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Hepatitis A Virus 3C Proteinase: Inhibitor Design, Synthesis and Testing

by

Manjinder Singh Lall 

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Chemistry

Edmonton, Alberta

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To my wife, Baljeet, and children, Navjoth and Amrita

ABSTRACT

Ten types of compounds were designed and synthesized as potential inhibitors of hepatitis A virus (HAV) 3C proteinase. Since this enzyme is essential for viral maturation, successful inhibitors may lead to antiviral therapeutic agents. Preparation of *N*-phthalimido-*N'*,*N'*-dimethylglutamine fluoromethyl ketone **9** in six steps (13% overall yield), provided a facile route to ^{13}C-labeled Ac-Leu-Ala-Ala-*N'*,*N'*-dimethyl-glutamine-fluoromethyl ketone **6** for NMR enzyme inactivation studies. Ac-Leu-Ala-Ala-*N'*,*N'*-Dimethylglutamine α,β-unsaturated methyl ester **10** was synthesized in seven steps (19% overall yield). Compound **10** is a time-dependent irreversible inhibitor of HAV 3C, k_{inact} / K_i = 137 $M^{-1}s^{-1}$. (4*RS*)-4-[(1*S*)-1-(*tert*-Butyloxycarbonylamino)-3-(*N*,*N*-dimethylcarbamoyl)propyl]-2-oxetanone **77** was synthesized in six steps (32% overall yield). (4*R* or *S*)-4-[(1*S*)-1-(*tert*-butyloxycarbonylamino)-3-(*N*,*N*-dimethylcarbamoyl)propyl]-3,3-dimethyl-2-oxetanone **93** was synthesized in seven steps (3% overall yield). Compound **93** is a weak inhibitor of HAV 3C (12% inhibition) at 100 μM. *N*-Cbz-Serine-β-lactone **13a** and its enantiomer **13b** were synthesized in two steps (30-40% overall yield). Compound **13a** was shown to irreversibly inactivate HAV 3C, k_{inact} / K_i = 63 M^{-1} s^{-1}. Mass spectrometric and HMQC NMR studies using [β-^{13}C]-**13a** show that the active site cysteine (Cys-172) thiol of the HAV 3C attacks the β-position (i.e. C-4) of the oxetanone ring, leading to ring opening and alkylation of the sulfur. In contrast, the enantiomer **13b**, is a reversible competitive inhibitor (K_i = 1.50 x 10^{-6} M). *N*-Phenethylsulfonyl-serine-β-lactone **14a** and its enantiomer **14b** are synthesized in four steps (9-11% overall yield). In a similar manner *N*-*trans*-*β*-styrenesulfonyl-L-serine-β-lactone **128a** and its enantiomer **128b** were synthesized in three steps 4-5% overall yield. Inhibition studies show the *N*-sulfonamide-

serine-β-lactones to be potent inhibitors of HAV 3C: **14a** (IC_{50} = 25 μM); **14b** (IC_{50} = 4 μM); **128a** (IC_{50} = 38 μM); and **128b** (IC_{50} = 3 μM). *N*-Phenethylsulfonyl-L-threonine-β-lactone **15a** and its stereoisomers D-threo-β-lactone **15b**, L-*allo*-threo-β-lactone **15c** and D-*allo*-threo-β-lactone **15d** were synthesized in five steps (35-54% overall yield). Inhibition studies show the *N*-sulfonamide-threonine-β-lactones to be time-dependent inhibitors of HAV 3C: **15a** (IC_{50} = 168 μM); **15b** (IC_{50} = 136 μM); **15c** (IC_{50} = 32 μM); and **15d** (IC_{50} = 12 μM). Ac-Leu-Ala-Ala-*N'*,*N'*-Dimethylglutamine-γ-lactone **16** was synthesized in eight steps (13% overall yield). Compound **16** is a time-dependent inhibitor of HAV 3C proteinase, k_{inact} / K_I = 48 $M^{-1}s^{-1}$. Ac-Leu-Ala-Ala-*N'*,*N'*-Dimethylglutamine-β-hydroxy acid **18** was synthesized in seven steps (47% overall yield). No significant inhibition of HAV 3C was observed at 100 μM. 4-(*N'*-Methyl)carbamoyl-*N*-methylphthalimide **25** was synthesized in two steps (42% overall yield). In addition, 4-(*N'*-methyl)carbamoyl-*N*-methylisoindolinone **27a** and 5-(*N'*-methyl)carbamoyl-*N*-methylisoindolinone **27b** were synthesized in three steps (13% and 4% overall yield, respectively). No significant inhibition of HAV 3C was observed with **25**, **27a** or **27b** at 100 μM. The results show for effective HAV 3C proteinase inhibition, serine or threonine β-lactones which possess a hydrophobic moiety such as phenyl attached to the α-amino group as in compounds **13a-b**, **14a-b** and **15a-d** are required. The β-lactone motif represents a new class of inhibitors of cysteine proteinases.

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To my supervisor, Professor John C. Vederas, I am most grateful for his excellent guidance, support, and encouragement throughout my studies. I thank all the members in our research group, past and present, for their help at various points during this work. I would like to especially thank Dr. Joanna M. Harris and Dr. Andrew Sutherland for proof-reading this manuscript. Dr. Michael N. G. James, Dr. Ernst M. Bergmann, Dr. Jonathan Parrish and Dr. Bruce A. Malcolm are gratefully acknowledged for their collaborative efforts and helpful discussions. For their technical expertise, I extend my gratitude to Constantine Karvellas, Colin Luo and Trevor Hart. The staff in spectral and analytical services in the Department of Chemistry is gratefully acknowledged for their assistance in characterizing compounds. Finally, I would like to thank my wife, Baljeet, and all family members, especially Jagir, Sukhjit, Jaswant, Hardeep, Raminder and Bhupender for their patience and invaluable support during this work.

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$[\alpha]$	specific rotation
Ac	acetyl
AIDS	acquired immune deficiency syndrome
Ala	alanine
APT	attached proton test
Arg	arginine
Ar	aryl
Bn	benzyl
Boc	butyloxycarbonyl
BOP	benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
BOPCl	bis(2-oxo-3-oxazolidinyl)phosphinic chloride
bp	boiling point
br	broad
BSA	bovine serum albumin
t-BuOK	potassium *tert*-butoxide
c	concentration
Cbz	benzyloxycarbonyl
CDI	1,1'-carbonyldiimidazole
CI	chemical ionization
CIC_{95}	culture inhibitor concentration at 95% (antiviral activity)
Cys	cysteine
δ	chemical shift in parts per million downfield from TMS
d	doublet

Dabcyl	4-(4-dimethylaminophenylazo)benzoyl
DMAD	dimethyl azodicarboxylate
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DTT	*DL*-dithiothreitol
Edans	5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid
EDTA	ethylenediaminetetraacetic acid
EI	electron impact ionization
Enz	enzyme
ES	electrospray ionization
Et	ethyl
EtOCOCl	ethyl chloroformate
EtOH	ethanol
FAB	fast atom bombardment
Gln	glutamine
Gly	glycine
HAV	hepatitis A virus
HBTU	*N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylanaminium hexafluorophosphate *N*-oxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
His	histidine
HMQC	heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
HRV	human rhinovirus
IC_{50}	inhibitor concentration at 50% (enzyme inhibition)
IR	infrared

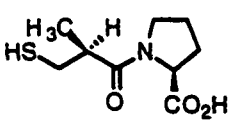
J	coupling constant
K_I	dissociation constant of mechanism-based inactivator
K_i	dissociation constant of enzyme-reversible inhibitor complex
k_{inact}	rate of enzyme inactivation
Leu	leucine
m	multiplet
Me	methyl
MeOH	methanol
MHz	megahertz
mp	melting point
MS	mass spectrometry
m/z	mass to charge ratio
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
Ph	phenyl
Phe	phenylalanine
Ph_3P	triphenylphosphine
Pht	phthalimido
ppm	parts per million
Py	pyridine
q	quartet
Rf	retention factor
RNA	ribonucleic acid
ROESY	rotating frame nuclear Overhauser and exchange spectroscopy
rt	room temperature
s	singlet
Ser	serine

t	triplet
$t_{1/2}$	half life
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thr	threonine
TLC	thin layer chromatography
TNBS	2,4,6-trinitrobenzenesulfonic acid
TsOH	*p*-toluenesulfonic acid
Tr	trityl
UV	ultraviolet
Val	valine

INTRODUCTION

Proteinases have long been recognized as valid targets for the development of inhibitors as therapeutics in a number of serious human diseases.[1] For example, the antihypertensive agent Captopril **1** (Figure 1) targets the metalloproteinase angiotension-converting enzyme (ACE),[2] whereas ICI 200 880 **2** is an inhibitor of human leukocyte elastase (HLE), a serine proteinase involved in inflammation and tissue degradation.[2] Proteinases which have received much attention in recent years are those encoded by viruses, with the focus having been on the aspartyl proteinase of human immunodeficiency virus (HIV).[3] The recent approval of HIV proteinase inhibitors such as Indinivar (Merck) **3** and Saquinavir (Roche) **4** for the therapy of acquired immune deficiency syndrome (AIDS), confirms the validity of viral proteinases as promising antiviral targets.[1] The hepatitis A virus (HAV) 3C cysteine proteinase of the viral family Picornaviridae has been of interest in our group.

Figure 1 Several selective proteinase inhibitors



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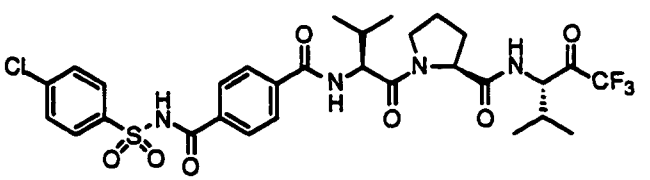

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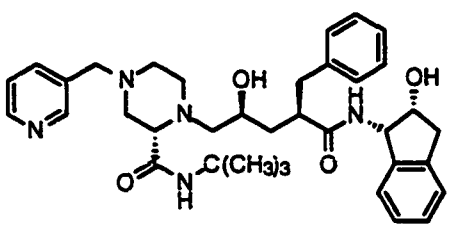

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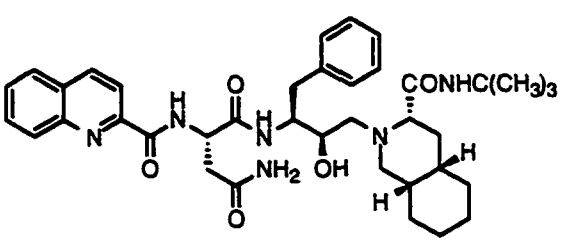


4

1. The Picornaviridae Family

The Picornaviridae, among the smallest icosahedral positive-sense single stranded RNA containing viruses known, comprise one of the largest and most important families of human and animal pathogens.[4] There are more then 200 known viruses which belong to this family, which are classified into six genera containing members such as human poliovirus (HPV), human rhinovirus (HRV), foot-and-mouth disease virus (FMDV), encephalomyocarditis virus (EMCV), hepatitis A virus (HAV) and human echovirus (Table 1).[5]

Table 1 The Picornaviridae Family

Genus	Serotypes	Examples	Disease	Proteinase
Entero	93	HPV	myelitis	2A, 3C
Rhino	105	HRV	common cold	2A, 3C
Aphtho	7	FMDV	foot-and-mouth	L, 3C
Cardio	2	EMCV	myocarditis	3C
Hepato	1	HAV	hepatitis A	3C
Orphano	2	echo 22 & 23	myocarditis	3C

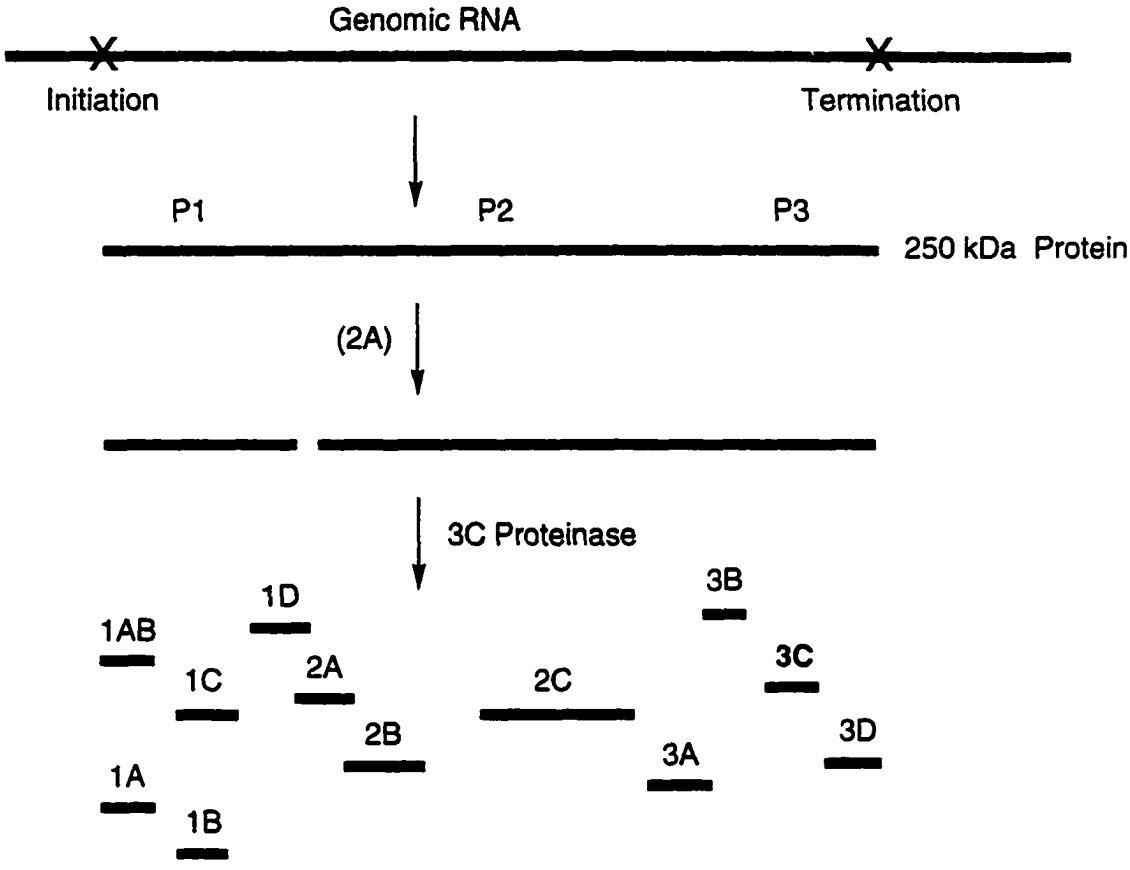
Picornaviruses cause a wide variety of diseases in humans, ranging from transient and benign (common cold) to severe and life threatening (polio). In a similar sporadic manner, picornaviral associated disease in North America extends from occasional outbreaks (polio) to endemic pools (common cold), and in many underdeveloped parts of the world HAV associated disease is still epidemic. The largest HAV epidemic in recent years occurred in

Shanghai (mollusk-linked) in 1988, involving ~300,000 people.[6] Although HPV and HAV vaccination have been very effective, subsequent outbreaks in industrialized nations, as recently occurred in Finland, are still a major concern to epidemiologists and public health officials.[7,8]

Immunization is the major alternative to the development of therapeutic or prophylactic agents for picornaviruses. Vaccine strategies have been successful for poliovirus and hepatitis A virus.[9,10] However, for many picornaviruses the development of effective vaccines is impractical due to the high mutation rate of the virus capsid proteins, which can lead to the generation of escape mutants capable of infecting previously vaccinated individuals.[11] In contrast to the high mutation rate observed in the capsid proteins, the processing proteinases appear relatively invariant and thus provide unique and highly susceptible targets for therapeutic intervention.[12] To date, there are no effective antiviral agents for the treatment or prophylaxis of any picornavirus. Hence, considerable importance lies in the development of antiviral agents to aid in the combat of picornaviral disease. In addition to the medicinal use of antiviral agents, these compounds play a pivotal role as tools in molecular virology to elucidate viral processes such as viral genome replication.[13]

Picornaviruses share the major features of the viral replication cycle, including the central role of the specific proteolytic processing of a viral polyprotein.[5] Individual details of viral replication and polyprotein processing distinguish the genera of the Picornaviridae family (Table 1).[5] The life cycle of the Picornaviridae is initiated by the virus attaching to and entering the host cell *via* some form of endocytosis.[14] The virus then uncoats, releasing its positive sense single-stranded RNA into the cytosol, where the latter functions as messenger RNA to direct the synthesis of a single polyprotein of approximately 250 kilodaltons (Figure 2).[15]

Figure 2 Generalized schematic representation of polyprotein translation and cleavage in the Picornaviridae



This polyprotein undergoes a co-translational cleavage into a capsid (P1) and nonstructural protein (P2-P3) precursor. In the case of the entro- and rhinoviruses, this is initially mediated by the 2A proteinase.[16,17] In other Picornaviridae, how this cleavage is accomplished remains unclear. The 3C proteinase is released from the polyprotein and the remainder of the polyprotein is cleaved into its component nonstructural products and capsid proteins, which then assemble into new virions. Studies of chimeric and mutant picornaviruses have demonstrated that interruption of 3C proteolytic processing prevents the formation of new virions.[15]

2. Properties of Hepatitis A (HAV) 3C Proteinase

The HAV 3C processing enzyme is a cysteine proteinase, which catalyzes peptide-bond cleavage through nucleophilic attack by the sulfur atom of the active site cysteine residue upon the substrate carbonyl carbon atom of the scissile bond to form a covalent tetrahedral intermediate.[18] The wild type HAV 3C enzyme has 219 amino acids with a molecular weight of 24 kilodaltons and exists as an active monomer. For ideal peptide substrates mimicking the 2B / 2C junction, the k_{cat} is typically about 1.8 sec^{-1} with an approximate K_m of 2.1 mM at pH 7.5. Attempts to crystallize the wild type HAV 3C for structural studies were unsuccessful due to the presence of two cysteines in the molecule (at position 24 and at the active site position 172). However, site-specific mutagenesis generated an inactive C24S-C172A mutant which crystallized and the structure was determined to 2.3 Å resolution.[18] Recently, a refined crystal structure of an active HAV 3C mutant, wherein only the external cysteine-24 has been replaced by alanine, was determined to 2.0 Å resolution (Figure 3).[19] In addition, the crystal structure of the 3C proteinase from human rhinovirus-14 (HRV) has been elucidated, by Matthews *et al.*,[20] at Agouron Pharmaceuticals. In HAV 3C the active site cysteine nucleophile (Cys-172) is in close proximity to a histidine residue (His-44), which acts as a general base to form the thiolate; an ordered water molecule is thought to complete the catalytic triad (Figure 4).[21] Although the HAV 3C and HRV-14 3C represent different subtypes of the 3C enzyme, the critical active site geometry of the nucleophilic cysteine side chain as well as the histidine general base are virtually superimposable with the equivalent residues, serine-195 and histidine-57 of chymotrypsin.[18] These structural studies show that topologies of the HAV and HRV 3C proteinases resemble β-barrel fold serine proteinases such as chymotrypsin and trypsin, respectively, rather than the papain family of cysteine proteinases.[18]

From the standpoint of inhibitor design, it is essential to understand how these enzymes obtain their high degree of specificity. In the case of HAV 3C, synthetic peptides

Figure 3 Ribbon secondary structure of HAV 3C (C24S) proteinase, figure prepared by Dr. Ernst M. Bergmann

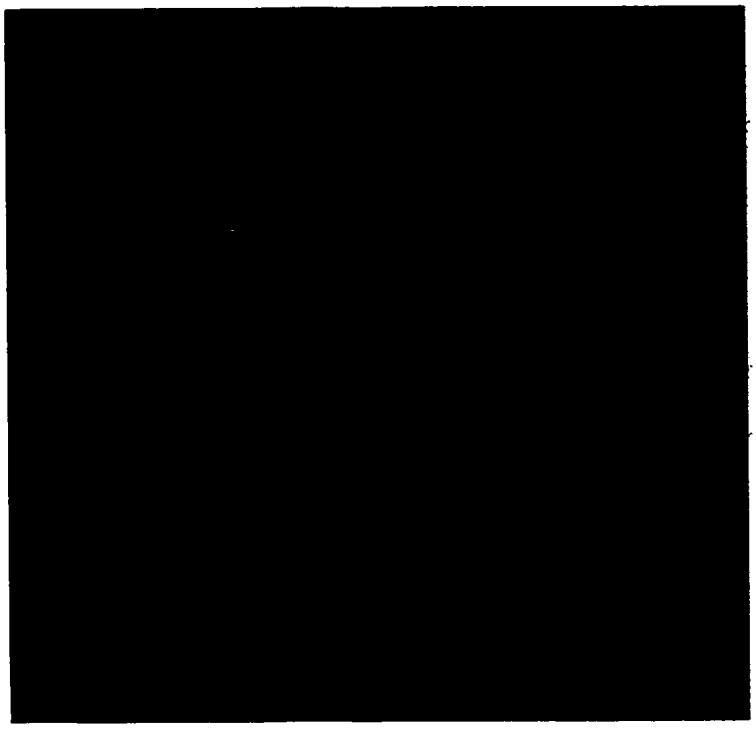
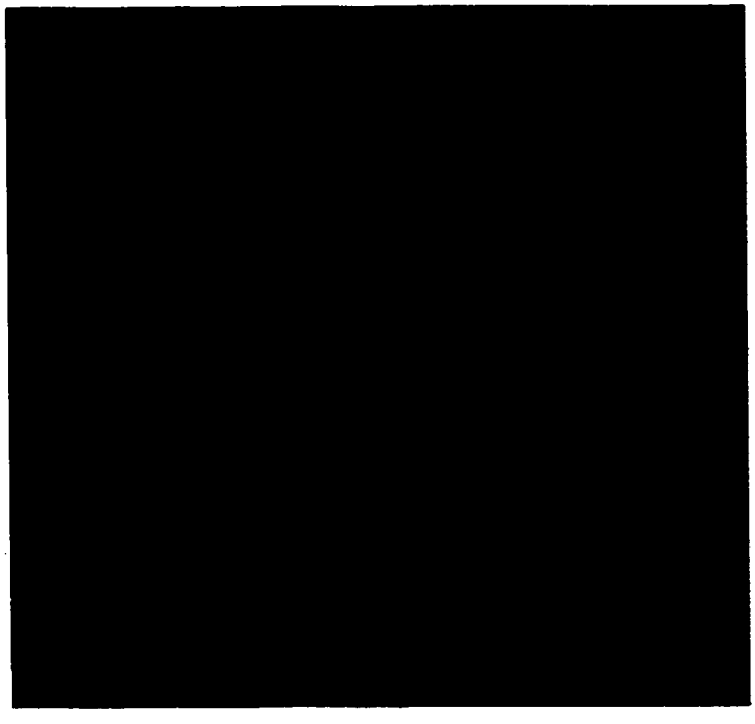
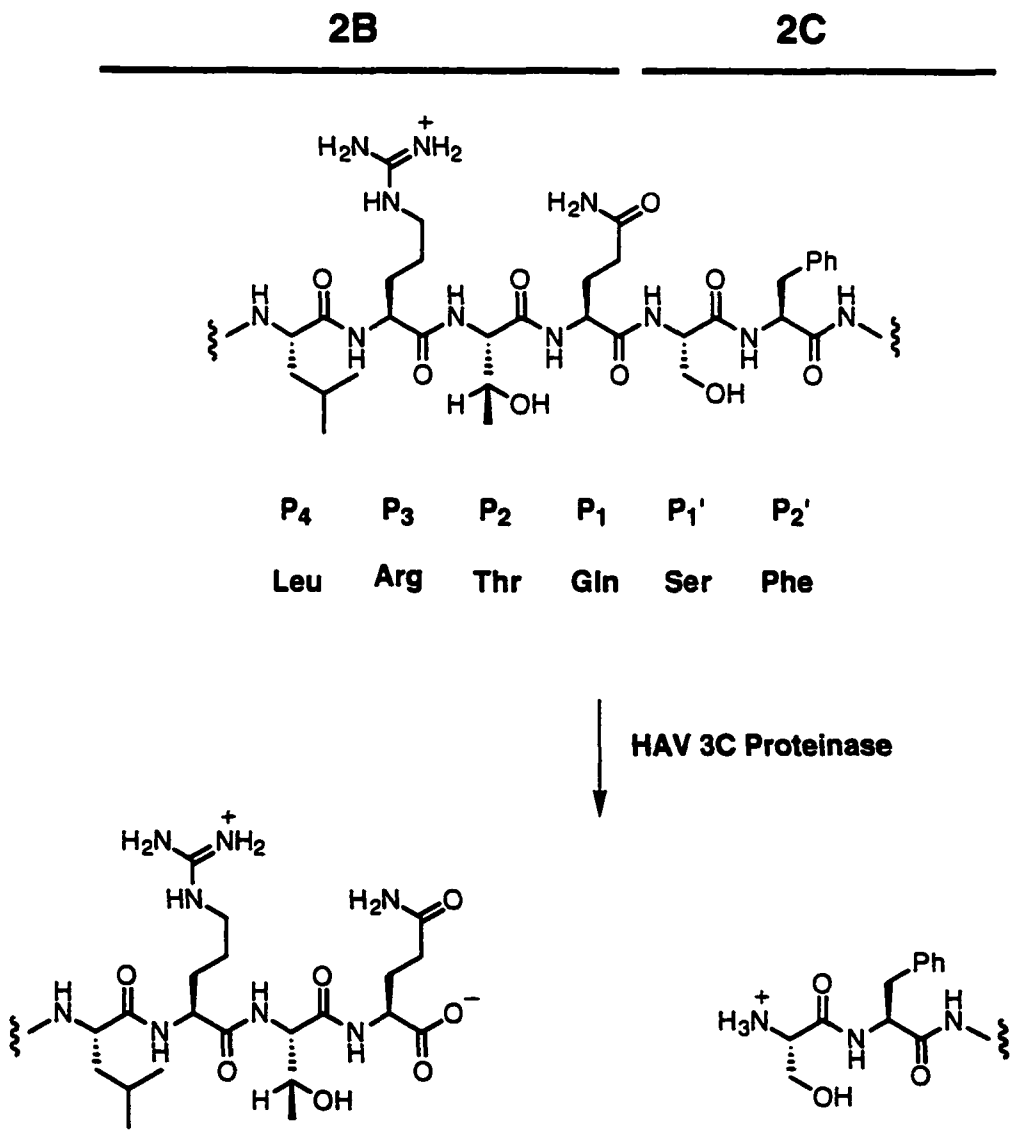


Figure 4 Active site of HAV 3C (C24S) proteinase, figure prepared by Dr. Ernst M. Bergmann



that correspond to the 2B / 2C and 2C / 3A junctions in the large precursor protein have been employed to elucidate substrate specificity (Figure 5).[22]

Figure 5 A preferred cleavage site of HAV 3C proteinase



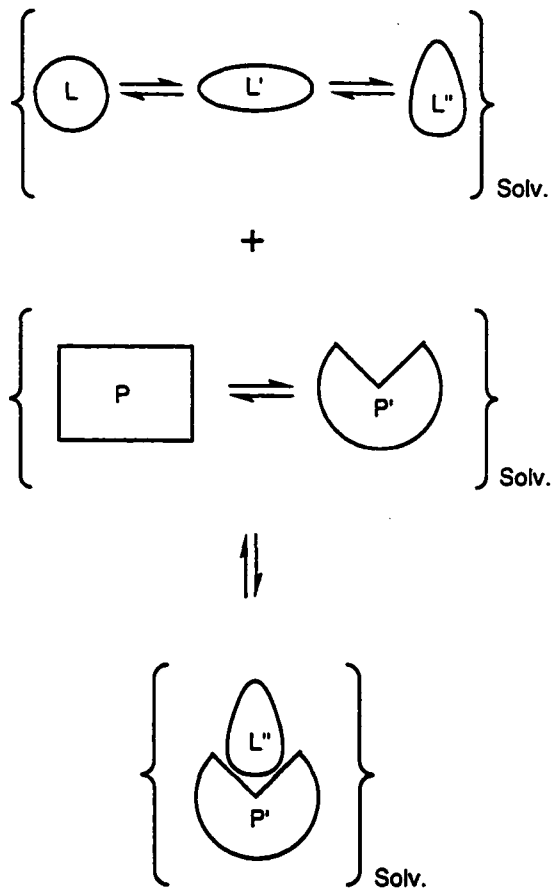
Any small amino acid (Gly, Ala or Ser) is accepted in the P_1' (in the nomenclature of Schechter and Berger)[23] position and virtually any amino acid is suitable in the P_2' position

with the exception of proline and arginine. Residue discrimination is apparent for the S_4 subsite: large side chains such as the branched aliphatic or aromatic side chains of leucine, isoleucine or tryptophan are preferred. There is high specificity for a glutamine residue at the P_1 cleavage site. The S_1 subsite is a shallow hydrophobic pocket, and at the base of this pocket resides a histidine residue (His-191) in an appropriate position to form a hydrogen bond with the side chain carbonyl oxygen of the glutamine in the substrate (Figure 4).

3. Inhibitor Design: Insight to Drug Discovery

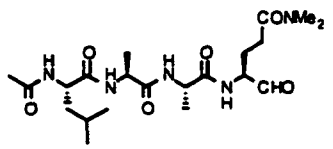
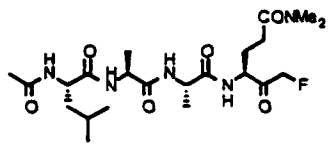
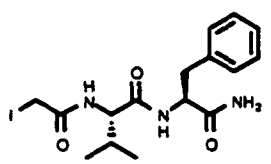
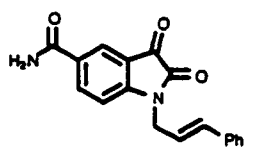
Considerable effort is devoted to study and inhibition of cysteine proteinases because they are targets for the development of therapeutic agents for many diseases including: viral infections, parasitic ailments, arthritis, cancer and osteoporosis.[24] Advances in molecular biology have provided availability of protein and protein structures, thus aiding the drug discovery process.[25] In an ideal situation, quantitative computational methods would greatly influence the protein structure-based drug design process. However, to date no methods are reliable enough, relative to qualitative human design, to be used in a reliable manner. The process of forming a complex between a small ligand and a protein is a complicated equilibrium process (Figure 6). A solvated ligand may exist as an equilibrium mixture of several conformers, and the protein may also have conformational freedom in solution. Thus, the design process currently relies on an iterative process of chemical synthesis of ligands and tests for binding.

Figure 6 Schematic of protein-ligand complexation



A number of agents have been described as inhibitors of HAV 3C and HRV 3C proteinases as potential therapeutic leads, including peptide aldehydes,[26,27] peptide fluoromethyl ketones,[28] β-lactams,[29] isatins,[30] homophthalimides,[31] vinylogous esters and sulfones,[32-34] halomethyl carbonyls,[35,36] and azapeptide compounds.[35,37,38] These agents were employed to block the formation of mature viral proteins arresting the viral life-cycle, and a few representatives are illustrated in Table 2. These inhibitors can be broadly classified into two categories: peptide-based inhibitors **5-7**; and non-peptide inhibitors **8**.

Table 2 Effect of inhibitor on the activity of picornavirus 3C proteinase

	Inhibitor	Inhibition	
		HAV 3C	HRV-14 3C
5		$K_i^* = 42$ nM	~2100 nM
6		$k_{inact} / K_I = 330\ M^{-1}s^{-1}$	n.d.
7		$k_{inact} / K_I = 200\ M^{-1}s^{-1}$	n.d.
8		n.d.	$K_i = 11$ nM

K_i^* = overall dissociation constant of the tight enzyme-inhibitor (slow binding) complex EI^*
k_{inact} / K_I = rate of enzyme inactivation
K_i = overall dissociation constant of the tight enzyme-inhibitor complex EI
n.d. = not determined

3.1 Peptide-Based Inhibitors

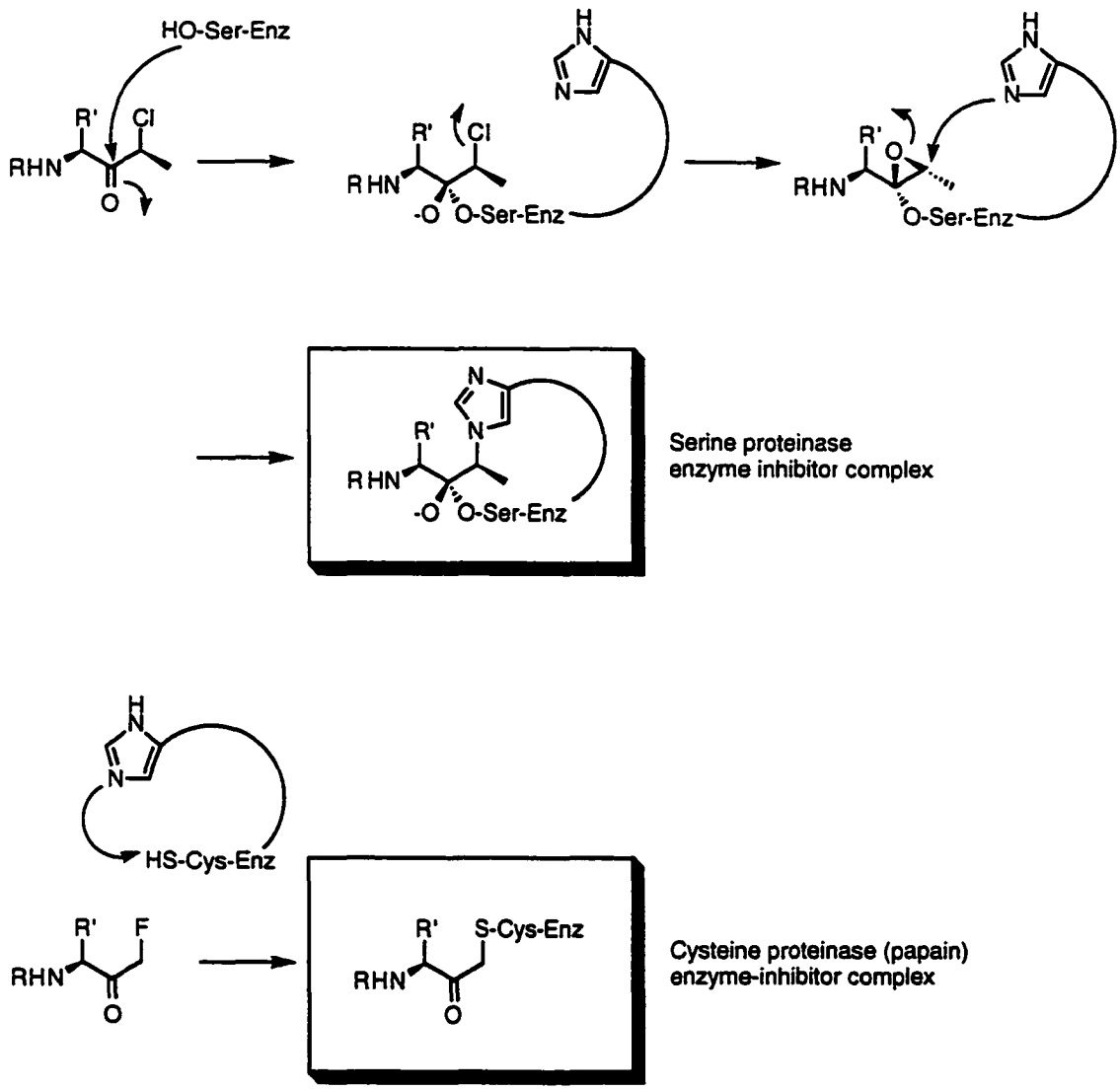
Since peptide-based inhibitors mimic natural substrates, an attractive feature of these compounds is that the selectivity and specificity can be designed to avoid cross reactivity with other essential human proteinases, thereby minimizing the occurrence of side-effects. Unfortunately, peptides are rapidly cleaved *in vivo*, are rarely bioavailable or orally active drugs. However, in combination with crystallographic studies of enzyme-inhibitor complexes, peptide-based inhibitors can provide a very useful picture of protein-ligand interactions for design of non-peptide drugs.

3.1.1 Peptidyl Halomethyl Ketones

Peptidyl halomethyl ketones behave as affinity labels of cysteine proteinases by blocking the essential thiol group *via* alkylation.[39] Although selectivity can be achieved by varying the peptidyl residue, the high chemical reactivity of the halomethyl moiety towards general nucleophilic attack can cause severe toxic effects in cellular systems.[40] Attempts to overcome nonspecific side-reactions employ fluorine, since the rate of thiol alkylation by fluoromethyl ketone is 0.2% of that observed with a chloromethyl ketone.[41] In addition, peptidyl fluoromethyl ketones have high selectivity toward cysteine proteinases, and display lower reactivity with serine proteinases.[42]

Recently, the groups of Ringe and Abeles[43] reported the mechanism of interaction between chymotrypsin (a serine proteinase) and an α-chloroethyl ketone (Figure 7).

Figure 7 Modes of inhibition for serine and cysteine proteinases by halomethyl ketones

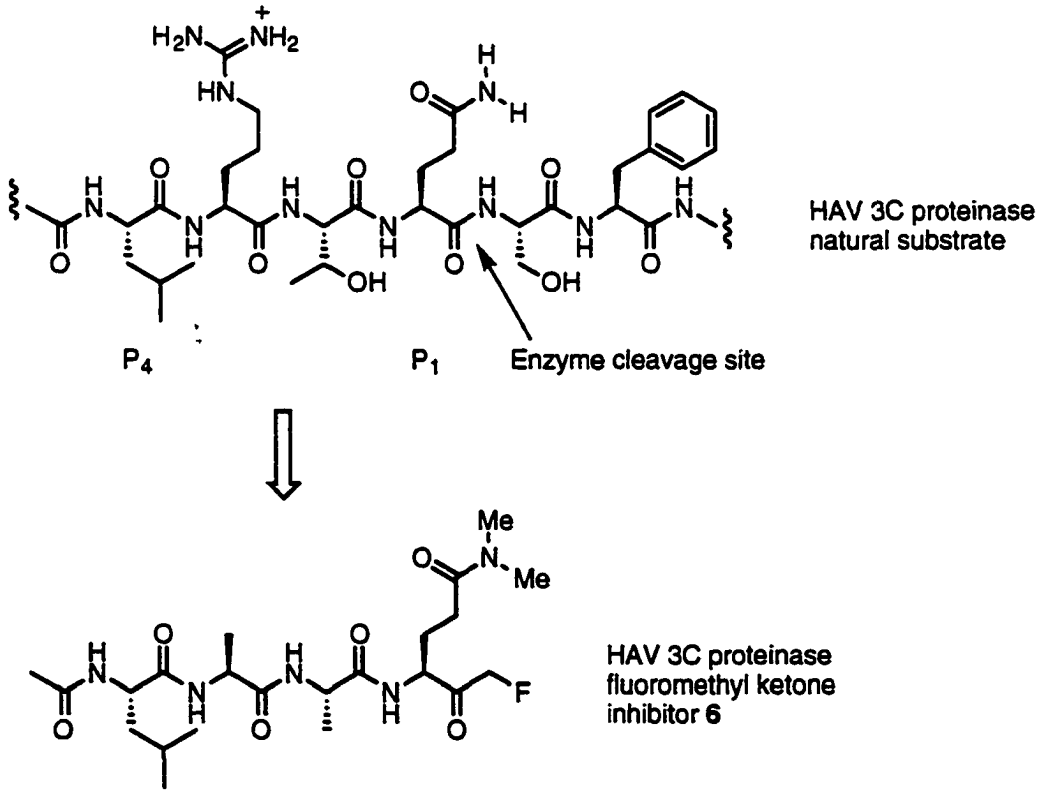


The serine hydroxyl attacks the haloketone carbonyl, internal displacement of halide then proceeds, followed by oxirane ring opening at the less hindered position by the histidine nitrogen. This results in an *N*-alkylated acetal as shown in Figure 7. However, crystallographic studies of the papain-like proteinase cruzain[44] inhibited by a fluoromethyl ketone, and NMR studies of papain inactivated by a chloromethyl ketone[45] clearly demonstrate that these cysteine proteinases have the active site sulfhydryl replacing the

halogen to form an α-keto sulfide (Figure 7). This could, in principle, occur *via* a mechanism similar to that proposed for chymotrypsin and chloroethyl α-ketone,[43] namely attack of sulfhydryl on the carbonyl and generation of an epoxide, which would then be followed by 1,2-sulfur migration. However, direct halogen displacement by the thiolate, which is much more nucleophilic than the serine hydroxyl appears more likely.

Since HAV 3C employs a nucleophilic thiol (Cys-172) within the enzyme active site, the peptidyl fluoromethyl ketone Ac-Leu-Ala-Ala-Gln(NMe_2)CH_2F **6** (Figure 8) was recently prepared in our group by Drs. Sven Frormann and Christopher Lowe.[28]

Figure 8 Preferred HAV 3C proteinase cleavage site and fluoromethyl ketone inhibitor **6**

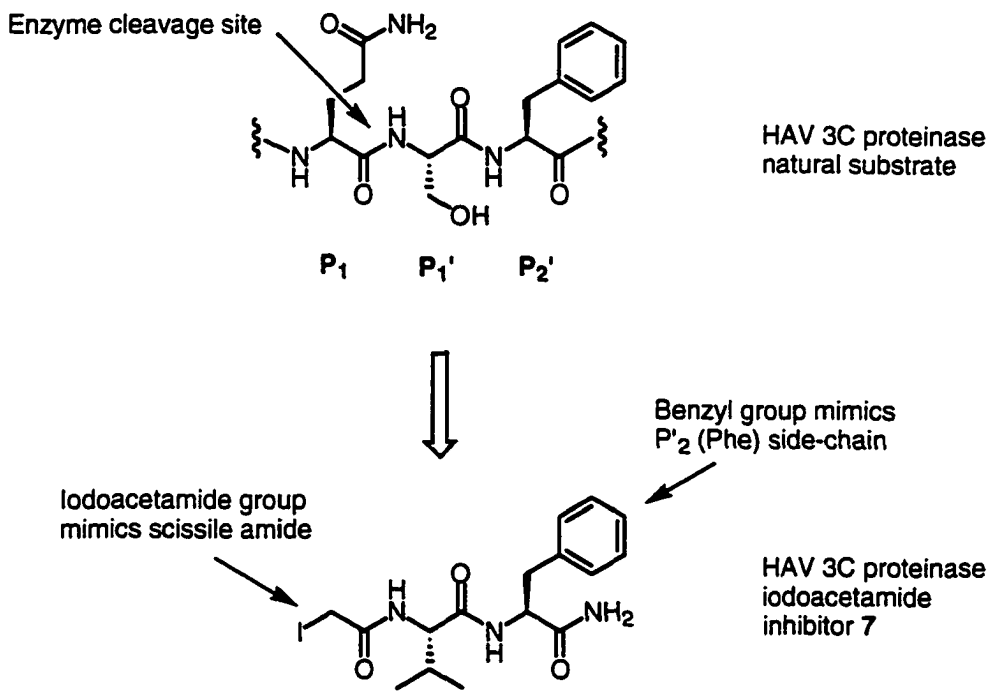


Compound **6** is a P-side inhibitor containing the essential P_4 (Leu) and P_1 (Gln) side chains required for enzyme recognition. In addition, the side-chain primary amide functionality of glutamine at P_1 is protected as the dimethyl amide for synthetic convenience and to prevent cyclization onto the reactive ketone carbonyl. Fluoromethyl ketone **6** is a potent irreversible inhibitor of HAV 3C proteinase, with a rate of enzyme inactivation $k_{inact} / K_I = 330\ M^{-1}s^{-1}$ ([E] = 0.07 μM, [I] = 1.0 μM).[28]

3.1.2 Peptidyl Halomethyl Carbonyls (P'-Side Inhibitors)

Peptides that mimic the 3C cleavage site from the P' recognition residues (carboxy terminal side of the scissile bond) also provide the potential for the development of potent inhibitors. Peptidyl *N*-iodoacetamides, recently synthesized in our group by Drs. Sven Frormann and John E. McKendrick,[36a] are P'-side inhibitors with modified N-termini. Iodoacetamide itself is a cysteine proteinase inhibitor, and hence shows inhibition towards HAV, HRV and poliovirus 3C proteinases.[36] Attachment of peptides that mimic the P_1'-P_2' side-chains can increase selectivity. For example, ICH_2CO-Val-Phe-NH_2 **7** (Figure 9) inhibits HAV 3C with a rate of enzyme inactivation $k_{inact} / K_I = 200\ M^{-1}s^{-1}$ ([E] = 0.07 μM, [I] = 1.0 μM), sixty times that of the parent iodoacetamide (ICH_2CONH_2) $k_{inact} / K_I = 3.1\ M^{-1}s^{-1}$.[36a]

Figure 9 P'-Side inhibitor



Recently, at the University of Alberta in the group of Dr. Michael N. James, a co-crystal structure of inhibitor **7** and an HAV 3C (C24S, F82A) double mutant has been diffracted to 1.9 Å resolution.[46] The complex has an acetyl-Val-Phe-amide group covalently attached to the sulfur-atom of the active site Cys-172, with the dipeptide side chains bound in their appropriate S_1' and S_2' specificity subsites (Figure 10). The crystal structure reveals that the HAV 3C proteinase possesses a defined S_2' subsite specificity pocket and suggests that the P_2' phenylalanine residue could be an important determinant for the selection of the primary cleavage site in HAV.

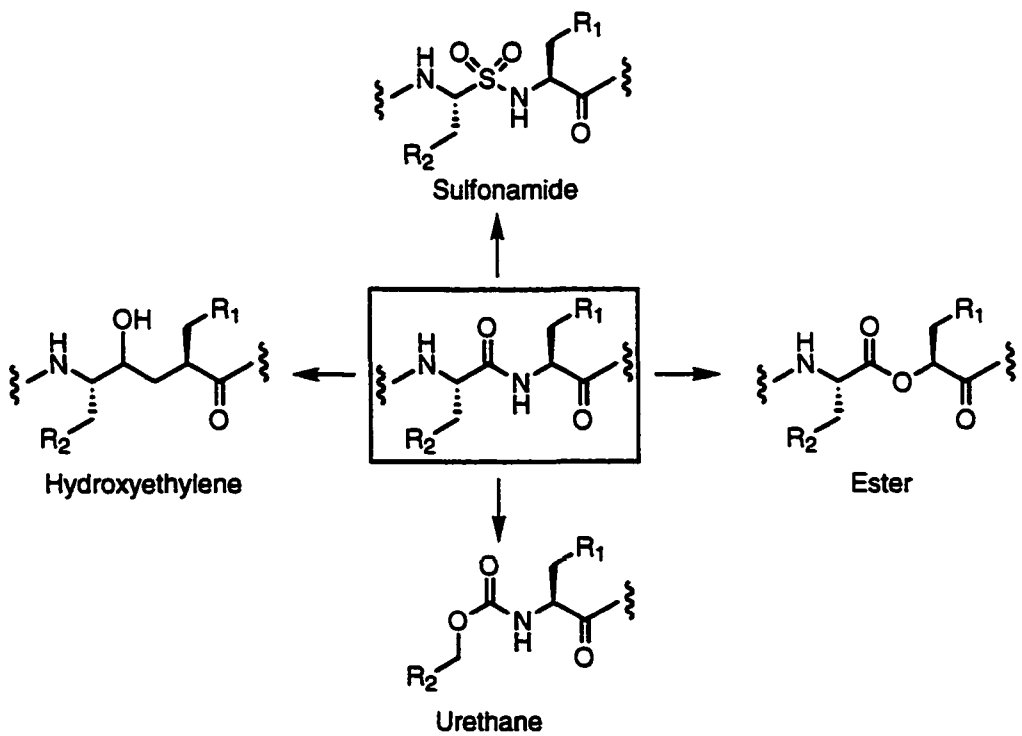
Figure 10 Active site of HAV 3C (C24S, F82A) proteinase (light gray) bound to acetyl-Val-Phe-amide (dark gray), figure prepared by Dr. Ernst M. Bergmann

3.2 Non-Peptide Inhibitors

In terms of bioavailability, proteolytic stability and pharmacokinetics, most peptides do not make good drugs. In contrast, mimetics having critical recognition domains may retain specificity for the target and have therapeutic potential.[47] There has been an increasing effort to design and synthesize active analogues of biologically significant peptides, to attain greater selectivity and fewer side effects than their present-day drugs.[48] Conformationally constrained molecules (rigid analogues) with appropriate recognition sites pay a lower entropy cost upon binding to their enzyme / receptor and therefore should adhere more strongly.[49] They may also have better proteolytic *in vivo* stability.[50]

Hydrolytically sensitive carboxamides can be isosterically mimicked by conversion to sulfonamides, esters, urethanes or hydroxyethylenes (Figure 11).[48]

Figure 11 Established isosteric replacements for peptide bonds

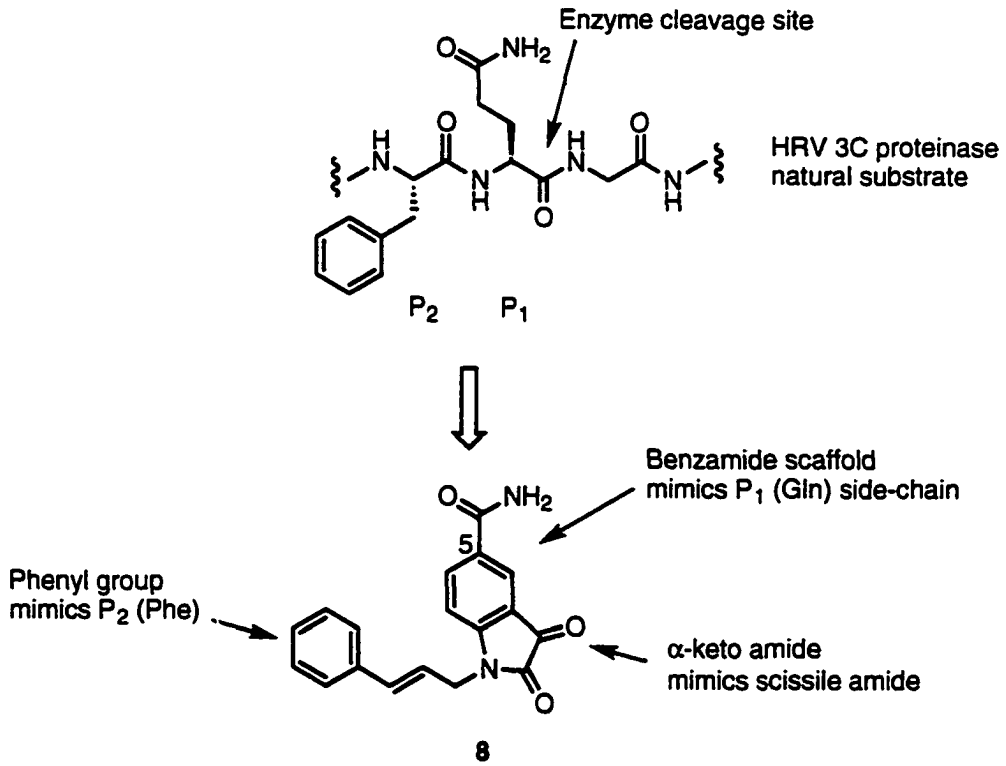


3.2.1 Isatins as Non-Peptide Inhibitors of HRV 3C Proteinase

Human rhinovirus (HRV) is the causative agent for more than fifty percent of incidents of the common cold.[4] The HRV 3C proteinase adopts a similar overall β-barrel topology to the HAV 3C proteinase, and both enzymes prefer a glutamine residue for the S_1 subsite. Having the coordinates for the X-ray crystal structure of HRV-14 3C and using molecular modeling, the group at Agouron Pharmaceuticals has developed selective, low molecular weight, non-peptidic inhibitors of several serotypes of the HRV 3C proteinases.[30] They focused their attention to the scissile cleavage, S_1 recognition, and the S_2 subsite. The HRV-14 3C proteinase specifically recognizes the primary carboxamide in the glutamine P_1 side-chain and cleaves the Gln (P_1)-Gly (P_1') amide bond by nucleophilic attack by the active site Cys-146 (serotype 14 numbering). This proteinase prefers the aromatic amino acids Phe and Tyr at P_2. The cyclic α-keto amide isatin structure appears to be a good scaffold which incorporates these features (Figure 12). Peptidyl α-keto amides are known reversible inhibitors of cysteine and serine proteinases.[51] In a similar fashion, the isatin heterocycle possesses an electrophilic ketone carbonyl, but in a conformationally restricted form. If the α-keto group of isatin is superimposed upon the scissile amide carbonyl of an octapeptide (Glu-Thr-Leu-Phe-Gln-Gly-Pro-Val) substrate, a carboxamide group at C-5 can occupy the S_1 recognition site, whereas substitution at N-1 accesses the S_2 binding pocket (Figure 12). Isatin **8** is a potent inhibitor of HRV-14 3C proteinase with K_i = 11 nM. Replacement of the N-1 *trans*-cinnamyl group, required for S_2 subsite recognition, with a methyl group increases the K_i by greater then four fold. Replacement of the C-5 carboxamide, required for S_1 recognition, with hydrogen reduces the inhibition by two thousand times. Unfortunately the most active isatins were toxic to cell cultures

infected with HRV, presumably because they are non-specifically reactive with thiols and amines.

Figure 12 Isatin **8** resemblance to HRV 3C substrate



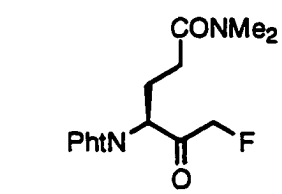
4. Project Goal: Design and Synthesis of HAV 3C Proteinase Inhibitors

The objective of this thesis is to investigate new types of warheads (thiol reactive functionality) for highly selective HAV 3C proteinase inhibition. Since there are structural features common to all members of the picornavirus family, it is likely that insights gained into the mechanism and specificity of HAV 3C proteinase will aid in the development of specific inhibitors of other human picornaviruses.

Ten types of substrate based inhibitors for HAV 3C were designed, as illustrated in Figure 13 (**A-D**), 14 (**E-I**) and 15(**J**). Target **A** is a phthalimido-protected intermediate in

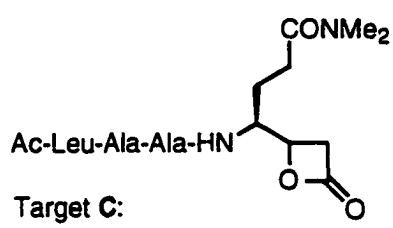
the synthesis of the established HAV 3C proteinase fluoromethyl ketone inhibitor **6** (Figure 8).[28] Preparation of target **A** using an alternative halogen exchange process,[52] would provide a facile synthetic route to a ^{13}C labeled **6** for enzyme inactivation studies. Target **B** contains an α,β-unsaturated ester to mimic the scissile amide bond in the substrate; thiol attack by the 3C enzyme would be expected to proceed in a Michael fashion to give an enzyme inhibitor adduct.[32-34] Targets **C** and **D** are modifications of the P_1 residue bearing an adjacent β-lactone. Since β-lactones are readily opened by thiols by attack at the β-position,[53] such peptides could irreversibly alkylate the active site Cys-172 of HAV 3C.

Figure 13 Targets **A-D**



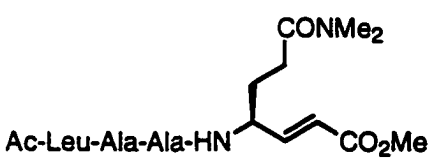
Target **A**:

Fluoromethyl ketone **9**



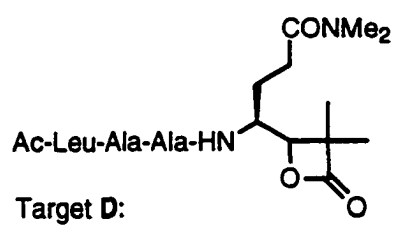
Target **C**:

β-Lactone **11**



Target **B**:

α,β-Unsaturated ester **10**



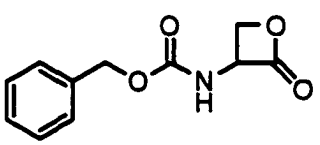
Target **D**:

Dimethyl β-lactone **12**

Targets **E-I** (Figure 14) also contain the thiol reactive β-lactone warhead, as well as a phenyl side chain to probe the importance of the P_2' side-chain, seen in the co-crystal complex of HAV 3C with compound **7** (Figure 9).[46] The synthesis and enzyme inhibition properties of target **F** will provide precedence for the preparation of target **G** compounds.

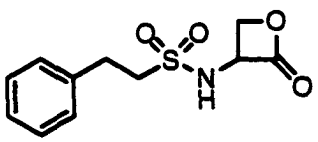
The four stereoisomers of target **G** may provide aqueous stability,[53] structural diversity and insight into the preferred trajectory of HAV 3C thiolate attack at the β-position of the oxetanone ring. In addition, γ-lactones such as target **H** are well-known to undergo ring opening as a result of nucleophilic attack at the carbonyl, but they can also react with nucleophiles (especially thiols) at the γ-position.[54] Target **I**, β-hydroxy acids, are precursors to β-lactones and could behave as transition state analogues, mimicking the tetrahedral intermediate of the natural peptide substrate en route to a hydrolytic pathway.

Figure 14 Targets **E-I**



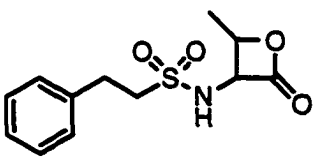
Target **E**:

N-Cbz-Serine-β-lactone **13**



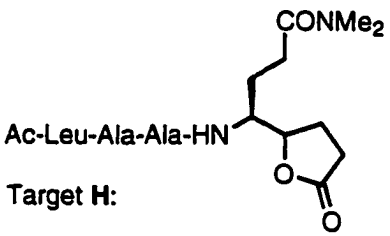
Target **F**:

N-Sulfonamide-serine-β-lacone **14**



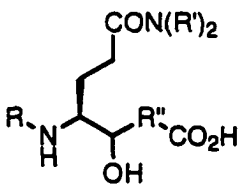
Target **G**:

N-Sulfonamide-threonine-β-lacone **15**



Target **H**:

γ-Lactone **16**



Target **I**:

β-Hydroxy acids

17: R = Boc, R' = Me, R" = CH_2

18: R = Ac-LAA, R' = Me, R" = CH_2

19: R = Boc, R' = Me, R" = $C(CH_3)_2$

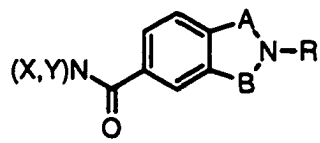
20: R = Boc, R' = Me, R" = $CHCH_3$

21: R = Cbz, R' = Me, R" = CH_2

22: R = Boc, R' = H, R" = CH_2

Target **J** (**23-26**) employs a rigid phthalimide scaffold, similar to the isatin analogue **8** (Figure 12) for HRV 3C,[30] on which a C-4 carboxamide is placed to mimic the essential P_1 glutamine substrate residue for HAV 3C. If HAV 3C inhibition occurs with compound **25**, isoindolinones **27a** and **27b** can be used to probe which imido carbonyl on **25** enters the HAV 3C oxyanion hole. In the following sections, the design and results of both synthetic studies and biological assays of these targets are described.

Figure 15 Target **J**



Target **J**:

Phthalimide and Isoindolinone

23: A = CO, B = CO, R = Me, X = H, Y = H

24: A = CO, B = CO, R = Me, X = Me, Y = Me

25: A = CO, B = CO, R = Me, X = Me, Y = H

26: A = CO, B = CO, R = NHCOPh, X = Me, Y = Me

27a: A = CH_2, B = CO, R = Me, X = Me, Y = H

27b: A = CO, B = CH_2, R = Me, X = Me, Y = H

RESULTS AND DISCUSSION

1. Peptide-Based Inhibitors of HAV 3C Proteinase

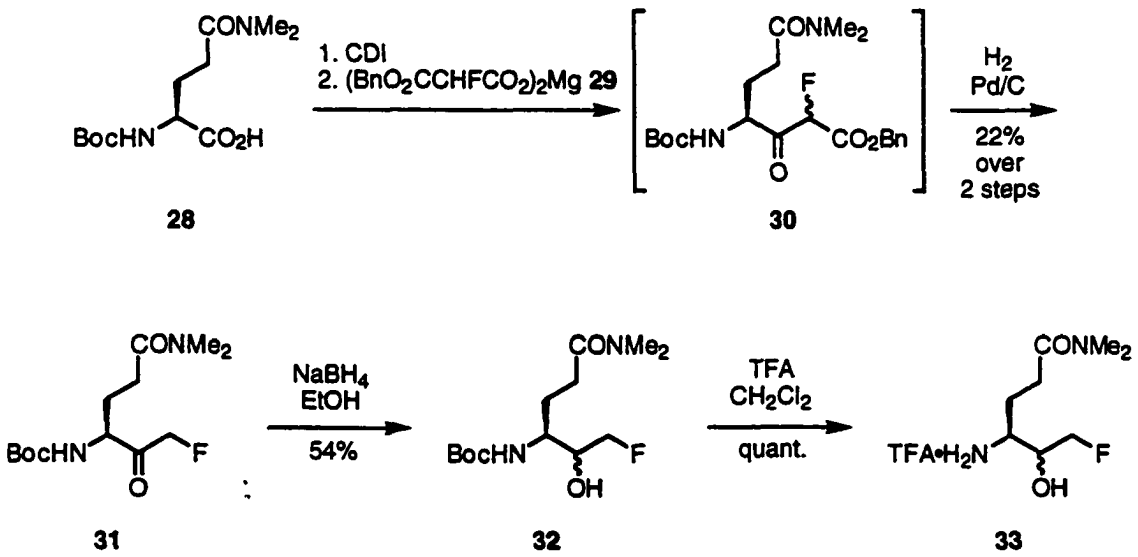
1.1 Peptidyl Fluoromethyl Ketones Target A

Affinity labeling is a powerful tool for the study of enzyme active sites. A substrate-like molecule with structural features adequate to form a complex with the enzyme under consideration, similar to the enzyme-substrate complex, is designed with a reactive group in its structure. Such an active site-directed reagent can then covalently modify the enzyme active site upon binding. Information gained from the enzyme-inhibitor complex can provide insight into enzyme-substrate function, enzyme mediated biological processes and overall understanding of metabolic integration. This powerful technique has been the subject of numerous reviews.[55-58]

Electron-withdrawing groups adjacent to a carbonyl group enhance the electrophilicity of the carbonyl functionality. Therefore introduction of an electron-withdrawing group next to the cleavage amide of a substrate holds promise in the design of inhibitors of proteinases. Fluorine is a promising electron-withdrawing substituent in this context, because of its electronegativity, minimal steric demands and the overall stability of the target structures.[59] Peptidyl fluoromethyl ketones have been shown to be highly potent proteinase inhibitors[60,61] and also show efficacy *in vivo* in several systems.[62-64] As indicated previously, fluoromethyl ketone **6** (Figure 8) is a potent time-dependent, irreversible inhibitor of HAV 3C proteinase, with a rate of enzyme inactivation k_{inact} / K_I = 330 $M^{-1}s^{-1}$ ([E] = 0.07 μM, [I] = 1.0 μM).[28] Inhibitor **6** was prepared according to the method developed previously in our group by Drs. Sven Frormann and Christopher Lowe (Schemes 1 and 2).[28]

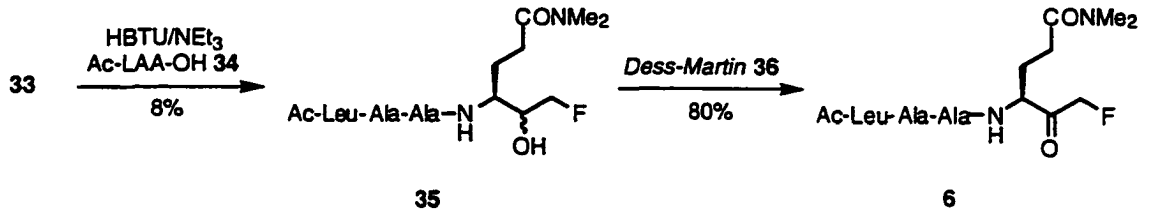
Activation of carboxylic acid **28** with 1,1'-carbonyldiimidazole (CDI) in THF, followed by enolate condensation with magnesium benzyl fluoromalonate **29**, affords intermediate **30** as a mixture of diastereoisomers (Scheme 1)[65]. Direct hydrogenation of **30** using 10% palladium on charcoal, without isolation, yields the fluoromethyl ketone monomer **31**.[66] Reduction of ketone **31** with $NaBH_4$ in ethanol followed by acidic work-up provides alcohol **32**.[67] Treatment of **32** with 50% trifluoroacetic acid in dichloromethane gives trifluoroacetate salt **33**.

