work-up with Et_2O. Salt **4.7** is decomposed to give impure (+)-(*R*)-2-benzylsuccinic acid **2.13** which is then further resolved by treatment with strychnine, the resulting salt **4.7** requiring no purification to give pure (***R***)-**2.13** ($[\alpha]_D$ = +27.0°) which is obtained as described for (***S***)-**2.13**.



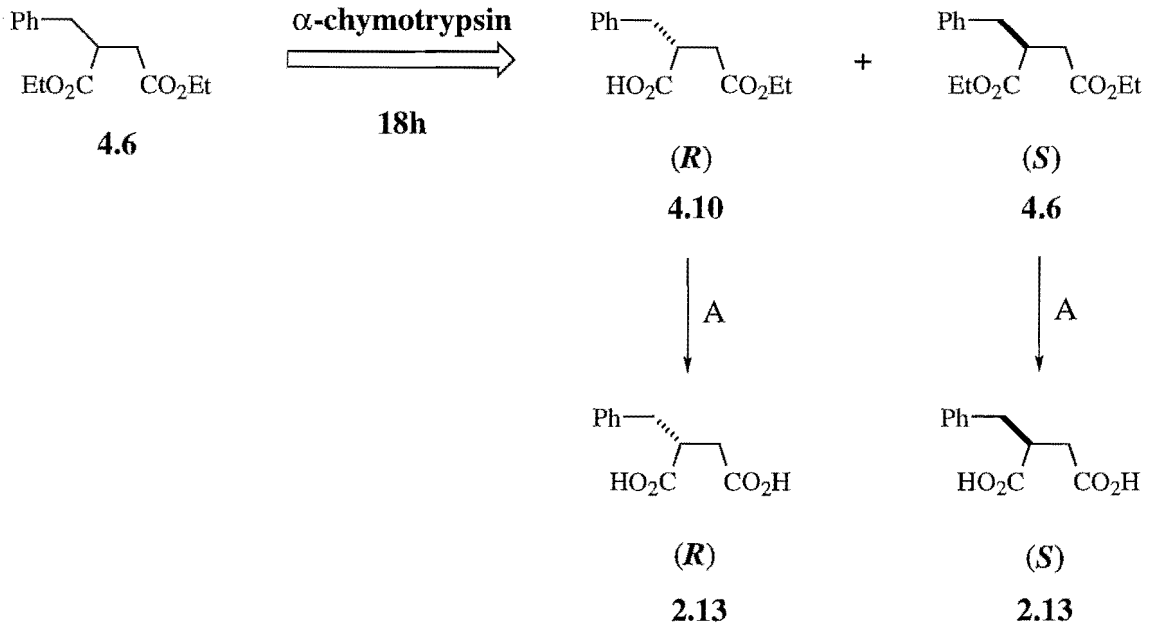
Scheme 4.1 Classical resolution of racemic 2-benzylsuccinic acid **2.13** by (+)-methylbenzylamine (MBA) and (-)-strychnine.

In addition to developing resolution procedures for 2-substituted succinic acids Fredga has undertaken the groundwork in determining the absolute configurations of the (+)- and (-)-enantiomers of the acids obtained. The method he has exclusively used has been that of quasi-racemates.[227] In brief, it has been shown that (+)-2-benzylsuccinic acid has the same configuration as (+)-malic acid whose absolute (*R*) configuration has been unequivocably established.[228]

The specific rotations obtained by Fredga for the above resolution are considered to be the maximum specific rotations for (+)-(***R***)- and (-)-(***S***)-**2.13** and have therefore been used routinely in the determination of the optical purity of samples prepared using other methods as seen below.

KINETIC ENZYMIC RESOLUTION

The length of a classical resolution however does not present itself in the resolution of racemates by enzymes. On presentation of a racemic substrate the enzyme due to its chirality behaves in an stereoselective manner reacting exclusively with one stereoisomer leaving the other untouched. As discussed earlier the (+)-(*R*) enantiomer of racemic diethyl 2-benzylsuccinate **4.6** can only be accommodated in the active site of α-chymotrypsin and is hydrolysed to (+)-(*R*)-ethyl 2-benzylsuccinate (**3.11**). The (-)-(*S*) enantiomer of **4.6** is left untouched as observed in **Scheme 4.2** which depicts the enzymic kinetic resolution of **4.6** by α-chymotrypsin.

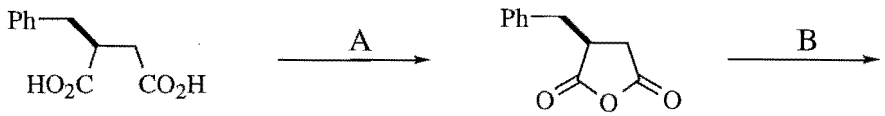


Scheme 4.2 A, NaOEt, EtOH, rt

Both (***R***)-**4.10** and (***S***)-**4.6** can be hydrolysed with ethanolic sodium hydroxide to give the corresponding (***R***)- and (***S***)- acids **2.13** whose rotations ($[\alpha]^{25}_D$ = +27° and $[\alpha]^{25}_D$ = -27° respectively) were found to be comparable to the definitive rotations determined by Fredga above; (***R***)-**2.13** ($[\alpha]^{25}_D$ = 27.0°) and (***S***)-**2.13** ($[\alpha]^{25}_D$ = -27.2°) indicating that the products obtained by the enzyme kinetic resolution were of high purity.

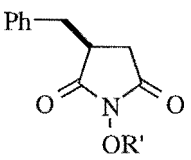
This resolution procedure has the potential to return amounts of both enantiomers of the racemic substrate in a day period. However high concentrations of α-chymotrypsin have to be used making this method prohibitive considering the supply of this enzyme in our laboratory and the hydrolysis of **4.6** is slow and with no suitable pH-Stat apparatus available, the 18h duration of the hydrolysis course to be followed manually makes this approach attractive to only an insomnic chemist.

Enantiopure inhibitors can then be prepared from the enantiopure 2-benzylsuccinic acid obtained from the above classical and enzymic resolutions employing the methodology of **Scheme 4.3** used in the synthesis of racemic inhibitors of type **1.41** discussed in Chapter 2. The reactions of **Scheme 4.3** which depicts the representative synthesis of (***S***)-**1.41d** have been shown to be devoid of racemization by Groutas *et al*[87e] who found the product inhibitors prepared by this route to be enantiopure by chiral shift reagent determination.[229]



(*S*) (*S*)

2.13 **2.27**



(*S*)

C → **2.25** R' = CH_2Ph

C → **2.26** R' = H

D → **1.41d** R' = SO_2CH_3

Scheme 4.3 A Ac_2O, reflux

B H_2NOCH_2Ph, toluene, reflux

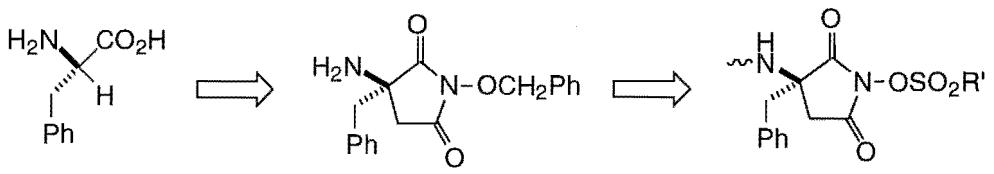
C H_2/10% Pd-C/THF

D CH_3SO_2Cl, pyridine, CH_2Cl_2

ASYMMETRIC SYNTHESIS

Asymmetric synthesis provides the chemist with the power to employ synthetic methods that not only result in the desired transformations of starting materials to products but control the absolute stereochemistry of the stereogenic centres which are created as a result of the synthetic operation.

An asymmetric synthesis of chiral succinates is therefore more flexible than the previous methods discussed. Methods that have been employed include the synthesis of chiral succinates from naturally occurring substrates,[230] a strategy widely known as the chiron approach.[231] This method has been used in the synthesis of imide containing psuedo peptide sequences **2.37** from (*L*)-phenylalanine (**4.11**). The stereochemistry residing in **4.11** is incorporated into the key intermediate **2.36** which mediates the stereochemistry in the final product **2.37**. However, to its disadvantage the chiron approach often involves multi-step syntheses.



4.11 **2.36** **2.37**

Scheme 4.4 Synthesis of 3-benzylsuccinimide containing pseudo peptides

The asymmetric hydrogenation of unsaturated succinate derivatives has been undertaken[232] however methods based on the principle of relayed asymmetric induction have proved popular. In this process asymmetric information is introduced to the racemic substrate before the reaction where the new asymmetric centre is created and then the chiral originator, a so-called chiral auxiliary is removed. Two auxiliaries that have furnished short syntheses of chiral alkyl succinates are iron acyl complexes[233] and the chiral oxazolidinones developed by Evans.[234]

Evan's chiral oxazolidinones prepared from naturally ocurring amino acids such as valine and phenylalanine are readily available. Of the chiral auxiliaries derived from these two amino acids those prepared from phenylalanine are crystalline not requiring the extra purification steps associated with the valinol derived auxiliaries and contain a good chromophore facilitating TLC analysis. The phenylalanine oxazolidinones are easily attached to the racemic substrate and recycled and hence were the chiral auxiliary of choice presenting an attractive route to the desired chiral alkyl succinate intermediates.

The mode of asymmetric induction by Evans chiral auxiliaries lies in the consideration of enolate chemistry. For a reaction at the α-position to be stereoselective several conditons have to be fulfilled among the most important being the geometry of the enolate formed. Factors which can control the stereoselectivity of enolate formation include choice of base, solvent, temperature and metal counter ion but most importantly from the viewpoint of this thesis the chiral auxiliary. In this respect the Evans oxazolidinones are attractive in that they give exclusively (Z)-enolates.

A second factor in determining the stereochemical outcome in the reaction is that the chiral auxiliary imposes a strong facial bias for the approach of the eletrophilic alkylating agent towards the enolate. It is here the substitution present the chiral auxiliary comes into play as the approach of the electrophile occurs on the less hindered face of the enolate.

Alkylation and aldol additions of *N*-acyl compounds derived from chiral oxazolidinones proceed with high diastereoselectivity as illustrated by relevant literature examples of the synthesis of chiral succinates. In 1981 Evans[235] prepared (2*R*)-benzylsuccinic acid employing the aldol addition of a *N*-acyloxazolidinone boron enolate derived from(*L*)-valinol. The key aldol adduct was prepared in 99% diastereoisomeric excess as determined by gas capillary chromatography which was degraded to give (2*R*)-benzylsuccinic acid whose optical purity was judged to be 98% by comparison to the maximum specific rotation obtained by Fredga.[225]

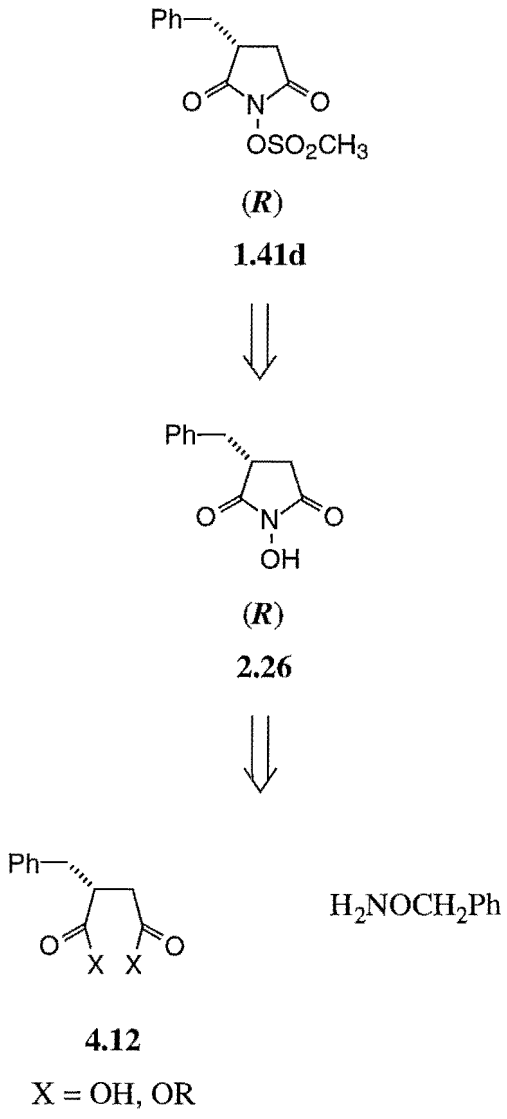
In 1988, Fadel and Salaun[236] reported the preparation of chiral succinic acid diesters employing the distereoselective alkylation (>95% as determined by ^{1}H NMR) of (*L*)-valinol derived *N*-acyloxazolidinones. The enantiomeric excess of the esters was determined by chiral shift reagent to be >95%.

In 1993, Crimmins *et al*[237] described a stereoselective synthesis (>95% diastereoisomeric excess) of succinic acid mono esters employing sodium base mediated alkylation of a chiral *N*-acyloxazolidinone derived from (*L*)-phenylalanine. This methodology was repeated in 1998 by Levy *et al*[226] and McClure *et al*[238] who reported the synthesis of chiral succinate derivatives with high diastereoselectivity.

The trend in these examples illustrates how Evans oxazolidinones are widely utilised for the synthesis of chiral compounds and that the stereochemistry of these reactions has been so rigorously established that present day synthetic communications rarely involve a critique on the stereoselectivity of these reactions-an example being patents filed for the synthesis of renin inhibitors using oxazolidinone methodology.[239]

4.3 SYNTHESIS AND INHIBITORY ACTIVITY OF AN ENANTIOPURE *N*-[(ALKYLSULFONYL)OXY] SUCCINIMIDE

To incorporate the Evans oxazolidinone methodology into a synthesis of an enantiopure inhibitor of type **1.41** a retrosynthetic analysis was carried out. The disconnection of **(*R*)-1.41d** serves as a representative example and is shown below (**Scheme 4.5**).

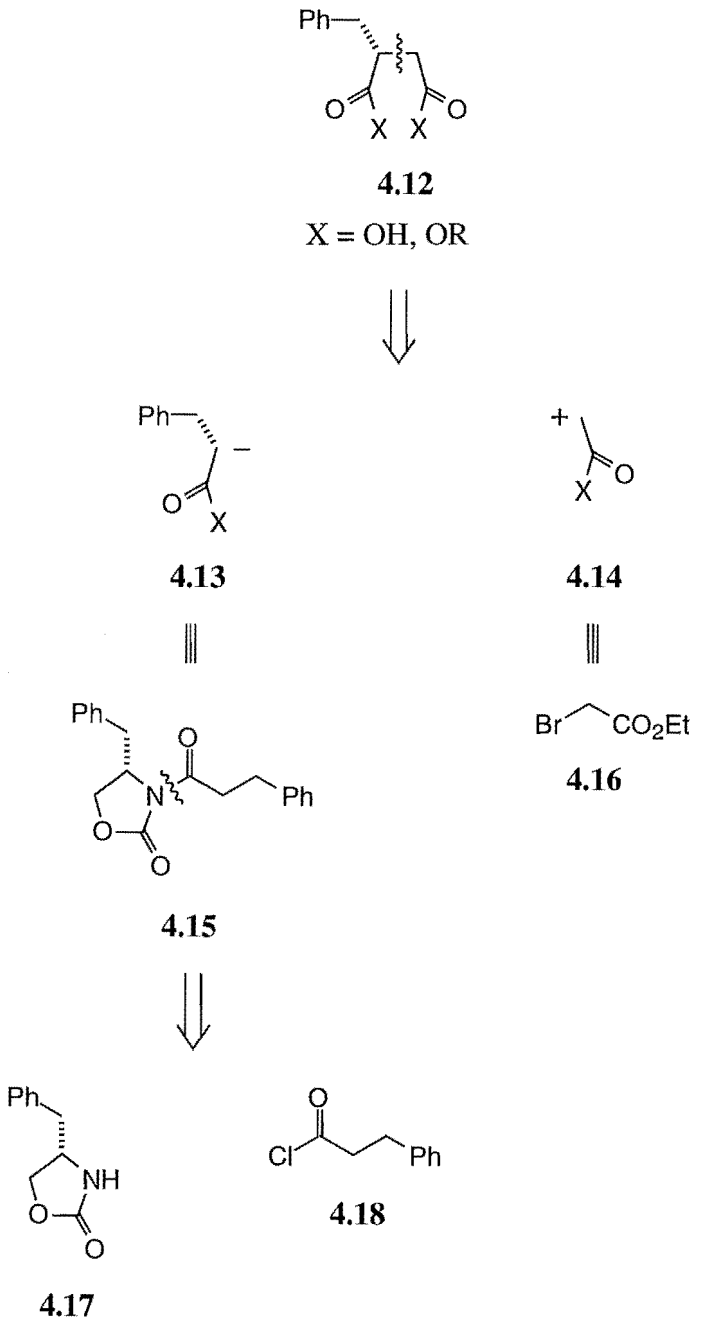


Scheme 4.5 Retrosynthetic analysis of **(*R*)-1.41d**

The retrosynthetic analysis proceeds with disconnections discussed earlier in the synthesis of the racemic counterpart. Disconnection of the bond between the oxygen and the reactive moiety L of **(*R*)-1.41d** gives (*R*)-3-substituted *N*-hydroxysuccinimide **(*R*)-2.26** and alkylsulfonic and alkylcarboxylic acid derivatives as reagents.

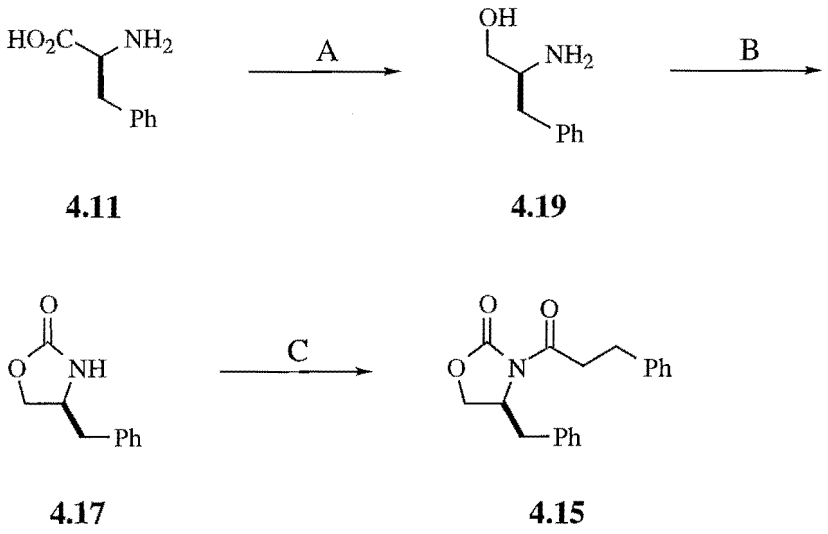
Disconnection of (***R***)-**2.26** proceeds by cleavage of the imide bonds, as indicated, to give hydroxylamine and the chiral 3-alkylsuccinate reagent **4.12**.

Retrosynthetic analysis of the key chiral succinate reagent **4.12** proceeded as shown in **Scheme 4.6**. Dissection of **4.12** as indicated gives the two synthons **4.13** and **4.14** which correspond to the chiral 2-benzylpropionate reagent **4.15** and bromoacetate esters **4.16**. Disconnection of **4.15** gives the chiral auxiliary **4.17** and 3-phenylpropionyl chloride **4.18**.



Scheme 4.6 Disconnection of **4.12** using a chiral propionate synthon

The starting point of the synthesis of an enantiopure inhibitor was the preparation of the chiral oxazolidinone **4.17** in two steps (Steps A-B of **Scheme 4.7**) from (*L*)-phenylalanine **4.11**.



4.11 **4.19**

4.17 **4.15**

Scheme 4.7 A 1) BF_3.OEt, 0.75h, rt, 2h reflux, 2) $BH_3.S(CH_3)_2$, 12.25h rt, 11.25h reflux, 3) 5N NaOH, overnight
B diethyl carbonate, K_2CO_3, 4h, 136 °C
C 1) n-BuLi, THF, 0.5h, -78 °C 2) 3-phenylpropionyl chloride, overnight -78 °C - rt

An initial preparation of (*L*)-phenyalaninol **4.19** was carried out using lithium aluminium hydride reduction of phenylalanine. Work up of this reaction was hindered by a persistent emulsion, which was removed by filtration through celite but probably contributed to the low observed yield for the reaction (10%).

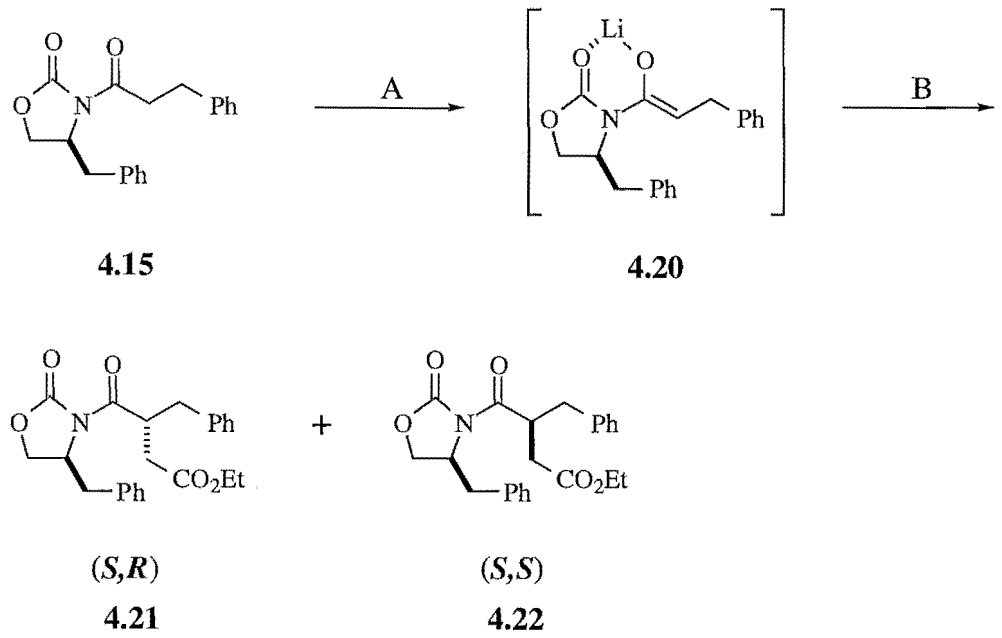
The low yield prompted the use of another method and hence the synthesis of (*L*)-phenylalaninol was carried out by borane reduction of (*L*)-phenylalanine according to the literature procedure (Step A, **Scheme 4.7**).[240] Thus (*L*)-phenylalanine was treated with boron trifluoride etherate followed by borane-dimethyl sulfide (1.15 equiv.). Aqueous alkali was added and the resulting reaction slurry refluxed overnight. Extractive work-up gave (*S*)-phenylalaninol **4.19** in 91% yield. Little emulsion was observed with this method and although this intricate procedure was lengthy (three days in total) and involved hazardous reagents and conditions a higher yield was observed. An added advantage is that borane reductions typically proceed with less racemisation.[240]

A mixture of **4.19** and diethyl carbonate (2 equiv.) and K_2CO_3 (0.5 equiv.) was heated for 4h at 136 °C to give the oxazolidinone chiral auxiliary **4.17** in 98% yield.

However when this reaction was repeated the results above proved difficult to reproduce; charring of the reaction mixture occurred on heating which on work-up gave a crude brown mass which proved difficult to purify by recrystallisation due to oiling problems. However we found the sucess of the subsequent acylation step such that these unfavourable samples of **4.17** could be used without hindering the yield of the reaction.

The chiral oxazolidinone **4.17** was acylated following literature directions[239,241] by treatment with *n*-BuLi (1.05 equiv.) followed by 3-phenylpropionyl chloride (1.1 equiv.), itself obtained from treatment of 3-phenylpropionic acid with $SOCl_2$ (1.2 equiv.) under refluxing conditions, to give crude **4.15**. This was purified by flash chromatography to give pure **4.15** as a white solid in 83% yield. The sucess of this reaction depended much on the quality of the *n*-BuLi and thus the reagent was frequently titrated to determine an accurate active concentration. The reaction was repeated in similar high yields illustrating the ease in which these compounds can be prepared highlighting the advantageous use of chiral auxiliaries derived from phenylalanine.

The key stereoselective reaction of the synthetic scheme was then carried out by treating **4.15** with LDA at -78 °C to give the corresponding chelated lithium enolate **4.17** which was alkylated by excess ethyl bromoacetate as the reaction solution was allowed to warm to room temperature overnight (**Scheme 4.8**).



4.15 **4.20**

(*S,R*) (*S,S*)

4.21 **4.22**

Scheme 4.8 A 1) LDA -78 °C 1h 2) ethylbromoacetate, overnight, -78 °C - rt

Work-up gave a brown residue which was purified by flash chromatagraphy. Proton NMR analysis of this product gave a spectrum consistent with the predicted (*S,R*) diastereoisomer **4.21** but also indicated the presence of a second component in *ca* 5% by ^{1}H NMR. This was determined to be either the minor diastereoisomer **4.22** or starting material **4.15**. Homonuclear decoupling experiments were carried out to make a definitive identification especially concentrating on locating a second triplet and quartet which would only be attrituable to the minor diastereoisomer **4.22**. These experiments proved difficult to analyse due to overlapping signals especially the methylene protons of the ethyl ester and those of the oxazolidinone ring of **4.21** since the irradiations in this region of the spectrum (δ 4.00ppm) were to be of important diagnostic value.

Confirmation of the presence of staring material in the alkylation product was obtained by comparison to an authentic spectrum of **4.15**. Of note, the ^{13}C NMR spectrum revealed no presence of the minor diastereoisomer **4.22**. Further evidence for the presence of **4.15** in the alkylation product was obtained from LiOH/30% H_2O_2 hydrolysis of a sample of the alkylation product. Proton NMR analysis of the product obtained indicated the presence of 3-phenyl propionic acid presumably arising from hydrolysis of **4.15**.

Attempts were made to further purify the alkylation product by repeating the chromatography lowering the polarity of the eluting solvent, using more silica, and collecting smaller fractions. However no effective separation of **4.15** and **4.21** could be effected.

The presence of starting material in the alkylation product suggested that formation of the lithium enolate **4.20** had not been quantative. Possible causes included not maintaining the reaction at -78 °C during enolisation such that the enolate formed could be undergoing decomposition via a ketene pathway. A product of this decomposition would be the chiral oxazolidinone **4.17** however inspection of the ^{1}H NMR spectrum of the crude product for the NH resonance diagnostic for **4.17** indicated its absence. This placed the quality of the LDA into question and consequently a new solution was employed which was then titrated according to literature procedure[242] and found to be 1.8M a significant deviation from the quoted 2.0M.

The alkylation procedure was repeated and in addition to purification by chromatography the product was recrystallised. The white solid obtained possessed a sharp melting point (60-62 °C) however ^{1}H NMR analysis indicated the presence of the disastereoisomers **4.21** and **4.22** in the ratio of 10:1, by examining the integrals of the resonances assigned as the methyl protons of the ethyl esters of this diastereoisomeric

pair, equivalent to a diastereoisomeric excess of 91% (see **Figure 4.2** for the appropriate ^{1}H NMR spectrum).

The assignment of the spectrum displayed in **Figure 4.2** was undertaken by carrying out several homonuclear decoupling experiments aided by comparison with spectra obtained for the chiral oxazolidinone **4.17** and *N*-acyloxazolidinone **4.15**. The spectrum can be divided into three parts which at several points overlap. The triplets (δ~1.25 ppm) according to the methyl protons of the ethyl esters of the diastereoisomers **4.21** and **4.22** are easily distinguished and due to their chemical equivalence, unlike the resonances of the diastereotopic protons featured in the rest of the spectrum, appear as a triplet. Further downfield (δ 2.4-3.3 ppm) are signals resulting from the merging of two spin systems made up of a series of doublets of doublets. The first doublet of doublets downfield is due to the one of the benzylic protons of the 4-benzyl substituent of the oxazolidinone ring. The other proton of this type then merges with the resonances for the benzylic protons of the 2'-benzyl substituent which in turn merge with the resonances for the methylene protons in the α-positon relative to the carbonyl of the ethyl ester. The protons of the oxazolidinone ring overlap with the quartet of the ethyl ester at δ~4.0ppm. Of the two protons at the 3-postion of the oxazolidinone ring one is a triplet due to coupling constants around this ring being of the same magnitude. The 4-H of the oxazolidinone ring overlaps with the 2'-H at δ~4.5 ppm which is shifted considerably downfield from the spin system it is a part of.

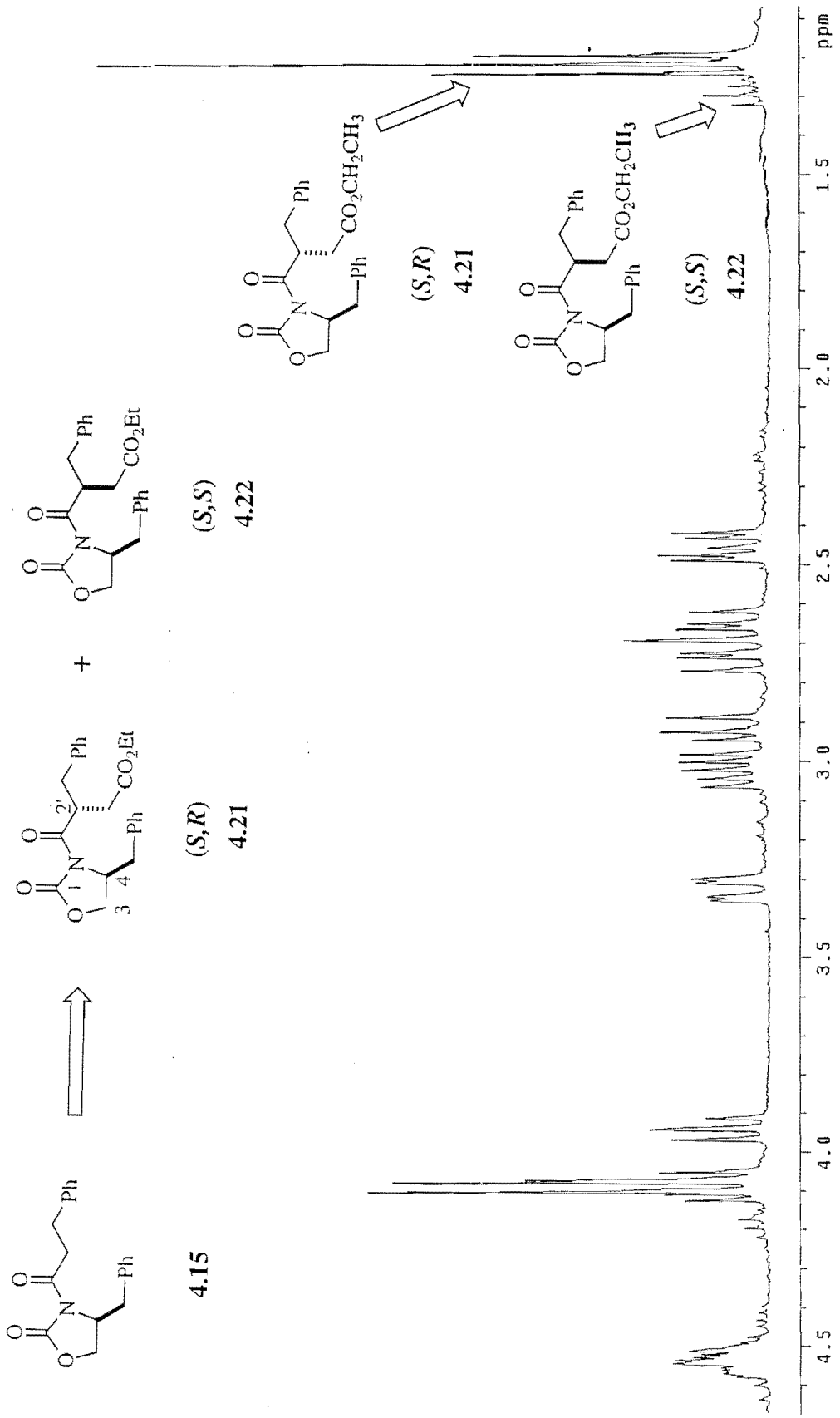
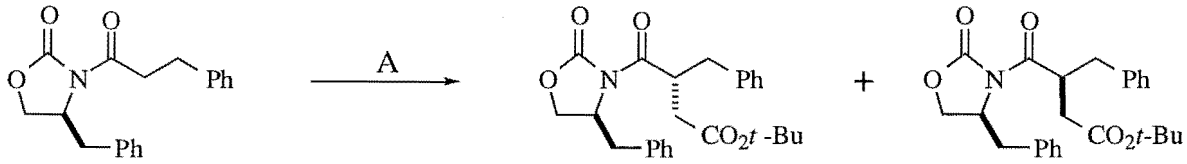


Figure 4.2 Selected region of ^{1}H NMR spectrum for product obtained on alkylation of **4.15** by ethyl bromoacetate

To further probe the nature of the alkylation reaction the *t*-butyl ester **4.23** was prepared. The purpose of its preparation lay in generating a simpler spectrum which could give a clearer picture as to the presence of the minor diastereoisomer. It was also considered that the increased bulk of the *t*-butyl ester relative to the ethyl ester may increase the diastereoisomeric excess observed for this reaction.



4.15 (*S,R*) **4.23** (*S,S*) **4.24**

Scheme 4.9 A, 1) LDA -78 °C 1h 2) *tert*-butyl bromoacetate, overnight, -78 °C - rt

Hence the *N*-acyloxazolidinone was treated with LDA (1.2 equiv.) and the resulting anion was alkylated with *t*-butyl bromoacetate to give a crude product which was purified by flash chromatography to give a white solid in 44% yield. This white solid was further purified by recrystallisation to a give a product that as observed with the ethyl ester had a sharp melting point (105-7 °C). Proton NMR analysis of this product provided a spectrum consistent with formation of **4.23**. Inspection of the region of the spectrum for **4.23** displayed in **Figure 4.3** indicated no presence of the minor isomer **4.24**.

The region of the spectrum for **4.23** displayed in **Figure 4.3** contains the same featues as that discussed for the spectrum of **4.22**. A series of doublets of doublets (δ 2.35-3.3 ppm) precedes the two protons at the 3-position of the oxazolidinone ring further downfield. The coupling existing between the protons of the oxazolidinone ring is made clearer in the case of the *t*-butyl ester confirming that two of the constants are of the same magnitude. As observed in the spectrum of **4.23** the NCH proton of the ring and that at the 2' position overlap.

Further confirmation of the alkylation product containing only the major diastereoisomer **4.23** was provided by inspection of the alkyl region of the ^{13}C NMR spectrum (see **Figure 4.4**), which showed no signs of the minor diastereoisomer **4.24**.