Scheme 1



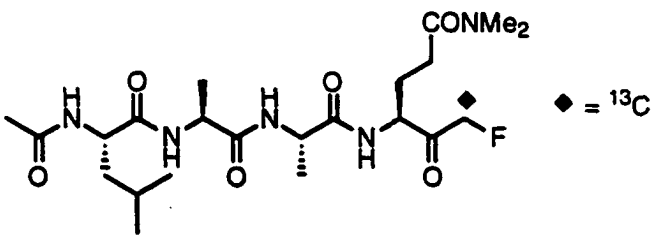
Coupling of tripeptide **34** to trifluoroacetate salt **33** is accomplished with HBTU, in the presence of triethylamine in DMF, and yields alcohol **35** (Scheme 2).[68] Treatment of alcohol **35** with Dess-Martin periodinane **36** gives desired fluoromethyl ketone tetrapeptide **6**.[69]

Scheme 2



A convenient method for the detection of covalent enzyme-substrate / inhibitor adducts is nuclear magnetic resonance (NMR) spectroscopy.[45] This technique, employing carbon-13 as the reporter nuclei has been used to directly observe and characterize several covalent enzyme-inhibitor adducts of proteinases.[26,28,45,70] To determine experimentally the type of adduct formed between the peptidyl fluoromethyl ketone **6** and the HAV 3C enzyme, the ^{13}C-labeled fluoromethyl ketone **6a** (Figure 16) is a useful tool.

Figure 16 ^{13}C-Labeled peptidyl fluoromethyl ketone **6a**

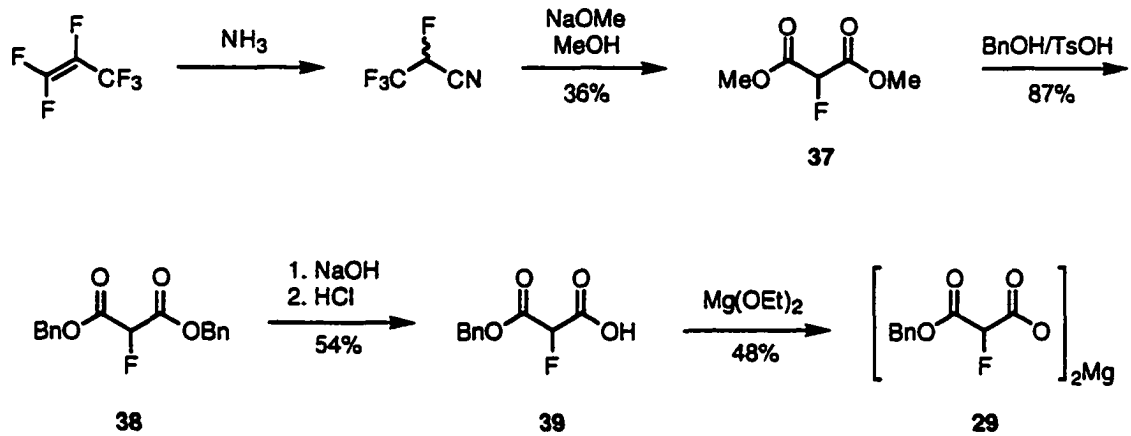


6a

The key feature of the fluoromethyl ketone synthesis in Scheme 1 is the use of magnesium benzyl fluoromalonate **29** as a means to introduce the fluoromethyl ketone unit.[65] Unlabeled magnesium benzyl fluoromalonate **29** is prepared by the lengthy procedure of Ishikawa and Ibrahim (Scheme 3).[71] Ammonolysis of hexafluoropropene gives 2,3,3,3-tetrafluoropropanenitrile. The nitrile is converted to dimethyl fluoromalonate **37** by treatment with sodium methoxide in methanol followed by hydrolysis with HCl.

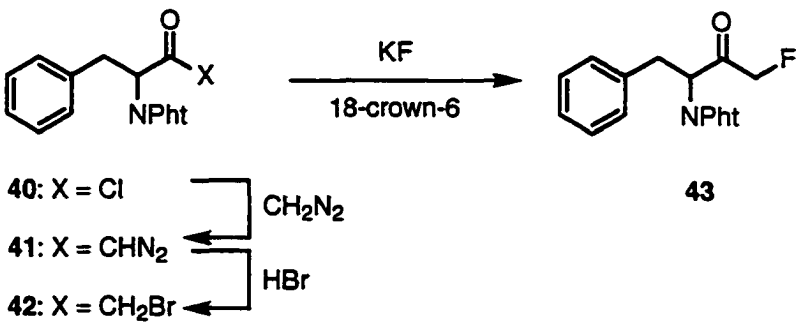
Transesterification of **37** with benzyl alcohol gives the dibenzyl fluoromalonate **38**. The half ester **39** is then obtained by treatment with one equivalent of base and is subsequently treated with magnesium ethoxide to generate the magnesium salt **29**. Unfortunately, [2-^{13}C]-hexafluoropropene is not commercially available and the synthesis of the corresponding 2-^{13}C-labeled magnesium benzyl malonate by this method appeared lengthy and impractical.

Scheme 3



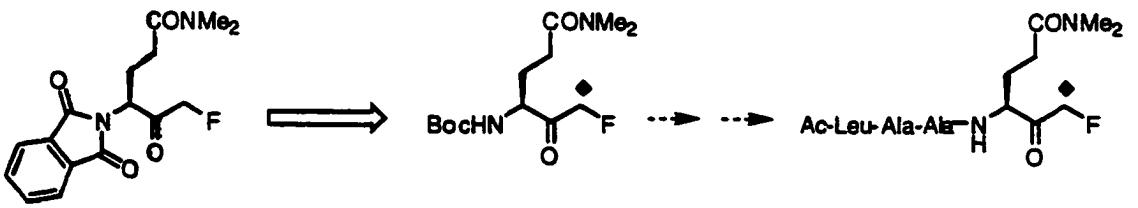
Alternatively, synthesis of fluoromethyl ketones using a variation of the halogen exchange method developed by Kolb,[52] can be used to place a single ^{13}C label specifically at the fluoromethyl carbon from the corresponding [^{13}C]-diazomethane (Scheme 4). The approach of Kolb and co-workers uses a bromine / fluorine exchange reaction adjacent to the ketone functionality as the key transformation. The required bromo ketone **42** is obtained from the acid chloride **40** of *N*-protected phenylalanine *via* the diazo ketone **41**. Halogen exchange is then achieved in 45% yield with dry potassium fluoride in acetonitrile in the presence of 18-crown-6, to afford the fluoroketone **43**.

Scheme 4



Synthesis of modified dimethyl glutamine analogue **9** (target **A**) was used as a synthetic model to explore whether the chemistry developed by Kolb (Scheme 4) can provide access to a fluoromethyl ketone with a derivative other than phenylalanine. If the methodology is successful in preparing fluoromethyl ketone **9** it could be applied to the synthesis of ^{13}C-labeled fluoromethyl ketone monomer **31a**, en route to the desired ^{13}C-labeled fluoromethyl ketone tetrapeptide **6a** (Scheme 5).

Scheme 5



Target **A**:
Fluoromethyl ketone **9**

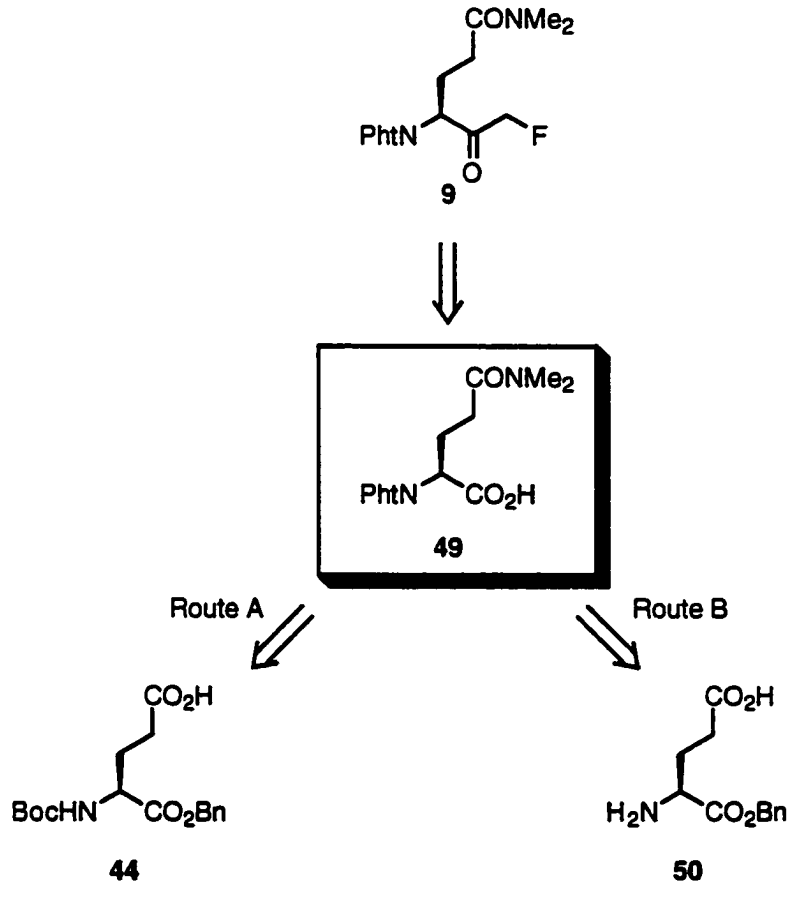
31a
◆ = ^{13}C

6a

Retrosynthetic analysis of compound **9** (Scheme 6) indicates that its preparation can be realized by two routes A and B; a key intermediate of both routes is acid **49** which provides the starting material to introduce the Kolb methodology (Scheme 4). Route A provides acid **49** from diprotected glutamic acid **44** and route B provides intermediate **49**

from benzyl ester protected glutamic acid **50**. Both routes outlined in Scheme 6 were attempted in order to obtain key intermediate acid **49**.

Scheme 6

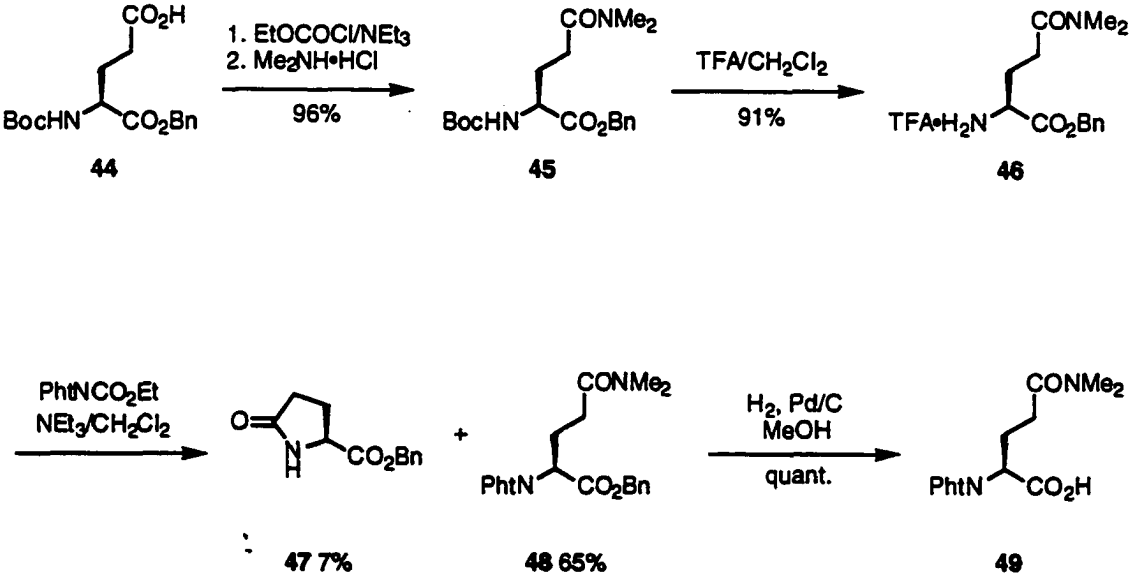


1.1.1 Route A: Synthesis of Acid 49

Dimethyl glutamine **45** was synthesized from protected glutamic acid **44** by modification of the literature procedure of Greenstein and Winitz (Scheme 7).[72] Protected glutamic acid **44** reacts with ethyl chloroformate in the presence of triethylamine to produce the expected mixed anhydride, which upon treatment with dimethylamine hydrochloride affords dimethyl glutamine **45**. Deprotection of **45** with 50% trifluoroacetic acid in

dichloromethane provides trifluoroacetate salt **46**. Salt **46** is heated under reflux in the presence of *N*-carbethoxyphthalimide,[73] to give phthalimide **48** as the major product and the cyclized side-product γ-lactam **47**. Initially, phthalic anhydride[74] was used to introduce the phthalimido protecting group onto **46**, however, unsatisfactory yields of **48** convinced us to abandon this approach. Hydrogenation of phthalimide **48** in the presence of 10% Pd/C catalyst in methanol provides key intermediate acid **49**.

Scheme 7

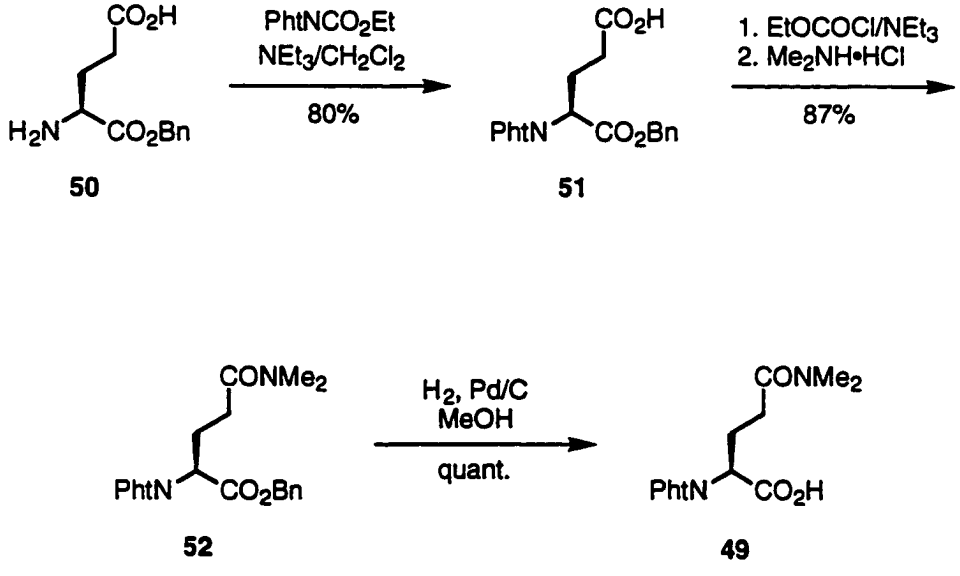


1.1.2 Route B: Synthesis of Acid 49

To improve upon overall yield and acquire acid **49** in a more efficient manner, route B (Scheme 8) was explored as an alternative synthetic path. Nitrogen protection of amine **50** proceeds with *N*-carbethoxyphthalimide[73] to give protected phthalimido **51** in reasonable yield (80%). Activation of acid **51** with ethyl chloroformate[72] in the presence of triethylamine gives the mixed anhydride, which is condensed with dimethylamine hydrochloride to give dimethyl glutamine **52**. Hydrogenation of dimethyl glutamine **52** in

the presence of 10% Pd/C catalyst in methanol provides acid **49**. The overall yield of route B is 70%, an improvement to the yield of route A (57%). In addition, route B has one less synthetic step. Nevertheless, both routes A and B provide **49** in good yield to test the Kolb methodology[52] (Scheme 4), for preparation of fluoromethyl ketone **9** (target A, Scheme 5).

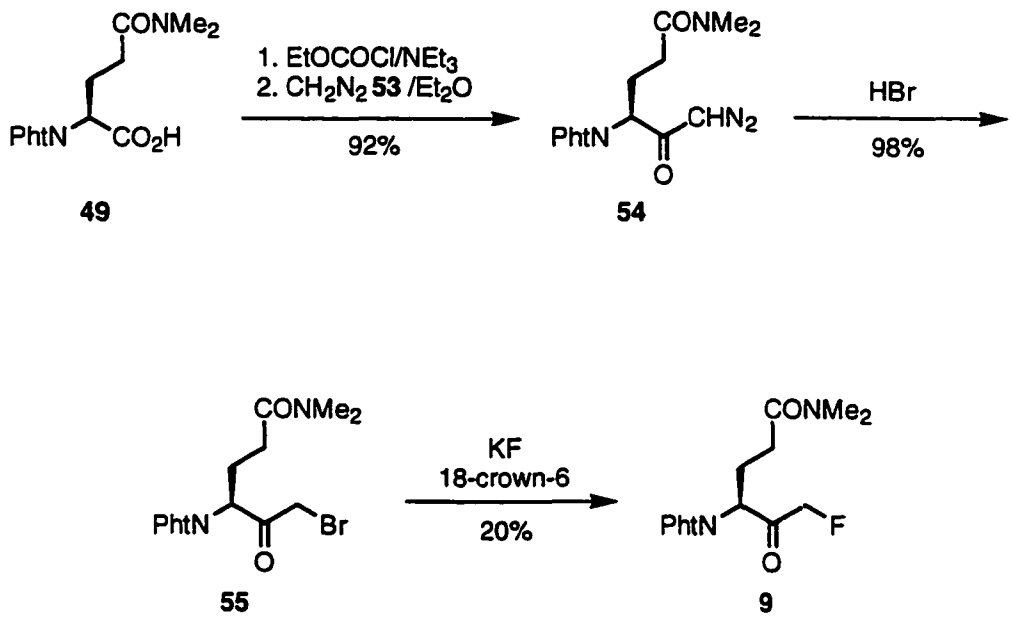
Scheme 8



1.1.3 Synthesis of Target A

The Kolb methodology was applied to **49** (Scheme 9).[52] Acid **49** reacts with ethyl chloroformate in the presence of triethylamine to produce the expected mixed anhydride, which upon treatment with ethereal diazomethane **53** affords diazo ketone **54**. Subsequent bromination of **54** with HBr provides bromomethyl ketone **55**. Halogen exchange is achieved in 20% yield, with dry potassium fluoride in acetonitrile in the presence of 18-crown-6, to afford fluoromethyl ketone **9** (target A).

Scheme 9



49 54 55 9

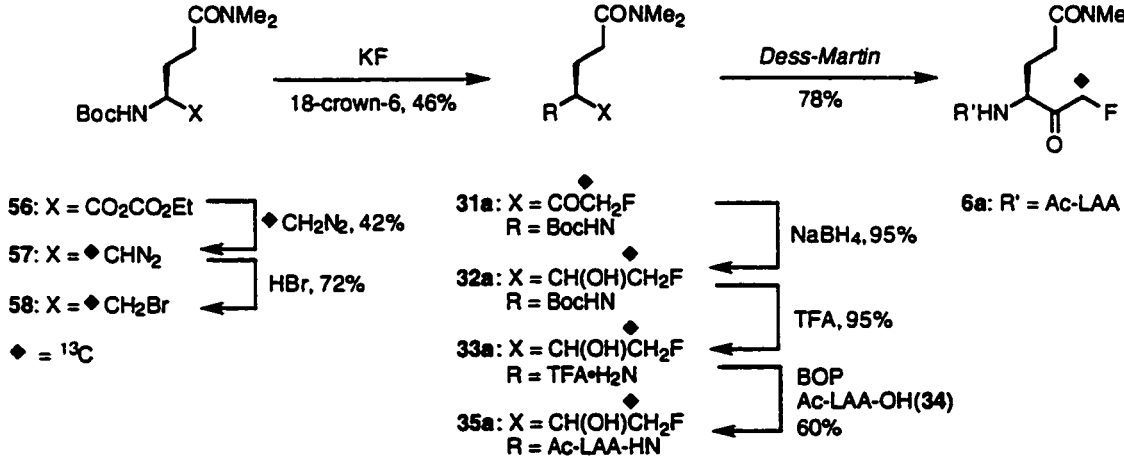
1.1.4 Synthesis of ^{13}C-Labeled 6a and NMR Studies

Following this procedure for the preparation of target **A**, the Kolb methodology was successfully applied to the synthesis of fluoromethyl ketone tetrapeptide **6a** (Scheme 5) by Dr. Sven Frormann in our group (Scheme 10). As the yields in the final step with the *N*-Pht derivative **9** (Scheme 9) were unsatisfactory, the synthesis was repeated with the *N*-Boc-protected derivative (Scheme 10). Initially, unlabeled material was used for the development of the synthesis shown in Scheme 10, then ^{13}C-labeled material ([^{13}C]-diazomethane) was used in the optimized procedure.

In Scheme 10, the mixed anhydride **56** is prepared by treating the acid with ethyl chloroformate in the presence of triethylamine. An ethereal solution of [^{13}C]-diazomethane (>95% isotopic purity) is condensed with mixed anhydride **56**, to give diazo ketone **57** (42%). Diazo ketone **57** is treated with HBr to afford the bromomethyl ketone **58** in 72% yield. Halogen exchange is achieved in 46% yield, with dry potassium fluoride in

acetonitrile in the presence of 18-crown-6, to afford fluoromethyl ketone **31a**. Reduction of ketone **31a** with sodium borohydride in ethanol gives fluoro alcohol **32a**, as a mixture of diastereoisomers, in 95% yield; subsequent reactions were performed on the diastereomeric mixture. Deprotection of fluoro alcohol **32a** proceeds with 50% trifluoroacetic acid in dichloromethane to afford the trifluoroacetate salt **33a** in 95% yield. Salt **33a** is coupled to tripeptide **34** (Ac-Leu-Ala-Ala-OH) in the presence of BOP reagent and triethylamine in DMF to furnish tetrapeptide fluoro alcohol **35a** (60%). Carbon-13 labeled fluoromethyl ketone **6a** is then produced in 78% yield upon Dess-Martin periodinane oxidation of fluoro alcohol **35a**.

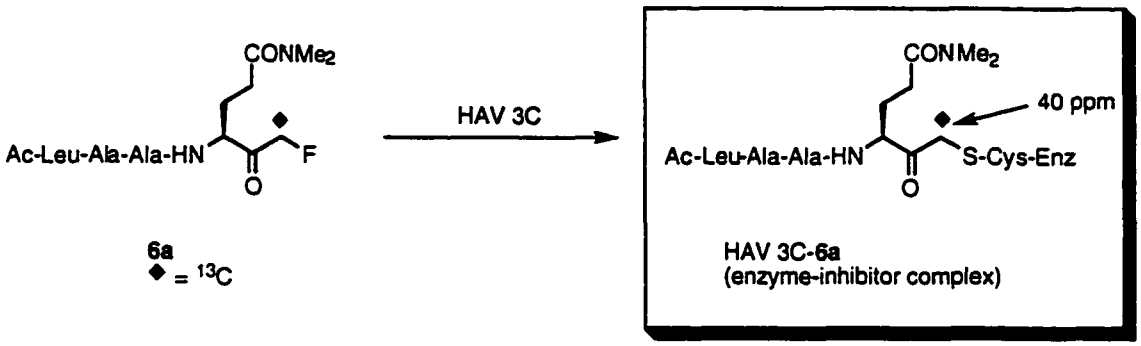
Scheme 10

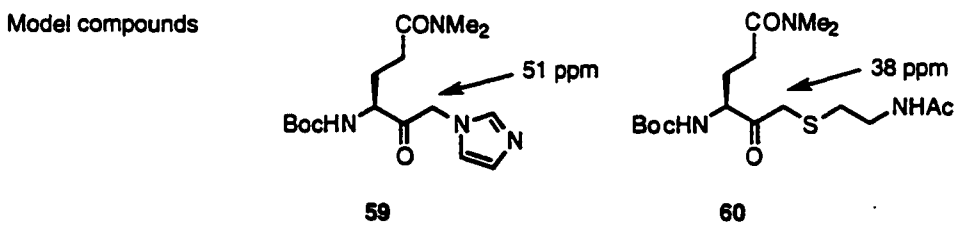


Two model compounds **59** and **60** were also synthesized (Figure 17) by Dr. Sven Frormann to assist analysis of whether alkylation of HAV 3C occurs with the active site nitrogen (histidine-44) or sulfur (cysteine-172) nucleophile. The chemical shifts of the methylene carbons adjacent to the heteroatoms in the model compounds are 51 ppm for the imidazoloketone **59** and 38 ppm for the (alkylthio)ketone **60**; the latter value is in good agreement with the literature values for such sulfides.[45] Reaction of HAV 3C with the ^{13}C-labeled tetrapeptide fluoromethyl ketone **6a** rapidly releases fluoride ion (^{19}F NMR

chemical shift -120 ppm)[28,60] and produces an irreversible adduct whose mass spectrum is shifted to higher mass by the expected 471 Da. The ^{13}C-NMR spectrum of the enzyme-inhibitor complex displays a new peak at 40 ppm, suggesting the formation of an (alkylthio)ketone. Hence, despite an active site geometry which resembles a serine proteinase, the active site sulfhydryl of HAV 3C dominates the inactivation chemistry with fluoromethyl ketone analogous to that of other cysteine proteinases such as papain.[44,45]

Figure 17 HAV 3C inactivation with **6a** and chemical shifts of model compounds





59 60

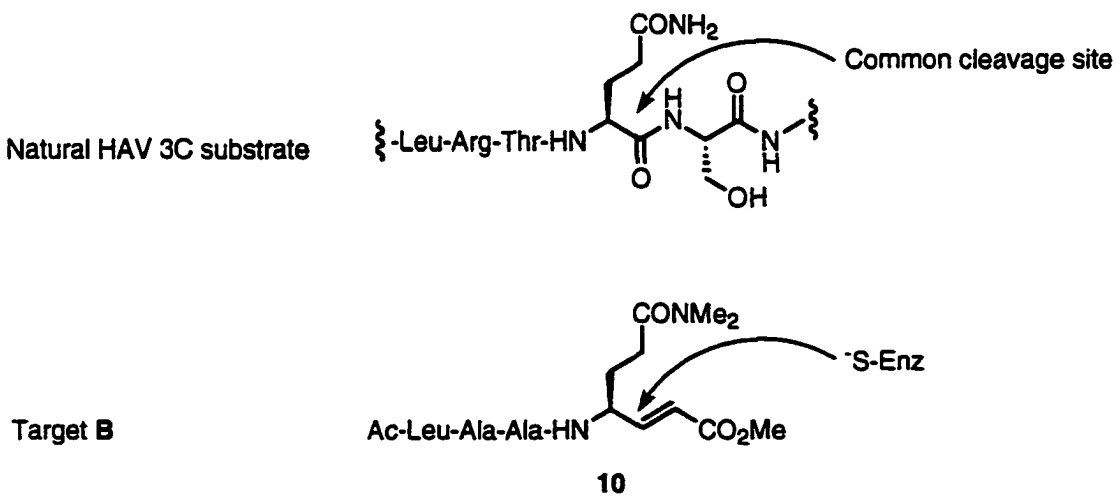
In summary, target A (compound **9**) was synthesized. Two synthetic routes were used which gave key intermediate acid **49**, the overall yield of route B is 70%, an improvement to route A (57%). The facile and efficient synthesis of **9** using a halogen exchange process provides access to ^{13}C labeled fluoromethyl ketone inhibitor **6a** for NMR enzyme inactivation studies. The ^{13}C-NMR spectrum of the enzyme-inhibitor complex displays a new peak at 40 ppm, indicating the formation of an (alkylthio)ketone.

1.2 Peptidyl Michael Acceptors

1.2.1 Michael Acceptor Design

Michael acceptors are useful synthetic intermediates and α,β-unsaturated carbonyl and sulfones act as Michael acceptors for soft nucleophiles such as thiol compounds,[75] leading to the formation of a covalent carbon-sulfur bond. Peptidyl Michael acceptors as inactivators of the cysteine proteinase papain were first introduced by Hanzlik and Thompson.[76] They showed that papain is inhibited irreversibly by their Michael acceptors but the serine proteinase chymotrypsin is not. Based on this observation and molecular modeling,[77] it seemed reasonable that a truncated peptide substrate in which the scissile amide carbonyl is replaced with a Michael acceptor might function as an active site irreversible HAV 3C inhibitor (Figure 18). Hence, target **B** incorporates the tetrapeptide analogue **10** as a mimic of the substrate, bearing an α,β-unsaturated ester functionality for specific recognition and binding to HAV 3C (Figure 18).

Figure 18 Rationale for target **B**

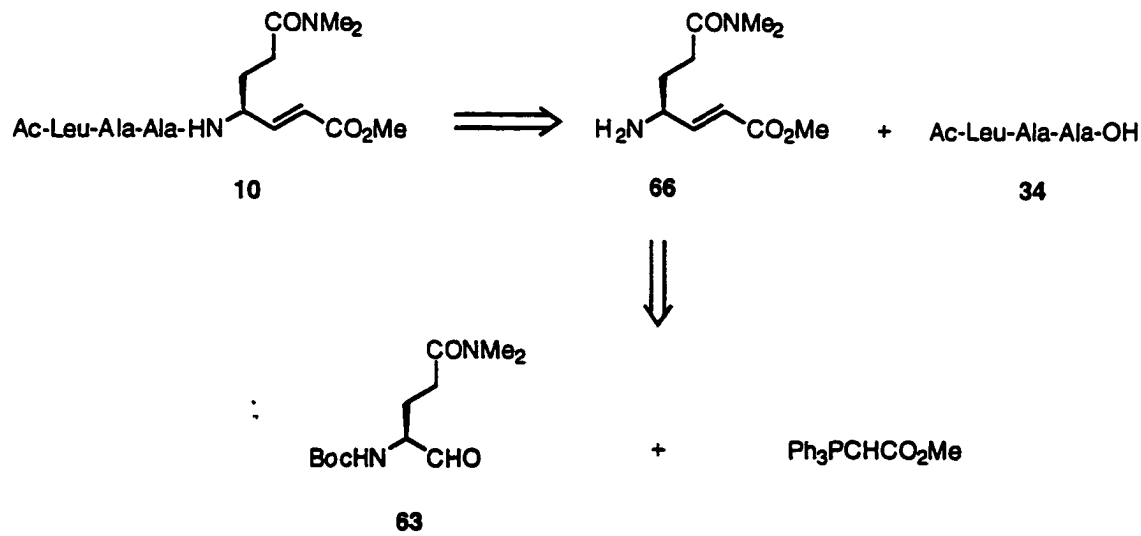


10

1.2.2 Synthesis of Target B

The strategy for the construction of target **B** (**10**) is based on the retrosynthetic analysis outlined in Scheme 11. Target molecule **10** can be derived from tripeptide **34** (Ac-Leu-Ala-Ala-OH) and the dimethyl glutamine α,β-unsaturated ester **66**. Tripeptide **34** is readily prepared by solid phase peptide synthesis using standard Fmoc chemistry on Wang resin. The key α,β-unsaturated ester **66** could, in principle, be synthesized by a Wittig reaction from aldehyde **63** and a stabilized ylide.

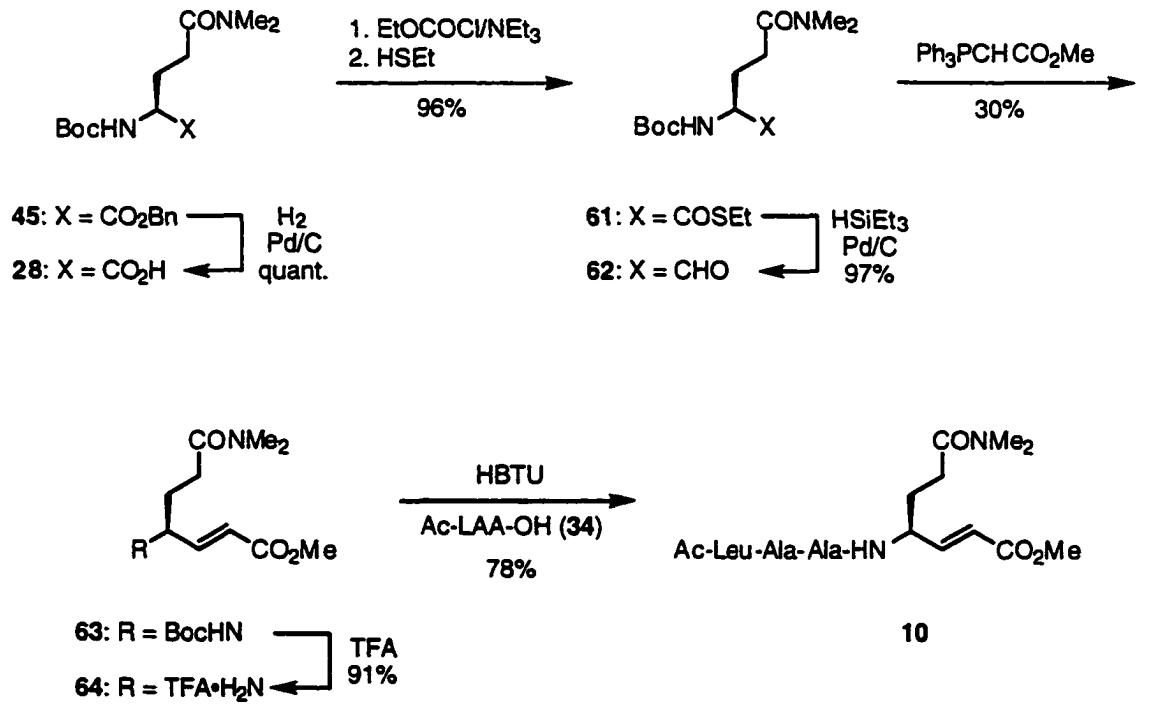
Scheme 11



Thus, the peptidyl Michael acceptor **10** is synthesized by the procedure outlined in Scheme 12. Dimethyl glutamine **45** is prepared as shown previously in Scheme 7. Hydrogenation of benzyl ester **45** in the presence of 10% palladium on charcoal, in methanol, affords acid **28**.[66] Acid **28** is readily converted to thioester **61** by reaction with ethyl chloroformate and ethanethiol in the presence of triethylamine.[78] Reduction of thioester **61** with triethylsilane and a catalytic amount of 10% palladium on charcoal, in dichloromethane, generates aldehyde **62** in good yield (97%).[79] The Wittig reaction then

proceeds with aldehyde **62** and methyl (triphenylphosphoranylidene)acetate, in THF, to give α,β-unsaturated ester **63**.[80] Deprotection of **63** in 50% trifluoroacetic acid in dichloromethane affords the trifluoroacetate salt **64**. Subsequent coupling of salt **64** with tripeptide **34**, *via* the active ester formed on treatment with HBTU, in the presence of triethylamine in DMF, produces the desired peptidyl Michael acceptor **10**.

Scheme 12



1.2.3 Inhibition of HAV 3C Proteinase by Target B

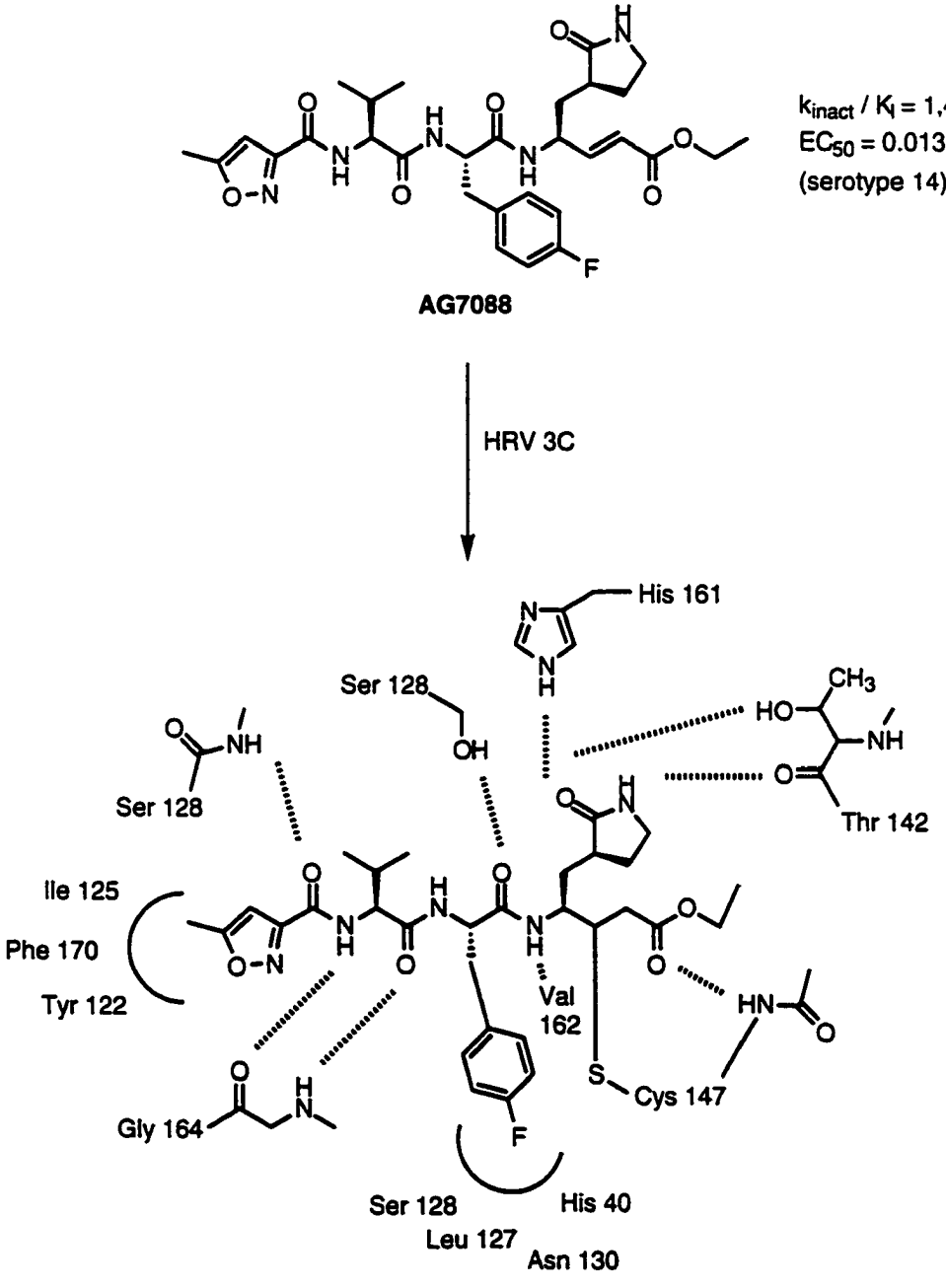
Target **B** (**10**) was assayed by the standard method,[81,82] which is described in the experimental section and employs a discontinuous 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay.[82] The enzyme inhibition studies were performed by Colin Luo (Department of Biochemistry).[81b] Compound **10** proved to be a time-dependent, irreversible inhibitor of HAV 3C proteinase, with a rate of enzyme inactivation k_{inact} / K_I = 137 $M^{-1}s^{-1}$ ([E] = 0.07

μM, [I] = 10.0 μM). This promising result warranted further study. Hence, inhibitor **10** was subjected to a continuous fluorogenic assay.[83] Only 15% inhibition (no enzyme inhibitor pre-incubation) and 46% inhibition (15 min enzyme inhibitor pre-incubation) of HAV 3C was observed at an enzyme concentration of 0.1 μM and inhibitor concentration of 100 μM.

The mode of action of inhibitor **10** on HAV 3C proteinase was not elucidated further. Shortly after this part of the work was completed a flurry of publications (by Dragovich *et al.*[32,34] at Agouron Pharmaceuticals and Wang *et al.*[33] at Eli Lilly and Company) was released regarding Michael acceptors as inhibitors for HRV 3C proteinase. The studies demonstrate the ability of peptidyl Michael acceptors to function as potent inhibitors of HRV 3C proteinase, irreversibly inhibiting the 3C proteinase from several HRV serotypes and exhibiting antiviral activity when tested against these serotypes in cell culture (Figure 19). In addition, crystallographic analysis of an enzyme-inhibitor complex confirmed the binding orientation of these compounds and revealed that enzymatic thiol attack proceeds in an expected Michael fashion. These studies established Michael acceptor **AG7088** as a highly potent, nontoxic antirhinoviral agent with broad efficacy against multiple virus serotypes. Compound **AG7088** has been formulated for intranasel delivery and has recently entered clinical trails.[34d]

In summary, target **B** compound **10** was synthesized. Inhibition studies showed that it is a potent inhibitor of HAV 3C proteinase with a rate of enzyme inactivation k_{inact} / K_I = 137 $M^{-1}s^{-1}$ ([E] = 0.07 μM, [I] = 10.0 μM) when tested using a discontinuous TNBS assay;[82] and a weak inhibitor diplaying 46% inhibition (15 min enzyme inhibitor pre-incubation) at an enzyme concentration of 0.1 μM and inhibitor concentration of 100 μM using a continuous fluorogenic assay.[83]

Figure 19 HRV-2 3C inactivation by **AG7088** and schematic diagram of co-crystal structure

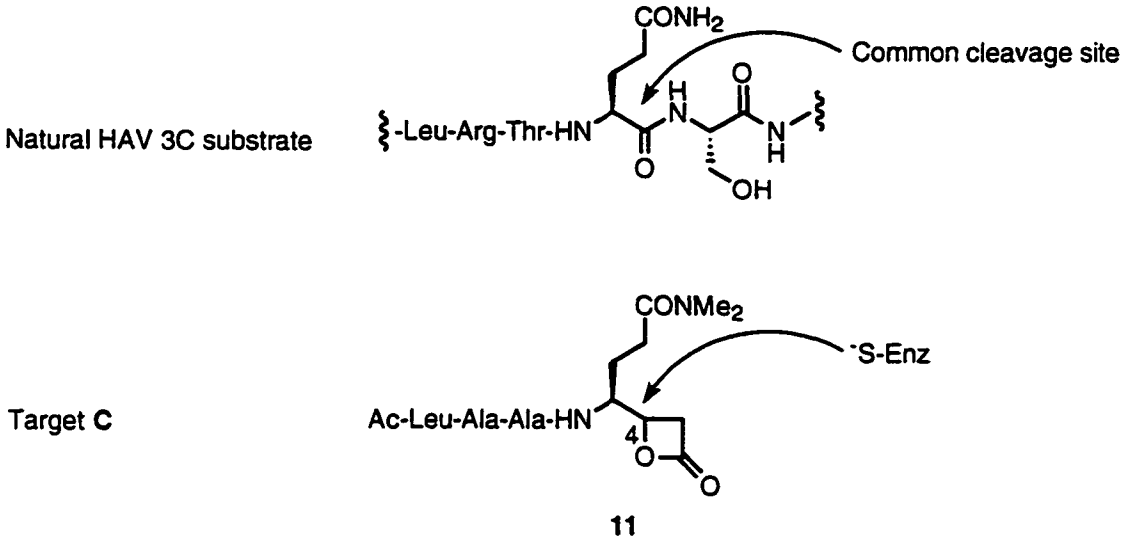


1.3 Peptidyl β-Lactones

1.3.1 β-Lactone Design

β-Lactones occur naturally in a variety of organisms, and many possess potent biological activity.[53e-g] The ability of thiols to open the four-membered ring by nucleophilic attack at either the carbonyl or at the β-position,[53a-d] suggests that cysteine proteinases could be irreversibly inactivated by β-lactones having correct substitution and stereochemistry (Figure 20). The β-lactone functionality is generally quite stable below pH 7.5. However, thiolate reacts at the β-position, to form sulfides, under neutral or slightly acidic (pH 5.5) aqueous conditions, or at the carbonyl, to form thiol esters, in non-aqueous environments.[84] A wide variety of carbon, nitrogen, phosphorous and oxygen nucleophiles are also known to attack the methylene group at the β-position of serine β-lactone.[53] It is feasible that the β-lactone functionality may react irreversibly with the thiol group at the active site of HAV 3C proteinase. Therefore, target **C (11)** (Figure 20) incorporates the tetrapeptide analogue, Ac-Leu-Ala-Ala-Gln(NMe_2), as a mimic of the substrate possessing a β-lactone functionality for specific recognition and binding to the HAV 3C proteinase. Both stereoisomers at C-4 are interesting targets since either could, in principle, form a covalent bond with the active site thiol. Molecular modeling also suggests that such tetrapeptide β-lactones could be potential inhibitors of the HAV 3C enzyme.[77]

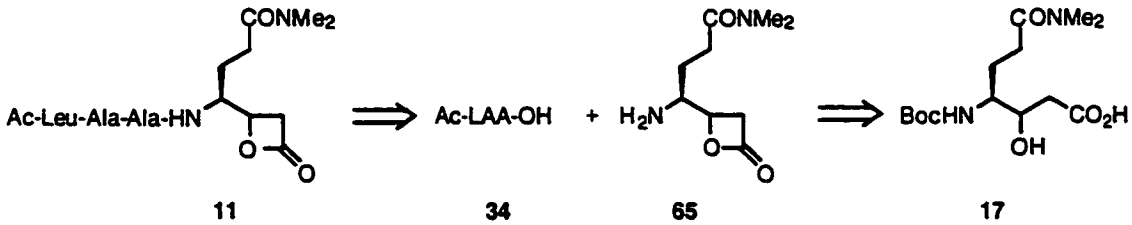
Figure 20 Rationale for target **C**



1.3.2 Synthetic Studies Towards Target C

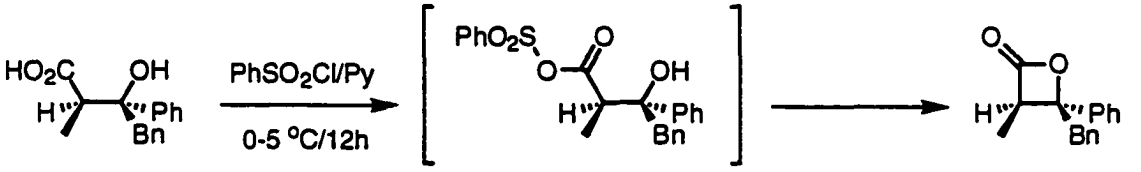
Retrosynthetic analysis for the construction of target **C** (**11**) is based on the strategy outlined in Scheme 13. The target molecule could be derived from a tripeptide Ac-Leu-Ala-Ala-OH **34** and a dimethyl glutamine β-lactone **65**. The key β-lactone **65** could, in principle, be synthesized by cyclization of β-hydroxy carboxylic acid **17**.

Scheme 13



Over the past fifty years, much attention has been focused on the preparation of β-lactones.[53] A major improvement occurred with the introduction by Adam *et al.*[85] of benzenesulfonyl chloride / pyridine as a lactonizing reagent. The conversion of β-hydroxy acids to β-lactones proceeds *via* a mechanism involving carboxy group activation (formation of a mixed anhydride intermediate) and its attack by the hydroxyl group. For example, β-lactone **68** is prepared, in excellent yield (93%), from acid **66** *via* mixed anhydride **67** (Scheme 14).[85] Benzenesulfonyl chloride / pyridine is presently the most commonly used reagent for the preparation of β-lactones.[53g,86]

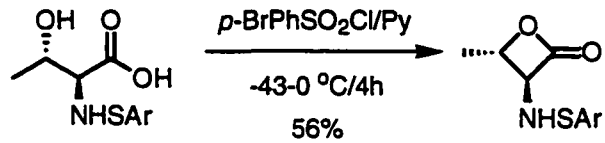
Scheme 14



66 **67** **68**

Other sulfonyl chlorides have also been successfully used, for example, tosyl chloride / pyridine[87] and *p*-bromobenzenesulfonyl chloride / pyridine.[88] Vederas and co-workers observed that the best cyclization conditions for β-hydroxy amino acid derivatives which bear a β-alkyl substituent are *p*-bromobenzenesulfonyl chloride / pyridine at -43 °C to 0 °C.[88a] Thus, optically pure β-hydroxy amino acid derivative **69** (Ar = *o*-nitrophenyl) cyclizes to the corresponding β-lactone **70** on treatment with *p*-bromobenzenesulfonyl chloride / pyridine, in 56% yield (Scheme 15).

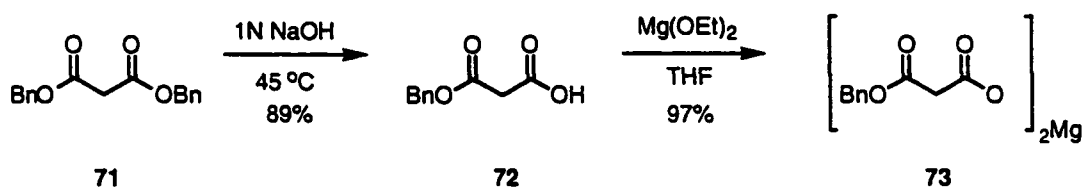
Scheme 15



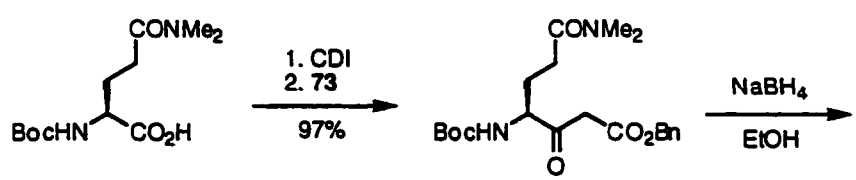
69 **70**

To adapt this procedure for the preparation of β-lactone synthon **65**, the precursor β-hydroxy amino acid derivative **17** was synthesized as shown in Scheme 16.

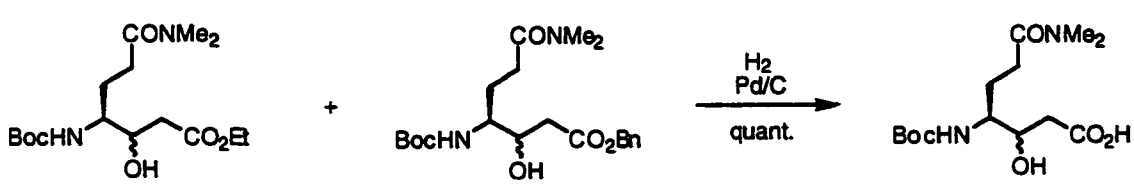
Scheme 16



71 **72** **73**



28 **74**

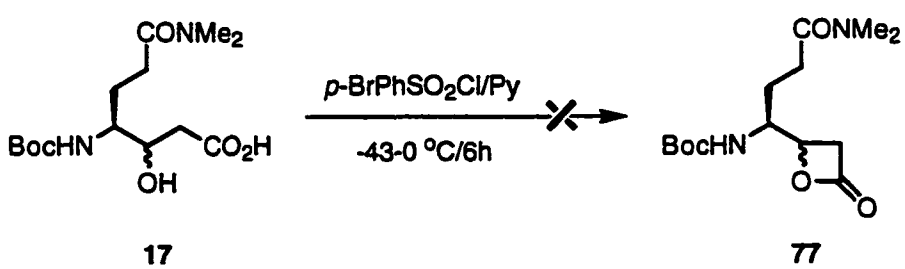


75 6% **76** 85% **17**

Treatment of dibenzyl malonate **71** with aqueous 1N NaOH in 2-propanol, at 45 °C, affords the monobenzyl malonate **72**, which is readily converted to the magnesium salt **73** by reaction with magnesium ethoxide in THF. The acid **28** is activated with 1,1'-carbonyl diimidazole in THF and condensed with magnesium salt **73**, followed by decarboxylative acidic work-up to provide β-keto ester **74**.[89] Reduction of **74** with $NaBH_4$ in ethanol, followed by acidic work-up, generates the required β-hydroxy ester **76** and the transesterified side-product **75**.[67] Hydrogenation of benzyl ester **76**, catalyzed by 10% palladium on charcoal, in methanol, provides the desired β-hydroxy acid **17**.[66]

Cyclization of β-hydroxy acid **17**, in the presence of *p*-bromobenzenesulfonyl chloride / pyridine to give β-lactone **77** was monitored by thin layer chromatography (TLC) and IR spectrometry (Scheme 17). Generally, β-lactones appear as a yellow spot on a blue background after heating when developed with alkaline bromocresol green spray.[90] In addition, β-lactones are characterized by an intense IR absorption at 1840-1820 cm^{-1}, due to the carbonyl stretch, which is at a higher frequency than observed with acyclic esters or larger-ring lactones.[91] However, neither TLC nor IR spectrometry showed any indication for the lactonization of **17** to give β-lactone **77**. Therefore, the cyclization approach of **17** with the reagent *p*-bromobenzenesulfonyl chloride in pyridine was abandoned.

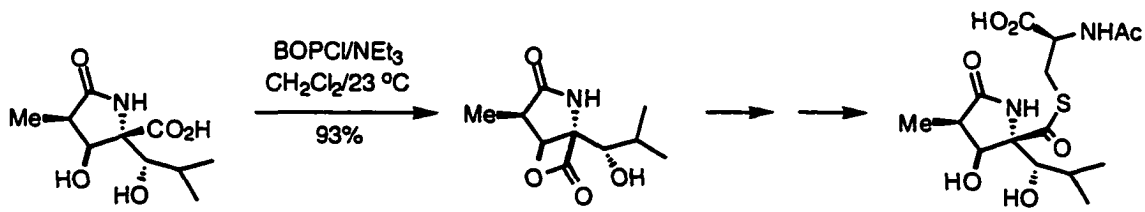
Scheme 17



17 **77**

Recently, Corey and co-workers have completed the total synthesis of lactacystin **80** (Scheme 18), an irreversible inhibitor of the proteolytic activity of the 20*S* proteasome.[92] An intermediate in the synthesis of lactacystin **80** is β-lactone **79**, which is prepared by cyclization of **78** with BOPCl, illustrated in Scheme 18.

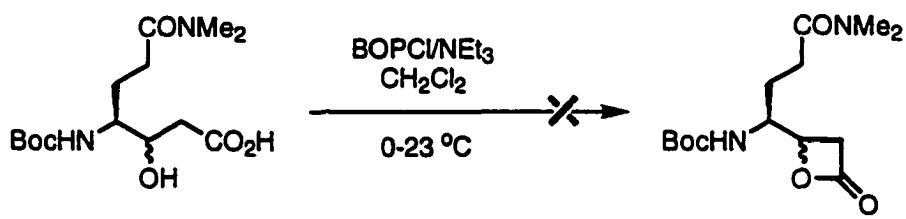
Scheme 18



78 **79** **80**

Therefore, an attempt was made to cyclize **17** using the reagent BOPCl (Scheme 19). Unfortunately, treatment of β-hydroxy acid **17** with BOPCl, in the presence of triethylamine in dichloromethane, failed to give any desired β-lactone **77**.

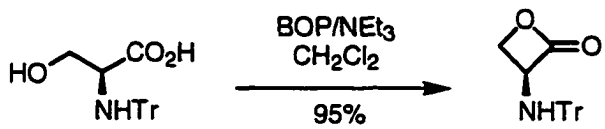
Scheme 19



17 **77**

In a final attempt to prepare β-lactone **77**, the modified procedure of Liskamp *et al.* was applied.[93] Liskamp and co-workers have found success in utilizing BOP reagent for the lactonization of *N*-Tr-serine **81** to its corresponding *N*-Tr-serine-β-lactone **82**, in good yield (Scheme 20).

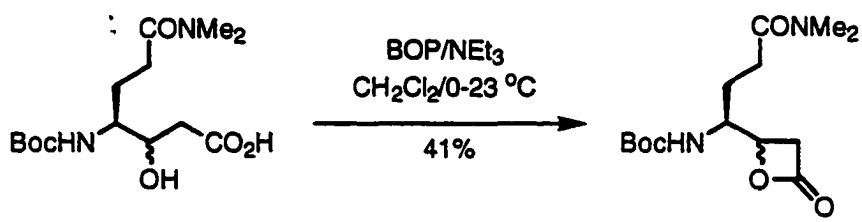
Scheme 20



81 **82**

Hence, treatment of β-hydroxy acid **17** with BOP reagent, in the presence of triethylamine in dichloromethane produces the desired yellow spot on TLC upon staining with bromocresol green spray, in addition to an intense IR carbonyl absorbance at 1830 cm^{-1}; observations which are attributed to the formation of β-lactone **77** (Scheme 21). Although, β-lactone **77** could be characterized, it is quite unstable and prolonged exposure to organic solvent ($CHCl_3$) results in an insoluble white precipitate of high molecular weight (by electrospray mass spectrometry), which is most likely the product of β-lactone polymerization.[94]

Scheme 21

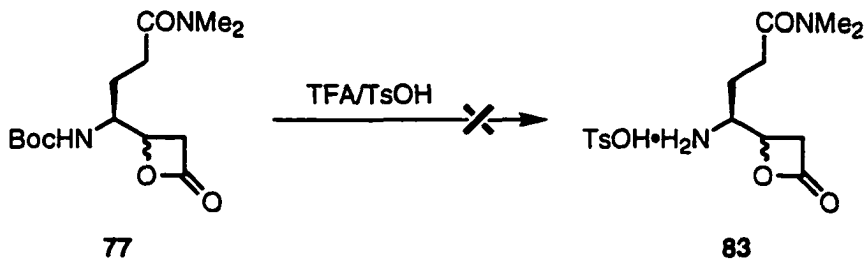


17 **77**

With β-lactone **77** available, only two steps remain to reach target **C** (**11**) in Scheme 13, deprotection of β-lactone **77** and its coupling to tripeptide **34** (Ac-Leu-Ala-Ala-OH). Methodology for *N*-Boc deprotection of β-lactone amino acids is well precedented in our group, using a mixture of trifluoroacetic acid and *p*-toluenesulfonic acid.[95,96] Treatment of β-lactone **77** with anhydrous trifluoroacetic acid and one equivalent

of *p*-toluenesulfonic acid gave none of the expected product **83** and resulted in loss of the IR carbonyl absorbance at 1830 cm^{-1} (Scheme 22).

Scheme 22



77 **83**

The instability of β-lactone **77** during *N*-Boc deprotection, compounded by its tendency to polymerize in organic solvent, prompted us to explore the stability of **77** in different solvent environments (Table 3). The presence or absence of β-lactone **77**, immediately upon exposure to varying solvent conditions is indirectly detected using the unique IR carbonyl stretch absorbance at 1830 cm^{-1}.

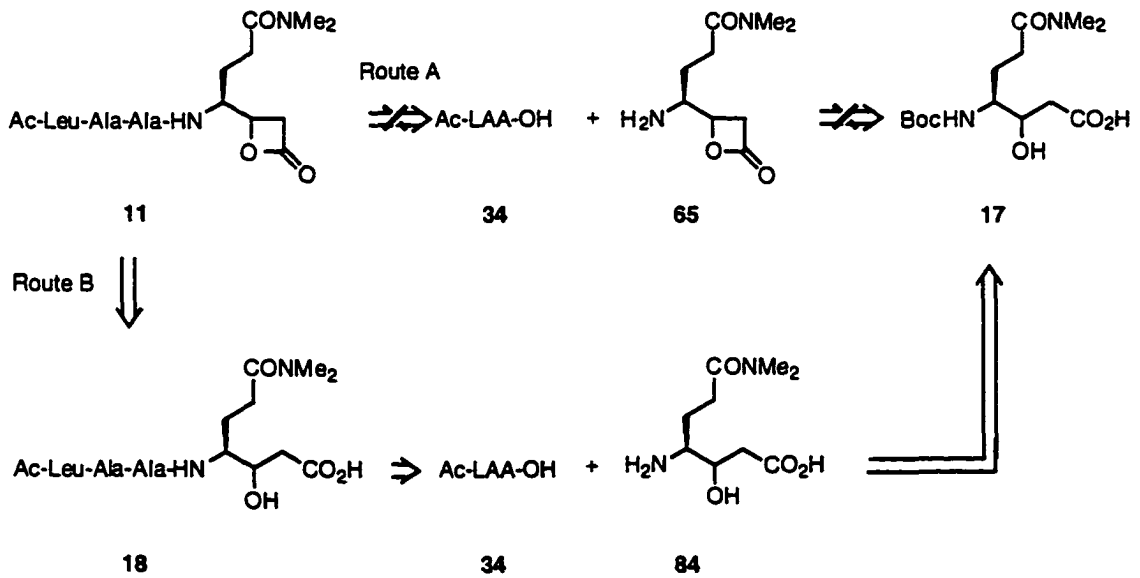
Table 3 Stability of β-lactone **77** in different solvent environments

Solvent	IR carbonyl absorbance at 1830 cm^{-1}
$CHCl_3$	present
CH_3CN / H_2O (1 : 1)	absent
CH_3CN / H_2O / TFA (1 : 1 : 0.001)	absent
CH_3OH / H_2O / TFA (1 : 1 : 0.001)	absent
$(CH_3)_2CHOH$ / H_2O / TFA (1 : 1 : 0.001)	absent

It is clear that β-lactone **77** decomposes rapidly when exposed to moisture, possibly as a consequence of very rapid hydrolysis of the β-lactone functionality. As a result of the instability of **77**, a different strategy was employed for the construction of target **C (11)**.

An alternative retrosynthetic path to target **C (11)** is presented in Scheme 23 (route B), which uses common intermediate **17** from route A. Preparation of the β-lactone functionality within the framework of a tetrapeptide could potentially stabilize **11**, avoiding the aqueous instability of **77**, and minimizing manipulations of compounds bearing this sensitive moiety.

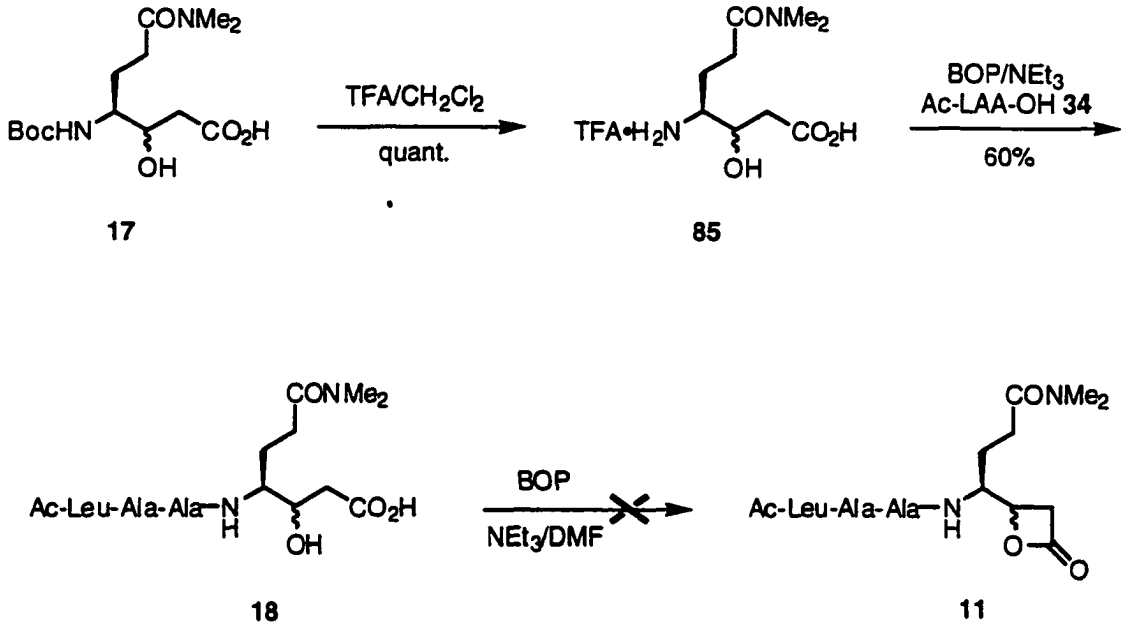
Scheme 23



Trifluoroacetate salt **85** is prepared by treating β-hydroxy acid **17** with 50% trifluoroacetic acid in dichloromethane, as outlined in Scheme 24. Coupling of tripeptide **34** with trifluoroacetate salt **85** is accomplished with BOP reagent in the presence of triethylamine in 60% yield. Treatment of tetrapeptide β-hydroxy acid **18** with BOP reagent in the

presence of triethylamine gave no indication, by TLC (bromocresol green spray) or IR spectroscopy, for the formation of β-lactone **11**.

Scheme 24



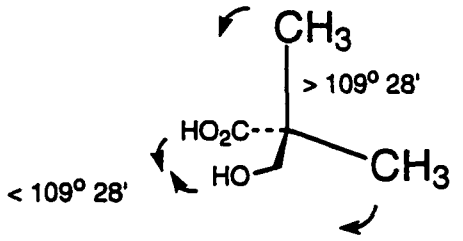
17 **85** **18** **11**

The inability to prepare target **C (11)** by either route A or B outlined in Scheme 23, combined with the instability associated with β-lactone **77** (hydrolysis under aqueous conditions and polymerization in organic solvent) is problematic for drug development. However, if related β-lactone molecules with greater stability can be designed, they may demonstrate inhibition of cysteine proteinases and show promise as drug leads.

1.3.3 Dimethyl β-Lactone Design

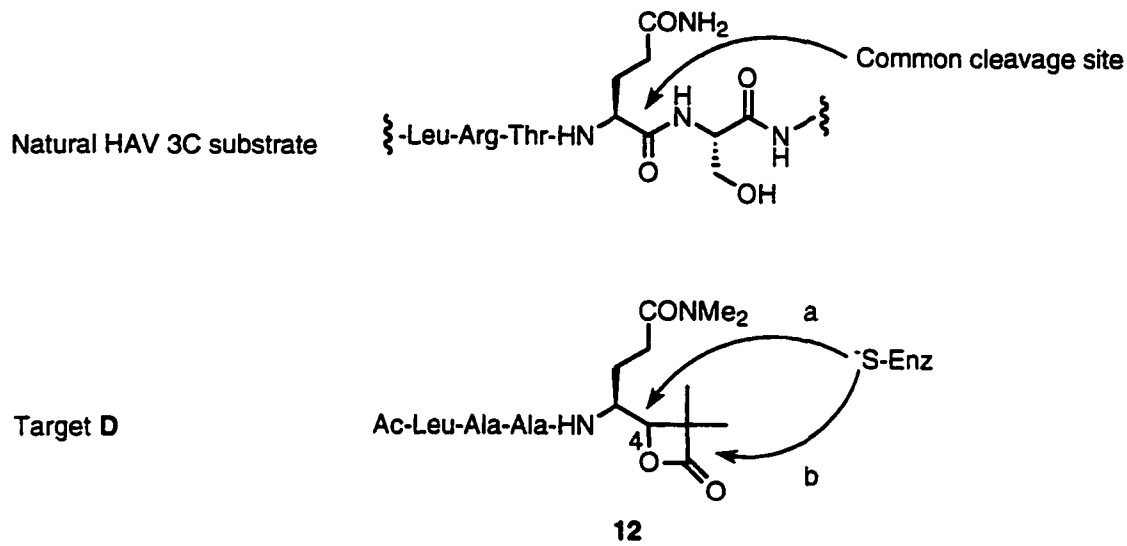
In order to stabilize β-lactone molecules such as **11** and **77**, methyl substituents can be placed at the α-position of the oxetanone ring to decrease the angle strain of the four membered ring. If two bulky groups attached to a tetrahedral carbon atom are separated by a distance approaching the sum of their van der Waals radii they tend to repel one another, and consequently the bond angle between one pair of groups on the tetrahedral carbon atom is increased to a value greater than 109° 28' causing the bond angle between the other groups to decrease below the tetrahedral angle. This observation is called the *Thorpe-Ingold* effect,[97] and is shown schematically in Figure 21.

Figure 21 Schematic illustration of the *Thorpe-Ingold* effect



Based on the *Thorpe-Ingold* effect, a modified dimethyl β-lactone target **D** molecule (**12**) was designed (Figure 22). The methyl groups at the α-position may potentially provide sufficient steric hindrance at the β-position to direct thiolate attack to the carbonyl during interaction with HAV 3C (path b, Figure 22).

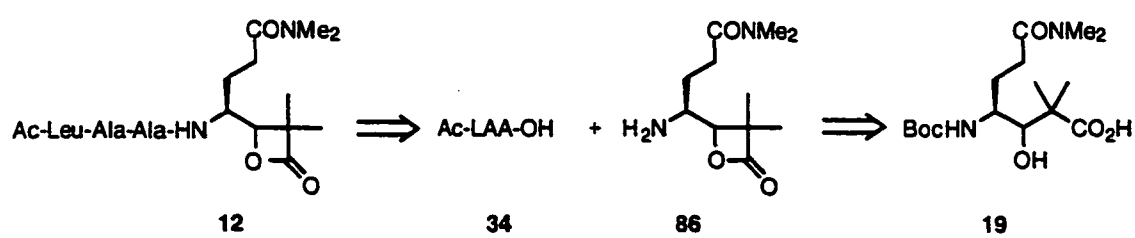
Figure 22 Rationale for target **D**



1.3.4 Synthetic Studies Towards Target D

Retrosynthetic analysis of target **D** (**12**) is based on the strategy outlined in Scheme 25. The target molecule can be derived from the tripeptide Ac-Leu-Ala-Ala-OH **34** and Gln(NMe_2) dimethyl β-lactone **86**. The key dimethyl β-lactone **86** could, in principle, be synthesized by cyclization of β-hydroxy carboxylic acid **19**.

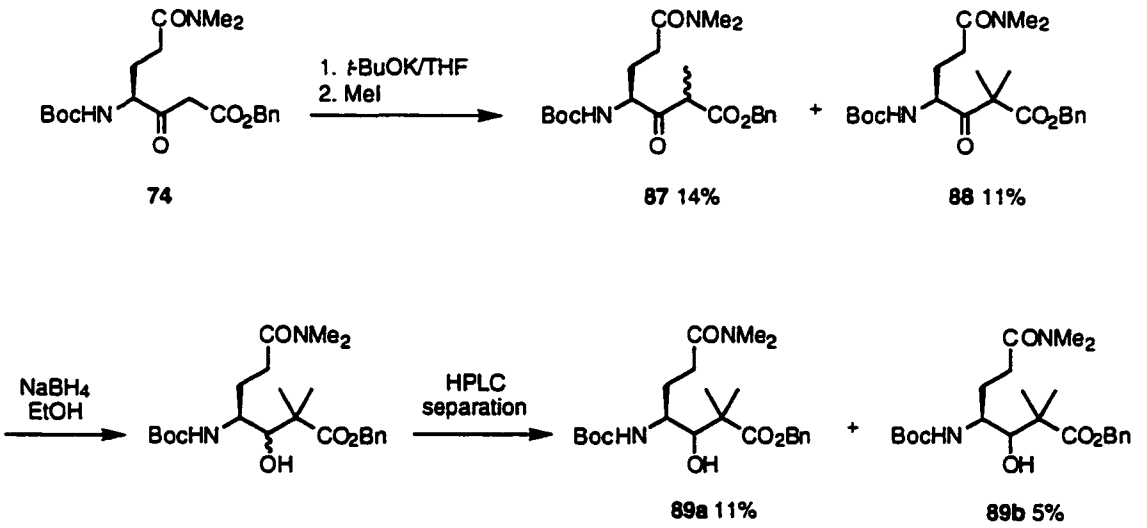
Scheme 25



Treatment of β-keto ester **74** with potassium *tert*-butoxide in THF, followed by two equivalents of iodomethane, provides dimethyl β-keto ester **88** and monomethyl β-keto

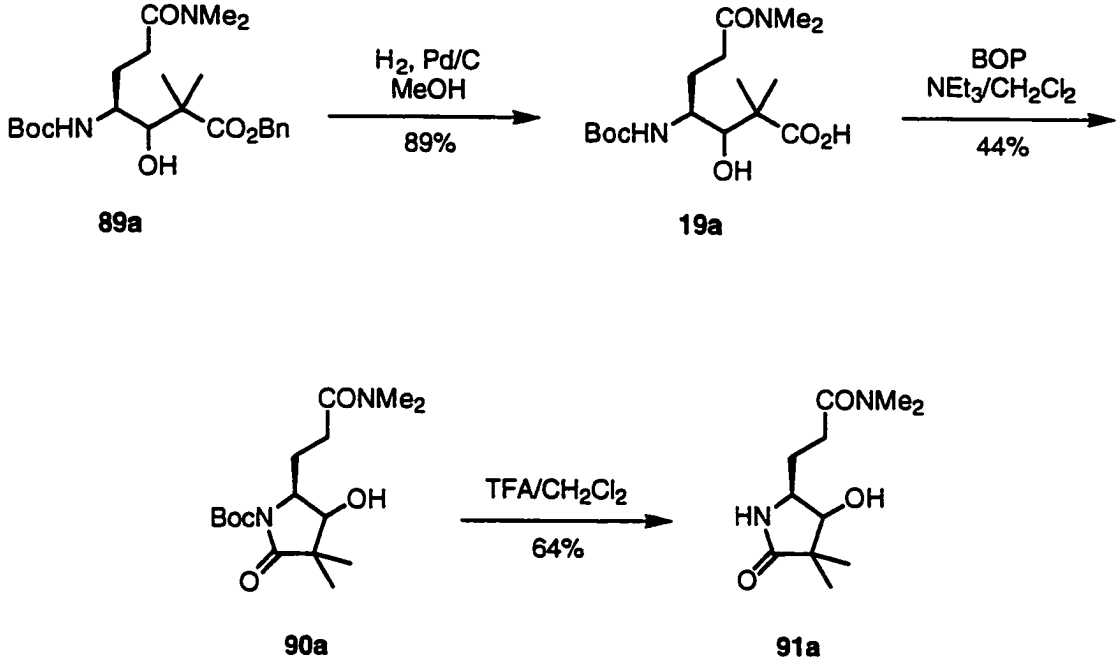
ester **87**, in low yields (Scheme 26).[98] Reduction of **88** with $NaBH_4$ in ethanol, followed by acidic work-up, generates the required dimethyl β-hydroxy ester as a mixture of diastereoisomers, which are separated by HPLC to afford **89a** and **89b**.[67] The relative stereochemistry at the carbon bearing the hydroxyl group for diastereomers **89a** and **89b** could not be unambiguously assigned by NMR.

Scheme 26



The synthesis was continued with diastereoisomer **89a** (Scheme 27). Hydrogenation of benzyl ester **89a** using 10% palladium on charcoal in methanol provides the desired dimethyl β-hydroxy acid **19a**.[66] Cyclization of β-hydroxy acid **19a**, with BOP reagent in the presence of triethylamine gives *N*-Boc-γ-lactam **90a** with no detectable desired dimethyl β-lactone.

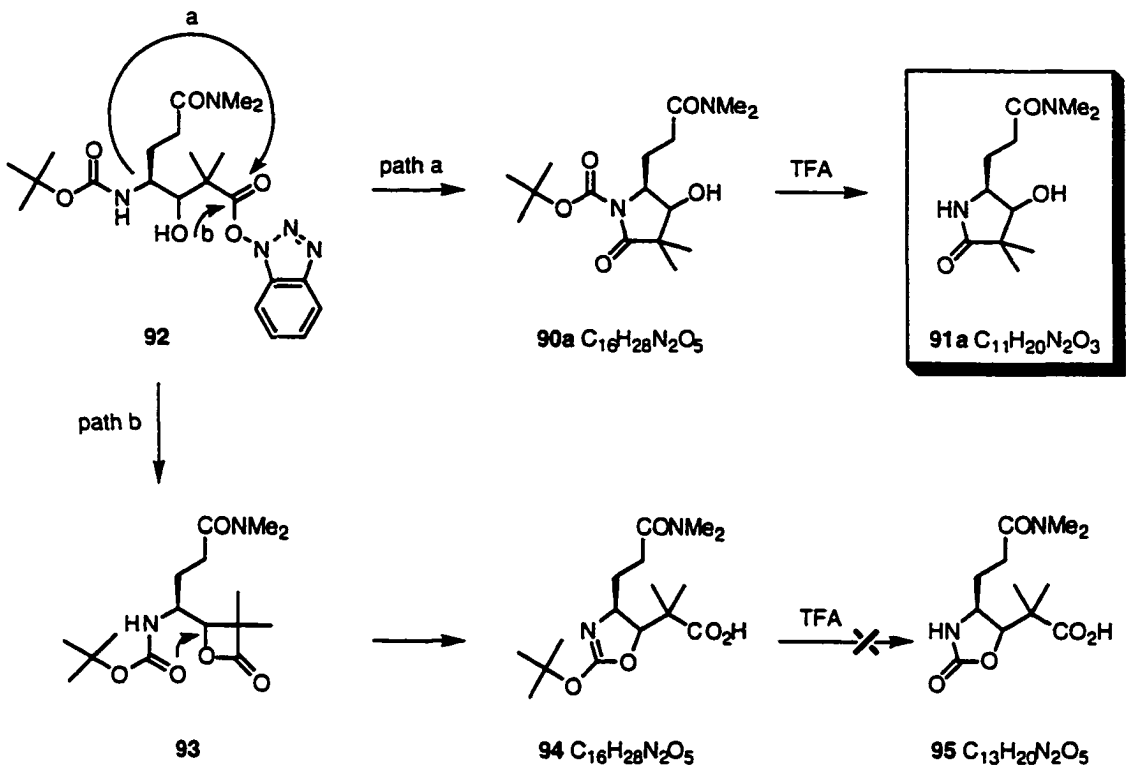
Scheme 27



89a **19a** **90a** **91a**

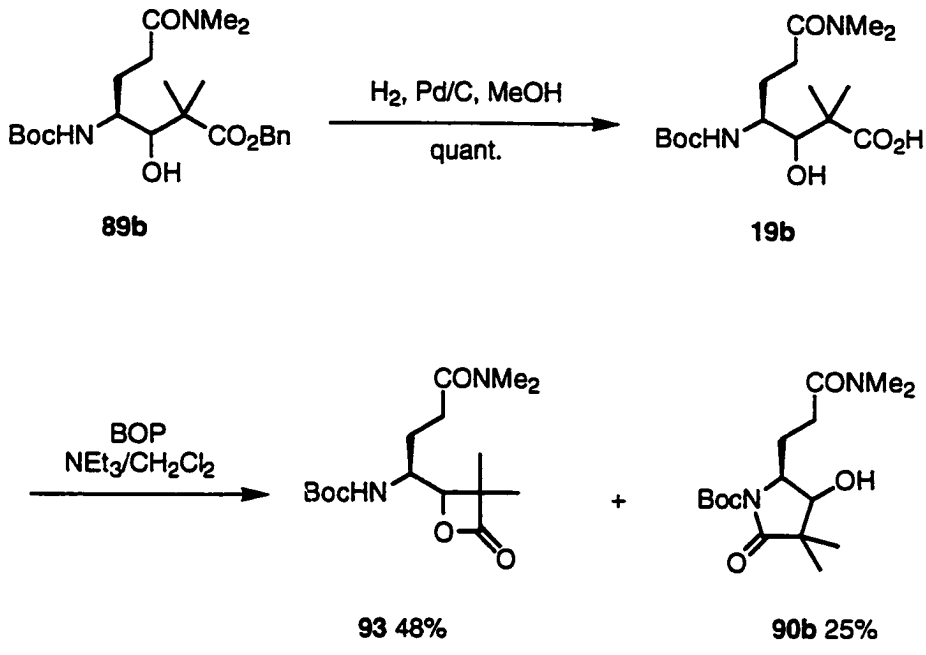
Product **90a** may arise from intramolecular nitrogen cyclization of active ester **92** to give the favored five-membered ring (**90a**) (path a, Figure 23), rather than the desired four-membered β-lactone ring. However, an alternative structure to **90a** is molecule **94**, arising from initial formation of the desired dimethyl β-lactone **93** (kinetic product) which then undergoes a rearrangement to oxazoline **94** (thermodynamic product) (path b, Figure 23). Both molecule **90a** and **94** have the same molecular formula ($C_{16}H_{28}N_2O_5$), although they can be easily distinguished from one another upon deprotection (Figure 23). Hence, treatment of **90a** with 50% trifluoroacetic acid in dichloromethane provides γ-lactam **91a** (Scheme 27). γ-Lactam **91a** has the molecular formula ($C_{11}H_{20}N_2O_3$), which distinguishes it from **95** ($C_{13}H_{20}N_2O_5$), suggesting that the favored route is path a in Figure 23.

Figure 23 Plausible side-product structures **90a** and **94**



With no formation of desired dimethyl β-lactone **93** from diastereoisomer **89a** (Scheme 27), the analogous synthesis was repeated with diastereoisomer **89b**, as shown in Scheme 28. Hydrogenation of benzyl ester **89b**, using 10% palladium on charcoal in methanol, provides the desired dimethyl β-hydroxy acid **19b**.[66] Cyclization of β-hydroxy acid **19b** then ensues, with BOP reagent in the presence of triethylamine, to give *N*-Boc-γ-lactam **90b** and the desired dimethyl β-lactone **93**, in 25% and 48% yields respectively. Similar to β-lactone **77**, molecule **93** displayed a similar tendency to polymerize[94] when exposed to organic solvent ($CHCl_3$) for a long duration, although β-lactone **93** showed enhanced stability to moisture (purified by HPLC in acetonitrile / water).

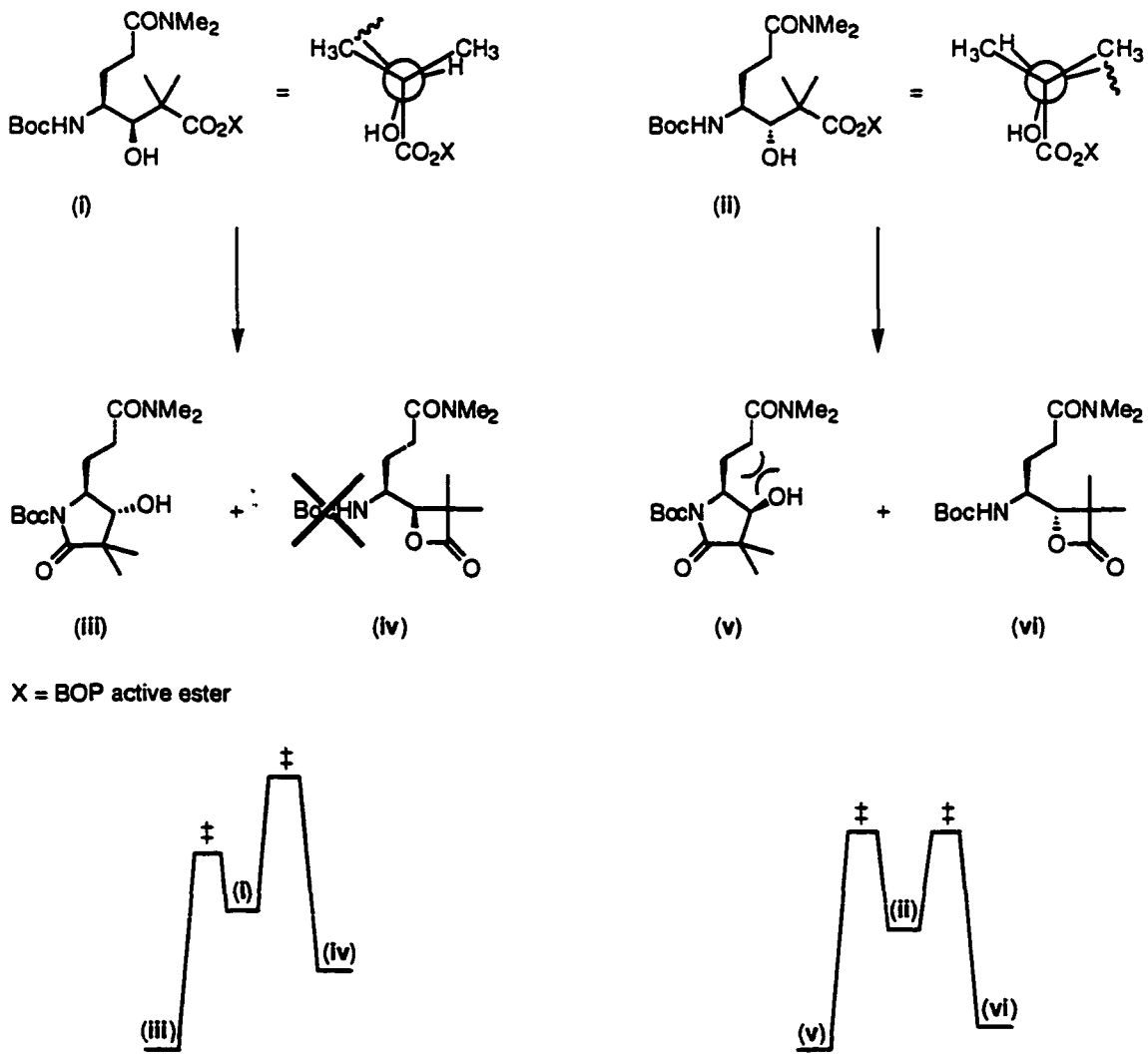
Scheme 28



Although the relative stereochemistry of diastereoisomers **19a / 19b** and their transformed products cannot be unambiguously assigned at this stage (Schemes 26-28), insight into their possible stereochemistry can be gained from their product distribution (Figure 24). The *Curtin-Hammett principle*, states that for competing reactions the product distribution is determined by the difference in activation energies for the two paths.[99] The fact that only one of the **19a / 19b** diastereoisomers gives desired dimethyl β-lactone product provides information. In Figure 24, the Newman projections (**i**) and (**ii**) suggest that ring strain and steric hindrance in both diastereoisomers hinder the formation of β-lactone from the BOP active ester; the activation energies that lead to dimethyl β-lactones (**iv**) and (**vi**) are high in energy. The presence of an alternative reaction path which provides products (**iii**) and (**v**), suggests that the activation energies to form these products are not equal to those that lead to the corresponding β-lactones. This may be the result of additional steric hindrance that product (**v**) encounters as a result of the syn relationship between the hydroxyl group and the adjacent groups, which is present to a lesser degree in

product (**iii**) (Figure 24). Therefore, the activation energy to generate (**iv**) must be higher than that giving product (**iii**), thus accounting for the formation of only product (**iii**) and no desired dimethyl β-lactone (**iv**). The situation is very different for products (**v**) and (**vi**). Their corresponding activation energies are probably both very high as a result of the syn relationship of the hydroxyl and adjacent groups in the transition-state to form product (**v**), and the ring strain in the transition-state to form product (**vi**). This could account for the formation of both products (**v** and **vi**) without any product discrimination.

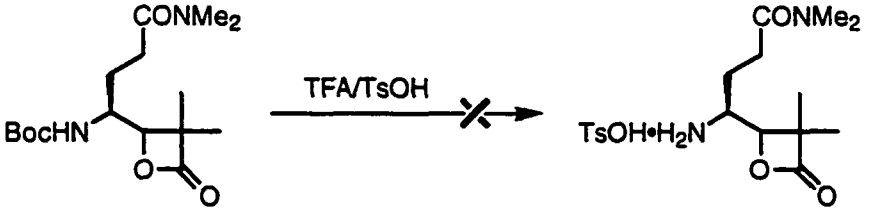
Figure 24 Stereochemistry inference from product distribution



The inferred stereochemical assignments in Schemes 26-28 from the products isolated would be as follows: (i) = **19a**, if x = H; (ii) = **19b**, if x = H; (iii) = **90a**, (v) = **90b**, (vi) = **93**. Hence, the stereogenic carbon assignment bearing the hydroxyl group for diastereoisomer **89a** would be *R*-configuration, and for **89b** *S*-configuration (Scheme 26). Unfortunately, such stereochemical assignment from product distribution is not definitive and the above configuration assignments are not without ambiguity.

Nevertheless, continuing with the synthesis of target **D** (**12**), an attempt was made to deprotect dimethyl β-lactone **93**. Treatment of β-lactone **93** with anhydrous trifluoroacetic acid and one equivalent of *p*-toluenesulfonic acid gave no desired product **96** and resulted in loss of the IR carbonyl absorbance at 1825 cm^{-1} (Scheme 29).[95,96]

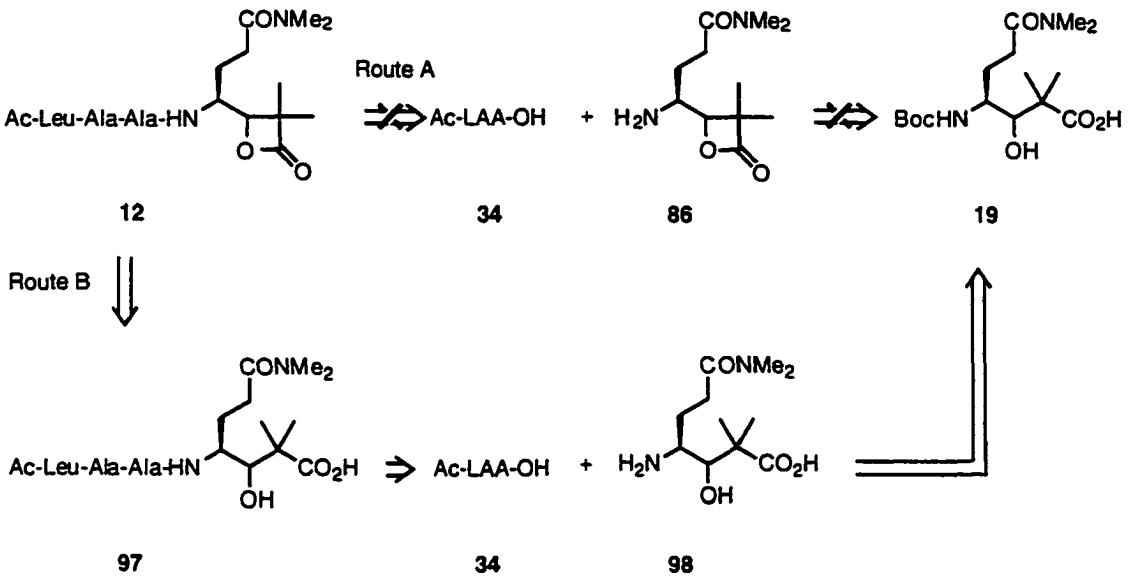
Scheme 29



93 **96**

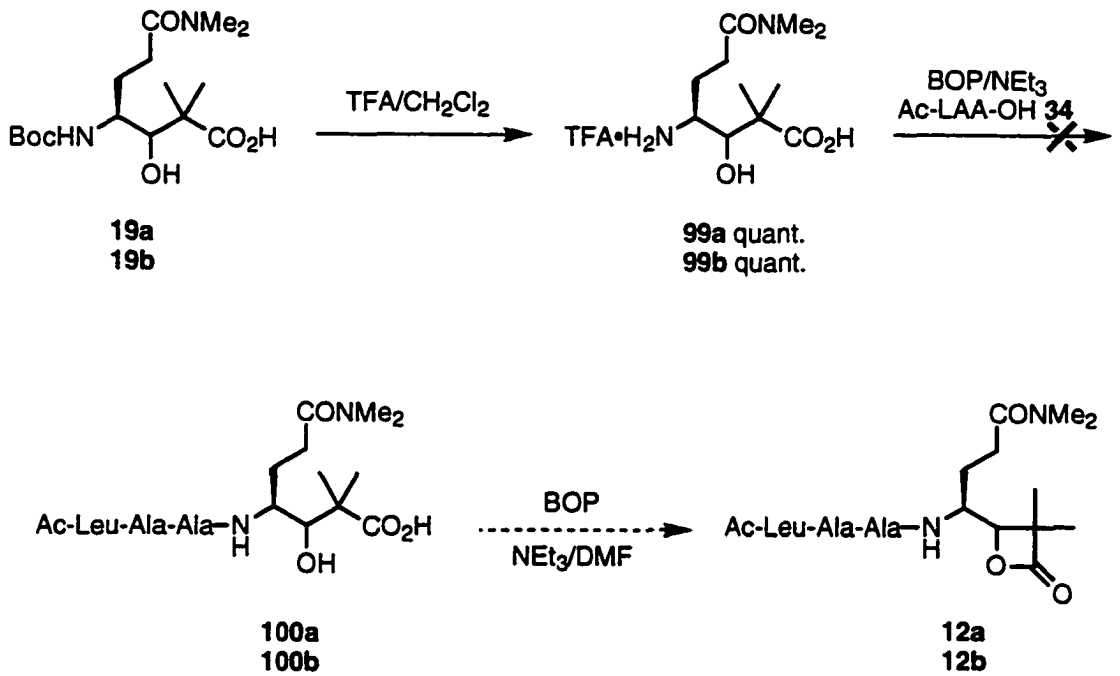
With this failure to successfully deprotect dimethyl β-lactone **93** an alternative retrosynthetic path, similar to that tried in the synthesis of target **C** (**11**), was investigated. This is outlined in Scheme 30 (route B) and uses a common intermediate (**19**) from route A. Again, preparation of the dimethyl β-lactone functionality within the framework of the tetrapeptide may induce inherent stability in molecule **12**, avoiding instability problems observed with molecule **93**.

Scheme 30



Deprotection of individual diastereoisomers **19a** and **19b** proceeds smoothly upon treatment with 50% trifluoroacetic acid in dichloromethane to afford trifluoroacetate salts **99a** and **99b** in quantitative yield (Scheme 31). Unfortunately, coupling of tripeptide **34** to either diastereoisomer salt **99a** or **99b** is unsuccessful in the presence of BOP reagent and triethylamine. The inability to couple either diastereoisomer salt **99a** or **99b** to tripeptide **34** may reflect the increased steric bulk the dimethyl substituents bestow upon **99a** and **99b**. Despite inability to couple tripeptide **34** to give **12**, monomer Gln(NMe_2) dimethyl β-lactone **93** was assayed against HAV 3C proteinase for inhibition.

Scheme 31



19a
19b

99a quant.
99b quant.

100a
100b

12a
12b

1.3.5 Inhibition of HAV 3C Proteinase by Target D Monomer

Target **D** monomer **93** was assayed against HAV 3C proteinase using a continuous fluorogenic assay,[83] which is described in the experimental section. Compound **93** gave a disappointing, 12% inhibition value (no enzyme inhibitor pre-incubation) against HAV 3C at an enzyme concentration of 0.1 μM and an inhibitor concentration of 100 μM.

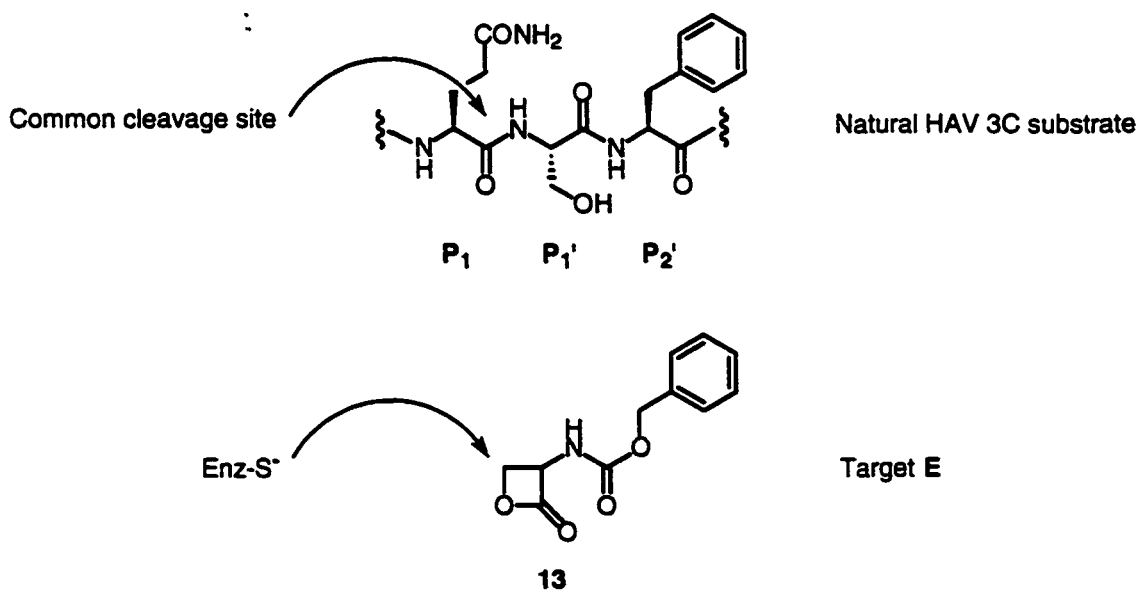
In summary, β-lactone target **C** and **D** monomer compounds (**77** and **93**), were designed as potential inhibitors of HAV 3C proteinase. They were synthesized through key intermediate, β-hydroxy acids **17** and **19b**. Target **C** type compound **77** showed poor stability in aqueous environments, limiting its use for inhibition studies. Whereas, target **D** compound **93** displayed increased aqueous stability, it showed only a disappointing 12% inhibition of against HAV 3C. Although the exact reasons for the failure to inhibit HAV 3C remain unknown, it may be that the lack of binding is due to lack of the tripeptide (Ac-Leu-Ala-Ala-OH) substrate recognition portion. Also the steric bulk introduced by the dimethyl

substituents may hinder accommodation of β-lactone **93** in the active site and / or attack by thiolate. Nevertheless, such compounds could still be tested with other cysteine proteinases.[39] In addition, modification of the β-lactone moiety may furnish compounds which have increased aqueous stability and show efficacy against HAV 3C proteinase.

1.3.6 *N*-Cbz-Serine-β-Lactone Design

The attempted syntheses of target **C** and target **D** type β-lactone molecules suggest that the design of a P-side inhibitor is problematic, due to nitrogen-carbonyl cyclization (side-products **90a** and **90b**), polymerization and hydrolytic sensitivity. Therefore, the design of a β-lactone which utilizes essential structural features of the HAV 3C substrate P'-side may show promise as an inhibitor. It seemed that *N*-Cbz-serine-β-lactone **13** in Figure 25, would be a reasonable initial target for 3C proteinase inhibition because its benzyl group may mimic the P_2' phenylalanine side chain in HAV 3C substrates.[22,36,46] As discussed previously, the enzyme-inhibitor complex formed with peptidyl iodoacetamide **7** (P'-side inhibitor) suggests that this side chain plays a key role in substrate recognition in HAV 3C.[46] β-Lactone **13** is also appealing because of its facile preparation by Mitsunobu cyclization of *N*-Cbz-serine,[53] and the fact that its simple scaffold would permit variation for subsequent structure-activity studies.

Figure 25 Rationale for target **E**

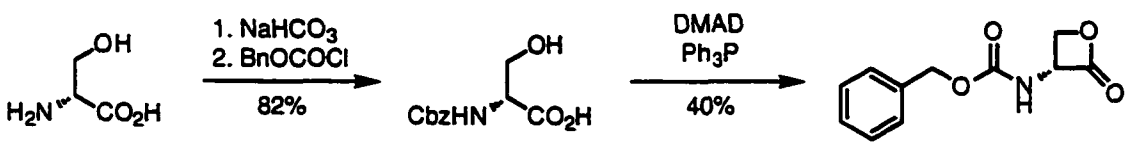


1.3.7 Synthesis of Target E and Analogues

N-Cbz-Serine-β-lactone target **E** (**13**) is not a peptide, although the molecule does contain the core α-amino acid building block. *N*-Acyl-α-amino-β-lactones are useful synthetic intermediates for the synthesis of stereochemically pure β-substituted α-amino acids,[100-102] and are readily available by cyclization of the appropriately protected β-hydroxy-α-amino acids.[102-104]

Target **E** (**13**) is readily prepared following the literature procedure (Scheme 32).[102] Nitrogen protection is accomplished by treating D-serine **101b** with benzyl chloroformate in the presence of aqueous sodium bicarbonate to afford *N*-benzyloxycarbonyl-D-serine **102b**. Cyclization of *N*-Cbz-D-serine **102b** under modified Mitsunobu conditions, using the preformed adduct of triphenylphosphine and dimethyl azodicarboxylate, gives β-lactone **13b** without loss of optical purity. The corresponding *N*-Cbz-L-serine-β-lactone enantiomer **13a** was prepared previously in our group by Dr. Lee Arnold, a former graduate student, in a similar manner to that presented in Scheme 32. Polymerization of **13a** or **13b** was not detected under prolonged exposure to organic solvent ($CHCl_3$).

Scheme 32

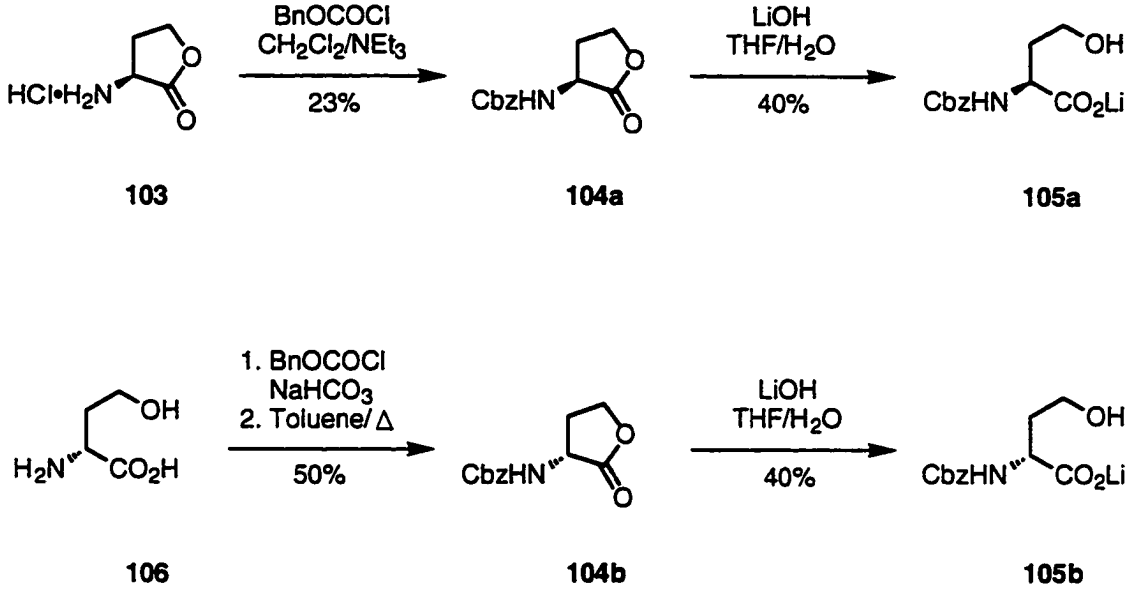


101b **102b** **13b**

As observed earlier with β-lactone **77**, a possible concern with β-lactones **13a** and **13b** is their susceptibility to hydrolysis, since α-amino-β-lactones bearing no β-substituent display low stability in basic aqueous media.[53] Utilizing IR spectroscopy, the rate of β-lactone hydrolysis in the enzyme buffer solution was determined by following the disappearance of the unique β-lactone carbonyl stretch (~1830 cm^{-1}), as described in the experimental section. The half-life for hydrolysis of **13a** in phosphate buffer at pH 7.5 is 76 min, which is sufficiently long for enzyme inhibition studies.

To provide structural diversity and probe the importance of the β-lactone ring for HAV 3C inhibition, *N*-Cbz-homoserine **105a**, **105b** and γ-lactone **104a**, **104b** analogues were prepared by Constantine Karvellas, a summer student in our group (Scheme 33). γ-Lactones are well known to undergo ring opening as a result of nucleophilic attack at the carbonyl,[106] and some show biological activity against serine proteinases[107] as well as thiol containing enzymes.[108] In addition, nucleophilic attack by thiol[109] and hydroxyl[110] has also been observed at the γ-position of the γ-lactone ring. Treatment of L-homoserine lactone **103** with benzyl chloroformate, in the presence of triethylamine, produces desired γ-lactone **104a**.[105] Lithium salt **105a** is prepared by hydrolysis of γ-lactone **104a** with lithium hydroxide. The enantiomer γ-lactone **104b** is obtained by treating D-homoserine **106** with benzyl chloroformate in the presence of aqueous sodium bicarbonate, followed by heating under reflux in a Soxhlet apparatus (Scheme 33).[105] Hydrolysis of γ-lactone **104b** with lithium hydroxide affords lithium salt **105b**.

Scheme 33

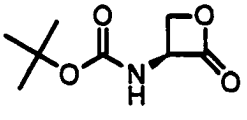


103 **104a** **105a**

106 **104b** **105b**

To determine the importance of the benzyl group, the *N*-Boc-L-serine-β-lactone **107** analogue (Figure 26), which was prepared previously by Dr. Lee Arnold,[111] in a manner analogous to that in Scheme 32, using *N*-Boc-L-serine **108** in place of *N*-Cbz-L-serine, was also tested in inhibition studies with HAV 3C proteinase.

Figure 26 *N*-Boc-L-Serine-β-lactone

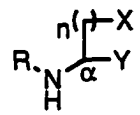


107

1.3.8 Inhibition of HAV 3C and HRV-14 3C Proteinases by Target E and Analogues

Target E compounds **13a**, **13b** and analogues **101a**, **101b**, **104a**, **104b**, **105a**, **105b**, **107** and **108** were assayed with HAV 3C proteinase using a continuous fluorogenic assay,[83] as described in the experimental section. The IC_{50} values were measured without pre-incubation with the enzyme, (Table 4).

Table 4 HAV 3C inhibition results for target **E** β-lactones and analogues



Compd.	n	R	X	Y	α-config.	$IC_{50}(\mu M)^a$	$t_{1/2}(min)^b$
13a	1	Cbz	O----------CO		*S*	35	76
13b	1	Cbz	O----------CO		*R*	6	n.d.
101a	1	Cbz	OH	CO_2H	*S*	>>100	n.d.
101b	1	Cbz	OH	CO_2H	*R*	>>100	n.d.
104a	2	Cbz	O----------CO		*S*	>>100	n.d.
104b	2	Cbz	O----------CO		*R*	>>100	n.d.
105a	2	Cbz	OH	CO_2Li	*S*	>>100	n.d.
105b	2	Cbz	OH	CO_2Li	*R*	>>100	n.d.
107	1	Boc	O----------CO		*S*	>100	285
108	1	Boc	OH	CO_2H	*S*	>>100	n.d.

[a] ([HAV 3C] = 0.1 μM, [Compd.] = 0.5-500 μM), see experimental section for details.
[b] β-Lactone hydrolysis half-life in phosphate buffer pH 7.5; n.d. = not determined

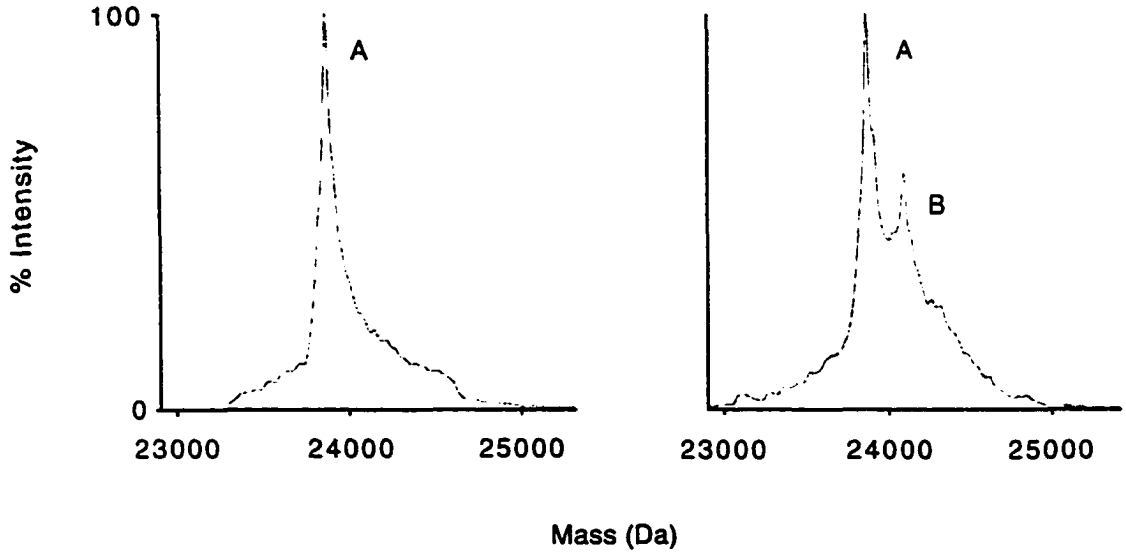
Despite the absence of the P_1 glutamine side chain important for HAV 3C substrate recognition, β-lactones **13a** and **13b** are potent inhibitors of HAV 3C proteinase with IC_{50} values of 35 and 6 μM respectively (Table 4). Further studies revealed that **13a** is a time-dependent *irreversible* inhibitor of HAV 3C proteinase ($k_{inact} = 0.012$ sec^{-1}, $K_I = 1.84 \times 10^{-4}$ M, $k_{inact} / K_I = 63$ M^{-1} sec^{-1}) at an enzyme concentration of 0.1 μM. Interestingly, the enantiomer **13b** is a competitive *reversible* inhibitor of HAV 3C proteinase ($K_I = 1.50 \times 10^{-6}$ M). The possibility that compound **13b** may in fact be a time-dependent inhibitor, but that this is not observed under the assay conditions, can be eliminated because studies at different pH conditions (e.g. pH 6) and with varying concentrations of inhibitor **13b** also display simple competitive behavior. Clearly the HAV 3C active site shows different modes of binding for enantiomers **13a** and **13b**, with only the former leading to permanent covalent modification of the active site. Further, the inhibitory properties of **13a** and **13b** are not affected by short exposure to 10-fold molar excess of dithiothreitol, suggesting that β-lactones of this type could be specific enzyme inhibitors that would not react inadvertently with ubiquitous biological thiols (e.g. glutathione).

The acyclic analogues **101a**, **101b**, **105a**, **105b** and **108**, at a concentration of 100 μM, show no significant inhibition of HAV 3C proteinase. The lack of inhibition could potentially be due to the anionic charge of the carboxylate at pH 7.5, however, the non-charged cyclic analogues **104a** and **104b** at 100 μM also fail to show any inhibition of this enzyme. An interesting observation is that *N*-Boc-serine-β-lactone **107** displays weak ($IC_{50} > 100$ μM) time-dependent inhibition of HAV 3C, suggesting that the benzyl group of **13a** plays an important binding role in HAV 3C inhibition. This provides further evidence for the essential role the P_2' phenylalanine side-chain may play in recognition of HAV 3C substrates.[46]

The kinetic observations with **13a** are consistent with a rapid covalent inactivation of HAV 3C proteinase. To confirm this, electrospray mass spectrometry was used to examine the enzyme after treatment with **13a**. The spectra of the uncomplexed enzyme and the HAV

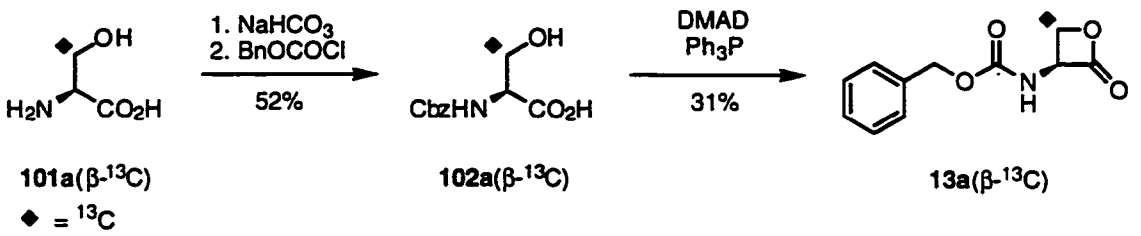
3C-**13a** complex are shown in Figure 27. The mass difference between the enzyme-inhibitor complex and the uninhibited enzyme (219 Da) is within experimental error of the calculated mass of the inhibitor (221 Da). A control dialysis experiment was performed on the HAV 3C-**13a** complex and uninhibited HAV 3C. After dialysis with buffer for 8 h at 4 °C, the uninhibited enzyme retained activity, but the HAV 3C-**13a** complex showed no recovery of proteinase activity, thus confirming that **13a** is probably covalently attached rather than tightly held in a non-covalent complex.

Figure 27 Electrospray mass spectra of HAV 3C proteinase (left spectra: A = 23,880 Da) and HAV 3C-**13a** enzyme inhibitor complex (right spectra: A = 23,882 Da, B = 24,101 Da).



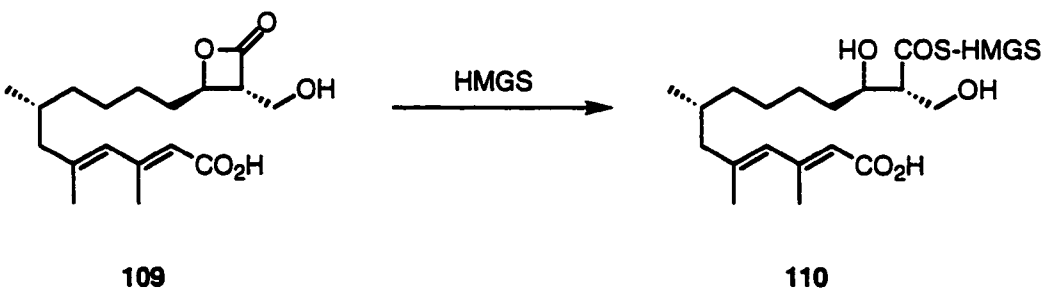
To determine by NMR spectroscopy the type of adduct formed between **13a** and the HAV 3C enzyme, **13a**(β-^{13}C) was synthesized, from the corresponding commercially available labeled L-serine (Scheme 34) by the same procedure used previously.[72,102]

Scheme 34



Cysteine residues in proteins can act as nucleophiles to open β-lactones by attack at the carbonyl, giving acylated products. For example, β-lactones such as the hypocholesterolemic agent (1233A) **109** may acylate a cysteine residue of 3-hydroxy-3-methylglutaryl CoA synthase (HMGS) to give **110**, illustrated in Figure 28.[112]

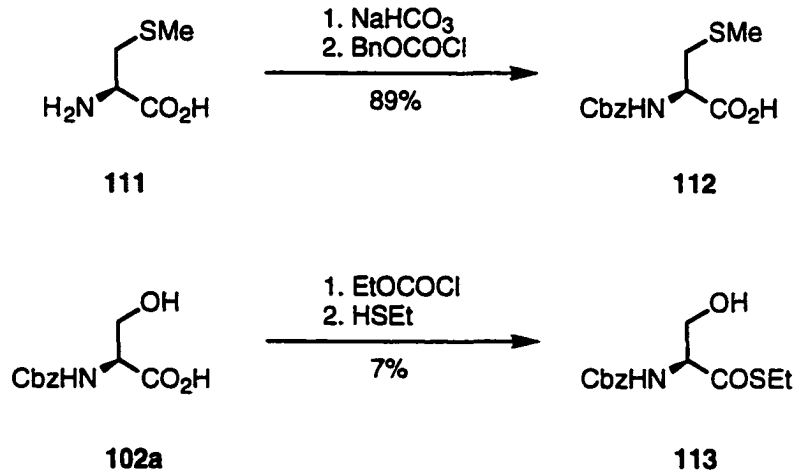
Figure 28 Inactivation of HMG CoA Synthase by the hypocholesterolemic agent **109**



109 **110**

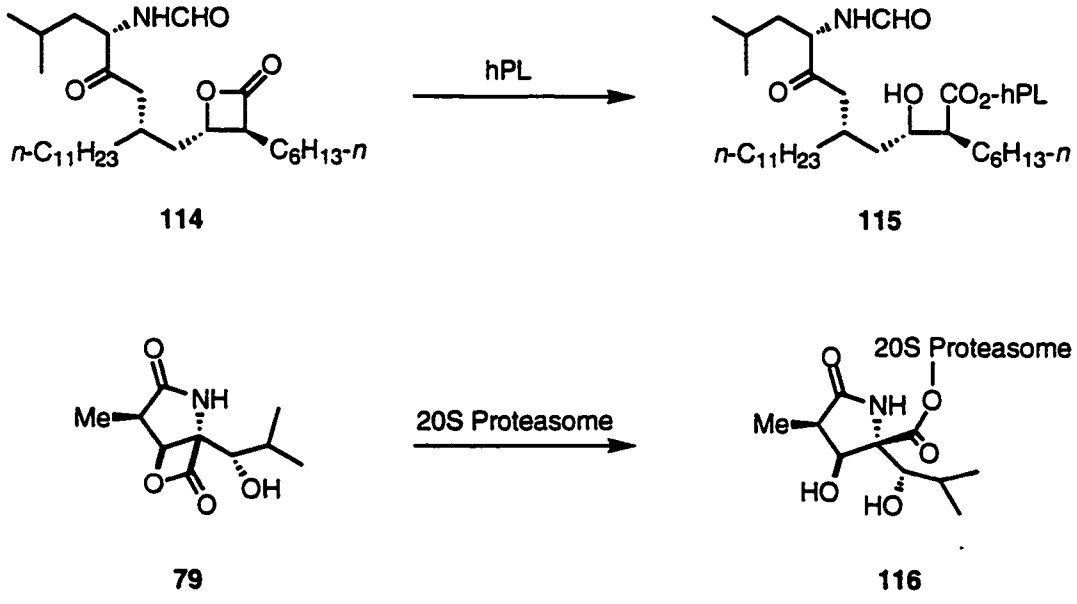
Therefore, model compounds sulfide **112** and thioester **113** were synthesized to assist in ascertaining, by their β-position chemical shifts, whether enzymatic thiolate attack on **13a** proceeds at the β-position to give the expected sulfide or at the carbonyl of the β-lactone to form a thioester (Scheme 35). Treatment of *S*-methyl cysteine **111** with benzyl chloroformate in the presence of aqueous sodium bicarbonate provides sulfide **112**. Thioester **113** is prepared by treating *N*-Cbz-L-serine **102a** in dichloromethane with ethyl chloroformate in the presence of triethylamine to form the mixed anhydride, which is then condensed with ethanethiol.

Scheme 35



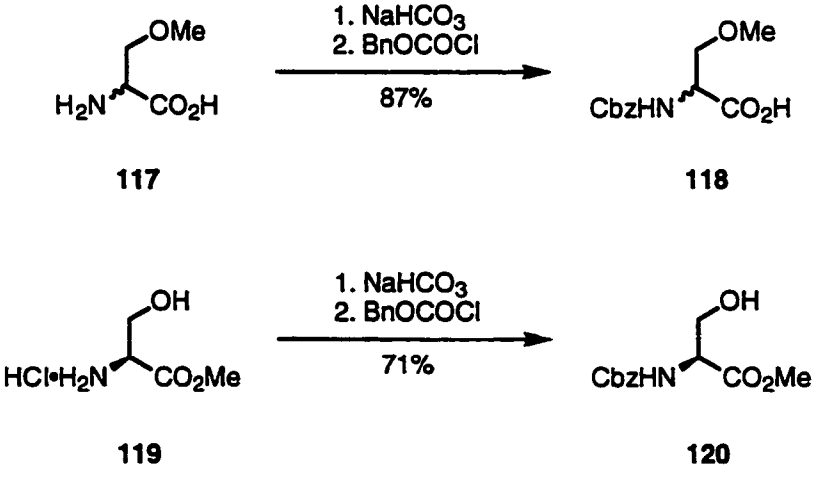
In addition, it is known that serine or threonine residues in proteins can also act as nucleophiles to open β-lactones by attack at the carbonyl to give acylated products. For instance, tetrahydrolipstatin (Orlistat) **114** inactivates human pancreatic lipase (hPL) by acylating a serine hydroxyl of the enzyme to give **115** (Figure 29)[113] and Omuralide **79**, the β-lactone precursor to lactacystin, has been shown to inhibit the 20S proteasome by *O*-acylation of a threonine residue, to give **116** (Figure 29).[114]

Figure 29 Acylation of hPL by **114** and 20S proteasome by **79**



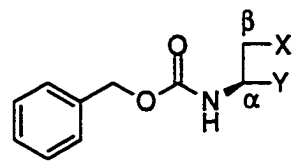
Hence, the chemical shifts of model serine ether **118** and ester **120** derivatives were also examined (Scheme 36). Treatment of *O*-methyl serine **117** with benzyl chloroformate in the presence of aqueous sodium bicarbonate affords serine ether **118**. Model serine ester **120** is prepared in a similar manner to **118** from L-serine methyl ester **119**.

Scheme 36



The carbon chemical shifts of the β-carbons for model NMR compounds **13a**(β-^{13}C), **102a**(β-^{13}C), **112**, **113**, **118** and **120** are presented in Table 5.

Table 5 Model β-carbon chemical shifts



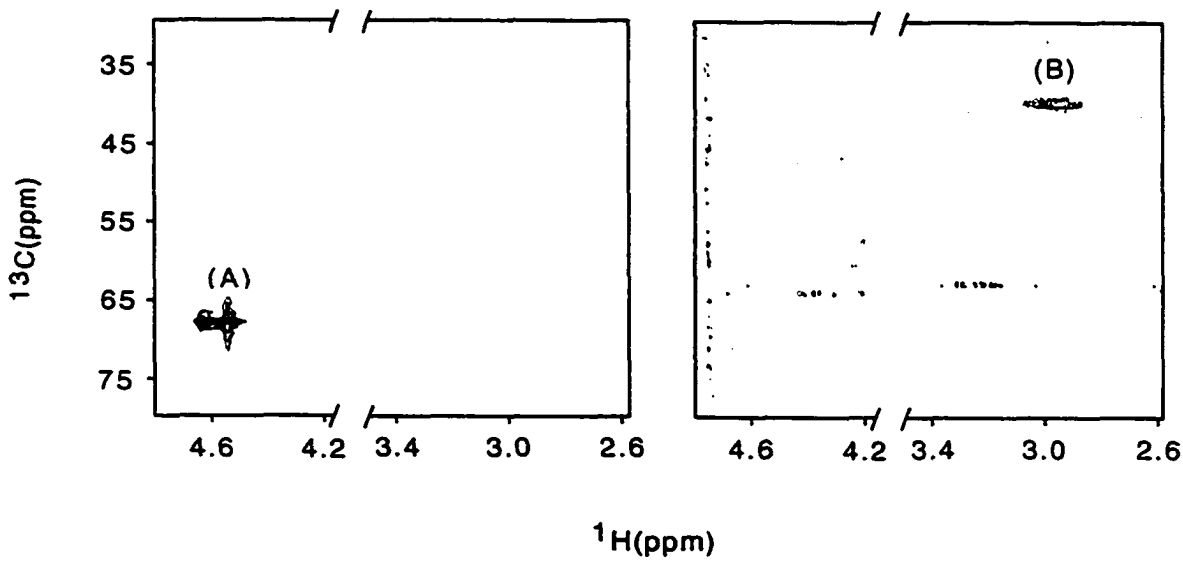
entry	X	Y	β-carbon(ppm)
13a(β-^{13}C)$^{\blacklozenge a}$	O———	CO	68
102a(β-^{13}C)$^{\blacklozenge b}$	OH	CO_2H	62
112^{b}	SMe	CO_2H	37
113^{b}	OH	COSEt	64
120^{b}	OH	CO_2Me	62
118^{b,c}	OMe	CO_2H	73

$^{\blacklozenge}$ = ^{13}C-Labeled at β-position. ^{1}H / ^{13}C HMQC
Conditions: ^{a} D_2O at pD 5.0, 6% DMSO-d_6. ^{b} 20mM Na_2PO_4 / D_2O at pD 7.5, 6% DMSO-d_6.
^{c} Racemic.

The heteronuclear multiple quantum coherence (HMQC) spectrum (Figure 30) of **13a**(β-^{13}C) shows a cross peak at 68 ppm for the labeled methylene carbon; upon addition of HAV 3C proteinase, this signal disappears and a new peak appears at 40 ppm on the carbon chemical shift axis. This signal for the enzyme inhibitor complex, i.e. HAV 3C-**13a**(β-^{13}C), clearly demonstrates formation of a thioether and thus *alkylation* of the active site cysteine by attack at the β-carbon of **13a**. The observed chemical shift is in good

agreement with that of the β-carbon of *N*-(benzyloxycarbonyl)-*S*-methyl-L-cysteine **112** (37 ppm) presented in Table 5.

Figure 30 ^{1}H / ^{13}C HMQC spectra of **13a**(β-^{13}C) inhibitor alone (left spectra) and in complex with HAV 3C proteinase (right spectra). Cross peak (A) shows the proton-carbon correlation of the unreacted inhibitor **13a**(β-^{13}C) and peak (B) is the cross peak for the β-thioalkylated adduct, i.e. HAV 3C-**13a**(β-^{13}C) enzyme inhibitor complex.

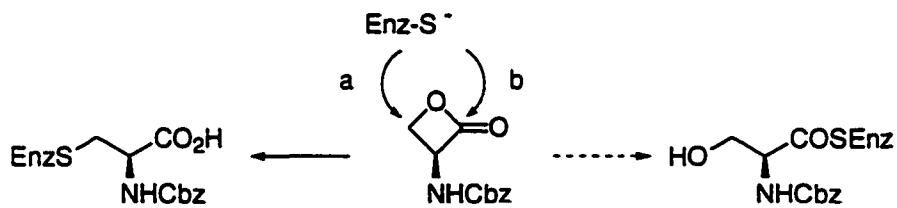


Target **E** compounds **13a** and **13b** were assayed with HRV-14 3C proteinase using a continuous colorimetric assay,[115] as described in the experimental section. Preliminary results with HRV-14 3C proteinase, an enzyme that has similar substrate specificity to HAV 3C and is a potential therapeutic target for the common cold,[22] show that β-lactones **13a** and **13b** have comparable potency. At an inhibitor concentration of 100 μM and HRV-14 3C concentration of 0.4 μM, β-lactones **13a** and **13b** gave 49% and 90% enzyme inhibition, respectively.

In summary, β-lactones **13a**, **13b** and analogues were synthesized. HAV 3C cysteine proteinase is inactivated by target **E** β-lactone **13a**, *via* nucleophilic ring opening

of the oxetanone ring at the β-position by the cysteine thiolate, path a, Figure 31. β-Lactone **13a** is a time-dependent *irreversible* inhibitor of HAV 3C proteinase with k_{inact} = 0.012 min^{-1}, K_I = 1.84 x 10^{-4} M, k_{inact} / K_I = 63 M^{-1} sec^{-1} at an enzyme concentration of 0.1 μM. In contrast, the enantiomer **13b** is a competitive *reversible* inhibitor of HAV 3C proteinase (K_i = 1.50 x 10^{-6} M), presumably because its β-methylene carbon is not correctly placed with respect to the required trajectory of the incoming cysteine thiolate nucleophile. The observation that γ-lactones **104a** and **104b** do not perturb HAV 3C activity, further exemplifies the unique reactivity of the oxetanone ring towards thiol containing biological molecules. Additional studies on structure-activity relationships for β-lactone inhibition of viral cysteine proteinases will help further define the medicinal potential of this new class of cysteine proteinase inhibitor.

Figure 31 Mode of HAV 3C nucleophilic attack on **13a**

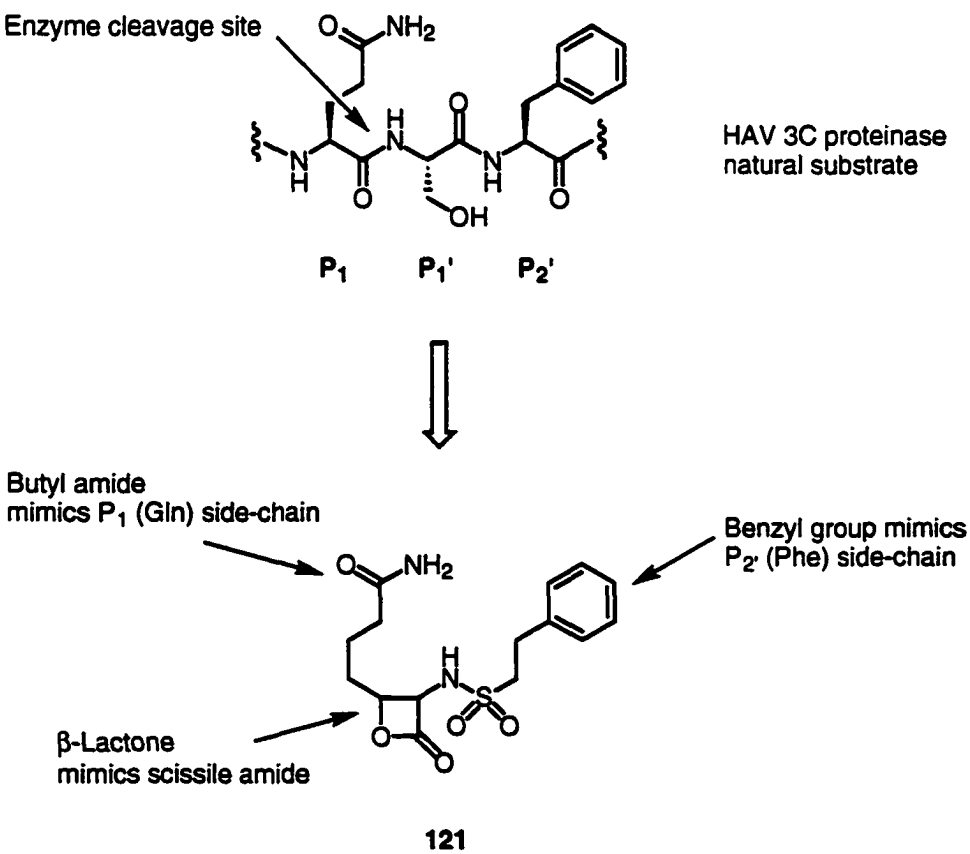


13a

1.3.9 *N*-Sulfonamide-Serine / Threonine-β-Lactone Design

With the successful synthesis of β-lactone leads **13a** and **13b**, which show efficacy against HAV and HRV 3C proteinases, the next step in inhibitor development is the introduction of enhanced specificity, stability and potency. Ideally, the β-lactone candidate that should be prepared next is β-lactone **121**, which introduces the essential P_1 side-chain required for recognition (Figure 32).