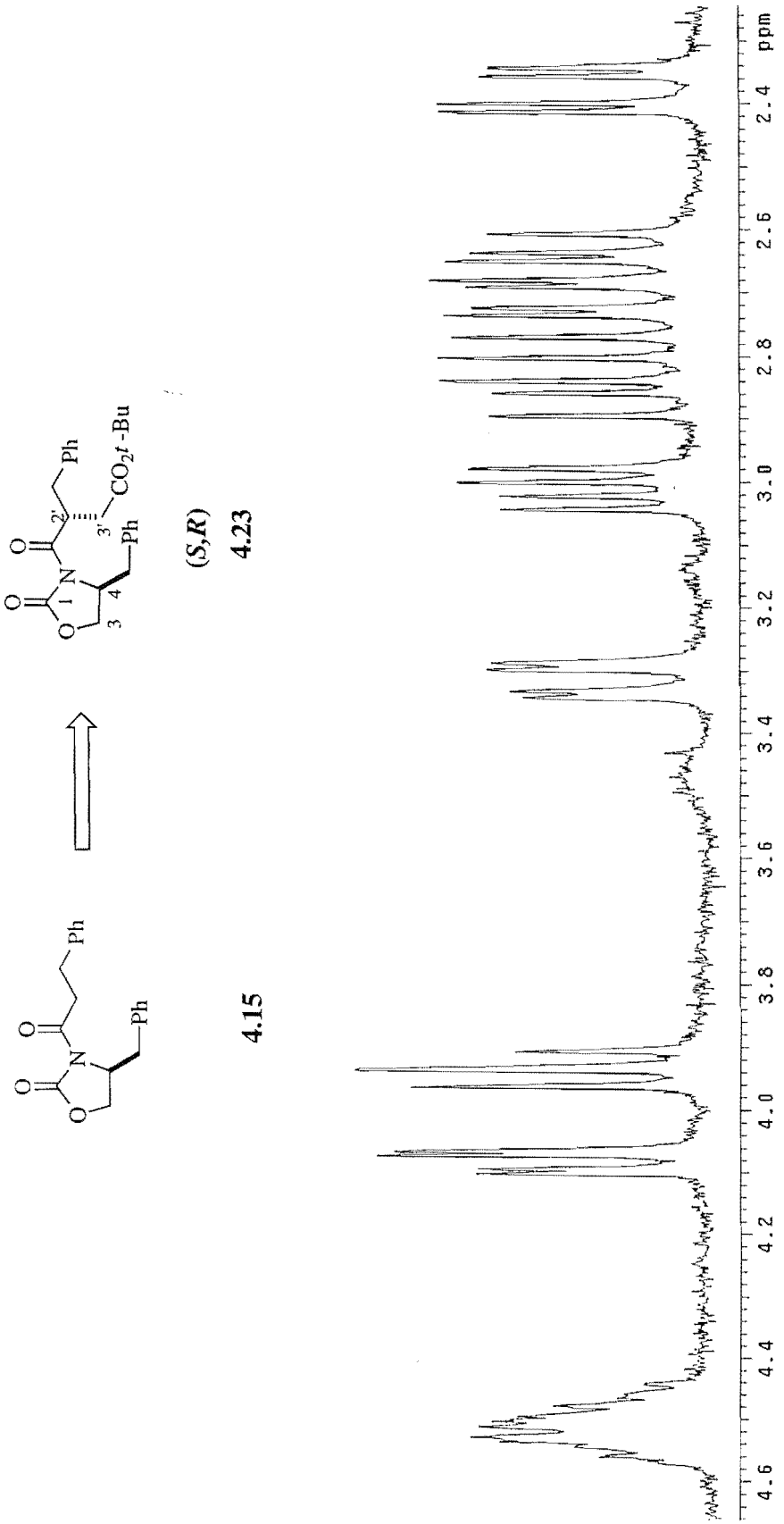


Figure 4.3 Selected region of ^{1}H NMR spectrum of product obtained on alkylation of **4.15** by *tert*-butyl bromoacetate

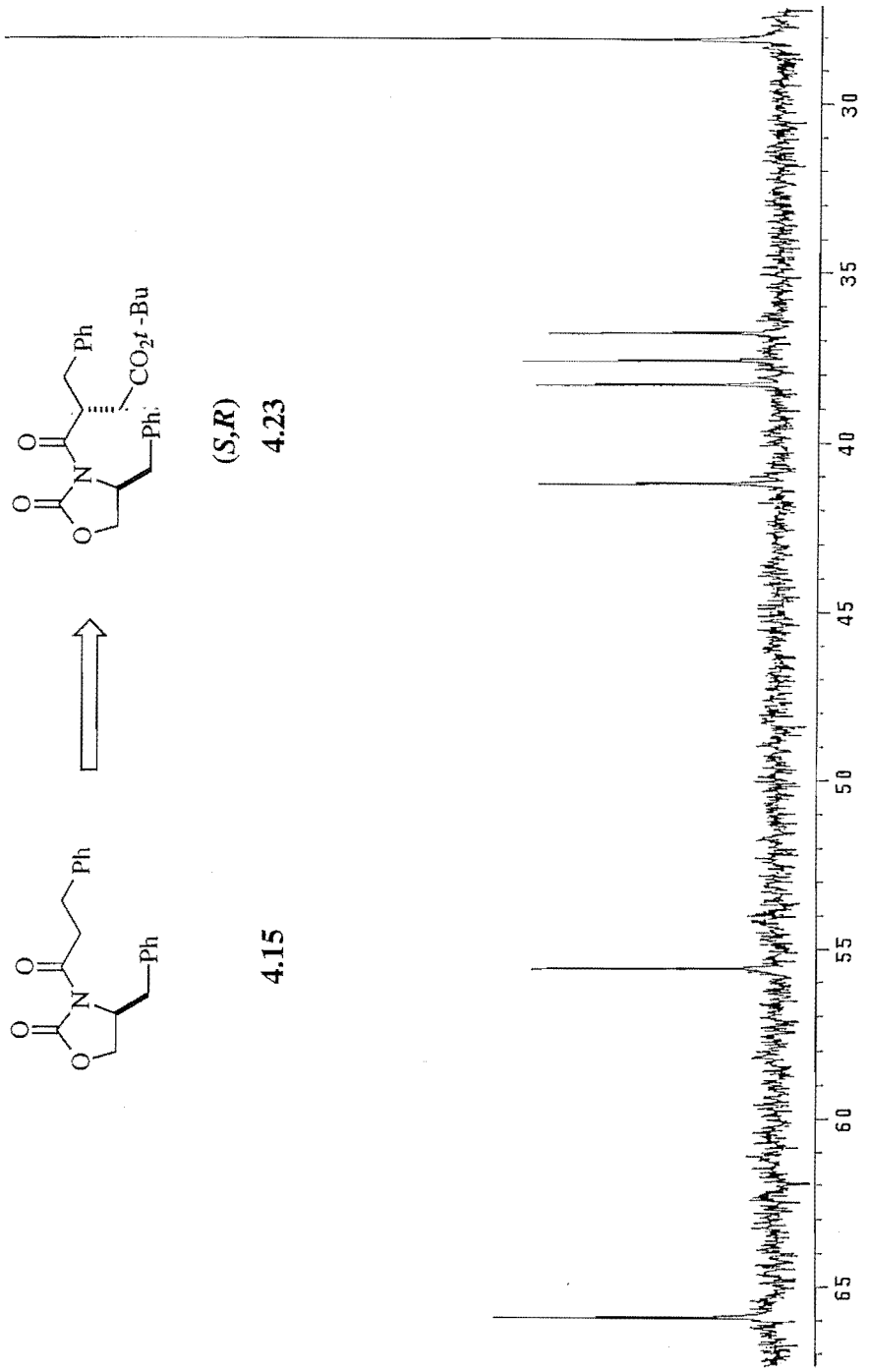
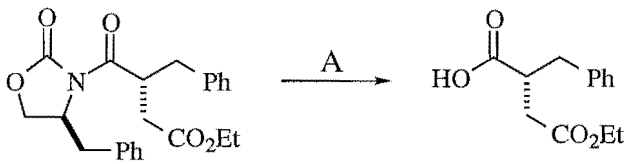


Figure 4.4 Selected region of ^{13}C NMR spectrum of product obtained on alkylation of **4.15** by *tert*-butyl bromoacetate

The absolute stereochemistry of the ethyl ester **4.21** was determined by subsequent LiOH/30% H_2O_2 hydrolysis to give **4.25** (**Scheme 4.10**) whose measured positive specific rotation ($[\alpha]_D^{24}$ = 20.3° (c = 1.6, EtOAc)) correlates to the (*R*) enantiomer on the basis of the work of Fredga discussed earlier.

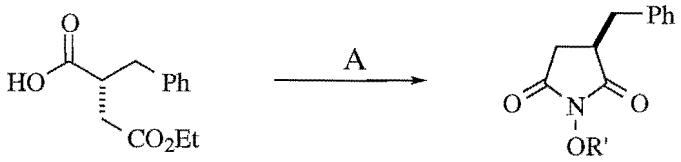


(*S,R*) **(*R*)**

4.21 **4.25**

Scheme 4.10 A, LiOH, 30% H_2O_2, 1h, 0-5 °C

The synthesis of **(*R*)-2.26** then proceeded using methodology developed in the synthesis of racemic **2.26** (**Scheme 4.11**). DCC/HOBt mediated coupling of *O*-benzyloxyamine to **(*R*)-4.25** followed by reflux in DCE gave **(*R*)-2.25** ($[\alpha]^{21}_D$ = -67.8° (c =4.5, EtOAc) in 58% yield over two steps. Hydrogenolysis in the presence of 10% Pd-C then gave (-)-(*R*)-3-benzyl-*N*-hydroxysuccinimide, **(*R*)-2.26**, ($[\alpha]^{24}_D$ = -78.0° (c = 3.0, EtOAc) in 91% yield.



(*R*) **(*R*)**

4.25 B ⌐→ **2.25** R' = CH_2Ph

2.26 R' = H

Scheme 4.11 A 1) $HCl.H_2NOCH_2Ph$, Et_3N, DCC, HOBt, CH_2Cl_2, overnight, 0-5 °C-rt 2) reflux, DCE, 20h

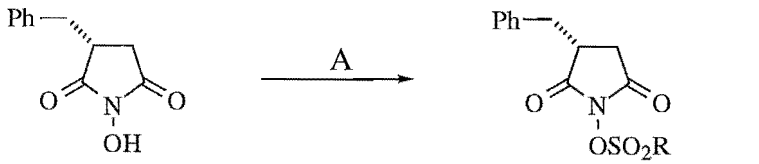
B H_2, 10% Pd-C, THF

To stringently determine the optical purity of **(*R*)-2.26** the optical purity or enantiomeric excesses of the intermediates in the synthesis of **(*R*)-2.26** would have to be determined. Although Step B of **Scheme 4.11** has been reported to proceed free of racemisation[87e] no such study has been made for the reactions of Step A. A definitive analysis of the optical purity of **(*R*)-2.26** could be obtained from coupling to a chiral amino acid and inspection of the diastereoisomeric excess obtained or by chiral shift determination. However the high diastereoisomeric excess (>90 %) observed in the

alkylation of **4.15** by ethyl bromoacetate, and the high optical rotations observed indicated that (***R***)-**2.26** had been obtained in good optical purity.

To examine the stereospecificity of action of an inhibitor of type **1.41** we needed to chose an inhibitor which had good binding affinity to α-chymotrypsin so that the stereoselectivity of action was high (invoking Pfeiffers rule)[217] and of a magnitude that there would exist a measurable difference in the activities between the racemate and the enantiopure derivative. The R substituent also had to be of greater size than methyl, as both (+)-(***R***)- and (-)-(*S*)-**1.41d** exhibited the same potency towards α-chymotrypsin which has been rationalised in the terms that the methyl substituent is small enough to allow both enantiomers to be accomodated in the active site.

Of the racemates whose inhibitory activity was discussed in Chapter 3 compound **1.41f** suited our needs. This compound was shown to have high binding affinity towards α-chymotrypsin and inhibited this enzyme at a measurable rate compared to other aromatics which were of such potency that they could not be measured using the progress curve method. The *trans*-styryl substituent is larger than the methyl substituent and it was therefore predicted that there would be an observed difference in actvity between (***R***)-**1.41f** and its racemate.



(***R***) (***R***)

2.26 **1.41f** R = *trans*-styryl

1.41d R = CH_3

Scheme 4.12 A, *trans*-styrylsulfonyl chloride, pyridine, overnight 50 °C

The inhibitory activity of (***R***)-**1.41f** against α-chymotrypsin was then assayed using the progress curve method. When experiments were carried out at an inhibitor concentration at 20-fold excess over enzyme a difference in the activity of (***R***)-**1.41f** and its racemate was observed. At this concentration the racemate was found to inactivate α-chymotrypsin totally within the mixing period whereas the (*R*)-enantiomer gave a typical progress curve indicating the enzyme was still active. These preliminary inhibition studies indicate that the the racemate is more potent than the (*R*)-enantiomer.

These preliminary inhibition studies indicate that the the racemate is more potent than the (*R*)-enantiomer. This result suggests that the (*S*)-enatiomer is also active, and

of greater potency than the (*R*)-enantiomer enriching the activity of the racemate. It appears also that both enantiomers are active towards α-chymotrypsin. On the basis of $k_{obs}/[I]$ values the less active (*R*)-enantiomer ($k_{obs}/[I]$ = 6 944 $M^{-1}s^{-1}$) still compares favourably with the succinimide and glutarimide inhibitors discussed in Chapter 3 being equipotent to the aromatic succinimide **1.41k** ($k_{obs}/[I]$ = 6 427 $M^{-1}s^{-1}$). The observed activity of **(*R*)-1.41f** reflects the high binding affinity to α-chymotrypsin that compounds exhibiting aromatic substitution possess.

For a definitive analysis of the stereoselectivity of inhibition of α-chymotrypsin by compound **1.41f** and other inhibitors of this type a route to the (*S*)-enantiomer of the important *N*-hydroxysuccinimide precursor **2.26** is required.

As depicted in **Figure 4.13** this may be achieved by using (*R*)-phenylalanine (***Strategy 1***) as the starting material or by using a different metal counter ion (***Strategy 2***) so that the enolate is not chelated. Both have the effect of inducing the opposite sense of alkylation to the route described above.

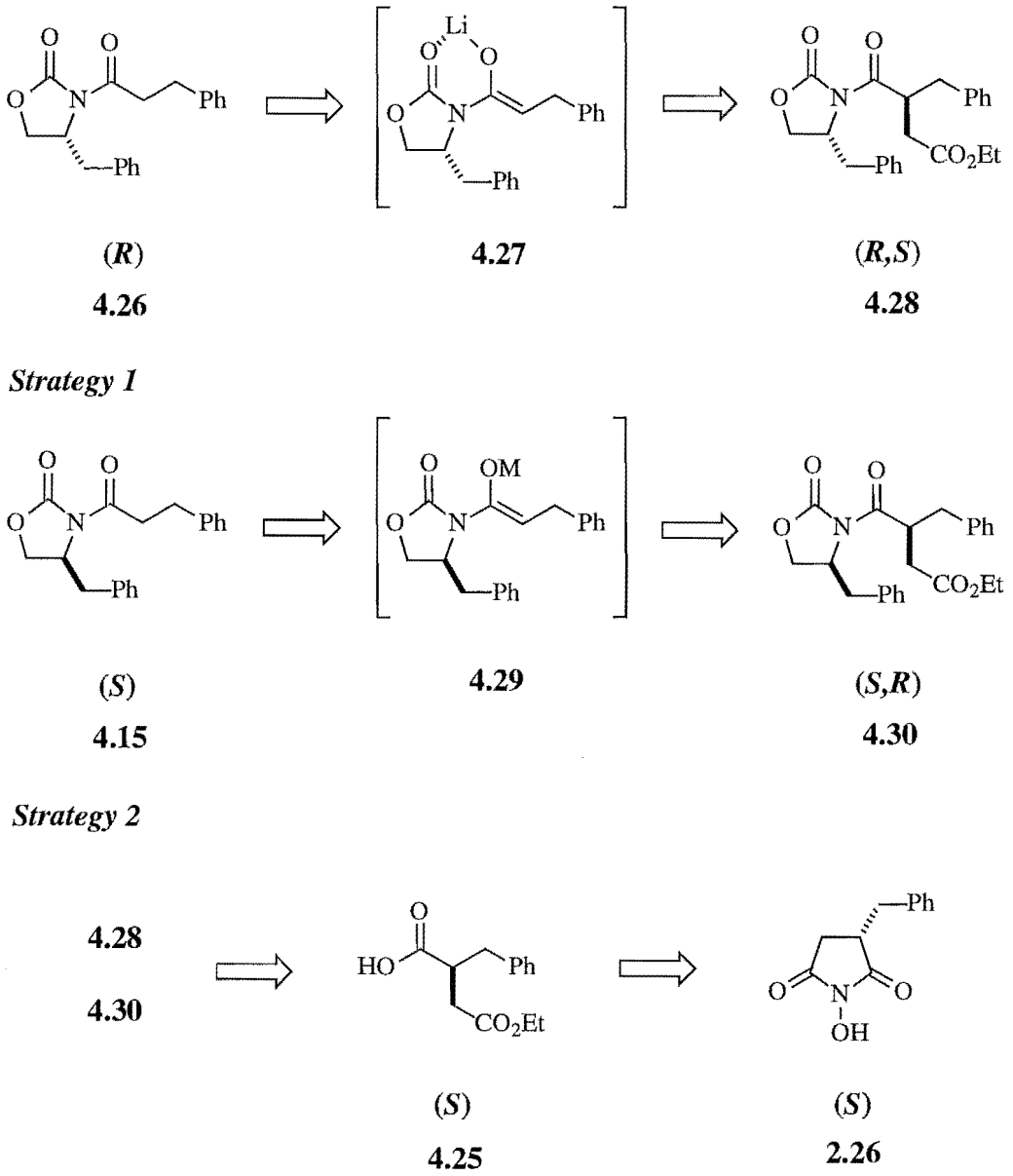
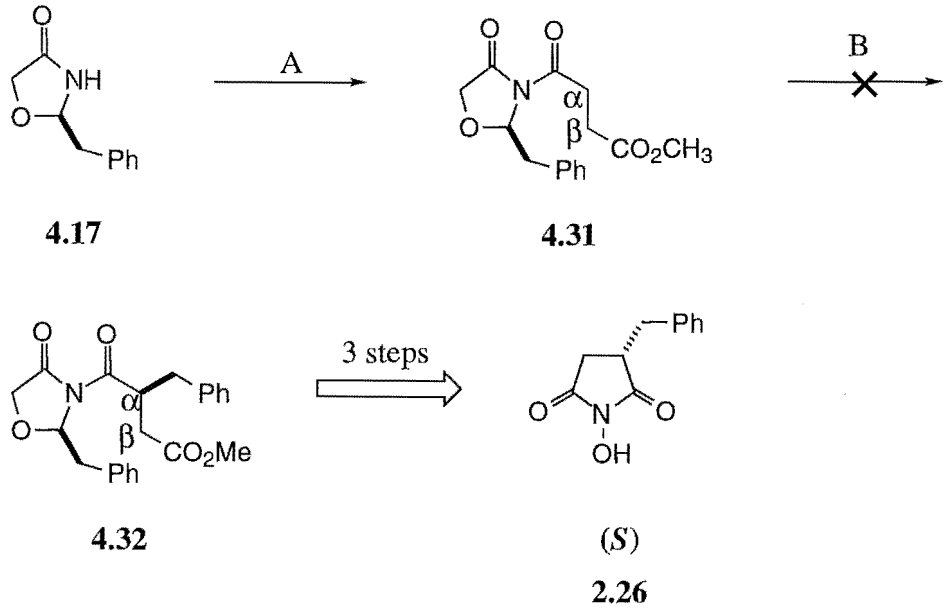


Figure 4.13 Strategies for the synthesis of (*S*)-**2.26**

A route towards (*S*)-**2.26** employing ***Strategy 1*** would require the synthesis of the (*R*)-*N*-acyloxazolidinone **4.26** using the lengthy borane reduction of (*R*)-phenylalanine and subsequent treatment with diethyl carbonate and acylation steps described for the preparation of the (*S*)-*N*-acyloxazolidinone **4.15**. An improved route to chiral oxazolidinones came to our attention during the above work which involved a one-pot two-step synthesis.[243] Employment of such methodology would lead to a short synthesis of (*S*)-**2.26**.

A route to (*S*)-**2.26** using ***Strategy 2*** was more practical as it made use of reagents already prepared above.

An attractive alternative synthesis of chiral succinates would be one where a number of derivatives could be prepared by substituting the alkylating agent used. To achieve this goal a different type of chiral enolate (**4.31**) to **4.27** and **4.29** was employed.



4.17 **4.31**

4.32 (*S*) **2.26**

Scheme 4.14 A, 1) *n*-BuLi, THF, 0.5h, -78 °C 2) 3-carbomethoxy-propionyl chloride, overnight -78 °C - rt
B 1) $TiCl_4$, Hunigs Base, 2) $PhCH_2Br$

Acylation of the chiral oxazolidinone with 3-carbomethoxy-propionyl chloride, prepared according to literature procedure,[244] gave the *N*-acyloxazolidinone **4.32**. Attempted enolisation of this by $TiCl_4$ and Hunigs base and subsequent alkylation with benzyl bromide gave a product whose ^1H NMR indicated the presence of a mixture of alkylated products and starting material. The mixture of the alkylation products may be explained in terms that the ester also activates the β-position towards alkylation as well as the desired α-position. Hence future development of this route may involve methods of directing the alkylation to one position.

In summary a synthetic route to enantiopure *N*-[(sulfonyl)oxy]imide inhibitors of type **1.41**was undertaken employing well established Evans oxazolidinone chemistry. An appropriately substituted racemic propionate substrate was attached to a chiral oxazolidinone to give a key *N*-acyloxazolidinone. This was treated with LDA to give the corresponding lithium enolate which was alkylated by both ethyl bromoacetate and *tert*-butyl acetate, reactions which proceeded with high stereoselectivity as evidenced by both ^1H and ^{13}C NMR. Hydrolysis of the alkylation product obtained with ethyl bromo acetate with LiOH/30% H_2O_2 gave enantiopure (*R*)-succinic acid mono ethyl ester. Employing methodology developed in the synthesis of racemic inhibitors the key enantiopure *N*-hydroxysuccinimide precursor (***R***)-**2.26** was prepared and then coupled

to *trans*-styryl sulfonyl chloride to give an enantiopure inhibitor **(*R*)-1.41f** with sufficient sterically demanding substitution that a difference in inhibitory activities between itself and its racemate may be observed. Preliminary inhibition studies undertaken employing the progress curve method indicated that the racemic inhbitor **1.41f** was more potent than **(*R*)-1.41f** suggesting that the (*S*)-enantiomer is the more active of the two enantiomers.

CHAPTER 5

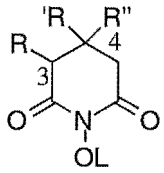
SUBSTITUTED *N*-[(4-HYDROXYMETHYL) PHENYL]IMIDES: NOVEL INHIBITORS

5.1 DESIGN AND PROPOSED MECHANISM OF INHIBITION

The glutarimide and succinimide based inhibitors **1.41** and **2.1** which were discussed in previous chapters, all operate with the same mode of inactivation. The key step in their proposed mechanism of inactivation is a Lossen rearrangement of an *N*-acyl or sulfonyloxy carboxamide to give a reactive electrophilic isocyanate that reacts further with the enzyme to give a doubly bound inactive enzyme-inhibitor complex (see **Section 1.3**, Chapter 1).

1.41

L = SO_2R'



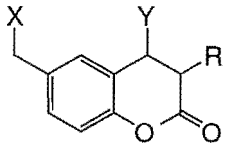
2.1

L = SO_2R'

= C(O)R'

Other classes of mechanism-based inhibitors operate by presenting a reactive species other than isocyanate in the active site of the enzyme, for example halo enol and ynenol lactones contain α-halo ketones and allenones respectively (see **Section 1.3**, Chapter 1). With the goal of designing new classes of mechanism-based imide inhibitors we aimed at incorporating electrophilic species previously observed to be involved in mechanism-based inhibition into the succinimide and glutarimide skeletons.

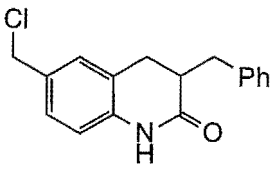
Halomethyl coumarins of type **1.27**, are known mechanism-based inhibitors of serine proteases. These operate by unmasking a 4-hydroxybenzylhalide which decomposes to give a reactive quinone methide which alkylates His-57 inactivating the enzyme. The functionalised 3,4-dihydroquinoline **1.29** was proposed to inactivate serine proteases by unmasking a quinone imine methide but it was found to be inactive probably because the *cis*-lactam ring was not hydrolysed by the enzyme. However incorporation of the latent methide of **1.29** into a macrocyclic peptide possessing the correct *trans* configuration resulted in a series of active inhibitors represented by **1.30**.



1.27

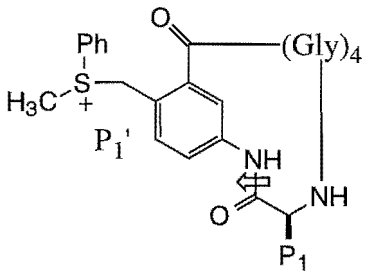
1.27c R = $PhCH_2$, Y = H, X = Cl

$k_{obs}/[I]$ >20 000 $M^{-1}s^{-1}$ / α-CT[58b]



1.29

inactive[61]

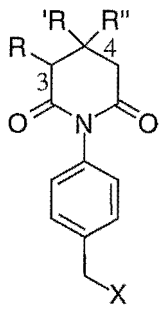


1.30

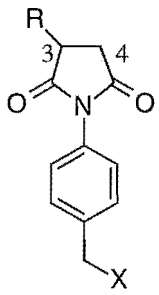
P_1 = (*L*)-Arg

$k_{obs}/[I]$ = 2 300 $M^{-1}s^{-1}$ / urokinase[62c]

Based on the knowledge that imides readily undergo enzyme induced ring opening, the imides **2.2** and **5.1** containing the latent quinone imine methide were proposed to be able to liberate activity on hydrolysis. The compounds would then be potential mechanism-based inhibitors of serine proteases.

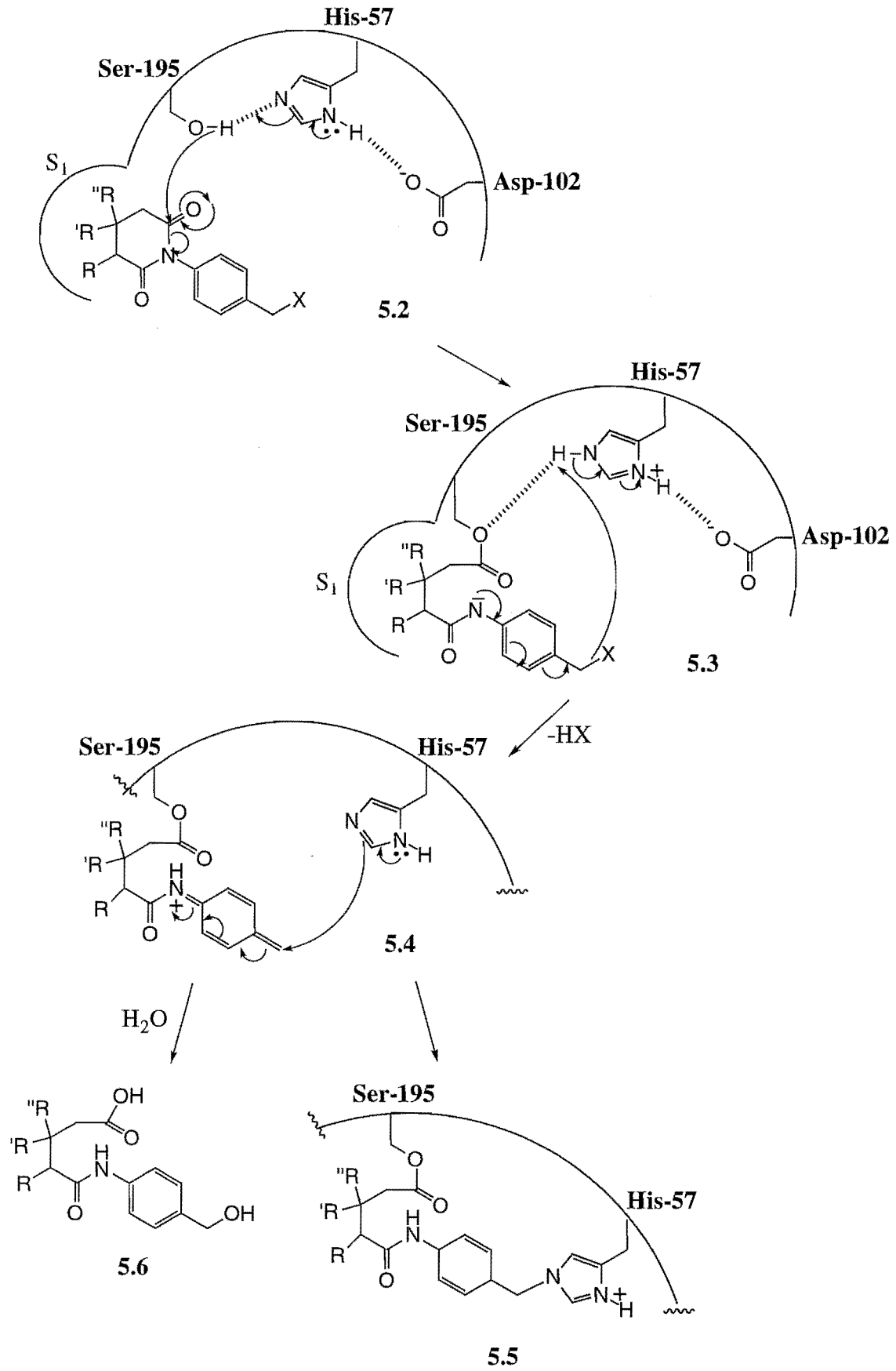


2.2



5.1

The proposed mechanism of the release of latent reactivity (**Scheme 5.1**), shown for **2.2**, would proceed with accommodation of the imide inhibitor in the active site of the enzyme through favourable interaction of the imide ring substitution with the S_1 pocket of the enzyme to give the Michaelis enzyme -inhibitor complex **6.2**. Ser-195 induced opening of imide ring to gives the acyl enzyme species **6.3**. Charge transfer through the aromatic ring as shown with release of the leaving group results in a reactive quinone imine methide tethered in the active site of the enzyme, see structure **5.4**. This will then

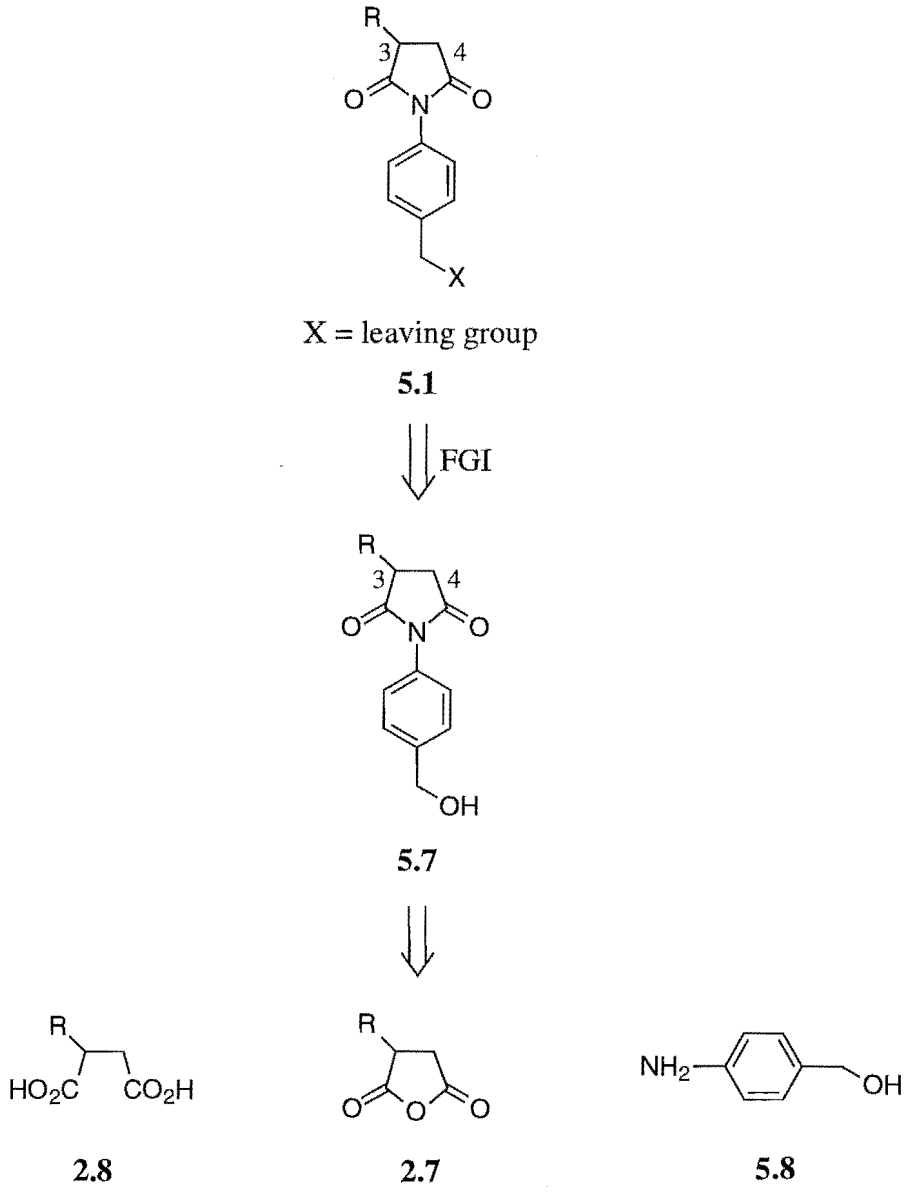


Scheme 5.1 Proposed mechanism of inactivation of α-chymotrypsin by representative inhibitor **2.2**

undergo reaction with an enzyme nucleophile such as His-57 to give the likely inactive enzyme species **5.5**. The quinone imine methide may also undergo hydrolysis to give **5.6** and liberating free enzyme in a turnover pathway.

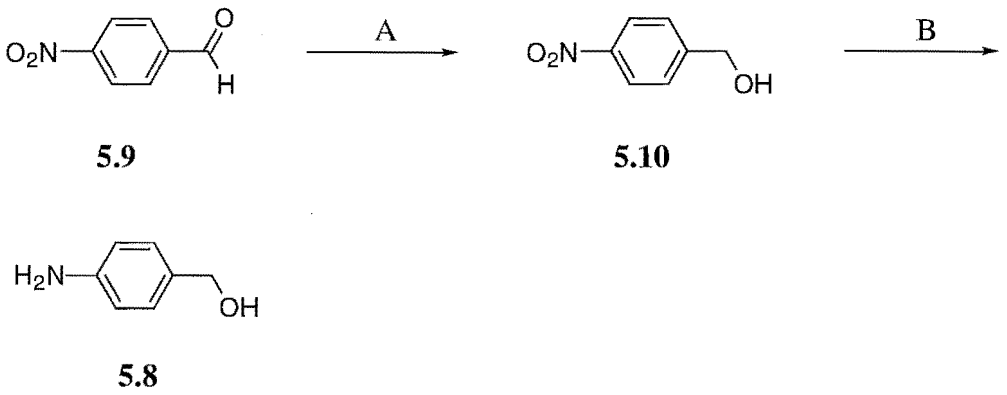
5.2 SYNTHESIS

A retrosynthetic analysis of **5.1** (**Scheme 5.2**) identified a synthetic strategy similar to that used for the preparation of substituted racemic *N*-hydroxyglutarimides and succinimides discussed in Chapter 2. The first disconnection of **5.1** involves a functional group interconversion to the *N*-(4-hydroxymethyl)phenyl imide **5.7**. Disconnection of the imide ring then follows, as shown, to give the diacid derivatives **2.7** and **2.8** and *p*-hydroxymethylbenzylamine **5.8** as reagents. It was considered therefore that the synthesis of the target inhibitors of types **2.2** and **5.1** could be wrought using the methodology developed for preparation of the related *N*-benzyloxyimides (see Chapter 2) replacing *O*-benzyloxyamine with *p*-hydroxymethylaniline, or a potential analogue.



Scheme 5.2 Retrosynthetic analysis of representative imide **5.1**

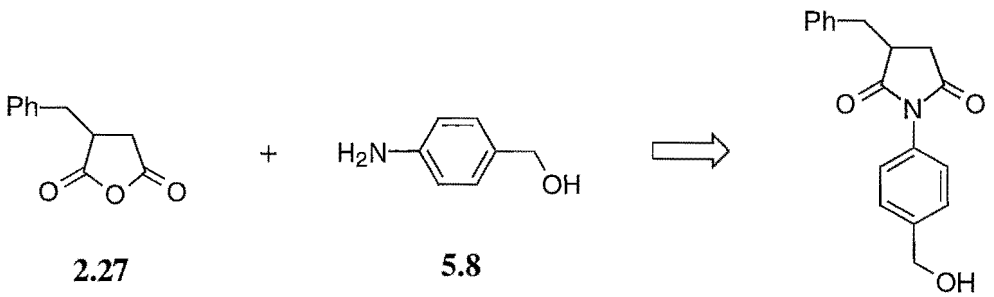
The preparation of 4-hydroxymethylaniline **5.8** (**Scheme 5.3**) was undertaken by a two step reduction of 4-nitrobenzaldehyde **5.9**. Reaction of this with an alkaline solution of sodium borohydride gave 4-nitrobenzyl alcohol **5.10** which was then reduced with hydrogen and Adam's catlayst to give **5.8**.