Figure 32 β-Lactone **121** resemblance to HAV 3C substrate

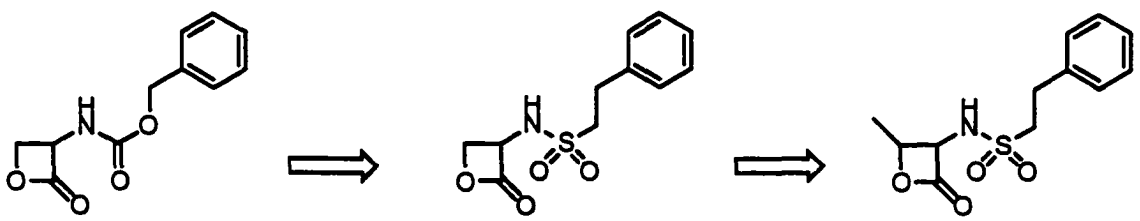


121

To determine, whether alkyl substitution is permitted at the β-position of **13a** and **13b**, the threonine analogue target **G** (**15**) was prepared (Figure 33). The sulfonamide isostere rather than the urethane was selected to avoid azlactone formation in the target **G** series.

The required carboxy group activation readily forms such unstable azlactones with *N*-acyl protecting groups.[116] Prior to the synthesis of targets **G**, the serine sulfonamide target **F** (**14**) was prepared to ascertain whether the enzyme accepts the sulfonamide for urethane replacement. The four possible stereoisomers of target **G** β-lactones should provide aqueous stability,[53] structural diversity and insight into the preferred trajectory of the HAV 3C thiolate attack at the β-position of the oxetanone ring.

Figure 33 Resemblance of targets **E**, **F** and **G** β-lactones



Target **E**
13

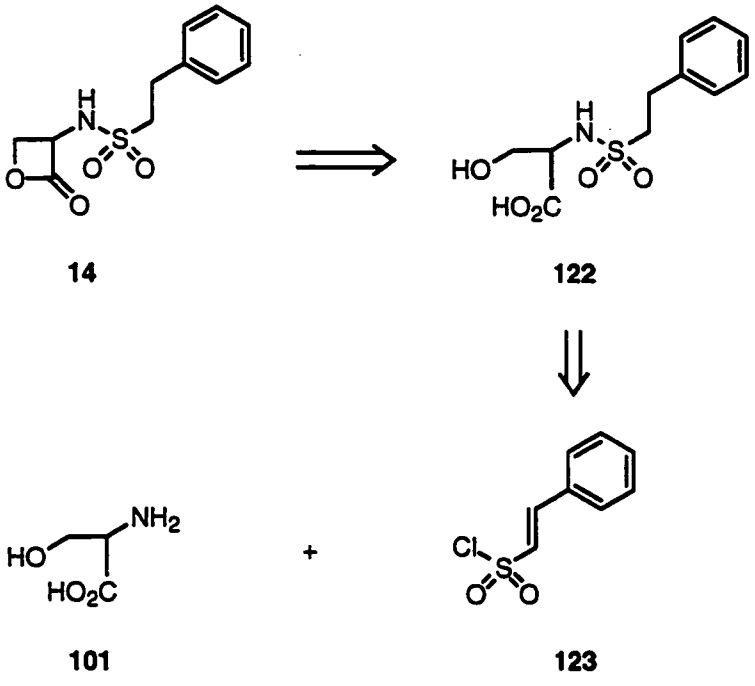
Target **F**
14

Target **G**
15

1.3.10 Synthesis of Target F and Analogues

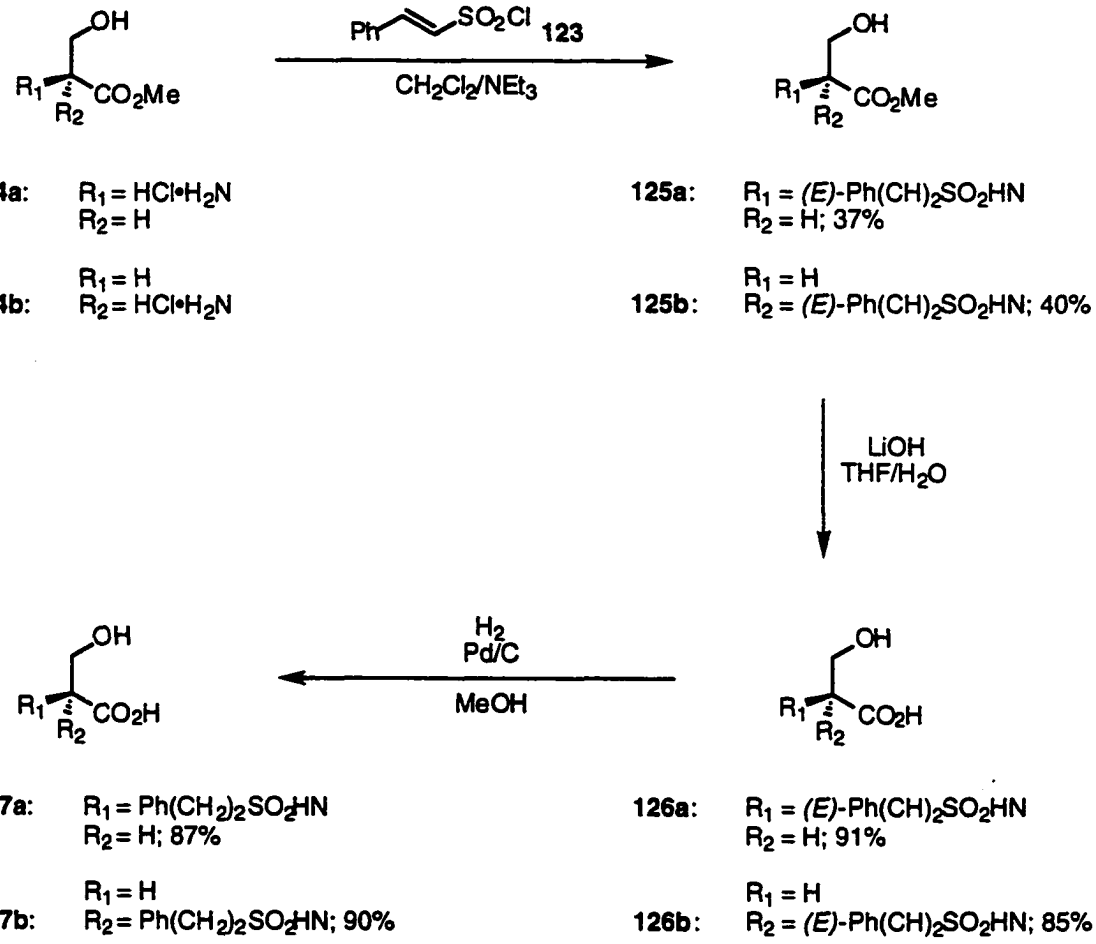
The strategy for the construction of target **F** (**14**) is based on the retrosynthetic analysis outlined in Scheme 37. The molecule can be derived from β-hydroxy carboxylic acid **122**, which in turn originates from the coupling of serine **101** and sulfonyl chloride **123**. *trans*-β-Styrenesulfonyl chloride **123** is used rather than the unknown (phenethylsulfonyl chloride) saturated analogue in order to avoid intramolecular electrophilic aromatic substitution.[117]

Scheme 37



Stereochemically pure sulfonamides **125a** and **125b** are available by coupling serine methyl ester **124a** and **124b**, respectively, to *trans*-β-styrenesulfonyl chloride (**123**) in dichloromethane in the presence of triethylamine (Scheme 38). Initially the sulfonamide formation was attempted with L-serine, rather than serine methyl ester, in THF / H_2O (1 : 3) in the presence of sodium bicarbonate; unfortunately, no desired product was obtained.[72] Hydrolysis of sulfonamides **125a** and **125b** with lithium hydroxide followed by acidic work-up affords β-hydroxy acids **126a** and **126b**, respectively. Hydrogenation of **126a** and **126b** catalyzed by 10% palladium on charcoal in methanol provides the desired β-hydroxy acids **127a** and **127b**,[66] respectively.

Scheme 38



124a: $R_1 = HCl \cdot H_2N$, $R_2 = H$

124b: $R_1 = H$, $R_2 = HCl \cdot H_2N$

125a: $R_1 = (E)\text{-}Ph(CH)_2SO_2HN$, $R_2 = H$; 37%

125b: $R_1 = H$, $R_2 = (E)\text{-}Ph(CH)_2SO_2HN$; 40%

127a: $R_1 = Ph(CH_2)_2SO_2HN$, $R_2 = H$; 87%

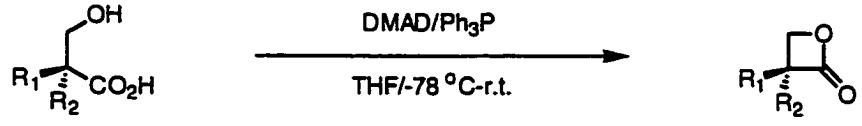
127b: $R_1 = H$, $R_2 = Ph(CH_2)_2SO_2HN$; 90%

126a: $R_1 = (E)\text{-}Ph(CH)_2SO_2HN$, $R_2 = H$; 91%

126b: $R_1 = H$, $R_2 = (E)\text{-}Ph(CH)_2SO_2HN$; 85%

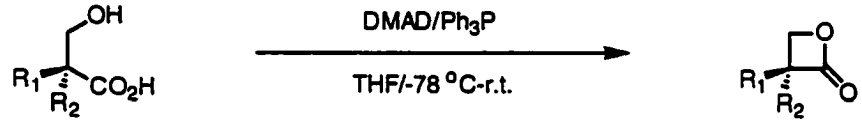
With β-hydroxy acids **127a** and **127b** available, cyclization under modified Mitsunobu conditions using the preformed *N*-phosphonium adduct of triphenylphosphine and dimethyl azodicarboxylate gives sulfonamides β-lactone **14a** and **14b**, respectively, without loss of optical purity (Scheme 39).[102] To introduce structural diversity into target **F**, the styrenyl sulfonamide β-lactones **128a** and **128b** (Scheme 39) were prepared in an analogous manner.

Scheme 39



127a: $R_1 = Ph(CH_2)_2SO_2HN$, $R_2 = H$ — **14a:** $R_1 = Ph(CH_2)_2SO_2HN$, $R_2 = H$; 32%

127b: $R_1 = H$, $R_2 = Ph(CH_2)_2SO_2HN$ — **14b:** $R_1 = H$, $R_2 = Ph(CH_2)_2SO_2HN$; 37%

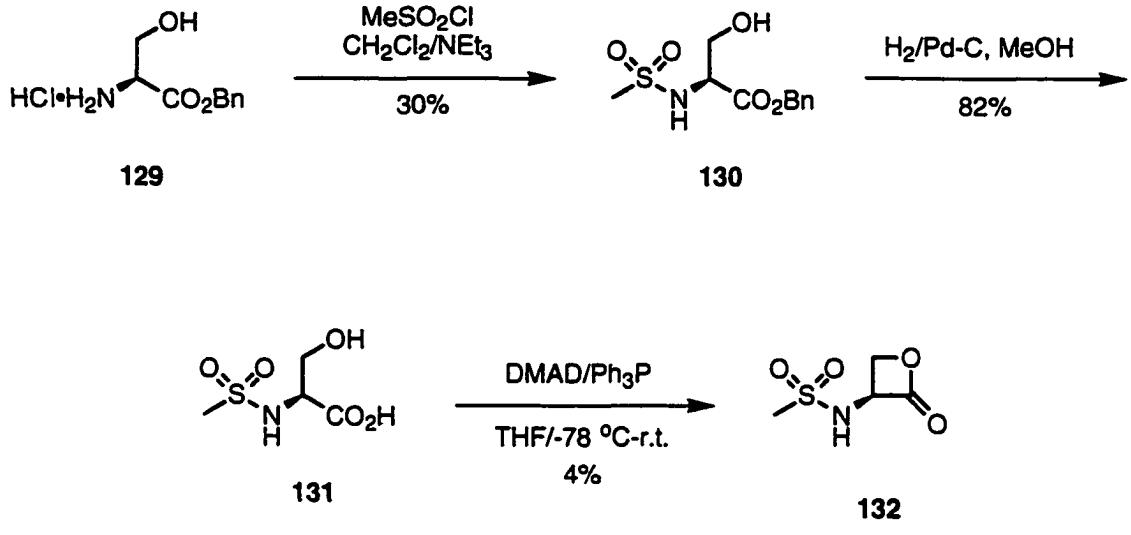


126a: $R_1 = (E)$-$Ph(CH)_2SO_2HN$, $R_2 = H$ — **128a:** $R_1 = (E)$-$Ph(CH)_2SO_2HN$, $R_2 = H$; 15%

126b: $R_1 = H$, $R_2 = (E)$-$Ph(CH)_2SO_2HN$ — **128b:** $R_1 = H$, $R_2 = (E)$-$Ph(CH)_2SO_2HN$; 14%

To further define the significance of the phenyl group and its potential to mimic the phenylalanine side-chain, the methyl sulfonamide β-lactone **132** was prepared as outlined in Scheme 40. Benzyl ester **129** was treated with methylsulfonyl chloride in the presence of triethylamine, to give sulfonamide **130**. Hydrogenation of **130** catalyzed by 10% palladium on charcoal in methanol provides β-hydroxy acid **131**.[66] Cyclization of β-hydroxy acid **131** under modified Mitsunobu conditions using the preformed *N*-phosphonium adduct of triphenylphosphine and dimethyl azodicarboxylate gives sulfonamide β-lactone **132** without loss of optical purity.[102]

Scheme 40

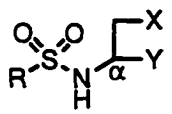


129 130 131 132

1.3.11 Inhibition of HAV 3C Proteinase by Target F and Analogues

Target **F** compounds **14a**, **14b** and analogues **125a**, **125b**, **126a**, **126b**, **127a**, **127b**, **128a**, **128b**, **131** and **132** were assayed with HAV 3C proteinase using a continuous fluorogenic assay,[83] as described in the experimental section. The IC_{50} values were measured without pre-incubation with the enzyme unless otherwise stated (Table 6).

Table 6 HAV 3C inhibition results for target **F** sulfonamide β-lactones and analogues



Compd.	R	X	Y	α-config.	$IC_{50}(\mu M)^a$	$t_{1/2}(min)^b$
14a	$Ph(CH_2)_2$	O----------CO		*S*	25	32
14b	$Ph(CH_2)_2$	O----------CO		*R*	4	n.d.
127a	$Ph(CH_2)_2$	OH	CO_2H	*S*	>>100	n.d.
127b	$Ph(CH_2)_2$	OH	CO_2H	*R*	>>100	n.d.
128a	*(E)*-$Ph(CH)_2$	O----------CO		*S*	38	n.d.
128b	*(E)*-$Ph(CH)_2$	O----------CO		*R*	3, (0.5)[c]	64
126a	*(E)*-$Ph(CH)_2$	OH	CO_2H	*S*	>>100	n.d.
126b	*(E)*-$Ph(CH)_2$	OH	CO_2H	*R*	>>100	n.d.
132	Me	O----------CO		*S*	>100	15
120	Me	OH	CO_2H	*S*	>>100	n.d.
125a	*(E)*-$Ph(CH)_2$	OH	CO_2Me	*S*	>>100	n.d.
125b	*(E)*-$Ph(CH)_2$	OH	CO_2Me	*R*	>>100	n.d.

[a] ([HAV 3C] = 0.1 μM, [Compd.] = 0.1-100 μM).

[b] β-Lactone hydrolysis half-life in phosphate buffer pH 7.5; n.d. = not determined

[c] 15 min inhibitor enzyme pre-incubation.

Target **F** sulfonamide serine β-lactones **14a** and **14b** are potent inhibitors of HAV 3C proteinase, with IC_{50} values of 25 and 4 μM, respectively (Table 6), which is comparable to the corresponding β-lactones **13a** and **13b**. Unexpectedly, kinetic analysis reveals that neither **14a** nor **14b** are time-dependent inhibitors of HAV 3C proteinase. Inhibitor **128a** (IC_{50} = 38 μM) does not display time-dependent behavior. However, its enantiomer **128b**

(IC_{50} = 3 μM) does show time-dependent inhibition of HAV 3C proteinase. The HAV 3C enzyme apparently shows different modes of binding for the different β-lactones, as the β-lactone **13a** is an irreversible inhibitor and the enantiomer **13b** is a reversible competitive inhibitor. The methyl sulfonamide β-lactone **132** displays weak (IC_{50} > 100 μM) time-independent inhibition of HAV 3C, suggesting that the aromatic group may play an important binding role in HAV 3C inhibition.

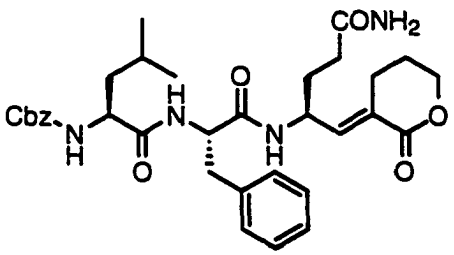
The stability of the sulfonamide β-lactones in aqueous buffer ranges from low (**132**), to reasonably stable (**128b**), as shown by β-lactone hydrolysis half-life ($t_{1/2}$) in phosphate buffer pH 7.5, Table 6. Introduction of a β-substituent on the oxetanone ring may increase the hydrolytic stability of the β-lactone ring.[53]

The corresponding carboxylic acids **127a**, **127b** and **131**, show no significant inhibition of HAV 3C proteinase at 100 μM concentration. In addition, the potential Michael acceptors **126a**, **126b**, **125a** and **125b** show no inhibition of HAV 3C proteinase at a inhibitor concentration of 100 μM. The above results, confirm the importance of the oxetanone ring for inhibitory activity.

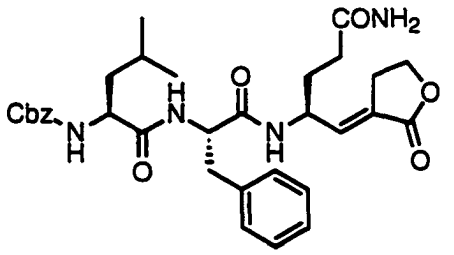
In summary, target **F** β-lactones **14a**, **14b**, **128a**, **128b** and **132** have been synthesized by cyclization of the corresponding β-hydroxy acids.[102] The sulfonamide serine β-lactone series does not exhibit enhanced stability in aqueous media compared to their urethane analogues. However, the introduction of an alkyl β-substituent, exemplified in the target **G** series (Figure 33), may improve the aqueous stability. Inhibition studies show that target **F** β-lactones are good inhibitors of HAV 3C proteinase.

The factors which determine whether a given β-lactone functions as a time-dependent or competitive inhibitor are currently not understood. Interestingly, a similar situation was observed with δ-lactone **133** which is a reversible inhibitor of HRV 3C (Figure 34), whereas, the corresponding γ-lactone **134** (Figure 34) is an irreversible inhibitor of HRV 3C.[32]

Figure 34 Reversible **133** and irreversible **134** Michael acceptors of HRV 3C proteinase



133 (K_i = 30 nM)

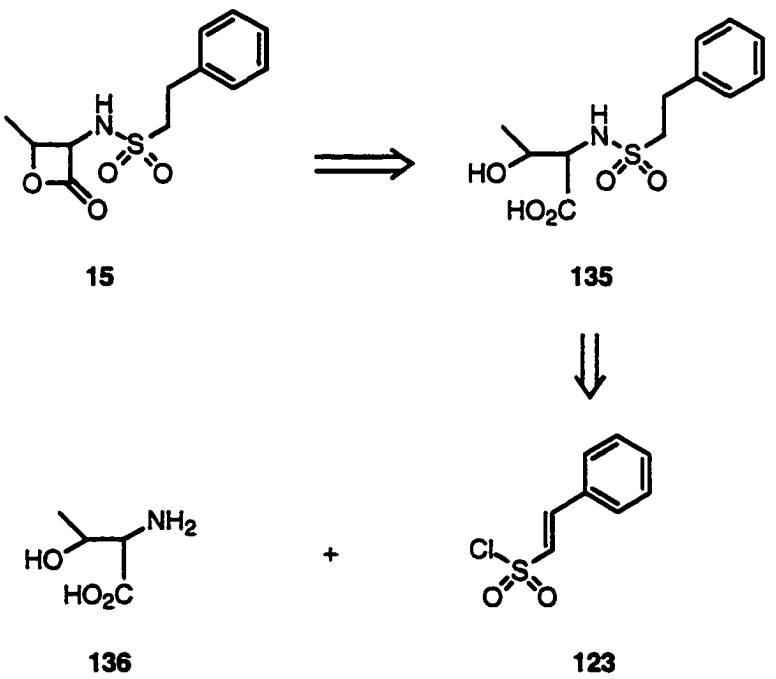


134 (k_{inact} / K_I = 10 900 M^{-1} s^{-1})

1.3.12 Synthesis of Target G and Analogues

The successful syntheses of sulfonamide serine β-lactones and the results of inhibition studies provide precedent for the synthesis of target **G**, sulfonamide threonine β-lactone. The presence of the methyl substituent at the β-position in threonine β-lactone should improve the hydrolytic stability in basic media[53] and would introduce structural diversity. In contrast to serine derivatives, cyclization of β-alkyl substituted β-hydroxy-α-amino acids (e.g. threonine) requires carboxy group activation to avoid decarboxylative elimination.[95] Hence, strategy for the construction of target **G** (**15**) is based on the retrosynthetic analysis outlined in Scheme 41. Target molecule **15** can be derived from β-hydroxy carboxylic acid **135**, which in turn originates from the coupling of threonine **136** and sulfonyl chloride **123**.

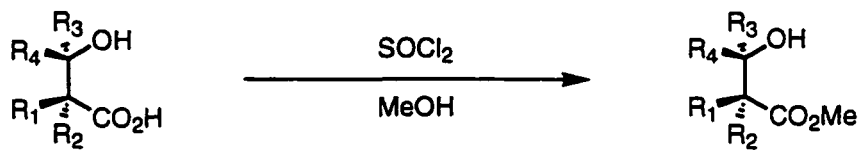
Scheme 41



15 **135** **136** **123**

Treatment of each of the threonine stereoisomers **136a-d** with thionyl chloride in methanol provides the corresponding threonine methyl esters **137a-d** in good yield (Scheme 42).[118]

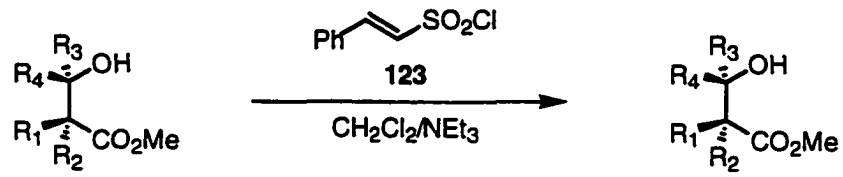
Scheme 42



136a:	$R_1 = H_2N$ $R_2 = H$, $R_3 = H$, $R_4 = Me$	**137a:**	$R_1 = HCl \cdot H_2N$ $R_2 = H$, $R_3 = H$, $R_4 = Me$; 98%
136b:	$R_1 = H$, $R_3 = Me$, $R_4 = H$ $R_2 = H_2N$	**137b:**	$R_1 = H$, $R_3 = Me$, $R_4 = H$ $R_2 = HCl \cdot H_2N$; 99%
136c:	$R_1 = H_2N$ $R_2 = H$, $R_3 = Me$, $R_4 = H$	**137c:**	$R_1 = HCl \cdot H_2N$ $R_2 = H$, $R_3 = Me$, $R_4 = H$; 99%
136d:	$R_1 = H$, $R_3 = H$, $R_4 = Me$ $R_2 = H_2N$	**137d:**	$R_1 = H$, $R_3 = H$, $R_4 = Me$ $R_2 = HCl \cdot H_2N$; 98%

Coupling of esters **137a-d** to *trans*-β-styrenesulfonyl chloride (**123**) in the presence of triethylamine affords the corresponding threonine sulfonamides **138a-d** (Scheme 43). Hydrolysis of sulfonamides **138a-d** with lithium hydroxide followed by acidic work-up affords β-hydroxy acids **139a-d.** Hydrogenation of β-hydroxy acids **139a-d** catalyzed by 10% palladium on charcoal in methanol provides desired acids **140a-d.**[66]

Scheme 43



137a: $R_1 = HCl \cdot H_2N$; $R_2 = H$, $R_3 = H$, $R_4 = Me$

137b: $R_1 = H$, $R_3 = Me$, $R_4 = H$; $R_2 = HCl \cdot H_2N$

137c: $R_1 = HCl \cdot H_2N$; $R_2 = H$, $R_3 = Me$, $R_4 = H$

137d: $R_1 = H$, $R_3 = H$, $R_4 = Me$; $R_2 = HCl \cdot H_2N$

138a: $R_1 = (E)\text{-}Ph(CH)_2SO_2HN$; $R_2 = H$, $R_3 = H$, $R_4 = Me$; 80%

138b: $R_1 = H$, $R_3 = Me$, $R_4 = H$; $R_2 = (E)\text{-}Ph(CH)_2SO_2HN$; 61%

138c: $R_1 = (E)\text{-}Ph(CH)_2SO_2HN$; $R_2 = H$, $R_3 = Me$, $R_4 = H$; 58%

138d: $R_1 = H$, $R_3 = H$, $R_4 = Me$; $R_2 = (E)\text{-}Ph(CH)_2SO_2HN$; 64%

LiOH
THF/H_2O

H_2
Pd/C
MeOH

140a: $R_1 = Ph(CH_2)_2SO_2HN$; $R_2 = H$, $R_3 = H$, $R_4 = Me$; 93%

140b: $R_1 = H$, $R_3 = Me$, $R_4 = H$; $R_2 = Ph(CH_2)_2SO_2HN$; 81%

140c: $R_1 = Ph(CH_2)_2SO_2HN$; $R_2 = H$, $R_3 = Me$, $R_4 = H$; 87%

140d: $R_1 = H$, $R_3 = H$, $R_4 = Me$; $R_2 = Ph(CH_2)_2SO_2HN$; 87%

139a: $R_1 = (E)\text{-}Ph(CH)_2SO_2HN$; $R_2 = H$, $R_3 = H$, $R_4 = Me$; 99%

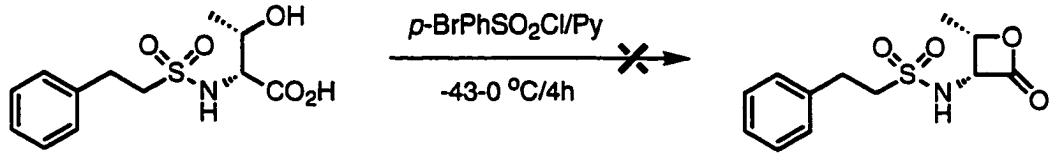
139b: $R_1 = H$, $R_3 = Me$, $R_4 = H$; $R_2 = (E)\text{-}Ph(CH)_2SO_2HN$; 96%

139c: $R_1 = (E)\text{-}Ph(CH)_2SO_2HN$; $R_2 = H$, $R_3 = Me$, $R_4 = H$; 96%

139d: $R_1 = H$, $R_3 = H$, $R_4 = Me$; $R_2 = (E)\text{-}Ph(CH)_2SO_2HN$; 98%

Treatment of **140b** with *p*-bromobenzenesulfonyl chloride / pyridine[116] did not give desired β-lactone **15b** (Scheme 44).

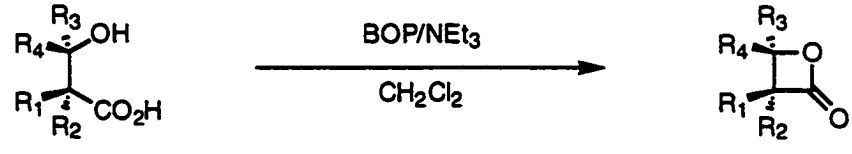
Scheme 44



140b **15b**

However, treatment of β-hydroxy acids **140a-d** with BOP reagent generates stereochemically pure sulfonamide L-, D-, L-*allo*- , and D-*allo*-threonine-β-lactones **15a-d**, respectively, (Scheme 45).[93]

Scheme 45



140a: R_1 = $Ph(CH_2)_2SO_2HN$, R_2 = H, R_3 = H, R_4 = Me

140b: R_1 = H, R_3 = Me, R_4 = H, R_2 = $Ph(CH_2)_2SO_2HN$

140c: R_1 = $Ph(CH_2)_2SO_2HN$, R_2 = H, R_3 = Me, R_4 = H

140d: R_1 = H, R_3 = H, R_4 = Me, R_2 = $Ph(CH_2)_2SO_2HN$

15a: R_1 = $Ph(CH_2)_2SO_2HN$, R_2 = H, R_3 = H, R_4 = Me; 75%

15b: R_1 = H, R_3 = Me, R_4 = H, R_2 = $Ph(CH_2)_2SO_2HN$; 75%

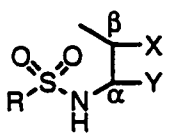
15c: R_1 = $Ph(CH_2)_2SO_2HN$, R_2 = H, R_3 = Me, R_4 = H; 78%

15d: R_1 = H, R_3 = H, R_4 = Me, R_2 = $Ph(CH_2)_2SO_2HN$; 89%

1.3.13 Inhibition of HAV 3C Proteinase by Target G and Analogues

Target G compounds **15a-d** and the carboxylic acids **140a-d** were assayed with HAV 3C proteinase using a continuous fluorogenic assay,[83] as described in the experimental section. The IC_{50} values were measured without pre-incubation with the enzyme, unless otherwise stated (Table 7).

Table 7 HAV 3C inhibition results for target G sulfonamide β-lactones and analogues



Compd.	R	X	Y	α,β-config	IC_{50}(μM)[a]	$t_{1/2}$(min)[b]
15a	$Ph(CH_2)_2$	O----------CO		S,R	168	n.d.
15b	$Ph(CH_2)_2$	O----------CO		R,S	136	358
15c	$Ph(CH_2)_2$	O----------CO		S,S	32	136
15d	$Ph(CH_2)_2$	O----------CO		R,R	12(1.4)[c]	n.d.
140a	$Ph(CH_2)_2$	OH	CO_2H	S,R	>>100	n.d.
140b	$Ph(CH_2)_2$	OH	CO_2H	R,S	>>100	n.d.
140c	$Ph(CH_2)_2$	OH	CO_2H	S,S	>>100	n.d.
140d	$Ph(CH_2)_2$	OH	CO_2H	R,R	>>100	n.d.

[a] ([HAV 3C] = 0.1 μM, [Compd.] = 0.25-100 μM).

[b] β-Lactone hydrolysis half-life in phosphate buffer pH 7.5; n.d. = not determined

[c] 15 min inhibitor enzyme pre-incubation.

Target G sulfonamide threonine-β-lactones **15a-d** are time-dependent inhibitors of HAV 3C. The most potent inhibitors in the series are the *allo*-threonine-β-lactones **15c** and **15d**

with IC_{50} values of 32 and 12, respectively (Table 7). In contrast, the threonine-β-lactones **15a** and **15b** are less active against HAV 3C, with IC_{50} values of 168 and 136, respectively. In general, alkyl β-substitution on the oxetanone ring decreases susceptibility to hydrolysis. Compound **15b** with *syn*-configuration about the β-lactone ring, has a longer half-life ($t_{1/2}$ = 358 min) than its *anti*-configuration diastereoisomer **15c** ($t_{1/2}$ = 136 min). The data presented in Table 7, suggests that β-lactone inhibitors of HAV 3C proteinase should have *R*-configuration at the α and β-positions, with a hydrophobic moiety attached to the α-amino group (eg. **15d**). However, comparison of (Tables 4, 6 and 7) indicates that the most hydrolysis-susceptible β-lactones are also the most potent HAV 3C proteinase inhibitors.

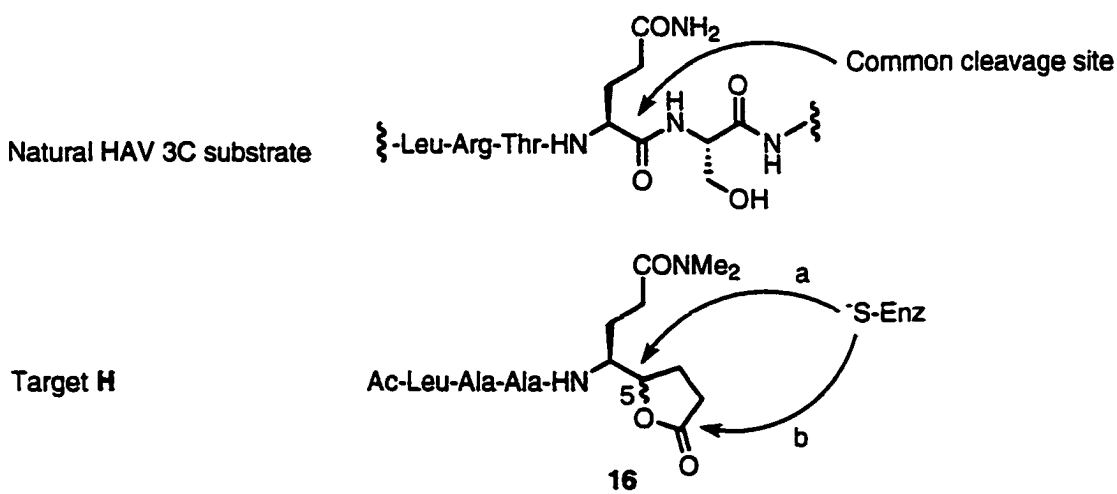
In summary, target G β-lactones **15a-d** and analogues have been synthesized. The synthetic strategy is based on the lactonization of the corresponding β-hydroxy acids using carboxy group activation.[93] The sulfonamide D-*allo*-threonine-β-lactone **15d** exhibits potent time-dependent inhibition of HAV 3C proteinase (IC_{50} = 12 μM), in addition to enhanced stability in basic aqueous media ($t_{1/2}$ ~ 136 min) compared to the parent urethane β-lactone **13a** (IC_{50} = 35 μM, $t_{1/2}$ = 76 min). These results show that β-substituted β-lactones retain inhibitory properties and have increased stability in aqueous media. Furthermore, β-lactone time-dependent HAV 3C inhibition appears to be dictated by the electrophilic nature of the oxetanone ring.

1.4 Peptidyl γ-Lactones

1.4.1 γ-Lactone Design

A number of γ-lactones are flavor components, insect sex-attractant pheromones and plant-growth regulators.[119-121] γ-Lactones undergo ring opening as a result of nucleophilic attack at the carbonyl,[106] and some show biological activity against serine proteinases[107] as well as thiol containing enzymes.[108] In addition, nucleophilic attack by thiol[109] and hydroxyl[110] has also been observed at the γ-position. The weak HAV 3C inhibition found with *N*-Cbz-homoserine-γ-lactones (**104a** and **104b**) may be due to poor recognition by the enzyme. Therefore, target **H** (**16**) (Figure 35) incorporates the tetrapeptide analogue as a mimic of the substrate. The target could form a covalent bond with the active site thiol, *via* γ-attack (path a) or carbonyl attack (path b).[77]

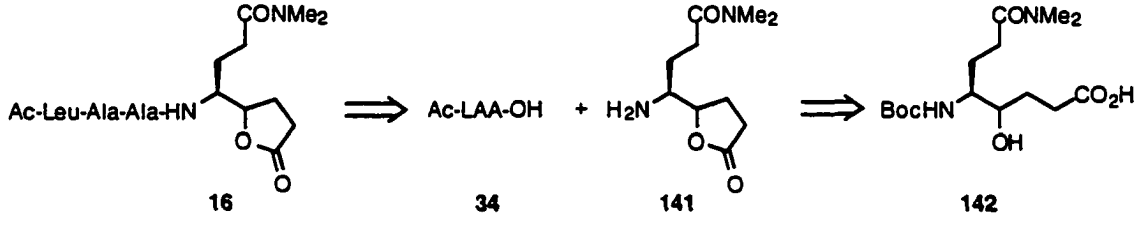
Figure 35 Rationale for target **H**



1.4.2 Synthesis of Target H

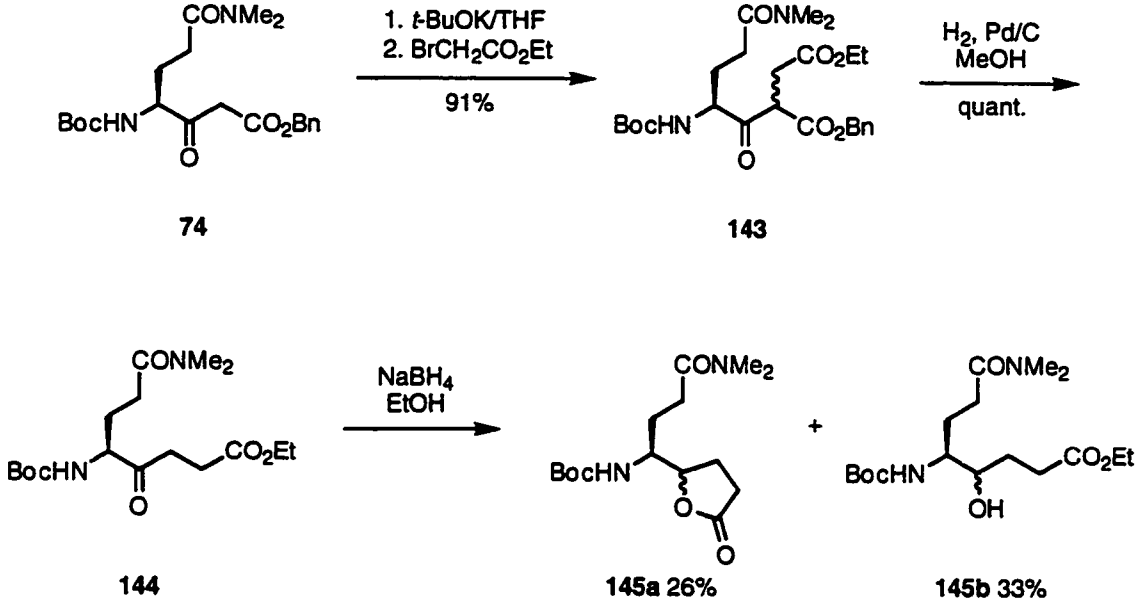
The strategy for the construction of target **H** (**16**) is based on the retrosynthetic analysis outlined in Scheme 46. Target molecule **16** can be derived from tripeptide **34** and the dimethyl glutamine γ-lactone **141**. The key γ-lactone **141** could in principle be synthesized by cyclization of γ-hydroxy carboxylic acid **142**.

Scheme 46



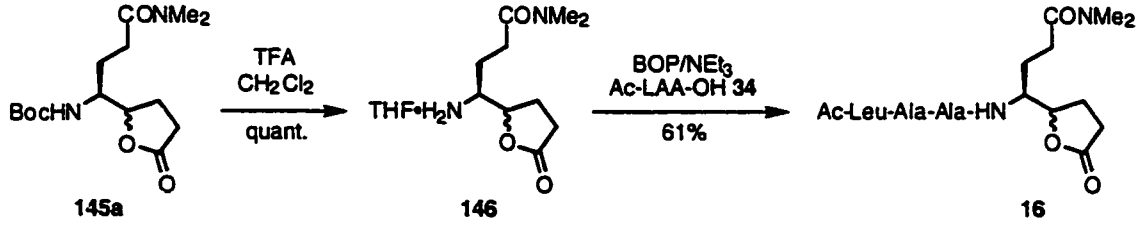
Treatment of β-keto ester **74**, in THF, with potassium *tert*-butoxide followed by ethyl bromoacetate, provides ethyl ester **143** in good yield (Scheme 47).[98] Hydrogenation of ethyl ester **143** provides γ-keto ester **144**.[66] Reduction of **144** with $NaBH_4$ in ethanol followed by acidic work-up generates the desired γ-lactone monomer **145a** plus the γ-hydroxy ester **145b**.[67]

Scheme 47



Deprotection of γ-lactone **145a** with 50% trifluoroacetic acid in dichloromethane affords the trifluoroacetate salt **146** (Scheme 48). Subsequent coupling of salt **146** with tripeptide **34** using BOP reagent in the presence of triethylamine in DMF produces the desired peptidyl γ-lactone **16**.

Scheme 48



1.4.3 Inhibition of HAV 3C Proteinase by Target H

Target **H** (**16**) was assayed by the standard method,[81,82] which is described in the experimental section and employs a discontinuous TNBS assay.[82] The enzyme inhibition studies were performed by Colin Luo (Department of Biochemistry).[81b] Compound **16** proved to be a time-dependent, irreversible inhibitor of HAV 3C proteinase, with a rate of enzyme inactivation $k_{inact} / K_I = 48\ M^{-1}s^{-1}$ ([E] = 0.07 μM, [I] = 50.0 μM). This promising result warranted further study, and thus **16** was subjected to continuous fluorogenic assay.[83] Only 17% inhibition (no enzyme inhibitor pre-incubation) and 39% inhibition (15 min enzyme inhibitor pre-incubation) of HAV 3C were observed at enzyme concentration of 0.1 μM and inhibitor concentration of 100 μM, respectively.

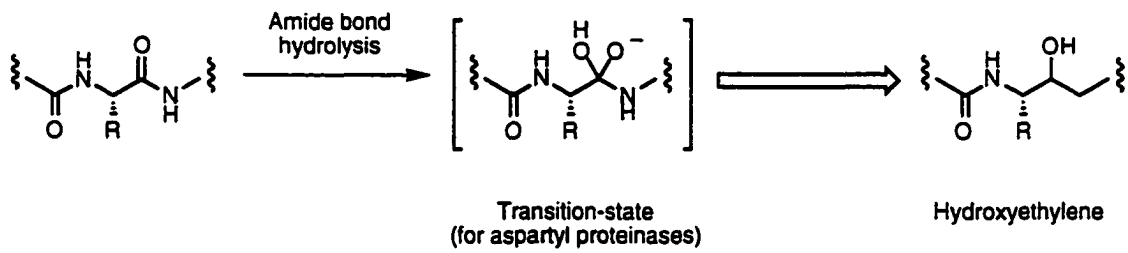
In summary, the results show that a γ-lactone on the substrate peptide backbone does not effectively inhibit the HAV 3C enzyme. Such compounds may potentially be effective against other cysteine proteinases, including other picornaviral 3C proteinases.

1.5 Peptidyl β-Hydroxy Acids

1.5.1 β-Hydroxy Acid Design

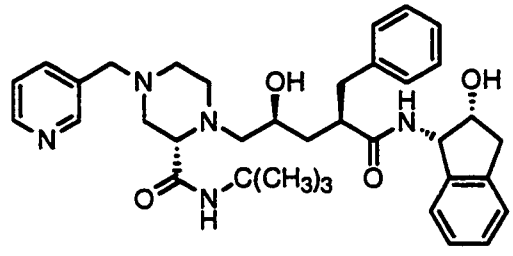
Transition-state isosteres that mimic intermediates in hydrolysis of the amide bond are important proteinase inhibitors used to develop therapeutic agents and to generate catalytic antibodies.[122] Peptidyl hydroxyethylenes, have been employed as transition-state isosteres for aspartyl proteinases (Figure 36).[123,124]

Figure 36 Hydroxyethylene unit for amide transition-state



For example, Indinivar **3** (Figure 37), is a potent competitive inhibitor of HIV-1 (human immunodeficiency virus-1) proteinase with a K_i = 0.14 nM[125] and displays antiviral activity (CIC_{95} = 25-50 nM) in cell cultures.[123] The HIV proteinase employs enzyme-bound water as the nucleophile to cleave the peptide bond.[125,126]

Figure 37 Indinivar **3** HIV-1 proteinase inhibitor



3

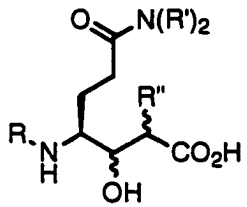
The precursors to β-lactones are β-hydroxy acids (or hydroxyethylene carboxylic acids), which bear a structural resemblance to the hydroxyethylene unit (Figure 38). An attractive feature of using a β-hydroxy acid as a "war-head" rather than a β-lactone is that the former is relatively chemically inert compared to the latter. Although the *N*-Cbz-serine, *N*-sulfonamide-serine and threonine acids show no enzyme inhibition of HAV 3C, the presence of the amide backbone and P_1 side-chain may enhance binding. To examine whether the β-hydroxy acid unit can inhibit cysteine proteinases, several previously prepared peptidyl β-lactone precursors (**17**, **18**, **19a** and **19b**) were tested as potential inhibitors of HAV 3C.

Figure 38 β-Hydroxy acid transition-state analogue

Hydroxyethylene β-Hydroxy acid

In addition, target I β-hydroxy acids **20**, **21** and **22** (Figure 39) were synthesized. All of these compounds possess an L-glutamine-related P_1 side-chain required for recognition.

Figure 39 Target I β-hydroxy acids



20: R = Boc, R' = Me, R" = Me

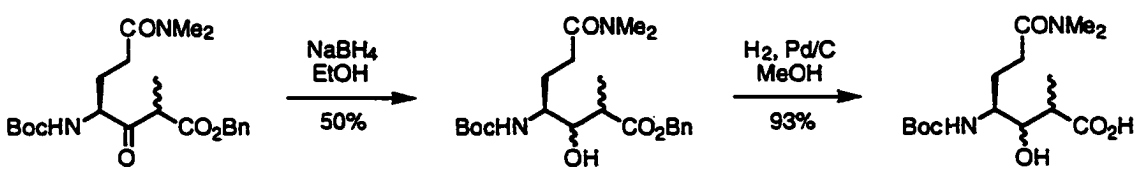
21: R = Cbz, R' = Me, R" = H

22: R = Boc, R' = H, R" = H

1.5.2 Synthetic Studies Towards Target I

Taking advantage of the side-product **87** shown in Scheme 26, β-hydroxy acid **20** was prepared as illustrated in Scheme 49. Reduction of β-keto ester **87** with $NaBH_4$ in ethanol followed by acidic work-up generates β-hydroxy ester **147** as a mixture of diastereoisomers.[67] Hydrogenation of **147**, using 10% palladium on charcoal in methanol provides the desired β-hydroxy acid **20**.[76]

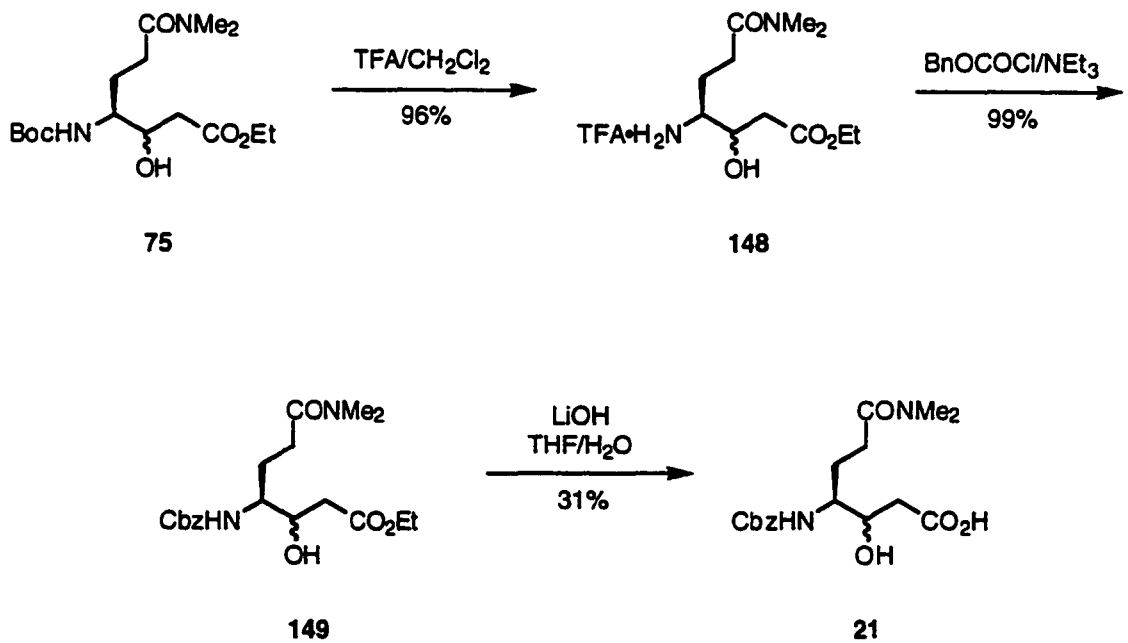
Scheme 49



87 **147** **20**

Compound **21** was synthesized from side-product **75** (Scheme 16) as shown in Scheme 50. Trifluoroacetate salt **148** is prepared by treating β-hydroxy ester **75** with 50% trifluoroacetic acid in dichloromethane. Coupling of trifluoroacetate salt **148** with benzyl chloroformate is accomplished in the presence of triethylamine in dichloromethane to afford *N*-Cbz protected **149**. Hydrolysis of **149** with lithium hydroxide followed by acidic work-up provides β-hydroxy acid **21**.

Scheme 50

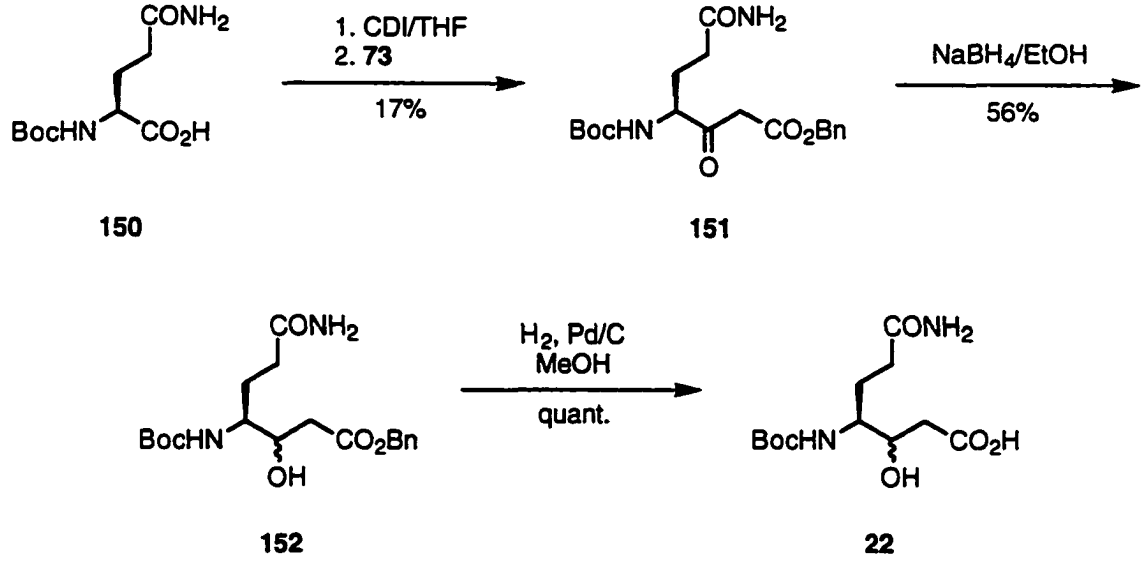


75 **148** **149** **21**

Analogue **22** was prepared as presented in Scheme 51. *N*-Boc-L-Glutamine **150** is activated with CDI in THF and condensed with magnesium salt **73** which is followed by decarboxylative acidic work-up to give β-keto ester **151**.[89] Reduction of **151** with $NaBH_4$ in ethanol with acidic work-up generates β-hydroxy ester **152**.[67] Hydrogenation of **152**

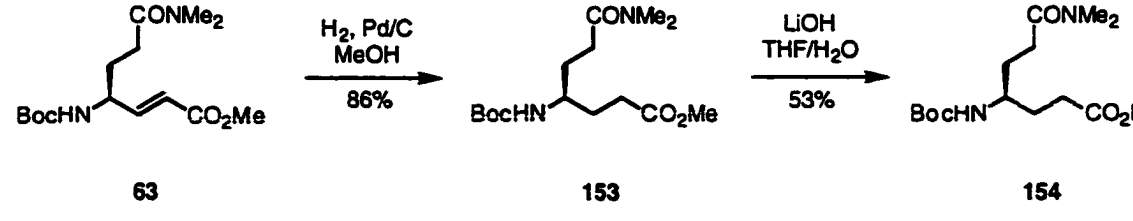
catalyzed by 10% palladium on charcoal in methanol, provides the desired β-hydroxy acid **22**.[66]

Scheme 51



To explore further the structural requirements for inhibition, compound **154** which lacks the hydroxyl group was synthesized (Scheme 52). The previously prepared unsaturated ester **63** (Scheme 12) was hydrogenated to provide ester **153**.[127] Hydrolysis of ester **153** with lithium hydroxide gives desired acid **154**.

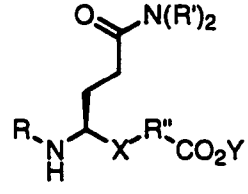
Scheme 52



1.5.3 Inhibition of HAV 3C Proteinase by Target I and Analogues

Target I β-hydroxy acids **17**, **18**, **19a**, **19b**, **20**, **21**, **22**, **74**, **75** and **154** (Table 8) were assayed against HAV 3C using a continuous fluorogenic assay.[83] The results show that the β-hydroxy acid moiety and its derivatives are poor inhibitors of HAV 3C.

Table 8 HAV 3C Inhibition results for target I β-hydroxy acids and analogues



Compd.	R	R'	R''	X[a]	Y	% Inh.[b]
17	Boc	Me	CH_2	CHOH	H	16
18	Ac-LAA	Me	CH_2	CHOH	H	13
19a	Boc	Me	$C(Me)_2$	CHOH	H	11
19b	Boc	Me	$C(Me)_2$	CHOH	H	20
20	Boc	Me	CHMe	CHOH	H	8
21	Cbz	Me	CH_2	CHOH	H	20
22	Boc	H	CH_2	CHOH	H	27
154	Boc	Me	CH_2	CH_2	H	20
74	Boc	Me	CH_2	CO	Bn	5
76	Boc	Me	CH_2	CHOH	Et	17

[a] *R / S* configuration except for **74** and **154**. [b] ([HAV 3C] = 0.1 μM, [Comp.] = 100 μM).

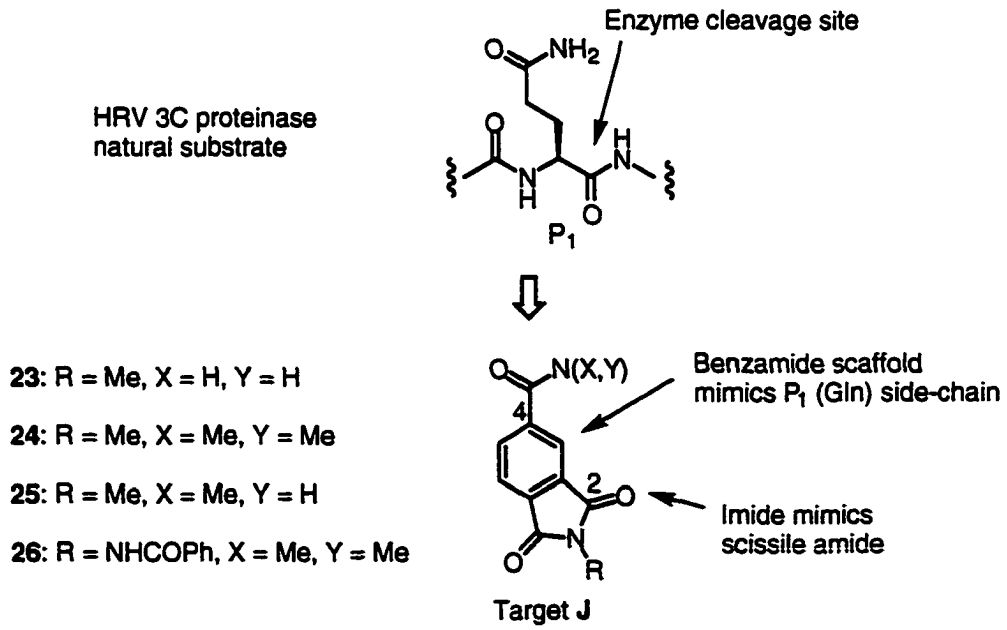
2. Phthalimide and Isoindolinone Inhibitors of HAV 3C Proteinase

2.1 Phthalimides Target J

2.1.1 Phthalimide Design

Conformationally constrained inhibitors of proteinases are found to bind much more tightly than the unconstrained inhibitors.[49,50,128] Replacement of peptide bonds by their isosteric equivalents can also greatly increase bioavailability by decreasing susceptibility to hydrolytic enzymes.[48] Based on these concepts, molecular modeling and a publication that described successful inactivation of HRV 3C proteinase by isatins (Figure 12),[30] target **J** phthalimides were designed for HAV 3C proteinase inhibition (Figure 40).

Figure 40 Rationale for target **J**

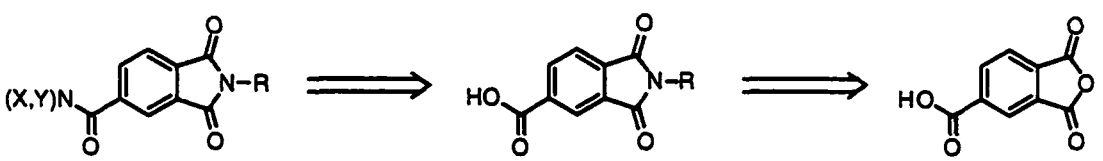


Thus, the conformationally restricted scaffold mimics the P_1 glutamine side-chain and positions an amide into the S_1 recognition site. Similarly, the 2-carbonyl group of the phthalimides is superimposable over the P_1 scissile amide carbonyl in the natural HAV 3C substrate.

2.1.2 Synthesis of Target J and Analogues

Retrosynthetic analysis (Scheme 53) of target **J** compounds (**23-26**) indicates that the starting building block is trimellitic anhydride **160**. This could be coupled with amine **161** or hydrazide **162** to produce the key phthalimide carboxylic acids **155** and **156**, respectively. Further reaction **155** and **156** with specific amines (**157-159**) would give the desired target **J** phthalimides **23-26**.

Scheme 53



Target J

23: R = Me, X = H, Y = H
24: R = Me, X = Me, Y = Me
25: R = Me, X = Me, Y = H
26: R = NHCOPh, X = Me, Y = Me

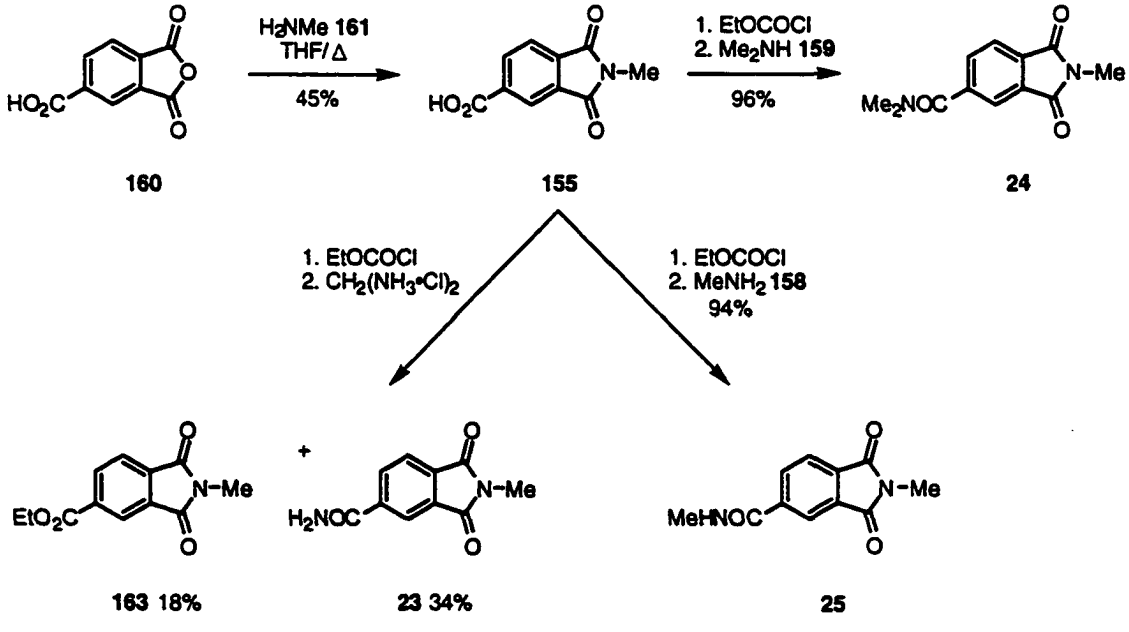
155: R = Me
156: R = NHCOPh
+
(X,Y)NH
157: (H, H)NH
158: (Me, H)NH
159: (Me, Me)NH

160
+
H_2N–R
161: R = Me
162: R =NHCOPh

Synthesis of phthalimides **23-25** is presented in Scheme 54. Preparation of intermediate **155** is accomplished by treating trimellitic anhydride **160** with methylamine hydrochloride **161** in the presence of triethylamine in THF under reflux.[74] Acid **155** reacts

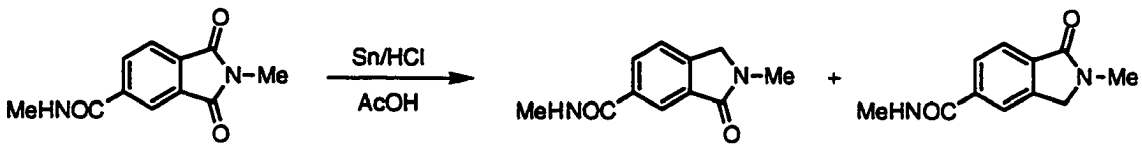
with ethyl chloroformate in the presence of triethylamine to produce the expected mixed anhydride, which upon treatment with diaminomethane dihydrochloride produces desired phthalimide **23** plus side-product **163**.[129] Initially, the primary amide of **23** was introduced by activation of acid **155** with CDI followed by treatment with ammonium hydroxide.[130] However, this route was abandoned due to unsatisfactory yields (11%). Similarly, compounds **24** and **25** can be synthesized by preparing the mixed anhydride of **155**, followed by condensation with dimethylamine and methylamine hydrochloride, respectively.

Scheme 54



In order to probe which imido carbonyl on target **J** (**25**) is the preferred carbonyl entering the HAV 3C oxyanion hole, isoindolinones **27a** and **27b** were synthesized (Scheme 55). Treatment of phthalimide **25** with tin powder in glacial acetic acid and concentrated hydrochloric acid under reflux affords isoindolinones **27a** and **27b**.[131]

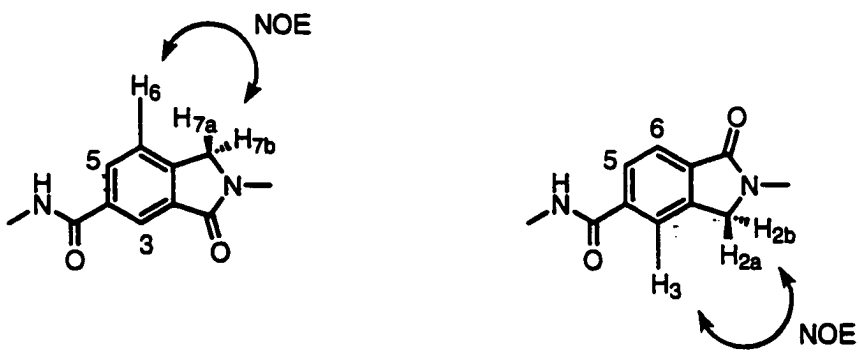
Scheme 55



25 27a 30% 27b 9%

Structural isomers **27a** and **27b** are distinguishable by ^{1}H ROESY experiments (Figure 41). The two dimensional ^{1}H ROESY spectrum of isoindolinone **27a** shows a distinct cross-peak between protons H_6 and H_7 on the proton chemical shift axis and the ROESY spectrum of **27b** shows a cross-peak between protons H_3 and H_2, providing evidence for the structural assignment.

Figure 41 NOE cross-peaks observed for isoindolinones **27a** and **27b**

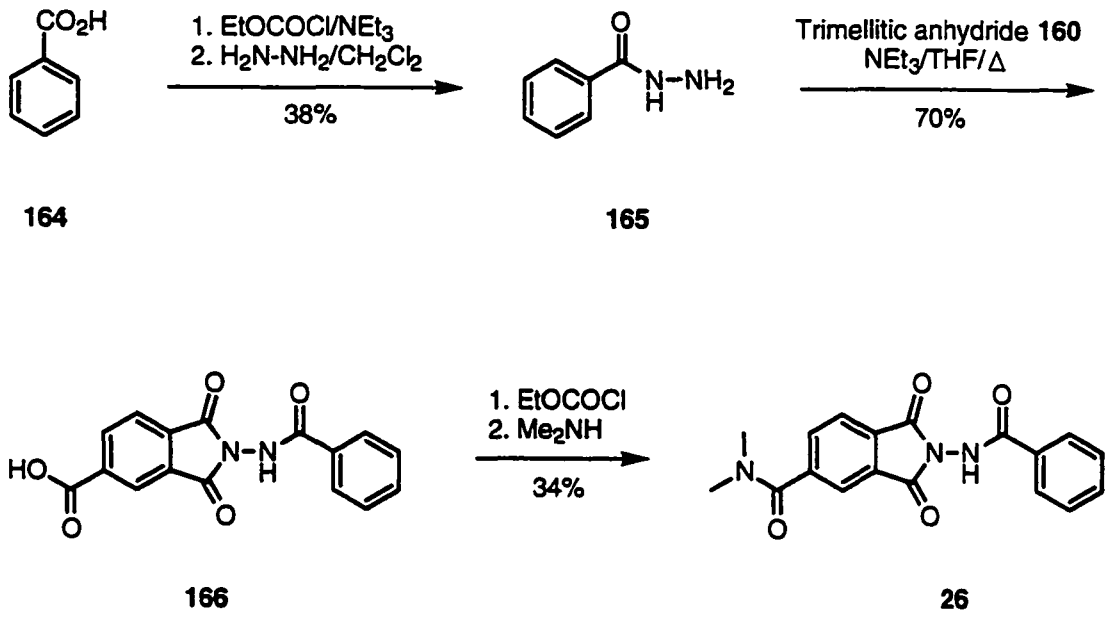


27a **27b**

Molecular modeling[77] suggested that phthalimide **26** would bind favorably in the active site of HAV 3C, and its synthesis is presented in Scheme 56. Benzoic acid **164** is treated with ethyl chloroformate in the presence of triethylamine in dichloromethane to generate the mixed anhydride, which is condensed with hydrazine to give hydrazide **165**. Treatment of **165** with trimellitic anhydride **160** in the presence of triethylamine in THF

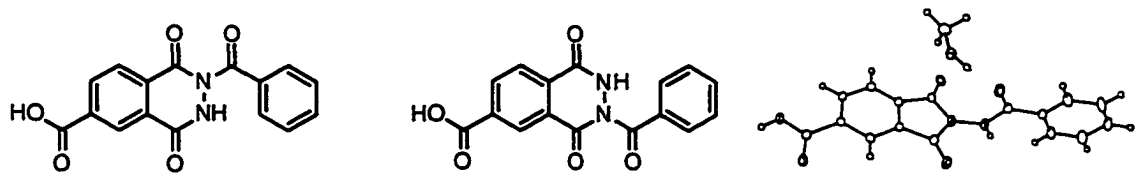
under reflux, affords **166**. Acid **166** is activated with ethyl chloroformate in the presence of triethylamine and treated with dimethylamine hydrochloride to produce desired phthalimide **26**.

Scheme 56



In Scheme 56, plausible alternative structures to **166** in the phthaloylation step, are the phthalhydrazide structural isomers (**167** and **168**) shown in Figure 42.[132] However, an X-ray crystallographic analysis of the product isolated confirms the structure of **166**.

Figure 42 Phthalhydrazide structural isomers **167**, **168** and X-ray structure of **166**

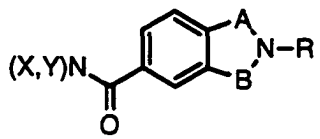


167 **168** **166**

2.1.3 Inhibition of HAV 3C and HRV-14 3C Proteinases by Target J and Analogues

Target J phthalimides **23-26**, and isoindolinones **27a** and **27b** (Table 9) were assayed against HAV 3C proteinase using a continuous fluorogenic assay[83] and HRV-14 3C proteinase using a continuous colorimetric assay,[115] which is described in the experimental section. Inhibitors tested were assayed at a concentration of 100 μM and enzyme concentration of 0.1 μM and 0.4 μM, for HAV 3C and HRV-14 3C, respectively. Phthalimides **23-26** and isoindolinone compounds **27a** and **27b** gave a disappointing 12-30% inhibition range (no enzyme inhibitor pre-incubation) against HAV 3C; however, phthalimide **23** shows reasonable inhibition (42%) against HRV-14 3C proteinase. Further structural elaboration of phthalimide **23** may provide improved inhibitory potency against HRV 3C.

Table 9 HAV and HRV 3C inhibition results of target **J** phthalimides and isoindolinones



Compd.	A	B	R	X	Y	% Inhibition	
						HAV 3C[a]	HRV 3C[b]
23	CO	CO	Me	H	H	19	42
24	CO	CO	Me	Me	Me	15	n.d.
25	CO	CO	Me	Me	H	30	n.d.
26	CO	CO	NHCOPh	Me	Me	12	n.d.
27a	CH_2	CO	Me	Me	H	18	n.d.
27b	CO	CH_2	Me	Me	H	15	n.d.

[a] Percent inhibition at ([HAV 3C] = 0.1 μM, [Comp.] = 100 μM).
[b] Percent inhibition at ([HRV 3C] = 0.4 μM, [Comp.] = 100 μM).
n.d. = not determined

In summary, target **J** compounds **23-26** and isoindolinones **27a** and **27b** were synthesized. The results show that these types of rigidified backbones are insufficient to inhibit either HAV or HRV 3C proteinases. A possible difficulty may be unfavorable positioning of vital pharmacophoric groups required for inhibition, as a result of conformational constraints imposed by the phthalimide and isoindolinone scaffolds. Nevertheless, such compounds may be effective against other cysteine proteinases, including other picornaviral proteinases.

Summary and Future Work

A series of inhibitors of HAV 3C proteinase have been synthesized and tested as potential lead compounds for the design of therapeutic agents for human picornaviral pathogens. This research shows that thiol reactive groups such as fluoromethyl ketones, α,β-unsaturated esters, γ-lactones and β-lactones can be used as effective tools to inhibit the HAV 3C enzyme. Various active site directed P-side tetrapeptide inhibitors were designed based on substrate specificity at P_1 (Gln) and P_4 (Leu). Preparation of fluoromethyl ketone **9**, provided a facile route to ^{13}C-labeled tetrapeptide fluoromethyl ketone **6** for NMR enzyme inactivation studies. The ^{13}C-NMR spectrum of the enzyme-inhibitor complex displays a new peak at 40 ppm, suggesting the formation of an (alkylthio)ketone. The α,β-unsaturated ester **10** proved to be a time-dependent irreversible inhibitor of HAV 3C proteinase, with a rate of enzyme inactivation $k_{inact} / K_i = 137\ M^{-1}s^{-1}$ ([E] = 0.07 μM, [I] = 10.0 μM). A similar α,β-unsaturated ester, compound **AG7088**, was shown by Agouron Pharmaceuticals[32,34] to be a highly potent, nontoxic antirhinoviral agent with broad efficacy against multiple HRV serotypes. Compound **AG7088** has been formulated for intranasel delivery and has recently entered clinical trails.[34d] γ-Lactone **16** was a time-dependent irreversible inhibitor of HAV 3C proteinase, with a rate of enzyme inactivation $k_{inact} / K_i = 48\ M^{-1}s^{-1}$ ([E] = 0.07 μM, [I] = 50.0 μM). Further functionalization of the γ-lactone ring possible by the addition of appropriately placed electron with-drawing groups may provide more potent inhibitors of HAV 3C.

In addition, active site directed P'-side inhibitors were designed based on substrate specificity at P_2' (Phe). β-Lactone **13a** is a time-dependent irreversible inhibitor of HAV 3C proteinase with $k_{inact} = 0.012\ s^{-1}$, $K_i = 1.84 \times 10^{-4}$ M, $k_{inact} / K_i = 63\ M^{-1}\ s^{-1}$. HAV 3C

proteinase is inactivated by β-lactone **13a**, *via* nucleophilic ring opening of the oxetanone ring at the β-position by the cysteine thiolate. In contrast, the enantiomer **13b** is a competitive reversible inhibitor of HAV 3C proteinase (K_i = 1.50 x 10^{-6} M). Additional studies on β-lactone **13** structure-activity relationships identified sulfonamide D-*allo*-threonine-β-lactone **15d** as a potent time-dependent inhibitor of HAV 3C proteinase (IC_{50} = 12 μM). Compound **15d** showed enhanced stability in basic aqueous media ($t_{1/2}$ ~ 136 min) compared to the parent urethane β-lactone **13a** ($t_{1/2}$ = 76 min). These results show that β-substituted β-lactones retain inhibitory properties and have increased stability in aqueous media. Furthermore, β-lactone time-dependent HAV 3C inhibition appears to be dictated by the electrophilic nature of the oxetanone ring.

Work is currently in progress to obtain an enzyme-inhibitor complex of HAV 3C proteinase with β-lactone **13a** to confirm β-attack on the oxetanone ring by the sulfur atom of Cys-172, in addition to advancing the inhibitor design process.

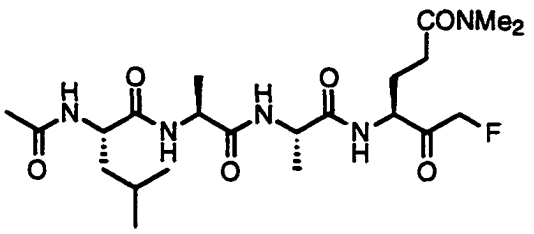
EXPERIMENTAL PROCEDURES

General Procedures

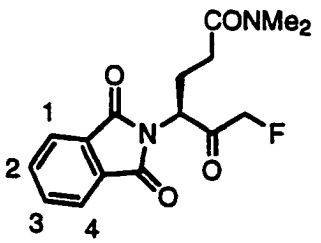
All processes involving air or moisture sensitive reactants were performed under an atmosphere of dry argon using oven-dried glassware. Reagents and solvents were reagent grade and used as supplied unless otherwise stated. Solvents for anhydrous reactions were dried according to Perrin *et al.*[133] Tetrahydrofuran (THF) and diethyl ether were distilled over sodium under an argon atmosphere. Acetonitrile, dichloromethane, triethylamine and pyridine were distilled over calcium hydride. *N,N*-Dimethylformamide (DMF) was distilled under reduced pressure over calcium hydride. Methanol and ethanol were distilled over magnesium turnings and a catalytic amount of iodine. Dimethyl sulfoxide (DMSO) was distilled over calcium hydride and stored over CaH_2. Water was obtained from a Milli-Q reagent water system. "Brine" refers to a saturated aqueous solution of NaCl. Unless otherwise specified, solutions of HCl, $NaHCO_3$, KOH and NaOH refer to aqueous solutions. Solvent evaporation was performed under reduced pressure below 40 °C using a Büchi rotary evaporator, followed by evacuation (<0.1 torr) to constant sample weight. Isotopically labeled L-serine (3-^{13}C, 99%) was purchased from Cambridge Isotope Laboratories (Andover MA) and was used directly without further purification.

Reactions and fractions from column chromatography were monitored and analyzed by thin-layer chromatography (TLC) using glass plates with a UV fluorescent indicator (silica gel, Merck 60 F_{254}; Merck RP-8 and Merck RP-18 F_{254}). One or more of the following methods were used for visualization: UV absorption by fluorescence quenching; iodine staining; phosphomolybdic acid / ceric sulfate / sulfuric acid (10 g : 1.25 g : 8% 250 mL) spray; Ninhydrin / methanol (1 g : 100 mL) spray; bromocresol green / ethanol / sodium hydroxide (0.04 g : 100 mL : 0.1 N added until the blue color appears) spray. Flash column chromatography was performed by the method of Still[134] using 230-400 mesh silica (Merck, silica gel).

Melting points were determined on a Thomas-Hoover oil immersion apparatus using open capillary tubes and are uncorrected. Infrared spectra (IR) were recorded on a Nicolet Magna 750 FT-IR spectrometer. Cast refers to the evaporation of a solution on a NaCl plate and (μscope) refers to microscope. Mass spectra (MS) were recorded on Kratos AEIMS-50 high resolution mass spectrometry (HRMS), electron impact ionization (EI), MS-12 chemical ionization ((CI), NH_3), MS-9 fast atom bombardment ((FAB), argon) and Micromass ZabSpec Hybrid Sector-TOF positive mode electrospray ionization ((ES), 0.5% solution of formic acid in acetonitrile : water (1 : 1)) instruments. Cleland matrix was used in FAB experiments and refers to a 5 : 1 mixture of dithiothreitol and dithioerythritol. Microanalyses were obtained on Perkin Elmer 240 or Carlo Erba 1180 elemental analyzers. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker WH-200, AM-300, WM-360, WH-400 and Inova Varian 300, 500 and 600 instruments. ^{1}H NMR chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS) using the solvent resonance as the reference: $CDCl_3$, δ 7.24; CD_2Cl_2, δ 5.32; D_2O, δ 4.72; CD_3OD, δ 3.30; *N,N*-$(CD_3)_2NCDO$, δ 2.74; $(CD_3)_2SO$, δ 2.49; $(CD_3)_2CO$, δ 2.04; CD_3CN, δ 1.93 and C_4D_8O, δ 1.73. ^{13}C NMR shifts are reported relative to: $CDCl_3$, δ 77.0; C_4D_8O, δ 67.4; CD_2Cl_2, δ 53.8; CD_3OD, δ 49.0; $(CD_3)_2CO$, δ 39.5; *N,N*-$(CD_3)_2NCDO$, δ 30.1; $(CD_3)_2CO$, δ 29.8 and CD_3CN, δ 1.3. Selective homonuclear decoupling, attached proton test (APT), ^{1}H-^{1}H, ^{1}H-^{13}C and nuclear Overhauser effect (NOE) correlation experiments were occasionally used for signal assignments. ^{1}H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet), number of protons, coupling constant(s) in Hertz (Hz), and assignment. When appropriate the multiplicity is preceded by br, indicating that the signal was broad. All literature compounds had IR, ^{1}H NMR and mass spectra consistent with the reported data.

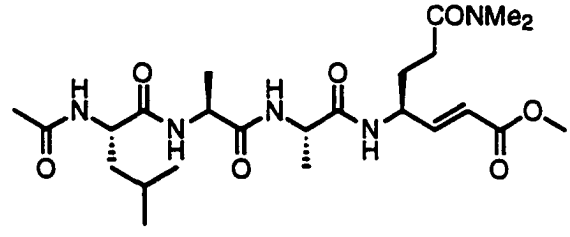


(4*S*)-*N*,*N*-Dimethyl-4-(acetylleucylalanylalanyl)amino-6-fluoro-5-oxohexanamide (6).[28] Dess-Martin periodinane **36** (31 mg, 72.9 μmol) was added to a solution of the fluoroalcohol **35** (11.9 mg, 24.3 μmol) in DMF (0.5 mL) at room temperature. The mixture was stirred for 2 h, then evaporated *in vacuo*. The residue was purified by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-30% acetonitrile : water, t_R 17.0 min) to yield the fluoroketone **6** (9.4 mg, 80%) as a white solid: IR ($CHCl_3$ cast) 3286, 2918, 1694, 1651, 1537, 1150, 1054, 667 cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 5.09 (dd, 2H, J = 46, 11 Hz, $CF\underline{H}_2$), 4.2-4.0 (m, 4H, α-H Leu, 2α-H Ala and α-H dimethyl Gln), 2.89 (s, 3H, $NC\underline{H}_3$), 2.77 (s, 3H, $NC\underline{H}_3$), 2.29 (m, 2H, $COC\underline{H}_2$), 2.08 (m, 1H, $C\underline{H}_2$ dimethyl Gln), 1.98 (m, 1H, $C\underline{H}_2$ dimethyl Gln), 1.88 (s, 3H, $COC\underline{H}_3$), 1.56 (m, 1H, C<u>H</u> Leu), 1.43 (m, 2H, $C\underline{H}_2$ Leu), 1.27 (d, 3H, J = 7 Hz, $C\underline{H}_3$ Ala), 1.25 (d, 3H, J = 7 Hz, $C\underline{H}_3$ Ala), 0.79 (d, 3H, J = 6 Hz, $C\underline{H}_3$ Leu), 0.72 (m, 3H, J = 6 Hz, $C\underline{H}_3$ Leu); MS (FAB) *m/z* (relative intensity) 488.0 (MH^+, 60%).



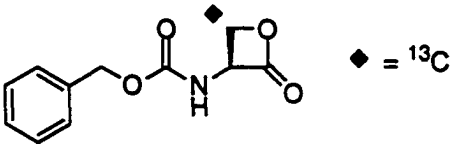
(4*S*)-*N*,*N*-Dimethyl-6-fluoro-5-oxo-4-phthalimidohexanamide (9). Potassium fluoride (145 mg, 2.5 mmol, dried at 100 °C under high vacuum for 24 h) and 18-crown-6 (16.4 mg, 0.06 mmol) were heated in acetonitrile (10 mL) for 30 min with vigorous stirring (oil bath at 90 °C). The mixture was allowed to cool and then the bromoketone **55** (237 mg, 0.62 mmol) was added. The mixture was heated with vigorous

stirring (in an oil bath at 90 °C) for an additional 15 h. The mixture was allowed to cool and then the solvent was evaporated *in vacuo*. Purification by HPLC (Resolve C-18 Prepak R 25 x 100 mm, 15 mL min^{-1} gradient elution, 8-44% acetonitrile : water) gave the fluoroketone **9** (40.5 mg, 20%) as a yellow oil: IR (CH_2Cl_2 cast) 3500, 2933, 1778, 1740, 1737, 1639, 1468, 1386, 724 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.88 (m, 2H, H_1 and H_4), 7.78 (m, 2H, H_2 and H_3), 5.09 (m, 1H, C<u>H</u>), 5.08 (dd, 1H, J = 47, 16 Hz, CF<u>H</u>), 4.95 (dd, 1H, J = 47, 16 Hz, CF<u>H</u>), 2.95 (s, 3H, NC<u>H</u>$_3$), 2.85 (s, 3H, NC<u>H</u>$_3$), 2.59 (m, 1H, C<u>H</u>$_2$), 2.41 (m, 1H, C<u>H</u>$_2$), 2.38 (m, 2H, COC<u>H</u>$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.1 (d, $^2J_{C\text{-}F}$ = 18 Hz), 171.1, 167.7, 134.4, 131.6, 123.7, 84.4 (d, $J_{C\text{-}F}$ = 185 Hz), 56.1, 37.1, 35.4, 29.2, 23.1; HRMS (ES) Calcd for $C_{16}H_{18}N_2O_4F$ 321.1251, found 321.1247.



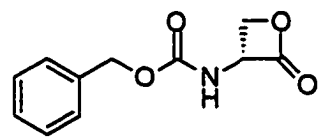
Methyl (2*E*,4*S*)-4-(acetylleucylalanylalanyl)amino-7-(*N*,*N*-dimethylamino)-7-oxohepten-2-oate (10). Triethylamine (4.0 µl, 29.2 µmol) was added to a solution of *N*-acetylleucylalanylalanine **34** (4.5 mg, 14.1 µmol) and HBTU (5.0 mg, 14.6 µmol) in DMF (1.0 mL) at 0 °C. The solution was stirred at 0 °C for 5 min, then added dropwise over 10 min to a solution of the trifluoroacetate salt **64** (4.8 mg, 14.6 µmol) and triethylamine (4.0 µl, 29.2 µmol) in DMF (1.0 mL) also at 0 °C. The mixture was stirred at 0 °C for 2 h, then the cold bath was removed and stirring was continued for an additional 3 h. The mixture was dried *in vacuo* overnight, and the residue was purified by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-30% acetonitrile : water, t_R 28.2 min) and recrystallized from $CHCl_3$-hexane to yield **10** (5.7 mg, 78%) as a white solid: mp 154-156 °C; $[\alpha]_D^{26}$ -21.62° (*c* 3.0, $CHCl_3$); IR (µscope)

3274, 2954, 1726, 1690, 1627, 1535, 1091 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.80-7.00 (br s, 4H, NH Leu, NH Ala, NH Ala, NH dimethyl Gln), 6.85 (dm, 1H, J = 16 Hz, CHCHCO$_2$Me), 5.98 (dm, 1H, J = 16 Hz, CHCHCO$_2$Me), 4.60-4.40 (m, 4H, α-H Leu, 2α-H Ala and α-H dimethyl Gln), 3.77 (2, 3H, CO_2CH_3), 3.05 (s, 3H, NCH_3), 2.95 (s, 3H, NCH_3), 2.40 (m, 2H, $COCH_2$ dimethyl Gln), 2.02 (s, 3H, $COCH_3$), 1.50-1.80 (m, 5H, CH Leu, CH_2 Leu and CH_2 dimethyl Gln), 1.42 (m, 3H, CH_3 Ala), 1.42 (m, 3H, CH_3 Ala), 0.95 (m, 3H, CH_3 Leu), 0.95 (m, 3H, CH_3 Leu); ^{13}C NMR (125 MHz, CD_3OD) δ 173.4, 173.2, 173.3, 172.3, 173.4, 166.7, 146.7, 132.4, 69.5, 51.8, 51.7, 50.6, 49.9, 40.7, 29.3, 24.9, 24.5, 23.5, 22.9, 22.8, 22.5, 21.9, 21.5, 17.7; HRMS (ES) Calcd for $C_{24}H_{42}N_5O_7$ 512.3084, found 512.3081.

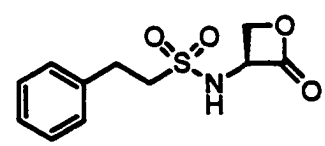


[3-^{13}C]-*N*-(Benzyloxycarbonyl)-L-serine-β-lactone, (13a(β-^{13}C)).[53a] A 25 mL round-bottom flask was equipped with a magnetic stirring bar, argon inlet adaptor, low temperature thermometer and rubber septum. The flask was charged with THF (5 mL) and triphenylphosphine (0.17 g, 0.67 mmol), the triphenylphosphine was dissolved with stirring and the flask was cooled to -78 °C. Dimethyl azodicarboxylate (80.0 μL, 0.73 mmol) was added dropwise with a syringe over 10 min. The resulting pale yellow solution was stirred at -78 °C for 10 min at which point a milky white slurry was obtained. The rubber septum on the flask was replaced with a pressure equalizing dropping funnel containing a solution of [3-^{13}C]-*N*-Cbz-L-serine **102a**(β-^{13}C) (0.16 g, 0.67 mmol) in THF (2 mL), which was added dropwise to the mixture over 30 min. After completion of the addition, the mixture was stirred at -78 °C for 20 min, the cooling bath was removed and the mixture was slowly warmed with stirring to room temperature over 2.5 h. The solvent was removed *in vacuo*, the residual pale yellow syrup was suspended in hexane-ethyl

acetate (4 : 1) and purified by flash chromatography (hexane-ethyl acetate, 4 : 1) which gave **13a**(β-^{13}C) (45.4 mg, 31%) as a white solid after recrystallization from ($CHCl_3$-hexane): mp 133-134 °C (lit. mp 133-134 °C)[53a]; $[\alpha]_D^{26}$ -6.45° (*c* 1.6, $CHCl_3$); IR (μscope) 3366, 1842, 1686, 1532, 1268, 751, 701 cm^{-1}; ^{1}H NMR (360 MHz, CD_2Cl_2) δ 7.38 (m, 5H, Ph), 5.54 (br s, 1H, NH), 5.14 (br s, 2H, PhCH_2), 5.06 (m, 1H, CH), 4.43 (dm, 2H, $^1J_{13C-H}$ = 160 Hz, $^{\bullet}$CH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.5, 155.2, 135.5, 128.7, 128.6, 128.4, 67.9, 66.4, 59.5; HRMS (EI) Calcd for $^{13}CC_{10}H_{11}NO_4$ 222.0722, found 222.0721.

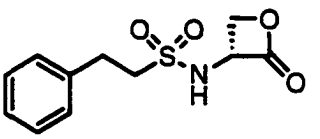


***N*-(Benzyloxycarbonyl)-D-serine-β-lactone (13b).**[53a] Cyclization of *N*-Cbz-D-serine **102b** (5.0g, 20.0 mmol) by Mitsunobu procedure[53a] (dimethyl azodicarboxylate (2.30 mL, 20.0 mmol) and triphenylphosphine (5.50 g, 20.0 mmol)) as described for **13a**(β-^{13}C) gave β-lactone **13b** (1.82 g, 40%): mp 133-134 °C (lit. mp 133-134 °C)[53a]; $[\alpha]_D^{26}$ +3.45° (*c* 6, $CHCl_3$); IR (μscope) 3361, 1827, 1684, 1529, 1266, 753, 702 cm^{-1}; ^{1}H NMR (360 MHz, CD_2Cl_2) δ 7.36 (m, 5H, Ph), 5.49 (br s, 1H, NH), 5.14 (br s, 2H, PhCH_2), 5.06 (m, 1H, CH), 4.44 (m, 2H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.6, 155.2, 135.5, 128.6, 128.5, 128.4, 67.8, 66.3, 59.7; HRMS (EI) Calcd for $C_{11}H_{11}NO_4$ 221.0688, found 221.0693; Anal. Calcd for $C_{11}H_{11}NO_4$: C, 59.72; H, 5.01; N, 6.33. Found: C, 59.71; H, 4.87; N, 6.29.

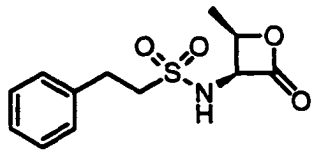


***N*-(Phenethylsulfonyl)-L-serine-β-lactone (14a).** Cyclization of *N*-(phenethylsulfonyl)-L-serine **127a** (0.50 g, 1.85 mmol) by Mitsunobu procedure[53a] (dimethyl azodicarboxylate (0.20 mL, 1.85 mmol) and triphenylphosphine (0.49 g, 1.85

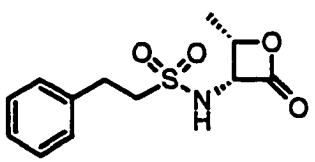
mmol)) as described for **13a**(β-^{13}C) gave β-lactone **14a** (0.15 g, 32%): mp 119-120 °C; $[\alpha]_D^{26}$ -35.21° (*c* 1.5, $CHCl_3$); IR ($CHCl_3$ cast) 3300, 3050, 2990, 1827, 1496, 1343, 1150, 743, 699 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.35 (m, 5H, Ph), 5.16 (ddd, 1H, *J* = 7, 6, 4 Hz ,CH), 5.13 (d, 1H, *J* = 7 Hz, NH), 4.58 (dd, 1H, *J* = 11, 6 Hz, CH_2), 4.31 (dd, 1H, *J* = 11, 4 Hz, CH_2), 3.48 (m, 2H, $PhCH_2CH_2$), 3.22 (m, 2H, $PhCH_2CH_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.7, 137.8, 129.7, 129.3, 127.9, 68.2, 61.4, 56.7, 30.6; HRMS (EI) Calcd for $C_{11}H_{13}NO_4S$ 255.0565, found 255.0562; Anal. Calcd for $C_{11}H_{13}NO_4S$: C, 51.75; H, 5.13; N, 5.49. Found: C, 51.63; H, 5.04; N, 5.42.



***N*-(Phenethylsulfonyl)-D-serine-β-lactone (14b).** Cyclization of *N*-(phenethylsulfonyl)-D-serine **127b** (0.50 g, 1.85 mmol) by Mitsunobu procedure[53a] (dimethyl azodicarboxylate (0.20 mL, 1.85 mmol) and triphenylphosphine (0.49 g, 1.85 mmol)) as described for **13a**(β-^{13}C) gave β-lactone **14b** (0.17 g, 37%): mp 119-120 °C; $[\alpha]_D^{26}$ +29.19° (*c* 1.4, $CHCl_3$); IR ($CHCl_3$ cast) 3281, 3050, 2990, 1829, 1496, 1320, 1150, 743, 699 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.35 (m, 5H, Ph), 5.16 (ddd, 1H, *J* = 9, 6, 5 Hz ,CH), 5.13 (d, 1H, *J* = 9 Hz, NH), 4.58 (dd, 1H, *J* = 11, 6 Hz, CH_2), 4.31 (dd, 1H, *J* = 11, 5 Hz, CH_2), 3.48 (m, 2H, $PhCH_2CH_2$), 3.22 (m, 2H, $PhCH_2CH_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.9, 137.9, 129.7, 129.3, 127.8, 68.2, 61.4, 56.7, 30.6; HRMS (EI) Calcd for $C_{11}H_{13}NO_4S$ 255.05653, found 255.05464; Anal. Calcd for $C_{11}H_{13}NO_4S$: C, 51.75; H, 5.13; N, 5.49. Found: C, 51.42; H, 5.37; N, 5.81.

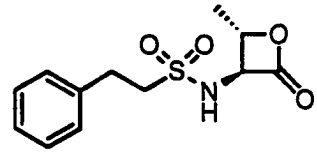


***N*-(Phenethylsulfonyl)-L-threonine-β-lactone (15a).** A suspension of *N*-(phenethylsulfonyl)-L-threonine-β-lactone **140a** (0.5 g, 1.74 mmol) in CH_2Cl_2 (30 mL) was cooled to 0 °C and treated with triethylamine (0.72 mL, 5.22 mmol) followed by BOP (0.92 g, 2.11 mmol). The cooling bath was removed and the reaction mixture was stirred at room temperature for 3 h. The solvent was removed *in vacuo* and the residue purified by flash chromatography (ethyl acetate : hexane, 1 : 1), followed by recrystallization from ($CHCl_3$-hexane) to give β-lactone **15a** (0.35 g, 75%) as a white solid: mp 139-141 °C; $[\alpha]_D^{26}$ -25.0° (*c* 1.2, $CHCl_3$); IR ($CHCl_3$ cast) 3319, 3050, 2980, 1813, 1497, 1338, 1150, 750, 698 cm^{-1}; ^1H NMR (360 MHz, CD_2Cl_2) δ 7.31 (m, 5H, Ph), 5.19 (dd, 1H, *J* = 10, 6 Hz, NCH), 5.65 (d, 1H, *J* = 10 Hz, NH), 4.23 (quintet, 1H, *J* = 6 Hz, CH(OH)), 3.46 (m, 2H, $PhCH_2CH_2$), 3.15 (m, 2H, $PhCH_2CH_2$), 1.47 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.0, 137.8, 129.7, 129.3, 128.0, 75.0, 62.3, 56.4, 30.7, 16.0; HRMS (EI) Calcd for $C_{12}H_{15}NO_4S$ 269.0722, found 269.0719; Anal. Calcd for $C_{12}H_{15}NO_4S$: C, 53.52; H, 5.61; N, 5.20. Found: C, 53.53; H, 5.52; N, 5.16.

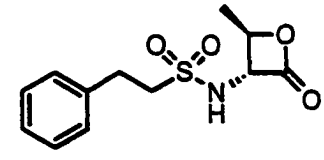


***N*-(Phenethylsulfonyl)-L-threonine-β-lactone (15b).** Cyclization of *N*-(phenethylsulfonyl)-D-threonine **140b** (0.50 g, 1.74 mmol) using BOP (0.92 g, 2.11 mmol) and triethylamine (0.72 mL, 5.22 mmol) as described for **15a** gave β-lactone **15b** (0.35 g, 75%) as a white solid: mp 139-141 °C; $[\alpha]_D^{26}$ -12.50° (*c* 0.8, $CHCl_3$); IR ($CHCl_3$ cast) 3317, 3025, 2979, 1808, 1497, 1329, 1146, 748, 696 cm^{-1}; ^1H NMR (360 MHz, CD_2Cl_2) δ 7.29 (m, 5H, Ph), 5.19 (m, 1H, NCH), 5.18 (br s, 1H, NH), 4.88 (dq, 1H, *J*

= 6, 3 Hz, C<u>H</u>(OH)), 3.43 (m, 2H, PhCH$_2$C<u>H$_2$</u>), 3.15 (m, 2H, PhC<u>H$_2$</u>CH$_2$), 1.45 (d, 3H, J = 6 Hz, CH(C<u>H$_3$</u>)); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.9, 137.8, 129.3, 128.9, 127.4, 74.9, 61.9, 56.0, 30.2, 15.5; HRMS (EI) Calcd for $C_{12}H_{15}NO_4S$ 269.0722, found 269.0712; Anal. Calcd for $C_{12}H_{15}NO_4S$: C, 53.52; H, 5.61; N, 5.20. Found: C, 53.46; H, 5.48; N, 5.12.

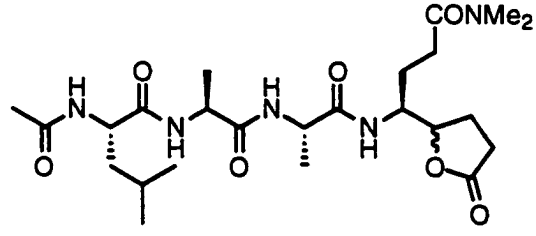


***N*-(Phenethylsulfonyl)-L-*allo*-threonine-β-lactone (15c).** Cyclization of *N*-(phenethylsulfonyl)-L-*allo*-threonine **140c** (0.50 g, 1.74 mmol) using BOP (0.92 g, 2.11 mmol) and triethylamine (0.72 mL, 5.22 mmol) as described for **15a** gave β-lactone **15c** (0.36 g, 78%) as a white solid: mp 130-132 °C; $[\alpha]_D^{26}$ -70.0° (*c* 1.1, $CHCl_3$); IR ($CHCl_3$ cast) 3302, 3030, 2971, 1828, 1496, 1360, 1147, 760, 696 cm^{-1}; ^{1}H NMR (360 MHz, CD_2Cl_2) δ 7.31 (m, 5H, <u>Ph</u>), 5.01 (d, 1H, J = 8 Hz, N<u>H</u>), 4.63 (dq, 1H, J = 6, 4 Hz, C<u>H</u>(OH)), 4.54 (dd, 1H, J = 8, 4 Hz, NC<u>H</u>), 3.46 (m, 2H, PhCH$_2$C<u>H$_2$</u>), 3.15 (m, 2H, PhC<u>H$_2$</u>CH$_2$), 1.59 (d, 3H, J = 6 Hz, CH(C<u>H$_3$</u>)); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.8, 137.9, 129.7, 129.2, 127.9, 78.6, 66.0, 56.6, 30.7, 19.3; HRMS (ES) Calcd for $C_{12}H_{15}NO_4SNa$ 292.0611, found 292.0623; Anal. Calcd for $C_{12}H_{15}NO_4S$: C, 53.52; H, 5.61; N, 5.20. Found: C, 53.16; H, 5.44; N, 5.05.



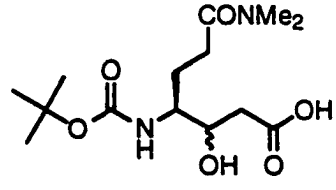
***N*-(Phenethylsulfonyl)-D-*allo*-threonine-β-lactone (15d).** Cyclization of *N*-(phenethylsulfonyl)-D-*allo*-threonine **140d** (0.50 g, 1.74 mmol) using BOP (0.92 g, 2.11 mmol) and triethylamine (0.72 mL, 5.22 mmol) as described for **15a** gave β-lactone **15d** (0.42 g, 89%) as a white solid: mp 130-132 °C; $[\alpha]_D^{26}$ +41.54° (*c* 1.3, $CHCl_3$); IR

($CHCl_3$ cast) 3303, 3031, 2972, 1838, 1497, 1326, 1148, 762, 697 cm^{-1}; ^1H NMR (360 MHz, CD_2Cl_2) δ 7.31 (m, 5H, Ph), 5.00 (d, 1H, J = 9 Hz, NH), 4.63 (dq, 1H, J = 6, 4 Hz, CH(OH)), 4.54 (dd, 1H, J = 9, 4 Hz, NCH), 3.46 (m, 2H, $PhCH_2CH_2$), 3.15 (m, 2H, $PhCH_2CH_2$), 1.59 (d, 3H, J = 6 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.1, 137.2, 129.1, 128.6, 127.3, 77.9, 65.4, 55.9, 30.0, 18.7; HRMS (EI) Calcd for $C_{12}H_{15}NO_4S$ 269.0722, found 269.0709; Anal. Calcd for $C_{12}H_{15}NO_4S$: C, 53.52; H, 5.61; N, 5.20. Found: C, 53.14; H, 5.49; N, 5.09.



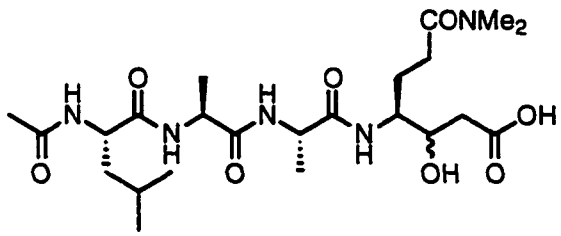
(5*RS*)-5-[(1*S*)-1-(acetylleucylalanylalanyl)amino-3-(*N*,*N*-dimethylcarbamoyl)propyl]oxolan-2-one (16). Triethylamine (15.0 µl, 0.10 mmol) was added to a solution of *N*-acetylleucylalanylalanine **34** (20.0 mg, 0.061 mmol) and BOP (28.0 mg, 0.063 mmol) in DMF (2.0 mL) at 0 °C. The solution was stirred at 0 °C for 5 min, then added dropwise over 10 min to a solution of the trifluoroacetate salt **146** (30.1 mg, 0.091 mmol) and triethylamine (15.0 µl, 0.10 mmol) in DMF (2.0 mL) also at 0 °C. The mixture was stirred at 0 °C for 2 h, then the cooling bath was removed and stirring was continued for an additional 3 h. The mixture was dried *in vacuo* overnight, and the residue was purified by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-20% acetonitrile : water, t_R 23.5 min) and recrystallized from ($CHCl_3$-diethyl ether) to yield **16** (19.7 mg, 61%) as a white solid mixture of diastereoisomers. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 2 : 1): IR (µscope) 3280, 2955, 1777, 1648, 1295, 1169, 1035 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 7.60-6.80 (br s, 4H, NH Leu, NH Ala, NH Ala, NH dimethyl Gln), 4.70-4.00 (m, 5H, α-H Leu, 2α-H Ala, α-H dimethyl Gln and

$CH_2CH_2CO_2CH$), 3.09 (s, 3H, NCH_3), 2.93 (s, 3H, NCH_3), 2.60-2.20 (m, 6H, $CH_2CH_2CO_2CH$, $COCH_2$ dimethyl Gln, $CH_2CH_2CO_2CH$), 2.20 (s, 3H, $COCH_3$), 1.95 (m, 1H, CH_2 dimethyl Gln), 1.80-1.60 (m, 4H, CH Leu, CH_2 Leu, CH_2 dimethyl Gln), 1.40 (m, 6H, CH_3 Ala, CH_3 Ala), 0.95 (m, 6H, CH_3 Leu, CH_3 Leu); (isomer B) δ 7.60-6.80 (br s, 5H, NH Leu, NH Ala, NH Ala, NH dimethyl Gln), 4.70-4.00 (m, 5H, α-H Leu, 2α-H Ala, α-H dimethyl Gln and $CH_2CH_2CO_2CH$), 3.09 (s, 3H, NCH_3), 2.93 (s, 3H, NCH_3), 2.60-2.20 (m, 6H, $CH_2CH_2CO_2CH$, $COCH_2$ dimethyl Gln, $CH_2CH_2CO_2CH$), 2.00 (s, 3H, $COCH_3$), 1.95 (m, 1H, CH_2 dimethyl Gln), 1.80-1.60 (m, 4H, CH Leu, CH_2 Leu, CH_2 dimethyl Gln), 1.40 (m, 6H, CH_3 Ala, CH_3 Ala), 0.95 (m, 3H, CH_3 Leu), 0.95 (m, 3H, CH_3 Leu); ^{13}C NMR (125 MHz, CD_3OD) (isomer A) δ 177.7, 177.2, 174.4, 173.6, 173.4, 173.1, 81.9, 52.5, 51.9, 50.2, 49.8, 45.4, 40.2, 36.8, 29.7, 28.5, 25.0, 24.9, 24.5, 22.9, 22.8, 17.5, 16.8, 15.5; (isomer B) δ 177.7, 177.2, 174.4, 173.6, 173.4, 172.4, 81.4, 50.3, 50.2, 49.9, 49.6, 45.4, 40.2, 36.8, 29.7, 28.5, 25.0, 24.9, 24.5, 22.9, 22.8, 17.5, 16.8, 15.5; HRMS (ES) Calcd for $C_{24}H_{42}N_5O_7$ 512.3084, found 512.3092.



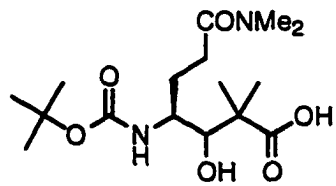
(3*RS*,4*S*)-4-(*tert*-Butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3-hydroxy-7-oxoheptanoic acid (17). To a solution of β-hydroxy benzyl ester **76** (1.05 g, 2.57 mmol) in methanol (20 mL) under argon was added 10% palladium on charcoal (0.10 g). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give the β-hydroxy acid **17** (0.82 g, quantitative) as a white solid. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR ($CHCl_3$ cast) 3356, 2976, 1707, 1688,

1624, 1452, 1249, 1168, 1044, cm^{-1}; ^1H NMR (360 MHz, CD_3OD) (isomer A) δ 3.63 (m, 1H, NC<u>H</u>), 3.18 (m, 1H, C(OH)<u>H</u>), 2.79 (s, 3H, NC<u>H</u>$_3$), 2.65 (s, 3H, NC<u>H</u>$_3$), 2.22 (m, 2H, CH(OH)C<u>H</u>$_2$), 2.15 (m, 2H, NCOC<u>H</u>$_2$), 1.75 (m, 1H, NCHC<u>H</u>$_2$), 1.35 (m, 1H, NCHC<u>H</u>$_2$), 1.14 (s, 9H, C(C<u>H</u>$_3$)$_3$); (isomer B) δ 3.94 (m, 1H, NC<u>H</u>), 3.45 (m, 1H, C(OH)<u>H</u>), 2.79 (s, 3H, NC<u>H</u>$_3$), 2.65 (s, 3H, NC<u>H</u>$_3$), 2.22 (m, 2H, CH(OH)C<u>H</u>$_2$), 2.15 (m, 2H, NCOC<u>H</u>$_2$), 1.79 (m, 1H, NCHC<u>H</u>$_2$), 1.69 (m, 1H, NCHC<u>H</u>$_2$), 1.14 (s, 9H, C(C<u>H</u>$_3$)$_3$); ^{13}C NMR (75 MHz, CD_3CN) (isomer A) δ 174.5, 174.1, 157.3, 79.6, 71.6, 55.8, 39.5, 37.7, 35.8, 30.1, 28.6, 26.0; (isomer B) δ 174.5, 174.1, 157.3, 79.6, 70.5, 55.1, 39.5, 37.7, 35.8, 30.3, 28.6, 26.0; HRMS (ES) Calcd for $C_{14}H_{26}N_2O_6Na$ 341.1689, found 341.1695.



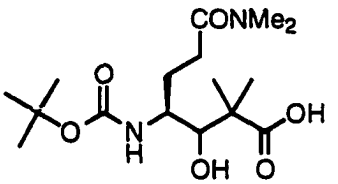
(3*RS*,4*S*)-*N*,*N*-Dimethyl-4-(acetylleucylalanylalanyl)amino-3-hydroxy-7-oxoheptanoic acid (18). Triethylamine (21.35 μl, 0.16 mmol) was added to a solution of *N*-acetylleucylalanylalanine **34** (27.6 mg, 0.088 mmol) and BOP (40.6 mg, 0.091 mmol) in DMF (1.5 mL) at 0 °C. The solution was stirred at 0 °C for 5 min, then added dropwise over 10 min to a solution of the trifluoroacetate salt **85** (30.1 mg, 0.091 mmol) and triethylamine (21.35 μl, 0.16 mmol) in DMF (2.5 mL) also at 0 °C. The mixture was stirred at 0 °C for 2 h, then the cold bath was removed and stirring was continued for an additional 3 h. The mixture was dried *in vacuo* overnight, and the residue was purified by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-40% acetonitrile : water (0.1% TFA), t_R 19.7 min) and recrystallized from CH_2Cl_2-diethyl ether to yield **18** (27.3 mg, 60%) as a white solid mixture of diastereoisomers. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A :

isomer B, 5 : 1): IR (μscope) 3291, 2958, 1714, 1655, 1648, 1547, 1253, 1174 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) (isomer A) δ 4.29-3.89 (m, 4H, α-H Leu, 2α-H Ala and α-H dimethyl Gln), 3.75 (m, 1H, CH(OH)), 2.94 (s, 3H, NCH_3), 2.83 (s, 3H, NCH_3), 2.57 (m, 2H, $CH(OH)CH_2$), 2.31 (m, 2H, $COCH_2$ dimethyl Gln), 2.02 (m, 1H, CH_2 dimethyl Gln), 1.94 (s, 3H, $COCH_3$), 1.62 (m, 1H, CH_2 dimethyl Gln), 1.49 (m, 1H, CH Leu), 1.39 (m, 2H, CH_2 Leu), 1.31 (m, 3H, CH_3 Ala), 1.31 (m, 3H, CH_3 Ala), 0.85 (m, 3H, CH_3 Leu), 0.85 (m, 3H, CH_3 Leu); (isomer B) δ 4.29-3.89 (m, 4H, α-H Leu, 2α-H Ala and α- H dimethyl Gln), 3.75 (m, 1H, CH(OH)), 2.94 (s, 3H, NCH_3), 2.83 (s, 3H, NCH_3), 2.57 (m, 2H, $CH(OH)CH_2$), 2.31 (m, 2H, $COCH_2$ dimethyl Gln), 2.02 (m, 1H, CH_2 dimethyl Gln), 1.94 (s, 3H, $COCH_3$), 1.62 (m, 1H, CH_2 dimethyl Gln), 1.49 (m, 1H, CH Leu), 1.39 (m, 2H, CH_2 Leu), 1.31 (m, 3H, CH_3 Ala), 1.31 (m, 3H, CH_3 Ala), 0.85 (m, 3H, CH_3 Leu), 0.85 (m, 3H, CH_3 Leu); ^{13}C NMR (125 MHz, CD_3OD) (isomer A) δ 175.8, 175.7, 175.2, 175.1, 174.8, 174.6, 54.8, 53.9, 53.1, 51.4, 50.9, 41.6, 37.8, 35.8, 30.6, 26.7, 25.9, 23.5, 22.9, 22.5, 21.7, 17.8, 16.8; (isomer B) δ 175.8, 175.7, 175.2, 175.1, 174.8, 174.6, 54.8, 53.9, 53.1, 51.4, 50.7, 40.0, 37.8, 35.8, 30.5, 26.5, 25.9, 23.3, 22.9, 22.5, 21.7, 17.8, 16.8; HRMS (ES) Calcd for $C_{23}H_{42}N_5O_8$ 516.3033, found 516.3027.

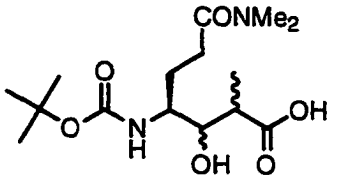


(3*R* or *S*,4*S*)-4-(*tert*-Butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3-hydroxy-2,2-dimethyl-7-oxoheptanoic acid (19a). To a solution of β-hydroxy benzyl ester **89a** (67.6 mg, 0.155 mmol) in methanol (1 mL) under argon was added 10% palladium on charcoal (6.76 mg). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give crude β-

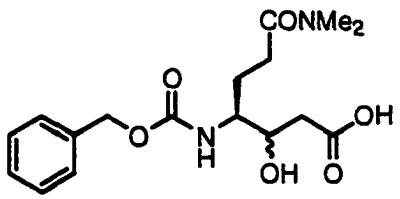
hydroxy acid. Purification by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-30% acetonitrile : water (0.1% TFA), t_R 10.7 min) gave β-hydroxy acid **19a** (47.9 mg, 89%) as a clear oil: $[\alpha]_D^{26}$ -6.86° (*c* 1.8, $CHCl_3$); IR ($CHCl_3$ cast) 3313, 2977, 1694, 1627, 1476, 1255, 1168, 1042 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 3.73 (d, 1H, *J* = 9 Hz, C(OH)H), 3.57 (m, 1H, NCH), 3.07 (s, 3H, NCH_3), 2.65 (s, 3H, NCH_3), 2.38 (m, 2H, $NCOCH_2$), 2.14 (m, 1H, $NCHCH_2$), 1.58 (m, 1H, $NCHCH_2$), 1.44 (s, 9H, $C(CH_3)_3$), 1.60 (s, 3H, $C(CH_3)_2$), 1.50 (s, 3H, $C(CH_3)_2$); ^{13}C NMR (75 MHz, CD_3CN) δ 181.2, 175.6, 157.6, 80.2, 78.3, 53.6, 46.9, 37.8, 35.9, 30.5, 29.4, 28.8, 25.0, 18.8; HRMS (ES) Calcd for $C_{16}H_{31}N_2O_6$ 347.2182, found 347.2188.



(3*R* or *S*,4*S*)-4-(*tert*-Butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3-hydroxy-2,2-dimethyl-7-oxoheptanoic acid (19b). Reaction of β-hydroxy ester **89b** (54.0 mg, 0.12 mmol) as described for **19a** gave the title compound **19b** (43.1 mg, quantitative) as a light-brown oil: $[\alpha]_D^{26}$ -9.26° (*c* 1.8, $CHCl_3$); IR ($CHCl_3$ cast) 3320, 2977, 1694, 1651, 1633, 1496, 1260, 1169, 1076 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 3.62 (m, 1H, NCH), 3.59 (br s, 1H, C(OH)H), 2.94 (s, 3H, NCH_3), 2.82 (s, 3H, NCH_3), 2.29 (m, 2H, $NCOCH_2$), 1.69 (m, 2H, $NCHCH_2$), 1.34 (s, 9H, $C(CH_3)_3$), 1.12 (s, 3H, $C(CH_3)$), 1.07 (s, 3H, $C(CH_3)$); ^{13}C NMR (75 MHz, CD_3CN) δ 180.9, 175.3, 157.7, 80.1, 77.9, 51.7, 47.3, 37.7, 35.8, 31.3, 30.5, 28.7, 22.4, 22.1; HRMS (ES) Calcd for $C_{16}H_{31}N_2O_6$ 347.2182, found 347.2188.

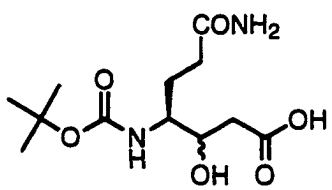


(2*RS*,3*RS*,4*S*)-4-(*tert*-Butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3-hydroxy-2-methyl-7-oxoheptanoic acid (20). To a solution of β-hydroxy benzyl ester **147** (11.0 mg, 0.026 mmol) in methanol (1.0 mL) under argon was added 10% palladium on charcoal (1.0 mg). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give the β-hydroxy acid **20** (8.1 mg, 93%) as a colorless oil. Spectroscopic characterization was performed on a mixture of diastereoisomers: IR ($CHCl_3$ cast) 3326, 2977, 1699, 1633, 1456, 1249, 1170, 1045 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 3.73 (m, 1H, NC<u>H</u>), 3.59 (m, 1H, C(OH)<u>H</u>), 2.99 (s, 3H, NC<u>H</u>$_3$), 2.85 (s, 3H, NC<u>H</u>$_3$), 2.50 (m, 1H, C<u>H</u>(CH_3)), 2.34 (m, 2H, NCOC<u>H</u>$_2$), 2.05 (m, 1H, NCHC<u>H</u>$_2$), 1.58 (m, 1H, NCHC<u>H</u>$_2$), 1.35 (s, 9H, C(C<u>H</u>$_3$)$_3$), 1.04 (d, 3H, J = 7 Hz, CH(C<u>H</u>$_3$)); ^{13}C NMR (125 MHz, CD_3CN) δ 175.6, 165.5, 158.1, 80.1, 75.4, 53.9, 43.6, 37.4, 35.8, 28.8, 28.0, 25.7, 11.4; HRMS (ES) Calcd for $C_{15}H_{29}N_2O_6$ 333.2026, found 333.2030.



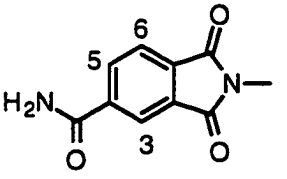
(3*RS*,4*S*)-4-(Benzyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3-hydroxy-7-oxoheptanoic acid (21). To a solution of β-hydroxy ester **149** (14.0 mg, 36.7 μmol) in THF (1.0 mL) and water (1.0 mL) at room temperature was added lithium hydroxide monohydrate (1.85 mg, 44.1 μmol). The mixture was stirred at room temperature for 2 h. The solvent was removed *in vacuo* and the residue was purified by flash chromatography (ethyl acetate : methanol : acetic acid, 100 : 5 : 1) to give **21** (4.0 mg,

31%) as a clear oil. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR (μscope) 3373, 3050, 2924, 1695, 1685, 1651, 1450, 1025, 750, 690 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 7.28 (m, 5H, Ph), 5.10 (d, 1H, J = 13 Hz, $PhCH_2$), 4.95 (d, 1H, J = 13 Hz, $PhCH_2$), 3.83 (m, 1H, NCH), 3.51 (m, 1H, C(OH)H), 2.84 (s, 3H, NCH_3), 2.80 (s, 3H, NCH_3), 2.28 (m, 2H, $CH(OH)CH_2$), 1.95 (m, 2H, $NCOCH_2$), 1.55 (m, 2H, $NCHCH_2$); (isomer B) δ 7.28 (m, 5H, Ph), 5.10 (d, 1H, J = 13 Hz, $PhCH_2$), 4.95 (d, 1H, J = 13 Hz, $PhCH_2$), 3.83 (m, 1H, NCH), 3.51 (m, 1H, C(OH)H), 3.00 (s, 3H, NCH_3), 2.78 (s, 3H, NCH_3), 2.28 (m, 2H, $CH(OH)CH_2$), 1.95 (m, 2H, $NCOCH_2$), 1.55 (m, 2H, $NCHCH_2$); ^{13}C NMR (125 MHz, CD_3OD) (isomer A) δ 175.3, 174.5, 158.9, 138.5, 129.4, 128.9, 128.8, 72.5, 67.5, 56.2, 35.8, 33.1, 30.7, 26.9, 26.8; (isomer B) δ 175.3, 174.5, 158.9, 138.5, 129.9, 128.8, 127.9, 72.5, 67.5, 56.5, 35.8, 33.1, 30.3, 26.8, 23.7; MS (ES) *m/z* (relative intensity) 353.5 (MH^+, 100%).

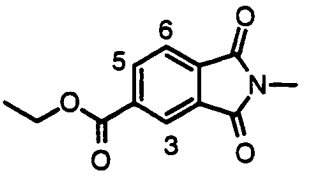


(3*RS*,4*S*)-7-(amido)-4-(*tert*-Butyloxycarbonylamino)-3-hydroxyheptanoic acid (22). To a solution of β-hydroxy benzyl ester **152** (56.1 mg, 15 mmol) in methanol (5 mL) under argon was added 10% palladium on charcoal (5.6 mg). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give the β-hydroxy acid **22** (42.0 mg, quantitative) as a white solid. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR ($CHCl_3$ cast) 3350, 2973, 1676, 1624, 1447, 1171, 1045, cm^{-1}; ^1H NMR (360 MHz, CD_3OD) (isomer A) δ 3.77 (m, 1H, NCH), 3.32 (m, 1H, C(OH)H), 2.35 (m, 2H, $CH(OH)CH_2$), 2.20 (m, 2H, $NCOCH_2$), 1.92 (m, 1H,

NCHC$\underline{H}_2$), 1.50 (m, 1H, NCHC$\underline{H}_2$), 1.32 (s, 9H, C(C$\underline{H}_3$)$_3$); (isomer B) δ 3.90 (m, 1H, NC$\underline{H}$), 3.42 (m, 1H, C(OH)$\underline{H}$), 2.35 (m, 2H, CH(OH)C$\underline{H}_2$), 2.20 (m, 2H, NCOC$\underline{H}_2$), 1.92 (m, 1H, NCHC$\underline{H}_2$), 1.50 (m, 1H, NCHC$\underline{H}_2$), 1.32 (s, 9H, C(C$\underline{H}_3$)$_3$); ^{13}C NMR (75 MHz, CD_3CN) (isomer A) δ 178.7, 175.9, 158.4, 80.2, 72.0, 56.1, 40.2, 33.1, 28.8, 27.6; (isomer B) δ 178.7, 175.9, 158.3, 80.2, 70.7, 55.4, 39.9, 33.3, 28.8, 27.6; HRMS (ES) Calcd for $C_{12}H_{22}N_2O_6Na$ 313.1376, found 313.1380.



23

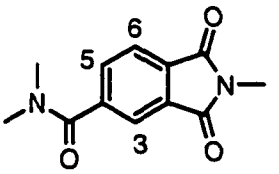


163

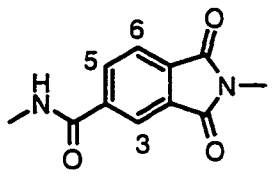
4-Carbamoyl-*N*-methylphthalimide (23) and 4-Ethyloxycarbonyl-*N*-methylphthalimide (163). Carboxylic acid **155** (20.0 mg, 97.1 μmol) was dissolved in dry CH_2Cl_2 (5 mL), with cooling to 0 °C under an argon atmosphere. Triethylamine (16.0 μL, 0.12 mmol) was added, followed by ethyl chloroformate (11.0 μL, 0.12 mmol). The mixture was stirred for 20 min at 0 °C, followed by the addition of diaminomethane dihydrochloride (11.6 mg, 97.2 μmol) and triethylamine (40.0 μL, 0.29 mmol). After 30 min at 0 °C, the reaction mixture was warmed to room temperature and stirring was continued for 3 h. The solvent was removed *in vacuo* and the residue purified by flash chromatography (ethyl acetate) to give the title compound **23** (6.7 mg, 34%) and **163** (4.2 mg, 18%), both as white solids.

For **23**: mp 257-260 °C; IR (μscope) 3322, 2950, 1777, 1672, 1525, 1070, 727, 545 cm^{-1}; ^{1}H NMR (360 MHz, CD_3CN) δ 8.19 (d, 1H, J = 8 Hz, H_5), 8.17 (s, 1H, H_3), 7.87 (d, 1H, J = 8 Hz, H_6), 6.95 (br s, 1H NH), 6.20 (br s, 1H NH), 3.09 (s, 3H, NC$\underline{H}_3$); ^{13}C NMR (75 MHz, d_7-DMF) δ 168.3, 168.2, 167.2, 140.7, 134.9, 134.2, 133.2, 123.5, 122.3, 24.1; HRMS (EI) Calcd for $C_{10}H_8N_2O_3$ 204.0535, found 204.0536.

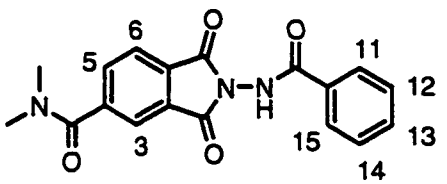
For **163**: mp 75-77 °C; IR (μscope) 3350, 2981, 1776, 1715, 1603, 1077, 727, 650 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 8.35 (dd, 1H, J = 8, 1 Hz, H_5), 8.31 (d, 1H, J = 1 Hz, H_3), 7.89 (d, 1H, J = 8 Hz, H_6), 4.40 (q, 2H, J = 7 Hz, $OC\underline{H}_2CH_3$), 3.09 (s, 3H, $NC\underline{H}_3$), 1.40 (t, 3H, J = 7 Hz, $OCH_2C\underline{H}_3$); ^{13}C NMR (125 MHz, CD_3OD) δ 168.1, 166.5, 165.3, 136.3, 136.1, 135.4, 133.1, 123.8, 123.4, 62.3, 23.5, 13.8; HRMS (EI) Calcd for $C_{12}H_{11}NO_4$ 233.0688, found 233.0689.



4-(*N'*,*N'*-Dimethyl)carbamoyl-*N*-methylphthalimide (24).[135] Carboxylic acid **155** (20.0 mg, 97.4 μmol) was dissolved in dry CH_2Cl_2 (5 mL), with cooling to 0 °C under an argon atmosphere. Triethylamine (16.0 μL, 0.12 mmol) was added, followed by ethyl chloroformate (11.0 μL, 0.12 mmol). The mixture was stirred for 20 min at 0 °C, followed by the addition of dimethylamine hydrochloride (9.50 mg, 0.12 mmol) and triethylamine (32.0 μL, 0.23 mmol). After 30 min at 0 °C, the reaction mixture was warmed to room temperature and stirring was continued for 3 h. The solvent was removed *in vacuo* and the residue purified by flash chromatography (ethyl acetate) to give the title compound **24** (21.7 mg, 96%) as a white solid: mp 95-97 °C; IR ($CHCl_3$ cast) 2927, 1776, 1634, 1504, 1065, 745, 666 cm^{-1}; ^1H NMR (360 MHz, CD_3OCD_3) δ 7.89 (d, 1H, J = 8 Hz, H_5), 7.84 (s, 1H, H_3), 7.82 (d, 1H, J = 8 Hz, H_6), 3.12 (s, 3H, $NC\underline{H}_3$), 3.07 (s, 3H, $N(C\underline{H}_3)_2$), 2.98 (s, 3H, $N(C\underline{H}_3)_2$); ^{13}C NMR (75 MHz, CD_3OD) δ 171.6, 169.3, 169.1, 143.2, 134.3, 133.9, 133.7, 124.3, 122.5, 39.8, 35.7, 24.1; HRMS (EI) Calcd for $C_{12}H_{12}N_2O_3$ 232.0848, found 232.0842.

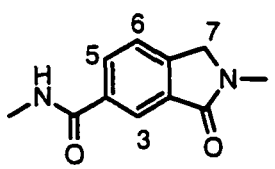


4-(*N'*-Methyl)carbamoyl-*N*-methylphthalimide (25). Carboxylic acid **155** (20.0 mg, 97.4 μmol) was dissolved in dry CH_2Cl_2 (5 mL), with cooling to 0 °C under an argon atmosphere. Triethylamine (16.0 μL, 0.12 mmol) was added, followed by ethyl chloroformate (11.0 μL, 0.12 mmol). The mixture was stirred for 20 min at 0 °C, followed by the addition of methylamine hydrochloride (7.90 mg, 0.12 mmol) and triethylamine (32.0 μL, 0.23 mmol). After 30 min at 0 °C, the reaction mixture was warmed to room temperature and stirring was continued for 3 h. The solvent was removed *in vacuo* and the residue purified by flash chromatography (ethyl acetate) to give the title compound **25** (18.4 mg, 94%) as a white solid: mp 205-207 °C; IR ($CHCl_3$ cast) 3396, 2939, 1773, 1668, 1539, 1088, 750, 665 cm^{-1}; ^1H NMR (400 MHz, CD_3CN) δ 8.15 (dd, 1H, J = 8, 1 Hz, H_5), 8.13 (s, 1H, H_3), 7.86 (dd, 1H, J = 8, 1 Hz, H_6), 7.20 (br s, 1H, NH), 3.09 (s, 3H, NCH_3), 2.89 (s, 3H, $NH(CH_3)$); ^{13}C NMR (75 MHz, CD_3OD) δ 169.5, 169.1, 168.5, 141.3, 135.7, 134.2, 133.9, 124.2, 122.6, 27.0, 24.1; HRMS (EI) Calcd for $C_{11}H_{10}N_2O_3$ 218.06914, found 218.06883.

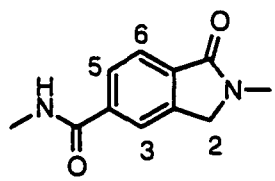


***N*-Benzamido-4-(*N',N'*-dimethyl)carbamoylphthalimide (26).** Hydrazide **166** (20.0 mg, 64.4 μmol) was dissolved in dry CH_2Cl_2 (2 mL), with cooling to 0 °C under an argon atmosphere. Triethylamine (10.7 μL, 77.0 μmol) was added, followed by ethyl chloroformate (7.4 μL, 77.0 μmol). The mixture was stirred for 20 min at 0 °C, followed by the addition of dimethylamine hydrochloride (6.3 mg, 77.0 μmol) and triethylamine (10.7 μL, 77.0 μmol). After 30 min at 0 °C, the reaction mixture was

warmed to room temperature and stirring was continued overnight. The solvent was removed *in vacuo* and the residue purified by flash chromatography (ethyl acetate : hexane, 1 : 1) to give the title compound **26** (7.3 mg, 34%) as a white solid: mp 260-262 °C; IR ($CHCl_3$ cast) 3187, 3055, 2961, 1740, 1685, 1513, 1069, 754, 693 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 9.48 (br s, 1H, CONH), 7.95 (d, 1H, J = 8 Hz, H_5), 7.95 (d, 1H, J = 7 Hz, H_{11}), 7.95 (d, 1H, J = 7 Hz, H_{15}), 7.88 (s, 1H, H_3), 7.84 (d, 1H, J = 7 Hz, H_6), 7.67 (t, 1H, J = 7 Hz, H_{13}), 7.58 (t, 1H, J = 7 Hz, H_{12}), 7.56 (t, 1H, J = 7 Hz, H_{14}), 3.10 (s, 3H, NCH_3), 2.90 (s, 3H, NCH_3); ^{13}C NMR (75 MHz, CD_3OD) δ 171.5, 168.3, 165.5, 158.5, 135.5, 135.2, 134.7, 134.0, 129.9, 129.7, 128.9, 128.7, 126.5, 125.5, 39.8, 35.7; HRMS (EI) Calcd for $C_{18}H_{15}N_3O_4$ 337.1063, found 337.1064.