5.9 **5.10** **5.8**

Scheme 5.3 A, alkaline $NaBH_4$
B, H_2, PtO_2, EtOH 3d

The synthesis of **5.11** was then attempted from the corresponding anhydride **2.27** using a number of reaction conditions. A first attempt involved refluxing a solution of the anhydride in benzene with a slight excess of **5.8** proved unsuccessful. Solubility problems were encountered and unreacted anhydride was recovered on work-up. Two approaches were employed to find a solution to these problems and effect a synthesis of **5.11**. The first involved optimising the reaction conditions and the second was based on employing a protected form of the amine.



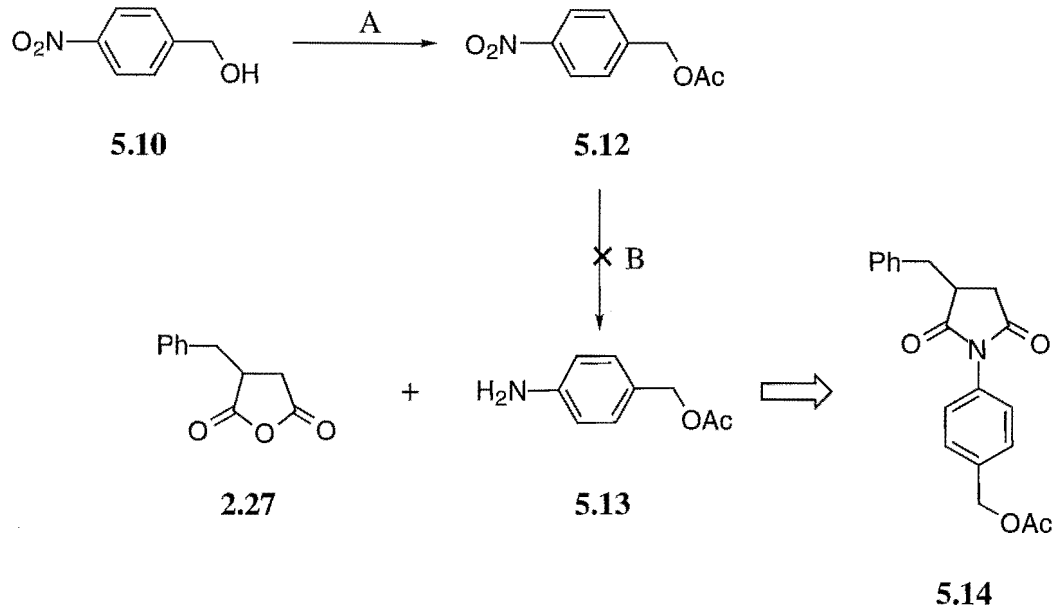
2.27 **5.8** **5.11**

Scheme 5.4 Attempted preparation of novel succinimide inhibitor **5.11**

Following the first approach subsequent reactions were attempted in toluene and 1,2-dichloroethane, however, only starting material and decomposition products were obtained. Additional reactions carried out with the addition of molecular sieves to the reaction mixture designed to scavenge water were also unsuccessful. The reaction was

also attempted with two equivalents of *p*-hydroxymethylaniline and in pyridine however this was method was also unsuccessful.

Considering the prior success of these reactions when using *O*-benzyloxyamine focus turned to consideration of using a protected form of the aniline **5.8** such that the modified properties may facilitate the reaction. The choice of an acetate protecting group was influenced by the desire to also incorporate a leaving group at this position so that a potential inhibitor may be obtained. The attempted synthesis of **5.14** is shown below (**Scheme 5.5**).



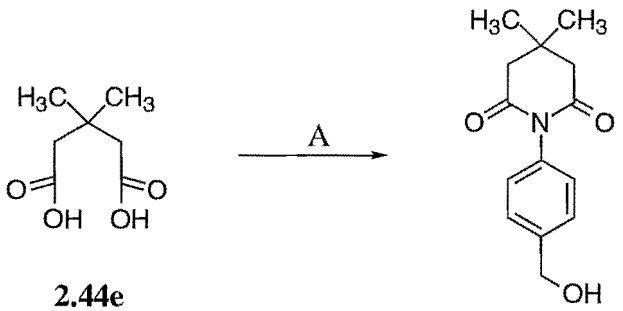
Scheme 5.5 A Ac_2O, pyridine, overnight, rt
B PtO_2, EtOH, overnight

The starting material for the above synthesis 4-nitrobenzylalcohol **5.10** was acetylated by reaction with acetic anhydride in pyridine to give *p*-nitrobenzylacetate **5.12**. Subsequent hydrogenation to give 4-acetoxymethylaniline **5.13** the key precursor to **5.14** however was unsuccessful with the molecule fragmenting during the reaction as indicated by the presence of 4-nitrotoluene in the isolated product as analysed by ^1H NMR.

With the proposed routes to the desired succinimide inhibitors **5.1** unsuccessful, focus turned to the preparation of a glutarimide derivative of type **2.2**. A mild method for the preparation of *N*-substituted glutarimides involves the reaction of amines with the appropriate acids mediated by coupling reagents such as dicyclohexylcarbodiimide (DCC)[246] and 1-(3-dimethylamino)-propyl)-3-ethyl-carbodiimide hydrochloride

(EDAC)[159] which was used in a successful preparation of a *N* -benzyloxyglutarimide (see **Section 2.4**, Chapter 2 for a discussion) following the directions of Zhu *et al.*[159]

The method of Zhu *et al*[159] was then applied to the synthesis of **5.15** (**Scheme 5.6**). This was undertaken by the EDAC mediated coupling of the amine **5.8** to 3,3-dimethylglutaric acid **2.44e** in the presence of triethylamine. Work-up of the reaction mixture gave a crude solid which was purified by flash SiO_2 chromatography to give pure **5.15** in 12% yield.



2.44e **5.15**

Scheme 5.6 A, EDAC, HOBt, Et_3N, **6.8**, overnight 0-5 °C - rt

Compound **5.15** was then assayed against α-chymotrypsin (**Figure 5.1**) using the incubation method as described in Chapter 3 to make a preliminary determination of the inhibitory activity.

The activity versus time profile of compound **5.15** (**Figure 5.1**) indicates that over a period of thirty minutes there is no net loss of activity. However as the inhibitor concentration was increased to 50-fold excess over enzyme it appears there is an initial loss of enzyme activity followed quickly, however by a regain in enzyme activity. This suggests that compound **5.15** is acting as a transient alternate substrate inhibitor of α-chymotrypsin in which the acyl-enzyme species (structure **5.3** of **Scheme 5.1**) inhibits the enzyme for a period equal to its lifetime. This description of the activity displayed by **5.15** is tentative for as discussed previously the error involved in incubation method measurements is significant (± 20%).

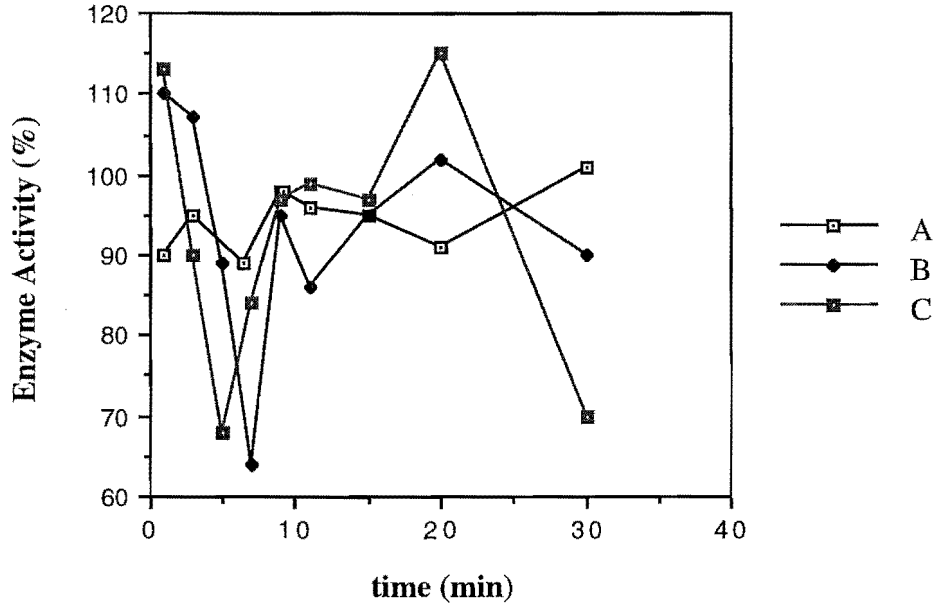
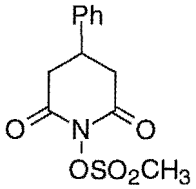


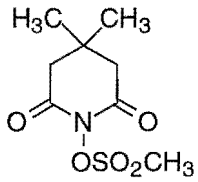
Figure 5.1 Time dependent inactivation of α-chymotrypsin by **5.15**. α-Chymotrypsin (4μM) was incubated with **6.15** (A, 10μM; B, 100μM; C, 200μM) and enzymic activity assayed using either *N*-Succ-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (A, 117μM) or BTEE (B, C, 117μM).

Study of the mechanism of inactivation proposed for these compounds (**Scheme 5.1**) indicates design efforts to enhance the activity of these compounds will be based on increasing the rate of formation of the initial Michaelis inhibitor enzyme complex (K_I) and the rates of formation of the reactive quinone methide and alkylation of the active site imidazole ring of His-57. Modification of the substituents on the imide ring to mimic the substitution present in a natural substrate of α-chymotrypsin would be expected to enhance activity. The α or β substituents of the ring are supposed to interact with the S_1 subsite of α-chymotrypsin and hence it is forecast that the introduction of aromatic substituents on the ring would lead to increased activity. This SAR trend was observed in the *N*-[(methanesulfonyl)oxy]glutarimide series assayed in Chapter 3. The glutarimide inhibitor **2.1f**, which possesses dimethyl substitution was found to be a poor inhibitor of α-chymotrypsin ($k_{obs}/[I]$ = 25 $M^{-1}s^{-1}$) when assayed by the incubation method, whereas compound **2.1d**, which possesses aromatic substitution was too potent to be measured accurately by the incubation method. On this basis, it is perhaps not suprising that **5.15** did not show significant activity.



2.1d

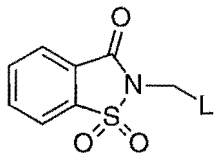
Too potent to be measured by incubation method



2.1f

$k_{obs}/[I] = 25\ M^{-1}s^{-1}$ / α-CT

The inhibitory activity may also be enhanced by increasing the rate of formation of the quinone imine methide. This may be accomplished by substituting the OH group of **5.15** with a better leaving group. Determination of a good leaving group can be made by comparison of the relative strengths of the parent acids hence it was supposed that incorporation of X = halogen into structure **5.15** in the place of the hydroxy goup would result in more potent inhibitors (compare the strengths of HCl and H_2O). This trend has been observed in the series of saccharin inhibitors of type **1.48** developed by Groutas *et al*[99] as seen by comparing the inhibitory activities of **1.48b** and **1.48a**.



1.48

1.48a L = OH

inactive[99]

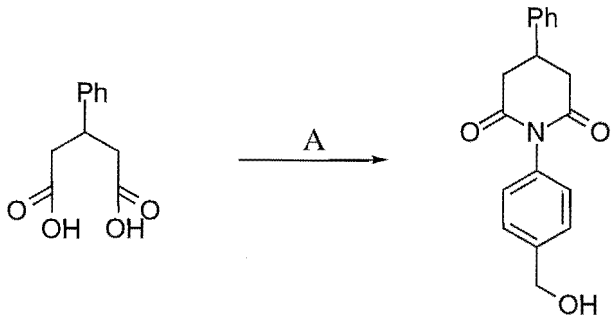
1.48b L = Cl

$k_{obs}/[I] > 49\ 000\ M^{-1}s^{-1}$ / HLE[99]

1.48c L = F

$k_{obs}/[I] > 4\ 750\ M^{-1}s^{-1}$ / HLE[99]

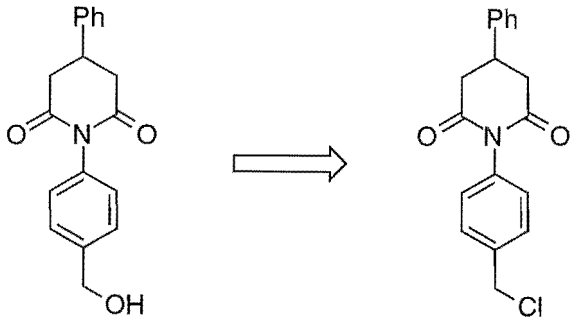
These SAR considerations suggested that compound **5.17** may be a more potent inhibitor of α-chymotrypsin than **5.15**, because it has both more suitable substitution to interact favourably with α-chymotrypsin and a better leaving group. Hence **5.17** became a synthetic target. EDAC mediated coupling of 4-hydroxymethylaniline **5.8** to the commercially available 3-phenylglutaric acid (**Scheme 5.7**) gave a solid that on analysis by thin layer chromatography (TLC) was found to be unsuitable for purification by flash SiO_2 chromatography. Purification was then carried out following the directions of Seth *et al.*[246] The solid was dissolved in 5% aqueous sodium carbonate and this aqueous solution was washed with dichloromethane to remove impurities such as **5.8**. Acidification of the aqueous layer and organic extraction gave **5.16** which was further purified by recrystallisation.



2.44a **5.16**

Scheme 5.7 A, EDAC, HOBt, **5.8**, Et_3N, 0-5 °C - rt overnight

Chlorination of **5.16** to afford **5.17** was then attempted using the method adopted by Groutas *et al*[99] in the preparation of **1.48b** from *N*-hydroxymethylsaccharin **1.48a**. A solution of **5.16** in ether was treated with thionyl chloride (5 equiv.) for two days at room temperature however ^1H NMR analysis of the product indicated the reaction was unsuccessful and an altenative procedure was sought.



5.16 **5.17**

The chlorination of primary alcohols using CCl_4-PPh_3 has been well documented[247] and was attractive as the reaction is both mild and rapid. A trial chlorination of **6.15** was undertaken employing this methodology however this too was unsuccessful. Although only preliminary results, the lack of success observed in these reactions may be due to the inherent reactivity of **5.17**. This may undergo ring-opening to give the quinone imine methide which reacts with any nucleophiles present in the reaction mixture. Proton NMR analysis of the product isolated from the attempted chlorination using thionyl chloride indicated the presence of a charateristic triplet and quartet of an ethyl ester perhaps formed from reaction of the methide with ethanol.

In summary we have developed a preliminary synthetic approach to a glutarimide designed to liberate reactivity on hydrolysis. Initial studies have failed to identify an inhibitor of α-chymotrypsin. The lack of inhibitory activity may be reasoned in that this

glutarimide does not act as a substrate of α-chymotrypsin and therefore cannot undergo the required ring-opening necessary to unmask the quinone imine methide. Considering the structure of the acyl enzyme intermediate in the formation of the methide the lone pair on the nitrogen may not be available to move through the aromatic ring to displace the leaving group as it is delocalised by the neighbouring carbonyl group.

Attempts to introduce activity into this series involved incorporation of ring substitution able to interact with α-chymotrypsin and improving the leaving group. Synthesis of a compound purporting both these features was unsuccessful maybe due to the irony that the synthesis is undone by the activity it is trying to create.

CHAPTER 6

EXPERIMENTAL

6.1 GENERAL

Melting points (mp) were measured using an Electrothermal apparatus and are uncorrected. Optical rotations were measured on a JASCO J-20C recording spectropolarimeter at the Na line (λ = 589nm). The specific optical rotation at the stated temperature $[\alpha]^{T}_{D}$ is given in units of 10^{-1} degcm^2 g^{-1} with the sample concentration, c, in the indicated solvent given in units of g $100cm^{-3}$.

IR spectra were recorded on a Schimadzu FTIR-8201PC spectrophotometer. Samples were run as KBr discs (KBr), Nujol mulls between KBr plates (Nujol/KBr), $CHCl_3$ and $CDCl_3$ solutions, or as a $CHCl_3$ film between NaCl plates ($CHCl_3$/NaCl). Data is presented as the wavenumbers (cm^{-1}) at which the maxima absorptions of diagnostic value occur.

Proton (300MHz) and ^{13}C (75MHz) nuclear magnetic resonance (NMR) spectroscopy was performed with either a Varian CFT300 or XL300 spectrometer. $CDCl_3$, with Me_4Si as an internal standard, a mixture of $CDCl_3$ and a small amount of DMSO-d_6 ($CDCl_3$/DMSO-d_6), DMSO-d_6, and D_2O (^1H NMR only) were used as solvents.

Proton $CDCl_3$ and $CDCl_3$/DMSO-d_6 spectra were referenced to the $(C\underline{H}_3)_4Si$ signal at a chemical shift (δ) of 0.00 parts per million (ppm) and ^{13}C NMR spectra to the central peak of the $\underline{C}DCl_3$ triplet at δ 77.0ppm. Proton DMSO-d_6 spectra were referenced to the residual $(C\underline{H}_3)_2SO$ signal at δ 2.50ppm and ^{13}C NMR spectra to the central peak of $(C\underline{H}_3)_2SO$ quintuplet at δ 39.6. Proton D_2O spectra were referenced to the residual $\underline{H}_2O$ signal at δ 4.70ppm.

Proton NMR data are recorded as follows: chemical shift in parts per million (δ) accurate to two decimal places (multiplicity (s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, dd: doublet of doublets, ABq: AB quartet, etc., b indicates some broadening of the signal), integrated intensities, coupling constant(s) in Hz accurate to one decimal place, and assignment as the underlined proton(s) within the given structural fragment). Data for ^{13}C NMR are recorded as the chemical shifts observed accurate to one decimal place. If required, 2D NMR experiments were carried out using a Varian CFT300 spectrometer to aid assignment.

Mass spectra were obtained using a Kratos MS80RFAspectrometer operating at 70eV. The molecular ion (M^+) only, if present, is reported accurate to four decimal places when using High Resolution Mass Spectrometry (HRMS) in the indicated operated mode +CI (Chemical Ionisation), +EI (Electron Ionisation) or FAB (Fast Atom

Bombardment). If the molecular ion was sufficiently weak so as not to be recorded significantly high mass ions and the more intense low mass ions accurate to one decimal place are reported. The molecular ion (M^+), if present, significantly high mass ions and the more intense low mass ions accurate to the nearest mass unit are reported when using Low Resolution Mass Spectrometry (LRMS) in the indicated operation mode as above. Data are presented in the folowing order: m/z value, the attributed fragmentation; relative intensity as a percentage of the base peak.

Elemental analyses were performed by the Campbell Microanalytical Laboratory, University of Otago, Dunedin.

Solvents were freshly dried and distilled according to the following well established procedures[248,249] and then stored in N_2 immediately prior to use. Tetrahydrofuran (THF), and diethyl ether (ether, Et_2O) were purified by distillation from sodium benzophenone ketyl. Ethanol (EtOH) and Methanol (MeOH) were purified by distillation from magnesium ethoxide or methoxide respectively. Benzene, dichloromethane (CH_2Cl_2) and toluene were purified by distillation from CaH_2. *N,N*-Dimethylformamide (DMF) was dried by the method of Burfield and Smithers.[250] Petroleum ether refers to the fraction of boiling range 50-70 °C. Reagents were purified prior to use according to literature procedures.[249] The commonly used bases *N,N*-diisopropylethylamine (Hunigs base), pyridine, and triethylamine (Et_3N) were purified by distillation from CaH_2 or P_2O_5.

Organic reactions requiring anhydrous conditions were performed with solvents and reagents as prepared above under a dry N_2 atmosphere with glassware baked in an oven set at 110 °C overnight then assembled hot and 'flame' dried with a bunsen burner or heat gun under N_2 or vacuum immediately prior to use. Reaction temperatures refer to the external bath temperature. A reaction carried out at room temperature (rt) refers to a temperature of *ca* 20 °C. A workup procedure carried out in the usual manner involved separation of the organic extract or phase, drying with Na_2SO_4 or $MgSO_4$, filtration to remove the dessicant and then evaporation of the bulk of the solvent using a rotary evaporator, the last traces being removed under high vacuum. Flash chromatography was conducted according to the method of Still *et al* [251] using Merck 60 PF254 type silica gel.

Diethyl benzyl malonate (**2.10**), 2-phenylsuccinic acid (**2.30**), 3-phenylglutaric acid (**2.44c**), 2-phenylglutaric anhydride (**2.43a**), β-*trans*-styryl sulfonyl chloride, 2-naphthalene sulfonyl chloride, 4,4'-biphenyldisulfonyl chloride were obtained from Aldrich Chemical Company, Milwaukee, USA. 2-Phenylglutaric anhydride **2.43a** was purified by treatment with Ac_2O before use. 3-Chlorosulfonyl benzoic acid and 1,3-

bis(chlorosulfonyl)benzene were obtained from Maybridge Chemical Co. Ltd, Tintagel, Cornwall, UK.

4,4-Cyclohexyl-3,5-dicyanoglutarimide (**2.48b**) was generously gifted by Dr. R. H. McKeown.

6.2 CHAPTER 2 EXPERIMENTAL

SYNTHESIS OF 2-SUBSTITUTED SUCCINIC ACIDS

Method A: Malonate Ester Route

Diethyl 2-carboethoxy-2-benzylsuccinate (2.11)[152]

A solution of sodium ethylate was prepared by slowly dissolving sodium (2.332g, 0.101mol) in EtOH (35mL). Diethyl benzylmalonate (**2.10**) (24.890g, 0.100mol) and ethyl bromoacetate (11mL, 16.566g, 0.099mol) were added slowly in succession. The resulting solution was refluxed for 14.25h, concentrated, made slightly acidic (pH=6, Universal Indicator Paper) with 3N H_2SO_4 and extracted with Et_2O. The Et_2O extract was worked up in the usual manner to give a yellow oil (28.49g) containing a 4:5 mixture, by ^1H NMR, of unreacted **2.10** and **2.11**. This crude product was used without further purification. ^1H NMR **2.11** ($CDCl_3$) δ 1.17-1.29 (m, 9H, 3x$OCH_2C\underline{H}_3$), 2.85 (s, 2H, $C\underline{H}_2CO_2Et$), 3.39 (s, 2H, $C\underline{H}_2Ph$), 4.12-4.24 (m, 6H, 3x$OC\underline{H}_2CH_3$), 7.08-7.26 (m, 5H, Ar$\underline{H}$).

2-Carboxyl-2-benzylsuccinic acid (2.12)[152]

The above crude product (28.49g) was boiled under reflux with KOH (21.338g, 0.380mol) in EtOH (120ml) for 4h. The reaction mixture was diluted with H_2O (55ml), and extracted with Et_2O. The aqueous layer was acidified (pH=1, Universal Indicator Paper) with 6M HCl, and extracted with Et_2O. The extracts were worked up in the usual manner to give a brown residue (13.127g) containing a 12:15 mixture, by ^1H NMR, of benzyl malonic acid (from the hydrolysis of **2.10** present in the starting material) and **2.12**: ^1H NMR **2.12** ($CDCl_3$) δ 2.85 (s, 2H, $C\underline{H}_2Ph$), 3.33 (s, 2H, $C\underline{H}_2CO_2H$), 7.14-7.58 (m, 5H, Ar$\underline{H}$), 9.66 (bs, 3H, 3x$CO_2\underline{H}$).

2-Benzylsuccinic acid (2.13)[152]

The above mixture (13.127g) was heated in an oil bath set at 160-170 ^0C for 2.25h. The reaction mixture was allowed to cool to rt and then refridgerated. The resultant brown mass was treated with boiling H_2O to give an immiscible mixture consisting of an upper aqueous phase and lower brown oil. The aqueous phase was decanted and allowed to cool to rt during which **2.13** crystallised as a white solid. More acid was obtained by repeated treatments and concentration of the mother liquors. Total yield (3.307g, overall yield 17%); mp 160-1 ^oC (H_2O, glittering white rectangular plates) lit.[252] mp 160-1 ^oC; IR (KBr) υ_{max} 3022.1 (OH), 1697.8 (C=O) cm^{-1}; ^1H NMR

($CDCl_3$/DMSO-d_6) δ 2.31 (dd, 1H, J = 4.9, 17.1Hz, HCHPh), 2.58 (dd, 1H, J = 8.3, 16.6Hz, HCHPh), 2.78 (dd, 1H, J = 4.9, 10.2Hz, HCHC(O)), 3.01-3.08 (m, 2H, HCHC(O) and CH), 7.18-7.30 (m, 5H); HRMS (+EI, M^+) calcd. 208.0736, found 208.0736.

Method B: Amide base alkylation of succinimide

2-Benzylsuccinimide (2.14)[144]

Liquid NH_3 (60mL) was transferred to a three necked reaction flask cooled to -78 °C (dry-ice/acetone bath) equipped with a dry-ice/acetone condenser and a droppping funnel. A small amount of Na was added followed by $FeNO_3.9H_2O$ (90mg). After the blue colour was discharged to give a charcoal like reaction solution, Na (total amount added 1.634g, 0.071mol) was then added portionwise to give a grey suspension of $NaNH_2$. This was stirred for a 1h at -78 °C after which succinimide (3.178g, 0.032mol) was added portionwise and the resulting grey suspension stirred for a further hour. The dropping funnel was charged with a solution of benzyl bromide (3.826mL, 5.490g, 0.032mol) in anhydrous Et_2O (10mL) and this was added rapidly to the reaction suspension. The dropping funnel was rinsed with an additional quantity of Et_2O (1mL) and the resulting pale yellow suspension stirred for 1h and the reaction quenched by pouring the reaction mixture into a beaker containing NH_4Cl (5.649g, 0.106mol, 3.3 equiv.). The NH_3 was allowed to evaporate while being replaced by the slow addition of Et_2O (50mL). A mixture of conc HCl (27mL) and ice (250mL) was added and the resulting mixture was stirred until the ice melted. The Et_2O phase was separated and the aqueous phase extracted with Et_2O. The organics were combined and washed with H_2O and worked up in the usual manner to give a creamy yellow solid. This was dissolved in Et_2O and extracted thoroughly with 5% aqueous NaOH. The alkaline extracts were washed with Et_2O and then acidified (pH=1, Universal Indicator Paper) with 6N HCl and then extracted with Et_2O. Work-up in the usual manner gave a white solid (755mg, 12%): mp 81-4 °C (lit.[144] mp 92.5-95 °C); IR (KBr) υ_{max} 3170.8 (NH), 1776.3 and 1705.0 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.48 (dd, 1H, J = 4.9, 18.5Hz, HCH$_2$Ph) 2.71 (dd, 1H, J = 8.8, 18.5Hz, HCHPh) 2.84 (dd, 1H, J = 9.5, 14.4Hz, HCHC(O)) 3.13-3.25 (m, 2H, HCHC(O) and CHC(O)), 7.17-7.34 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 34.4, 36.1, 42.7, 127.0, 128.8, 128.9, 137.1, 180.1, 189.6; LRMS m/z (+EI) 189 (67, M^+), 161 (3), 146 (17), 117 (2), 103 (5), 91 (100), 78 (8), 65 (13), 51 (8).

2-Benzylsuccinic acid (2.13)

2.14 (684mg, 4mmol) was refluxed with 6N HCl (15mL) for 26h. The reaction solution was allowed to cool to rt during which a white solid cystallised. This reaction mixture was evaporated to dryness and sufficient Et_2O and saturated aqueous NH_4Cl solution were added to dissolve the white solid. The Et_2O phase was worked up in the usual manner to give **2.13** as a white solid (760mg, quantitative yield): mp 152-155 °C (lit.[252] mp 160-1 °C). Spectral data as given above.

Method C: Stobbe Condensation Route

Ethyl 2-benzylidenesuccinate (2.17)[157d]

A solution of sodium ethoxide was prepared by slowly dissolving sodium (1.476g, 0.064mol, 1.23 equiv.) in EtOH (25ml) under a N_2 atmosphere. Diethyl succinate (8.6ml, 9.004g, 0.052mol) was added dropwise and the resulting milky mixture heated until reflux at which point benzaldehyde (5.3ml, 5.533g, 0.052mol) was added dropwise. The resulting yellow reaction mixture was refluxed for 12h. The now brown reaction mixture was concentrated and dissolved in H_2O (100mL) and then extracted with Et_2O (2x100mL). The aqueous layer was acidified (pH=1, Universal Indicator paper) with 6N HCl (20mL) and this was extracted with Et_2O (2x100mL). The organic extracts were worked up in the usual manner to give crude ethyl 2-benzylidenesuccinate **2.17** as a viscous brown syrup (9.262g, 76%): ^{1}H NMR δ 1.35 (t, 3H, *J* = 7.1Hz, $CH_2C\underline{H}_3$), 3.58 (s, 2H, $C\underline{H}_2CO_2H$), 4.31 (q, 2H, *J* = 7.1, 7.3Hz, $C\underline{H}_2CH_3$), 7.34-7.44 (m, 5H, Ar$\underline{H}$), 7.93 (bs, 1H, N$\underline{H}$); ^{13}C NMR δ 14.1, 33.6, 61.4, 125.5, 128.7, 129.0, 134.7, 142.3, 167.4, 177.2; HRMS (+EI, M^+) calcd. 234.0893, found 234.0892.

Ethyl 2-benzylsuccinate (2.18)

A solution of ethyl 2-benzylidenesuccinate **2.17** (9.144g, 0.039mol) in EtOAc (20mL) was hydrogenated in the presence of 10% palladium on carbon (1.141g) for 19h. The reaction mixture was filtered through a column packed with both celite and Na_2SO_4. The filter cake was washed with a small quantity of EtOAc and the filtrate and washings were combined and evaporated to give **2.18** as a yellow oil (5.541g, 60%): ^{1}H NMR ($CDCl_3$) δ 1.18 (t, 3H, *J* = 7.1Hz, $OCH_2C\underline{H}_3$), 2.43 (dd, 1H, *J* = 4.4, 17.1Hz, $\underline{H}$CHPh), 2.66-2.81 (m, 2H, HC$\underline{H}$Ph and $\underline{H}$CHC(O)), 3.01-3.13 (m, 2H, HC$\underline{H}$C(O) and C$\underline{H}$C(O)), 4.12 (q, 2H, *J* = 6.8, 7.4Hz, $OC\underline{H}_2CH_3$), 7.14-7.31 (m, 5H, Ar$\underline{H}$); ^{13}C NMR δ 14.0, 34.7, 37.6, 42.8, 60.8, 126.7, 128.5, 129.0, 138.0, 174.0, 177.8.

2-Benzylsuccinic acid (2.13)[152]

A solution of ethyl 2-benzylsuccinate **2.18** (2.416g, 0.010mol) in 1:1 EtOH/H_2O (20mL) with KOH (2.23g, 0.040mol, 4 equiv.) was refluxed for 5h. The reaction solution was concentrated and extracted with EtOAc. The aqueous layer was acidified (pH=1, Universal Indicator paper) with 6N HCl and then extracted with EtOAc. The organic extracts were worked up in the usual manner to give **2.13** as a white solid (1.745g, 82%). Spectral data data as given above.

THE SYNTHESIS OF 2-SUBSTITUTED SUCCINIC ACID ANHYDRIDES

2-Phenylsuccinic anhydride (2.30)

A mixture of 2-phenylsuccinic acid **2.29** (5.000g, 26mmol) and acetyl chloride (10.5mL, 11.592g, 129mmol) was refluxed for 1h. The excess acetyl chloride was evaporated and the resultant liquid distilled at reduced pressure to give a pale yellow solid. Recrystallisation from toluene/petroleum ether gave **2.30** as a white solid (3.123g, 69%): mp 51-3 °C (lit.[166] mp 53-5 °C); IR (KBR) υ_{max} 1867.0 and 1791.7 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.13 (dd, 1H, *J* = 6.9, 19.1Hz, HCH), 3.47 (dd, 1H, *J* = 10.3, 19.1Hz, HCH), 4.35 (dd, 1H, J = 6.8, 10.2Hz, CH), 7.26-7.29 (m, 2H, ArH), 7.40-7.45 (m, 3H, ArH); ^{13}C NMR ($CDCl_3$) δ 36.5, 46.5, 127.4, 128.6, 129.4, 134.7, 169.9, 172.0.

2-Benzylsuccinic anhydride (2.27)[84]

A mixture of 2-benzylsuccinic acid (**2.13**) (2.055g, 0.010mol) and Ac_2O (9.6ml, 10.387g, 0.102mol, 10.2 equiv.) was refluxed for 1h. Evaporation of solvent gave a crude white solid which was triturated with ice-cold petroleum ether to give **2.27** as a white solid (1.766g, 94%): mp 97-9 °C (lit.[84] mp 95-7 °C); IR ($CDCl_3$) υ_{max} 1868.9 and 1785.0 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.72 (dd, 1H, *J* = 6.3, 18.5Hz, HCHPh), 2.95 (dd, 1H, *J* = 5.3, 14.1Hz, HCHPh), 3.02 (dd, 1H, *J* = 8.3, 14.2Hz, HCHC(O)), 3.24 (dd, 1H, *J* = 4.9, 14.2Hz, HCHC(O)), 3.40-3.50 (m, 1H, CH), 7.17 (d, 2H, *J* = 6.3Hz, ArH), 7.27-7.38 (m, 3H, ArH); ^{13}C NMR ($CDCl_3$) δ 32.9, 36.0, 42.1, 127.6, 135.8, 169.6, 173.1, 128.9, 129.1; HRMS (+EI, M^+) calcd. 190.0630, found 190.0630.

THE SYNTHESIS OF 3-SUBSTITUTED *N*-BENZYLOXY SUCCINIMIDES

General Method: From anhydride

A dispersion of *O*-benzyloxyamine (1 equiv.) and the respective anhydride (stated amount) in toluene or xylenes (stated volume) dissolved on heating until reflux and the resulting solution was refluxed with azeotropic removal of H_2O for the stated time. The solvent was evaporated to yield either a crude solid or more typically a brown residue. Either was dissolved in EtOAc and washed with 10% aqueous $NaHCO_3$ and H_2O. The organic phase was worked up in the usual manner to give the title compounds. These were purified by recrystallisation from the stated solvent system.

3-Phenyl-*N*-benzyloxysuccinimide (2.31)

By General Method: a dispersion of *O*-benzyloxyamine (154mg, 1.3mmol, 1.2 equiv.) and 2-phenylsuccinic anhydride **2.30** (200mg, 1.1mmol) in toluene (2.5mL) dissolved with heating and the resulting solution was refluxed with azeotropic removal of H_2O for 7h. Work-up gave **2.31** as a pale yellow solid (223mg, 70%): mp 111 °C (1:1:2 Et_2O/pentane/EtOAc, pale yellow-white crystals); IR (KBr) υ_{max} 1718.5 and 1201.6 (C=O) cm^{-1}; ^1H NMR δ 2.60 (dd, 1H, *J* = 4.3, 18.5Hz, HCHC(O)), 3.03 (dd, 1H, *J* = 9.3, 18.1Hz, HCHC(O)), 3.84 (dd, 1H, *J* = 4.4, 9.3Hz, CH), 5.16 (s, 2H, OCH_2Ph), 6.95-6.98 (m, 2H, ArH), 7.26-7.28 (m, 3H, ArH), 7.34-7.36 (m, 3H, ArH), 7.45-7.48 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$) δ 34.3, 42.8, 78.2, 127.1, 127.8, 128.4, 128.9, 129.3, 129.9, 1333.0, 136.3, 170.2, 171.8. Anal. Calcd. for $C_{17}H_{15}NO_3$: C, 72.59; H, 5.37; N, 4.98. Found: C, 72.45; H, 5.41; N, 5.13.

3-Benzyl-*N*-benzyloxysuccinimide (2.25)[84]

By General Method: a dispersion of *O*-benzyloxyamine (0.370g, 3.0mmol, 1.07 equiv.) and 2-benzylsuccinic anhydride **2.27** (525mg, 2.8mmol) in xylenes (6mL) dissolved with heating and the resulting solution was refluxed with azeotropic removal of H_2O for 18.25h. Work up gave a white solid which wa recrystallised from toluene/petroleum ether to give **2.25** as a white solid (635mg, 78%): mp 90-1 °C (lit.[84] mp 130-2 °C); IR (KBr) υ_{max} 1785.9 and 1719.5 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.35 (dd, 1H, *J* = 4.4, 18.1Hz, HCHPh), 2.60 (dd, 1H, *J* = 8.8, 18.1Hz, HCHPh), 2.70 (dd, 1H, *J* = 8.8, 18.1Hz, HCHC(O)), 2.98-3.07 (m, 1H, CH), 3.13 (dd, 1H, *J* = 4.4, 13.7Hz, HCHC(O)), 3.40-3.50 (AB_q, 2H, *J* = 9.8Hz, OCH_2), 7.13 (d, 2H, *J* = 6.3Hz, ArH), 7.23-7.45 (m, 3H, ArH); ^{13}C NMR ($CDCl_3$) δ 30.5, 36.2, 38.3, 78.6, 127.3,

128.5, 128.9, 129.1, 129.4, 130.0, 133.2, 136.3, 170.4, 173.3; HRMS (+CI, MH^+) calcd. 296.1287, found 296.1287.