27a

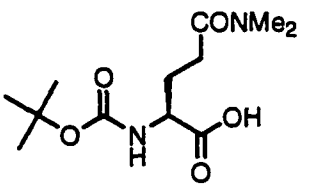


27b

4-(*N'*-Methyl)carbamoyl-*N*-methylisoindolinone (27a) and 5-(*N'*-methyl)carbamoyl-*N*-methylisoindolinone (27b). To a 5 mL round-bottom flask charged with phthalimide **25** (71.0 mg, 0.33 mmol) was added glacial acetic acid (0.25 mL), concentrated HCl (0.25 mL) and tin powder (92.7 g, 0.78 mmol). The creamy slurry was heated in an oil bath under reflux for 2 h with stirring. The solution was filtered hot and the tin shavings were washed with acetic acid (5 mL), the solvent was removed *in vacuo*. The residue was taken up in ethyl acetate (15 mL) and washed with saturated $NaHCO_3$ (2 x 10 mL). The organic extracts were washed with brine (10 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash chromatography ($CHCl_3$: methanol, 10 : 1) gave the isoindolinone isomers **27a** (19.8 mg, 30%) and **27b** (5.9 mg, 9%), both as white solids.

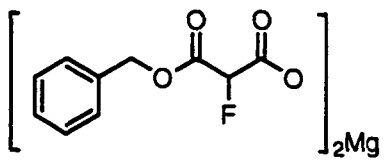
For **27a**: IR (μscope) 3343, 2929, 1692, 1656, 1552, 1106, 756 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 8.17 (d, 1H, J = 1 Hz, H_3), 7.98 (dd, 1H, J = 8, 1 Hz, H_5), 7.58 (d, 1H, J = 8 Hz, H_6), 7.10 (br s, 1H, NH), 4.44 (s, 2H, H_7), 3.12 (s, 3H, NCH_3), 2.89 (s, 3H, $NH(CH_3)$); ^{13}C NMR (125 MHz, CD_3OD) δ 169.2, 168.9, 145.6, 135.2, 133.2, 131.1, 123.7, 121.9, 52.6, 28.9, 26.3; HRMS (EI) Calcd for $C_{11}H_{12}N_2O_2$ 204.0899, found 204.0889.

For **27b**: IR (μscope) 3440, 2922, 1682, 1622, 1591, 1050, 750 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.90 (d, 1H, J = 1 Hz, H_3), 7.81 (dd, 1H, J = 8, 1 Hz, H_5), 7.76 (d, 1H, J = 8 Hz, H_6), 7.05 (br s, 1H, NH), 4.44 (s, 2H, H_2), 3.12 (s, 3H, NCH_3), 2.89 (s, 3H, $NH(CH_3)$); ^{13}C NMR (125 MHz, CD_3OD) δ 169.2, 169.1, 142.8, 138.2, 135.4, 127.4, 123.4, 122.5, 52.6, 29.0, 26.3; HRMS (EI) Calcd for $C_{11}H_{12}N_2O_2$ 204.0899, found 204.0897.

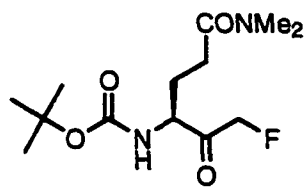


***N*-(*tert*-Butyloxycarbonyl)-γ-(*N*,*N*-dimethyl)-L-glutamine (28).**[136] To a solution of benzyl ester **45** (4.9 g, 13.7 mmol) in methanol (100 mL) under argon was added 10% palladium on charcoal (0.49 g). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give **28** (3.7 g, quantitative) as a white solid: mp 119-121 °C; $[\alpha]_D^{26}$ -4.74° (*c* 2.5, $CHCl_3$); IR (μscope) 3323, 2977, 1709, 1612, 1510, 1251 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 4.04 (dd, 1H, J = 8, 9 Hz, CH), 2.97 (s, 3H, NCH_3), 2.89 (s, 3H, NCH_3), 2.38 (m, 2H, CH_2CO), 2.08 (m, 1H, CH_2), 1.83 (m, 1H, CH_2), 1.36 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.9, 173.7, 155.7, 79.9, 53.3, 37.5, 35.9, 30.2, 28.9, 28.3; HRMS (EI) Calcd for

$C_{12}H_{22}N_2O_5$ 274.15286, found 274.15197; Anal. Calcd for $C_{12}H_{22}N_2O_5$: C, 52.54; H, 8.08; N, 10.21. Found: C, 52.12; H, 8.32 N, 10.01.

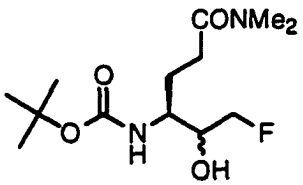


Benzyl fluoromalonate, magnesium salt (29).[28] A solution of the monoester **39** (3.0 g, 14.3 mmol) in THF (20 mL) was treated with magnesium ethoxide (0.82 g, 7.08 mmol). The mixture was stirred vigorously for 2 h and then filtered through a Celite pad. The solid was washed with THF (2 x 7 mL). Hexane (150 mL) was added dropwise over a period of 30 min to the THF filtrate with vigorous stirring. The white precipitate was immediately filtered, washed with hexane (2 x 10 mL), and the filter cake was dried under vacuum overnight, to give **29** (3.03 g, 48%) as a white solid: IR ($CHCl_3$ cast) 3441, 1752, 1671, 1454, 1272, 1181, 738, 698 cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 7.39 (m, 5H, Ph), 5.24 (d, 1H, J = 50 Hz, CH), 5.13 (s, 2H, CH_2); MS (FAB) *m/z* (relative intensity) 446.7 (MH^+, 0.31%); Anal. Calcd for $C_{20}H_{16}F_2MgO_8$: C, 53.78; H, 3.61. Found: C, 54.06; H, 3.71.



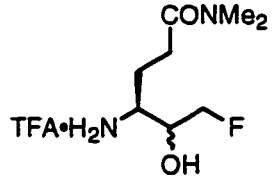
(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-fluoro-5-oxo-hexanamide (31).[28] 1,1'-Carbonyldiimidazolide (0.74 g, 4 mmol) was added to a solution of **28** (1.1 g, 4 mmol) in THF (20 mL) and stirred for 1 h. Magnesium salt **29** (0.94 g, 2 mmol) was added as a fine powder, and the mixture was stirred for 6 h. The solution was washed with 1 N HCl (4 mL). The aqueous layer was extracted with toluene (2 x 8 mL), the combined organic extracts were washed with saturated aqueous $NaHCO_3$ (4 mL) and brine (4 mL), dried over $MgSO_4$ and concentrated *in vacuo* to approximately 8

mL. The resulting solution in toluene was hydrogenated overnight under an atmosphere of hydrogen in the presence of palladium catalyst (200 mg, 10% on charcoal). The solution was filtered, washed with 1 N HCl (50 mL), saturated aqueous $NaHCO_3$ (30 mL) and brine (30 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give the fluoroketone **31** (0.26 g, 22% over both steps) as a clear oil: IR ($CHCl_3$ cast) 3303, 2977, 1740, 1635, 1507, 1252, 1168 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 5.65 (br s, 1H, NH), 5.10 (dd, 2H, J = 47, 16 Hz, CFH_2), 4.51 (m, 1H, NCH), 3.03 (s, 3H, NCH_3), 2.97 (s, 3H, CH_3), 2.48 (m, 2H, $COCH_2$), 2.21 (m, 1H, CH_2), 1.99 (m, 1H, CH_2), 1.48 (s, 9H, $C(CH_3)_3$); MS (FAB) *m/z* (relative intensity) 291.0 (MH^+, 43.5%).

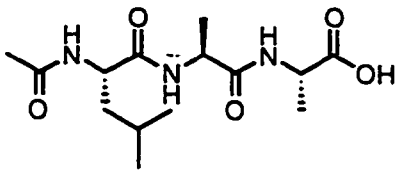


(4*S*,5*RS*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-fluoro-5-hydroxyhexanamide (32).[28] Sodium borohydride (16.2 mg, 0.42 mmol) in ethanol (2 mL) was added to a cooled (0 °C) solution of the ketone **31** (298.5 mg, 1.03 mmol) in ethanol (2 mL). The mixture was stirred at room temperature for 1 h, concentrated *in vacuo*, dissolved in water (2.5 mL) and acidified to pH 1.5 with 1 N sulfuric acid. The mixture was immediately extracted with ethyl acetate (3 x 5 mL). The combined organic layers were washed with brine (5 mL), then dried over $MgSO_4$ and the solvent evaporated *in vacuo* to yield crude fluoroalcohol **32** as a mixture of diastereoisomers. The crude mixture was purified by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 8-32% acetonitrile : water) and collected as a mixture of diastereoisomers, removal of solvent *in vacuo* gave **32** (161.3 mg, 54%) as a white solid. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 3 : 1): IR (μscope) 3331, 2982, 1698, 1625, 1562, 1252, 1165, 1030 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 5.19 (br s, 1H, NH), 4.51 (dm, 2H, J = 47 Hz, CFH_2), 3.82

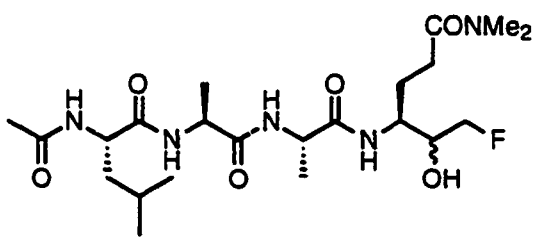
(m, 1H, NC<u>H</u>), 3.71 (m, 1H, C(OH)<u>H</u>), 3.05 (s, 3H, NC<u>H</u>$_3$), 2.95 (s, 3H, NC<u>H</u>$_3$), 2.42 (m, 2H, COC<u>H</u>$_2$), 1.96 (m, 2H, C<u>H</u>$_2$), 1.41 (s, 9H, C(C<u>H</u>$_3$)$_3$); (isomer B) δ 5.05 (br s, 1H, N<u>H</u>), 4.51 (dm, 2H, J = 47 Hz, CF<u>H</u>$_2$), 3.82 (m, 1H, NC<u>H</u>), 3.71 (m, 1H, C(OH)<u>H</u>), 3.05 (s, 3H, NC<u>H</u>$_3$), 2.98 (s, 3H, NC<u>H</u>$_3$), 2.42 (m, 2H, COC<u>H</u>$_2$), 1.96 (m, 2H, C<u>H</u>$_2$), 1.41 (s, 9H, C(C<u>H</u>$_3$)$_3$); MS (FAB) *m/z* (relative intensity) 293.0 (MH^+, 53.9%).



(4*S*,5*RS*)-*N*,*N*-Dimethyl-4-amino-6-fluoro-5-hydroxyhexanamide, trifluoroacetate salt (33).[28] Trifluoroacetic acid (0.9 mL) was added to a cooled (0 °C) solution of **32** (103.4 mg, 0.353 mmol) in CH_2Cl_2 (3.7 mL). The solution was stirred and warmed to 10 °C over 1 h. The solvent was evaporated *in vacuo* and the residue was dissolved in CH_2Cl_2 (3.7 mL) and cooled to 0 °C. Further trifluoroacetic acid (0.9 mL) was added and the mixture was allowed to warm to room temperature over 1.5 h. The solvent was evaporated *in vacuo*, toluene (3 mL) and diethyl ether (3 mL) were added and evaporated and the residue was dried under high vacuum to yield the trifluoroacetate salt **33** (117.3 mg, quantitative) as a white solid mixture of diastereoisomers. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 3 : 1): IR ($CHCl_3$ cast) 3200, 2950, 1677, 1626, 1504, 1263, 1183, 1063 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 8.05 (br s, 3H, N<u>H</u>$_3$), 4.55 (dm, 2H, J = 47 Hz, CF<u>H</u>$_2$), 4.12 (m, 1H, NC<u>H</u>), 3.39 (m, 1H, C(OH)<u>H</u>), 3.05 (s, 3H, NC<u>H</u>$_3$), 2.95 (s, 3H, NC<u>H</u>$_3$), 2.78 (m, 1H, C<u>H</u>$_2$), 2.55 (m, 1H, C<u>H</u>$_2$), 1.98 (m, 2H, COC<u>H</u>$_2$); (isomer B) δ 8.05 (br s, 3H,N<u>H</u>$_3$), 4.55 (dm, 2H, J = 47 Hz, CF<u>H</u>$_2$) 3.91 (m, 1H, NC<u>H</u>), 3.27 (m, 1H, C(OH)<u>H</u>), 2.99 (s, 3H, NC<u>H</u>$_3$), 2.89 (s, 3H, NC<u>H</u>$_3$), 2.78 (m, 1H, C<u>H</u>$_2$), 2.55 (m, 1H, C<u>H</u>$_2$), 1.98 (m, 2H, COC<u>H</u>$_2$); MS (FAB) *m/z* (relative intensity) 193.0 (MH^+, 100%).

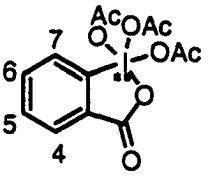


Acetyl-L-leucyl-L-alanyl-L-alanine (34).[26] Peptide **34** was prepared on a Rainin PS-3 solid-phase peptide synthesizer using standard Fmoc chemistry on Wang resin. The N-terminus was capped using acetic anhydride. Purification by HPLC (gradient elution over 40 min of acetonitrile and 0.1% TFA in water, from 0-30%, t_R 12.1 min) gave peptide **34** (42%) as a white solid: IR (μscope) 3333, 3061, 2975, 1730, 1655, 1580, 1265, 1162, 608 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 4.2-4.0 (m, 3H, α-H Leu and 2α-H Ala), 1.72 (s, 3H, $COCH_3$), 1.45 (m, 1H, CH Leu), 1.33 (m, 2H, CH_2 Leu), 1.14 (d, 3H, J = 7 Hz, CH_3 Ala), 1.12 (d, 3H, J = 7 Hz, CH_3 Ala), 0.75 (d, 3H, J = 6 Hz, CH_3 Leu), 0.65 (d, 3H, J = 6 Hz, CH_3 Leu); MS (FAB) *m/z* (relative intensity) 316.0 (MH^+, 22.8%).



(4*S*,5*RS*)-*N*,*N*-Dimethyl-4-(acetylleucylalanylalanyl)amino-6-fluoro-5-hydroxyhexanamide (35).[28] Triethylamine (86.5 μl, 0.624 mmol) was added to a solution of *N*-acetylleucylalanylalanine **34** (95.3 mg, 0.302 mmol) and HBTU (118.6 mg, 0.313 mmol) in DMF (2.5 mL) at 0 °C. The solution was stirred at 0 °C for 5 min, then added dropwise over 10 min to a solution of the trifluoroacetate salt **33** (95.8 mg, 0.312 mmol) and triethylamine (86.5 μl, 0.624 mmol) in DMF (2.5 mL) also at 0 °C. The mixture was stirred at 0 °C for 2 h, then the cold bath was removed and stirring was continued for an additional 3 h. The mixture was dried *in vacuo* overnight, and the residue

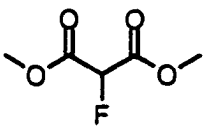
was purified by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-40% acetonitrile : water, t_R 12.1 min) to yield **35** (12 mg, 8%) as a white solid mixture of diastereoisomers. Spectroscopic characterization was performed on a mixture of diastereoisomers: IR ($CHCl_3$ cast) 3286, 2956, 1651, 1574, 1245, 1161 cm^{-1}: ^1H NMR (360 MHz, D_2O) δ 4.38 (dm, 2H, J = 47 Hz, $CF\underline{H}_2$), 4.2-4.0 (m, 4H, α-H Leu and 2α-H Ala and α-H dimethyl Gln), 3.75 (m, 1H, C(OH)H), 2.92 (s, 3H, $NC\underline{H}_3$), 2.78 (s, 3H, $NC\underline{H}_3$), 2.32 (m, 2H, $COC\underline{H}_2$), 2.28 (m, 1H, $C\underline{H}_2$ dimethyl Gln), 1.98 (m, 1H, $C\underline{H}_2$ dimethyl Gln), 1.88 (s, 3H, $COC\underline{H}_3$), 1.58 (m, 1H, C<u>H</u> Leu), 1.45 (m, 2H, $C\underline{H}_2$ Leu), 1.27 (m, 3H, $C\underline{H}_3$ Ala), 1.25 (m, 3H, $C\underline{H}_3$ Ala), 0.79 (m, 3H, $C\underline{H}_3$ Leu), 0.72 (m, 3H, $C\underline{H}_3$ Leu); MS (FAB) *m/z* (relative intensity) 490.4 (MH^+, 3.83%).



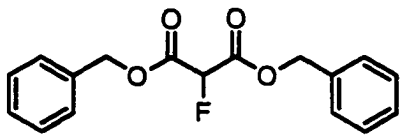
1,1,1-Triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one (36).[69] To a suspension of 2-iodobenzoic acid (5.65 g, 22.6 mmol) in a solution of 1 N sulfuric acid (35 mL) was added $KBrO_3$ (4.95 g, 29.5 mmol) over a period of 30 min. The temperature was maintained below 55 °C with an ice bath. The round-bottom flask was equiped with a reflux condenser and the temperature of the solution was raised to 65 °C with an oil bath, stirring was continued for 4 h. Bromine was given off as copious brown fumes which were dispersed with a stream of argon. The mixture was cooled to 0 °C and filtered. The solid was washed with water (50 mL), ethanol (2 x 5 mL) and diethyl ether (2 x 5 mL), and dried *in vacuo* to give the Dess-Martin precursor oxide (hydroxyiodinane oxide) as a white solid (5.41 g, 85%).

CAUTION! The Dess-Martin precursor oxide was reported to be explosive under excessive heating (>200 °C) or impact.[137]

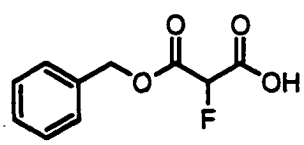
To a solution of *p*-toluenesulfonic acid (60 mg, 0.315 mmol) in acetic anhydride (48 mL) was added the previously prepared hydroxyiodinane oxide (5.40 g, 19.31 mmol). The mixture was heated in an oil bath maintained at 80 °C for 2 h. The brown solution was cooled in an ice bath and the precipitate collected by filtration to afford a white powder. This was washed with diethyl ether (5 x 5 mL) to give Dess-Martin periodinane **36** (4.88 g, 60%) which was stored under argon with refrigeration: IR (μscope) 3067, 2989, 1636, 1564, 1248, 1164, 781 cm^{-1}; ^1H NMR (300 MHz, D_2O) δ 8.91 (dd, 1H, J = 8, 0.9 Hz, H_7), 8.03 (dd, 1H, J = 7, 1.5 Hz, H_4), 7.92 (ddd, 1H, J = 8, 7, 1.5 Hz, H_6), 7.75 (ddd, 1H, J = 8, 7, 0.9 Hz, H_5), 1.95 (s, 9H, 3($C\underline{H}_3$)); MS (FAB) *m/z* (relative intensity) 424.9 (MH^+, 0.23%).



Dimethyl fluoromalonate (37).[71] To a solution of dry methanol (125 mL) at 0 °C was added sodium (12.7 g, 0.55 mmol) over 30 min with stirring. 2,3,3,3-tetrafluoropropanenitrile (20 g, 0.16 mol) was added dropwise, maintaining the temperature below 10 °C with an icebath. The reaction mixture was allowed to warm to room temperature and stirring was continued for 1 h. The resulting solution was acidified to pH 2 with concentrated HCl over a period of 1 h. After stirring at room temperature for another 1 h the mixture was poured into ice water. The oily product was extracted with diethyl ether (2 x 20 mL), the combined organic layers were washed with brine (20 mL), dried over $MgSO_4$ and filtered. The filtrate was distilled under reduced pressure to give **37** (8.62 g, 36%) as a light yellow liquid: bp 90-92 °C / 15 mmHg (lit. bp 80-83 °C / 13 mmHg)[71]; IR (neat) 2963, 1774, 1439, 1209, 1117, 706 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 5.28 (d, 1H, J = 42 Hz, C$\underline{H}$), 3.86 (s, 6H, 2($C\underline{H}_3$)).

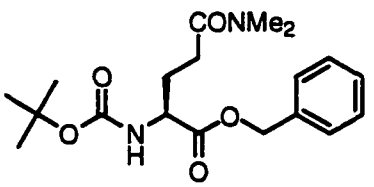


Dibenzyl fluoromalonate (38).[28] A mixture of dimethyl ester **37** (4.67 g, 31.08 mmol), toluene (13 mL), benzyl alcohol (16 mL, 151 mmol) and *p*-toluenesulfonic acid (0.35 g, 1.84 mmol) in a 3-neck 50 mL round-bottom flask connected to a Dean-Stark apparatus was heated under reduced pressure (45 °C / 30 mmHg) until all of the toluene had distilled, then for an additional 5 h at 115 °C / 70 mmHg. The mixture was allowed to cool to 55 °C and isopropanol (7.5 mL) was added, followed by hexane (15 mL). The product crystallized and was placed in the freezer overnight. The product was filtered, washed with hexane (2 x 10 mL), and dried overnight *in vacuo* to afford dibenzyl ester **38** (8.2 g, 87%) as a white solid: mp 45.5-47.5 °C; IR (CH_2Cl_2 cast) 2990, 1744, 1455, 1271, 1186, 1176, 740, 696 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.39 (m, 10H, 2Ph), 5.38 (d, 1H, J = 48 Hz, CH), 5.23 (s, 4H, 2(CH_2)); HRMS (EI) Calcd for $C_{17}H_{15}FO_4$ 302.0954, found 302.0956; Anal. Calcd for $C_{17}H_{15}FO_4$: C, 67.54; H, 5.00. Found: C, 67.82; H, 4.88.



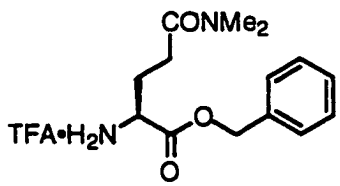
Benzyl fluoromalonate (39).[28] Dibenzyl ester **38** (7.3 g, 24.2 mmol) was suspended in isopropanol (40 mL) and heated with stirring to 45 °C, by which time the solid material had dissolved. Over a 1 h period, 1 N aqueous NaOH (25 mL, 25.4 mmol) was added dropwise. After an additional 10 min, the solution was concentrated *in vacuo* to a volume of approximately 17 mL. Water (25 mL) was added, and the pH of the solution was adjusted to 8.5 using saturated aqueous $NaHCO_3$. The mixture was washed with CH_2Cl_2 (2 x 10 mL) to remove the benzyl alcohol. The pH of the aqueous layer was adjusted to 2 with 6 N HCl. The mixture was extracted with ethyl acetate (20 mL). The pH

of the aqueous layer was adjusted to 2 with 2 N HCl, and a second ethyl acetate (20 mL) extraction was performed. The combined extracts were washed with brine (15 mL), dried over $MgSO_4$, filtered and evaporated to dryness. The oily residue was triturated with hexane (25 mL) for 1 h, which gave **39** (2.75 g, 54%) as a white solid: mp 121 °C dec; IR (CH_2Cl_2 cast) 3200, 2961, 1760, 1456, 1277, 1191, 751, 697 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 9.27 (br s, 1H, OH), 7.41 (m, 5H, Ph), 5.42 (d, 1H, J = 48 Hz, CH), 5.34 (s, 2H, CH_2); HRMS (EI) Calcd for $C_{10}H_9FO_4$ 212.0485, found 212.0487; Anal. Calcd for $C_{10}H_9FO_4$: C, 56.61; H, 4.27. Found: C, 56.97; H, 4.07.

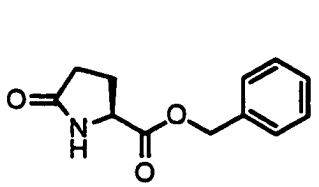


***N*-(*tert*-Butyloxycarbonyl)-γ-(*N*,*N*-dimethyl)-L-glutamine benzyl ester (45).**[136] *N*-*t*-Boc-L-Glutamic acid α-benzyl ester **44** (Sigma) (1.0 g, 3.0 mmol) was dissolved in dry CH_2Cl_2 (10 mL), with cooling to 0 °C under an argon atmosphere. Triethylamine (0.40 mL, 3.0 mmol) was added, followed by ethyl chloroformate (0.30 mL, 3.0 mmol). The mixture was stirred for 20 min at 0 °C, followed by the addition of dimethylamine hydrochloride (0.25 g, 0.30 mmol) and triethylamine (0.40 mL, 3.0 mmol). After 30 min at 0 °C, the reaction mixture was warmed to room temperature and stirring was continued overnight. The solvent was removed *in vacuo*, the residue was partitioned between ethyl acetate (10 mL) and water (10 mL). The aqueous phase was extracted with ethyl acetate (2 x 5 mL) and the combined organic extracts were washed with saturated aqueous $NaHCO_3$ (5 mL) and brine (5 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give a white solid. Purification by recrystallization (CH_2Cl_2-hexane) gave the title compound **45** (1.04 g, 96%) as a white solid: mp 93-95 °C ; $[\alpha]_D^{26}$ +9.02° (*c* 1.2, $CHCl_3$); IR ($CHCl_3$ cast) 3305, 2976, 1744, 1711, 1640, 1500, 1251, 751, 698 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.35 (m, 5H, Ph), 5.42 (d, 1H, J = 6 Hz, NH), 5.19 (d, H, J = 12

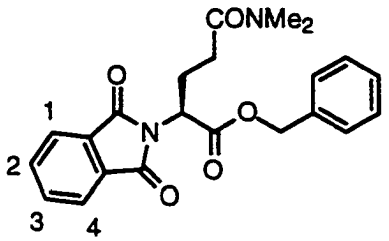
Hz, PhC$\underline{H}_2$), 5.17 (d, H, J = 12 Hz, PhC$\underline{H}_2$), 4.31 (m, 1H, C$\underline{H}$), 2.88 (s, 3H, NC$\underline{H}_3$), 2.86 (s, 3H, NC$\underline{H}_3$), 2.37 (m, 2H, C$\underline{H}_2$CO), 2.21 (m, 1H, C$\underline{H}_2$), 2.11 (m, 1H, C$\underline{H}_2$), 1.43 (s, 9H, C(C$\underline{H}_3$)$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.3, 171.8, 155.6, 135.5, 128.6, 128.4, 128.3, 79.8, 67.0, 53.5, 37.1, 35.6, 29.3, 28.3, 27.6; HRMS (EI) Calcd for $C_{19}H_{28}N_2O_5$ 364.1998, found 364.1997; Anal. Calcd for $C_{19}H_{28}N_2O_5$: C, 62.62; H, 7.74; N, 7.69. Found: C, 62.45; H, 7.94; N, 7.40.



L-Glutamic acid-α-benzyl ester-γ-dimethylamide, trifluoroacetate salt (46). To a solution of *N*-(*tert*-butyloxycarbonyl)-γ-(*N,N*-dimethyl)-L-glutamine benzyl ester **45** (0.50 g, 1.40 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added trifluoroacetic acid (10 mL); the mixture was stirred for 1 h at 0 °C. The ice bath was removed and the solution was allowed to warm to room temperature over a 1 h period. The solvent was removed *in vacuo* and the residue was triturated with hexane (3 x 10 mL) and dried under high vacuum to give the trifluoroacetate salt **46** (0.47 g, 91%) as a yellow oil: IR (CH_2Cl_2 cast) 3036, 2946, 1779, 1673, 1556, 1260, 1084, 754, 701 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.38 (br s, 3H, N$\underline{H}_3$), 7.39 (m, 5H, $\underline{Ph}$), 5.15 (d, 1H, J = 12 Hz, PhC$\underline{H}_2$), 5.34 (d, 1H, J = 12 Hz, PhC$\underline{H}_2$), 4.21 (m, 1H, C$\underline{H}$), 2.89 (s, 6H, (NC$\underline{H}_3$)$_2$), 2.55 (m, 1H, C$\underline{H}_2$), 2.45 (m, 1H, C$\underline{H}_2$), 2.26 (m, 2H, COC$\underline{H}_2$); HRMS (EI) Calcd for $C_{14}H_{20}N_2O_3$ 264.1474, found 264.1468.



47

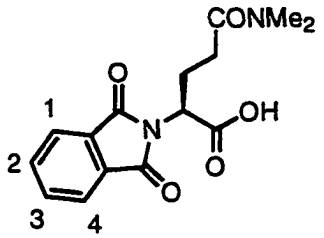


48

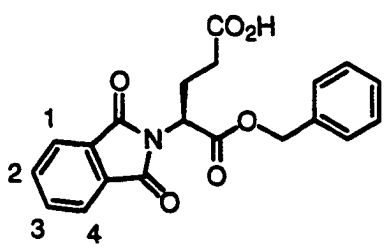
(5*S*)-5-Benzyloxycarbonyl-2-azolidinone (47) and *N*-phthaloyl-L-glutamic acid-α-benzyl ester-γ-dimethylamide (48).[138] To a solution of the trifluoroacetate salt **46** (1.0 g, 2.64 mmol) in THF (25 mL) was added triethylamine (0.52 mL, 3.75 mmol) and *N*-carbethoxyphthalimide (0.58 g, 2.65 mmol) at room temperature. The mixture was heated at reflux, under argon for 24 h and then concentrated *in vacuo*. Purification by flash chromatography (ethyl acetate) gave the side-product L-glutamine α-benzyl ester-γ-lactam **47** (40 mg, 7%) as a clear oil and compond **48** (0.68 g, 65%) as a white solid.

For **47**: IR (CH_2Cl_2 cast) 3226, 3091, 2957, 1704, 1498, 1193, 750, 698 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.39 (m, 5H, Ph), 6.15 (br s, 1H, NH), 5.19 (s, 2H, $PhCH_2$), 4.28 (dd, 1H, J = 9, 5 Hz, CH), 2.49 (m, 1H, CH_2), 2.36 (m, 2H, $COCH_2$), 2.27 (m, 1H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 128.5, 66.5, 55.5, 29.7; HRMS (EI) Calcd for $C_{12}H_{13}NO_3$ 219.0895, found 219.0877.

For **48**: mp 63-65 °C; IR (CH_2Cl_2 cast) 3090, 2937, 1775, 1743, 1716, 1644, 1387, 753, 698 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.85 (m, 2H, H_1 and H_4), 7.72 (m, 2H, H_2 and H_3), 7.27 (m, 5H, Ph), 5.19 (d, 1H, J = 13 Hz, $PhCH_2$), 5.12 (d, 1H, J = 13 Hz, $PhCH_2$), 5.01 (dd, 1H, J = 6, 4 Hz, CH), 2.89 (s, 3H, NCH_3), 2.83 (s, 3H, NCH_3), 2.64 (m, 1H, CH_2), 2.55 (m, 1H, CH_2), 2.37 (m, 2H, $COCH_2$); HRMS (EI) Calcd for $C_{22}H_{22}N_2O_5$ 394.1529, found 394.1525; Anal. Calcd for $C_{22}H_{22}N_2O_5$: C, 66.99; H, 5.62; N, 7.10. Found: C, 66.77; H, 5.96; N, 7.08.

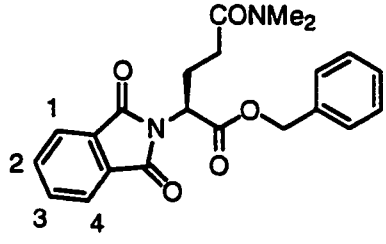


***N*-Phthaloyl-L-glutamic acid-γ-dimethylamide (49).** To a solution of benzyl ester **48** (1.63 g, 4.13 mmol) in methanol (30 mL) under argon was added 10% palladium on charcoal (0.16 g). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give **49** (1.24 g, quantitative) as a white solid: mp 134-136 °C; IR (CH_2Cl_2 cast) 3434, 3026, 2935, 1720, 1620, 1387, 723 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.89 (m, 2H, H_1 and H_4), 7.71 (m, 2H, H_2 and H_3), 5.18 (br s, 1H, $CO_2\underline{H}$), 4.88 (dd, 1H, J = 6, 4 Hz, C$\underline{H}$), 3.03 (s, 3H, NC$\underline{H}_3$), 2.96 (s, 3H, NC$\underline{H}_3$), 2.69 (m, 1H, C$\underline{H}_2$), 2.51 (m, 2H, COC$\underline{H}_2$), 2.46 (m, 1H, C$\underline{H}_2$); HRMS (EI) Calcd for $C_{15}H_{16}N_2O_5$ 304.1059, found 304.1058; Anal. Calcd for $C_{15}H_{16}N_2O_5$: C, 59.21; H, 5.29; N, 9.20. Found: C, 59.28; H, 4.91; N, 9.04.



***N*-Phthaloyl-L-glutamic acid-α-benzyl ester (51).**[139] To a solution of L-glutamic acid-α-benzyl ester **50** (3.0 g, 12.6 mmol) in THF (100 mL) was added *N*-carbethoxyphthalimide (3.33 g, 15.2 mmol) and then triethylamine (2.1 mL, 15.2 mmol). The solution was heated at reflux for 24 h under argon. The solvent was removed *in vacuo*, the residue was dissolved in 10% aqueous $NaHCO_3$ (15 mL) and washed with ethyl acetate (3 x 10 mL). The aqueous layer was acidified to pH 2.5 with 5 N HCl and extracted with ethyl acetate (3 x 10 mL), dried over Na_2SO_4 and concentrated *in vacuo* to yield **51** (3.7 g,

80%) as a yellow oil: IR (CH_2Cl_2 cast) 3064, 2950, 1777, 1743, 1716, 1455, 1388, 1193 720, 698 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.82 (m, 2H, H_1 and H_4), 7.72 (m, 2H, H_2 and H_3), 7.29 (m, 5H, Ph), 5.21 (d, 1H, J = 13 Hz, $PhCH_2$), 5.17 (d, 1H, J = 13 Hz, $PhCH_2$), 5.01 (dd, 1H, J = 7, 4 Hz, CH), 2.63 (m, 1H, CH_2), 2.57 (m, 1H, CH_2), 2.38 (m, 2H, $COCH_2$); HRMS (ES) Calcd for $C_{20}H_{18}N_1O_6$ 368.1134, found 368.1137.

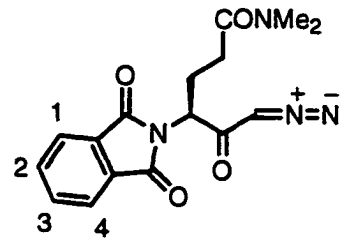


***N*-Phthaloyl-L-glutamic acid-α-benzyl ester-γ-dimethylamide (52).** Reaction of *N*-phthaloyl-L-glutamic acid-α-benzyl ester **51** (3.39 g, 9.23 mmol) with triethylamine (1.6 mL, 11.5 mmol), ethyl chloroformate (1.1 mL, 11.5 mmol), and dimethylamine hydrochloride (0.94 g, 11.5 mmol) with triethylamine (1.6 mL, 11.5 mmol) as described for **45** gave the title compound **52** (3.16 g, 87%) as a white solid; data as **48**: mp 63-65 °C; IR (CH_2Cl_2 cast) 3090, 2934, 1775, 1743, 1716, 1644, 1387, 753, 698 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.85 (m, 2H, H_1 and H_4), 7.75 (m, 2H, H_2 and H_3), 7.27 (m, 5H, Ph), 5.21 (d, 1H, J = 13 Hz, $PhCH_2$), 5.18 (d, 1H, J = 13 Hz, $PhCH_2$), 5.03 (dd, 1H, J = 6, 4 Hz, CH), 2.89 (s, 3H, NCH_3), 2.83 (s, 3H, NCH_3), 2.63 (m, 1H CH_2), 2.55 (m, 1H CH_2), 2.37 (m, 2H, $COCH_2$); HRMS (EI) Calcd for $C_{22}H_{22}N_2O_5$ 394.1529, found 394.1522; Anal. Calcd for $C_{22}H_{22}N_2O_5$: C, 66.99; H, 5.62; N, 7.10. Found: C, 66.73; H, 5.45; N, 6.95.

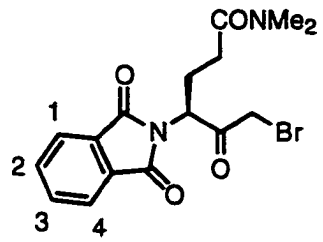
$H_2C{=}\overset{+}{N}{=}\overset{-}{N}$

Diazomethane (53).[140] Only non-ground glassware was used. To a solution of 2-ethoxy ethanol (10.5 mL) in diethyl ether (15.5 mL) was added a solution of KOH (2.11 g, 37.7 mmol) in water (3.5 mL), the mixture was heated under reflux. A solution of

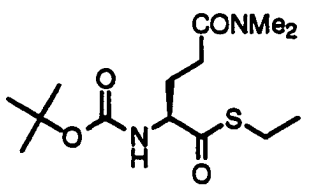
Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) (7.43 g, 34.7 mmol) in diethyl ether (78.5 mL) was added dropwise to the solution at reflux, producing a yellow ethereal solution of diazomethane (ca. 0.37 M) which was distilled into a receiving flask cooled at 0 °C. The ethereal solution of diazomethane was used immediately.



(4*S*)-*N*,*N*-Dimethyl-6-diazo-5-oxo-4-phthalimidohexanamide (54). Triethylamine (0.58 mL, 4.3 mmol) and ethyl chloroformate (1.1 mL, 4.2 mmol) were added to a cooled (0 °C) solution of *N*-phthaloyl-L-glutamic acid-γ-dimethylamide **49** (1.05 g, 3.5 mmol) in THF (50 mL). The mixture was stirred at 0 °C for 5 min and then filtered quickly into a ca. 0.3 M ethereal diazomethane solution **53** (47 mL, ca. 17 mmol) at 0 °C. The yellow mixture slowly paled on warming to room temperature overnight. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate / water (20 mL, 1 : 1) and washed with ethyl acetate (3 x 10 mL). The sample was dried over $MgSO_4$ and concentrated *in vacuo*, giving the diazoketone **54** (1.05 g, 92%) as a yellow oil: IR (CH_2Cl_2 cast) 3093, 2108, 1713, 1638, 1385, 1339, 720 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.88 (m, 2H, H_1 and H_4), 7.79 (m, 2H, H_2 and H_3), 5.61 (s, 1H, $N_2C\underline{H}$), 4.92 (dd, 1H, J = 6, 4 Hz, $C\underline{H}$), 2.95 (s, 3H, $NC\underline{H}_3$), 2.89 (s, 3H, $NC\underline{H}_3$), 2.59 (m, 2H, $COC\underline{H}_2$), 2.39 (m, 2H, $C\underline{H}_2$); HRMS (ES) Calcd for $C_{16}H_{16}N_4O_4Na$ 351.1064, found 351.1070.

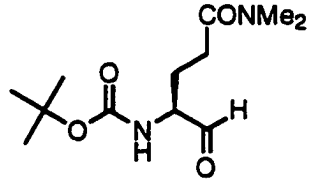


(4*S*)-*N*,*N*-Dimethyl-6-bromo-5-oxo-4-phthalimidohexanamide (55). Aqueous hydrobromic acid (48%) (0.23 mL, 2.05 mmol) was added to a cooled (0 °C), vigorously stirred solution of the diazoketone **54** (0.561 g, 1.71 mmol) in THF (20 mL). The solution was stirred for 1 h until gas evolution ceased, and then CH_2Cl_2 (20 mL) was added. The solution was washed with water (3 x 10 mL), dried over $MgSO_4$ and the solvent evaporated *in vacuo* to give the bromoketone **55** (0.638 g, 98%) as an orange oil: IR ($CHCl_3$ cast) 3050, 2938, 1774, 1714, 1641, 1412, 1384, 721, 529 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.89 (m, 2H, H_1 and H_4), 7.78 (m, 2H, H_2 and H_3), 5.19 (dd, 1H, *J* = 6, 4 Hz, C<u>H</u>), 4.09 (d, 1H, *J* = 11 Hz, CBr<u>H</u>), 4.05 (d, 1H, *J* = 11 Hz, CBr<u>H</u>), 2.91 (s, 3H, NC<u>H</u>$_3$), 2.81 (s, 3H, NC<u>H</u>$_3$), 2.62 (m, 1H C<u>H</u>$_2$), 2.48 (m, 1H, C<u>H</u>$_2$), 2.38 (m, 2H, COC<u>H</u>$_2$); HRMS (ES) Calcd for $C_{16}H_{18}BrN_2O_4$ 381.0450, found 381.0445.



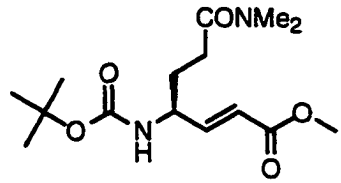
***N*-(*tert*-Butyloxycarbonyl)-γ-(*N*,*N*-dimethyl)-L-glutamine α-ethyl thioester (61).**[26] *N*-(*tert*-butyloxycarbonyl)-γ-(*N*,*N*-dimethyl)-L-glutamine **28** (1.0 g, 3.65 mmol) was dissolved in dry CH_2Cl_2 (20 mL), with cooling to 0 °C under an argon atmosphere. Triethylamine (0.60 mL, 4.40 mmol) was added, followed by ethyl chloroformate (0.42 mL, 4.40 mmol). The mixture was stirred for 20 min at 0 °C, followed by the addition of ethane thiol (0.32 ml, 4.40 mmol) and triethylamine (0.60 mL, 4.40 mmol). After 30 min at 0 °C, the reaction mixture was warmed to room temperature and stirring was continued overnight. To the mixture was added CH_2Cl_2 (20 mL) then the

solution was washed with 0.5 M HCl (2 x 5 mL), saturated aqueous $NaHCO_3$ (5 mL) and brine (5 mL), and dried over $MgSO_4$. The solvent was removed *in vacuo* to give crude product which was recrystallized from (CH_2Cl_2-hexane) to yield the title compound **61** (1.12 g, 97%) as a white solid: mp 145-146 °C; $[\alpha]_D^{26}$ -10.97° (*c* 1.6, $CHCl_3$); IR ($CHCl_3$ cast) 3214, 2980, 1711, 1674, 1617, 1545, 1254, 1164 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 5.72 (d, 1H, *J* = 7 Hz, NH), 4.31 (m, 1H, CH), 2.99 (s, 3H, NCH_3), 2.95 (s, 3H, NCH_3), 2.84 (q, 2H, *J* = 7 Hz, SCH_2CH_3), 2.43 (m, 2H, CH_2CO), 2.19 (m, 1H, CH_2), 2.02 (m, 1H, CH_2), 1.43 (s, 9H, $C(CH_3)_3$), 1.22 (t, 3H, *J* = 7 Hz, SCH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 202.4, 172.7, 156.2, 80.5, 61.2, 37.8, 36.3, 29.9, 29.1, 28.9, 27.9, 23.7; MS (FAB) *m/z* (relative intensity) 319.0 (MH^+, 100%); Anal. Calcd for $C_{14}H_{26}N_2O_4S$: C, 52.80; H, 8.23; N, 8.80. Found: C, 52.40; H, 8.42; N, 8.68.

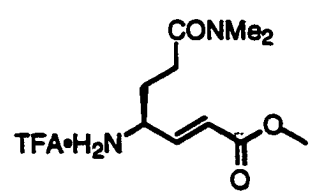


***N*-(*tert*-Butyloxycarbonyl)-γ-(*N*,*N*-dimethyl)-L-glutaminal (62).** To a stirred solution of thioester **61** (0.10 g, 0.31 mmol) in CH_2Cl_2 (2 mL) at room temperature was added 10% palladium on charcoal (5.0 mg) under argon and then triethylsilane (0.25 mL, 1.57 mmol). The mixture was stirred for 2 h. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give a clear oil. Purification by flash chromatography (ethyl acetate) gave aldehyde **62** (64.8 mg, 80%) as a white solid: mp 142-144 °C; $[\alpha]_D^{26}$ +23.20° (*c* 8.5, $CHCl_3$); IR ($CHCl_3$ cast) 3300, 2976, 1733, 1707, 1634, 1511, 1055 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 9.60 (s, 1H, CHO), 5.59 (br s, 1H, NH), 4.18 (m, 1H, CH), 3.02 (s, 3H, NCH_3), 2.92 (s, 3H, NCH_3), 2.43 (m, 2H, CH_2CO), 2.24 (m, 1H, CH_2), 1.99 (m, 1H, CH_2), 1.44 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 200.8, 172.7, 156.6, 80.4, 60.1, 37.8, 36.1, 29.3, 29.0,

26.6; MS (FAB) *m/z* (relative intensity) 258.9 (MH^+, 100%); Anal. Calcd for $C_{12}H_{22}N_2O_4$: C, 55.80; H, 8.58; N, 10.85. Found: C, 55.41; H, 8.52; N, 10.52.

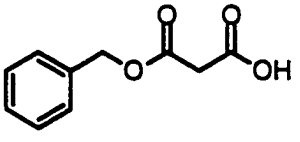


Methyl (2*E*,4*S*)-4-(*tert*-butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-7-oxohepten-2-oate (63). To a solution of **62** (35.9 mg, 0.14 mmol) in THF (2 mL) at room temperature under argon was added methyl (triphenylphosphoranylidene)acetate (51.0 mg, 0.15 mmol). The reaction mixture was stirred overnight. The solvent was removed *in vacuo* and the residue was purified by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-30% acetonitrile : water, t_R 25.9 min) to give **63** (12.8 mg, 30%) as a white solid: mp 137-139 °C; $[\alpha]_D^{26}$ -4.23° (*c* 2.1, $CHCl_3$); IR ($CHCl_3$ cast) 3221, 2981, 1723, 1710, 1658, 1539, 1027 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 6.84 (dd, 1H, *J* = 15, 5 Hz, C<u>H</u>CHCO$_2$Me), 5.95 (dd, 1H, *J* = 15, 1 Hz, CHC<u>H</u>CO$_2$Me), 5.10 (br s, 1H, N<u>H</u>), 4.38 (m, 1H, NC<u>H</u>), 3.73 (s, 3H, CO$_2$C<u>H</u>$_3$), 3.00 (s, 3H, NC<u>H</u>$_3$), 2.97 (s, 3H, NC<u>H</u>$_3$), 2.41 (m, 2H, C<u>H</u>$_2$CO), 1.97 (m, 2H, C<u>H</u>$_2$), 1.43 (s, 9H, C(C<u>H</u>$_3$)$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.5, 167.4, 156.1, 149.1, 121.3, 95.4, 52.3, 30.2, 30.1, 29.8, 29.1, 29.1, 29.0; HRMS (EI) Calcd for $C_{15}H_{26}N_2O_5$ 314.1842, found 314.1843.

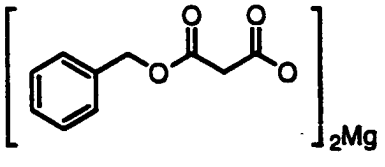


Methyl (2*E*,4*S*)-4-amino-7-(*N*,*N*-dimethylamino)-7-oxohepten-2-oate, trifluoroacetate salt (64). To a solution of α,β-unsaturated ester **63** (45.0 mg, 0.14 mmol) in CH_2Cl_2 (0.5 mL) at 0 °C was added trifluoroacetic acid (0.5 mL), and the

mixture was stirred for 1 h at 0 °C. The ice bath was removed and the solution was allowed to warm to room temperature over a 1 h period. The solvent was removed *in vacuo* and the residue dried under high vacuum to give the trifluoroacetate salt **64** (42.70 g, 91%) as a yellow oil: $[\alpha]_D^{26}$ +9.92° (*c* 2.1, H_2O); IR ($CHCl_3$ cast) 3220, 2954, 1726, 1676, 1623, 1507, 1202, 1034 cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 6.72 (dd, 1H, *J* = 16, 8 Hz, C<u>H</u>CHCO$_2$Me), 6.08 (dd, 1H, *J* = 16, 1 Hz, CHC<u>H</u>CO$_2$Me), 3.95 (ddd, 1H, *J* = 8, 5, 3 Hz, NC<u>H</u>), 3.65 (s, 3H, CO$_2$C<u>H</u>$_3$), 2.93 (s, 3H, NC<u>H</u>$_3$), 2.79 (s, 3H, NC<u>H</u>$_3$), 2.40 (m, 2H, C<u>H</u>$_2$CO), 2.04 (m, 1H, C<u>H</u>$_2$), 1.93 (m, 1H, C<u>H</u>$_2$); ^{13}C NMR (125 MHz, D_2O) δ 174.4, 168.4, 142.4, 125.9, 53.2, 52.3, 37.9, 36.2, 29.1, 28.0; HRMS (ES) Calcd for $C_{10}H_{19}N_2O_3$ 215.1396, found 215.1400.

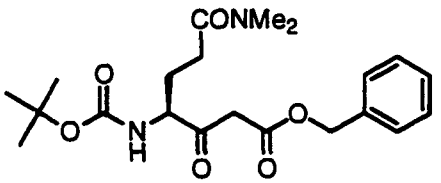


Benzyl malonate (72).[141] Reaction of dibenzyl malonate (16.83 g, 52.2 mmol) and 1 N aqueous NaOH (62.2 mL, 62.2 mmol) as described for **39** gave the title compound **72** (10.2 g, 89%) as a white solid: mp 38-42 °C; IR (CH_2Cl_2 cast) 3035, 2950, 1746, 1498, 1215, 1105, 750, 698 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.28 (m, 5H, <u>Ph</u>), 5.17 (s, 2H, PhC<u>H</u>$_2$), 3.42 (s, 2H, C<u>H</u>$_2$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.9, 166.4, 134.9, 128.6, 128.5, 128.3, 67.5, 40.9; HRMS (EI) Calcd for $C_{10}H_{10}O_4$ 194.05791, found 194.05704; Anal. Calcd for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19. Found: C, 62.20; H, 5.49.



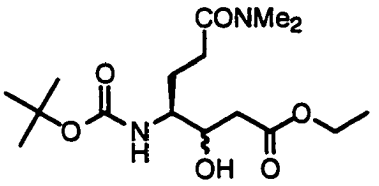
Benzyl malonate, magnesium salt (73).[141] Reaction of benzyl malonate (9.66 g, 49.75 mmol) and magnesium ethoxide (2.85 g, 24.88 mmol) as described for **29**

gave the title compound **73** (9.89 g, 97%) as a white solid: mp 130-135 °C; IR (CH_2Cl_2 cast) 3500, 3064, 2959, 1721, 1659, 1498, 1228, 1106, 754, 682 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.33 (m, 5H, Ph), 5.07 (s, 2H, $PhCH_2$), 3.22 (s, 2H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.9, 171.1, 135.6, 128.8, 128.6, 128.2, 67.3, 20.8; MS (FAB) *m/z* (relative intensity) 411.0 (MH^+, 1.5%); Anal. Calcd for $C_{20}H_{18}MgO_8$: C, 58.49; H, 4.42. Found: C, 58.41; H, 4.55.

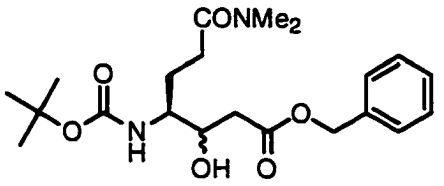


Benzyl (4*S*)-4-(*tert*-butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3,7-dioxoheptanoate (74). To a solution of *N*-(*tert*-butyloxycarbonyl)-γ-(*N*,*N*-dimethyl)-L-glutamine **28** (3.40 g, 12.38 mmol) in THF (60 mL) was added 1,1'-carbonyl diimidazole (2.41 g, 14.85 mmol). The clear solution was stirred for 1 h at room temperature under argon. Magnesium benzyl malonate **73** (6.1 g, 14.85 mmol) was added. The mixture was stirred overnight at room temperature. The pH was adjusted to 2 with 0.5 N HCl, and the product was extracted with ethyl acetate (2 x 50 mL). The combined extracts were washed with saturated aqueous $NaHCO_3$ (25 mL), and brine (25 mL), dried over $MgSO_4$ and concentrated *in vacuo*. Recrystallization (CH_2Cl_2-hexane) gave benzyl ester **74** (4.88 g, 97%) as a white solid: mp 94-96 °C; IR ($CHCl_3$ cast) 3296, 2976, 1747, 1712, 1634, 1505, 1252, 1165, 1024, 750, 698 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.38 (m, 5H, Ph), 5.65 (d, 1H, J = 6 Hz, NH), 5.17 (s, 2H, $PhCH_2$), 4.36 (m, 1H, CH), 3.68 (s, 2H, $COCH_2CO$), 2.97 (s, 3H, NCH_3), 2.93 (s, 3H, NCH_3), 2.50 (m, 1H, $NCOCH_2$), 2.37 (m, 1H, $NCOCH_2$), 2.19 (m, 1H, $NCHCH_2$), 1.96 (m, 1H, $NCHCH_2$), 1.41 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.4, 172.1, 166.9, 155.8, 135.4, 128.6, 128.6, 128.3, 80.0, 67.1, 59.7, 45.9, 37.1, 35.6,

28.9, 28.3, 25.9; HRMS (EI) Calcd for $C_{21}H_{30}N_2O_6$ 406.2104, found 406.2099; Anal. Calcd for $C_{21}H_{30}N_2O_6$: C, 62.05; H, 7.44; N, 6.89. Found: 61.98; H, 7.43; N, 6.85.



75



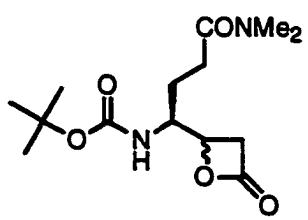
76

Ethyl (3*RS*,4*S*)-4-(*tert*-butyloxycarbonylamino)-3-hydroxy-7-(*N*,*N*-dimethylamino)-7-oxoheptanoate (75) and benzyl (3*RS*,4*S*)-4-(*tert*-butyloxycarbonylamino)-3-hydroxy-7-(*N*,*N*-dimethylamino)-7-oxoheptanoate (76). To a stirred solution of β-keto ester **74** (3.01g, 7.38 mmol) in ethanol (37 mL) under argon at 0 °C, was added dropwise a solution of $NaBH_4$ in absolute ethanol (0.1 M, 73 mL). The reaction mixture was stirred at 0 °C for 30 min, then at room temperature for 30 min. The solution was acidified to pH 2 with 1 N $KHSO_4$ and the solvent was removed *in vacuo*. The residue was dissolved in water (50 mL), extracted with ethyl acetate (3 x 25 mL), dried over $MgSO_4$ and concentrated *in vacuo* to yield crude alcohol. Purification by flash chromatography (ethyl acetate : hexane, 9 : 1) gave **75** (0.15 g, 6%) and β-hydroxy esters **76** (2.57 g, 85%) as a clear oil and white solid, respectively.

For **75**: Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR ($CHCl_3$ cast) 3345, 2977, 1732, 1711, 1694, 1249, 1043 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 5.20 (br s, 1H, NH), 4.18 (q, 2H, J = 7 Hz, OCH_2CH_3), 3.99 (m, 1H, NCH), 3.47 (m, 1H, C(OH)H), 3.01 (s, 3H, NCH_3), 2.97 (s, 3H, NCH_3), 2.50 (m, 2H, $CH(OH)CH_2$), 2.40 (m, 2H, $NCOCH_2$), 1.99 (m, 1H, $NCHCH_2$), 1.81 (m, 1H, $NCHCH_2$), 1.41 (s, 9H, $C(CH_3)_3$), 1.23 (t, 3H, J = 7 Hz, OCH_2CH_3); (isomer B) δ 5.20 (br s, 1H, NH), 4.17 (q, 2H, J = 7 Hz, OCH_2CH_3), 4.01 (m, 1H, NCH), 3.47 (m, 1H, C(OH)H), 3.01 (s, 3H, NCH_3), 2.97 (s, 3H, NCH_3), 2.50 (m, 2H, $CH(OH)CH_2$), 2.40 (m, 2H, $NCOCH_2$), 1.99 (m, 1H, $NCHCH_2$), 1.81 (m, 1H,

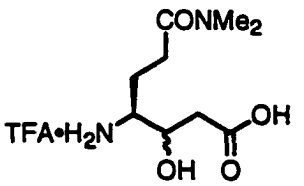
NCHC$\underline{H}_2$), 1.41 (s, 9H, C(C$\underline{H}_3$)$_3$), 1.21 (t, 3H, J = 7 Hz, OCH$_2$C$\underline{H}_3$); ^{13}C NMR (75 MHz, $CDCl_3$) (isomer A) δ 172.9, 172.6, 156.2, 79.2, 70.9, 60.6, 54.6, 38.8, 37.1, 35.5, 29.6, 28.3, 24.9, 14.1; (isomer B) δ 172.9, 172.4, 156.1, 79.0, 68.7, 60.5, 54.0, 38.5, 37.1, 35.5, 29.6, 28.3, 24.9, 0.9; HRMS (EI) Calcd for $C_{16}H_{30}N_2O_6$ 346.2104, found 346.2140.

For **76**: Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR ($CHCl_3$ cast) 3331, 3030, 2974, 1719, 1710, 1649, 1500, 1247, 1163, 1043, 738, 694 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) (isomer A) δ 7.39 (m, 5H, $\underline{Ph}$), 5.18 (s, 2H, PhC$\underline{H}_2$), 5.13 (br s, 1H, N$\underline{H}$), 3.99 (m, 1H, NC$\underline{H}$), 3.59 (m, 1H, C(OH)$\underline{H}$), 3.01 (s, 3H, NC$\underline{H}_3$), 2.95 (s, 3H, NC$\underline{H}_3$), 2.61 (m, 2H, CH(OH)C$\underline{H}_2$), 2.41 (m, 2H, NCOC$\underline{H}_2$), 1.99 (m, 1H, NCHC$\underline{H}_2$), 1.82 (m, 1H, NCHC$\underline{H}_2$), 1.43 (s, 9H, C(C$\underline{H}_3$)$_3$); (isomer B) δ 7.39 (m, 5H, $\underline{Ph}$), 5.18 (s, 2H, PhC$\underline{H}_2$), 4.98 (br s, 1H, N$\underline{H}$), 3.99 (m, 1H, NC$\underline{H}$), 3.59 (m, 1H, C(OH)$\underline{H}$), 3.01 (s, 3H, NC$\underline{H}_3$), 2.95 (s, 3H, NC$\underline{H}_3$), 2.61 (m, 2H, CH(OH)C$\underline{H}_2$), 2.41 (m, 2H, NCOC$\underline{H}_2$), 1.99 (m, 1H, NCHC$\underline{H}_2$), 1.82 (m, 1H, NCHC$\underline{H}_2$), 1.43 (s, 9H, C(C$\underline{H}_3$)$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) (isomer A) δ 172.9, 172.7, 156.5, 135.7, 128.6, 128.3, 128.2, 79.5, 68.5, 66.5, 54.2, 38.4, 37.2, 35.7, 29.6, 28.4, 25.0; (isomer B) δ 172.9, 172.7, 156.5, 135.7, 128.6, 128.3, 128.2, 79.5, 70.9, 66.5, 54.8, 38.7, 37.2, 35.7, 29.6, 28.4, 25.0; HRMS (EI) Calcd for $C_{21}H_{32}N_2O_6$ 408.2260, found 408.2248; Anal. Calcd for $C_{21}H_{32}N_2O_6$: C, 61.75; H, 8.14; N, 6.86. Found: 61.52; H, 8.27; N, 7.04.



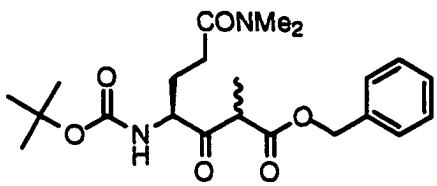
(4*RS*)-4-[(1*S*)-1-(*tert*-Butyloxycarbonylamino)-3-(*N*,*N*-dimethylcarbamoyl)propyl]-2-oxetanone (77). A suspension of **17** (0.11 g, 0.32 mmol) in CH_2Cl_2 (5 mL) was cooled to 0 °C and treated with triethylamine (0.14 mL, 0.97

mmol) followed by BOP (0.17 g, 0.39 mmol). The cooling bath was removed and the reaction mixture was stirred at room temperature for 24 h. The reaction was quenched with water (6 mL) and extracted with CH_2Cl_2 (3 x 5 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated *in vacuo*. Purification by flash chromatography (ethyl acetate) followed by crystallization from ($CHCl_3$-hexane) gave β-lactone **77** (1.25 g, 41%) as a white solid. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 9 :1): IR ($CHCl_3$ cast) 3299, 2975, 1830 1710, 1630, 1624, 1248, 1168, 1049, cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) (isomer A) δ 5.12 (br s, 1H, NH), 4.34 (m, 1H, CH_2OCOCH), 3.85 (m, 1H, NCH), 3.48 (m, 2H, CH_2OCOCH), 3.02 (s, 3H, NCH_3), 2.96 (s, 3H, NCH_3), 2.44 (m, 1H, $NCOCH_2$), 2.03 (m, 1H, $NCHCH_2$), 1.88 (m, 1H, $NCHCH_2$), 1.43 (s, 9H, $C(CH_3)_3$); (isomer B) δ 5.12 (br s, 1H, NH), 4.34 (m, 1H, CH_2OCOCH), 3.85 (m, 1H, NCH), 3.48 (m, 2H, CH_2OCOCH), 3.05 (s, 3H, NCH_3), 2.95 (s, 3H, NCH_3), 2.44 (m, 1H, $NCOCH_2$), 2.03 (m, 1H, $NCHCH_2$), 1.88 (m, 1H, $NCHCH_2$), 1.47 (s, 9H, $C(CH_3)_3$); ^{13}C NMR-(HMQC) (125 MHz, $CDCl_3$) (isomer A) δ 71.9, 53.1, 41.2, 37.5, 36.3, 28.9, 27.8, 24.1; (isomer B) δ 71.9, 53.1, 41.2, 37.5, 36.3, 28.9, 27.8, 24.1; HRMS (ES) Calcd for $C_{14}H_{24}N_2O_5Na$ 323.1583, found 323.1583.

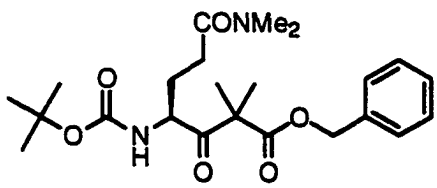


(3*RS*,4*S*)-4-amino-7-(*N*,*N*-dimethylamino)-3-hydroxy-7-oxoheptanoic acid, trifluoroacetate salt (85). To a solution of β-hydroxy acid **17** (0.10 g, 0.31 mmol) in CH_2Cl_2 (5 mL) at 0 °C was added trifluoroacetic acid (5 mL), the mixture was stirred for 1 h at 0 °C. The ice bath was removed and the solution was allowed to warm to room temperature over a 1 h period. The solvent was removed *in vacuo* and the residue was crystallized from methanol-$CHCl_3$ and dried under high vacuum to give the

trifluoroacetate salt **85** (81.0 mg, quantitative) as a white solid. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR (μscope) 3261, 2945, 1725, 1666, 1612, 1417, 1204, 1066 cm^{-1}; ^1H NMR (360 MHz, D_2O) (isomer A) δ 4.23 (m, 1H, NC<u>H</u>), 3.29 (m, 1H, C<u>H</u>(OH)), 2.93 (s, 3H, NC<u>H</u>$_3$), 2.81 (s, 3H, NC<u>H</u>$_3$), 2.53 (m, 2H, CH(OH)C<u>H</u>$_2$), 2.52 (m, 2H, NCOC<u>H</u>$_2$), 1.87 (m, 1H, NCHC<u>H</u>$_2$), 1.75 (m, 1H, NCHC<u>H</u>$_2$); (isomer B) δ 4.23 (m, 1H, NC<u>H</u>), 3.29 (m, 1H, C<u>H</u>(OH)), 2.93 (s, 3H, NC<u>H</u>$_3$), 2.81 (s, 3H, NC<u>H</u>$_3$), 2.53 (m, 2H, CH(OH)C<u>H</u>$_2$), 2.52 (m, 2H, NCOC<u>H</u>$_2$), 1.87 (m, 1H, NCHC<u>H</u>$_2$), 1.75 (m, 1H, NCHC<u>H</u>$_2$); ^{13}C NMR (75 MHz, D_2O) (isomer A) δ 175.5, 174.8, 67.9, 55.7, 38.1, 37.9, 36.3, 30.0, 23.2; (isomer B) δ 175.5, 174.8, 67.5, 55.7, 38.1, 37.9, 36.3, 29.7, 25.6; HRMS (ES) Calcd for $C_9H_{19}N_2O_4$ 219.1345, found 219.1343.



87



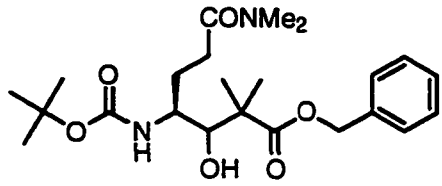
88

Benzyl (2*RS*,4*S*)-4-(*tert*-butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-2-methyl-3,7-dioxoheptanoate (87) and benzyl (4*S*)-4-(*tert*-butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-2,2-dimethyl-3,7-dioxoheptanoate (88). To a solution of benzyl (4*S*)-4-(*tert*-butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3,7-dioxoheptanoate **74** (15.0 g, 36.9 mmol) in THF (200 mL) was added potassium *tert*-butoxide (8.28 g, 73.8 mmol), the solution was stirred for 10 min at 0 °C under argon. Iodomethane (4.59 mL, 73.8 mmol) was added dropwise over 15 min, the mixture was stirred for 30 min at 0 °C and then warmed to room temperature overnight. The solvent was removed *in vacuo* to give a residue which was suspended in water (100 mL) and washed with ethyl acetate (3 x 50 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a brown oil. Purification by flash

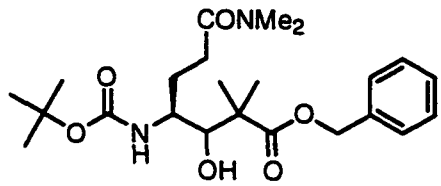
chromatography (ethyl acetate : hexane, 1 : 1) produced the mono-methyl analogue **87** (1.7 g, 11%) and compound **88** (2.18 g, 13.6%), both as white solids after recrystallization from diethyl ether-hexane.

For **87**: Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 1 : 1): IR ($CHCl_3$ cast) 3303, 2975, 1744, 1708, 1640, 1455, 1170, 1088, 751, 699 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 7.33 (m, 5H, Ph), 5.48 (br s, 1H, NH), 5.13 (m, 2H, $PhCH_2$), 4.49 (m, 1H, CH), 3.97 (m, 1H, $CH(CH_3)$) 3.01 (s, 3H, NCH_3), 2.99 (s, 3H, NCH_3), 2.45 (m, 1H, $NCHCH_2$), 2.34 (m, 2H, $NCOCH_2$), 1.73 (m, 1H, $NCHCH_2$), 1.48 (s, 9H, $C(CH_3)_3$); 1.12 (s, 3H, $CH(CH_3)$); (isomer B) δ 7.33 (m, 5H, Ph), 5.45 (br s, 1H, NH), 5.13 (m, 2H, $PhCH_2$), 4.49 (m, 1H, CH), 3.97 (m, 1H, $CH(CH_3)$) 2.92 (s, 3H, NCH_3), 2.88 (s, 3H, NCH_3), 2.45 (m, 1H, $NCHCH_2$), 2.34 (m, 2H, $NCOCH_2$), 1.73 (m, 1H, $NCHCH_2$), 1.47 (s, 9H, $C(CH_3)_3$); 1.09 (s, 3H, $CH(CH_3)$); ^{13}C NMR (75 MHz, $CDCl_3$) (isomer A) δ 205.5, 171.9, 170.1, 155.2, 135.6, 128.4, 128.3, 128.2, 67.2, 67.0, 57.3, 54.8, 37.4, 35.6, 28.9, 28.0, 26.4, 17.7; (isomer B) δ 205.5, 171.9, 170.1, 155.2, 135.6, 128.4, 128.3, 128.2, 67.2, 67.0, 57.3, 54.8, 37.1, 35.6, 28.9, 28.3, 26.0, 19.1; HRMS (EI) Calcd for $C_{22}H_{32}N_2O_6$ 420.2260, found 420.2269.

For **88**: mp 77-79 °C; $[\alpha]_D^{26}$ -14.29° (*c* 0.7, $CHCl_3$); IR ($CHCl_3$ cast) 3300, 2977, 1742, 1708, 1643, 1498, 1165, 1082, 752, 698 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.37 (m, 5H, Ph), 5.45 (br s, 1H, NH), 5.18 (s, 2H, $PhCH_2$), 4.62 (m, 1H, CH), 2.96 (s, 6H, $N(CH_3)_2$), 2.34 (m, 2H, $NCOCH_2$), 2.17 (m, 1H, $NCHCH_2$), 1.79 (m, 1H, $NCHCH_2$), 1.46 (s, 3H, $C(CH_3)$), 1.43 (s, 3H, $C(CH_3)$) 1.41 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 208.0, 172.8, 171.9, 155.4, 135.6, 128.6, 128.3, 128.2, 79.8, 67.1, 55.5, 54.8, 37.1, 35.6, 28.9, 28.3, 27.7, 22.4, 22.1; HRMS (EI) Calcd for $C_{23}H_{34}N_2O_6$ 434.24170, found 406.24100; Anal. Calcd for $C_{23}H_{34}N_2O_6$: C, 63.57; H, 7.89; N, 6.45. Found: 63.20; H, 7.93; N, 6.54.



89a



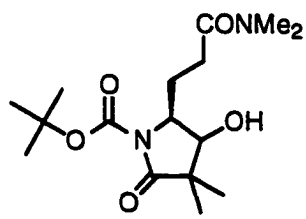
89b

Benzyl (3*R* or *S*,4*S*)-4-(*tert*-butyloxycarbonylamino)-3-hydroxy-7-(*N*,*N*-dimethylamino)-2,2-dimethyl-7-oxoheptanoate (89a & 89b). To a stirred solution of β-keto ester **88** (2.16 g, 4.98 mmol) in ethanol (25 mL) under argon at 0 °C, was added dropwise a solution of $NaBH_4$ (0.1 M, 50 mL) in absolute ethanol. The reaction mixture was stirred at 0 °C for 30 min, followed by 30 min at room temperature. The solution was acidified to pH 2 with 1 N $KHSO_4$ and the solvent was removed *in vacuo*. The residue was dissolved in water (25 mL), extracted with ethyl acetate (3 x 15 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield crude alcohol **89**. Purification by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-30% acetonitrile : water, t_R 30.2 and 32.6 min) separated the β-hydroxy ester diastereoisomers **89a** (230 mg, 11%) and **89b** (110 mg, 5%) respectively, as yellow oils.

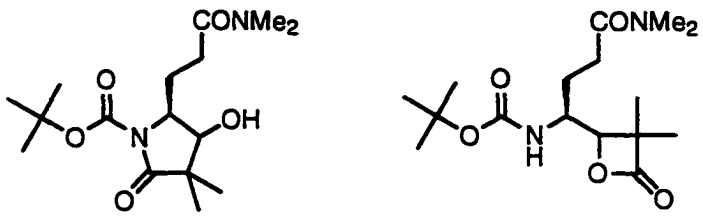
For **89a**: $[\alpha]_D^{26}$ -36.93° (*c* 1.8, $CHCl_3$); IR ($CHCl_3$ cast) 3363, 3030, 2975, 1710, 1632, 1261, 1167, 1041, 753, 697 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.37 (m, 5H, Ph), 5.19 (d, 1H, *J* = 12 Hz, $PhCH_2$), 5.12 (d, 1H, *J* = 12 Hz, $PhCH_2$), 4.69 (br s, 1H, NH), 3.73 (m, 1H, NCH), 3.69 (br s, 1H, C(OH)H), 2.99 (s, 3H, NCH_3), 2.93 (s, 3H, NCH_3), 2.34 (m, 2H, $NCOCH_2$), 1.92 (m, 1H, $NCHCH_2$), 1.69 (m, 1H, $NCHCH_2$), 1.43 (s, 9H, $C(CH_3)_3$), 1.29, (s, 3H, $C(CH_3)$), 1.22, (s, 3H, $C(CH_3)$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.9, 172.9, 155.4, 135.9, 128.6, 128.1, 127.9, 79.2, 78.2, 66.4, 52.2, 45.4, 37.2, 35.6, 29.2, 28.4, 26.6, 23.5, 20.5; HRMS (EI) Calcd for $C_{23}H_{36}N_2O_6$ 436.2573, found 436.2573.

For **89b**: $[\alpha]_D^{26}$ -76.60° (*c* 0.43, $CHCl_3$); IR ($CHCl_3$ cast) 3432, 3032, 2975, 1708, 1650, 1626, 1260, 1167, 1050, 752, 698 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.38 (m, 5H,

Ph), 5.19 (d, 1H, J = 12 Hz, PhC$\underline{H}_2$), 5.09 (d, 1H, J = 12 Hz, PhC$\underline{H}_2$), 4.92 (d, 1H, N$\underline{H}$), 3.84 (m, 1H, NC$\underline{H}$), 3.64 (br s, 1H, C(OH)$\underline{H}$), 2.97 (s, 3H, NC$\underline{H}_3$), 2.92 (s, 3H, NC$\underline{H}_3$), 2.35 (m, 2H, NCOC$\underline{H}_2$), 1.96 (m, 2H, NCHC$\underline{H}_2$), 1.44 (s, 9H, C(C$\underline{H}_3$)$_3$), 1.29, (s, 3H, C(C$\underline{H}_3$)), 1.21, (s, 3H, C(C$\underline{H}_3$)); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 173.2, 155.7, 135.8, 128.6, 128.2, 127.8, 79.2, 78.5, 66.6, 50.3, 46.1, 37.3, 35.7, 29.9, 25.5, 28.4, 23.1, 20.8; HRMS (EI) Calcd for $C_{23}H_{36}N_2O_6$ 436.2573, found 436.2578.



(4*R* or *S*,5*S*)-1-(*tert*-Butyloxycarbonyl)-4-hydroxy-3,3-dimethyl-5-[(2-*N*,*N*-dimethylcarbamoyl)ethyl]azolidin-2-one (90a). A suspension of **19a** (47.6 mg, 0.14 mmol) in CH_2Cl_2 (5 mL) was cooled to 0 °C and treated with triethylamine (57 μL, 0.41 mmol) followed by BOP (73.0 mg, 0.16 mmol). The cold bath was removed and the reaction mixture was stirred at room temperature for 24 h. The reaction was quenched with water (6 mL) and extracted with CH_2Cl_2 (3 x 5 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-20% acetonitrile : water, t_R 18.5 min) gave **90a** (19.4 mg, 44%) as a white solid: $[\alpha]_D^{26}$ +39.82° (*c* 3.4, $CHCl_3$); IR ($CHCl_3$ cast) 3338, 2975, 1775, 1714, 1628, 1257, 1154, 1087 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.96 (br s, 1H, CH(O$\underline{H}$)), 3.79 (d, 1H, J = 9 Hz, C(OH)$\underline{H}$), 3.59 (ddd, 1H, J = 9, 6, 3 Hz, NC$\underline{H}$), 3.08 (s, 3H, NC$\underline{H}_3$), 2.97 (s, 3H, NC$\underline{H}_3$), 2.68 (m, 1H, NCOC$\underline{H}_2$), 2.58 (m, 1H, NCOC$\underline{H}_2$), 2.19 (m, 1H, NCHC$\underline{H}_2$), 2.01 (m, 1H, NCHC$\underline{H}_2$), 1.57 (s, 9H, C(C$\underline{H}_3$)$_3$), 1.23 (s, 3H, C(C$\underline{H}_3$)), 1.12 (s, 3H, C(C$\underline{H}_3$)); ^{13}C NMR (75 MHz, CD_3CN) δ 173.4, 171.5, 150.9, 83.4, 78.4, 62.3, 45.7, 37.4, 35.8, 30.8, 28.7, 28.1, 23.2, 18.5; HRMS (ES) Calcd for $C_{16}H_{28}N_2O_5Na$ 351.1896, found 351.1902.



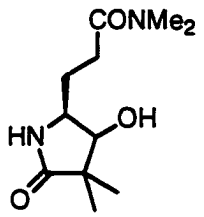
90b **93**

(4*R* or *S*,5*S*)-1-(*tert*-Butyloxycarbonyl)-4-hydroxy-3,3-dimethyl-5-[(2-*N*,*N*-dimethylcarbamoyl)ethyl]azolidin-2-one (90b) and (4*R* or *S*)-4-[(1*S*)-1-(*tert*-butyloxycarbonylamino)-3-(*N*,*N*-dimethylcarbamoyl)propyl]-3,3-dimethyl-2-oxetanone (93). A suspension of **19b** (43.1 mg, 0.12 mmol) in CH_2Cl_2 (5 mL) was cooled to 0 °C and treated with triethylamine (52 μL, 0.37 mmol) followed by BOP (66.0 mg, 0.15 mmol). The cooling bath was removed and the reaction mixture was stirred at room temperature for 24 h. The reaction was quenched with water (6 mL) and extracted with CH_2Cl_2 (3 x 5 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-20% acetonitrile : water, t_R 26.9 and 30.5 min) gave **90b** (10.0 mg, 25%) and β-lactone **93** (19.7 mg, 48%) respectively, as white solids.

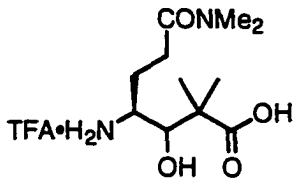
For **90b**: $[\alpha]_D^{26}$ +11.35° (*c* 2.3, $CHCl_3$); IR ($CHCl_3$ cast) 3355, 2970, 1756, 1714, 1633, 1255, 1155, 1094 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 4.03 (d, 1H, *J* = 9 Hz, C(OH)H), 3.91 (ddd, 1H, *J* = 9, 7, 3 Hz, NCH), 3.07 (s, 3H, NCH_3), 2.98 (s, 3H, NCH_3), 2.72 (m, 1H, $NCOCH_2$), 2.59 (m, 1H, $NCOCH_2$), 1.75 (m, 1H, $NCHCH_2$), 1.57 (s, 9H, $C(CH_3)_3$), 1.42 (m, 1H, $NCHCH_2$), 1.21 (s, 3H, $C(CH_3)$), 1.17 (s, 3H, $C(CH_3)$); ^{13}C NMR (75 MHz, CD_3CN) δ 175.1, 171.5, 150.9, 84.5, 73.4, 60.8, 47.7, 37.8, 35.8, 31.7, 28.7, 25.3, 24.2, 19.3; HRMS (ES) Calcd for $C_{16}H_{28}N_2O_5Na$ 351.1896, found 351.1902.

For **93**: $[\alpha]_D^{26}$ +6.19° (*c* 1.1, CH_3OH); IR ($CHCl_3$ cast) 3313, 2972, 1825, 1711, 1651, 1247, 1168, 1047 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 4.65 (d, 1H, *J* = 9 Hz, NH), 4.18

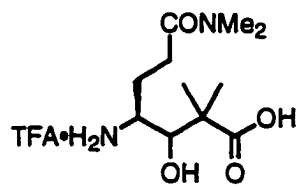
(d, 1H, J = 5 Hz, OCOCH), 3.99 (m, 1H, NCH), 3.02 (s, 3H, NCH_3), 2.97 (s, 3H, NCH_3), 2.42 (m, 2H, $NCOCH_2$), 1.89 (m, 1H, $NCHCH_2$), 1.79 (m, 1H, $NCHCH_2$), 1.43 (s, 3H, $C(CH_3)$), 1.41 (s, 9H, $C(CH_3)_3$)), 1.32 (s, 3H, $C(CH_3)$); ^{13}C NMR (75 MHz, CD_3CN) δ 174.7, 171.9, 155.6, 83.7, 73.5, 53.7, 49.5, 37.1, 35.6, 28.7, 28.5, 28.3, 23.4, 16.1; HRMS (ES) Calcd for $C_{16}H_{28}N_2O_5Na$ 351.1896, found 351.1887.



(4*R* or *S*,5*S*)-4-Hydroxy-3,3-dimethyl-5-[(2-*N*,*N*-dimethylcarbamoyl)ethyl]azolidin-2-one (91a). A 5 mL single-necked round-bottom flask was equipped with a magnetic stirring bar and an argon inlet. The flask was charged with a mixture of **90a** (8.50 mg, 25.80 μmol) and anhydrous *p*-toluenesulfonic acid (4.70 mg, 27.1 μmol), the flask was cooled in an ice bath for 15 min. Anhydrous TFA (0.3 mL) was added *via* a syringe over 5 min. The pale yellow solution was stirred at 0 °C for 25 min, the TFA was removed *in vacuo* below 30 °C and the resulting yellow oil was placed under high vacuum. The residue was purified by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-30% acetonitrile : water, t_R 6.2 min) to yield γ-lactam **91a** (3.80 mg, 64%) as a clear oil: $[\alpha]_D^{26}$ +35.71° (*c* 0.84, H_2O); IR ($CHCl_3$ cast) 3275, 2933, 1778, 1679, 1123, 1034 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 6.18 (br s, 1H, NH), 3.67 (d, 1H, J = 7 Hz, C(OH)H), 3.38 (m, 1H, NCH), 3.09 (s, 3H, NCH_3), 2.97 (s, 3H, NCH_3), 2.51 (m, 2H, $NCOCH_2$), 1.99 (m, 2H, $NCHCH_2$), 1.19 (s, 3H, $C(CH_3)$), 1.04 (s, 3H, $C(CH_3)$); ^{13}C NMR (75 MHz, CD_3CN) δ 175.3, 157.1, 85.2, 59.2, 34.4, 33.1, 29.1, 26.1, 23.9, 21.4, 18.9; HRMS (ES) Calcd for $C_{11}H_{20}N_2O_3Na$ 251.1372, found 251.1369.

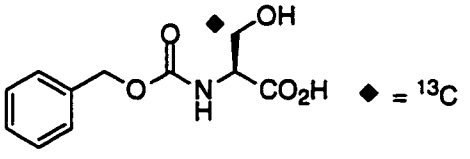


(3*R* or *S*,4*S*)-4-amino-7-(*N*,*N*-dimethylamino)-3-hydroxy-2,2-dimethyl-7-oxoheptanoic acid, trifluoroacetate salt (99a). To a solution of β-hydroxy acid **19a** (17.1 mg, 49.3 μmol) in CH_2Cl_2 (0.5 mL) at 0 °C was added trifluoroacetic acid (0.5 mL), the mixture was stirred for 1 h at 0 °C. The ice bath was removed and the solution was allowed to warm to room temperature over a 1 h period. The solvent was removed *in vacuo* and the residue dried under high vacuum to give the trifluoroacetate salt **99a** (81.0 mg, quantitative) as a yellow oil: $[\alpha]_D^{26}$ -1.92° (*c* 4.7, H_2O); IR ($CHCl_3$ cast) 3350, 2934, 1674, 1625, 1407, 1137, 1059 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 3.59 (d, 1H, *J* = 2 Hz, C(OH)H), 3.34 (ddd, 1H, *J* = 6, 4, 2 Hz, NCH), 3.02 (s, 3H, NCH_3), 2.87 (s, 3H, NCH_3), 2.48 (m, 2H, $NCOCH_2$), 1.84 (m, 2H, $NCHCH_2$), 1.19 (s, 3H, $C(CH_3)$), 1.16 (s, 3H, $C(CH_3)$); ^{13}C NMR (75 MHz, CD_3CN) δ 185.5, 174.8, 76.8, 52.8, 37.9, 36.0, 30.9, 30.7, 29.2, 23.3, 18.3; HRMS (ES) Calcd for $C_{11}H_{23}N_2O_4$ 247.1658, found 247.16626.

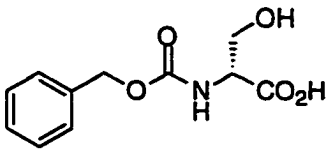


(3*R* or *S*,4*S*)-4-amino-7-(*N*,*N*-dimethylamino)-3-hydroxy-2,2-dimethyl-7-oxoheptanoic acid, trifluoroacetate salt (99b). Reaction of β-hydroxy acid **19b** (22.0 mg, 63.5 μmol) as described for **19a** gave the title compound **99b** (23.0 mg, quantitative) as a light-brown oil: $[\alpha]_D^{26}$ -21.27° (*c* 6.6, H_2O); IR ($CHCl_3$ cast) 3450, 2961, 1725, 1694, 1651, 1427, 1140 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 3.52 (d, 1H, *J* = 7 Hz, C(OH)H), 3.36 (m, 1H, NCH), 2.94 (s, 3H, NCH_3), 2.85 (s, 3H, NCH_3), 2.48 (m, 2H, $NCOCH_2$), 1.85 (m, 1H, $NCHCH_2$), 1.73 (m, 1H,

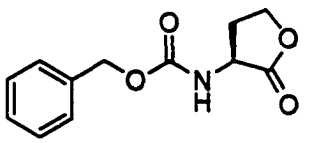
NCHC$\underline{H}_2$), 1.04 (s, 3H, C(C$\underline{H}_3$)), 0.92 (s, 3H, C(C$\underline{H}_3$)); ^{13}C NMR (75 MHz, CD_3CN) δ 185.5, 172.5, 62.6, 58.7, 37.8, 36.1, 30.5, 29.3, 28.5, 22.9, 18.1; HRMS (ES) Calcd for $C_{11}H_{23}N_2O_4$ 247.1658, found 247.16620.



[3-^{13}C]-*N*-(Benzyloxycarbonyl)-L-serine (102a(β-^{13}C)).[142] This material was prepared from the modified procedure of Greenstein and Winitz.[143] To a suspension of $NaHCO_3$ (0.30 g, 3.53 mmol) in THF (2 mL) and water (4 mL) at room temperature was carefully added L-serine **101a**(β-^{13}C) (3-^{13}C, 99%) (Cambridge Isotope Laboratories) (0.15 g, 1.41 mmol). After cessation of gas evolution, benzyl chloroformate (0.22 mL, 1.56 mmol) was added dropwise over a 30 min period and stirring was continued for a further 1 h. The reaction mixture was washed with diethyl ether (2 x 2 mL), and acidified to pH 2 by careful additon of 1 N HCl. The slurry was extracted with ethyl acetate (2 x 2 mL) and the organic phases were combined and washed with 1 N HCl (2 x 2 mL) and brine (3 mL), dried over $MgSO_4$, filtered and evaporated *in vacuo* to give **102a**(β-^{13}C) (176.6 mg, 52%) as a white solid: mp 117-119 °C (lit. mp 117-119 °C)[143b]; $[\alpha]_D^{26}$ +7.29° (*c* 2.5, CH_3CN); IR (μscope) 3317, 3028, 2950, 1748, 1690, 1533, 1247, 1056, 1018, 749, 696 cm^{-1}; ^{1}H NMR (360 MHz, CD_3OD) δ 7.35 (m, 5H, $\underline{Ph}$), 5.09 (br s, 2H, Ph$\underline{CH}_2$), 4.28 (dd, 1H, J = 8, 4 Hz, C$\underline{H}$), 3.85 (dddd, 2H, $^1J_{13C\text{-}H}$ = 145 Hz, J = 11, 8, 4 Hz, $^{\blacklozenge}C\underline{H}_2$); ^{13}C NMR (75 MHz, CD_3CN) δ 172.3, 157.2, 138.1, 129.4, 128.9, 128.7, 67.8, 62.7, 56.7; HRMS (ES) Calcd for $^{13}C_1{}^{12}C_{10}H_{13}N_1O_5Na$ 263.0725, found 263.0729.

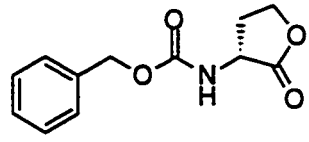


***N*-(Benzyloxycarbonyl)-D-serine (102b).**[143] Reaction of D-serine **101b** (Sigma) (5.0 g, 47.65 mmol), $NaHCO_3$ (10.0 g, 119.35 mmol) and benzyl chloroformate (7.5 mL, 52.5 mmol) as described for **13a**(β-^{13}C) gave the title compound **102b** (9.38 g, 82%) as a white solid: mp 117-119 °C (lit. mp 117-119 °C)[143b]; IR (μscope) 3336, 3317, 3208, 3061, 1747, 1690, 1534, 1248, 1060, 1029, 750, 697 cm^{-1}; ^{1}H NMR (360 MHz, CD_3OD) δ 7.38 (m, 5H, Ph), 5.08 (br s, 2H, $PhCH_2$), 4.27 (dd, 1H, J = 4, 4 Hz, CH), 3.88 (dd, 1H, J = 11, 4 Hz, CH_2), 3.83 (dd, 1H, J = 11, 4 Hz, CH_2); ^{13}C NMR (75 MHz, CD_3CN) δ 172.3, 157.2, 138.1, 129.4, 128.9, 128.7, 67.2, 62.7, 56.9; HRMS (EI) Calcd for $C_{11}H_{13}NO_5$ 239.0793, found 239.0788; Anal. Calcd for $C_{11}H_{13}NO_5$: C, 55.22; H, 5.47; N, 5.85. Found: C, 55.21; H, 5.31; N, 5.82.



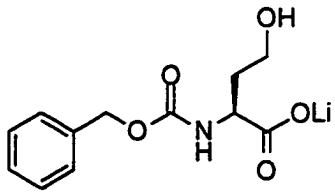
***N*-(Benzyloxycarbonyl)-L-homoserine γ-lactone (104a).**[144] To a suspension of L-homoserine lactone hydrochloride **103** (Sigma) (0.1 g, 0.73 mmol) in CH_2Cl_2 (20 mL) at 0 °C was added triethylamine (0.2 mL, 1.44 mmol), and benzyl chloroformate (0.125 mL, 0.88 mmol) dropwise. The reaction mixture was vigorously stirred for 3 h. The solution was washed with 1 N HCl (2 x 20 mL) and water (10 mL), dried over $MgSO_4$, filtered and evaporated *in vacuo* to give crude product as an off-white solid. Recrystallization from (chloroform-hexane) gave the title compound **104a** (40.0 mg, 23%) as a white solid: mp 118-120 °C; $[\alpha]_D^{26}$ -1.05° (*c* 2.85, $CHCl_3$); IR ($CHCl_3$ cast) 3329, 2949, 1777, 1692, 1543, 1298, 1074, 778, 693 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.38 (m, 5H, Ph), 5.39 (br s, 1H, NH), 5.13 (s, 2H, $PhCH_2$), 4.42 (m, 2H, OCH_2), 4.22 (m, 1H, CH), 2.79 (m, 1H, CH_2), 2.21 (dddd, 1H, J = 12, 12, 12, 9 Hz, CH_2); ^{13}C

NMR (75 MHz, $CDCl_3$) δ 174.9, 158.1, 135.9, 128.6, 128.4, 128.2, 67.4, 65.8, 50.6, 30.6; HRMS (EI) Calcd for $C_{12}H_{13}NO_4$ 235.0845, found 235.0845; Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.95. Found: C, 60.85; H, 5.25; N, 5.92.

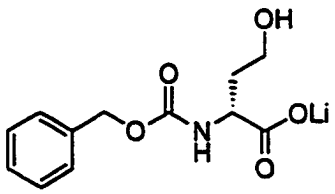


***N*-(Benzyloxycarbonyl)-D-homoserine δ-lactone (104b).**[144] To a suspension of $NaHCO_3$ (0.5 g, 5.95 mmol) in THF (2.5 mL) and water (5 mL) at room temperature was carefully added D-homoserine **106** (Sigma) (0.25 g, 2.09 mmol). After the cessation of gas evolution, benzyl chloroformate (0.375 mL, 2.71 mmol) was added dropwise over 30 min and stirring was continued for a further 1h. The reaction mixture was then washed with diethyl ether (2 x 2.5 mL), and acidified to pH 2 by careful addition of 1 N HCl. The slurry was extracted with ethyl acetate (2 x 2.5 mL) and the ethyl acetate layers were pooled. The combined ethyl acetate layer was washed with 1 N HCl (2 x 2.5 mL) and then brine (2.5 mL), dried over $MgSO_4$ and evaporated *in vacuo* to give crude δ-lactone **104b** plus *N*-(benzyloxycarbonyl)-D-homoserine as a white solid. This material was dissolved in toluene (10 mL) and heated at reflux with a soxhlet containing CaH_2 for 4 h. The solution was evaporated *in vacuo*, redissolved in EtOAc (5 mL), washed with saturated aqueous $NaHCO_3$ (2.5 mL) and brine (2.5 mL), dried over $MgSO_4$ filtered and evaporated *in vacuo* to give **104b** (0.1 g, 50%) as a white solid: mp 118-120 °C; $[\alpha]_D^{25}$ +1.75° (*c* 2.6, $CHCl_3$); IR ($CHCl_3$ cast) 3327, 2940, 1777, 1693, 1542, 1298, 1073, 741, 693 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.38 (m, 5H, Ph), 5.39 (br s, 1H, NH), 5.13 (s, 2H, $PhCH_2$), 4.42 (m, 2H, OCH_2), 4.22 (m, 1H, CH), 2.79 (m, 1H, CH_2), 2.21 (dddd, 1H, J = 12, 12, 12, 9 Hz, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.9, 156.1, 135.9, 128.6, 128.3, 128.2, 67.4, 65.8, 50.5, 30.3; HRMS (EI) Calcd for $C_{12}H_{13}NO_4$

235.08446, found 235.08462; Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.95. Found: C, 61.20; H, 5.49 N, 5.85.

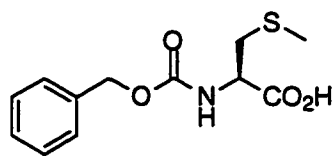


***N*-(Benzyloxycarbonyl)-L-homoserine, lithium salt (105a).** A solution of *N*-(benzyloxycarbonyl)-L-homoserine δ-lactone **104a** (50.0 mg, 0.197 mmol) suspended in THF / H_2O (20 mL, 1 : 1) was treated with lithium hydroxide monohydrate (8.3 mg, 0.197 mmol). The reaction mixture was stirred at room temperature for 2 h. The solution was evaporated *in vacuo* to give a white solid, this material was redissolved in water (10 mL) and extracted with diethyl ether (2 x 5 mL). The aqueous layer was evaporated *in vacuo* to afford the salt **105a** (20.0 mg, 40%) as a pale yellow oil: $[\alpha]_D^{26}$ -8.54° (*c* 4.7, H_2O); IR (MeOH cast) 3316, 2955, 1694, 1599, 1538, 1258, 1061, 697 cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 7.28 (m, 5H, Ph), 4.99 (d, 1H, *J* = 12 Hz, $PhCH_2$), 4.93 (d, 1H, *J* = 12 Hz, $PhCH_2$), 3.87 (dd, 1H, *J* = 9, 4 Hz, NCH), 3.48 (m, 2H, OCH_2), 1.88 (m, 1H, CH_2), 1.67 (m, 1H, CH_2); ^{13}C NMR (75 MHz, D_2O) δ 180.4, 158.8, 137.4, 129.6, 129.1, 128.5, 67.7, 59.4, 54.6, 34.9; HRMS (ES) Calcd for $C_{12}H_{15}N_1O_5Li$ 260.1110, found 260.1110.

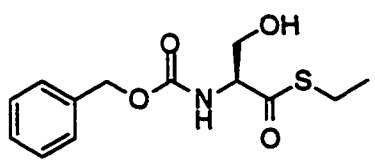


***N*-(Benzyloxycarbonyl)-D-homoserine, lithium salt (105b).** Reaction of *N*-(benzyloxycarbonyl)-D-homoserine γ-lactone **104b** (0.10 g, 0.394 mmol) and lithium hydroxide monohydrate (17.0 mg, 0.394 mmol) as described for **105a** afforded the salt **105b** (40.0 mg, 40%) as a pale yellow oil: $[\alpha]_D^{26}$ +10.21° (*c* 30.3, H_2O); IR (MeOH cast)

3378, 2956, 1695, 1598, 1538, 1261, 1060, 697 cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 7.28 (m, 5H, Ph), 4.98 (d, 1H, J = 12 Hz, $PhCH_2$), 4.92 (d, 1H, J = 12 Hz, $PhCH_2$), 3.88 (dd, 1H, J = 9, 4 Hz, NCH), 3.49 (m, 2H, OCH_2), 1.88 (m, 1H, CH_2), 1.67 (m, 1H, CH_2); ^{13}C NMR (75 MHz, D_2O) δ 180.4, 158.7, 137.4, 129.6, 129.1, 128.5, 67.7, 59.4, 54.6, 34.9; HRMS (ES) Calcd for $C_{12}H_{15}N_1O_5Li$ 260.1110, found 260.1110.

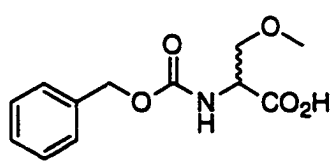


***N*-(Benzyloxycarbonyl)-*S*-methyl-L-cysteine (112).**[145] Reaction of *S*-methyl-L-cysteine **111** (Aldrich) (1 g, 7.39 mmol), $NaHCO_3$ (1.55 g, 18.53 mmol) and benzyl chloroformate (1.16 mL, 8.15 mmol) as described for **102a**(β-^{13}C) gave cysteine analogue **112** (1.76 g, 89%) as an oil: $[\alpha]_D^{26}$ -25.64° (*c* 44, MeOH); IR ($CHCl_3$ cast) 3311, 2920, 1718, 1586, 1215, 774, 697, 611 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 7.35 (m, 5H, Ph), 5.12 (br s, 2H, $PhCH_2$), 4.38 (dd, 1H, J = 8, 5 Hz, CH), 2.98 (dd, 1H, J = 14, 5 Hz, SCH_2), 2.82 (dd, 1H, J = 14, 8 Hz, SCH_2), 2.11 (s, 3H, SCH_3); ^{13}C NMR (75 MHz, CD_3CN) δ 172.3, 156.0, 136.9, 128.3, 127.7, 127.6, 65.4, 53.5, 34.8, 15.1; MS (ES) *m/z* (relative intensity) 270 (MH^+, 80%); Anal. Calcd for $C_{12}H_{15}NO_4S$: C, 53.51; H, 5.61; N, 5.20. Found: C, 53.11; H, 5.73; N, 5.36.



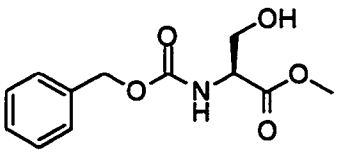
***N*-(Benzyloxycarbonyl)-L-serine ethyl thioester (113).** A solution of *N*-(benzyloxycarbonyl)-L-serine **102a** (1.0 g, 4.18 mmol) in CH_2Cl_2 (25mL) under argon at 0 °C was treated with triethylamine (0.7 mL, 5.01 mmol) and ethyl chloroformate (0.48 mL, 5.01 mmol) and stirred for 20 min. Upon formation of a white precipitate ethanethiol (0.37 mL, 5.01 mmol) was added followed by an additional equivalent of triethylamine

(0.7 mL, 5.01 mmol). The solution was stirred at 0 °C for an additional 30 min, then the ice-bath was removed and stirring was continued overnight. To the reaction mixture was added CH_2Cl_2 (25 mL), the solution was washed with 0.5 N HCl (2 x 10 mL) and then saturated aqueous $NaHCO_3$ (10 mL) which gave an emulsion. The emulsion was filtered through a pad of Celite and the organic layer was washed with brine (5 mL), dried over $MgSO_4$ filtered and concentrated *in vacuo*. Purification by flash chromatography (EtOAc : Hex, 1 : 4) gave the title compound **113** (77.1 mg, 7%) as a solid: mp 49-51 °C; $[\alpha]_D^{26}$ -26.75° (*c* 1.6, $CHCl_3$); IR ($CHCl_3$ cast) 3351, 2931, 1684, 1520, 1261, 1059, 737, 697 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.27 (m, 5H, Ph), 5.68 (br s, 1H, NH), 5.11 (s, 2H, $PhCH_2$), 4.42 (m, 1H, CH), 4.08 (dd, 1H, J = 11, 4 Hz, CH_2OH); 3.79 (dd, 1H, J = 11, 4 Hz, CH_2OH), 2.88 (q, 2H, J = 7 Hz, SCH_2), 1.83 (br s, 1H, OH), 1.21 (t, 3H, J = 7 Hz, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.0, 156.2, 136.1, 128.6, 128.3, 128.2, 67.4, 63.2, 62.4, 23.6, 14.3; HRMS (ES) Calcd for $C_{13}H_{17}NO_4S$ 284.0956, found 284.0955; Anal. Calcd for $C_{13}H_{17}NO_4S$: C, 55.10; H, 6.05; N, 4.94. Found: C, 54.91; H, 6.14; N, 4.90.

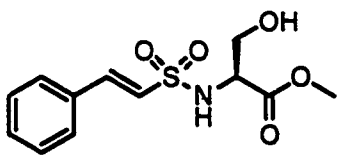


***N*-(Benzyloxycarbonyl)-*O*-methyl-DL-serine (118).**[146] Reaction of *O*-methyl-DL-serine **117** (Sigma) (1 g, 8.39 mmol), $NaHCO_3$ (1.76 g, (21.03 mmol) and benzyl chloroformate (1.32 mL, 9.25 mmol) as described for **102a**(β-^{13}C) gave **118** (1.85 g, 87%) as a yellow oil: IR ($CHCl_3$ cast) 3313, 2936, 1723, 1521, 1213, 775, 698, 623 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 7.34 (m, 5H, Ph), 5.11 (s, 2H, $PhCH_2$), 4.37 (dd, 1H, J = 4, 4 Hz, CH), 3.76 (dd, 1H, J =10, 4 Hz, CH_2), 3.64 (dd, 1H, J = 10, 4 Hz, CH_2), 3.38 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) δ 171.7, 156.0, 136.9, 128.3, 127.8, 127.7, 71.4, 65.5, 58.2, 54.1; MS (ES) *m/z* (relative intensity) 254 (MH^+,

100%); Anal. Calcd for $C_{12}H_{15}NO_5$: C, 56.91; H, 5.97; N, 5.53. Found: C, 56.83; H, 6.07; N, 5.69.

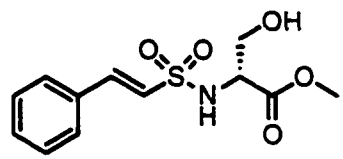


***N*-(Benzyloxycarbonyl)-L-serine methyl ester (120).**[147] Reaction of L-serine methyl ester hydrochloride **119** (Aldrich) (1 g, 6.43 mmol), $NaHCO_3$ (1.34 g, (16.1 mmol) and benzyl chloroformate (1.01 mL, 7.08 mmol) as described for **102a**(β-^{13}C). Purification by flash chromatography gave **120** (1.51 g, 71%) as a pale yellow oil: $[\alpha]_D^{26}$ +8.05° (*c* 14.7, $CHCl_3$); IR ($CHCl_3$ cast) 3373, 2955, 1722, 1527, 1214, 1062, 776, 698, 577 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.39 (m, 5H, Ph), 5.76 (br s, 1H, NH), 5.17 (s, 2H, $PhCH_2$), 4.48 (m, 1H, CH), 4.03 (dd, 1H, *J* = 11, 4 Hz, CH_2), 3.95 (dd, 1H, *J* = 11, 3 Hz, CH_2), 3.78 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.1, 156.3, 136.1, 128.6, 128.3, 128.1, 67.3, 63.2, 56.1, 52.7; MS (ES) *m/z* (relative intensity) 254 (MH^+, 53%); Anal. Calcd for $C_{12}H_{15}NO_5$: C, 56.91; H, 5.97; N, 5.53. Found: C, 56.65; H, 6.01; N, 5.47.

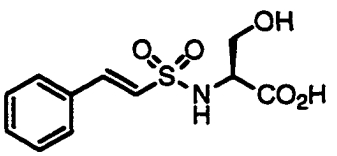


***N*-(*trans*-β-Styrenesulfonyl)-L-serine methyl ester (125a).** To a solution of L-serine methyl ester hydrochloride **124a** (Aldrich) (5.0 g, 32.14 mmol) in CH_2Cl_2 (50 mL) at room temperature was added triethylamine (11.14 mL, 80.34 mmol). The solution was stirred for 5 min and then *trans*-β-styrenesulfonyl chloride **123** (7.8 g, 38.57 mmol) dissolved in CH_2Cl_2 (20 mL) was added dropwise over 10 min. The resulting solution was stirred overnight. The reaction mixture was extracted with 1 N HCl (3 x 20 mL), washed with brine (20 mL), dried over $MgSO_4$ filtered and evaporated *in vacuo* to give a crude

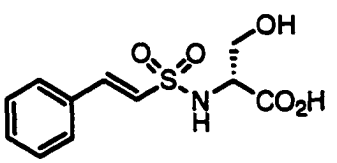
yellow oil. Purification by flash chromatography (ethyl acetate : hexane, 1 : 1) gave the title compound **125a** (3.37 g, 37%) as a pale yellow solid: mp 76-78 °C; $[\alpha]_D^{26}$ +14.29° (*c* 0.7, $CHCl_3$); IR ($CHCl_3$ cast) 3501, 3280, 2954, 1740, 1615, 1436, 1215, 1072, 747, 690 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.44 (m, 5H, Ph), 7.43 (d, 1H, *J* = 15 Hz, PhCHCH), 6.82 (d, 1H, *J* = 15 Hz, PhCHCH), 5.86 (d, 1H, *J* = 8 Hz, NH), 4.11 (ddd, 1H, *J* = 8, 8, 4, Hz, CH), 3.89 (br d, 2H, *J* = 4 Hz, CH_2), 3.72 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.5, 142.7, 133.1, 131.7, 129.8, 129.1, 125.7, 64.5, 58.4, 53.8; HRMS (ES) Calcd for $C_{12}H_{15}NO_5SNa$ 308.0569, found 308.0565; Anal. Calcd for $C_{12}H_{15}NO_5S$: C, 50.51; H, 5.29; N, 4.91. Found: C, 50.44; H, 5.21; N, 4.85.



***N*-(*trans*-β-Styrenesulfonyl)-D-serine methyl ester (125b).** Reaction of D-serine methyl ester hydrochloride **124b** (Aldrich) (4.50 g, 28.92 mmol), triethylamine (10.0 mL, 72.31 mmol) and *trans*-β-styrenesulfonyl chloride **123** (7.8 g, 38.57 mmol) as described for **125a** gave **125b** (3.21 g, 40%) as a pale yellow solid: mp 63-65 °C; $[\alpha]_D^{26}$ -8.79° (*c* 14.10, $CHCl_3$); IR ($CHCl_3$ cast) 3503, 3280, 2954, 1741, 1616, 1436, 1216, 1072, 747, 690 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.45 (m, 5H, Ph), 7.43 (d, 1H, *J* = 15 Hz, PhCHCH), 6.82 (d, 1H, *J* = 15 Hz, PhCHCH), 5.86 (d, 1H, *J* = 8 Hz, NH), 4.11 (ddd, 1H, *J* = 8, 4, 4, Hz, CH), 3.89 (br d, 2H, *J* = 4 Hz, CH_2), 3.72 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 141.9, 132.5, 131.0, 129.1, 128.4, 125.1, 63.8, 57.8, 53.1; HRMS (ES) Calcd for $C_{12}H_{15}NO_5SNa$ 308.0569, found 308.0566; Anal. Calcd for $C_{12}H_{15}NO_5S$: C, 50.51; H, 5.29; N, 4.91. Found: C, 50.24; H, 5.20; N, 4.85.

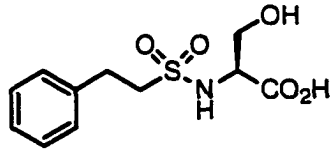


***N*-(*trans*-β–Styrenesulfonyl)-L-serine (126a).** To a solution of *N*-(*trans*-β-styrenesulfonyl)-L-serine methyl ester **125a** (3.16 g, 11.08 mmol) in THF and water (1 : 1, 50 mL) at room temperature was added lithium hydroxide monohydrate (0.93 g, 22.15 mmol). The resulting solution was stirred for 2 h. The solvent was removed *in vacuo*, the residue dissolved in saturated aqueous $NaHCO_3$ (20 mL), washed with diethyl ether (3 x 15 mL), acidified to pH 2 with 1 N HCl, extracted with ethyl acetate (3 x 15 mL), washed with brine (15 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give **126a** (2.73 g, 91%) as a white solid: mp 187-188 °C; $[\alpha]_D^{26}$ +3.33° (*c* 1.5, CH_3CN); IR (CH_3CN cast) 3266, 3059, 2933, 1732, 1614, 1448, 1197, 1066, 745, 689 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.49 (m, 5H, Ph), 7.42 (d, 1H, *J* = 15 Hz, PhCHCH), 6.92 (d, 1H, *J* = 15 Hz, PhCHCH), 5.85 (d, 1H, *J* = 8 Hz, NH), 3.99 (ddd, 1H, *J* = 11, 4, 4, Hz, CH), 3.82 (dd, 1H, *J* = 11, 4 Hz, CH_2), 3.75 (dd, 1H, *J* = 11, 4 Hz, CH_2); ^{13}C NMR (125 MHz, CD_3CN) δ 171.7, 141.3, 133.8, 131.5, 129.8, 129.1, 127.2, 63.7, 58.4; HRMS (EI) Calcd for $C_{11}H_{13}NO_5S$ 271.0515, found 271.0507; Anal. Calcd for $C_{11}H_{13}NO_5S$: C, 48.70; H, 4.83; N, 5.16. Found: C, 48.71; H, 4.83; N, 5.13.

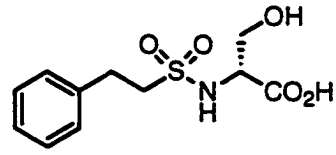


***N*-(*trans*-β–Styrenesulfonyl)-D-serine (126b).** Reaction of *N*-(*trans*-β-styrenesulfonyl)-D-serine methyl ester **125b** (2.10 g, 7.36 mmol) and lithium hydroxide monohydrate (0.62 g, 14.72 mmol) as described for **126a** gave **126b** (1.7 g, 85%) as a white solid: mp 187-188 °C; $[\alpha]_D^{26}$ +5.26° (*c* 0.95, CH_3CN); IR (CH_3CN cast) 3305, 3050, 2960, 1727, 1612, 1449, 1145, 1064, 746, 689 cm^{-1}; ^1H NMR (360 MHz,

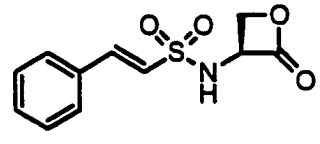
CD_3CN) δ 7.50 (m, 5H, Ph), 7.42 (d, 1H, J = 15 Hz, PhCHCH), 6.92 (d, 1H, J = 15 Hz, PhCHCH), 5.84 (d, 1H, J = 8 Hz, NH), 3.99 (ddd, 1H, J = 12, 4, 4, Hz, CH), 3.82 (dd, 1H, J = 12, 4 Hz, CH_2), 3.75 (dd, 1H, J = 12, 4 Hz, CH_2); ^{13}C NMR (75 MHz, CD_3CN) δ 171.9, 141.4, 134.0, 131.6, 130.0, 129.3, 127.3, 63.9, 58.6; HRMS (EI) Calcd for $C_{11}H_{13}NO_5S$ 271.0515, found 271.0508; Anal. Calcd for $C_{11}H_{13}NO_5S$: C, 48.70; H, 4.83; N, 5.16. Found: C, 48.58; H, 4.74; N, 5.05.



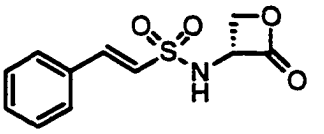
***N*-(Phenethylsulfonyl)-L-serine (127a).** To a solution of *N*-(*trans*-β-styrenesulfonyl)-L-serine **126a** (1.93 g, 7.11 mmol) in methanol (50 mL) under argon was added 10% palladium on carbon (0.5 g) at room temperature. The flask was evacuated and flushed with hydrogen, then stirred at room temperature under hydrogen overnight. The mixture was filtered through a pad of Celite and evaporated *in vacuo*. This procedure was repeated three times to yield a pale white solid which was recrystallized from (methanol / $CHCl_3$ / hexane) to give **127a** (1.68 g, 87%) as a white solid: mp 168-170 °C; $[\alpha]_D^{26}$ -4.65° (*c* 1.3, MeOH); IR ($CHCl_3$ cast) 3426, 3300, 2925, 1727, 1311, 1149, 1064, 741, 698 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.28 (m, 5H, Ph), 5.85 (d, 1H, J = 8 Hz, NH), 4.11 (ddd, 1H, J = 8, 5, 5, Hz, CH), 3.85 (dd, 1H, J = 11, 5 Hz, CH_2), 3.75 (dd, 1H, J = 11, 5 Hz, CH_2), 3.29 (m, 2H, $PhCH_2CH_2$), 3.09 (m, 2H, $PhCH_2CH_2$); ^{13}C NMR (125 MHz, CD_3CN) δ 173.7, 139.9, 129.7, 129.5, 127.6, 64.4, 59.6, 55.9, 30.9; HRMS (ES) Calcd for $C_{11}H_{15}NO_5SNa$ 296.0569, found 296.0565.



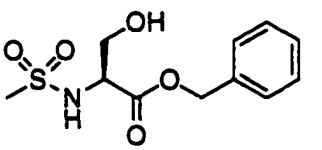
***N*-(Phenethylsulfonyl)-D-serine (127b).** Reaction of *N*-(*trans*-β-styrenesulfonyl)-D-serine **126b** (1.0 g, 3.67 mmol) and 10% palladium on carbon (0.25 g) as described for **127a** gave **127b** (0.91 g, 90%) as a white solid: mp 168-170 °C; $[\alpha]_D^{26}$ +8.14° (*c* 2.2, MeOH); IR ($CHCl_3$ cast) 3438, 3302, 2946, 1727, 1498, 1322, 1151, 1064, 736, 697 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.28 (m, 5H, Ph), 5.85 (d, 1H, *J* = 8 Hz, NH), 4.11 (ddd, 1H, *J* = 8, 5, 5, Hz, CH), 3.85 (dd, 1H, *J* = 11, 5 Hz, CH_2), 3.75 (dd, 1H, *J* = 11, 5 Hz, CH_2), 3.29 (m, 2H, $PhCH_2CH_2$), 3.09 (m, 2H, $PhCH_2CH_2$); ^{13}C NMR (125 MHz, CD_3CN) δ 172.3, 139.7, 129.6, 129.5, 127.6, 64.1, 58.8, 55.2, 30.5; HRMS (ES) Calcd for $C_{11}H_{15}NO_5SNa$ 296.0569, found 296.0570.



***N*-(*trans*-β–Styrenesulfonyl)-L-serine-β-lactone (128a).** Cyclization of *N*-(*trans*-β–styrenesulfonyl)-L-serine **126a** (0.50 g, 1.85 mmol) by Mitsunobu procedure[53a] (dimethyl azodicarboxylate (0.20 mL, 1.85 mmol) and triphenylphosphine (0.49 g, 1.85 mmol)) as described for **13a**(β-^{13}C) gave β-lactone **128a** (70.1 mg, 15%): mp 113-114 °C; $[\alpha]_D^{26}$ -44.12° (*c* 0.7, $CHCl_3$); IR ($CHCl_3$ cast) 3280, 3059, 2923, 1832, 1576, 1323, 1146, 745, 688 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.58 (d, 1H, *J* = 15 Hz, PhCHCH), 7.49 (m, 5H, Ph), 6.90 (d, 1H, *J* = 15 Hz, PhCHCH), 5.27 (d, 1H, *J* = 9 Hz, NH), 5.14 (ddd, 1H, *J* = 9, 7, 5 Hz , CH), 4.58 (dd, 1H, *J* = 12, 7 Hz, CH_2), 4.39 (dd, 1H, *J* = 12, 5 Hz, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.2, 143.8, 132.7, 129.8, 129.2, 128.9, 125.4, 68.4, 61.4; HRMS (ES) Calcd for $C_{11}H_{11}NO_4SNa$ 276.0307, found 276.0308.

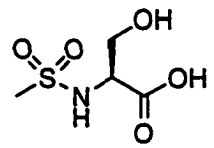


***N*-(*trans*-β–Styrenesulfonyl)-D-serine-β-lactone (128b).** Cyclization of *N*-(*trans*-β–styrenesulfonyl)-D-serine **126b** (0.45 g, 1.67 mmol) by Mitsunobu procedure[53a] (dimethyl azodicarboxylate (0.18 mL, 1.67 mmol) and triphenylphosphine (0.44 g, 1.67 mmol)) as described for **13a**(β-^{13}C) gave β-lactone **128b** (60.0 mg, 14%): mp 113-114 °C; $[\alpha]_D^{26}$ -64.0° (*c* 1.0, $CHCl_3$); IR ($CHCl_3$ cast) 3285, 3059, 2950, 1831, 1576, 1322, 1145, 745, 689 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.58 (d, 1H, *J* = 15 Hz, PhC<u>H</u>CH), 7.49 (m, 5H, <u>Ph</u>), 6.90 (d, 1H, *J* = 15 Hz, PhCHC<u>H</u>), 5.27 (d, 1H, *J* = 8 Hz, N<u>H</u>), 5.14 (ddd, 1H, *J* = 8, 6, 5 Hz , C<u>H</u>), 4.58 (dd, 1H, *J* = 12, 6 Hz, C<u>H</u>$_2$), 4.39 (dd, 1H, *J* = 12, 5 Hz, C<u>H</u>$_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.4, 143.7, 132.7, 132.1, 129.9, 129.3, 125.4, 68.3, 61.4; HRMS (ES) Calcd for $C_{11}H_{11}NO_4SNa$ 276.0307, found 276.0305.

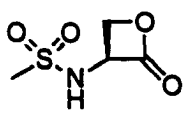


***N*-(Methylsulfonyl)-L-serine benzyl ester (130).** To a solution of L-serine benzyl ester **129** (2.5 g, 10.79 mmol) in CH_2Cl_2 (50 mL) at 0 °C was added triethylamine (3.6 mL, 25.9 mmol), and then methylsulfonyl chloride (1.0 mL, 12.95 mmol) dropwise over 10 min. The mixture was stirred and warmed to room temperature over 1 h. The solvent was removed *in vacuo* and the residue purified by flash chromatography (ethyl acetate : hexane, 1 : 1) to yield sulfonamide **130** (0.88 g, 30%) as a white solid: mp 68-70 °C; $[\alpha]_D^{26}$ -17.40° (*c* 11.3, $CHCl_3$); IR ($CHCl_3$ cast) 3501, 3033, 2938, 1739, 1498, 1326, 1153, 1066, 753, 699 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.39 (m, 5H, <u>Ph</u>), 5.62 (d, 1H, *J* = 7 Hz, N<u>H</u>), 5.24 (d, 1H, *J* =13 Hz, PhC<u>H</u>$_2$), 5.21 (d, 1H, *J* =13 Hz, PhC<u>H</u>$_2$), 4.25 (ddd, 1H, *J* = 4, 4, 7 Hz, C<u>H</u>), 4.05 (dd, 1H, *J* = 11, 4 Hz, C<u>H</u>$_2$), 3.95 (dd, 1H, *J* =

11, 4 Hz, C<u>H</u>$_2$), 2.98 (s, 3H, C<u>H</u>$_3$), 2.24 (br s, 1H, CH$_2$(O<u>H</u>)); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 134.9, 128.8, 128.4, 128.4, 68.0, 63.8, 58.1, 41.6; HRMS (EI) Calcd for $C_{11}H_{15}NO_5S$ 273.0671, found 273.0668; Anal. Calcd for $C_{11}H_{15}NO_5S$: C, 48.34; H, 5.53; N, 5.12. Found: C, 48.26; H, 5.61; N, 5.03.

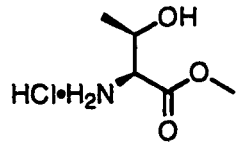


***N*-(Methylsulfonyl)-L-serine (131).** To a solution of sulfonamide **130** (0.68 g, 2.48 mmol) in methanol (10 mL) under argon was added 10% palladium on charcoal (7.0 mg). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give the *N*-sulfonamide serine **131** (0.37 g, 82%) as a white solid: mp 159-161 °C; $[\alpha]_D^{26}$ -17.30° (*c* 10.4, $CHCl_3$); IR (μscope) 3431, 2947, 1741, 1462, 1326, 1168, 1026 cm^{-1}; ^{1}H NMR (360 MHz, CD_3OD) δ 4.25 (m, 1H, C<u>H</u>), 3.79 (dd, 1H, *J* = 13, 5 Hz, C<u>H</u>$_2$), 3.72 (dd, 1H, *J* = 13, 7 Hz, C<u>H</u>$_2$), 2.81 (s, 3H, C<u>H</u>$_3$); ^{13}C NMR (75 MHz, CD_3OD) δ 173.5, 64.3, 59.6, 41.5; MS (CI) *m/z* (relative intensity) 201.4 ($MH^+ + NH_3$, 100%); Anal. Calcd for $C_4H_9NO_5S$: C, 26.23; H, 4.95; N, 7.65. Found: C, 26.34; H, 4.74; N, 7.33.

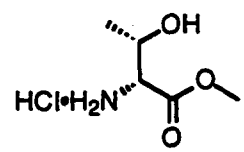


***N*-(Methylsulfonyl)-L-serine-β-lactone (132).** Cyclization of *N*-methylsulfonyl-L-serine **131** (0.20 g, 1.09 mmol) by Mitsunobu procedure[53a] (dimethyl azodicarboxylate (0.12 mL, 1.09 mmol) and triphenylphosphine (0.29 g, 1.09 mmol)) as described for **13a**(β-^{13}C) gave β-lactone **132** (6.4 mg, 4%): mp 107-109 °C; $[\alpha]_D^{26}$ -17.14° (*c* 0.7, $CHCl_3$); IR (μscope) 3283, 2934, 1828, 1348, 1151, 1071 cm^{-1}; ^{1}H NMR (360 MHz, CD_3CN) 6.41 (brs, 1H, N<u>H</u>), 5.19 (m, 1H, C<u>H</u>), 4.53 (dd, 1H, *J* = 12, 7 Hz,

C$\underline{H}_2$), 4.28 (dd, 1H, J = 12, 5 Hz, C$\underline{H}_2$), 3.03 (s, 3H, C$\underline{H}_3$); ^{13}C NMR (125 MHz, CD_3CN) δ 170.4, 68.4, 61.7, 42.6; HRMS (EI) Calcd for $C_4H_7NO_4S$ 165.0096, found 165.0097.

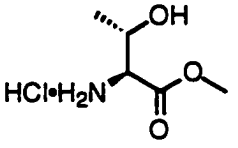


L-Threonine methyl ester hydrochloride (137a).[148] Methanol (25 mL) was cooled in an ice-NaCl bath, and thionyl chloride (1.80 mL, 25.20 mmol) was added dropwise. To the resultant solution of HCl in methanol was added L-threonine **136a** (3.0 g, 25.20 mmol) and the reaction mixture was heated under reflux for 1 h. The solvent was removed *in vacuo* and another 25 mL of a 2 M solution of HCl in methanol, prepared in the same manner as before, was added and the reaction mixture was heated under reflux for another 1 h. The solvent was removed *in vacuo* to yield **137a** (4.2 g, 98%) as a white solid: mp 160-163 °C; $[\alpha]_D^{26}$ -10.0° (*c* 1.0, CH_3OH); IR (μscope) 3366, 3050, 2958, 1746, 1442, 1047 cm^{-1}; ^{1}H NMR (360 MHz, CD_3OD) δ 4.27 (dq, 1H, J = 7, 4 Hz ,C$\underline{H}$(OH)), 3.92 (d, 1H, J = 4 Hz, NC$\underline{H}$), 3.84 (s, 3H, $CO_2C\underline{H}_3$), 1.31 (d, 3H, J = 7 Hz, CH(C$\underline{H}_3$)); ^{13}C NMR (125 MHz, CD_3OD) δ 169.6, 66.3, 59.9, 53.7, 20.5; HRMS (EI) Calcd for $C_5H_{12}NO_3$ 134.0817, found 134.0810.

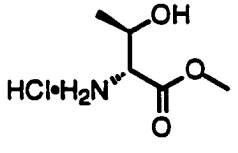


D-Threonine methyl ester hydrochloride (137b).[148] Reaction of D-threonine **136b** (5.0 g, 41.14 mmol) and thionyl chloride (3.0 mL, 41.14 mmol) in methanol (100 mL) as described for **137a** gave **137b** (7.1 g, 99%) as a white solid: mp 159-162 °C; $[\alpha]_D^{26}$ +7.78° (*c* 0.9, CH_3OH); IR (CH_3CN cast) 3350, 3050, 2956, 1744, 1440, 1042 cm^{-1}; ^{1}H NMR (360 MHz, CD_3OD) δ 4.27 (dq, 1H, J = 7, 4 Hz ,C$\underline{H}$(OH)),

3.92 (d, 1H, J = 4 Hz, NC<u>H</u>), 3.84 (s, 3H, $CO_2C\underline{H}_3$), 1.31 (d, 3H, J = 7 Hz, CH($C\underline{H}_3$)); ^{13}C NMR (125 MHz, CD_3OD) δ 169.6, 66.4, 59.9, 53.7, 20.5; HRMS (EI) Calcd for $C_5H_{12}NO_3$ 134.0817, found 134.0856; Anal. Calcd for $C_5H_{12}ClNO_3$: C, 35.41; H, 7.13; N, 8.26. Found: C, 35.07; H, 7.42; N, 8.01.

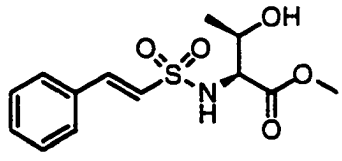


L-*allo*-Threonine methyl ester hydrochloride (137c).[148] Reaction of L-*allo*-threonine **136c** (3.0 g, 25.20 mmol) and thionyl chloride (1.80 mL, 25.20 mmol) in methanol (25 mL) as described for **137a** gave **137c** (4.22 g, 99%) as a white solid: mp 95-97 °C; $[\alpha]_D^{26}$ +28.0° (*c* 1.0, CH_3OH); IR (μscope) 3354, 3050, 2895, 1735, 1440, 1059 cm^{-1}; ^{1}H NMR (360 MHz, CD_3OD) δ 4.24 (dq, 1H, J = 7, 4 Hz ,C<u>H</u>(OH)), 4.08 (d, 1H, J = 4 Hz, NC<u>H</u>), 3.83 (s, 3H, $CO_2C\underline{H}_3$), 1.26 (d, 3H, J = 7 Hz, CH($C\underline{H}_3$)); ^{13}C NMR (125 MHz, CD_3OD) δ 168.8, 66.5, 59.4, 53.5, 18.7; MS (ES) *m/z* (relative intensity) 134 (MH^+, 100%); Anal. Calcd for $C_5H_{12}ClNO_3$: C, 35.41; H, 7.13; N, 8.26. Found: C, 35.13; H, 7.24; N, 8.13.

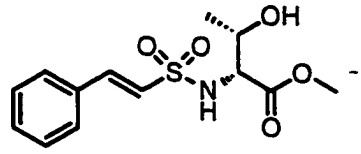


D-*allo*-Threonine methyl ester hydrochloride (137d).[148] Reaction of D-*allo*-threonine **136d** (3.0 g, 25.20 mmol) and thionyl chloride (1.80 mL, 25.20 mmol) in methanol (25 mL) as described for **137a** gave **137d** (4.20 g, 98%) as a white solid: mp 95-97 °C; $[\alpha]_D^{26}$ -26.0° (*c* 1.5, CH_3OH); IR (μscope) 3357, 3050, 2898, 1740, 1440, 1060 cm^{-1}; ^{1}H NMR (360 MHz, CD_3OD) δ 4.24 (dq, 1H, J = 6, 3 Hz ,C<u>H</u>(OH)), 4.08 (d, 1H, J = 3 Hz, NC<u>H</u>), 3.84 (s, 3H, $CO_2C\underline{H}_3$), 1.26 (d, 3H, J = 6 Hz, CH($C\underline{H}_3$)); ^{13}C NMR

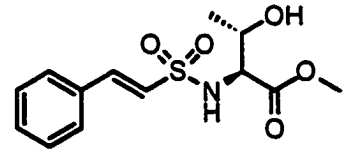
(75MHz, CD_3OD) δ 168.8, 66.5, 59.4, 53.5, 18.6; HRMS (EI) Calcd for $C_5H_{12}NO_3$ 134.0817, found 134.0813.



***N*-(*trans*-β-Styrenesulfonyl)-L-threonine methyl ester (138a).** To a solution of L-threonine methyl ester hydrochloride **137a** (4.32 g, 25.47 mmol) in CH_2Cl_2 (40 mL) at room temperature was added triethylamine (8.73 mL, 62.98 mmol), the solution was stirred for 5 min and then *trans*-β-styrenesulfonyl chloride **123** (6.20 g, 30.59 mmol) dissolved in CH_2Cl_2 (20 mL) was added over 10 min. The resulting solution was stirred overnight. The reaction mixture was washed with 1 N HCl (3 x 20 mL) and brine (20 mL), dried over $MgSO_4$ filtered and concentrated *in vacuo* to give a crude yellow oil. Purification by flash chromatography (ethyl acetate : hexane, 1:1) followed by recrystallization from ($CHCl_3$-hexane) gave the title compound **138a** (6.08 g, 80%) as a white solid: mp 100-102 °C; $[\alpha]_D^{26}$ +13.33° (*c* 1.5, $CHCl_3$); IR (μscope) 3526, 3275, 3025, 2981, 1738, 1387, 1149, 1082, 742, 687 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.44 (d, 1H, *J* = 15 Hz, PhC<u>H</u>CH), 7.42 (m, 5H, <u>Ph</u>), 6.79 (d, 1H, *J* = 15 Hz, PhCHC<u>H</u>), 5.32 (d, 1H, *J* = 9 Hz, N<u>H</u>), 4.11 (dq, 1H, *J* = 6, 3 Hz, C<u>H</u>(OH)), 3.88 (dd, 1H, *J* = 9, 3 Hz, NC<u>H</u>), 3.68 (s, 3H, $CO_2C\underline{H}_3$), 1.95 (br s, 1H, CH(O<u>H</u>)), 1.37 (d, 3H, *J* = 6 Hz, CH($C\underline{H}_3$)); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.4, 141.9, 132.5, 131.0, 129.2, 128.3, 125.2, 68.3, 60.9, 52.9, 20.1; HRMS (EI) Calcd for $C_{13}H_{18}NO_5S$ 300.0906, found 300.0893; Anal. Calcd for $C_{13}H_{17}NO_5S$: C, 52.16; H, 5.72; N, 4.68. Found: C, 51.76; H, 5.70; N, 4.64.