Alternatively **2.25** was prepared in the following manner from ethyl 3-benzylsuccinate **2.18**.

A solution of ethyl 2-benzylsuccinate **2.18** (193mg, 0.81mmol) in CH_2Cl_2 (2mL) was added to a solution of *O*-benzyloxyamine hydrochloride (130mg, 0.81mmol) and Et_3N (141.2μL, 103mg, 1.02mmol, 1.26 equiv.) in CH_2Cl_2 (2mL) that had been stirring for 10 min under N_2 at 0-5 °C (ice-salt bath). HOBt (110mg, 0.81mmol) and then DCC (165mg, 0.80mmol) were added successively and the resulting yellow solution was stirred for 21h while the temperature was allowed to come to rt. The resulting white slurry was filtered to remove the white DCU solid formed during the reaction and this was washed with a small quantity of CH_2Cl_2. The filtrate and the washings were combined and evaporated to give a brown residue. This was taken up into the minimum amount of EtOAc and the suspended DCU was filtered and washed with a small quantity of EOAc and the filtrate and washings were combined and washed with H_2O (10mL) and worked up in the usual manner to give a brown residue (287mg). A sample (233mg) of this was purified by flash SiO_2 chromatography eluting with 1:3 EtOAc/petroleum ether to give 3-benzyl-*N*-benzyloxysuccinimide **2.25** as a white solid (33mg) with physical data identical to that given above, and then eluting with EtOAc to give 3-(carboethoxy)-3-benzylpropionyl-*N*-benzyloxyamide **2.24** as a white solid (109mg): mp 90-91 °C; ^1H NMR ($CDCl_3$) δ 1.18 (t, 3H, *J* = 7.1Hz, $OCH_2\underline{CH_3}$), 2.81 (dd, 1H, *J* = 7.8, 13.7Hz, HCHPh), 2.97-3.03 (m, 2H, HCHPh and HCHC(O)), 3.17-3.65 (m, 2H, HCHC(O) and CHC(O)), 4.09 (q, 2H, *J* = 6.8, 7.3Hz, OCH_2CH_3), 4.86 (bs, 2H, OCH_2Ph), 7.15-7.37 (m, 10H, ArH), 8.16 (bs, 1H, NH).

A solution of **2.24** (92mg, 0.27mmol) and *p*-TsOH (26mg, 0.14mmol, 0.5 equiv.) in 1,2-dichloroethane (DCE) (5mL) was refluxed with azeotropic removal of EtOH for 13h. The solvent was evaporated to give **2.25** as a pale brown solid (106mg, quantative yield). Total yield **2.25** 139mg, (85%). Spectral data as above.

THE SYNTHESIS OF 3-SUBSTITUTED *N*-HYDROXY SUCCINIMIDES

Method A: From 3-substituted*N*-benzyloxysuccinimide

A solution of the respective substituted *N*-(benzyloxy)succinimide (stated amount) in the stated solvent (stated volume) was hydrogenated in the presence of the indicated palladium catalyst (stated amount) for the stated time. The reaction mixture was filtered

through celite and Na_2SO_4 and this cake washed with a small quantity of solvent. The filtrate and washings were combined and evaporated to give the title *N*-hydroxysucccinimides. These were purified, if required, by either chromatagraphy or recrystallisation using the stated solvent system.

Method B: From 2-substituted succinic acid anhydride

The respective anhydride (stated amount) was added to a solution of hydroxylamine hydrochloride (1.2 equiv.) and K_2CO_3 (0.6-1equiv.) in distilled H_2O (stated volume). This mixture was refluxed for the stated time. The hot solution was acidified (pH=1, Universal Indicator Paper) with 10% or conc. HCl, allowed to cool to rt, and then extracted with EtOAc. The combined extracts were worked up in the usual manner to give the title*N*-hydroxysuccinimide.

3-Phenyl-*N*-hydroxysuccinimide (2.32)

By Method A: a solution of 3-phenyl-*N*-benzyloxysuccinimide **2.31** (40mg, 0.14mmol) in THF (1mL) was hydrogenated for 4.25h in the presence of 10% palladium on carbon (5mg). Work-up gave a yellow oil which was purified by radial chromatography eluting with 1:4 EtOAc/petroleum ether to give **2.32** as a pale yellow oil (14mg, 53%): mp 109-111 °C (EtOAc/petroleum ether, white crystals); IR ($CHCl_3$/NaCl) υ_{max} 3118.7 (OH), 1787.9 and 1693.4 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.73 (dd, 1H, *J* = 4.4, 17.5Hz, HCH), 3.17 (dd, 1H, *J* = 9.2, 18.0Hz, HCH), 4.14 (dd, 1H, *J* = 4.4, 8.8Hz, CH), 7.31-43 (m, 5H, ArH), 10.78 (bs, 1H, NOH); ^{13}C NMR ($CDCl_3$) δ 34.2, 42.8, 127.7, 128.0, 129.0, 137.9, 171.8, 173.5; LRMS (+EI, m/z) 191 (M+, 18), 175 (25), 121 (35), 104 (100), 78 (45), 51 (55). Anal. Calcd. for $C_{10}H_9NO_3$: C, 62.83; H, 4.74; N, 7.33. Found: C, 62.67; H, 4.68; N, 7.36.

By Method A: a solution of 3-phenyl-*N*-benzyloxysuccinimide **2.31** (40mg, 0.14mmol) in MeOH (7.5mL) was hydrogenated at 50psi for 8.5h in the presence of 5% palladium on $BaSO_4$. Work up gave a pale yellow oil which was purified by radial chromatography eluting with 1:4 EtOAc/petroleum ether to give a colourless oil. This was recrystallised from EtOAc/petroleum ether to give **2.32** as a white solid (10mg, 37%): mp 108-9 °C. Physical data as given above.

By Method B: 2-phenylsuccinic anhydride **2.30** (500mg, 2.9mmol) was added to a solution of hydroxylamine hydrochloride (237mg, 3.4mmol, 1.2 equiv.) and K_2CO_3 (236mg, 1.7mmol, 0.6 equiv.) in distilled H_2O (7.5mL). This mixture was refluxed for 5h. Subsequent work up as described gave **2.32** as a pale yellow-white solid (440mg, 82%). Physical data as above.

3-Benzyl-*N*-hydroxysuccinimide (2.26)[84]

By Method A: a solution of 3-Benzyl-*N*-benzyloxysuccinimide **2.25** (202mg, 0.7mmol) in 1:1 MeOH/EtOH (7mL) hydrogenated in the presence of 10% palladium on carbon (71mg) for 19.5h. Work up as described gave **2.26** as a white solid (86mg, 61%): mp 105-110 °C (lit.[84] mp 120-1°C); IR (KBr) υ_{max} 3224.8 (OH), 1770.5 and 1697.2 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$/DMSO-d_6) δ 2.13 (dd, 1H, *J* = 4.4, 18.1Hz, HCHPh) 2.41 (dd, 1H, *J* = 8.8, 18.1Hz, HCHPh) 2.62 (dd, 1H, *J* = 8.6, 13.5Hz, HCHC(O)) 2.83-2.91 (m, 1H, CHC(O)), 2.97 (dd, 1H, *J* = 4.4, 13.7Hz, HCHC(O)), 6.92-7.25 (m, 5H, ArH): ^{13}C NMR ($CDCl_3$/MSO-d_6) δ 30.3, 35.9, 38.0, 126.7, 128.5, 128.7, 136.6, 171.3, 174.1; HRMS (+EI, M^+) calcd. 205.0739, found 205.0739. Anal. Calcd for $C_{11}H_{11}NO_3.1/2H_2O$: C, 61.66; H, 5.65. Found C, 62.08; H, 5.21.

By Method A: a solution of 3-benzyl-*N*-benzyloxysuccinimide **2.25** (5.565g, 19mmol) in THF (80mL) was hydrogenated in the presence of 10% palladium on carbon (1.896g). Work-up as described gave **2.26** as a white solid (2.780g, 72%): mp = 119-120 °C (lit.[84] mp = 120-1 °C). Physical data as above.

By Method B: 2-benzylsuccinic anhydride **2.27** (266mg, 1.4mmol) was added to a solution of hydroxylamine hydrochloride (201mg, 2.8mmol) and anhydrous K_2CO_3 (200mg, 1.4mmol, 1 equiv.) in H_2O (7mL) and the resulting mixture refluxed for 1.75h. Acidification with three drops of conc. HCl and subsequent work up gave **2.26** as a white solid (299mg, 51%): mp 110-17°C (lit.[84] mp = 120-1 °C). Physical data as above.

THE SYNTHESIS OF 3-SUBSTITUTED *N*-[(ALKYL AND ARYLSULFONY]OXY] SUCCINIMIDES

Method A: Sulfonylation at 0-5 °C then rt

The appropriate sulfonyl chloride (1.5 equiv.) was added slowly to a solution of the *N*-hydroxysuccinimide (stated amount) and either Et_3N or *i*-PrNEt or pyridine (1.1 equiv.) in CH_2Cl_2 or toluene (stated volume) stirring under N_2 at 0-5 °C (ice-salt bath). Stirring was continued for the stated time after which the ice-salt bath was removed and the reaction mixture allowed to come to rt while stirring over the stated period. In preparations where CH_2Cl_2 had been used as the solvent, the reaction solution was diluted and then washed with H_2O, 10% HCl or 10% aqueous citric acid, and 10% or saturated aqueous $NaHCO_3$. The organic phase was worked up in the usual manner to give the title compound. In preparations where toluene had been used the reaction solution was evaporated and the resulting residue dissolved in EtOAc and worked up in the same manner as described for a reaction carried out in CH_2Cl_2. Further purification if required was carried out as described per compound.

Method B: Sulfonylation at rt

The appropriate sulfonyl chloride was (1-1.5 equiv.) was added slowly to solution of N-hydroxy imide (stated amount) and either Et_3NH or ^{i}PrNEt (1.1 equiv.) or pyridine (2 equiv.) in CH_2Cl_2 or toluene (stated volume) stirring at rt in N_2. Stirring was continued for the stated time. Work up was carried out as described for **Method A**.

Method C: Sulfonylation at 50 °C

The appropriate sulfonyl chloride (1 equiv.) was added to a solution of *N*-hydroxyimide (stated amount) and pyridine (2 equiv.) in toluene (stated volume) stirring at 40 °C. The temperature was elevated to 50 °C and stirring was continued for the stated time. The reaction solution was then allowed to cool to rt and work up carried out as per **Methods A** and **B**.

3-Benzyl-*N*-[(methanesulfonyl)oxy]succinimide (1.41d)[84]

By Method B: $MeSO_2Cl$ (16.3μL, 24mg, 0.21mmol) was added to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (44mg, 0.21mmol) and pyridine (33.9μL, 33mg, 0.42mmol, 2 equiv.) in toluene (1mL). Stirring was continued for 12h. Work up in the manner described for a reaction carried out in toluene gave a white solid (59mg, quantatative yield); mp 70-1°C (lit.[84] mp 70-1°C); ^{1}H NMR ($CDCl_3$) δ 2.55 (dd, 1H, *J*

= 4.2, 16.1Hz, HCHPh), 2.80 (dd, 1H, *J* = 8.6, 18.4Hz, HCHPh) 2.93 (dd, 1H, *J* = 10.3, 15.1Hz, HCHC(O)) 3.22-3.30 (m, 2H, HCHC(O)) and CHC(O)), 3.40 (s, 3H, CH_3)2.97 (dd, 1H, *J* = 4.4, 13.7Hz, HCHC(O)), 7.18 (d, 2H, *J* = 7.6Hz, ArH), 7.26-7.36 (m, 3H, ArH):^{13}C NMR ($CDCl_3$) δ 30.4, 36.0, 38.4, 40.9, 127.3, 129.0, 135.9, 168.2, 171.2: HRMS (+EI, M^+) calcd. 283.0515, found 283.0515.

3-Phenyl-*N*-[(methanesulfonyl)oxy]succinimide (1.41e)

By Method A: CH_3SO_2Cl (60μL, 90mg, 0.78mmol, 1.5 equiv.) was added to a solution of 3-phenyl-*N*-hydroxysuccinimide **2.32** (100mg, 0.52mmol) and *i*-PrNEt (100μL, 74mg, 0.57mmol, 1.1 equiv.) in CH_2Cl_2 (2mL). Stirred 0-5 °C for 20 min and then at rt for 30 min. Work up gave **1.41e** as a pale yellow solid (138mg, 98%): mp 169-70°C (toluene/petroleum ether, white crystals); IR ($CHCl_3$/NaCl) υ_{max} cm^{-1} 1747.4 and 1720.4 (C=O), 1421.4 and 1209.3 (-OSO_2-) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.92 (dd, 1H, *J* = 4.4, 18.6, HCHC(O)), 3.35 (dd, 1H, *J* = 10.0, 18.6Hz, HCHC(O)), 3.47 (s, 3H, CH_3), 4.15 (dd, 1H, *J* = 4.4, 9.2, CHC(O)), 7.25-7.28 (m, 2H, ArH), 7.35-7.43 (m, 3H, ArH); ^{13}C NMR ($CDCl_3$) δ 34.6, 41.3, 43.1, 127.5, 128.6, 129.5, 135.7, 167.9, 169.9: HRMS (+EI) (M^+) calcd. 269.0358, found 269.0358.

3-Benzyl-*N*-[(*trans*-styrylsulfonyl)oxy]succinimide (1.41f)[87a]

By Method A: *trans*-styryl sulfonyl chloride (197mg, 0.97mmol, 1.5 equiv.) was added to an ice-cooled solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (134mg, 0.7mmol) and pyridine (60μL, 57mg, 0.72mmol) stirring under N_2. Stirring was continued overnight as the reaction solution was allowed to come to rt. Work-up gave **1.41f** as abrown oil (quanatative yield): ^{1}H NMR ($CDCl_3$) δ 2.53 (dd, 1H, *J* = 4.5, 18.5Hz, HCHPh), 2.78 (dd, 1H, *J* = 8.5, 18.0Hz, HCHPh), 2.95 (dd, 1H, *J* = 10.0, 15.0Hz, HCHC(O)), 3.20-3.28 (m, 2H, HCHC(O) and CHC(O)), 6.95 (d, 2H, *J* = 15.6Hz, ArH), 7.15-7.56 (m, 10H, ArH), 7.69 (d, 2H, *J* = 15.5Hz, ArH).

3-Benzyl-*N*-[(benzenesulfonyl)oxy]succinimide (1.41g)[87a]

By Method B: benzenesulfonyl chloride (26μL, 35mg, 0.2mmol) was added to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (40mg, 0.2mmol) and pyridine (32μL, 32mg, 0.4mmol) in toluene (1mL) stirring under N_2 at rt. Stired overnight. Work-up for a reaction carried out in toluene gave **1.41g** as a white solid: mp 83-5°C (lit.[87a] mp 80-2°C): IR ($CDCl_3$) υ_{max} 1814.9 and 1747.4 (C=O), 1452.3, 1404.1 and 1197.7 and 1180.4 (-OSO_2-) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.47 (dd, 1H, *J* = 4.7, 18.4Hz, HCHPh), 2.72 (dd, 1H, *J* = 8.8, 18.1Hz, HCHPh), 2.93 (dd, 1H, *J* = 8.3, 13.6Hz, HCHC(O)), 3.12-3.26 (m, 2H, HCHC(O)) and CHC(O)), 7.17 (d, 2H, *J* = 7.5Hz, ArH), 7.25-7.37

(m, 3H, Ar7 (m, 3H, ArH), 7.59 (t, 2H, J = 7.8Hz, ArH), 7.75 (t, 1H, J = 7.2Hz, ArH), 7.99 (d, 2H, J = 7.3Hz, ArH): ^{13}C NMR ($CDCl_3$) δ 30.3, 36.1, 38.5, 127.4, 129.0, 129.1, 129.4, 134.1, 135.5, 135.9, 167.7, 170.8: HRMS (+EI, M^+) calcd. 345.0671, found 345.0671.

3-Benzyl-*N*-[4-toluenesulfonyl)oxy]succinimide (1.41h)

By Method A: To a ice-cooled solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (52mg, 0.25mmol) and ^{i}PrNEt (48.1mL, 36mg, 0.28mmol, 1.1 equiv.) in CH_2Cl_2 (1mL) stirring in N_2, TsCl was added slowly portionwise. The resulting yellow solution was stirred at 0-5°C for 0.5h and then the ice bath was removed and the solution allowed to come to rt while stirring was continued for 3h. The reactionsolution was diluted with CH_2Cl_2 (5mL), washed with 10% HCl (5mL), saturated aqueous $NaHCO_3$ (5mL), and H_2O (5mL). Subsequent work up in the usual manner gave a white solid (?mg, ?%): mp 71-6°C (lit.[87a] mp 87-8°C): IR (KBr) υ_{max} cm^{-1} 1793.7 and 1739.7 (C=O): ^{1}H NMR ($CDCl_3$) δ 2.44-2.52 (m, 4H, HCHPh and CH_3), 2.73 (dd, 1H, J = 8.8, 18.3Hz, HCHPh), 2.94 (dd, 1H, J = 9.5, 15.0Hz, HCHC(O)), 3.16-3.25 (m, 2H, HCHC(O)) and CHC(O)), 7.17 (d, 2H, J = 6.5Hz, ArH), 7.26-7.37 (m, 3H, ArH), 7.84 (d, 2H, J = 8.5Hz, ArH); ^{13}C NMR ($CDCl_3$) δ 21.8, 30.3, 36.0, 38.5, 127.7, 128.9, 129.1, 129.3, 130.0, 130.9, 135.9, 147.0, 167.8, 170.9: HRMS (+EI, M^+) calcd. 359.0828, found 359.082828. Anal. Calcd for $C_{18}H_{17}NO_5S$: C, 60.15; H, 4.77; N, 3.90; S, 8.90. Found C, 60.26; H, 4.80; N, 3.94; S, 8.68.

3-Phenyl-*N*-[(4-toluenesulfonyl)oxy]succinimide (1.41i)

By Method A: a solution of TsCl (149mg, 0.78mmol, 1.5 equiv.) in CH_2Cl_2 (0.5 mL) added to a solution of 3-phenyl-*N*-hydroxysuccinimide **2.32** (100mg, 0.52mmol) and *i*-PrNEt (100μL, 74mg, 0.57mmol, 1.1 equiv.) in CH_2Cl_2 (2mL). Stirred at 0-5 °C for 20 min and then at rt for 30 min. Work up gave **1.41i** as a white solid (177mg, 98%); mp 119°C (toluene, white crystals); IR ($CHCl_3$/NaCl) υ_{max} 1801.4 and 1751.2 (C=O), 1421.4 and 1182.3 (-OSO_2-) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.46 (s, 3H, CH_3), 2.91 (dd, 1H, J = 4.4, 18.6Hz, HCHC(O)), 3.31 (dd, 1H, J = 9.8, 18.6Hz, HCHC(O)), 4.09 (dd, 1H, J = 4.4, 9.7Hz, CHC(O)), 7.24-7.27 (m, 2H, ArH), 7.35-7.44 (m, 5H, ArH), 7.93 (d, 2H, J = 7.8Hz, ArH): ^{13}C NMR ($CDCl_3$) δ 21.8, 34.6, 43.1, 110.9, 127.4, 128.4, 129.3, 129.4, 130.0, 137.0, 167.7, 169.4; HRMS (+EI, M^+) calcd. 345.0671, found 345.0671.

3-Benzyl-*N*-[(2-napthylsulfonyl)oxy]succinimide (1.41j)

By Method A: 2-napthylsulfonyl chloride (33mg, 0.15mmol, 1.1 equiv.) was added to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.25** (30mg, 0.15mmol) and *i*-PrNEt (26μL, 21mg, 0.16mmol, 1.1 equiv.) in CH_2Cl_2 (5mL). Stirred at 0-5 °C for 0.5h and then at rt for 1h. Work up by diluting with CH_2Cl_2 and washing with H_2O, 10% HCl and 10% aqueous $NaHCO_3$ gave **1.41j** as a white solid (57mg, 99%); ^1H NMR ($CDCl_3$) δ 2.50 (dd, 1H, *J* = 4.4, 18.5Hz, HCHPh), 2.74 (dd, 1H, *J* = 9.1, 18.4Hz, HCHPh), 2.95 (dd, 1H, *J* = 8.8, 14.7Hz, HCHC(O)) 3.16-3.27 (m, 2H, HCHC(O)) and CHC(O)), 7.15-7.18 (m, 2H, ArH), 7.26-7.37 (m, 3H, ArH), 7.63-7.7.75 (m, 2H, ArH), 7.93-8.05 (m, 2H, ArH), 8.56 (s, 1H, ArH):^{13}C NMR ($CDCl_3$) δ 30.3, 36.0, 38.5, 121.3, 123.2, 127.4, 128.0, 128.1, 128.9, 129.0, 12.1, 129.6, 129.7, 130.2, 130.8, 131.8, 131.9, 135.9, 136.0, 1648, 167.8, 170.9: HRMS (+EI, M^+) calcd. 395.0828, found 395.0828.

3-Benzyl-*N*-[(3-nitrobenzenesulfonyl)oxy]succinimide (1.41k)

By Method A: a solution of 3-nitrobenzene sulfonyl chloride (120mg, 0.55mmol, 1.5 equiv) was added to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.25** (75mg, 0.36mmol) and Et_3N (55.7mL, 40mg, 0.40mmol, 1.1equiv.) in CH_2Cl_2 (4mL). Stirred 0-5°C 1h and then rt 4.5h. Work up as described gave a brown residue which was purified by flash SiO_2 chromatography eluting with 3:7 EtOAc/petroleum ether to give a brown oil (quantative yield): IR ($CDCl_3$) υ_{max} 1813.0 and 1747.4 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.51 (dd, 1H, *J* = 4.4, 18.6Hz, HCHPh), 2.77 (dd, 1H, *J* = 8.8, 18.6Hz, HCHPh), 2.94 (dd, 1H, *J* = 8.8, 13.7Hz, HCHC(O)), 3.16 (dd, 1H, *J* = 4.9, 14.2Hz, HCHC(O)), 3.24-3.33 (m, 1H, CHC(O)), 7.17 (d, 2H, *J* = 7.3Hz, ArH), 7.23-7.34 (m, 3H, ArH), 7.83 (t, 1H, *J* = 7.8Hz, ArH), 8.24 (d, 1H, *J* = 8.3Hz, ArH), 8.55 (d, 1H, *J* = 8.3Hz, ArH), 8.75 (s, 1H, ArH); ^{13}C NMR ($CDCl_3$) δ 30.3, 36.0, 38.5, 124.5, 127.6, 129.1, 129.1, 129.7, 130.8, 134.5, 135.6, 136.6, 148.2, 167.5, 170.6: HRMS (+EI, M^+) calcd. 390.0522, found 390.0522.

Synthesis of 3-benzyl-*N*-[(2-carbethoxy-ethanesulfonyl)oxy]succinimide (1.41l)

1. Freshly distilled 2-bromopropionic acid **2.37** (18mL, 30.6g, 0.2mol) was added dropwise to $SOCl_2$ (20mL, 32.740g, 0.275mol, 1.4 equiv.) stirring at 30-40°C. After the addition was completed the resulting solution was refluxed for 2h. Excess $SOCl_2$ was removed by distilllation at aspirator pressure to give a dark brown liquid which was then distilled at reduced pressure to give the intermediate 2-bromopropionoyl chloride as a colourless liquid (10.192g, 30%). This acid chloride was transferred with toluene washings (2mL) to a dropping funnel and was added dropwise to dry EtOH (4mL, 3.140g, 0.068mol, 1.15 equiv.) stirring at 0-5°C (ice-salt bath). After the addition was

completed, stirring was continued at that same temperature for 2h at which point the ice-salt bath was removed and the reaction solution allowed to come to rt while being stirred for 2.75d. The reaction solution was poured into H_2O (10mL) and the organic layer that separated washed with the same volume of 10% aqueous $NaHCO_3$ and H_2O and worked up in the usual manner to give the ester **2.38** as a clear oil (6.629g, 18% overall): bp 22°C / 0.02mmHg; ^{1}H NMR ($CDCl_3$) δ 1.27 (t, 3H,*J* = 7.1Hz, $CH_2C\underline{H}_3$), 1.79 (d, 3H, *J* = 6.8Hz, $CHC\underline{H}_3$), 4.20 (q, 2H, *J* = 6.8, 7.4Hz, $C\underline{H}_2CH_3$), 4.33 (q, 1H, *J* = 6.8, 6.9Hz, $C\underline{H}CH_3$).

2. A solution of ethyl 2-bromopropionate **2.38** (1.31g, 7mmol) and K_2SO_3 (1.312g, 8mmol) in 12%:88% EtOH/H_2O (17mL) was refluxed for 1.5h. The solvent was evaporated *in vacuo* to give a white solid. This crude salt **2.39** (1.271g, 80%) was dried in an oven set at 110°C for 2d and then used without further purification. ^{1}H NMR (D_2O) δ 1.25 (t, 3H,*J* = 7.3Hz, $CH_2C\underline{H}_3$), 1.48 (d, 3H, *J* = 6.9Hz, $CHC\underline{H}_3$), 3.93 (q, 1H, *J* = 6.8, 7.1Hz, $C\underline{H}CH_3$), 4.22 (q, 2H, *J* = 7.1, 7.3Hz, $C\underline{H}_2CH_3$).

3. A suspension of potassium ethyl 2-sulfopropionate **2.39** (1.271g, 6mmol) and PCl_5 (1.250g, 6mmol) in $POCl_3$ (12mL) was stirred vigorously at 80°C for 2.5h under a N_2 atmosphere. The reaction mixture was allowed to cool to rt and then filtered under a N_2 atmosphere directly into a modified Claisen flask and short Vigreux column set up for distillation. The salt cake was washed with a small quantity of $POCl_3$ and this washing and the yellow coloured filtrate were distilled at aspirator pressure to give a mixture of a small quantity of white solid, presumably unremoved NaCl, and brown residue. This mixture was distilled at reduced pressure to give **2.40** as a yellow coloured liquid with a characteristic piquant odour (122mg, 11%): bp 52°C / 0.02mmHg; ^{1}H NMR ($CDCl_3$) δ 1.35 (t, 3H,*J* = 7.1Hz, $CH_2C\underline{H}_3$), 1.86 (d, 3H, *J* = 6.8Hz, $CHC\underline{H}_3$), 4.30-4.38 (m, 2H, $C\underline{H}_2CH_3$), 4.50 (q, 1H, *J* = 6.8, 7.4Hz, $C\underline{H}CH_3$).

4. By Method C: a solution of ethyl (2-chlorosulfonyl)propionate **2.40** (122mg, 0.61mmol) in toluene (1.5mL) was added dropwise to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (125mg, 0.61mmol) and pyridine (98.7μL, 97mg, 1.2mmol, 2 equiv.) in toluene (1.5mL) stirring in N_2 at rt. The resulting white slurry was stirred at rt for 15min after which the tenperature was raised to 50 °C during which the slurry cleared to give a brown coloured solution. Stirring was then continued for 18h. The reaction solution was evaporated to give a brown residue which was dissolved in EtOAc and wahed with 2N HCl. The aqueous layer was re-extracted with EtOAc and the organics combined and worked up in the usual manner to give a brown coloured residue (151mg, 67%). Purification of 101mg of this residue by flash SiO_2 chromatography eluting with 1:1 EtOAc/petroleum ether gave **1.41l** as a brown coloured residue (64mg, 28%); IR ($CDCl_3$) υ_{max} 1801.4 and 1747.4 (C=O), 1409.9 and 1190.0 (-SO_2-) cm^{-1};

^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, *J* = 7.3Hz, $CH_2C\underline{H}_3$), 1.85 (dd, 3H, *J* = 2.3, 7.3Hz, $CHC\underline{H}_3$), 2.55 (dd, 1H, *J* = 3,17.5Hz, HCHPh), 2.80 (dd, 1H, *J* = 8.8, 18.3Hz, HCHPh), 2.87-2.97 (m, 1H, $H_2CC\underline{H}CH_2$), 3.24-3.30 (m, 2H, $CHC\underline{H}_2$), 4.31 (q, 2H, *J* = 7.0, 7.3Hz) 4.67 (q, 1H, *J* = 6.0, 7.0Hz, $C\underline{H}CH_3$), 7.17-7.35 (m, 5H, Ar<u>H</u>); ^{13}C NMR ($CDCl_3$) δ 13.4, 13.9, 30.5, 36.1, 36.3, 38.5, 38.6, 63.2, 65.3, 127.4, 128.5, 129.0, 129.0, 129.2, 135.9, 164.3, 168.1, 171.0, 171.0; HRMS (+EI, M^+) calcd. 369.0883, found 369.0882.

3-Benzyl-*N*-[(3-{3-benzyl-*N*-succinimidylsulfonate}benzenesulfonyl)oxy] succinimide (1.41n)

By Method A: 1,3-bis(chlorosulfonyl)benzene (32mg, 0.12mmol) was added slowy to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (45mg, 0.22mmol, 2 equiv.) and *i*-PrNEt (42μL, 31mg, 0.24mmol, 2.2 equiv.) stirring under N_2 at 0-5 °C. Stirring continued at 0-5 °C for 0.5h and then for 11h at rt. Work-up gave **1.41n** as a white solid (22mg, 17%) after crystallisation from toluene/petroleum ether: mp 95-100 °C; IR ($CDCl_3$) υ_{max} 1813.0 and 1747.4 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.50 (dd, 1H, *J* = 4.4, 18.5Hz, HCHPh), 2.75 (dd, 1H, *J* = 9.0, 18.3Hz, HCHPh), 2.94 (dd, 1H, *J* = 8.6, 13.9Hz, HCHC(O)), 3.17-3.27 (m, 2H, HCHC(O) and CHC(O)), 7.13-7.36 (m, 5H, Ar<u>H</u>), 7.86 (t, 1H, *J* = 8.1Hz, Ar<u>H</u>), 8.35 (d, 2H, *J* = 8.3Hz, Ar<u>H</u>), 8.59 (s, 1H, Ar<u>H</u>); ^{13}C NMR ($CDCl_3$) δ 30.4, 36.0, 38.5, 127.4, 129.0, 130.1, 130.8, 135.5, 135.8, 136.1, 167.6, 170.7; HRMS (FAB, MH^+) calcd. 613.0951, found 613.0951.

3-Benzyl-*N*-[(4'-{3-benzyl-*N*-succinimidyl}sulfonatebiphenylsulfonyl)oxy]succinimide (1.41m)

By Method C: 4,4-biphenyl disulfonyl chloride (173mg, 0.5mmol) was added slowly portionwise to a solution 3-benzyl-*N*-hydroxysuccinimide (202mg, 1mmol, 2 equiv.) and pyridine (160.1mL, 157mg, 2mmol, 4 equiv.) in toluene (8mL) stirring at 40°C in N_2. The temperature was raised to 50°C and stirring continued for 2h. Solvent was evaporated and the resulting mixture consisting of a brown residue and a white solid was dissolved in the minimum amount of EtOAc and washed with H_2O. The aqueous layer was reextracted with EtOAc and the extracts combined and worked up in the usual manner to give a yellow solid. Purification by flash SiO_2 chromatography eluting with CH_2Cl_2 gave **1.41m** as a brown oil (117mg, 34%): IR ($CDCl_3$) υ_{max} 1813.0 and 1747.4 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.50 (dd, 1H, *J* = 4.4, 18.5Hz, HCHPh), 2.75 (dd, 1H, *J* = 9.0, 18.3Hz, HCHPh), 2.94 (dd, 1H, *J* = 8.6, 13.9Hz, HCHC(O)), 3.17-3.27 (m, 2H, HCHC(O) and CHC(O)), 7.13-7.36 (m, 5H, Ar<u>H</u>), 7.86 (t, 1H, *J* = 8.1Hz, Ar<u>H</u>), 8.35 (d, 2H, *J* = 8.3Hz, Ar<u>H</u>), 8.59 (s, 1H, Ar<u>H</u>); ^{13}C NMR ($CDCl_3$) δ 30.4, 36.0, 38.5, 127.4, 129.0, 130.1, 130.8, 135.5, 135.8, 136.1, 167.6, 170.7; HRMS (FAB, MH^+)

calcd. 613.0951, found 613.0951. Anal. Calcd. for $C_{34}H_{28}N_2O_{10}S_2.1.5H_2O$: C, 57.17; H, 3.95; N, 3.92. Found C, 56.96; H, 4.14; N, 3.93.

THE SYNTHESIS OF 3-SUBSTITUTED *N*-[(ACYL)OXY] SUCCINIMIDES

Method A: Et_3N/^{i}PrNEt and DMAP catalysed acylation.

Acetic anhydride or the appropriate acid chloride (1 equiv.) was added to a solution of 3-substituted *N*-hydroxysuccinimide (stated amount), DMAP (0.1 equiv.), and Et_3N or *i*-PrNEt (1 equiv.) in CH_2Cl_2 (stated volume) which had been stirring for 5min under N_2 at rt. Stirring was then continued at rt overnight. The reaction solution was diluted with EtOAc and washed with 10% aqueous citric acid and H_2O and then 0.5N $KHCO_3$ and H_2O again. The organic phase was then worked up in the usual manner to give the title compounds. These were purified by crystallisation from the stated solvent system.

MethodB: Et_3N /*i*-PrNEt catalysed acylation.

The appropriate acid chloride (1.5 equiv.) was added to a solution of *N*-hydroxy imide (stated amount) and Et_3NH or ^{i}PrNEt (1.1 equiv.) in CH_2Cl_2 stirring at 0-5^{o}C (ice-salt bath). Stirring was continued at that temperature for the stated time, (typically at least 20min), then the ice-salt bath removed and the reaction mixture allowed to come to rt while stirring for the stated period (typically at least 0.5h). The reaction solution was diluted with CH_2Cl_2 and washed with H_2O, 10% HCl and saturated $NaHCO_3$. The organic phase was worked up in the usual manner to give the title compounds in high purity by ^{1}HNMR.

Method C: TMSOTf catalysed acetylation.

TMSOTf (0.02 equiv.) was added to a solution of *N*-hydroxy imide (stated amount) and Ac_2O (1.5 equiv.) in CH_2Cl_2 which had been stirring in N_2 for 10min at 0-5^{o}C (ice-salt bath). The resulting solution was allowed to come to rt while stirring overnight. This was then treatedwith saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The organic extract was separated, washed with 10% aqueous $NaHCO_3$ and worked up in the usual manner to give the title compounds as crystalline solids.

Method D: TMSOTf catalysed acylation with *in situ* mixed anhydride

A solution of the appropriate acid (stated amount) in CH_2Cl_2 was treated with *N*-methylmorpholine and ethyl chloroformate to give an *in situ* mixed anhydride. After

this solution was stirred for the stated period the *N*-hydroxysuccinimide (stated amount) was added followed by TMSOTf (0.02 equiv.) and the reaction stirred while being allowed to come to rt over the stated period. Saturated aqueous $NaHCO_3$ was added to the reaction mixture and the organic phase then separated and washed with saturated aqueous NH_4Cl and H_2O. Work-up in the normal manner gave the title compound.

Method E: DCC, HOBt mediated acid coupling

HOBt (stated amount) and acid (stated amount) were added to an ice cooled solution of the *N*-hydroxysuccinimide (stated amount) in CH_2Cl_2 stirring under N_2. The resulting solution was stirred for the stated period under N_2 at 0-5 °C after which DCC (stated amount) was added and the resulting mixture stirred for the indicated period while the reaction mixture was allowed to come to rt. The reaction mixture was evaporated and the resulting product suspended in EtOAc (stated amount). This suspension was then filtered to remove the by-product DCU and the filter cake washed with a small quantity of EtOAc. The filtrate and washings were combined and washed with 0.5N aqueous $KHCO_3$ and H_2O and the organic phase worked up in the usual manner to give the title compound.

3-Benzyl-*N*-[(acetyl)oxy]succinimide (1.41o)

By Method A: Ac_2O (55.7μL, 60mg, 0.6mmol) was added to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (111mg, 0 6mmol), DMAP (8mg, 0.06mmol. 0.1 equiv.), and Et_3N (82.2mL, 60mg, 0.6mmol) in CH_2Cl_2 (6mL) which had been stirring for 5min under N_2 at rt. Work-up gave **1.41o** as a brown residue (108mg, 79%): mp 107-9°C (EtOAc/petroleum ether, amber coloured crystals); ^1H NMR ($CDCl_3$) δ 2.22 (s, 3H, C(O)C<u>H</u>$_3$), 2.43 (dd, 1H, *J* = 3.4, 18.1Hz, <u>H</u>CHPh), 2.62-2.81 (m, 2H, HC<u>H</u>Ph and <u>H</u>CHC(O)), 3.16 (bs, 2H, HC<u>H</u>C(O) and C<u>H</u>C(O)), 7.08-7.26 (m, 5H, Ar<u>H</u>); ^{13}C NMR ($CDCl_3$) δ 17.5, 30.7, 36.3, 38.7, 127.2, 128.6, 128.9, 136.3, 165.5, 168.5, 171.5; HRMS (+EI, M^+) calcd. 247.0845, found 247.0845.

By method C: TMSOTf (4μL, 5mg, 0.02mmol, 0.02 equiv.) was added to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (202mg, 0.99mmol) and Ac_2O (140μL, 151mg, 1.5mmol, 1.5 equiv.) in CH_2Cl_2 (6mL) that had been stirring at 0-5 °C under N_2 for 10 min. Stirrind was continued as the reaction solutionwas allowed to come to rt overnight. Work-up as described above gave **1.41o** as a brown coloured residue which on standing at rt over a period of one week solidified to afford **1.41o** as a white crystalline solid (209mg, 86%). Physical data as given above.

3-Phenyl-*N*-[(acetyl)oxy]succinimide (1.41p)

By Method B: AcCl (28μL, 31mg, 0.39mmol) was added to a solution of 3-phenyl-*N*-hydroxysuccinimide **2.32** (50mg, 0.26mmol) and *i*-PrNEt (49.9μL, 37mg, 0.29mmol, 1.1 equiv.) in CH_2Cl_2 (1mL). Work-up gave **1.41p** as an off-white solid (45mg, 74%): mp 74 °C (Et_2O/petroleum ether, colourless crystals); IR ($CDCl_3$) υ_{max} 1789.8 and 1743.5 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.37 (s, 3H, C$\underline{H}_3$), 2.89 (dd, 1H, J = 4.4, 19.0, $\underline{H}$CHC(O)), 3.33 (dd, 1H, J = 9.8, 18.5Hz, HC$\underline{H}$C(O)), 4.12 (bs, 1H, C$\underline{H}$C(O)), 7.31-7.43 (m, 5H, Ar$\underline{H}$); ^{13}C NMR ($CDCl_3$) δ 17.6, 35.3, 43.5, 126.6, 127.5, 128.3, 129.3, 129.6, 130.2, 165.6, 168.3; HRMS (+EI, M^+) calcd. 233.0688, found 233.0688. Anal. Calcd. for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 62.10; H, 4.81; N, 6.25.

3-Benzyl-*N*-[(*trans*-cinnamoyl)oxy]succinimide (1.41q)

By Method A: *trans*-cinnamoyl chloride (70mg, 0.42mmol, 1.05 equiv.) was added slowly portionwise to a solution of 3-benzyl-*N*-hydroxysuccinimide **2.26** (83mg, 0.4mmol), DMAP (4mg, 0.03mmol), and *i*-PrNEt (70.4μL, 52mg, 0.4mmol) in CH_2Cl_2 (7mL) that had been stirring for 5min under N_2 at rt. The resulting reaction solution was stirred for 14.5h. Work-up gave **1.41q** as a white soid (100mg, 75%): mp 105-7°C (EtOAc/petroleum ether, white crystals); ^1H NMR ($CDCl_3$) δ 2.57 (bd, 1H, J = 18.0Hz, $\underline{H}$CHPh), 2.76-2.93 (m, 2H, HC$\underline{H}$Ph and $\underline{H}$CHC(O)), 3.29 (bs, 2H, HC$\underline{H}$C(O) and C$\underline{H}$) 6.59 (d, 1H, J = 16.1Hz, C(H):C$\underline{H}$Ph), 7.93 (d, 1H, J = 16.1Hz, C(O)C$\underline{H}$:C(H)Ph); ^{13}C NMR ($CDCl_3$) δ 30.8, 36.2, 38.8, 111.5, 127.2, 127.8, 128.6, 128.9, 128.9 129.0, 131.5, 133.4, 149.9, 162.0, 168.7, 171.7; HRMS (+EI) (M^+) calcd 335.1158, found 335.1158. Anal. Calcd for $C_{20}H_{17}O_4N$: C, 71.64; H, 5.07; N, 4.18. Found: C, 71.12; H, 5.12; N, 4.10.

By Method D: *N*-methylmorpholine (115mL, 106mg, 1.05mmol) and ethyl chloroformate (104mL, 118mg, 1.09mmol, 1.1 equiv.) were added to an ice cooled solution of cinnamic acid (156mg, 1.05mmol, 1.05 equiv.) in CH_2Cl_2 (5mL). The resulting solution was stirred at 0-5 °C under N_2 for 10 min. 3-Benzyl-*N*-hydroxysuccinimide **2.26** (206mg, 1mmol) and TMSOTf (4mL, 5mg, 0.02mmol) were then added and the resulting reaction solution allowed to warm to rt while stirring overnight. Work-up gave **1.41q** as a white solid (289mg, 86%). Spectral data as above.

3-Benzyl-*N*-[(*N*-benzyloxycarbonyl-(*L*)-phenylalanyl)oxy]succinimide (1.41r)

By Method E: HOBt (66mg, 0.49mmol) and *N*-Cbz-(*L*)-phenylalanine (147mg, 0.49mmol) were added to an ice-cooled solution of 3-benzyl-*N*-hydroxysucccnimide **2.26** (101mg, 0.50mmol) in CH_2Cl_2 (9mL) stirring under N_2. This solution was stirred

at 0-5 °C under N_2 for 15min after which DCC (114mg, 0.55mmol) was added and the solution allowed to warm to rt while being stirred for 43h. The reaction solution was evaporated and the resulting mixture of a brown residue and white solid was suspended in the minimum amount of EtOAc. This was then filtered to remove the by-product DCU and the filer cake washed with a small quantity of EtOAc. The filtrate and washings were combined and washed with 0.5N $KHCO_3$ and H_2O and the organics worked-up in the normal manner to give a brown residue (198g, 83%): ^{1}H NMR ($CDCl_3$) δ 2.56 (1H, dd, *J* = 3.9, 18.1 Hz, HCHPh), 2.78-2.93 (2H, m, HCHPh and HCHC(O)), 3.29-3.43 (m, 2H, HCHC(O) and CH), 4.62-4.66 (m, 1H, C(O)CHNH), 5.10 (s, 2H, OCH_2Ph), 5.24 (d, 1H, *J* = 6.3Hz, NH), 7.09-7.38 (m, 10H, ArH); ^{13}C NMR δ 24.6, 25.3, 29.6, 30.6, 30.7, 33.3, 36.1, 37.6, 37.8, 38.1, 38.4, 38.7, 52.9, 54.6, 54.8, 67.0, 67.3, 77.2, 127.0, 127.1, 127.3, 127.4, 128.0, 128.1, 128.2, 128.4, 128.5, 128.7, 128.8, 128.9, 129.3, 129.5, 134.3, 135.6, 135.8, 136.0, 136.2, 136.6, 155.5, 155.9, 167.4, 168.2, 171.1, 171.8, 174.5, 174.88; HRMS (+EI) calcd. 486.1792, found 486.1791.

THE SYNTHESIS OF 2-ALKYLGLUTARIC ACIDS

Method A: Michael Reaction Route

Diethyl 2-benzyl-2-cyanoethyl malonate (2.45)[182a]

Acrylonitrile (2.4mL, 1.934g, 36mmol) was added slowly to a solution of diethyl benzyl malonate (8.7mL, 9.257g, 37mmol) and 30% methanolic KOH (201mg) in ^{t}BuOH (13mL) stirring in N_2 at such a rate that the temperature of the reaction mixture did not exceed 40 °C. Stirring was continued at rt for a further 4.5h. The reaction mixture was acidified (pH=1, Universal Indicator Paper) with glacial CH_3CO_2H and 6N HCl then concentrated and extracted with Et_2O. The Et_2O extract was washed with an equal volume of H_2O and worked up in the usual manner to give an oily brown residue. This was then distilled under reduced pressure (1mmHg) to give three liquid fractions. The first (1.620g) and second (1.045g) fractions were collected in the ranges 124-50 °C and 150-70 °C and contained, by ^{1}H NMR, 21:4 and 14:11 unreacted diethyl benzyl malonate/diethyl benzyl-2-cyanoethyl malonate respectively. The third fraction was found to contain pure product and after standing at rt for 19d slowly crystallised to give **2.45** as a white solid (5.616g, 50%): bp 170-96 °C/1mmHg (lit.[182a] bp 175-80 °C/1mmHg); ^{1}H NMR ($CDCl_3$) δ 1.27 (t, 6H, J = 7.3Hz, $2CH_2CH_3$), 2.11 (t, 2H, *J* = 7.8Hz, CH_2CN), 2.44 (t, 2H, *J* = 7.8Hz, CH_2CH_2), 3.26 (s, 2H, $PhCH_2$), 4.23 (q, 4H, *J* = 6.9, 7.3Hz, $2CH_2CH_3$), 7.06-7.09 (m, 2H, ArH), 7.24-7.31 (m, 3H, ArH): ^{13}C NMR ($CDCl_3$) δ 13.0, 13.8, 28.6, 39.4, 57.5, 61.7, 119.0, 127.3, 128.4, 129.7, 134.8, 169.9:

HRMS (+EI, M^+) calcd. 289.1315, found 289.1314. Anal. Calcd for $C_{17}H_{21}NO_4$: C, 66.41; H, 6.62; N, 4.84. Found: C, 66.17; H, 6.50; N, 4.71.

2-Benzylglutaric acid (2.44b)

A mixture of **2.45** (2.249g, 7mmol) and 68% HBr (18mL) was refluxed for 1d. The solution was then evaporated to give a mixture of a brown liquid and a white solid (presumably NH_4Br). Sufficient H_2O was added to dissolve the NH_4Br and the resulting mixture was extracted with Et_2O. Work up in the usual manner gave **2.44b** as a brown oil (1.174g, 72%): ^1H NMR ($CDCl_3$) δ 1.78 (q, 2H, *J* = 7.3, 7.8Hz), 2.26-2.39 (m, 2H), 2.67 (dd, 1H, *J* = 7.3, 14.1Hz), 2.80 (dd, 1H, *J* = 6.4, 13.2Hz), 2.94 (dd, 1H, *J* = 8.3, 13.6Hz), 7.26-7.39 (m, 5H, Ar<u>H</u>); ^{13}C NMR ($CDCl_3$) δ 25.9, 31.5, 37.8,46.2, 126.5, 128.4, 128.8, 138.2.

THE SYNTHESIS OF 3,3-DIALKYLGLUTARIC ACIDS

4,4-Dimethyl-3,5-dicyanoglutarimmide (2.48a)[185]

A solution of EtOH saturated with NH_3 was prepared by bubbling $NH_{3(g)}$ through EtOH (200mL) at *ca.* -5 °C for 6h. This solution was added to a mixture of ethyl cyanoacetate (106mL 113g, 1mol) and acetone (37mL, 29.267g, 0.5mol) and the resulting mixture was stored in the freezer for 3d. Cold EtOH (200mL) was added and the mixture stirred thoroughly and returned to the freezer. After this period the reaction mixture was allowed to warm to rt and the yellow salt that had deposited over this period was collected by suction filtration and washed with a small quantity of Et_2O to remove ethyl cyanoacetate and acetone and then air-dried. The resulting pale yellow solid was dissolved in boiling H_2O and conc. HCl added until this solution was acid to Congo Red Paper after which an additional volume of conc. HCl (50mL) was added. On cooling **2.48a** crystallised as a white solid (40.87g, 42%); IR (KBr) υ_{max} 3215.1 (NH), 1712.7 and 1749.3 (C=O); ^1H NMR (DMSO-d_6) δ 1.27 (s, 3H, C<u>H</u>$_3$), 1.44 (s, 3H, C<u>H</u>$_3$), 4.88 (s, 2H, 2xC<u>H</u>), 12.23 (bs, 1H, N<u>H</u>); ^{13}C NMR (DMSO-d_6) 19.9, 26.0, 39.1, 46.9, 114.6, 164.7; HRMS (FAB, M^+) calcd. 191.0695, found 191.0695.

3,3-Cyclohexylglutaric acid (2.44d)[253]

4,4-Cyclohexyl-3,5-dicyano-glutarimide **2.49b** (2.020g, 9mmol) was dissolved in conc. H_2SO_4 (5mL) with gentle warming. Water (5mL) was added slowy and the resultant solution heated to reflux becoming a black mixture containing a suspension of fine white solid. At this stage some foaming problems were experienced leaving material adhered to the insides of the reaction flask and reflux condenser. This was

washed back down into the reaction mixture with a small quantity of H_2O. Reflux was continued overnight during which period the described suspension dissolved to give a clear black solution. This was diluted carefully with H_2O (10mL) and extracted thoroughly with Et_2O. The Et_2O extracts were combined and washed with H_2O and then extracted with saturated aqueous $NaHCO_3$. The $NaHCO_3$ extracts were acidfied (pH=1, Universal Indicator Paper) with conc. HCl and then extracted with Et_2O. Work up in the usual manner gave **2.44d** as a white solid (1.259g, 72%): mp 179-82°C (lit.[253] mp 182-3 °C).

3,3-Dimethylglutaric acid (2.44e)[185]

Compound **2.48a** (23.22g, 0.12mol) was dissolved in conc. H_2SO_4 (62mL) with slight warming. Water (62mL) was then added slowly and the resulting mixture refluxed for 1.5d. The reaction mixture was diluted with H_2O (50mL) and extracted with Et_2O. The organic extracts were combined and washed with H_2O and then worked up in the normal manner to give a pale yellow solid (15.19g, 78%); mp 99-102 °C (lit.[185] mp 102-3 °C); IR (KBr) υ_{max} 2881.5 (OH), 1689.5 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.14 (s, 6H, 2xC<u>H</u>$_3$), 2.38 (s, 4H, 2xC<u>H</u>$_2$C(O)).

THE SYNTHESIS OF SUBSTITUTED GLUTARIC ANHYDRIDES

General Method

A mixture of the glutaric acid (stated amount) and excess AcCl or Ac_2O (stated amount) was refluxed for the stated time. The solvent - a mixture of the excess AcCl or Ac_2O and AcOH which was formed during reaction - was removed by downward distillaton at aspirator pressure to give either liquids or more typically brown to black residues. These were distilled at reduced pressure, or recrystallised from the stated solvent system, as indicated, to give the title anhydride.

2-Phenylglutaric anhydride (2.43a)

A mixture of 2-phenylglutaric acid **2.44a** (2.500g, 13mmol) and AcCl (5.200mL, 61mmol, 4.7equiv.) was refluxed for 1h. The excess AcCl was evaporated and the resultant liquid was distilled at reduced pressure to give a pale yellow solid. Recrystallisation from toluene/petroleum ether gave **2.43a** as a white solid (2.081g, 84%); IR ($CHCl_3$/NaCl) υ_{max} 1813.0 and 1762.8 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.28 (m, 2H, C<u>H</u>$_2$C(O)), 2.85 (m, 2H, CHC<u>H</u>$_2$), 3.92 (dd, 1H, J = 6.3, 8.7Hz, C<u>H</u>), 7.33 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 24.4, 29.4, 46.8, 127.8, 128.0, 128.9, 135.9, 166.5.

2-Benzylglutaric anhydride (2.43b)

A mixture of 2-benzylglutaric acid **2.44b** (2.131g, 9.6mmol) and Ac_2O (5.978mL, 6.468g, 0.063mol, 6.6 equiv.) was refluxed for 2d. Distillation of the reaction mixture at reduced pressure gave **2.43b** as a pale yellow solid (1.354g, 69%): mp 80-1 °C (toluene/petroleum ether); IR (KBr) υ_{max} 1801.4 and 1755.1 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.64-1.78 (m, 1H, HCHPh), 1.89-1.98 (m, 1H, HCHPh), 2.55-2.67 (m, 1H, $CHHCHCH_2$), 2.76-2.91 (m, 3H, $CHHCHCH_2$ and $C(O)CH_2$), 3.44 (q, 1H, J = 8.8, 18.1Hz, CHC(O)) 7.19-7.36 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 21.2, 29.9, 36.1, 42.3, 127.1, 128.8, 129.1, 137.2, 166.5, 168.7.

3-Phenylglutaric anhydride (2.43c)

A mixture of 3-phenylglutaric acid (764mg, 4mmol) and Ac_2O (1.900mL, 2.056g, 20mmol, 5 equiv.) was refluxed for 20.75h. The solvent was removed to give a brown mass. This was dissolved in toluene, activated carbon added and the mixture filtered with celite. The celite cake was washed with a small quantity of toluene and the filtrate and washings combined and evaporated to yield a pale yellow solid (629mg, 89%): mp 101-3 °C: ^1H NMR ($CDCl_3$) δ 2.87 (dd, 2H, J = 11.2, 17.6Hz, $CH_2C(O)$), 3.11 (dd, 2H, J = 4.4, 17.6Hz, $CH_2C(O)$), 3.38-3.48 (m, 1H, CH), 7.18-7.43 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 37.1, 41.0, 126.2, 128.1, 129.3, 139.1, 165.9.

3,3-Cyclohexylglutaric anhydride (2.43d)[253]

A mixture of 3,3-cyclohexylglutaric acid **2.44d** (848mg, 4mmol) and Ac_2O (0.793mL 858mg, 8mmol, 2equiv.) was refluxed for 2h 20min. The solvent was removed as per the general method to give a brown mass that was distilled at reduced pressure to give **2.43d** as a white solid (691mg, (91%): mp 68-9 °C (lit.[253] mp 73 °C (benzene/petroleum ether): bp 150 °C/5mmHg (lit.[253] bp 213 °C/36mmHg); IR (KBr) υ_{max} 1807.2 and 1755.1 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.43-1.53 (m, 10H, $5xCH_2$), 2.66 (s, 4H, $2xCH_2C(O)$); ^{13}C NMR ($CDCl_3$) δ 21.3, 25.2, 32.2, 35.9, 41.5, 166.3.

3,3-Dimethylglutaric anhydride (2.43e)

3,3-Dimethyl glutaric acid **2.44e** (14.945g, 0.093mol) and Ac_2O (55mL, 59.510g, 0.583mol, 6.3 equiv.) were placed in a 2-necked round bottom flask set up for downward distillation with a thermometer dipping into the reaction mixture. This was heated, with distillation of AcOH and Ac_2O, until the temperature reached 180 °C. The resultant dark brown mass was allowed to cool to rt while stirring and then dissolved in the minimum amount of boiling toluene. Activated carbon was added, celite added and

the mixture filtered. On cooling to rt a white solid crystallised from the filtrate. This was reheated then recooled and petroleum ether was added upon which **2.44e** precipitated as a white solid which was isolated by suction filtration. Yield 11.703g, (89%): mp 122-5 °C (lit.[254] mp 122-3°C); ^{1}H NMR ($CDCl_3$) δ 1.15 (s, 6H, 2C$\underline{H}_3$), 2.61 (s, 4H, 2xC$\underline{H}_2$C(O)): ^{13}C NMR ($CDCl_3$) δ129.1, 129.1, 130.0, 133.7, 139.8, 167.3.

THE SYNTHESIS OF SUBSTITUTED *N*-BENZYLOXYGLUTARIMIDES

Method A: From anhydride

A dispersion of *O*-benzyloxyamine (1 equiv.) and the respective anhydride (stated amount) in toluene or xylenes (stated volume) was refluxed with azeotropic removal of H_2O for the stated time. The solvent was evaporated to yield either a crude solid or more typically a brown residue. Subsequent work up depended on this result. A solid was purified by recrystalllisation from the stated solvent system to give the title compounds as white solids. A residue was dissolved in the minimum of EtOAc (stated volume) and washed with an equal volume of 10% aqueous $NaHCO_3$ and H_2O. The organic phase was worked up in the usual manner to give the title compounds as solids. These were purified, if required, by recrystallisation from the stated solvent system.

Method B: From acid

O-Benzyloxyamine (1.2 equiv.), Et_3NH (5 equiv.) and HOBt (2.2 equiv.) were added in successsion to a solution of acid (stated amount) in CH_2Cl_2 (stated volume) stirring in N_2 at 0-5 °C (ice-salt bath). Stirring was continued for ten min and then EDAC (2.08 equiv.) was added. The resulting solution was allowed to come to rt while stirring overnight. The reaction solution was washed with H_2O, 10% HCl, and saturated aqueous $NaHCO_3$. Work up in the usual manner gave the desired product as a crude oily solid. This was purified by flash chromatography eluting with the stated solvent system.

3-Benzyl-*N*-benzyloxyglutarimide (2.49b)

By Method A: a dispersion of 2-benzylglutaric anhydride **2.43b** (418mg, 2mmol) and *O*-benzyloxyamine (263mg, 2.1mmol, 1.05 equiv.) in xylenes (5mL) was heated until dissolution and refluxed with azeotropic removal of H_2O overnight. Work-up gave a white solid (436mg, 69%): mp 91-4 °C (toluene/petroleum ether); ^{1}H NMR ($CDCl_3$) δ 1.51-1.59 (m, 1H, $\underline{H}$CHPh), 1.65-1.85 (m, 1H, HC$\underline{H}$Ph), 2.45-2.57 (m, 1H, CH$\underline{H}$CHCH$_2$), 2.64-2.81 (m, CHHC$\underline{H}$CH$_2$ and C$\underline{H}_2$C(O)), 3.40 (dd, 1H, J = 3.5,

13.0Hz, CHC(O)), 5.01 (s, 2H, OCH_2Ph), 7.16-7.52 (m, 10H, ArH); ^{13}C NMR ($CDCl_3$) δ 21.5, 32.1, 36.1, 44.6, 77.95, 126.8, 128.3, 128.6, 129.1, 129.1, 130.0, 133.8, 137.9, 168.1, 170.3; HRMS (+CI, MH^+) calcd. 310.14440, found 310.1443.

4-Phenyl-*N*-benzyloxyglutarimide (2.49c)

By Method A: a dispersion of 3-phenylglutaric anhydride **2.43c** (286mg, 1.5mmol) and *O*-benzyloxyamine (196mg, 1.6mmol, 1.07 equiv.) in toluene (11ml) refluxed with azeotropic removal of H_2O for 19h. Work up gave **2.49c** as a white solid (425mg, 96%): mp 164-7 °C; IR υ_{max} (KBr) 1743.5 and 1691.5 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.83-3.05 (m, 4H, $2xCH_2C(O)$), 3.27-3.37 (m, 1H, CH), 5.03 (s, 2H, OCH_2Ph), 7.18 (d, 2H, J = 8.3Hz, ArH), 7.26-7.40 (m, 6H, ArH), 7.51-7.55 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$) δ 34.5, 40.2, 78.2, 126.2, 127.7, 128.4, 129.1, 129.1, 130.0, 133.7, 139.8, 167.3; HRMS (+EI, m/z) 190.1 (M - OCH_2Ph, 11), calcd. 289.1315, found 289.1314.

4,4-Cyclohexyl-*N*-benzyloxyglutarimide (2.49d)

A dispersion of 3,3-cyclohexylglutaric anhydride **2.43d** (478g, 2.6mmol) and *O*-benzyloxyamine (338mg, 2.7mmol) in xylene (6mL) was refluxed with azeotropic removal of H_2O overnight. The solvent was evaporated *in vacuo* to give a white solid which was dissolved in the minimum amount of EtOAc and washed with 10% aqueous $NaHCO_3$. Work-up in the usual manner gave **2.49d** as a purified white solid (618mg, 83%): IR (KBr) 1741.6 and 1705.0 (C=O) cm^{-1}; ^1H NMR ($CDCL_3$) δ 1.33-1.58 (m, 10H, $5xCH_2$), 2.61 (s, 2H, $CH_2C(O)$), 5.03 (s, 2H, OCH_2), 7.35-7.37 (m, 3H, ArH), 7.51-7.54 (m, 2H, ArH); ^{13}C NMR ($CDCL_3$) δ 21.4, 25.5, 32.2, 35.8, 44.4, 78.0, 128.4, 129.1, 129.9, 170.1; HRMS (+EI, M^+) calcd. 287.1522, found 287.1522.

4,4-Dimethyl-*N*-benzyloxyglutarimide (2.49e)

By Method A: a dispersion of 3,3-dimethyl glutaric anhydride **2.43e** (300mg, 2.11mmol) and *O*-benzyloxyamine (265mg, 2.15mmol, 1.02 equiv.) in xylene (4.3mL) was heated until dissolution and then refluxed with removal of H_2O for 15h. The resultant solution was allowed to cool to rt and then refridgerated overnight during which **2.49e** crystallised as a white solid. This was isolated by withdrawing the solvent with a pasteur pipette. Yield (375mg, 72%): mp 112-4 °C; IR (KBr) υ_{max} 1733.9 and 1693.4 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.07 (s, 6H, $2xCH_3$), 2.56 (t, 2H, $2xCH_2C(O)$), 5.02 (s, 2H, OCH_2Ph), 7.36-7.38 (m, 3H, ArH), 7.52-7.53 (m, 2H, ArH); ^{13}C NMR (CDCl3) δ 27.5, 29.4, 46.7, 78.0, 128.4, 129.1, 129.9, 133.8, 167.6; HRMS (FAB, MH^+) calcd. 248.1287, found 248.1287. Anal. Calcd. for $C_{14}H_{17}NO_3$: C, 68.02; H, 6.88; N, 5.67. Found: C, 67.72; H, 6.96; N, 5.63.

By Method B: with *O*-Benzyloxyamine (43mg, 0.35mmol, 1.2 equiv.) in CH_2Cl_2 (2mL), Et_3N (202μL, 147mg, 1.45mmol, 5 equiv.) and HOBt (88mg, 0.65mol, 2.2 equiv.) were added in successsion to a solution of 3, 3-dimethylglutaric acid (46mg, 0.29mmol) in CH_2Cl_2 (4mL). EDAC (115mg, 0.60mmol, 2.08 equiv.) was added. Work up gave an oily white solid which was purified by flash SiO_2 chromatography eluting with 1:4 EtOAc/petroleum ether to give **2.49e** as a white solid (10 mg, 13%). Physical data as above.

THE SYNTHESIS OF SUBSTITUTED *N*-HYDROXY GLUTARIMIDES

Method A: From 3-substituted*N*-benzyloxysuccinimide

A solution of the respective substituted *N*-(benzyloxy)succinimide (stated amount) in the stated solvent (stated volume) was hydrogenated in the presence of the indicated palladium catalyst (stated amount) for the stated time. The reaction mixture was filtered through celite and Na_2SO_4 and this cake washed with a small quantity of solvent. The filtrate and washings were combined and evaporated to give the title *N*-hydroxysuccinimides. These were purified, if required, by either chromatagraphy or recrystallisation using the stated solvent system.

Method B: From 2-substituted succinic acid anhydride

The respective anhydride (stated amount) was added to a solution of hydroxylamine hydrochloride (1.2 equiv.) and K_2CO_3 (0.6-1equiv.) in distilled H_2O (stated volume). This mixture was refluxed for the stated time. The hot solution was acidified (pH=1, Universal Indicator Paper) with 10% or conc. HCl, allowed to cool to rt, and then extracted with EtOAc. The combined extracts were worked up in the usual manner to give the title*N*-hydroxysuccinimide.

3-Phenyl-*N*-hydroxyglutarimide (2.42a)

By Method B: 2-phenylglutaric anhydride **2.43a** (500mg, 2.6mmol) was added to a solution of hydroxylamine hydrochloride (220mg, 3.2mmol, 1.2 equiv.) and K_2CO_3 (218mg, 1.6mmol, 0.6 equiv.) in distilled H_2O (7.5mL). This mixture was refluxed for 5h. Acidification with 10% HCl and subsequent work up gave a pale yellow oil (420mg, 77%). This oil was heated to 160 °C for 1h at *ca.* 2mmHg. Decolourisation of the resultant oil with charcoal gave **2.42a** as a pale yellow oil (218mg, 56%): IR ($CHCl_3$/NaCl) υ_{max} 1768.6 and 1643.2 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.27 (m,

2H, C$\underline{H}_2$C(O)), 2.86 (m, 2H, CHC$\underline{H}_2$), 4.07 (dd, 1H, J = 4.9, 11.2Hz, C$\underline{H}$), 7.29 (m, 5H, Ar$\underline{H}$), 10.35 (bs, 1H, NO$\underline{H}$); ^{13}C NMR ($CDCl_3$) δ 24.8, 31.5, 48.3, 126.9, 127.6, 128.3, 138.9, 168.6, 170.0; HRMS (+EI, M^+) calcd. 205.0739, found 205.0739.

3-Benzyl-*N*-hydroxyglutarimide (2.42b)

By Method A: a solution of 3-benzyl-*N*-benzyloxyglutarimide **2.49b** (198mg, 0.64mmol) in THF (5mL) was hydrogenated in the presence of 10% palladium on carbon (66mg) for 14h. Work-up gave **2.42b** as a white solid (116mg, 83%); mp 62-5 °C; IR ($CDCl_3$) υ_{max} 3350.1 (OH), 1739.7 and 1681.8 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.61-1.75 (m, 1H, $\underline{H}$CHPh), 1.83-1.93 (m, 1H, HC$\underline{H}$Ph), 2.55-2.67 (m, 1H, CH$\underline{H}$CHCH$_2$), 2.72-2.94 (m, 3H, CHHC$\underline{H}$CH$_2$ and C(O)C$\underline{H}_2$), 3.45 (dd, 1H, J = 2.9, 14.2Hz, C$\underline{H}$C(O)), 7.18-7.34 (m, 5H, Ar$\underline{H}$); ^{13}C NMR ($CDCl_3$) δ 21.5, 31.0, 36.0, 43.6, 126.9, 128.7, 129.1, 137.6, 166.9, 169.2; HRMS (+EI) calcd. 219.0896, found 219.0895.

4-Phenyl-*N*-hydroxyglutarimide (2.42c)

By Method A: a solution of 4-phenyl-*N*-benzyloxyglutarimide **2.49c** (347mg, 1.2mmol) in THF (6mL) was hydrogenated in the presence of 10% palladium on carbon for 17.25h. Work-up gave **2.42c** as a brown residue (221mg, 90%); ^{1}H NMR ($CDCl_3$/DMSO-d_6) δ 2.90-3.06 (m, 4H, 2xC$\underline{H}_2$), 3.37-3.49 (m, 1H, CH), 7.23-7.39 (m 5H, ArH), 10.03 (bs, 1H, NOH):^{13}C NMR (CDCl3/DMSO-d_6) δ 33.9, 39.2, 125.8, 126.9, 128.4, 139.8, 167.2.

4,4-Cyclohexyl-*N*-hydroxyglutarimide (2.42d)

By Method A: a solution of 4, 4-Cyclohexyl-N-(benzyloxy)glutarimide **2.49d** (618mg, 2.2mmol) in THF (5mL) was hydrogenated for 72h in the presence of 10% palladium on carbon (206mg). Work-up gave **2.42d** as a white solid (371mg, 86%) which was purified by crystallisation from toluene/petroleum ether to give a white solid (305mg, 82%): mp 99-102 °C; IR ($CDCl_3$) υ_{max} 3352.1 (OH), 1735.8 and 1678 (C=O) cm^{-1}; ^{13}C NMR ($CDCl_3$) δ 21.3, 25.5, 35.9, 43.4, 167.2: HRMS (+EI, M^+) calcd. 197.1053, found 197.1052.

4,4-Dimethyl-*N*-hydroxyglutarimide (2.42e)

By Method A: a solution of 3,3-dimethyl-*N*-(benzyloxy)glutarimide **2.49e** (95mg, 0.38mmol) in THF (5mL) was hydrogenated in the presence of 10% Pd/C (43mg) for 7h. Work up as described gave a brown oil which on standing at rt crystallised.

Recrystallisation from toluene/petroleum ether gave a purified white solid (35mg, 59%): mp 56-9 °C; IR (KBr) υ_{max} 3496.7 (OH), 1734.9 and 1676.0 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.17 (s, 6H, 2x$C\underline{H}_3$), 2.67 (s, 4H, 2x$C\underline{H}_2C(O)$); ^{13}C NMR ($CDCl_3$) δ 27.7, 29.9, 45.6, 166.5; HRMS (+EI, M^+) calcd. 157.0739, found 157.0739.

THE SYNTHESIS OF SUBSTITUTED *N*-[(ACYL AND ALKYL)OXY]GLUTARIMIDES

3-Phenyl-*N*-[(methanesulfonyl)oxy]glutarimide (2.1a)

Methane sulfonyl chloride (34mL, 50mg, 0.44mmol, 1.5 equiv.) was added to an ice-cooled solution of 3-phenyl-*N*-hydroxyglutarimide **2.42a** (60mg, 0.29mmol) and *i*-PrNEt (55μL, 41mg, 0.32mmol) in CH_2Cl_2 (2mL) stirring under N_2. The resulting solution was stirrd at 0-5 °C for 20min followed by 30min at rt. Work-up gave **2.1a** as a pale yellow oil (25mg, 30%): IR ($CHCl_3$/NaCl) υ_{max} 1793.7 and 1724.2 (C=O), 1375.2 and 1215.1 (-SO_2-) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.28-2.34 (m, 2H, $CHC\underline{H}_2$), 2.87-2.93 (m, 2H, $CH_2C\underline{H}_2$), 3.43 (s, 3H, $OSO_2C\underline{H}_3$), 4.05 (dd, 1H, J = 5.3, 9.2Hz, C$\underline{H}$), 7.23-7.40 (m, 5H, Ar$\underline{H}$); ^{13}C NMR ($CDCl_3$) 25.0, 31.5, 40.7, 49.5, 128.0, 128.1, 129.0, 129.1,166.6, 168.3; HRMS (+EI) calcd. 283.0515, found 283.0514.

3-Phenyl-*N*-[(acetyl)oxy]glutarimide (2.1b)

Acetyl chloride (52mL, 57mg, 0.73mmol, 1.5 equiv.) was added to an ice-cooled solution of 3-phenyl-*N*-hydroxyglutarimide **2.42a** (100mg, 0.49mmol) and *i*-PrNEt (94μL, 70mg, 0.54mmol, 1.1 equiv.). The resulting solution was stirred for 20min under N_2 at 0-5 °C and allowed to warm to rt while stirring for a further 0.5h. Work-up gave **2.1b** as a pale yellow oil (34mg, 28% yield): ^1H NMR ($CDCl_3$) δ 2.30 (m, 5H, $CHC\underline{H}_2$ and $C\underline{H}_3$), 2.85 (m, 2H, $C\underline{H}_2C(O)$), 4.06 (m, 1H, C$\underline{H}$), 7.32 (m, 5H, Ar$\underline{H}$); ^{13}C NMR ($CDCl_3$) δ 25.0, 31.5, 40.7, 49.5, 128.0, 128.1, 129.1, 136.4, 166.6, 168.3; IR ($CHCl_3$/NaCl) υ_{max} 1813.3 and 1751.2 (C=O) cm^{-1}; HRMS (+EI, M^+) calcd. 247.0845, found 247.0845.

3-Benzyl-N-[(methanesulfonyl)oxy]glutarimide (2.1c)

Methane sulfonyl chloride (39.3μL, 58mg, 0.51mmol, 1.5 equiv.) was added to an ice-cooled solution of 3-benzyl-*N*-hydroxyglutarimide **2.42b** (74mg, 0.34mmol) and Et_3N (51.8μL, 38mg, 0.37mmol) in CH_2Cl_2 (5mL) stirring under N_2. Stirred at 0-5 °C for 0.5h and then at rt overnight. Work-up gave a brown coloured residue which was purified by flash SiO_2 chromatography to give **2.1c** as a white solid (79mg, 79%): mp 86-7 °C; IR ($CDCl_3$) υ_{max} 1762.8 and 1720.0 (C=O), 1388.7 and 1247.9 (-SO_2-) cm^{-1};

^{1}H NMR ($CDCl_3$) δ 1.67-1.81 (m, 1H, HCHPh), 1.89-1.98 (m, 1H, HCHPh), 2.63-3.01 (m, 4H, $CHCH_2$ and $CH_2C(O)$), 3.42 (s, 3H, $ArCH_3$), 3.47 (dd, 1H, J = 4.2, 14.0 Hz, CH), 7.19-7.36 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 21.2, 32.0, 35.9, 40.7, 44.8, 127.0, 128.8, 129.1, 137.4, 166.7, 169.1; HRMS (+EI, M^+) calcd. 297.0671, found 297.0671. Anal. Calcd. for $C_{13}H_{15}NO_5S$: C, 52.51; H, 5.09; N, 4.71; S, 10.76. Found: C, 52.51; H, 5.30; N, 4.68; S, 10.71.

4-Phenyl-*N*-[(methanesulfonyl)oxy]glutarimide (2.1d)

Methane sulfonylchloride (13.2mL, 20mg, 0.17mmol) was added to a solution of 4-phenyl-*N*-hydroxyglutarimide **2.42c** (35mg, 0.17mmol) and pyridine (27.7mL, 27mg, 0.34mmol, 2 equiv.) in CH_2Cl_2 (3mL) and the resulting solution stirred overnight under N_2 at rt. The reaction solution was washed with H_2O, 10% aqueous HCl and saturated aqueous $NaHCO_3$. The organic phase was worked-up in the normal manner to give **2.1d** as a brown solid which was purified by trituration with ice-cold petroleum ether. Yield 46mg (94%): mp 101-3 °C; IR ($CDCl_3$) 1759.0 and 1720.0 (C=O), 1379.0 and 1226.6 (-SO_2-) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.99-3.18 (m, 4H, 2x$CH_2C(O)$), 3.43 (s, 3H, OSO_2CH_3), 3.46 -3.56 (1H, m, CH), 7.22-7.42 (m, 5H, ArH); ^{13}C NMR δ 34.2, 40.1, 40.7, 126.3, 128.0, 129.2, 139.2, 166.0; HRMS (+EI) calcd. 283.0515, found 283.0515.

4,4-Cyclohexyl-N-[(methanesulfonyl)oxy]glutarimide (2.1e)

Methane sulfonyl chloride (41.8μL, 62mg, 0.54mmol, 1.5 equiv.) was added to an ice-cooled solution of 4,4-cyclohexyl-*N*-hydroxyglutarimide **2.42d** (70mg, 0.36mmol) and Et_3NH (55.2μL, 40mg, 0.40mmol, 1.1 equiv.) in CH_2Cl_2 (3mL). Stirred at 0-5 °C for 20min then at rt overnight. Brown residue obtained on work up which was purified by flash chromatography eluting with 2:3 EtOAc/petroleum ether to give **2.1e** as a white solid (51mg, 52%): mp 80-81 °C; IR ($CDCl_3$) υ_{max} 1759.0 and 1724.2 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.50 (bs, 10H, $5CH_2$), 2.76 (s, 4H, $2CH_2C(O)$), 3.42 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 21.4, 25.4, 32.5, 35.7, 40.7, 44.2, 166.3; HRMS (+EI, M^+) calcd. 275.0828, found 275.0828. Anal. Calcd for $C_{11}H_{17}NO_5S$: C, 47.99; H, 6.23; N, 5.09; S, 11.62. Found: C, 48.12; H, 6.31; N, 5.10; S, 11.71.

4,4-Dimethyl-*N*-[(methanesulfonyl)oxy]glutarimide (2.1f)

Methane sulfonyl chloride (49.5mL, 73mg, 0.64mmol) was added to a solution of 4,4-dimethyl-*N* -hydroxyglutarimide **2.42e** (100mg, 0.64mmol) and pyridine (104μL, 101mg, 1.28mmol, 2 equiv.) in toluene (4mL) which had been stirring at 40 °C under N_2. The temperature was elevated to 50 °C and the reaction solution stirred for 3d at this temperature. The reactio solution was evaporated to give a brownresidue that was

dissolved in the minimum anount of EtOAc and washed with H_2O, 10% aqueous HCl, and saturated aqueous $NaHCO_3$ and the organic phase worked up in the normal manner to give **2.1f** as a white solid (quantative yield): mp 86-88 ^oC; IR ($CDCl_3$) υ_{max} 1764.7 and 1724.2 (C=O), 1386.7 and 1232.4 (-SO_2-) cm^{-1} ; ^1H NMR ($CDCl_3$) d 1.17 (s, 6H, 2x$C\underline{H}_3$), 2.70 (s, 4H, 2x$C\underline{H}_2C(O)$), 3.42 (s, 3H, $OSO_2C\underline{H}_3$); ^{13}C NMR ($CDCl_3$) d 27.4, 29.7, 40.6, 46.4, 166.3; HRMS (+EI) calcd. 235.0515, found 235.0515.

6.3 CHAPTER 4 EXPERIMENTAL

(*S*)-phenylalaninol (4.19)

Method A: $LiALH_4$ reduction of (*S*)-Phenylalanine

To a suspension of $LiAlH_4$ (1.723g, 0.045mol, 1.5 equiv.) in THF (100mL) (*L*)-phenylalanine (5.1g, 0.03mol) was added portionwise and the resulting mixture refluxed gently for 6h. The reaction mixture was allowed to cool to rt after the which 1N aqueous NaOH (5mL) was added and the reaction mixture refluxed for a further 0.5h. The reaction mixture was again allowed to cool to rt and diluted with 0.5N NaOH (*ca* 100mL). The aqueous and organic layers were then left to separate but due to an emulsion this mixture was filtered through celite. The aqueous and organic phases of the filtrate were then separated, the aqueous phase extracted with EtOAc and the organic extracts combined and worked up in the usual manner to give an oily white solid (454mg, 10%): ^{1}H NMR ($CDCl_3$) δ 1.89 (bs, 1H, NH), 2.53 (dd, 1H, *J* = 8.8, 13.5Hz, HCHPh), 2.79 (dd, 1H, *J* = 5.0, 13.5Hz, HCHPh), 3.09-3.17 (m, 1H, CH), 3.38 (dd, 1H, *J* = 7.0, 10.5Hz, HCHOH), 3.64 (dd, 1H, *J* = 3.8, 10.5Hz, HCHOH), 7.18-7.31 (m, 5H, ArH).

Method B: Borane reduction of (*S*)-Phenylalanine

Freshly distilled BF_3.OEt (0.760mL, 877mg, 6.2mmol) was added over a period of 40 min to a white slurry of (*S*)-Phenylalanine (1.001g, 6.1mmol) in THF (3.2mL) stirring in N_2 at rt. The mixture was heated uptil reflux. Reflux was continued for 2h, resulting in a colourless, homogeneous solution. BH_3.SMe_2 (0.670mL, 529mg, 7mmol, 1.15 equiv.) was then added via a dropping funnel to the refluxing solution followed by a THF (1mL) washing of the funnel over a period of 2.75h and reflux was continued for 4.5h. The solution was allowed to cool to rt while stirring for 12.25h. The reaction solution was again heated to reflux and this was maintained for 0.5h after which additional BH_3.SMe_2 (0.2mL, 158mg, 2.1mmol) was added dropwise and the resulting solution refluxed for a further 4.25h. THF (1mL) and 5N aqueous NaoH (4mL) were added in succession and the resulting mixture consisiting of an upper white phase and a clear lower phase was refluxed overnight. The reaction mixture was allowed to cool to rr and then filtered. The residual solids were washed with a 1:1 THF/H_2O (2mL) rinsing of the reaction flask and THF (2mL). The filtrate and washings were combined and evaporated to give a white slurry which was extracted with CH_2Cl_2. Work up in the usual manner gave **4.19** as a white solid (603mg, 65%). More product (232mg, 25%) was obtained on a second extraction procedure. Total yield 835mg (91%): mp 90-2 °C (EtOAc, fine white needles) (lit.[240] mp 88.5-91 °C); ^{1}H NMR ($CDCl_3$) δ 2.16 (bs, 1H,

NH), 2.51 (dd, 1H, J = 8.8, 13.6Hz, HCHPh), 2.79 (dd, 1H, J = 5.2, 13.5Hz, HCHPh), 3.07-3.15 (m, 1H, CPh), 3.07-3.15 (m, 1H, CH), 3.39 (dd, 1H, J = 7.1, 10.Hz, HCHOH), 3.63 (dd, 1H, J = 3.9, 10.7Hz, HCHOH), 7.18-7.34 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 40.8, 54.2, 66.3, 126.4, 128.6, 129.2, 138.6; HRMS (+EI, M^+) 149.0 (57), 120.1 (47), 103.1 (10), 91.1 (20), 77.0 (8), 60.1 (M - $PhCH_2$, 100).

(*S*)-4-Benzyl-2-oxazolidinone (4.17)[240]

A mixture of (S)-phenylalaninol **4.19** (407mg, 3mmol), K_2CO_3 (38mg, 0.3mmol, 0.1 equiv.) and diethyl carbonate (0.679mL, 662mg, 6mmol, 2 equiv.) was heated in an oil bath set at 136 °C for 4h. The resuling mixture consisting of a white solid suspended in a yellow solution was allowed to cool to rt and then diluted with CH_2Cl_2 and washed with H_2O. The organic phase was worked up in the usual manner to give **4.17** as a white solid (468mg, 98%): mp 85-7 °C (EtOAc/petroleum ether, white plates) (lit.[240] mp 84.5-86.5 °C); ^{1}H NMR ($CDCl_3$) δ 2.81-2.94 (m, 2H, CH_2Ph), 4.05-4.16 (m, 2H, HCHO and CH), 4.39-4.46 (m, 1H, HCHO), 6.04 (bs, 1H, NH), 7.15-7.19 (m, 2H, ArH), 7.24-7.36 (m, 3H, ArH); ^{13}C NMR ($CDCl_3$) δ 41.3, 53.7, 69.5, 127.1, 128.9, 129.0, 135.9, 159.6: HRMS (+EI, M^+) calcd. 177.0790, found 177.0790.

***N*-3-Phenylpropionyl-(*S*)-4-benzyl-2-oxazolidinone (4.15)[239a]**

n-BuLi (1.3M in hexane) (14.9mL, 19mmol, 1.05 equiv.) was added dropwise to a solution of (*S*)-4-benzyl-2-oxazolidinone **4.17** (3.245g, 18mmol) in THF (13mL) stirring in N_2 at -78 °C. The resulting dark red solution was stirred for 1h after which a solution of 3-phenylpropionyl chloride (3.360g, 20mmol, 1.11 equiv.) in THF (6mL) was added slowly dropwise. The resulting amber reaction solution was allowed to come to rt while stirring was continued overnight. Brine (20mL) was added and the solvent evaporated to give an immiscible mixture consisting of a brown oily residue and aqueous layer. This was extracted with CH_2Cl_2 to give a brown residue that was purified by flash chromatography eluting with 1:4 EtOAc/petroleum ether to give **4.15** as a white solid (4.623g, 83%): mp 105-6 °C (lit.[239a] mp 92-5 °C); IR ($CDCl_3$) υ_{max} 1782.1 and 1701.1 (C=O) cm^{-1}; ^{1}H NMR δ 2.75 (dd, 1H, J = 9.5, 13.4Hz, CHHCHPh), 2.95-3.10 (m, 2H, CHHCHPh and CH_2HCHPh), 3.19-3.38 (m, 3H, CH_2HCHPh and CH_2C(O)), 4.13-4.21 (m, 2H, CH_2O), 4.62-4.70 (m, 1H, NCH), 7.16-7.35 (m, 10H, ArH); ^{13}C NMR ($CDCl_3$) δ 30.2, 37.1,37.8, 55.1, 66.2, 126.2, 127.3, 128.4, 128.6, 128.9, 129.4, 135.2, 140.4, 153.4, 172.4; HRMS (+EI, M^+) calcd. 309.1366, found 309.1365.

_N_-[3-Ethoxycarbonyl-(2*R*)-benzyl-1-oxopropyl]-(*S*)-4-benzyl-2-oxazolidinone (4.21)

A 1.8M solution of LDA in THF/hexanes (0.64mL, 1.15mmol, 1.26 equiv.) was added slowly dropwise to a solution of (S)-4-benzyl-2-oxazolidinone (280mg, 0.91mmol) in THF (5mL) stirring under a N_2 atmosphere at -78 °C (dry ice-acetone bath). The resulting solution was stirred for 1h at -78 °C after which a solution of ethyl bromoacetate (212μL, 319mg, 1.91mmol) in THF (1mL) was added slowly dropwise. The bath was packed with dry ice and the temperature allowed to come to rt while stirring was continued overnight. Brine (6mL) was added and the bulk of the THF evaporated to give a white slurry which was extracted with EtOAc. Work up of the extract in the usual manner gave a brown residue which was purified by flash SiO_2 chromatography eluting with 1:4 EtOAc/petroleum ether to give **4.21** as a brown residue (177mg, 49%): mp 60-2 °C (EtOAc/petroleum ether); ^{1}H NMR ($CDCl_3$) δ 2.45 (dd, 1H, J = 4.2, 17.2Hz, NCHHCHPh), 2.62-2.77 (m, 2H, NCHHCHPh and C(O)CHHCHPh), 2.89-3.06 (m, 2H, C(O)CHHCHPh and HCHC(O)), 3.32 (dd, 1H, J = 3.2, 13.4Hz, HCHC(O)), 3.94 (t, 1H, J = 8.3Hz, HCHO), 4.05-4.12 (m, 3H, HCHO and OCH_2CH_3), 4.49-4.60 (m, 2H, NCH and C(O)CH), 713-7.37 (m, 10H, ArH); ^{13}C NMR ($CDCl_3$) δ 14.1, 35.5, 37.5, 38.3, 41.0, 55.6, 60.7, 65.9, 126.8, 127.2, 128.5, 128.9, 129.2, 129.5, 135.6, 137.8, 152.9, 171.8, 175.1; HRMS (+EI) calcd. 395.1734, found. 395.1733.

_N_-[3-*tert*-Butoxycarbonyl-(2*R*)-benzyl-1-oxopropyl]-(*S*)-(4*S*)-benzyl-2-oxazolidinone (4.23)

LDA 1.8 M solution in THF/hexanes (2.4mL, 4.3mmol, 1.2 equiv.) was slowly added dropwise to a solution of 4-(*S*)-benzyl-2-oxazolidinone **4.15** (1.116g, 3.6mmol) in THF (10mL) stirring in N_2 at -78 °C (dry ice-acetone bath). The resulting solution was stirred for 1h after which a solution of *tert*-butyl bromoacetate (1.458mL, 1.761g, 9mmol, 2.5 equiv.) in THF (2mL) was added slowly dropwise. The bath was packed with dry ice and the temperature allowed to come to rt while stirring was continued overnight. Brine (12mL) was added and the bulk of the THF evaporated to give an immiscible solution consisting of a brown residue and an aqueous phase. This was extracted with EtOAc (30mL) then washed with brine (30mL) and worked up in the usual manner to give a brown residue (94mg). Purification by flash SiO_2 chromatography eluting with 1:4 EtOAc/petroleum ether gave **4.23** as a pale yellow solid (675mg, 44%): mp 105-7 °C (EtOAc/petroleum ether, crystals); ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H, $O(CH_3)_3$), 2.37 (dd, 1H, J = 4.2, 16.8Hz, NHCHHCHPh), 2.64 (dd, 1H, J = 9.0, 12.9Hz, NHCHHCHPh), 2.73 (dd, 1H, J = 10.0, 13.4Hz, C(O)CHHCHPh), 2.84 (dd, 1H, J = 10.7, 17.1Hz, C(O)CHHCHPh), 3.01 (dd, J = 6.3,

12.7Hz, CHHCHC(O)), 3.30 (dd, 1H, J = 3.4, 13.7Hz, CHHCHC(O)), 3.92 (t, 1H, J = 8.3Hz, HCHO), 4.07 (dd, 1H, J = 2.4, 8.8Hz, HCHO), 4.44-4.57 (m, 2H, NCH and CHC(O)), 7.13-7.37 (m, 10H, ArH); ^{13}C NMR δ ($CDCl_3$) 28.0, 36.8, 37.6, 38.3, 41.2, 55.6, 65.9, 80.8, 126.7, 127.2, 128.4, 128.9, 129.3, 129.5, 135.7, 138.0, 152.9, 171.2, 175.4; HRMS (+EI) calcd. 423.2047 found. 423.2046.

(+)-(*R*)-Ethyl-3-Benzylsuccinate (4.25)

30% H_2O_2 (36μL, 0.4mmol, 4 equiv.) was added dropwise to a solution of (4*S*)-(-)-4-benzyl-*N*-[(2*R*)-benzyl-3-(ethoxycarbonyl)propionyl]-2-oxazolidinone **4.21** (35mg, 0.09mmol) in 4:1 THF/H_2O (4mL). $LiOH.H_2O$ (6mg, 0.14mmol, 1.6 equiv.) was added and the reslting mixture was stirred for 1h after the $LiOH.H_2O$ had dissolved. Aqueous 1.345M Na_2SO_3 (265μL, 0.4mmol, 4 equiv.) was added and the bulk of the THF evaporated to give a mixture consisting of a brown residue and an aqueous phase. This mixture was extracted with CH_2Cl_2 to remove the chiral oxazolidinone auxiliary **4.17** and the aqueous layer was acidified with 2N HCl and then extracted with EtOAc. The EtOAc extracts were worked up in the usual manner to give (*R*)-ethyl-3-benzylsuccinate **4.25** as a brown residue (17mg, 81%): $[\alpha]^{24}_D$ = 20.3° (c = 1.6, EtOAc); IR υ_{max} ($CDCl_3$) 1728.1 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.22 (t, 3, J = 7.z, OCH_2CH_3), 2.40 (dd, 1H, J = 4.4, 17.1Hz, HCHPh), 2.59-2.82 (m, 2H, HCHPh and HCHC(O)), 3.10-3.19 (m, 2H, HCHC(O) and CHC(O)), 4.10 (q, 2H, J = 6.8, 7.3Hz, OCH_2CH_3), 7.17-7.33 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 14.0, 34.7, 37.3, 42.8, 60.8, 126.8, 128.9, 129.0, 137.9, 171.7, 180.2; HRMS (+EI) calcd. 236.1049, found 236.1049.

(-)-(*R*)-3-Benzyl-*N*-benzyloxysuccinimide ((*R*)-2.25)

A solution of (*R*)-ethyl 2-benzylsuccinate **4.25** (140mg, 0.59mmol) in CH_2Cl_2 (7mL) was added to a solution of*O* -benzyloxyamine hydrochloride (94mg, 0.59mmol) and Et_3N (104μL, 76mg, 0.75mmol, 1.27 equiv.) in CH_2Cl_2 (4mL) which had been stirring for 0.25h under N_2 at 0-5 °C (ice-salt bath). HOBt (80mg, 0.59mmol) and then DCC (123mg, 0.59mmol) were added successively and the resulting solution stirred overnight while the temperature was allowed to come to rt. The reaction slurry was evaporated to give a white solid that was suspended in EtOAc (8mL) and this mixture was filtered to remove the DCU byproduct. The reaction flask was rinsed with EtOAC (2mL) and this was used to wash the DCU filter cake. The filtrate and the washings were combined and evaporated to give a brown residue (294mg) containing a 2:1 mixture, by ^{1}H NMR, of 3-ethoxycarbonyl-(3*R*)-benzylpropionyl *N*-benzyloxy amide **4.26** and (3*R*)-benzyl-*N*-benzyloxysuccinimide (***R***)-**2.25**.

A solution of this residue (294mg) and *p*-TsOH.1H_2O (57mg, 0.30mmol, 0.5 equiv.) in 1,2-DCE (10mL) was refluxed with azeotropic removal of EtOH for 20h. The solvent was evaporated to give a brown residue (0.335g) which was submitted to flash SiO_2 chromatography eluting with 1:4 EtOAc/petroleum ether to give (*R*)-3-benzyl-*N*-benzyloxysuccinimide (***R*)-2.25** as a white solid (101mg, overall yield 58%): mp 86-88 °C; $[\alpha]^{21}_D$ = -67.8° (c = 4.5, EtOAc); ^{1}H NMR ($CDCl_3$) δ 2.28 (dd, 1H, *J* = 4.4, 18.1Hz, HCHPh), 2.53 (dd, 1H, *J* = 8.8, 18.1Hz, HCHPh), 2.70 (dd, 1H, *J* = 8.3, 13.7Hz, HCHC(O)), 2.92-3.00 (m, 1H, CHC(O)), 3.06 (dd, 1H,*J* = 4.2, 13.4Hz, HCHC(O)), 7.06 (d, 2H, *J* = 6.8Hz, ArH), 7.17-7.38 (m, 8H, ArH); ^{13}C NMR δ 30.5, 36.2, 38.3, 78.5, 127.2, 128.5, 128.9, 129.1, 129.4, 130.0, 133.2, 136.3, 170.4, 173.3 .

(-)-(*R*)-3-Benzyl-*N*-hydroxysuccinimide ((*R*)-2.26)

A solution of (-)-(*R*)-3-benzyl-*N* -hydroxysuccinimide (***R*)-2.25** (101mg, 0.34mmol) in EtOAc (4mL) was hydrogenated in the presence of 10% palladium on carbon (33mg) for 7h. The reaction mixture was filtered through celite and Na_2SO_4. This filter cake was washed with a small quantity of EtOAc and the filtrate and the washings were combined and evaporated to give (***R*)-2.26** as a white solid (64mg, 91%): mp 142-3 °C; $[\alpha]^{24}_D$ = -78.0° (c = 3.0, EtOAc); ^{1}H NMR ($CDCl_3$/DMSO-d_6) δ 2.35-2.45 (m, 1H, HCHPh), 2.60-2.71 (m, 1H, HCHPh), 2.82-2.92 (m, 1H, HCHC(O)), 3.08-3.15 (m, 1H, HCHC(O)), 3.21-3.27 (m, 1H, CH), 7.17-7.39 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$/DMSO-d_6) δ 30.3, 36.0, 38.1, 126.8, 128.6, 128.8, 136.6, 171.1, 174.0.

(*R*)-3-Benzyl-*N*-[(*trans*-styrylsulfonyl)oxy]succinimide ((*R*)-1.41f)

Trans-Styrylsulfonyl chloride (90mg, 0.44mmol, 1.5 equiv.) was added to a solution of (-)-(*R*)-3-benzyl-*N*-hydroxysuccinimide (***R*)-2.26** (59mg, 0.29mmol) and pyridine (47μL, 46mg, 0.58mmol, 2 equiv.) in CH_2Cl_2 (5mL) which had been stirring under N_2 at rt. The resulting solution was then stirred overnight. The reaction solution was then diluted with CH_2Cl_2 (5mL) and then washed with H_2O, 10% HCl and saturated aqueous $NaHCO_3$. The organic phase was worked up in the normal manner to give (***R*)-1.41f** as a brown oil (quantative yield): ^{1}H NMR ($CDCl_3$) δ 2.53 (dd, 1H, *J* = 4.4, 18.6Hz, HCHPh), 2.78 (dd, 1H, *J* = 8.5, 18.3Hz, HCHPh), 2.95 (dd, 1H, *J* = 10.0, 15.4, HCHC(O)), 3.20-3.30 (m, 2H, HCHC(O) and CHC(O)), 6.94 (d, 1H, *J* = 15.6Hz, C:CHPh), 7.15-7.35 (m, 5H, ArH), 7.68 (d, 1H, *J* = 15.6Hz, HC:C); HRMS (+EI) calcd. 371.0828, found 371.0828.

_N_-3-Carbomethoxy-propionyl-(_S_)-4-benzyl-2-oxazolidinone (4.32)

n-BuLi (1.6M in hexane) (540μL, 0.86mmol, 1.02 equiv.) was added dropwise over 15min to a solution of (*S*)-4-benzyl-2-oxazolidinone **4.17** (150mg, 0.85mmol) in THF (3mL) stirring under N_2 at -78 °C. A solution of 3-carbomethoxy-propionyl chloride (140mg, 0.93mmol, 1.1 equiv.) in THF (1mL) was added in one portion and the resulting reaction solution was stirred overnight while being allowed to come to rt. The reaction solution was quenched with saturated aqueous NH_4Cl and the resulting mixture evaporated and then extracted with CH_2Cl_2. The organic extracts were washed with 1N aqueous NaOH and then worked up in the normal manner to give a brown residue (198mg, 80%); IR (KBr) υ_{max} 1782.1, 1735.8 and 1703.0 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.69-2.83 (m, 3H, HCHPh and $CH_2CH_2CO_2CH_3$), 3.24-3.31 (m, 3H, HCHPh and $C(O)CH_2CH_2$), 3.72 (s, OCH_3), 4.16-4.26 (m, 2H, $CHCH_2O$), 4.64-4.72 (m, 1H, NCH), 7.20-7.37 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 28.0, 30.8, 37.7, 51.8, 55.1, 66.3, 127.3, 128.9, 129.4, 135.1, 153.4, 171.8, 172.8.

6.4 CHAPTER 5 EXPERIMENTAL

4-Nitro-benzyl alcohol (5.10)

An alkaline solution of $NaBH_4$ was prepared by dissolving $NaBH_4$ (950mg, 25mmol, 0.38 equiv.) in 2N NaOH (1.33mL) and H_2O (12mL). This alkaline solution was added to a yellow solution of 4-nitrobenzaldehyde **5.9** (10g, 66mmol) in CH_3OH (65mL) at such a rate that the internal reaction temperature was maintained below 25 °C. The reaction solution turned orange almost immediately and when a sample withdrawn from the reaction mixture was treated with 3N H_2SO_4 $H_{2(g)}$ evolved indicating the reaction was complete. The reaction mixture was then evaporated to give a yellow oil which was diluted with H_2O (60mL) and extracted with Et_2O (2 x 60mL). The Et_2O extracts were worked-up in the normal manner to give a pale yellow solid (6.628g, 66%): mp 92-4 °C; ^{1}H NMR (DMSO-d_6) δ 4.74 (d, 2H, J = 5.4Hz, C$\underline{H}_2$OH), 5.65 (t, 1H, J = 5.9Hz, CH$_2$O$\underline{H}$), 7.66 (d, 2H, J = 8.8Hz, Ar$\underline{H}$), 8.24 (d, 2H, J = 7.3Hz, Ar$\underline{H}$); ^{13}C NMR (DMSO-d_6) δ 61.9, 123.0, 126.7, 146.1, 150.5.

4-Hydroxymethyl aniline (5.8)

A solution of 4-nitro-benzyl alcohol **6.10** (200mg, 1.3mmol) in EtOH (3mL) was hydrogenated inthe presence of PtO_2 (15mg) for 28h. The reaction mixture was filtered through celite and the filtrate was evaporated to give a brown residue that solidified overnight to afford **6.8** as a brown solid (129mg, 78%): mp 43 °C (lit.[245a] mp 63-4 °C); ^{1}H NMR ($CDCl_3$) δ 2.96 (bs, 2H, N$\underline{H}_2$), 4.54 (s, 2H, C$\underline{H}_2$OH), 6.67 (d, 2H, J = 8.3Hz), 7.15 (d, 2H, J = 8.3Hz); ^{13}C NMR ($CDCl_3$) δ 65.1, 115.1, 128.7, 131.1, 145.9; HRMS (+EI) calcd. 123.0685, found 123.0684.

4,4-Dimethyl-*N*-[(4-hydroxymethyl)phenyl]glutarimide (5.15)

EDAC (260mg, 1.36mmol, 2.09 equiv.) was added to a solution of 3,3-dimethylglutaric acid **2.44e** (104mg, 0.65mmol), 4-hydroxymethylaniline **5.8** (97mg, 0.78mmol, 1.2 equiv.) and HOBt (193mg, 1.43mmol, 2.2 equiv.) and Et_3N (453mL, 329mg, 3.25mmol, 5 equiv.) in CH_2Cl_2 (10mL) stirring under N_2 at 0-5 °C. Stirring was continued overnight as the reaction mixture was allowed to warm to rt. The reaction mixture was then washed with H_2O (10mL), and the aqueous phase extracted with CH_2Cl_2. The organics were combined and washed with 10% aqueous HCl and then worked up in the normal manner to give a brown residue which was purified by flash SiO_2 chromatography , eluting with 7:13 EtOAc/petroleum ether to give **5.15** as a brown residue (20mg, 12%): IR ($CDCl_3$) υ_{max} 1732.0 and 1683.7 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.21 (s, 6H, 2xC$\underline{H}_3$), 2.67 (s, 2xC$\underline{H}_2$C(O), 4.70 (s, 2H, OC$\underline{H}_2$Ph), 7.07

(d, 2H, J = 7.8Hz, ArH), 7.45 (d, 2H, J = 8.3Hz, ArH); ^{13}C NMR ($CDCl_3$) δ 27.7, 29.3, 46.5, 64.7, 127.7, 128.4, 134.1, 141.4, 172.0; HRMS (+EI) calcd. 247.1209, found 247.1208.

4-Phenyl-*N*-[(4-hydroxymethyl)phenyl]glutarimide (5.16)

Aniline **5.8** (108mg, 0.89mmol), HOBt (214mg, 1.6mmol, 2.2 equiv.) and Et_3N (502mL, 364mg, 3.6mmol, 5 equiv.) were added in succession to a solution of 3-phenylglutaric acid (150mg, 0.72mmol) in CH_2Cl_2 (10mL) stirring under N_2 at 0-5 °C. EDAC (288mg, 1.5 mmol, 2.08 equiv.) was added to the above solution and stirring was continued overnight whilethe reaction solution was allowed to come to rt. The reaction solution was washed with H_2O and 2N HCl and the organic phase worked up in the normal manner to give crude **5.16** as an orange solid (138mg, 64% mass recovery): mp 68 °C (EtOAc/petroleum ether); IR ($CDCl_3$) υ_{max} 1735.8 and 1687.6 (C=O); ^{1}H NMR ($CDCl_3$) δ 3.00 (dd, 2H, J = 11.0, 16.6Hz, CH$_2$C(O)), 3.16 (dd, 2H, J = 4.4, 17.1Hz, CH$_2$C(O)), 3.53-3.61 (m, 1H, CH), 4.74 (s, 2H, CH$_2$OH), 7.10 (d, 2H, J = 8.3Hz, ArH), 7.23-7.49 (m, 7H, ArH); HRMS (+EI) calcd. 295.1209 found 295.1208.

REFERENCES

1. a) Lynch, D. R.; Snyder, S. H. *Ann. Rev. Biochem.* **1986**, *55*, 773.
   b) McKelvy, J. F. *Ann. Rev. Neurosci.* **1986**, *9*, 415.
   c) Neurath, H. *Science* **1984**, *224*, 350.
   d) Thorsett, E. D.; Wyvratt, M. J. in *Neuropeptidase Inhibitors* Ed. Turner, A.J., Ellis Horwood, Chichester, **1987**, 229.

2. Polgar, L. *Mechanisms of Protease Action* CRC Press Inc, Boca Raton, **1989**.

3. Rich, D. H. in *Comprehensive Medicinal Chemistry, The Rational Design, Mechanistic Study and Therapeutic Application of Chemical Compounds, Vol.2: Enzymes and Other Molecular Targets*, Ed. Hansch, C.; Sammes, P. G.; Taylor, J. B. Pergamon Press, Oxford, **1990**, 391 and references therein.

4. Fischer, G. *Nat. Prod. Rep.*, **1988**, *5*, 465.

5. *Advances in Experimental Medicine and Biology, Vol. 240, Proteases II Potential Role in Health and Disease*, Ed. Horl, W. H.; Heidland, A., Plenum Press, New York London, **1988**.

6. Stroud, R. M. *Sci. Am.* **1974**, 74.

7. Walsh, C. *Enzymatic Reaction Mechanisms* W.H. Freeman and Company, San Francisco, **1979**, 53.

8. Kraut, J. in *Ann. Rev. Biochem*. Ed. Snell, E. E; **1977**, *46*, 331.

9. a) Blow, D. M. in *The Enzymes, Vol. III Hydrolysis: Peptide Bonds* Ed. Boyer, P. 3rd edn, Academic Press, New York and London, **1971**, 185.
   b) Hess, G. P. in *The Enzymes, Vol. III Hydrolysis: Peptide Bonds* Ed. Boyer, P. 3rd edn, Academic Press, New York and London, **1971**, 213.
   c) Blackburn, S. *Enzyme Structure and Function* Marcel Dekker, New York, **1976**, 11.
   d) Fink, A. L. in *Enzyme Mechanisms* Ed. Page, M. I.; Williams, A.The Royal Society of Chemistry, London, **1987**, 159.

10. Edwards, P. D.; Bernstein, P. R. *Med. Res. Rev.* **1994**, *14* , 127.

11. a) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun*. **1967**, *27*, 157.

b) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1968**, *32*, 898.

12. Umezawa, H.; Aoyagi, T.; Morishma, H.; Kunimotot, S.; Matsuzaki, M.; Hamada, M.; Takeuchi, T. *J. Antibiot.* **1970**, *23*, 425.

13. (a) Umezawa, H.; Aoyagi, T.; Okura, A.; Morishima, H.; Takeuchi, T.; Okami, Y. *J. Antibiot.* **1973**, *26*, 787.
(b) Okura, A.; Morishima, H.; Takita, T.; Aoyagi, T.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1975**, *28*, 337.

14. Aoyagi, T.; Miyata, S.; Nanbo, M.; Kojima, F.; Matsuzaki, M.; IshizkaM.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1969**, *22*, 558.

15. Umezawa, H.; Aoyagi, T. in *Proteinases in Mammalian Cells and Tissues* Ed. Barrett, A. J., Elsevier, Amsterdam, **1977**, 637.

16. Edwards, P. D.; Bernstein, P. R. *Med. Res. Rev.* **1994**, *14* , 133.

17. Feinstein, G.; Malemud, C. J.; Janoff, A. *Biochim. Biophys. Acta* **1976** , *429*, 925.

18. Zimmerman M.; Ashe, B. M.; Dorn, Jr., C. P.; Shu-Shu, Y.; Jones, H. in *Neutral Proteases Hum. Polymorphonucl. Leukocytes [Proc. Int. Symp.*] Eds, Havemann, K.; Janoff, A., Urban and Schwarzenburg, Munich, **1978**, 234.

19. Stein, R. L.; Trainor, D. A.; Wildonger, R. A. *Annu.Rep. Med. Chem.* **1985**, *20*, 237.

20. McRae, B.; Nakajima, K.; Travis, J.; Powers, J. C. *Biochemistry* **1980**, *19*, 3973.

21. Yasutake, A.; Powers, J. C. *Biochemistry* **1981**, *20*, 3675.

22. Chapters 7-20 in *Proteinase Inhibitors*, Eds. Barrett, A. J.; Salvesen, G., (Research Monographs in Cell and Tissue Physiology, Vol 12), Elsevier, Amsterdam, **1986**, 301.

23. Baugh, R. J.; Schnebli, H.P. in *Proteinases in Tumor Invasion* Ed. Straubli, P.; Barrett, A. J.; Baici, A., Raven Press, New York, **1986**, 157.

24. a) *Pulmonary Emphysema and Proteolysis* Ed. Mittman, C., Academic Press, New York, **1972**; 1-537.
b) Sprung, C. L.; Schultz, D. R.; Clerch, A. R. *New Engl. J. Med.* **1984**, *304*, 1301.
c) Velvart, M. *Rheumatol. Int.* **1981**, *1*, 121.

25. Silverman, R. B. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology Vol. I*, CRC Press, Boca Raton, FL, **1988.**

26. Lippert, B.; Jung, M. J.; Metcalf, B. W. *Brain. Res. Bull.* **1980**, *5* (2), 375.

27. Lentini, A.; Farchione, F.; Ternai, B.; KreuA-Ongarjnukool, N.; Tovivich, P. *Biol. Chem. Hoppe-Seyler* **1987**, *368*, 369.

28. Digenis, G. A.; Agha, B. J.; Tsuji, K.; Kato, M.; Shingoi, M. *J. Med. Chem.* **1986**, *29*, 1468.

29. Dutta, A. S.; Giles, M. B.; Gormley, J. J.; Williams, J. S.; Kusner, E. J. *J. Chem. Soc. Perkin Trans.* **1987**, *111*, 1.

30. Westerik, J. O.; Wolfenden, R. *J. Biol. Chem.* **1972**, *247*, 8195.

31. Edwards, P. D.; Meyer, Jr., E. F.; Vjayalakshmi, J.; Tuthill, P. A.; Andisik, D. A.; Gomes, B.; Strimpler, A. *J. Am. Chem. Soc.* **1992**, *114*, 1854.

32. Wiley, R. A.; Rich, D. H. *J. Med. Chem.* **1993**, *13*, 327.

33. Imperiali, B.; Abeles, R. H. *Tetrahedron Lett.* **1986**, *27*, 135.

34. Imperiali, B.; Abeles, R. H. *Biochemistry* **1986**, *25*, 3760.

35. Bergeson, S. H.; Edwards, P. D.; Krell, R. D.; Shaw, A.; Stein, R. L.; Stein, M. M.; Strimpler, A. M.; Trainor, D. A.; Wildonger, R. A.; Wolanin, D. J. 193 National Meeting of the American Chemical Society (Abstracts of Papers), Denver, Colorado, April 5-10, **1987**.

36. Edwards, P. D. *Tetrahedron Lett.* **1992**, *33*, 4279.

37. Stein, M. M.; Wildonger, R. A.; Trainor, D. A.; Edwards, P. D.; Yee, Y. K.; Lewis, J. J.; Zottola, M. A.; Williams, J. C.; Strimpler, A. M. Eleventh

American Peptide Symposium, (Abstracts of Papers), University of California, San Diego, California, July 9-14, **1989**.

38. Edwards, P. D.; Bernstein, P. R. *Med. Res. Rev.* **1994**, *14*, 145.

39. Peet, N. P.; Burkhart, J. P.; Angelastro, M. R.; Giroux, E. L.; Mehdi, S.; Bey, P.;Kolb, M.; Neises, B.; Schirlin, D. *J. Med. Chem.* **1990**, *33*, 394.

40. Hori, H.; Yasutake, A.; Minematsu, Y.; Powers, J. C. in *Peptides, Structure and Function (Proceedings of the Ninth American Peptide Symposium)*, Eds. Deber, C. M.; Hruby, V. J.; Kopple, K. D. Pierce Chem. Co., Rockford, Illinois, **1985**, 819.

41. Mehdi, S.; Angelastro, M. R.; Burkhart, J. P.; Koehl, J. P.; Peet, N. P.; Bey, P. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 595.

42. Stein, M. M.; Wildonger, R. A.; Trainor, D. A.; Edwards, P. D.; Yee, Y. K.; Lewis, J. J.; Zottola, M. A.; Williams, J. C.; Strimpler, A. M. in *Peptides, Structure and Function (Proceedings of the Eleventh American Peptide Symposium)*, Eds., River, J. E.; Marshall, G. R. ESCOM, Leiden, **1990**, 369.

43. (a) Kettner, C. A.; Shenvi, A. B. *J. Biol. Chem.* **1984**, *259*, 15106.
(b) Kettner, C. A.; Bone, R.; Agard, D. A.; Bachovchin, W. W. *Biochemistry* **1988**, *27*, 7682.

44. (a) Knabb, R. M.; Kettner, C. A.; Timmermans, P. B. M. W. M.; Reilly, T.M. *Thromb. Haemostas.* **1992**, *67*, 56.
(b) Kettner, C. A.; Mersinger, L. J.; Knabb, R. M. *J. Biol. Chem.* **1990**, *265*, 18289.
(c) Quan, M. L.; Wityak, J.; Dominguez, C.; Duncia, J. V.; Kettner, C. A.; Ellis, C. D.; Liauw, A. Y.; Park, J. M.; Santella, J. B.; Knabb, R. M.; Thoolen, M. J.; Weber, P. C.; Wexler, R. R. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1595.

45. (a) Tian, Z. -Q.; Brown, B. B.; Mack, D. P.; Hutton, C. A.; Bartlett, P. A. *J. Org. Chem.* **1997**, *62*, 514 and references therein.
(b) Edwards, P. D.; Berstein 148-152 and references therein.

46. Shaw, E. in *Enzyme Inhibitors as Drugs* Ed., Sandler, M. MacMillan Press, **1980**, 25.

47. (a) Lamden, L. A.; Bartlett, P. A. *Biochem. Biophys. Res. Commun.* **1983**, *112*, 1085.
(b) Bartlett, P. A.; Lamden, L. A. *Bioorg. Chem.* **1986**, *14, 356.*
(c) Oleksyszyn, J.; Powers, J. C. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 143.
(d) Oleksyszyn, J.; Powers, J. C. *Biochemistry* **1991**, *30*, 485.

48. (a) Lively, M. O.; Powers, J. C. *Biochim. Biophys. Acta* **1978**, *525*, 171.
(b) Yoshimura, T.; Barker, L. N.; Powers, J. C. *J. Biol. Chem.* **1982**, *257*, 5077.

49. (a) Brown, W. E.; Wold, F. *Science* **1971**, *174*, 608.
(b) Brown, W. E.; Wold, F. *Biochemistry* **1973**, *12*, 828.
(c) Brown, W. E.; Wold, F. *Biochemistry* **1973**, *12*, 835.
(d) Brown, W. E. *Biochemistry* **1975**, *14*, 5079.

50. Miyano, M.; Deason, J. R.; Nakao, A.; Stealey,M. A.; Villamil, C. I.; Sohn, D. D.; Meuller, R. A. *J. Med. Chem.* **1988**, *31*, 1052.

51. Kawabata, K.; Suzuki, M.; Sugitani, M.; Imaki, K.; Toda, M.; Miyamoto, T. *Biochem. Biophys. Res. Commun.* **1991**, *177*, 814.

52. Silverman, R. B. *The Organic Chemistry of Drug Design and Drug Action*, Academic Press, San Diego and London, **1992**, 185-7.

53. (a) Groutas, W. C.; Badger, R. C.; Ocain, T. D.; Felker, D.; Frankson, J.; Theodorakis, M. C. *Biochem. Biophys. Res. Commun.* **1080**, *95*, 1890.
(b) Walker, B.; Elmore, D. T. *Biochem. J.* **1984**, *221*, 277.
(c) Groutas, W. C.; Abrams, W. R.; Theodorakis, M. C.; Kasper, A. M.; Rude, S. A.; Badger, R. C.; Ocain, T. D.; Miller, K. E.; Moi, M. K.; Brubaker, M. J.; Davis, . S.; Zandler, M. E. *J. Med. Chem.* **1985**, *28*, 204.
(d) Groutas, W. C.; Brubaker, M. J.; Zandler, M. E.; Mazo-Gray, V.; Rude, S. A.; Crowley, J. P.; Castrisos, J. C.; Dunshee, D. A.; Giri, P. K. *J. Med. Chem.* **1986**, *29*, 1302.

54. Walsh, C. *Tetrahedron* **1982**, *38*, 871.

55. (a) Walsh, C.; Cromartie, T.; Marcotte, P.; Spencer, R. *Methods Enzymol.* **1978**, *53D*, 437.
(b) Wang, E.; Walsh, C. *Biochemistry* **1978**, *17*, 1313.

56. Alazard, R.; Bechet, J. J.; Dupaix, A.; Yon, J. *Biochim. Biophys. Acta* **1973**, *309*, 379.

57. (a) White, E. H.; Roswell, D. F.; Politzer, I. R.; Branchini, B. R. *J. Am. Chem. Soc.* **1975**, *97*, 2290.
(b) White, E. H.; Roswell, D. F.; Politzer, I. R.; Branchini, B. R. *Meth. Enzymol.* **1977**, *46*, 216.
(c) White, E. H.; Jelinski, Politzer, I. R.; Branchini, B. R.; Roswell, D. F. *J. Am. Chem. Soc.* **1981**, *103*, 4231.

58. (a) Bechet, J. J.; Dupaix, A.; Yon, J.; Wakselman, M.; Robert, J. -L.; Vilkas, M. *Eur. J. Biochem.* **1973**, *35*, 527.
(b) Bechet, J. -J.; Dupaix, A.; Blagoeva, I. *Biochimie* **1977**, *59*, 231.

59. Vilain, A. -C.; Okochi, V.; Vergely, I.; Reboud-Ravaux, M.; Mazaleyrat, J. -P.; Wakselman, M. *Biochim. Biophys. Acta* **1991**, *401*, 1076.

60. Bechet, J. -J.; Dupaix, A.; Roucous, C.; Bonamy, A. -M. *Biochimie* **1977**, *59*, 241.

61. Decodts, G.; Wakselman, M. *Eur. J. Med. Chem.* **1983**, *18*, 107.

62. (a) Wakselman, M. *Nouv. J. Chim.* **1983**, *7*, 439.
(b) Wakselman, M.; Mazalyerat, J. P.; Xie, J.; Montagne, J. J.; Vilain, A. -C.; Reboud-Ravaux, M. *Eur. J. Med. Chem.* **1991**, *26*, 699.
(c) Wakselman, M.; Xie, J.; Mazalyerat, J. P.; Boggetto, N.; Vilain, A. -C.; Montagne, J. J.; Reboud-Ravaux, M. *J. Med. Chem.* **1993**, *36*, 1539.

63. Powers, J. C.; Kam, C. -M.; Narasimhan, L.; Oleksyszym, J.; Hernandez, M. A.; Ueda, T. *J. Cell. Biochem.* **1989**, *39*, 33.

64. Harper J. W.; Hemmi, K.; Powers, J. C. *J. Am. Chem. Soc.* **1983**, *105*, 6518.

65. Harper J. W.; Hemmi, K.; Powers, J. C. *Biochemistry* **1985**, *24*, 1831.

66. Alpin, R. T.; Robinson, C. V.; Schofield, C. J.; Westwood, N. J. *J. Chem. Soc. Chem. Commun.* **1992**, 1650.

67. Harper, J. W.; Powers, J. C. *J. Am. Chem. Soc.* **1984**, *106*, 7618.

68. Harper, J. W.; Powers, J. C. *Biochemistry* **1985**, *24*, 7200.

69. Meyer, Jr., E. F.; Presta, L. G.; Radhkrishnan, R. *J. Am. Chem. Soc.* **1985**, *107*, 4091.

70. Vijayalakshmi, J.; Meyer, Jr., E. F.; Kam, C. -M.; Powers, J. C. *Biochemistry* **1991**, *30*, 2175.

71. Chakravarty, .K.; Krafft, G. A.; Katzenellenbogen J. A. *J. Biol. Chem.* **1982**, *257*, 610.

72. Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen J. A. *J. Biol. Chem.* **1983**, *258* , 15046.

73. Rando, R. R. *Science* **1974**, *185*, 321.

74. Krafft, G. A.; Katzenellenbogen, J. A. *J. Am. Chem. Soc.* **1981**, *103*, 5459.

75. Daniels, S. B.; Katzenellenbogen, J. A. *Biochemistry* **1986**, *25*, 1436.

76. Naruto, S.; Motoc, I.; Marshall, G. R.; Daniels, S. B.; Sofia, M. J.; Katzenellenbogen, J. A. *J. Am. Chem. Soc.* **1985**, *107*, 5262.

77. Katzenellenbogen, J. A.; Rai, R.; Dai, W. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1399.

78. Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Org. Chem.* **1983**, *48*, 3318.

79. Reed, P. E.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1991**, *266*, 13.

80. Hemmi, K.; Harper, J. W.; Powers, J. C. *Biochemistry* **1985**, *24*, 1841.

81. Tam, T. F.; Spencer, R. W.; Thomas, E. M.; Copp, L. J.; Krantz, A. *J. Am. Chem. Soc.* **1984**, *106*, 6849.

82. Copp, L. J.; Krantz, A.; Spencer, R. W. *Biochemistry* **1987**, *26*, 169.

83. Spencer, R. W.; Tam, T. F.; Thomas, E.; Robinson, V. J.; Krantz, A. *J. Am. Chem. Soc.* **1986**, *108*, 5589.

84. Groutas, W. C.; P. K. Giri, P. K.; J. P. Crowley, J. P.; Castrisos, J. C; Brubaker, M. J. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 741.

85. Groutas, W. C.; Stanga, M. A.; Brubaker, M. J. *J. Am. Chem. Soc.* **1989**, *111*, 1931.

86. (a) Lossen, H. *Liebigs Ann. Chem.* **1869**, *150*, 314.
(b) Lossen, W. *Liebigs Ann. Chem.* **1872**, *161*, 347.
(c) Lossen, W. *Liebigs Ann. Chem.* **1877**, *186*, 1.
(d) Lossen, W. *Liebigs Ann. Chem.* **1889**, *252*, 170.
(e) Lossen, W. *Liebigs Ann. Chem.* **1894**, *281*, 169.

87. (a) Groutas, W. C.; Brubaker, M. J.; Stanga, M. A.; Castrisos, J. C.; Crowley, J. P.; Schats, E. J. *J. Med. Chem.* **1989**, *32*, 1607.
(b) Groutas, W. C.; Hoidal, M. J.; Brubaker, M. J.; Stanga, M. A.; Venkataraman, R.; Gray, B. H. Rao, N. V. *J. Med. Chem.* **1990**, *33*, 1085.
(c) Groutas, W. C.; Stanga, M. A.; Castrisos, J. C.; Schatz, E. J.; Brubaker, M. J. *J. Pharm. Sci.* **1990**, *79*, 886.
(d) Groutas, W. C.; Brubaker, M. J.; Venkataraman, R.; Epp, J. B.; Stanga, M. A.; McClenahan, J. J. *Arch. Biochem. Biophys.* **1992**, *294*, 144.
(e) Groutas, W. C.; Brubaker, M. J.; Venkataraman, R.; Stanga, M. A. *Arch. Biochem. Biophys.* **1992**, *297*, 144.
(f) Groutas, W. C.; Venkataraman, R.; Brubaker, M. J.; Epp, J. B.; Chong, L. S.; Stanga, M. S.; McClenahan J.J.; Tagusagawa, F. *Biochim. Biophys. Acta.* **1993**, *1164*, 283.
(g) Groutas, W. C.; Brubaker, M. J.; Chong, L. S.; Epp, J. B.; Huang, H.; Keller, C. E.; McClenahan J.J.; Givens, R. S.; Singh, R.; Zandler, M. E.; Karr, P. A.; Tagusagawa, F. *Drug Design and Discovery* **1994**, *11*, 149.

88. Bauer, L.; Exner, O. *Angew. Chem. Int. Ed.* **1974**, *13*, 376.

89. Groutas, W. C.; Venkataraman, R.; Brubaker, M. J.; Stanga, M. A. *Biochemistry* **1991**, *30*, 4132.

90. Neumann, U.; Gutschow, M. *J. Biol. Chem.* **1994**, *269*, 21561.

91. Kerrigan, J. E.; Shirley, J. J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 451.

92. Groutas, W. C.; Castrisos J. C.; Stanga M. A.; Kuang, R.; Venkataraman, R.; Epp, J. B.; Brubaker, M. J.; Chong, L. S. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1163.

93. Groutas, W. C.; Huang, H.; Epp, J. B.; Bruaker, M. J.; Keller, C. E.; McClenahan, J. J. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1565.

94. Groutas, W. C.; Chong, L. S.; Epp, J. B.; Venkataraman, R.; Brubaker, M. J.; Stanga, M. A.; Eun-Hong, K.; Keller, C. E. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1571.

95. Pinto, I. P.; West, A.; Debouck, C. M.; DiLella, A. G.; Gorniak, J. G.; O'Donnell, K. C.; O'Shannessy, Patel, A.; Jarvest, R. L. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2467.

96. Hlasta, D. J.; Bell, M. R.; Boaz, N. W.; Court, J. J.; Desai, R. C.; Franke, C. A.; Mura, A. J.; Subramanyam, C.; Dunlap, R. P. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1801.

97. (a) Zimmerman, M.; Morman, H.; Mulvey, D.; Jones, H.; Frankshun, R.; Ashe, B. M. *J. Biol. Chem.* **1980**, *255*, 9848.
(b) Ashe, B. M.; Clark, R. L.; Jones, H.; Zimmerman, M. *J. Biol. Chem.* **1980**, *256*, 11603.

98. Reczek, J. A.; Welter, T. R. *Eur. Pat. Appl.* **1982** *56444*.

99. (a) Groutas, W. C.; Brubaker, M. J.; Venkataraman, R.; Epp, J. B.; Houser-Archield, N.; Chong, L. S.; McClenahan, J. J. *Bioorg. Med. Chem. Letts.* **1992**, *2*, 175.
(b) Groutas, W. C.; Houser-Archield, N.; Chong, L. S.; Venkataraman, R.; Epp, J. B.; Huang, H.; McClenahan, J. *J. Med. Chem.* **1993**, *36*, 3178.

100. Hlasta, D. J.; Court, J. J.; Desai, R. C.; Talomie, T. G.; Shen, J. *Bioorg. Med. Chem. Letts.* **1996**, *6*, 2941.

101. Subramanyam, C.; Bell, M. R.; Ferguson, E.; Gordon, R. J.; Dunlap, R. P.; Franke, C. A.; Mura, A. J. *Bioorg. Med. Chem. Letts.* **1995**, *5*, 319.

102. Desai, R. C.; Court, J. J.; Ferguson, E.; Gordon, R. J.; Hlasta, D. J. *J. Med. Chem.* **1995**, *38*, 1571.

103. Smith, R. A.; Copp, L. J.; Coles, P. J.; Pauls, H. W.; Robinson, V. J.; Spencer, R. W.; Heard, S. B.; Krantz, A. *J. Am. Chem. Soc.* **1988**, *110*, 4429.

104. Subramanyam, C.; Bell, M. R.; Carabateas, P. M.; Court, J. J.; Dority, Jr., J. A.; Ferguson, E. W.; Gordon, R. J.; Hlasta, D. J.; Kumar, V.; Saindane, M. *J. Med. Chem.* **1994**, *37*, 2623.

105. Edwards, P. D.; Bernstein, P. R. *Med. Res. Rev.* **1994**, *14*, 127 and references cited on pp. 180-5.

106. Williams, F. M. *Pharmac. Ther.* **1987**, *34*, 99.

107. Hlasta, D. J.; Bell, M. R.; Court, J. J.; Cundy, K. C.; Desai, R. C.; Ferguson, E. W.; Gordon, R. J.; Kumar, V.; Maycock, A. L.; Subramanyam, C. *Bioorg. Med. Chem. Letts.* **1995**, *5*, 331.

108. Desai, R. C.; Dunlap, R. P.; Farrell, R. P.; Ferguson, E.; Franke, C. A.; Gordon, R.; Hlasta, D. J.; Talomie, T. G. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 105.

109. Hlasta, D. J.; Subramanyam, C.; Bell, M. R.; Carabateas, P. M.; Court, J. J.; Desai, R. C.; Drozd, M. L.; Eickhoff, W. M.; Ferguson, E. W.; Gordon, R. J.; Johnson, J. J.; Kumar, V.; Maycock, A. L.; Mueller, K. R.; Pagani, E. D.; Robinson, D. T.; Saindane, M. T.; Silver, P. J.; Subramanian, S.; Dunlap R. P.; Franke, C. A.; Mura, A. J.; Rowlands, A. *J. Med. Chem.* **1995**, *38*, 739.

110. Subramanyam, C.; Bell, M. R.; Ghose, A. K.; Kumar, V.; Dunlap, R. P.; Franke, C. A.; Mura, A. J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 325.

111. Groutas,W. C.; Chong, L. S.; Venkataraman, R.; Epp, J. B.; Kuang, R.; Brubaker, M. J.; Houser-Archfield, N.; Huang, H.; McClenahan, J. J. *Biochem. Biophys. Res. Commun.* **1993**, *194*, 1491.

112. (a) Gabriel, S.; Colman, J. *Ber.* **1900**, *33*, 980.
(b) Hill, J. H. M. *J. Org. Chem.* **1965**, *30*, 620.
(c) Abe, K.; Yamamoto, S.; Matsui, K.; *Yakugaku Zasshi* **1956**, *76*, 1058.

113. Groutas, W. C.; Chong, L. S.; Venkataraman, R. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 730.

114. Groutas, W. C.; Chong, L. S.; Venkataraman, R.; Huang, H.; Epp, J. B.; Kuang, R. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2745.

115. Groutas, W. C.; Brubaker. M. J.; Venkataraman, R.; Epp, J. B.; Houser-Archield, N.; Chong, L. S.; McClenahan, J. J. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 175.

116. Kuang, R.; Venkataraman, R.; Ruan, S.; Groutas, W. C. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 539.

117. Konstau, M. W.; Vargo, K. M.; Davis, P. B. *Am. Rev. Resp. Dis.* **1990**, *141*, 186.

118. Groutas, W. C.; Kuang, R.; Venkataraman, R. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 341.

119. Groutas, W. C.; Kuang, R.; Venkataraman, R.; Epp, J. B.; Ruan, S.; Prakash, O. *Biochemistry* **1997**, *36*, 4739.

120. Ghuysen, J. -M. in *The Bacterial D-D Carboxypeptides/transpeptidase Enzyme System*, University of Tokyo Press, **1976**, 115-143.

121. (a) Brenner, D. G.; Knowles, J. R. *Biochemistry* ***1981***, *20*, 3680.
(b) Brenner, D. G.; Knowles, J. R. *Biochemistry* ***1984***, *23*, 5833.

122. Doherty, J. B.; Ashe, B. M.; Argenbright, L. W.; Barker, P. L.; Bonney, R. J.; Chandler, G. O.; Dahlgren, M. E.; Dorn, Jr., C. P.; Finke, P. E.; Firestone, R. A.; Fletcher, D.; Hagmann, W. K.; Mumford, R.; O'grady, L.; Maycock, A. L.; Pisano, J. M.; Shah, S. K.; Thompson, K. R.; Zimmerman, M. *Nature* **1986**, *322*, 192.

123. Green, B. G.; Weston, H.; Ashe, B. M.; Doherty, J. B.; Finke, P. E.; Hagmann, W. K.; Lark, M.; Mao, J.; Maycock, A. L.; Moore, V.; Mumford, R.; Shah, S. K.; Walaovits, L.; Knight, W. B. *Arch. Biochem. Biophys.* **1991**, *286*, 284.

124. Shah, S. K.; Brause, K. A.; Chandler, G. O.; Finke, P. E.; Ashe, B. M.; Weston, H.; Knight, W. B.; Maycock, A. L.; Doherty, J. B. *J. Med. Chem.* **1990**, *33*, 2529.

125. Navia, M. A.; Springer J. P.; Lin, T. -Y.; Williams, H. R.; Firestone, R. A.; Pisano, J. M.; Doherty, J. B.; Finke, P. E.; Hoogsteen, K. *Nature* **1987** *327*, 79.

126. Knight, W. B.; Maycock, A. L.; Green, B. G.; Ashe, B. M.; Gale P.; Weston, H.; Finke, P. E.; Hagmann, W. K.; Shah, S. K.; Doherty, J. B. *Biochemistry* **1992**, *31*, 4980.

127. Hagmann, W. K.; O'Grady, L. A.; Ashe, B. M.; Dahlgren, M. E.; Weston, H.; Maycock, A. L.; Knight, W. B.; Doherty, J. B.; Pisano, J. M.; Shah, S. K. *Eur. J. Med. Chem.* **1989**, *24*, 599.

128. Doherty, J. B.; Ashe, B. M.; Barker, P. L.; Blacklock, T. J.; Butcher, J. W.; Chandler, G. O.; Dahlgren, M. E.; Davies, P. ; Dorn, Jr., C. P.; Finke, P. E.; Firestone, R. A.; Hagmann, W. K.; Halgren, T.; Knight, W. B.; Maycock, A. L.; Navia, M. A.; O'Grady, L. A.; Pisano, J. M.; Shah, S. K.; Thompson, K. R.; Weston, H.; Zimmerman, M. *J. Med. Chem.* **1990**, *33*, 2513.

129. Finke, P. E.; Ashe, B. M.; Knight, W. B.; Maycock, A. L.; Navia, M. A.; Shah, S. K.; Thompson, K. R.; Underwood, D. J.; Weston, H.; Zimmerman, M.; Doherty J. B. *J. Med. Chem.* **1990**, *33*, 2522.

130. Finke, P. E.; Shah, S. K.; Ashe, B. M.; Ball, R. G.; Blacklock, T. J.; Bonney, R. J.; Brause, K.A.; Chandler, G. O.; Cotton, M.; Davies, P.; Dellea, P. S.; Dorn, Jr., C. P.; Fletcher, D. S.; O'Grady, L A.; Hagmann, W. K.; Hand, K. M.; Knight, W. B.; Maycock, A. L.; Mumford, R. A.; Osinga, D. G.; Sohar, P.; Thompson, K. R.; Weston, H.;Doherty, J. B. Firestone, R. A.; Halgren, T.; Knight, W. B.; Maycock, A. L.; Navia, M. A.; O'Grady, L.; Pisano, J. M.; Shah, S. K.; Thompson, K. R.; Weston, H.; Zimmerman, M. *J. Med. Chem.* **1992**, *35*, 3731.

131. Firestone, R. A.; Barker, P. L.; Pisano, J. M.; Ashe, B. M.; Dahlgren, M. E. *Tetrahedron* **1990**, *46*, 2255.

132. Shah, K. S.; Dorn, Jr., C. P.; Finke, P. E.; Hale, J. J.; Hagmann, W. K.; Brause, K. A.; Chandler, G. O.; Kissenger, A. L.; Ashe, B. M.; Weston, H.; Knight, W. B.; Maycock, A. L.; Dellea, P. S.; Flechter, D. S.; Hand, K. M.; Mumford, R. A.; Underwood, D. J.; Doherty J. B. *J. Med. Chem.* **1992**, *35*, 3745.

133. Han, T. W.; Trehan, A. K.; Wright, J. J. K.; Federici, M. E.; Seiler, S. M.; Meanwell, N. A. *Bioorg. Med. Chem.* **1995**, *3*, 1123.

134. Borthwick, A. D.; Weingarten, G.; Haley, T. M.; Tomaszewski, M.; Wang, W.; Hu, Z.; Bedared, J.; Jin, H.; Yuen, L.; Mansour, T. S. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 365.

135. (a) Tong, L.; Qian, C.; Massariol, M. -J.; Ronneau, P. R.; Cordingley, M. G.; Lagace, L. *Nature* **1996**, *383*, 272.
(b) Qiu, X.; Culp, J. S.; DiLella, A. G.; Helmig, B.; Hoog, S. S.; Jason, . A.; Smith, W. W.; Abdel-Meguid, S. S. *Nature* **1996**, *383*, 275.
(c) Shieh, H. -J.; Kurumbail, R. G.; Stevens A. M.; Stegean, R. A.; Sturman, E. J.; Pak, J. Y.; Wittwer, A. J.; Palmier, M.O.; Wiegand, R. C.; Holwerda, B. C.; Stallings, W. C. *Nature* **1996**, *383*, 279.
(d) Chen. P.; Tsue, H.; Almassy, R. J.; Gribskov, C. L.; Katoh, S; Vanderpool, D. L.; Margosiak, S. A.; Pinko, C.; Matthews, D. A.; Kan, C. -C. *Cell* **1996**, *86*, 835.

136. Hagmann, W. K.; Shah, S. K.; Dorn, Jr. C. P.; O'Grady, L. A.; Hale, J. J.; Finke, P. E.; Thompson, K. R.; Brause, K. A.; Ashe, B. M.; Weston, H.; Dahlgren, M. E.; Maycock, A. L.; Dellea, P. S.; Hand, K. M.; Osinga, D. G.; Bonney, R. J.; Davies, P.; Fletcher, D. S.; Doherty, J. B. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 545.

137. Knight, W. B.; Chabin, R.; Green, B. *Arch. Biochem. Biophys.* **1992**, *296*, 704.

138. Knight, W. B.; Swiderek, K. M.; Sakuma, T.; Calaycay, J.; Shively, J. E.; Lee, T. D.; Covey, T. R.; Shushan, B.; Green, B. C.; Chabin, R.; Shah, S. K.; Mumford, R.; Dickinson, T. A.; Griffin, P. R. *Biochemistry* **1993**, *32*, 2031.

139. Knight, W. B.; Green, B. G.; Chabin, R. M.; Gale, P.; Maycock, A. L. Weston, H.; Kuo, D. W.; Westler, W. M.; Dorn, Jr., C. P.; Finke, P. E.; Hagmann, W. K.; Hale, J. J.; Liesch, J.; MacCross, M.; Navia, M. A.; Shah,S. K.; Underwood, D.; Doherty, J. B. *Biochemistry* **1992**, *31*, 8160.

140. Edwards, P. D.; Bernstein, P. R. *Med. Res. Rev.* **1994**, *14*, 168.

141. Hagmann, W. K.; Thompson, K. R.; Shah, S. K.; Finke, P. E.; Ashe, B. M.; Weston, H.; Maycock, A. L.; Doherty, J. B. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 681.

142. Shah, S. K.; Dorn, Jr., C. P.; Finke, P. E.; Hale, J. J.; Hagmann, W. K.; Brause, K. A.; Chandler, G. O.; Kissinger, A. L.; Ashe, B. M.; Weston, H.; Knight, W. B.; Maycock, A. L.; Dellea P. S.; Fletcher, D. S.; Hand, K. M.; Mumford, R. A.; Underwood, D. J.; Doherty, J. B. *J. Med. Chem.* **1992**, *35*, 3745.

143. Hagmann, W. K.; Kissinger, A. L.; Shah, S. K.; Finke, P. E.; Dorn, Jr., C. P.; Brause, K. A.; Ashe, B. M.; Weston, H.; Maycock, A. L.; Knight, W. B.; Dellea, P. S.; Fletcher, D. S.; Hand, K. M.; Osinga, D.; Davies, P.; Doherty, J. B. *J. Med. Chem.* **1993**, *36*, 771.

144. Byrant, D. R.; Hauser, C. R. *J. Am. Chem. Soc.* **1961**, *83*, 3468.

145. Hargreaves, M. K.; Pritchard, J. G.; Dave, H. R. *Chem. Rev.* **1970**, *70*, 439.

146. Fujisaki, S.; Hamura, S.; Eguchi, H.; Nishida, A. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2426.

147. Kornblum, N.; Jones W. J.; Anderson, G. J. *J. Am. Chem. Soc.* **1959**, *81*, 4113.

148. Fieser L. F.; Fieser, M. *Reagents for Organic Synthesis*, Wiley, **1969** 2, 371.

149. Sandler, S. R.; Karo, W. in *Organic Functional Group Preparations* Academic Press, New York, 2nd ed, **1983**, 482.

150. Jencks, W. P. *J. Am. Chem. Soc.* **1958**, *80*, 4581.

151. (a) Fittig. R.; Roders. P. *Ann.* **1890**, *256*, 95.
(b) Lucus, R.; Papadakis, M. Z. *Ann. Chim.* **1032**, *18*, 32.
(c) Baeyer, A.; Perkin, W. H. *Ber.* **1884**, *17*, 449.

152. Cohen, S. G.; Milovanovic, A. *J. Am. Chem. Soc.* **1968**, *90*, 3495.

153. Marvel C. S. *Org. Syn. Coll. Vol. III*, Ed., Horning, E. C. **1955**, 705.

154. (a) Brown, R. F. C. *Aust. J. Chem.* **1964**, *17*, 154.
(b) Wolfe, J. F.; Rogers T. G. *J. Org. Chem.* **1970**, *35*, 3600.
(c) Goehring, R. R.; Greenwood, T. D.; Nwokogu, G. C.; Pisipati, J. S.; Rogers, T. G.; Wolfe, J. F. *J. Med. Chem* **1990**, *33*, 926.

155. Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*, Wiley, New York, **1966**, *1*, 1034-5 and references therein.

156. Stobbe, H. *Ann.* **1890**, *281*, 282.

157. (a) Overberger, C. G.; Roberts, C. W. *J. Am. Chem. Soc.* **1949**, *71*, 3618.
(b) Billet, Mlle. D. *Bull. Soc. Chim. Fr.* **1949**, *16*, 297.
(c) Johnson, W. S.; Daub, G. H. *Organic Reactions* Eds., Adams, R.; Adkins, H. (*decu*); Blatt, A. H.; Cope, A. C.; McGrew, F. C.; Niemann, C.; Snyder, H. R., Wiley, New York, **1951**, *6*, 1.
(d) Doulut, S.; Dubuc, I.; Rodriguez, M.;Vecchini, F.; Fulcrand, H.; Barelli, H.; Checler, F.; Bourdel, E.; Aumelas, A.; Lallement, J. C.; Kitabgi, P.; Costentin J.; Martinez, J. *J. Med. Chem.* **1993**, *36*, 1369.

158. Bauer, L.; Miarka, S. V. *J. Am. Chem. Soc.* **1957**, *79*, 1983.

159. Zhu, Z.; Chuong, P.M.; Lemoine, P.; Tomas, T.; Galons, H. *Heterocycles* **1996**, *43*, 1923.

160. Cohn, L. *Ann.* **1880**, *205*, 295.

161. Morrell, G. J. *J. Am. Chem. Soc.* **1914**, *105*, 1733.

162. Oldham, M. D. *Ph. D. 12 Month Progress Report*, Department of Chemistry, University of Canterbury, **1993**.

163. Park, M.; Lee, J.; Choi, J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1297.

164. Ames, D. E.; Grey, T.F.; *J. Chem. Soc.* **1955**, 631.

165. Aldrich Catalogue Handbok of Fine Chemicals, **1996-7**.

166. Price, C. C.; Tomisek, A. J. *J. Org. Chem.* **1943**, *65*, 439.

167. Nikam, S. S.; Kornberg, B. E.; Johnson, D. R.; Doherty, A. M. *Tetrahedron Lett.* **1995**, *36*, 197.

168. Wang, K.-T.; Braltesani, D. N.; Weinstein, B. *J. Het. Chem.* **1966**, *3*, 98.

169. Orndoff, W. R.; Pratt, D. S.; *Am. Chem. J.* **1912**, *47*, 89.

170. (a) Buess, C. M.; Bauer, L. *J. Org. Chem.* **1955**, *20*, 33.
(b) Kuhle, Wegler. *Ann.* **1985**, *616*, 183.

171. Mitsunobu, O. *Synthesis* **1981**, 1.

172. Growchowski, E.; Jurczak, J. *Synthesis* **1977**, 277.

173. Sammes, M. P.; Wylie, C. M.; Hoggett, J. G. *J. Chem. Soc.* **1971**, 2151.

174. Abell A. D.; Oldham, M. D. *J. Org. Chem.* **1997**, *62*, 1509.

175. Oldham, M. D. *Ph. D. Thesis* Department of Chemistry, University of Canterbury, **1998**.

176. Le Berre, A.; Etienne, A.; Desmazieres, B. *Bull. Soc. Chim. Fr.* **1975**, *3-4*, 807.

177. Strecker, *Ann.* **1868**, *148*, 90.

178. Rougny, A.; Daudon, M. *Bull. Soc. Chim. Fr.* **1976**, *5*, 833.

179. Procopiou, P. A.; Baugh S. P. D.; Flack S. S.; Inglis G. G. A. *J. Chem. Soc. Chem. Commun.* **1996**, 2625.

180. Puterbaugh, W. H. *J. Am. Chem. Soc.* **1962**, *27*, 4010.

181. Michael, J. *J. Prakt. Chem.* **1887**, *35*, 349.

182. (a) Bruson, H. A.; Reiner, T. W. *J. Am. Chem. Soc.* **1943**, *65*, 23.
(b) Ansell, M. F.; Hey, H. *J. Chem. Soc.* **1950**, 1683 and references therein.

183. I. Guareschi, I.*Chem. Zbl.* **1901**, *1*, 579.

184. (a) Knoevenagel, *Ber.* **1894**, *27*, 2346.
(b) Jones, G. *Organic Reactions* **1967**, *15*, 204.

185. Handley, G. J.; Nelson, E. R.; Somers, T. C. *J. Aust. Chem.* **1960**, *13*, 129.

186. (a) Bergmann, E. D.; Ginsburg, D.; Pappo, R. *Organic Reactions* **1959**, *10*, 179-555.

(b) Kon, G. A. R.; Thorpe, J. F. *J. Chem. Soc.* **1919**, *115*, 686.

187. (a) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245.
(b) Jung, M. J.; Metcalf, B. W. *Biochem. Biophys. Res. Commun.* **1975**, *67*, 301

188. Oleksyszyn, J.; Powers, J. C. *Biochemistry* **1991**, *30*, 485.

189. Moorman, A. R.; Abeles, R. H. *J. Am. Chem. Soc.* **1982**, *104*, 6785.

190. Tian, W.-X.; Tsou, C. L. *Biochemistry* **1982**, *21*, 1028.

191. Morrison, J. F.; Walsh, C. T. *Adv. Enzymol* **1988**, *61*, 201.

192. Cha, S. *Biochem. Pharmacol.* **1975**, *24*, 217.

193. Waley, S. G. *Biochem. J.* **1980**, *185*, 771.

194. Berezin, I. V.; Kazanshaya, N. F.; Klyosev, A. A. *FEBS Lett.* **1971**, *15*, 121.

195. (a) Hummel, B. C. W. *Can. J. Biochem.* **1959**, *37*, 1393.
(b) Walsh, K. A.; Wilcox, P. E. *Meth. Enzymol.* **1970**, *19*, 33.

196. Schwert, G. W.; Kaufman, S. *J. Biol. Chem.* **1951**, *190*, 807.

197. (a) Baek, D. -J.; Reed, P. E.; Daniels, S. B.; Katzenellenbogen, J. A. *Biochemistry* **1990**, *29*, 4305.
(b) Krantz, A.; Spencer, R. W.; Tam. T. F.; Liak, T. J.; Copp, L. J.; Thomas, E. M.; Rafferty, S. P. *J. Med.Chem.* **1990**, *33*, 464.
(c) Reed, P. E.; Katzenellenbogen, J. A. *J. Org. Chem.* **1991**, *56*, 2624.
(d) Baek, D. -J.; Daniels, S. B.; Reed, P. E.; Katzenellenbogen, J. A. *J. Org. Chem.* **1989**, *54*, 3963.
(e) Stein, R. L.; Strimpler, A. M.; Viscarello, B. R.; Wildonger, R. A.; Mauger, R. C.; Trainor, D. A. *Biochemistry* **1987**, *26*, 4126.
(f) Hedstrom, E.; Moorman, A. R.; Dobbs, J.; Abeles, R. H. *Biochemistry* **1984**, *23*, 1753.

198. Bieth, J. G. *Meth. Enzymol.* **1995**, *248*, 59.

199. Navia, M. A.; McKeever, B. M.; Springer, J. P.; Lin, T. -Y.; Williams, H. R.; Fluder, E. M.; Doprn, Jr., C. P.; Hoogsteen, K. *Proc. Natl. Acad. Sci.* **1989**, *86*, 7.

200. Zydowsky, T.M.; Dellaria, Jr., J. F; Nellans, H. N. *J. Org. Chem.* **1988**, *53*, 5607.

201. Karle, I. L.; Flippen-Anderson, J. L. *Acta Crystallogr.* **1989**, *C45*, 791.

202. Krause, J. A.; Baures, P. W.; Eggleston, D. S. *Acta Crystallogr.* **1993**, *B49*, 123.

203. Delettre, P. J.; Berthou, J.; Lifchitz, A.; Jolles, P. *Acta Crystallogr.* **1988** , *C44*, 902.

204. (a) Bode, W.; Meyer, E.; Powers, J. C. *Biochemistry* **1989**, *28*, 1951.
(b) Brubaker, M. J.; Groutas, W. C.; Hoidal, J. R. Rao, N. V. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 1318.

205. Procter, G. *Asymmetric Synthesis*, Oxford University Press, Oxford, **1996**, 1.

206. Eliel, E. L.; Wilen, S. H. *Stereochemistry of Organic Compounds*, Wiley, New York, **1994**, 201-208.

207. Craig, D. P.; Mellor, D. P. *Top. Curr. Chem.* **1976**, *63*, 1.

208. Holmstedt, B. *The Use of Eantiomers in Biological Studies: A Historical Review* in *Chirality and Biological Activity*, Eds. Holmstedt, B.; Frank, H.; Testa, B. Liss, New York, **1990**, Chapter 1.

209. Piutti, A. *Heabd. Seances. Acad. Sci.* **1886**, *103*, 134.

210. Bentley, R. *Molecular Asymmetry in Biology*, Academic, New York, **1969**, *1*, 286.

211. Ohloff, G. *Experientia* **1986**, *42*, 271.

212. Powell, J. R.; Ambre, J. J.; Ruo, T. I. *The Efficacy and Toxicity of Drug Stereoisomers* in *Drug Stereochemistry. Analytical Methods and Pharmacology*, Eds. Wainer, I. W.; Drayer, D. E., Marcel Dekker, New York, **1988**, 245.

213. Lehmann, F. P. A.; Rodrigues de Miranda, J. F.; Ariens, E. J. *Prog. Drug. Res.* **1976**, *20*, 101.

214. Ariens, E. J. *Trends Pharmacol. Sci* . **1986**, *7*, 200.

215. Abell, A. D.; Erhard, K. F.; Hwa-Kwo, Y.; Yamashita, D. S.; Brandt, M.; Mohammed, H.; Levy, M. A.; Holt, D. A. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1365.

216. Stein, R. L.; Srimpler, A. M.; Edwards, P. D.; Lewis, J. J.; Mauger, R. C.; Schwartz, J. A.; Stein, M. M.; Trainor, D. A.; Wildonger, R. A.; Zottola, M. A. *Biochemistry* **1987**, *26*, 2682.

217. Pfeiffer, C. C. *Science* **1956**, *124*, 29.

218. Snoke, J. E.; Neurath, H. *Chem. Rev.* **1950**, *46*, 69.

219. Cohen, S. G.; Schultz, R. M. *Proc. Natl. Acad. Sci.* **1967**, *57*, 243.

220. Schallenberger, R. S.; Acree, T. E.; Lee, C. Y. *Nature* **1969**, *221*, 555.

221. Levin, G. V. US Patent 4 262 032, Apr. 14, 1981; *Chem. Abstr.* **1981**, *95*, 78771h.

222. Cushny, A. R. *The Biological Relations of Optically Isomeric Substances*, Williams and Wilkins, Baltimore, **1926**.

223. Eliel, E. L.; Wilen, S. H. *Stereochemistry of Organic Compounds*, Wiley, New York, **1994**, 205 and references therein.

224. (a) Mander, L. N. *Stereoselective Synthesis* in W Eliel, E. L.; Wilen, S. H. *Stereochemistry of Organic Compounds*, Wiley, New York, **1994**, Chapter 12, 835 and references therein.
(b) Koskinen, A. *Asymmetric Synthesis of Natural Products*, Wiley, Chichester, **1993**, 29.

225. Fredga, A. *Arkiv Kemi Mineral. Geol.* **1948**, *26B*, (11) in Newman, P. *Optical Resolution Procedures for Chemical Compounds*, **2**, Acids, **1981**, *2*, (1), 542.

226. Levy, D.E.; Lapierre, F.; Liang, W.; Ye, W.; Lange, C. W.; Li, X.; Grobelny, D.; Casabonne, M.; Tyrrell, D.; Holme, K.; Nadzan, A.; Galardy, R. E. *J. Med. Chem.* **1998**, *41*, 199.

227. (a) Fredga, A.; Palm, O. *Arkiv Kemi Mineral. Geol.* **1949**, *26A*, (26), 9; *Chem. Abstr.* **1949**, *43*, 6611.
(b) Fredga, A. *Arkiv Kemi* **1953**, *6*, 277.
(c) Matell, M. *Arkiv Kemi* **1953**, *5*, 17.
(d) Fredga, A. *Arkiv Kemi Mineral. Geol.* **1941**, *14B*, (27), 8.
(e) Fredga, A. *Arkiv Kemi Mineral. Geol.* **1942**, *15B*, (23), 1.
(f) Fredga, A. *Tetrahedron* **1960**, *8*, 126.

228. Eliel, E. L.; Wilen, S. H. *Stereochemistry of Organic Compounds*, Wiley, New York, **1994**, 126.

229. (a) Whitesides, G. M.; Lewis, D. W. *J. Am. Chem. Soc.*, **1970**, *92*, 6979.
(b) Parker, D. *Chem. Rev.* **1991**, *91*, 1441.

230. (a) Bajwa, J. S.; Miller, M. J. *J. Org. Chem.* **1983**, *48*, 1114.
(b) Mori, K.; Iwasawa, H. *Tetrahedron* **1980**, *36*, 87.

231. Hanessian, S. *Total Synthesis of Natural Products: The 'Chiron' Approach* Pergamon Press, Oxford, **1986**.

232. (a) Kawano, H.; Ishii, Y.; Ikariya, T.; Saburi, M.; Yoshikawa, S.; Uchida, Y.; Kumobayashi, H. *Tetrahedron Lett.* **1987**, *28*, 1905 and references cited therein.
(b) Hung, C. W.; Wong, H. N. C. *Tetrahedron Lett.* **1987**, *28*, 2393.

233. (a) Bashiardes, G.; Collingwood, S. P.; Davies, S. G.; Preston, S. C. *J. Organomet. Chem.* **1989**, *364*, C29.
(b) Bashiardes, G.; Collingwood, S. P.; Davies, S. G.; Preston, S. C. *J. Chem. Soc. Perkin Trans.* **1989**, 1162.

234. (a) Evans, D. A. *Aldrichimica Acta*. **1982**, *15*, 23.
(b) Evans, D. A. in *Asymmetric Synthesis*, Ed. Morrison, J. D., Academic Press, New York, **1984**, *3*, 87-90.
(c) Evans, D. A.; Takacs, J. M.; McGee, L. R.; Mathre, D. J.; Bartroli, J. *Pure and Appl. Chem.* **1981**, *53*, 1109.
(d) Evans, D. A.; Ennis, M. D.; Mathre, D. J. *J. Am. Chem. Soc.* **1982**, *104*, 1737.

235. Evans, D. A.; Bartroli, J.; Shih, T. L. *J. Am. Chem. Soc.* **1981**, *103*, 2127.

236. Fadel, A.; Salaun, J. *Tetrahedron Lett.* **1988**, *29*, 6257.

237. Beckett, R. P.; Crimin, M. J.; Davis, M. H.; Spavold, Z. *Synlett* **1993**, 137.

238. McClure, K. F.; Axt, M. Z. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 143.

239. (a) Sanko Company Limited, *Eur. Pat.* 0 383 635 A2, **1986**.
(b) Sanko Company Limited, *Eur. Pat.* 0 228 192 A2, **1986**.

240. Gage J. R.; Evans, D. A. *Org. Synth.* **1989**, *68*, 77 and references therein.

241. Gage J. R.; Evans, D. A. *Org. Synth.* **1989**, *68*, 83 and references therein.

242. Ireland, R. E.; Meissner, R. S. *J. Org. Chem.* **1991** *56*, 4566.

243. Sudharshan, M.; Hultin, P. G. *Synlett.* **1997**, 171.

244. Cason, J. *Org. Synth. Coll. Vol. III*, Wiley, New York, **1955**, 169.

245. (a) Nystrom R. F.; Brown, W. G. *J. Am. Chem. Soc.* **1947**, *69*, 2548.
(b) Grice, R.; Owen, L. H. *J. Chem. Soc.* **1963**, 1947.

246. Witiak, D. T.; Seth, S. K.; Baizman, E. R.; Weibel, S. L.; Wolf, H. H. *J. Med. Chem.* **1972**, *15*, 1117.

247. Kocienski, P. J.; Cernigliaro, G.; Feldsyein, G. *J. Org. Chem.* **1977**, *42*, 353.

248. *Vogel's Textbook of Practical Organic Chemistry*, 5th edn, Furniss, B. S.; Hannaford, A. J.; Smith, P. W. G.; Tatchell, A. R. **1989**.

249. Perrin, D. D.; Armarego, W. L. F. *Purfication of Laboratory Chemicals*, 3rd edn, Pergamon Press, London, **1988**.

250. Burfield, D. R.; Smithers, R. H. *J. Org. Chem.* **1978**, *43*, 3966.

251. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.*, **1978**, *43*, 2923.

252. Weizman, A. *J. Org. Chem.,* **1943**, *8*, 285.

253. Thole, F. B.; Thorpe, J. F. *J. Chem. Soc.* **1911**, *99*, 466.

254. Benica, W. S.; Wilson, C. O. *J. Amer. Pharm. Assn. [Sci. Edn.]*, **1950**, *39*, 451.

APPENDIX

DATA OBTAINED FROM CRYSTAL STRUCTURE DETERMINATION OF 3-BENZYL-*N*-[(ACETYL)OXY] SUCCINIMIDE (1.41o)

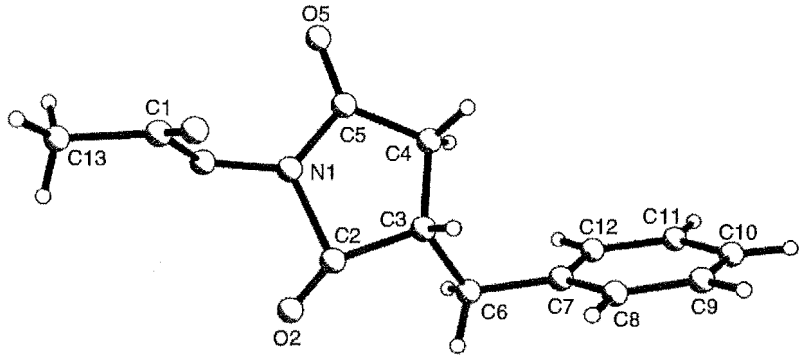


Figure A.1 Labelled drawing of **1.41o**

Atom	X	Y	Z	U(eq)
O1	7989(4)	692(4)	3430(2)	38
O2	6068(4)	-837(4)	4275(2)	42
O5	9951(4)	2018(4)	4888(2)	46
O11	6554(5)	2638(4)	3261(3)	68
N1	7958(8)	720(5)	4408(3)	30
C1	7256(5)	1869(6)	2895(3)	36
C2	6958(5)	-100(5)	4766(3)	30
C3	7260(5)	169(5)	5844(3)	29
C4	8676(5)	960(5)	6054(3)	30
C5	8985(5)	1356(5)	5089(3)	30
C6	7182(6)	-1234(5)	6415(3)	38
C7	7376(5)	-946(5)	7495(3)	31
C8	6366(6)	-227(5)	7880(3)	38
C9	6556(7)	40(5)	8864(4)	47
C10	7720(11)	-408(6)	9477(4)	45
C11	8793(7)	-1122(6)	9108(4)	49
C12	8605(6)	-1374(6)	8112(3)	40
C13	7280(7)	1618(7)	1868(3)	53

Table A.1 Atomic coordinates [x 10^4] (standard deviation) and equivalent displacement parameters [$Å^2$ x 10^3] for **1.41o**. U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

O1-N1	1.383(5)	O1-C1	1.419(5)
O2-C2	1.202(5)	O5-C5	1.201(5)
O11-C1	1.171(5)	N1-C2	1.403(7)
N1-C5	1.368(7)	C1-C13	1.470(6)
C2-C3	1.507(6)	C3-C4	1.534(6)
C3-C6	1.527(6)	C4-C5	1.495(6)
C6-C7	1.517(6)	C7-C8	1.387(6)
C7-C12	1.387(7)	C8-C9	1.382(6)
C9-C10	1.344(10)	C10-C11	1.418(9)
C11-C12	1.396(7)		

Table A.2 Bond lengths for **1.41o**

N1-O1-C1	113.8(4)	O1-N1-C2	120.2(5)
O1-N1-C5	123.0(6)	C2-N1-C5	116.1(4)
O1-C1-O11	120.0(4)	O1-C1-C13	107.2(4)
O11-C1-C13	130.8(5)	O2-C2-N1	124.6(5)
O2-C2-C3	130.0(5)	N1-C2-C3	105.4(4)
C2-C3-C4	104.9(3)	C2-C3-C6	111.8(4)
C4-C3-C6	115.8(4)	C3-C4-C5	106.1(3)
O5-C5-N1	123.2(5)	O5-C5-C4	130.4(4)
N1-C5-C4	106.3(4)	C3-C6-C7	111.8(4)
C6-C7-C8	121.4(5)	C6-C7-C12	119.7(5)
C8-C7-C12	118.9(4)	C7-C8-C9	121.0(5)
C8-C9-C10	121.0(6)	C9-C10-C11	119.6(5)
C10-C11-C12	119.2(6)	C7-C12-C11	120.3(5)

Table A.3 Bond Angles [°] (standard deviation) for **1.41o**

Atom	U11	U22	U33	U23	U13	U12
O1	45(2)	49(2)	23(2)	4(1)	11(2)	10(2)
O2	36(2)	51(2)	39(2)	-9(2)	5(2)	-9(2)
O5	45(2)	51(2)	44(2)	-2(2)	18(20	-10(2)
O11	92(4)	63(2)	49(2)	10(2)	13(2)	22(3)
N1	28(3)	40(3)	23(2)	1(2)	9(2)	-6(3)
C1	41(3)	50(3)	17(2)	4(2)	7(2)	23(3)
C2	26(3)	29(3)	40(3)	2(2)	14(2)	1(2)
C3	33(3)	31(2)	28(2)	-4(2)	14(2)	2(2)
C4	33(3)	30(2)	27(2)	-4(2)	5(2)	5(2)
C5	32(3)	28(2)	31(2)	-2(2)	8(2)	3(2)
C6	50(4)	28(3)	38(3)	-1(2)	17(3)	-6(2)
C7	41(3)	27(2)	27(2)	5(2)	9(2)	-3(2)
C8	46(3)	30(3)	40(3)	4(2)	12(2)	1(2)
C9	72(4)	32(3)	41(30	-3(2)	23(3)	-1(3)
C10	75(8)	34(3)	28(3)	-7(2)	14(4)	-8(4)
C11	52(4)	43(3)	44(3)	7(3)	-9(3)	-13(3)
C12	42(3)	38(3)	40(3)	-2(2)	7(2)	-1(3)
C13	57(4)	68(4)	36(3)	14(3)	15(3)	3(4)

Table A.4 Anisotropic displacement parameters [$Å^2$ x 10^3] for **1.41o**. The anisotropic displacement factor exponent takes the form: $-2\pi^2$ [$(ha^*)^2U_{11}$ + . . . + $2hka^*b^*U_{12}$]

Atom	X	Y	Z	U(eq)
H3	6548	842	5989	35
H4A	8625	1829	6438	36
H4B	9401	327	6406	36
H6A	7902	-1903	6299	45
H6B	6279	-1694	6187	45
H8	5546	80	7470	46
H9	5868	538	9105	56
H10	7821	-251	10140	54
H11	9611	-1419	9524	59
H12	9307	-1830	7861	48
H13A	8015	2187	1689	79
H13B	6396	1900	1476	79
H13C	7444	602	1767	79

Table A.5 Hydrogen coordinates [x 10^4] and isotropic displacement parameters [$Å^2$ x 10^3] for **1.41o**

CRYSTAL STRUCTURE DETERMINATION OF 3-PHENYL-*N*-[(ACETYL)OXY]SUCCINIMIDE (1.41p)

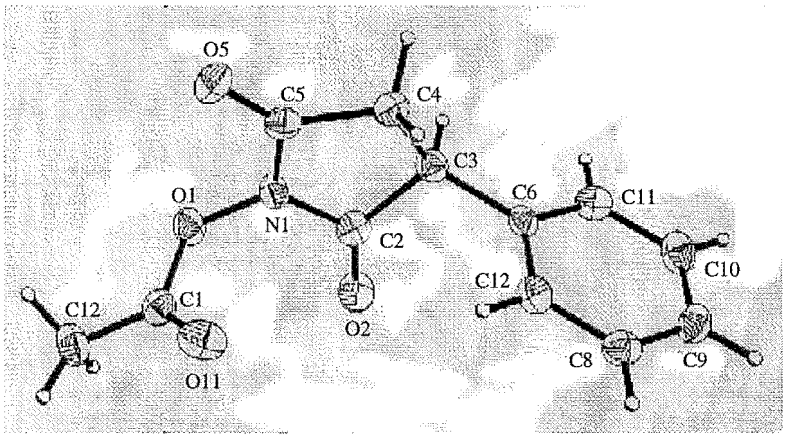


Figure A.2 Labelled drawing of **1.41p** showing 50% thermal ellipsoid probability for non-hydrogen atoms, with hydrogens as small spheres of arbitrary size

Single-crystal data collection was performed at 293K on a Siemens P4 diffractometer using graphite-monochromatised Mo-Kα radiation (λ = 0.71073Å). The crystal used was a rectangular prism with dimensions 0.23 x 0.30 x 0.63 mm. Compound **3** $C_{12}H_{11}NO_4$ (M_r = 233.22) crystallised from diethyl ether-petroleum ether in the monoclinic system, space group C2/c, a = 19.542(3), b = 5.7830(10), c = 20.626(4) Å, α = γ = 90°, β = 107.570(10)°, V = 2222.2(7) Å^3, Z = 8, D_{calc} = 1.394 Mg m^{-3}. Absorption coefficient 0.106mm^{-1}, θ range for data collection 2.07 - 24.99, index ranges -22 ≤ h ≤ 8, 0 ≤ k ≤ 6, -24 ≤ l ≤ 23, data/restraints/parameters 1946/0/155, goodness of fit on F^2 was 0.817, final R indices [I>2σ(I)] R_1 = 0.0404, wR_2 = 0.0752, R indices (all data) R_1 = 0.0853, wR_2 = 0.0845, largest difference peak and hole 0.187 and -0.169eÅ^{-3}.

The unit cell parameters were obtained by least-squares refinement of the setting angles of 42 reflections with 6.894° ≤ 2θ ≤ 24.910° from a Siemens P4 diffractometer. A unique data set was measured at 293K within $2\theta_{max}$ = 57° limit (Omega scans). Of the 2908 reflections obtained, 1946 were unique (R_{int} = 0.0473) and were used in the full-matrix least-squares refinement.[A.1] The intensities of 3 standard reflections, measured every 97 relections throughout the data collection, showed only 1.55% decay. The structure was solved by direct methods.[A.2] 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.[A.3]

Atom	X	Y	Z	$U_{(eq)}$
O1	613(1)	7595(3)	4080(1)	43(1)
O2	248(1)	7382(3)	5279(1)	40(1)
O5	1992(1)	5656(3)	4316(1)	37(1)
O11	1230(1)	10772(3)	4552(1)	43(1)
N1	1011(1)	6460(4)	4665(1)	30(1)
C1	774(1)	9944(4)	4099(1)	30(1)
C2	772(1)	6399(4)	5228(1)	31(1)
C3	1290(1)	4806(4)	5728(1)	30(1)
C4	1951(1)	4712(4)	5463(1)	29(1)
C5	1689(1)	5608(4)	4745(1)	29(1)
C6	1426(1)	5593(4)	6459(1)	25(1)
C7	1750(1)	7667(4)	6683(1)	32(1)
C8	1867(1)	8363(4)	7343(1)	34(1)
C9	1665(1)	6950(5)	7793(1)	36(1)
C10	1331(1)	4880(5)	7581(1)	39(1)
C11	1208(1)	4198(5)	6905(1)	34(1)
C12	289(1)	11080(5)	3487(1)	40(1)

Table A.6 Atomic coordinates [x 10^4] and equivalent isotropic displacement parameters [$Å^2$ x 10^3] for **1.41p**. $U_{(eq)}$ is defined as one third of the trace of the orthogonalized U_{ij} tensor.

O11-C1	1.180(3)	O1-N1	1.388(2)
O1-C1	1.393(3)	O5-C5	1.205(3)
O2-C2	1.203(3)	N1-C2	1.376(3)
N1-C2	1.377(3)	C12-C1	1.483(3)
C5-C4	1.505(3)	C4-C3	1.548(3)
C3-C6	1.520(3)	C3-C2	1.520(3)
C6-C7	1.370(3)	C6-C11	1.384(3)
C11-C10	1.398(3)	C10-C9	1.371(4)
C9-C8	1.379(3)	C8-C7	1.372(3)

Table A.7 Selected bond lengths [Å] for **1.41p**

N1-O1-C1	112.0(2)	C2-N1-C5	116.7(2)
C2-N1-O1	120.1(2)	C5-N1-O1	122.6(2)
O11-C1-O1	121.9(2)	O11-C1-C12	129.0(2)
O1C1-C12	109.1(2)	O5-C5-N1	124.9(2)
O5-C5-C4	129.4(2)	N1-C5-C4	105.6(2)
C5-C4-C3	105.3(2)	C6-C3-C2	111.9(2)
C6-C3-C4	116.5(2)	C2-C3-C4	103.8(2)
O2-C2-N1	125.2(2)	O2-C2-C3	129.6(2)
N1-C2-C3	105.2(2)	C7-C6-C11	119.3(2)
C7-C6-C3	121.4(2)	C11-C6-C3	119.2(2)
C6-C11-C10	120.3(3)	C9-C10-C11	119.1(3)
C10-C9-C8	120.6(2)	C7-C8-C9	119.8(2)
C6-C7-C8	120.9(2)		

Table A.8 Selected bond angles [°] for **1.41p**

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O1	38(1)	33(1)	25(1)	1(1)	0(1)	-3(1)
O2	29(1)	54(1)	38(1)	4(1)	11(1)	8(1)
O5	30(1)	23(2)	34(1)	-6(1)	19(1)	-5(1)
O11	35(1)	35(1)	54(1)	-5(1)	3(1)	-5(1)
N1	29(1)	33(1)	25(1)	5(1)	6(1)	3(1)
C1	29(1)	33(2)	31(1)	0(1)	13(1)	-2(1)
C2	23(1)	34(2)	33(1)	-3(1)	7(1)	-3(1)
C3	31(1)	27(2)	31(1)	2(1)	9(1)	-1(1)
C4	28(1)	32(2)	30(1)	-4(1)	8(1)	1(1)
C5	30(1)	23(2)	34(1)	-8(1)	7(1)	-5(1)
C6	22(1)	28(1)	24(1)	4(1)	5(1)	6(1)
C7	30(1)	33(2)	34(1)	3(1)	10(1)	-2(12)
C8	30(2)	32(2)	39(2)	-3(1)	7(1)	1(1)
C9	31(2)	49(2)	2791)	-2(1)	10(1)	7(1)
C10	36(2)	48(2)	3692)	10(1)	16(1)	1(2)
C11	30(2)	32(2)	39(2)	-3(1)	7(1)	1(1)
C12	30(1)	33(2)	34(1)	3(1)	10(1)	-2(1)

Table A.9 Anisotropic displacement parameters [$Å^2$ x 10^3] for **1.41p**. The anisotropic displacement factor exponent takes the form: $-2\pi^2$ [$(ha^*)^2U_{11}$ + . . . + $2hka^*b^*U_{12}$]

Atom	X	Y	Z	U(eq)
H3	1076(1)	3258(4)	5682(1)	36
H4A	2336(1)	5676(4)	5739(1)	36
H4B	2126(1)	3139(4)	5472(1)	36
H7	1893(1)	8615(4)	6384(1)	39
H8	2081(1)	9785(4)	7488(1)	41
H9	1757(1)	7406(5)	8243(1)	43
H10	1187(1)	3942(5)	7882(1)	47
H11	979(1)	2800(5)	6755(1)	41
H12A	-191(2)	11087(22)	3517(4)	48
H12B	301(6)	10247(14)	3088(1)	48
H12C	445(5)	12642(9)	3460(4)	48

Table A.10 Hydrogen coordinates [x10^4] and isotropic displacement parameters (Åx10^3) for **1.41p**

DATA OBTAINED FROM CRYSTAL STRUCTURE DETERMINATION OF 3-BENZYL-*N*-[(*TRANS*-CINNAMOYL) OXY]SUCCINIMIDE (1.41q)

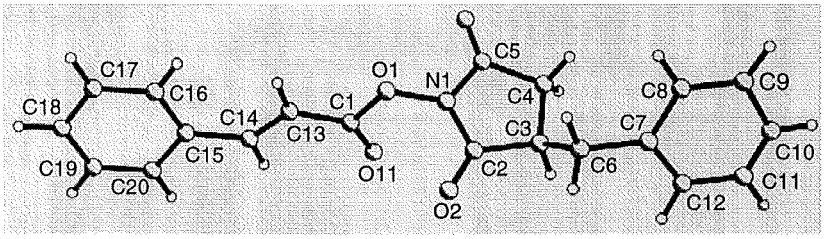


Figure A.3 Labelled drawing of **1.41q**

Atom	X	Y	Z	U(eq)
O1	1608(1)	5045(6)	1(1)	50
O2	1982(1)	5138(7)	1255(1)	59
O5	1876(1)	908(6)	-377(1)	57
O11	818(1)	3915(6)	129(1)	57
N1	1857(1)	3276(7)	370(1)	47
C1	1032(2)	5266(9)	-92(2)	41
C2	2051(2)	3518(10)	991(2)	51
C3	2362(2)	1383(8)	1241(2)	51
C4	2339(2)	-29(9)	687(2)	48
C5	2003(2)	1344(9)	149(2)	46
C6	2939(2)	1941(9)	1672(2)	61
C7	3293(2)	-24(9)	1941(2)	47
C8	3202(2	1411(10)	2359(2)	61
C9	3533(2)	-3173(10)	2604(2)	48
C10	3991(2)	-3693(8)	2437(2)	54
C11	4084(2)	2286(9)	2022(2)	60
C12	3746(2)	-5639(10)	1774(2)	57
C13	822(2)	7236(8)	445(2)	42
C14	302(2)	7865(9)	556(2)	49
C15	36(2)	9834(8)	-892(2)	41

Table A.11 Atomic coordinates [x 10^4] (standard deviation) and equivalent isotropic displacement parameters [$Å^2$ x 10^3] for **1.41q**. $U_{(eq)}$ is defined as one third of the trace of the orthogonalized U_{ij} tensor.

Atom	X	Y	Z	U(eq)
C16	271(2)	11210(8)	-1211(2)	48
C17	-9(2)	13023(9)	1523(2)	58
C18	539(2)	13536(9)	-1522(2)	62
C19	-766(2)	12219(10)	-1211(2)	60
C20	-504(2)	10388(9)	-904(2)	51

Table A.11 (cont.) Atomic coordinates [x 10^4] (standard deviation) and equivalent isotropic displacement parameters [$Å^2$ x 10^3] for **1.41q**. $U_{(eq)}$ is defined as one third of the trace of the orthogonalized U_{ij} tensor.

O1-N1	1.380(4)	O1-C1	1.419(5)
O2-C2	1.201(6)	O5-C5	1.204(5)
O11-C1	1.198(5)	N1-C2	1.388(6)
N1-C5	1.376(6)	C1-C13	1.436(6)
C2-C3	1.513(7)	C3-C4	1.549(6)
C3-C6	1.508(6)	C4-C5	1.508(6)
C6-C7	1.485(7)	C7-C8	1.376(6)
C7-C12	1.392(6)	C8-C9	1.349(7)
C9-C10	1.399(6)	C10-C11	1.381(6)
C11-C12	1.342(7)	C13-C14	1.320(5)
C14-C15	1.451(6)	C15-C16	1.391(6)
C15-C20	1.413(6)	C16-C17	1.365(6)
C17-C18	1.392(6)	C18-C19	1.347(7)
C19-C20	1.357(7)		

Table A.12 Bond lengths [Å] (standard deviation) for **1.41q**

N1-O1-C1	113.0(3)	O1-N1-C2	121.9(3)
O1-N1-C5	116.8(4)	C2-N1-C5	120.5(4)
O1-C1-O11	118.7(4)	O1-C1-C13	108.7(4)
O11-C1-C13	132.5(4)	O2-C2-N1	125.2(5)
O2-C2-C3	129.4(4)	N1-C2-C3	105.7(4)
C2-C3-C4	105.7(4)	C2-C3-C6	109.4(4)
C4-C3-C6	115.5(4)	C3-C4-C5	105.2(4)
O5-C5-N1	124.7(5)	O5-C5-C4	128.8(5)
N1-C5-C4	106.5(4)	C3-C6-C7	114.8(5)
C6-C7-C8	123.7	C6-C7-C12	120.3(4)
C8-C7-C12	115.9(4)	C7-C8-C9	122.8(4)
C8-C9-C10	120.9(4)	C9-C10-C11	116.2(4)
C10-C11-C12	122.3(4)	C7-C12-C11	121.8(5)
C1-C13-C14	119.6(4)	C13-C14-C16	125.8(5)
C14-C15-C16	125.0(4)	C14-C15-C20	117.7(4)
C16-C15-C20	117.3(4)	C15-C16-C17	121.5(4)
C16-C17-C19	119.9(5)	C17-C18-C19	118.9(5)
C18-C19-C20	122.7(5)	C15-C20-C19	119.7(5)

Table A.13 Bond Angles [°] (standard deviation) for **1.41q**

Atom	U11	U22	U33	U23	U13	U12
O1	3292)	71(3)	49(2)	22(2)	16(1)	6(2)
O2	59(2)	69(3)	52(2)	3(2)	24(2)	15(2)
O5	51(2)	82(3)	39(2)	-1(2)	16(1)	-13(2)
O11	43(2)	75(3)	56(2)	11(2)	21(2)	-3(2)
N1	37(3)	68(3)	36(2)	13(2)	1492)	10(2)
C1	37(3)	53(3)	34(2)	0(2)	13(2)	-8(2)
C2	35(3	78(4)	44(3)	2(3)	18(2)	2(3)
C3	44(3)	58(4)	49(3)	9(2)	15(2)	4(3)
C4	45(3)	55(3)	43(2)	1(2)	14(2)	-0(2)
C5	29(3)	65(4)	47(3)	-2(3)	19(2)	-11(2)
C6	28(3)	63(4)	37(2)	3(2)	10(2)	-4(2)
C7	39(3)	63(4)	32(2)	1(2)	5(2)	7(3)
C8	33(3)	105(5)	45(3)	12(3)	14(2)	13(3)
C9	49(3)	89(5)	51(3)	22(3)	15(2)	-6(3)

Table A.14 Anisotropic displacement parameters [$Å^2$ x 10^3] for **1.41q**. The anisotropic displacement factor exponent takes the form: $-2\pi^2$ [$(ha^*)^2U_{11}$ + . . . + 2hka*b*U_{12}]

Atom	U11	U22	U33	U23	U13	U12
C10	53(3)	50(3)	49(3)	5(3)	7(2)	9(3)
C11	50(3)	79(4)	56(3)	8(3)	25(3)	17(3)
C12	49(3)	81(4)	44(3)	98(3)	20(2)	10(3)
C13	28(3)	63(4)	37(2)	3(2)	10(2)	-4(2)
C14	41(3)	72(4)	35(2)	-5(2)	15(2)	-7(3)
C15	49(3)	36(3)	30(2)	-3(2)	4(2)	1(2)
C16	40(3)	56(4)	45(3)	2(3)	10(2)	-4(3)
C17	50(3)	55(4)	60(3)	12(3)	7(2)	-6(3)
C18	58(4)	48(4)	59(3)	0(3)	-4(3)	5(3)
C19	52(3)	67(4)	54(3)	-15(3)	11(3)	13(3)
C20	45(3)	69(4)	42(2)	-1(3)	20(2)	3(3)

Table A.14 (cont.) Anisotropic displacement parameters [$Å^2$ x 10^3] for **1.41q**. The anisotropic displacement factor exponent takes the form: $-2\pi^2$ [$(ha^*)^2U_{11}$ + . . . + $2hka^*b^*U_{12}$]

Atom	X	Y	Z	U(eq)
H3	2161	583	1462	61
H4A	2712	-296	686	58
H4B	2160	-1455	688	58
H6A	3127	2832	1461	73
H6B	2904	2851	1994	73
H8	2900	-1121	2478	73
H9	3456	-4054	2887	77
H10	4219	-4916	2596	65
H11	4393	-2543	1911	72
H12	3817	295	1483	68
H13	1052	8063	-593	50
H14	85	6967	-41	59
H16	626	10888	-1212	58
H17	155	13914	-1736	70
H18	-732	14767	-1733	74
H19	-1118	12581	-1207	72
H20	626	10888	-1212	58

Table A.15 Hydrogen coordinates [x 10^4] and isotropic displacement parameters [$Å^2$ x 10^3] for **1.41q**

REFERENCES

A.1 Sheldrick, G. M. SHELXL 93, *J. Appl. Crystallogr.*, in press.

A.2 Sheldrick, G. M. SHELXL 86, *Acta Crystallogr., Sect. A.* **1990**, *46*, 467.

A.3 *International Tables for Crystallography*, Vol C, Eds. Ibers, J. A.; Hamilton, W. C., Kynoch Press, Birmingham, **1992**.