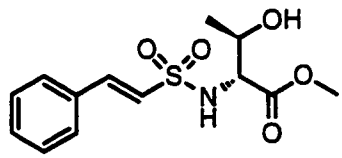


***N*-(*trans*-β-Styrenesulfonyl)-D-threonine methyl ester (138b).** Reaction of D-threonine methyl ester hydrochloride **137b** (4.94 g, 29.13 mmol), triethylamine (10.0 mL, 72.83 mmol) and *trans*-β-styrenesulfonyl chloride **123** (7.08 g, 34.96 mmol) as described for **138a** gave **138b** (5.29 g, 61%) as a white solid: mp 100-102 °C; $[\alpha]_D^{26}$ -20.0° (*c* 0.8, $CHCl_3$); IR ($CHCl_3$ cast) 3498, 3278, 3059, 2953, 1739, 1383, 1141, 1026, 747, 691 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.43 (d, 1H, *J* = 15 Hz, PhCHCH), 7.42 (m, 5H, Ph), 6.79 (d, 1H, *J* = 15 Hz, PhCHCH), 5.39 (d, 1H, *J* = 9 Hz, NH), 4.25 (dq, 1H, *J* = 6, 3 Hz, CH(OH)), 3.88 (dd, 1H, *J* = 9, 3 Hz, NCH), 3.65 (s, 3H, CO_2CH_3), 1.88 (br s, 1H, CH(OH)), 1.37 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.2, 142.5, 133.2, 131.7, 129.8, 128.9, 125.9, 69.0, 61.6, 53.6, 20.8; HRMS (ES) Calcd for $C_{13}H_{17}NO_5SNa$ 322.0725, found 322.0728; Anal. Calcd for $C_{13}H_{17}NO_5S$: C, 52.16; H, 5.72; N, 4.68. Found: C, 52.40; H, 5.74; N, 4.67.

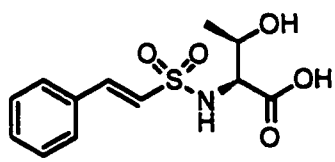


***N*-(*trans*-β-Styrenesulfonyl)-L-*allo*-threonine methyl ester (138c).** Reaction of L-*allo*-threonine methyl ester hydrochloride **137c** (4.37 g, 25.79 mmol), triethylamine (8.85 mL, 64.48 mmol) and *trans*-β-styrenesulfonyl chloride **123** (6.27 g, 30.95 mmol) as described for **138a** gave **138c** (4.46 g, 58%) as a white solid: mp 94-96 °C; $[\alpha]_D^{26}$ +22.22° (*c* 0.9, $CHCl_3$); IR (μscope) 3431, 3264, 3021, 2974, 1737, 1496, 1381, 1154, 1031, 746, 690 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.49 (d, 1H, *J* = 15 Hz, PhCHCH), 7.43 (m, 5H, Ph), 6.78 (d, 1H, *J* = 15 Hz, PhCHCH), 5.49 (d, 1H, *J* = 9 Hz, NH), 4.18 (dq, 1H, *J* = 6, 4 Hz, CH(OH)), 4.02 (dd, 1H, *J* = 9, 4 Hz, NCH), 3.67

(s, 3H, CO_2CH_3), 2.23 (br s, 1H, CH(OH)), 1.22 (d, 3H, J = 6 Hz, CH(CH_3)); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.7, 142.5, 132.3, 131.1, 129.1, 128.3, 124.6, 68.6, 60.9, 52.9, 19.0; HRMS (EI) Calcd for $C_{13}H_{18}NO_5S$ 300.0906, found 300.0896; Anal. Calcd for $C_{13}H_{17}NO_5S$: C, 52.16; H, 5.72; N, 4.68. Found: C, 52.02; H, 5.72; N, 4.58.

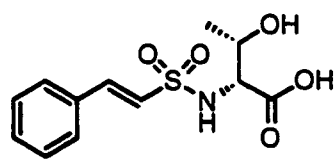


***N*-(*trans*-β-Styrenesulfonyl)-D-*allo*-threonine methyl ester (138d).** Reaction of D-*allo*-threonine methyl ester hydrochloride **137d** (4.0 g, 23.62 mmol), triethylamine (8.18 mL, 59.04 mmol) and *trans*-β-styrenesulfonyl chloride **123** (5.74 g, 28.34 mmol) as described for **138a** gave **138d** (4.51 g, 64%) as a white solid: mp 94-96 °C; $[\alpha]_D^{26}$ -37.0° (*c* 1.0, $CHCl_3$); IR (μscope) 3391, 3263, 3041, 2973, 1737, 1576, 1336, 1153, 1095, 750, 690 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.50 (d, 1H, J = 15 Hz, PhCHCH), 7.42 (m, 5H, Ph), 6.78 (d, 1H, J = 15 Hz, PhCHCH), 5.49 (d, 1H, J = 9 Hz, NH), 4.18 (dq, 1H, J = 6, 4 Hz, CH(OH)), 4.02 (dd, 1H, J = 9, 4 Hz, NCH), 3.67 (s, 3H, CO_2CH_3), 2.23 (br s, 1H, CH(OH)), 1.22 (d, 3H, J = 6 Hz, CH(CH_3)); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8, 142.5, 132.4, 131.1, 129.2, 128.4, 124.7, 68.6, 61.0, 52.9, 19.1; HRMS (EI) Calcd for $C_{13}H_{18}NO_5S$ 300.0906, found 300.0891; Anal. Calcd for $C_{13}H_{17}NO_5S$: C, 52.16; H, 5.72; N, 4.68. Found: C, 52.21; H, 5.61; N, 4.58.



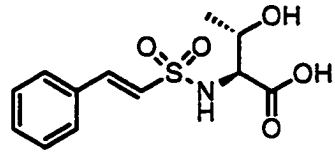
***N*-(*trans*-β-Styrenesulfonyl)-L-threonine (139a).** To a solution of *N*-(*trans*-β-styrenesulfonyl)-L-threonine methyl ester **138a** (3.0 g, 10.02 mmol) in THF and water (1 : 1, 150 mL) at room temperature was added lithium hydroxide monohydrate (0.84 g, 20.04 mmol). The resulting solution was stirred for 4 h. The solvent was removed

in vacuo, the residue dissolved in saturated aqueous $NaHCO_3$ (30 mL), washed with diethyl ether (3 x 20 mL), acidified to pH 2 with 1 N HCl, extracted with ethyl acetate (3 x 20 mL), washed with brine (20 mL), dried over $MgSO_4$ filtered and concentrated *in vacuo* to give **139a** (2.83 g, 99%) as a white solid: mp 145-147 °C; $[\alpha]_D^{26}$ +45.0° (*c* 1.0, CH_3OH); IR (μscope) 3447, 3304, 3026, 2982, 1720, 1576, 1449, 1372, 1132, 1030, 746, 690 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.50 (m, 5H, Ph), 7.41 (d, 1H, *J* = 15 Hz, PhCHCH), 6.91 (d, 1H, *J* = 15 Hz, PhCHCH), 5.69 (d, 1H, *J* = 9 Hz, NH), 4.19 (dq, 1H, *J* = 6, 3 Hz, CH(OH)), 3.81 (dd, 1H, *J* = 9, 3 Hz, NCH), 2.25 (br s, 1H, CH(OH)), 1.21 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, CD_3CN) δ 172.2, 141.3, 133.9, 131.6, 130.0, 129.3, 127.4, 68.5, 61.9, 20.3; HRMS (EI) Calcd for $C_{12}H_{16}NO_5S$ 286.0749, found 286.0739; Anal. Calcd for $C_{12}H_{15}NO_5S$: C, 50.52; H, 5.30; N, 4.91. Found: C, 50.24; H, 5.25; N, 4.88.

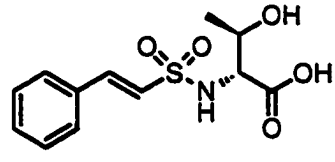


***N*-(*trans*-β-Styrenesulfonyl)-D-threonine (139b).** Reaction of *N*-(*trans*-β-styrenesulfonyl)-D-threonine methyl ester **138b** (3.0 g, 10.02 mmol) and lithium hydroxide monohydrate (0.84 g, 20.04 mmol) as described for **139a** gave **139b** (2.74 g, 96%) as a white solid: mp 145-147 °C; $[\alpha]_D^{26}$ -37.67° (*c* 0.6, CH_3OH); IR (μscope) 3445, 3302, 3026, 2960, 1718, 1576, 1449, 1371, 1131, 1029, 745, 689 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.50 (m, 5H, Ph), 7.41 (d, 1H, *J* = 15 Hz, PhCHCH), 6.91 (d, 1H, *J* = 15 Hz, PhCHCH), 5.69 (d, 1H, *J* = 9 Hz, NH), 4.19 (dq, 1H, *J* = 6, 3 Hz, CH(OH)), 3.81 (dd, 1H, *J* = 9, 3 Hz, NCH), 2.20 (br s, 1H, CH(OH)), 1.21 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, CD_3CN) δ 172.1, 141.3, 133.9, 131.5, 130.0, 129.2, 127.3, 68.4, 61.8, 20.2; HRMS (EI) Calcd for $C_{12}H_{16}NO_5S$ 286.0749, found 286.0749;

Anal. Calcd for $C_{12}H_{15}NO_5S$: C, 50.52; H, 5.30; N, 4.91. Found: C, 50.45; H, 5.37; N, 4.82.

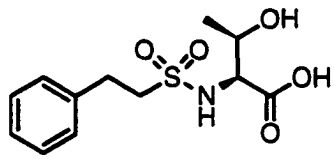


***N*-(*trans*-β-Styrenesulfonyl)-L-*allo*-threonine (139c).** Reaction of *N*-(*trans*-β-styrenesulfonyl)-L-*allo*-threonine methyl ester **138c** (3.0 g, 10.02 mmol) and lithium hydroxide monohydrate (0.84 g, 20.04 mmol) as described for **139a** gave **139c** (2.74 g, 96%) as a white solid: mp 140-142 °C; $[\alpha]_D^{26}$ +26.67° (*c* 1.8, CH_3OH); IR (μscope) 3430, 3255, 3050, 2982, 1719, 1576, 1449, 1327, 1135, 1031, 745, 690 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.50 (m, 5H, Ph), 7.41 (d, 1H, *J* = 15 Hz, PhCHCH), 6.90 (d, 1H, *J* = 15 Hz, PhCHCH), 5.83 (d, 1H, *J* = 9 Hz, NH), 4.00 (quintet, 1H, *J* = 6 Hz, CH(OH)), 3.84 (dd, 1H, *J* = 9, 6 Hz, NCH), 1.18 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (75 MHz, CD_3CN) δ 171.8, 141.6, 134.0, 131.7, 130.0, 129.3, 127.3, 68.8, 62.3, 19.6; HRMS (ES) Calcd for $C_{12}H_{16}NO_5S$ 286.0749, found 286.0747.

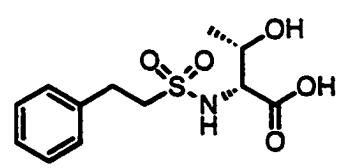


***N*-(*trans*-β-Styrenesulfonyl)-D-*allo*-threonine (139d).** Reaction of *N*-(*trans*-β-styrenesulfonyl)-D-*allo*-threonine methyl ester **138d** (3.0 g, 10.02 mmol) and lithium hydroxide monohydrate (0.84 g, 20.04 mmol) as described for **139a** gave **139d** (2.80 g, 98%) as a white solid: mp 140-142 °C; $[\alpha]_D^{26}$ -32.63° (*c* 1.9, CH_3OH); IR (μscope) 3523, 3286, 3062, 2985, 1722, 1576, 1449, 1321, 1157, 1083, 746, 689 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.50 (m, 5H, Ph), 7.41 (d, 1H, *J* = 15 Hz, PhCHCH), 6.90 (d, 1H, *J* = 15 Hz, PhCHCH), 5.83 (d, 1H, *J* = 9 Hz, NH), 3.99 (dq, 1H, *J* = 6, 5 Hz, CH(OH)), 3.84 (dd, 1H, *J* = 9, 5 Hz, NCH), 1.18 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C

NMR (125 MHz, CD_3CN) δ 171.8, 141.6, 133.9, 131.7, 130.0, 129.3, 127.2, 68.8, 62.3, 19.6; HRMS (EI) Calcd for $C_{12}H_{16}NO_5S$ 286.0749, found 286.0753; Anal. Calcd for $C_{12}H_{15}NO_5S$: C, 50.52; H, 5.30; N, 4.91. Found: C, 50.77; H, 5.23; N, 4.84.

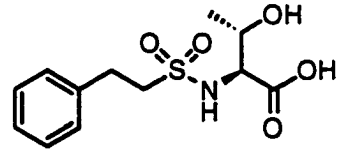


***N*-(Phenethylsulfonyl)-L-threonine (140a).** To a solution of *N*-(*trans*-β-styrenesulfonyl)-L-threonine **139a** (2.0 g, 7.01 mmol) in methanol (50 mL) under argon was added 10% palladium on carbon (0.5 g) at room temperature. The flask was evacuated and flushed with hydrogen, then stirred at room temperature under hydrogen overnight. The mixture was filtered through a pad of Celite and evaporated *in vacuo*. This procedure was repeated three times to yield a pale white solid which was recrystallized from (methanol / $CHCl_3$ / hexane) to give **140a** (1.88 g, 93%) as a white solid: mp 157-159 °C; $[\alpha]_D^{26}$ -19.0° (*c* 1.0, CH_3OH); IR (μscope) 3470, 3303, 2979, 1718, 1335, 1152, 1068, 750, 690 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.28 (m, 5H, Ph), 5.65 (d, 1H, *J* = 9 Hz, NH), 4.23 (dq, 1H, *J* = 6, 3 Hz, CH(OH)), 3.93 (dd, 1H, *J* = 9, 3 Hz, NCH), 3.26 (m, 2H, $PhCH_2CH_2$), 3.09 (m, 2H, $PhCH_2CH_2$), 1.21 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, CD_3CN) δ 172.6, 139.7, 129.6, 129.5, 127.6, 68.5, 62.1, 55.2, 30.5, 20.3; HRMS (EI) Calcd for $C_{12}H_{18}NO_5S$ 288.0906, found 288.0898; Anal. Calcd for $C_{12}H_{17}NO_5S$: C, 50.16; H, 5.96; N, 4.87. Found: C, 49.88; H, 5.97; N, 4.90.

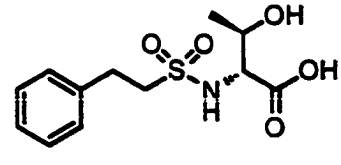


***N*-(Phenethylsulfonyl)-D-threonine (140b).** Reaction of *N*-(*trans*-β-styrenesulfonyl)-D-threonine **139b** (1.97 g, 6.90 mmol) and 10% palladium on carbon (0.5 g) as described for **140a** gave **140b** (1.61 g, 81%) as a white solid: mp 157-159 °C;

$[\alpha]_D^{26}$ +10.0° (*c* 1.0, CH_3OH); IR (μscope) 3470, 3302, 2979, 1717, 1325, 1152, 1068, 747, 700 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.28 (m, 5H, Ph), 5.68 (d, 1H, *J* = 9 Hz, NH), 4.23 (dq, 1H, *J* = 6, 3 Hz, CH(OH)), 3.93 (dd, 1H, *J* = 9, 3 Hz, NCH), 3.28 (m, 2H, $PhCH_2CH_2$), 3.09 (m, 2H, $PhCH_2CH_2$), 1.21 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (75 MHz, CD_3CN) δ 172.6, 139.7, 129.6, 129.5, 127.6, 68.5, 62.1, 55.2, 30.5, 20.3; HRMS (EI) Calcd for $C_{12}H_{18}NO_5S$ 288.0906, found 288.0899; Anal. Calcd for $C_{12}H_{17}NO_5S$: C, 50.16; H, 5.96; N, 4.87. Found: C, 49.84; H, 6.14; N, 4.92.

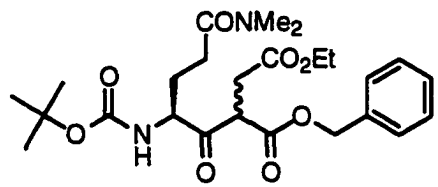


***N*-(Phenethylsulfonyl)-L-*allo*-threonine (140c).** Reaction of *N*-(*trans*-β-styrenesulfonyl)-L-*allo*-threonine **139c** (1.79 g, 6.28 mmol) and 10% palladium on carbon (0.5 g) as described for **140a** gave **140c** (1.57 g, 87%) as a white solid: mp 122-124 °C; $[\alpha]_D^{26}$ +4.34° (*c* 4.61, $CO(CH_3)_2$); IR (μscope) 3482, 3291, 2973, 1726, 1350, 1127, 1087, 745, 697 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.28 (m, 5H, Ph), 5.74 (d, 1H, *J* = 9 Hz, NH), 4.08 (quintet, 1H, *J* = 6, 5 Hz, CH(OH)), 4.01 (dd, 1H, *J* = 9, 5 Hz, NCH), 3.29 (m, 2H, $PhCH_2CH_2$), 3.06 (m, 2H, $PhCH_2CH_2$), 1.18 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, CD_3CN) δ 171.9, 139.6, 129.5, 129.4, 127.5, 68.6, 62.3, 55.1, 30.4, 19.1; HRMS (EI) Calcd for $C_{12}H_{18}NO_5S$ 288.0906, found 288.0880; Anal. Calcd for $C_{12}H_{17}NO_5S$: C, 50.16; H, 5.96; N, 4.87. Found: C, 49.69; H, 6.03; N, 4.93.



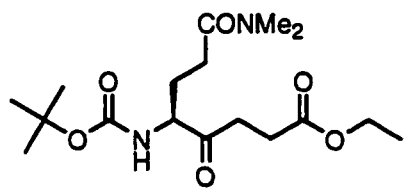
***N*-(Phenethylsulfonyl)-D-*allo*-threonine (140d).** Reaction of *N*-(*trans*-β-styrenesulfonyl)-D-*allo*-threonine **139d** (2.0 g, 7.01 mmol) and 10% palladium on carbon

(0.5 g) as described for **140a** gave **140d** (1.74 g, 87%) as a white solid: mp 122-124 °C; $[\alpha]_D^{26}$ -3.80° (*c* 5.3, $CO(CH_3)_2$); IR (μscope) 3483, 3292, 2973, 1726, 1351, 1127, 1087, 748, 698 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.27 (m, 5H, Ph), 5.72 (d, 1H, *J* = 9 Hz, NH), 4.07 (m, 1H, CH(OH)), 4.02 (m, 1H, NCH), 3.29 (m, 2H, $PhCH_2CH_2$), 3.06 (m, 2H, $PhCH_2CH_2$), 1.18 (d, 3H, *J* = 6 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, CD_3CN) δ 172.0, 139.7, 129.6, 129.5, 127.6, 68.7, 62.3, 55.1, 30.5, 19.2; HRMS (EI) Calcd for $C_{12}H_{17}NO_5S$ 287.0827, found 287.0710.



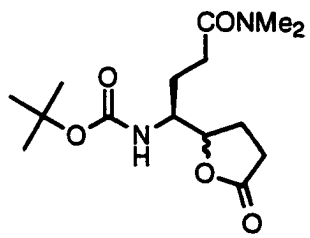
Ethyl (3*RS*,5*S*)-3-(benzyloxycarbonyl)-5-(*tert*-butyloxycarbonylamino)-8-(*N*,*N*-dimethylamino)-4,8-dioxooctanoate (143). To a solution of benzyl (4*S*)-4-(*tert*-butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3,7-dioxoheptanoate **74** (4.0 g, 9.8 mmol) in THF (80 mL) cooled in an ice bath at 0 °C was added potassium *tert*-butoxide (1.33 g, 11.8 mmol). The solution was stirred for 10 min at 0 °C under argon. Ethyl bromoacetate (1.31 mL, 11.8 mmol) was added dropwise over 15 min, the mixture was stirred for 30 min at 0 °C and then warmed to room temperature overnight. The solvent was removed *in vacuo* to give a residue, which was suspended in water (100 mL) and washed with ethyl acetate (3 x 50 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a lime-green oil. Purification by flash chromatography (ethyl acetate) produced the title compound **143** (4.4 g, 91%) as a clear oil. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 2 : 1): IR ($CHCl_3$ cast) 3364, 2979, 1735, 1643, 1498, 1499, 1167, 1096, 752, 699 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 7.35 (m, 5H, Ph), 5.62 (br s, 1H, NH), 5.19 (d, 1H, *J* = 11 Hz, $PhCH_2$), 5.17 (d, 1H, *J* = 11 Hz, $PhCH_2$), 4.17 (m, 2H, OCH_2CH_3), 4.39 (m, 1H, COCHCO), 4.03 (m, 1H, NCH),

2.99 (m, 2H, $C\underline{H}_2CO_2Et$), 2.97 (s, 3H, $NC\underline{H}_3$), 2.94 (s, 3H, $NC\underline{H}_3$), 2.28 (m, 2H, $NCOC\underline{H}_2$), 1.97 (m, 2H, $NCHC\underline{H}_2$), 1.42 (s, 9H, $C(C\underline{H}_3)_3$), 1.23 (m, 3H, $OCH_2C\underline{H}_3$); (isomer B) δ 7.35 (m, 5H, Ph), 5.62 (br s, 1H, N<u>H</u>), 5.18 (d, 1H, J = 11 Hz, $PhC\underline{H}_2$), 5.16 (d, 1H, J = 11 Hz, $PhC\underline{H}_2$), 4.39 (m, 1H, COC<u>H</u>CO), 4.03 (m, 1H, NC<u>H</u>), 4.17 (m, 2H, $OC\underline{H}_2CH_3$), 2.99 (m, 2H, $C\underline{H}_2CO_2Et$), 2.97 (s, 3H, $NC\underline{H}_3$), 2.94 (s, 3H, $NC\underline{H}_3$), 2.28 (m, 2H, $NCOC\underline{H}_2$), 1.97 (m, 2H, $NCHC\underline{H}_2$), 1.42 (s, 9H, $C(C\underline{H}_3)_3$), 1.23 (m, 3H, $OCH_2C\underline{H}_3$); ^{13}C NMR (75 MHz, $CDCl_3$) (isomer A) δ 203.5, 170.3, 168.1, 167.8, 155.2, 134.9, 128.3, 128.2, 128.1, 79.6, 67.2, 60.7, 55.4, 50.5, 37.9, 37.8, 32.8, 28.1, 26.2, 25.3, 13.8; (isomer B) δ 203.5, 170.9, 170.6, 169.2, 155.2, 134.9, 128.3, 128.2, 128.1, 79.6, 67.8, 60.7, 55.4, 50.5, 37.9, 38.2, 32.8, 28.1, 26.2, 25.3, 13.8; HRMS (EI) Calcd for $C_{25}H_{36}N_2O_8$ 492.2472, found 492.2462.

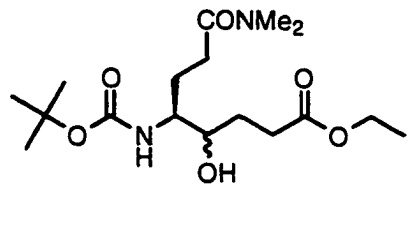


Ethyl (5*S*)-5-(*tert*-butyloxycarbonylamino)-8-(*N*,*N*-dimethylamino)-4,8-dioxooctanoate (144). To a solution of **143** (3.76 g, 7.64 mmol) in methanol (75 mL) under argon was added 10% palladium on charcoal (0.37 g). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give ketone **144** (2.73 g, quantitative) as a clear oil: $[\alpha]_D^{26}$ +18.67° (*c* 3.0, $CHCl_3$); IR ($CHCl_3$ cast) 3300, 2977, 1709, 1637, 1651, 1167, 1056 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 5.62 (br s, 1H, N<u>H</u>), 4.30 (m, 1H, NC<u>H</u>), 4.13 (q, 2H, J = 7 Hz, $OC\underline{H}_2CH_3$), 2.99 (s, 3H, $NC\underline{H}_3$), 2.92 (s, 3H, $NC\underline{H}_3$), 2.82 (m, 1H, $C\underline{H}_2CH_2CO_2Et$), 2.63 (m, 2H, $CH_2C\underline{H}_2CO_2Et$), 2.48 (m, 1H, $C\underline{H}_2CH_2CO_2Et$), 2.31 (m, 2H, $NCOC\underline{H}_2$), 2.25 (m, 1H, $NCHC\underline{H}_2$), 1.88 (m, 1H, $NCHC\underline{H}_2$), 1.44 (s, 9H, $C(C\underline{H}_3)_3$) 1.21 (t, 3H, J = 7 Hz, $OCH_2C\underline{H}_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 209.3, 171.9, 170.1, 155.3 79.7, 60.3, 57.1,

36.6, 34.9, 34.1, 33.5, 28.6, 27.7, 25.7, 13.6; HRMS (EI) Calcd for $C_{17}H_{30}N_2O_6$ 358.2104, found 358.2104.



145a



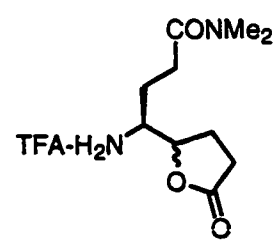
145b

(5*RS*)-5-[(1*S*)-1-(*tert*-butyloxycarbonylamino)-3-(*N*,*N*-dimethylcarbamoyl)propyl]oxolan-2-one (145a) and ethyl (4*RS*,5*S*)-5-(*tert*-butyloxycarbonylamino)-8-(*N*,*N*-dimethylamino)-4-hydroxy-8-oxooctanoate (145b). To a stirred solution of γ-keto ester **144** (0.75 g, 2.10 mmol) in ethanol (15 mL) under argon at 0 °C, was added dropwise a solution of $NaBH_4$ (0.1 M, 20 mL) in absolute ethanol. The reaction mixture was stirred at 0 °C for 30 min, followed by 30 min at room temperature. The solution was acidified to pH 2 with 1 N $KHSO_4$ and the solvent was removed *in vacuo*. The residue was dissolved in water (15 mL), extracted with ethyl acetate (3 x 10 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by HPLC (Waters Resolve C-18 reverse phase, 8 x 200 mm, gradient elution, 0-20% acetonitrile : water, t_R 39.2 and 46.4 min) gave **145a** (172 mg, 26%) and **145b** (249 mg, 33%) respectively, as white solids.

For **145a**: Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 2 : 1): IR ($CHCl_3$ cast) 3315, 2975, 1776, 1708, 1632, 1248, 1169, 1047 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 5.04 (br s, 1H, NH), 4.15 (m, 1H, $CH_2CH_2CO_2CH$), 3.70 (m, 1H, NCH), 2.99 (s, 6H, $N(CH_3)_2$), 2.48 (m, 2H, $CH_2CH_2CO_2CH$), 2.42 (m, 2H, $NCOCH_2$), 2.27 (m, 2H, $CH_2CH_2CO_2CH$), 2.02 (m, 1H, $NCHCH_2$), 1.82 (m, 1H, $NCHCH_2$), 1.42 (s, 9H, $C(CH_3)_3$); (isomer B) δ 5.04 (br s, 1H, NH), 4.15 (m, 1H, $CH_2CH_2CO_2CH$), 3.70 (m, 1H, NCH), 2.99 (s, 6H, $N(CH_3)_2$), 2.48 (m, 2H, $CH_2CH_2CO_2CH$), 2.42 (m, 2H, $NCOCH_2$), 2.27 (m, 2H,

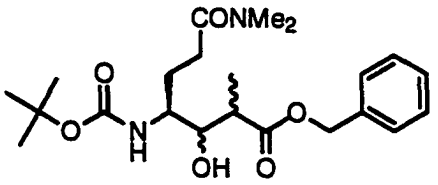
$C\underline{H}_2CH_2CO_2CH$), 2.02 (m, 1H, NCHC$\underline{H}_2$), 1.82 (m, 1H, NCHC$\underline{H}_2$), 1.42 (s, 9H, C($C\underline{H}_3$)$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) (isomer A) δ 176.9, 172.6, 172.0, 156.0, 82.4, 53.6, 37.2, 35.7, 29.3, 28.6, 28.3, 25.1, 24.4; (isomer B) δ 176.9, 172.6, 172.0, 156.0, 82.3, 52.8, 37.2, 35.7, 29.3, 28.6, 28.3, 25.1, 24.3; HRMS (ES) Calcd for $C_{15}H_{26}N_2O_5Na$ 337.1739, found 337.1741.

For **145b**: Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 2 : 1): IR ($CHCl_3$ cast) 3300, 2977, 1731, 1708, 1635, 1248, 1169, 1095 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) (isomer A) δ 5.16 (br s, 1H, N$\underline{H}$), 4.17 (q, 2H, J = 7 Hz, OC$\underline{H}_2CH_3$), 4.15 (m, 1H, NC$\underline{H}$), 3.70 (m, 1H, C$\underline{H}$(OH)), 3.01 (s, 3H, NC$\underline{H}_3$), 2.97 (s, 3H, NC$\underline{H}_3$), 2.85 (m, 2H, $CH_2C\underline{H}_2CO_2Et$), 2.40 (m, 2H, $C\underline{H}_2CH_2CO_2Et$), 2.40 (m, 2H, NCOC$\underline{H}_2$), 2.01 (m, 1H, NCHC$\underline{H}_2$), 1.82 (m, 1H, NCHC$\underline{H}_2$), 1.42 (s, 9H, C($C\underline{H}_3$)$_3$), 1.24 (t, 3H, J = 7 Hz, $OCH_2C\underline{H}_3$); (isomer B) δ 5.16 (br s, 1H, N$\underline{H}$), 4.17 (q, 2H, J = 7 Hz, OC$\underline{H}_2CH_3$), 4.15 (m, 1H, NC$\underline{H}$), 3.70 (m, 1H, C$\underline{H}$(OH)), 3.01 (s, 3H, NC$\underline{H}_3$), 2.97 (s, 3H, NC$\underline{H}_3$), 2.85 (m, 2H, $CH_2C\underline{H}_2CO_2Et$), 2.40 (m, 2H, $C\underline{H}_2CH_2CO_2Et$), 2.40 (m, 2H, NCOC$\underline{H}_2$), 2.01 (m, 1H, NCHC$\underline{H}_2$), 1.82 (m, 1H, NCHC$\underline{H}_2$), 1.42 (s, 9H, C($C\underline{H}_3$)$_3$), 1.24 (t, 3H, J = 7 Hz, $OCH_2C\underline{H}_3$); ^{13}C NMR (75 MHz, $CDCl_3$) (isomer A) δ 175.7, 170.9, 156.2, 86.3, 79.8, 60.9, 52.9, 37.9, 37.2, 36.7, 35.7, 29.5, 28.3, 25.1, 14.1; (isomer B) δ 175.5, 171.9, 155.9, 86.2, 79.6, 60.9, 51.1, 34.7, 34.3, 33.7, 33.5, 29.3, 28.2, 25.1, 14.1; MS (CI, NH_3) *m/z* (relative intensity) 361.3 (MH^+, 100%).



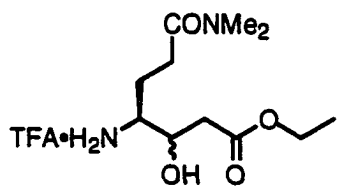
(5*RS*)-5-[(1*S*)-1-amino-3-(*N*,*N*-dimethylcarbamoyl)propyl]oxolan-2-one, trifluoroacetate salt (146). To a solution of γ-lactone **145a** (15.5 mg, 49.3

μmol) in CH_2Cl_2 (0.5 mL) at 0 °C was added trifluoroacetic acid (0.5 mL), the mixture was stirred for 1 h at 0 °C. The ice bath was removed and the solution was allowed to warm to room temperature over a 1 h period. The solvent was removed *in vacuo* and the residue dried under high vacuum to give the trifluoroacetate salt **146** (20.0 mg, quantitative) as a lime-green oil. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 2 : 1): IR ($CHCl_3$ cast) 3300, 2941, 1783, 1678, 1626, 1420, 1201, 1178, 1033 cm^{-1}; ^1H NMR (360 MHz, D_2O) (isomer A) δ 4.80 (m, 1H, NC<u>H</u>), 3.58 (m, 1H, $CH_2CH_2CO_2C\underline{H}$), 2.95 (s, 3H, $NC\underline{H_3}$), 2.81 (s, 3H, $NC\underline{H_3}$), 2.60 (m, 2H, $CH_2C\underline{H_2}CO_2CH$), 2.60 (m, 2H, $NCOC\underline{H_2}$), 2.35 (m, 1H, $NCHC\underline{H_2}$), 2.05 (m, 1H, $NCHC\underline{H_2}$), 1.90 (m, 1H, $C\underline{H_2}CH_2CO_2CH$), 1.77 (m, 1H, $C\underline{H_2}CH_2CO_2CH$); (isomer B) δ 4.80 (m, 1H, NC<u>H</u>), 3.58 (m, 1H, $CH_2CH_2CO_2C\underline{H}$), 2.95 (s, 3H, $NC\underline{H_3}$), 2.81 (s, 3H, $NC\underline{H_3}$), 2.60 (m, 2H, $CH_2C\underline{H_2}CO_2CH$), 2.60 (m, 2H, $NCOC\underline{H_2}$), 2.35 (m, 1H, $NCHC\underline{H_2}$), 2.05 (m, 1H, $NCHC\underline{H_2}$), 1.90 (m, 1H, $C\underline{H_2}CH_2CO_2CH$), 1.77 (m, 1H, $C\underline{H_2}CH_2CO_2CH$); ^{13}C NMR (75 MHz, $CDCl_3$) (isomer A) δ 181.1, 174.9, 81.0, 53.5, 38.1, 36.4, 30.1, 29.8, 23.1, 22.7; (isomer B) δ 180.9, 174.5, 80.9, 55.3, 36.3, 35.4, 30.0, 29.6, 24.2, 23.3; HRMS (ES) Calcd for $C_{10}H_{18}N_2O_3$ 215.1396, found 215.1394.



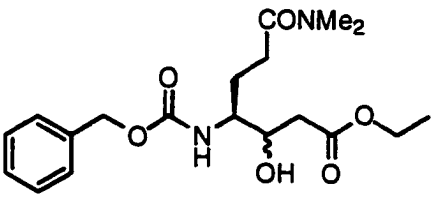
Benzyl (2*RS*,3*RS*,4*S*)-4-(*tert*-butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3-hydroxy-2-methyl-7-oxoheptanoate (147). To a stirred solution of β-keto ester **87** (50.0 mg, 0.12 mmol) in ethanol (0.60 mL), under argon at 0 °C, was added dropwise a solution of $NaBH_4$ (0.1 M, 1.20 mL) in absolute ethanol. The reaction mixture was stirred at 0 °C for 30 min, then at room temperature for 30 min. The solution was acidified to pH 2 with 1 N $KHSO_4$ and the solvent was removed *in vacuo*. The residue was dissolved in water (2 mL), extracted with ethyl acetate (3 x 2 mL), dried

over $MgSO_4$, filtered and concentrated *in vacuo* to yield crude alcohol **147**. Purification by flash chromatography (ethyl acetate) gave β-hydroxy ester **147** (25.0 mg, 50%) as a clear oil. Spectroscopic characterization was performed on a mixture of diastereoisomers: IR ($CHCl_3$ cast) 3372, 3030, 2975, 1732, 1696, 1651, 1560, 1170, 1042, 752, 698 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.38 (m, 5H, Ph), 5.15 (s, 2H, $PhCH_2$), 4.86 (br s, 1H, NH), 3.82 (m, 1H, NCH), 3.61 (m, 1H, C(OH)H), 3.02 (s, 3H, NCH_3), 2.98 (s, 3H, NCH_3), 2.61 (m, 1H, $CH(CH_3)$), 2.39 (m, 2H, $NCOCH_2$), 2.11 (m, 1H, $NCHCH_2$), 1.84 (m, 1H, $NCHCH_2$), 1.43 (s, 9H, $C(CH_3)_3$), 1.29 (d, 3H, J = 7 Hz, $CH(CH_3)$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 176.0, 173.3, 155.9, 135.9, 128.6, 128.3, 128.2, 79.5, 74.2, 66.5, 52.8, 42.3, 37.4, 35.8, 29.6, 28.4, 26.2, 11.2; HRMS (EI) Calcd for $C_{22}H_{34}N_2O_6$ 422.2417, found 422.2421.



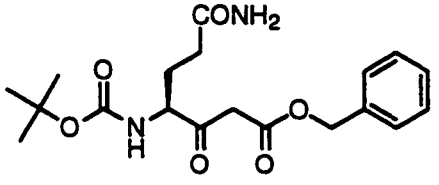
Ethyl (3*RS*,4*S*)-4-amino-7-(*N*,*N*-dimethylamino)-3-hydroxy-7-oxoheptanoic acid, trifluoroacetate salt (148). To a solution of β-hydroxy ester **75** (33.7 mg, 0.097 mmol) in CH_2Cl_2 (1 mL) at 0 °C was added trifluoroacetic acid (1 mL), the mixture was stirred for 1 h at 0 °C. The ice bath was removed and the solution was allowed to warm to room temperature over a 1 h period. The solvent was removed *in vacuo* and the residue was dried under high vacuum to give the trifluoroacetate salt **148** (33.6 mg, 96%) as an orange oil. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR (μscope) 3350, 2936, 1731, 1677, 1406, 1201, 1062 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) (isomer A) δ 4.16 (m, 1H, NCH), 4.02 (q, 2H, J = 7 Hz, OCH_2CH_3), 3.22 (m, 1H, C(OH)H), 2.93 (s, 3H, NCH_3), 2.83 (s, 3H, NCH_3), 2.52 (m, 2H, $CH(OH)CH_2$), 2.49 (m, 2H, $NCOCH_2$), 1.90 (m, 1H, $NCHCH_2$), 1.72 (m, 1H, $NCHCH_2$), 1.14 (t, 3H, J = 7 Hz, OCH_2CH_3); (isomer B)

δ 4.02 (q, 2H, J = 7 Hz, OCH_2CH_3), 3.99 (m, 1H, NCH), 3.10 (m, 1H, C(OH)H), 2.93 (s, 3H, NCH_3), 2.83 (s, 3H, NCH_3), 2.52 (m, 2H, $CH(OH)CH_2$), 2.49 (m, 2H, $NCOCH_2$), 1.90 (m, 1H, $NCHCH_2$), 1.72 (m, 1H, $NCHCH_2$), 1.14 (t, 3H, J = 7 Hz, OCH_2CH_3); ^{13}C NMR (75 MHz, D_2O) (isomer A) δ 174.3, 172.5, 68.5, 61.9, 56.6, 38.6, 37.6, 35.9, 30.7, 23.9, 14.5; (isomer B) δ 174.3, 172.5, 67.9, 61.9, 56.4, 40.0, 37.6, 35.9, 30.4, 26.7, 14.5; HRMS (ES) Calcd for $C_{11}H_{23}N_2O_4$ 247.1658, found 247.1658.

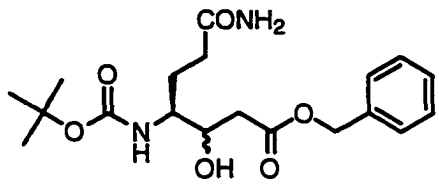


Ethyl (3*RS*,4*S*)-4-(benzyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-3-hydroxy-7-oxoheptanoate (149). To a solution of trifluoroacetate salt **148** (14.6 mg, 40.5 μmol) in CH_2Cl_2 (1.0 mL) at 0 °C was added triethylamine (20.0 μL, 142.0 μmol). The solution was stirred for 5 min and then benzyl chloroformate (7.0 μL, 48.6 μmol) was added dropwise over 5 min. The mixture was stirred at 0 °C for 30 min and then at room temperature for 2 h. The solvent was removed *in vacuo* and the residue was purified by flash chromatography (ethyl acetate) to yield **149** (15.2 mg, 99%) as a clear oil. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR (μscope) 3331, 3050, 2932, 1717, 1629, 1044, 740, 698 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) (isomer A) δ 7.38 (m, 5H, Ph), 5.57 (d, 1H, J = 8 Hz, NH), 5.05 (d, 1H, J = 12 Hz, $PhCH_2$), 5.01 (d, 1H, J = 12 Hz, $PhCH_2$), 4.18 (q, 2H, J = 7 Hz, OCH_2CH_3), 4.01 (m, 1H, NCH), 3.70 (m, 1H, C(OH)H), 2.95 (s, 3H, NCH_3), 2.91 (s, 3H, NCH_3), 2.58 (m, 2H, $CH(OH)CH_2$), 2.39 (m, 2H, $NCOCH_2$), 2.00 (m, 1H, $NCHCH_2$), 1.88 (m, 1H, $NCHCH_2$), 1.23 (t, 3H, J = 7 Hz, OCH_2CH_3); (isomer B) δ 7.38 (m, 5H, Ph), 5.28 (d, 1H, J = 8 Hz, NH), 5.05 (d, 1H, J = 12 Hz, $PhCH_2$), 5.01 (d, 1H, J = 12 Hz, $PhCH_2$), 4.18 (q, 2H, J = 7 Hz, OCH_2CH_3), 3.98 (m, 1H, NCH), 3.68 (m, 1H, C(OH)H), 2.95 (s, 3H, NCH_3), 2.91 (s,

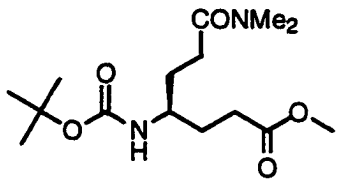
3H, NCH_3), 2.58 (m, 2H, $CH(OH)CH_2$), 2.39 (m, 2H, $NCOCH_2$), 2.00 (m, 1H, $NCHCH_2$), 1.88 (m, 1H, $NCHCH_2$), 1.25 (t, 3H, J = 7 Hz, OCH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) (isomer A) δ 172.9, 172.8, 156.8, 136.6, 128.5, 128.1, 128.0, 70.8, 68.3, 61.1, 55.3, 38.6, 35.5, 33.5, 29.5, 24.7, 14.2; (isomer B) δ 172.9, 172.8, 156.8, 136.6, 128.5, 128.1, 127.9, 69.7, 68.3, 60.7, 55.3, 38.3, 35.5, 33.5, 29.5, 24.7, 14.2; HRMS (ES) Calcd for $C_{19}H_{28}N_2O_6Na$ 403.1845, found 403.1856.



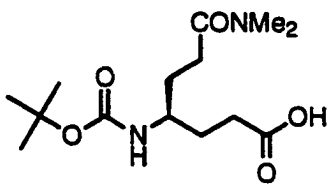
Benzyl (4*S*)-7-(amido)-4-(*tert*-butyloxycarbonylamino)-3-oxoheptanoate (151). To a solution of *N*-(*tert*-butyloxycarbonyl)-L-glutamine **150** (0.5 g, 2.03 mmol) in THF (10 mL) was added 1,1'-carbonyl diimidazole (0.40 g, 2.44 mmol). The clear solution was stirred for 1 h at room temperature under argon. Magnesium benzyl malonate **73** (1.0 g, 2.44 mmol) was added. The mixture was stirred overnight at room temperature. The pH was adjusted to 2 with 0.5 N HCl, and the product was extracted with ethyl acetate (2 x 5 mL). The combined extracts were washed with saturated aqueous $NaHCO_3$ (5 mL) and brine (5 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash chromatography (ethyl acetate) gave β-keto ester **151** (131.4 mg, 17%) as a white solid: IR ($CHCl_3$ cast) 3346, 2976, 1709, 1671, 1512, 1164, 1047, 750, 698 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 7.38 (m, 5H, Ph), 6.10 (br s, 1H, NH_2), 6.01 (d, 1H, J = 6 Hz, NH), 5.63 (br s, 1H, NH_2), 5.18 (s, 2H, $PhCH_2$), 4.08 (m, 1H, CH), 3.62 (s, 2H, $COCH_2CO$), 2.22 (m, 1H, $NCOCH_2$), 2.37 (m, 1H, $NCOCH_2$), 2.09 (m, 1H, $NCHCH_2$), 1.96 (m, 1H, $NCHCH_2$), 1.78 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 203.9, 175.2, 168.1, 157.5, 137.1, 129.5, 129.2, 129.1, 79.5, 67.5, 60.8, 46.6, 31.8, 28.5, 25.9; HRMS (ES) Calcd for $C_{19}H_{26}N_2O_6Na$ 401.1689, found 401.1700.



Benzyl (3*RS*,4*S*)-7-(amido)-4-(*tert*-butyloxycarbonylamino)-3-hydroxyheptanoate (152). To a stirred solution of β-keto ester **151** (0.13 g, 0.35 mmol) in isopropanol / THF (5 mL, 1 : 1) under argon at 0 °C, was added dropwise a solution of $NaBH_4$ (0.1 M, 3.47 mL) in isopropanol. The reaction mixture was stirred at 0 °C for 30 min, then at room temperature for 30 min. The solution was acidified to pH 2 with 1 N HCl and the solvent was removed *in vacuo*. The residue was dissolved in water (5 mL), extracted with ethyl acetate (3 x 5 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield crude alcohol. Purification by flash chromatography (ethyl acetate : hexane, 9 : 1) gave β-hydroxy ester **152** (74.4 g, 56%) as a white solid. Spectroscopic characterization was performed on a mixture of diastereoisomers (isomer A : isomer B, 5 : 1): IR ($CHCl_3$ cast) 3397, 3050, 2935, 1730, 1782, 1652, 1529, 1246, 1166, 1019, 748, 696 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) (isomer A) δ 7.39 (m, 5H, Ph), 6.13 (br s, 1H, NH_2), 5.55 (br s, 1H, NH_2), 5.34 (br s, 1H, J = 9 Hz, NH), 5.09 (s, 2H, $PhCH_2$), 3.89 (m, 1H, NCH), 3.56 (m, 1H, C(OH)H), 2.42 (m, 2H, $CH(OH)CH_2$), 2.15 (m, 2H, $NCOCH_2$), 1.82 (m, 1H, $NCHCH_2$), 1.59 (m, 1H, $NCHCH_2$), 1.40 (s, 9H, $C(CH_3)_3$); (isomer B) δ 7.39 (m, 5H, Ph), 6.13 (br s, 1H, NH_2), 5.55 (br s, 1H, NH_2), 5.34 (br s, 1H, J = 9 Hz, NH), 5.09 (s, 2H, $PhCH_2$), 3.99 (m, 1H, NCH), 3.42 (m, 1H, C(OH)H), 2.42 (m, 2H, $CH(OH)CH_2$), 2.15 (m, 2H, $NCOCH_2$), 1.82 (m, 1H, $NCHCH_2$), 1.59 (m, 1H, $NCHCH_2$), 1.40 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) (isomer A) δ 177.5, 173.3, 158.4, 137.6, 129.5, 129.2, 129.1, 80.3, 70.7, 67.4, 55.4, 40.0, 33.1, 28.8, 27.6; (isomer B) δ 177.5, 173.3, 158.4, 137.6, 129.5, 129.2, 129.1, 80.3, 72.1, 67.4, 56.2, 40.5, 33.1, 28.8, 27.6; HRMS (ES) Calcd for $C_{19}H_{28}N_2O_6Na$ 403.1845, found 403.1844.

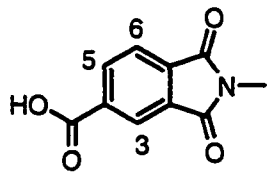


Methyl (4*S*)-4-(*tert*-butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-7-oxoheptanoate (153). To a solution of α, β-unsaturated ester **63** (25.0 mg, 0.080 mmol) in methanol (2.0 mL) under argon was added 10% palladium on charcoal (2.50 mg). The mixture was stirred under an atmosphere of hydrogen until gas absorption ceased. The catalyst was removed by filtration through a column of Celite and the filtrate was concentrated *in vacuo* to give the β-hydroxy acid **153** (21.7 mg, 86%) as a white solid: mp 92-94 °C; IR ($CHCl_3$ cast) 3312, 2974, 1737, 1708, 1638, 1052 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 4.60 (br s, 1H, NH), 3.67 (s, 3H, CO_2CH_3), 3.59 (m, 1H, NCH), 3.00 (s, 3H, NCH_3), 2.97 (s, 3H, NCH_3), 2.41 (m, 2H, CH_2CO_2Me), 2.40 (m, 2H, CH_2CON), 1.87 (m, 2H, CH_2CH_2CON), 1.72 (m, 2H, $CH_2CH_2CO_2Me$), 1.40 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.9, 172.7, 155.9, 79.1, 51.7, 50.6, 37.2, 35.6, 31.0, 30.8, 30.6, 29.9, 28.4; HRMS (ES) Calcd for $C_{15}H_{28}N_2O_5Na$ 339.1896, found 339.1911.

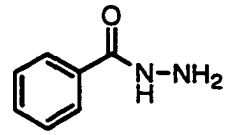


(4*S*)-4-(*tert*-Butyloxycarbonylamino)-7-(*N*,*N*-dimethylamino)-7-oxoheptanoic acid (154). To a solution of ester **153** (16.1 mg, 50.8 μmol) in THF (1.0 mL) and water (1.0 mL) at room temperature was added lithium hydroxide monohydrate (2.56 mg, 60.9 μmol). The mixture was stirred at room temperature for 2 h. The solvent was removed *in vacuo* and the residue was dissolved in water (3 mL) and acidified to pH 2 with 1 N HCl. The aqueous solution was extracted with ethyl acetate (2 x

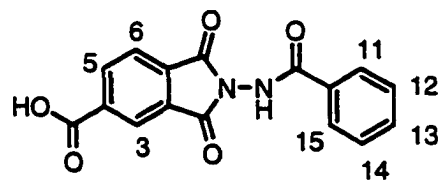
2 mL) and the combined organic layers were washed with brine (2 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* and the residue purified by flash chromatography (ethyl acetate : methanol : acetic acid, 100 : 5 : 1) to give **154** (8.2 mg, 53%) as a yellow oil: $[\alpha]_D^{26}$ +2.50° (*c* 2.0, CH_3OH); IR ($CHCl_3$ cast) 3313, 2926, 1707, 1708, 1634, 1450, 1248, 1059 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 5.23 (br s, 1H, J = 7 Hz, NH), 3.45 (m, 1H, NCH), 2.93 (s, 3H, NCH_3), 2.85 (s, 3H, NCH_3), 2.40-2.20 (m, 4H, CH_2CO_2H, CH_2CON), 2.00-1.50 (m, 4H, CH_2CH_2CON, $CH_2CH_2CO_2H$), 1.40 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, CD_3CN) δ 175.5, 173.4, 157.1, 79.1, 51.7, 38.1, 37.5, 30.9, 30.2, 28.6, 27.3, 25.7; HRMS (ES) Calcd for $C_{14}H_{26}N_2O_5Na$ 325.1739, found 325.1737.



4-Carboxyl-*N*-methylphthalimide (155). To a solution of methylamine hydrochloride **161** (1.75 g, 26.0 mmol) in THF (200 mL) was added triethylamine (8.6 mL, 62.0 mmol) at room temperature with stirring. Trimellitic anhydride **160** (5.0 g, 26.0 mmol) was added and the mixture was heated under reflux for 6 h. The solvent was removed *in vacuo* and the residue was purified by flash chromatography ($CHCl_3$: methanol : acetic acid, 100 : 10 : 1) to yield phthalimide **155** (2.42 g, 45%) as a white solid: mp 227-230 °C; IR ($CHCl_3$ cast) 3472, 3050, 2955, 1772, 1626, 1516, 1072, 775, 611 cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 8.33 (d, 1H, *J* = 8 Hz, H_5), 8.30 (s, 1H, H_3), 7.86 (d, 1H, *J* = 8 Hz, H_6), 3.08 (s, 3H, NCH_3); ^{13}C NMR (75 MHz, d_7-DMF) δ 167.9, 166.5, 162.7, 137.1, 136.1, 135.8, 133.2, 123.7, 123.6, 24.1; HRMS (EI) Calcd for $C_{10}H_7NO_4$ 205.0375, found 205.0366; Anal. Calcd for $C_{10}H_7NO_4$: C, 58.54; H, 3.44; N, 6.83. Found: C, 58.39; H, 3.20; N, 6.69.



Benzoic hydrazide (165).[149] Benzoic acid **164** (1.0 g, 8.20 mmol) was dissolved in dry CH_2Cl_2 (40 mL), with cooling to 0 °C under an argon atmosphere. Triethylamine (1.36 mL, 9.83 mmol) was added, followed by ethyl chloroformate (0.94 mL, 9.83 mmol). The mixture was stirred for 20 min at 0 °C, followed by the addition of hydrazine (0.31 mL, 9.83 mmol) and triethylamine (1.36 mL, 9.83 mmol). After 30 min at 0 °C, the reaction mixture was warmed to room temperature and stirring was continued overnight. The solvent was removed *in vacuo*, the residue was partitioned between ethyl acetate (20 mL) and water (20 mL). The aqueous phase was extracted with ethyl acetate (2 x 10 mL) and the combined organic extracts were washed with saturated aqueous $NaHCO_3$ (20 mL) and brine (20 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a white solid. Purification by flash chromatography (ethyl acetate : ammonium hydroxide, 100 : 1) gave the title compound **165** (0.42 g, 38%) as a white solid: mp 114-117 °C; IR ($CHCl_3$ cast) 3294, 3050, 1636, 1499, 1052, 750, 698 cm^{-1}; ^1H NMR (300 MHz, CD_3CN) δ 8.12 (br s, 1H, CONH), 7.74 (m, 2H, *meta*-Ph), 7.48 (m, 3H, *ortho* and *para*-Ph), 4.10 (br s, 2H, NH_2); ^{13}C NMR (75 MHz, CD_3OD) δ 169.6, 134.2, 132.6, 129.5, 128.1; HRMS (EI) Calcd for $C_7H_8N_2O$ 136.0637, found 136.0636; Anal. Calcd for $C_7H_8N_2O$: C, 61.75; H, 5.92; N, 20.57. Found: C, 61.46; H, 5.81; N, 20.32.



***N*-Benzamido-4-carboxylphthalimide (166).** To a solution of benzoic hydrazide **165** (1.0 g, 7.34 mmol) in THF (50 mL) was added triethylamine (3 mL, 22.03 mmol) at room temperature with stirring. Trimellitic anhydride **160** (1.70 g, 8.81 mmol) was added and the mixture was heated under reflux for 6 h. The solvent was removed *in*

vacuo and the residue was purified by flash chromatography ($CHCl_3$: methanol : acetic acid, 100 : 10 : 1) and recrystallized from methanol to yield hydrazide **166** (1.57 g, 70%) as a white solid: mp 255-257 °C; IR ($CHCl_3$ cast) 3304, 3053, 1750, 1653, 1508, 1076, 787, 698 cm^{-1}; ^1H NMR (360 MHz, CD_3CN) δ 9.28 (br s, 1H, CONH), 8.49 (d, 1H, J = 8 Hz, H_5), 8.42 (s, 1H, H_3), 8.04 (d, 1H, J = 8 Hz, H_6), 7.92 (d, 1H, J = 7 Hz, H_{11}), 7.92 (d, 1H, J = 7 Hz, H_{15}), 7.68 (t, 1H, J = 7 Hz, H_{13}), 7.55 (t, 1H, J = 7 Hz, H_{12}), 7.55 (t, 1H, J = 7 Hz, H_{14}); ^{13}C NMR (75 MHz, CD_3OD) δ 168.8, 167.4, 165.9, 165.9, 138.6, 137.3, 134.7, 134.0, 132.4, 131.8, 129.9, 128.9, 125.5, 125.0; HRMS (EI) Calcd for $C_{16}H_{10}N_2O_5$ 310.0590, found 310.0585; Anal. Calcd for $C_{16}H_{10}N_2O_5$ (plus 1 mol of MeOH): C, 59.65; H, 4.12; N, 8.18. Found: C, 59.76; H, 4.01; N, 8.13.

Crystallographic data for *N*-benzamido-4-carboxylphthalimide (166). Data were acquired on a Bruker P4/RA/SMART 1000 CCD diffractometer. All intensity measurements were performed using graphite monochromated Mo-Kα radiation (λ = 0.71073 Å). *N*-Benzamido-4-carboxylphthalimide **166** ($C_{17}H_{14}N_2O_6$ ($C_{16}H_{10}N_2O_5$-CH_3OH)) was obtained as white crystals, space group *C2/c* (No. 15), a = 31.114 (3), b = 7.0781 (6), c = 14.387 (2) Å, V = 3158.1 (5) $Å^3$, Z = 8, T = -60 °C, ρ_{calcd} = 1.440 g cm^{-3}, μ = 0.111 mm^{-1}. A total of 2836 reflections were collected, of these, 2781 were unique. The low value of the linear absorption coefficient and the range of transmission coefficients for a Gaussaian integration (face-indexed) absorption correction (0.9907-0.9838) suggested that an absorption correction was not warranted. The structure was solved by direct methods *(SHELXS-86),*[150] and refined by full-matrix least-squares methods on F^2 *(SHELXL-93).*[151] In the final refinement cycle 2781 reflections with $F_o^2 \geq -3\sigma(F_o^2)$ were used and 231 parameters varied; the model converged with unweighted and weighted agreement factors R_1 = 0.0714, (for 2781 data with $F_o^2 > 2\sigma(F_o^2)$) and xR_2 = 0.2187 (for

all data), with a goodness-of-fit indicator *(S)* of 1.034. Atomic coordinates, bond lengths and angles, and thermal parameters have been deposited at the Cambridge Crystallographic Data Center.

Materials and methods for inhibition studies with HAV 3C proteinases.

Proteinase Production and Purification. Recombinant C24S HAV 3C proteinase (a mutant in which the nonessential surface cysteine has been replaced with serine and which exhibits identical catalytic parameters to wild-type enzyme) was expressed in *E. coli* and purified.[82] Purity of the enzyme sample was greater than 90% as determined spectrophotometrically ε_{280} = 1.2 mg / mL.

Colorimetric Proteinase Assay. Peptide substrate was synthesized using solid phase Fmoc chemistry on Rink resin as previously described.[26] All peptides were purified by reverse-phase HPLC (C-18, 5 x 25 cm, Vydac, 2% / min linear gradient of 0.1% TFA / water adding 0.1% TFA / acetonitrile). Peptide structures were verified by NMR and mass spectrometry.

Peptide proteolysis was monitored using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay as previously described.[82] Reaction mixtures were incubated in reaction buffer at 20 °C. Aliquots (10 μL) were removed from the reaction mixture at timed intervals and peptide hydrolysis was quenched with 50 μL of 0.25 M sodium borate, pH 10. A solution of freshly prepared 0.14 M TNBS (12.5 μL) (Johnson-Mattey, Ward Hill, MA) in 0.25 M sodium borate solution was added to the quenched reaction mixture and incubated for 10 min at 20 °C. The color was stabilized by adding 200 μL of 3.5 mM Na_2SO_3 in 0.2 M KH_2PO_4. The concentration of free amine generated during peptide hydrolysis was determined by measuring the absorbance at 405 nm using a microtitre plate reader (Biorad, Richmond, CA).

Proteinase inactivation was quantitatively evaluated by progress curve analysis[152] as previously described.[26] The extent of peptide proteolysis (release of α-amino groups) was

monitored using the TNBS assay as described above. The concentration of inhibitor was varied from 0.5 to 500 μM; substrate (Ac-ELRTQSFS-amide) concentration was 2 mM and HAV 3C proteinase (C24S mutant) concentration was 0.07 μM. Enzyme was dialyzed against reaction buffer to remove DTT immediately prior to use. Reactions were initiated with enzyme and absorbances were converted into μmoles of product using a glycine standard curve. All determinations were performed in triplicate with different enzyme and inhibitor preparations.

Progress curves were fitted using least squares non-linear regression analysis using Mac Curve Fit 1.0.7, (K. Raner) to:

$$P = \frac{v_o(1-e^{-kt})}{k}$$

where v_o is the initial velocity and k is the apparent first order rate constant (k_{obs}) for the inactivation process. Parameter estimates from individual experiments (weighted by standard error) were averaged to obtain the final value.

Continuous Fluorogenic Assay. Enzyme was dialyzed against reaction buffer to remove DTT immediately prior to use. Cleavage reactions (700 μl) were performed at 25 °C in a solution containing 100 mM K_3PO_4 at pH 7.5, 2 mM EDTA, 0.1 mg / mL BSA, 10 μM fluorogenic substrate Dabcyl-GLRTQSFS-Edans (Bachem), 0.1 μM 3C proteinase and 1% DMF. Reactions were initiated by the addition of enzyme or substrate, depending on the kinetic assay performed. Fluorescence was continuously monitored by excitation at 336 nm and emission at 472 nm at bandwidths of 3 nm with a Shimadzu RF-5301PC spectrofluorophotometer.[83] DMF, THF or DMSO, in which the substrate and inhibitors were dissolved, did not have a significant effect on the 3C proteinase activity when used at a concentration of 10% or less. For proteinase inhibition studies, rates were derived from the initial 3 min of the reaction, inhibitor stock solutions were prepared at 10 mM in DMF and serial dilutions made in DMF. At least five different inhibitor concentrations were examined along with the control sample containing no inhibitor under the conditions

described above. The HAV 3C proteinase activity in the presence of the specified inhibitor was expressed as a percentage of that obtained from the respective control samples. For inhibitors displaying dose-dependent inhibition of the proteinase activity, IC_{50} values were determined from plots of the relative proteinase activity versus log inhibitor concentration. Time-dependent loss of enzyme activity was determined by the protocol of Silverman.[153] The rate of inactivation of β-lactone **13a** was determined by the method of Kitz and Wilson.[154] The competitive inhibition constant (K_i) for β-lactone **13b** was determined from a slope replot of the Lineweaver-Burke plot.[155]

The sensitivity of inhibitors **13a** and **13b** to DTT was evaluated using reactions similar to those described in the previous paragraph, but with the addition of up to 500 μM DTT to the inhibitor-containing mixture followed by the addition of enzyme.

Dialysis experiments with **13a** involved the preparation of two 0.1 μM enzyme solutions identical in all respects other than one contained inhibitor (100 μM of **13a**) and the other solution contained no inhibitor. The two solutions were independently assayed for initial enzyme activity and again after 8 h dialysis using a Centriprep-10 (Amicon) centricon ultrafiltration unit.

Materials and methods for inhibition studies with HRV 3C proteinases.

Proteinase Production and Purification. Recombinant HRV-14 3C protease was purified using the procedure published previously.[156]

Continuous Colorimetric Proteinase Assay. Peptide substrate was designed using the native 2C / 3A cleavage site of the viral polyprotein and was custom synthesized by American Peptide Co. (CA, USA). Peptide sequence was confirmed by amino acid sequence analysis and mass spectrometry.

A typical HRV 3C protease assay[115] was performed at 30 °C for the time indicated (15-30 min) in a 1 mL reaction mixture containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 250 μM peptide substrate EALFQ-pNA (American Peptide Co.), and 3C

proteinase at 0.4 μM. The reaction was started by the addition of either substrate or 3C proteinase. Cleavage of pNA peptides between glutamine at P1 and pNA at P1' by the proteinase releases yellow-colored free pNA, absorbance of free pNA was measured using a GBC Cintra 40 UV spectrometer at a visible wavelength (405 nm) against a blank where either substrate or enzyme was not included in the reaction mixture. DMSO, in which the substrate and inhibitors were dissolved, did not have a significant effect on the 3C proteinase activity when used at a concentration of 10% or less. For proteinase inhibition studies, rates were derived from initial velocity measurements at the time points when the cleavage reaction was proceeding in a linear fashion, inhibitor stock solutions were prepared at 10 mM in DMSO and serial dilutions made in DMSO. The HRV 3C proteinase activity in the presence of the specified inhibitor was expressed as a percentage of that obtained from the respective control samples.

Rate of Hydrolysis of β-Lactones in Phosphate Buffer.

Assuming pseudo-first order kinetics, the hydrolysis of β-lactone was followed by FT-IR with a Nicolet Magna 750 FT-IR instrument using a 0.1 mm IR-Trans 4 cell (Kodak, polycrystalline ZnS). A solution containing 100 mM K_3PO_4 pH 7.5, 2 mM EDTA, 20 mM β-lactone , and 20% DMF was prepared, an aliquot was removed and placed in the IR cell at 22 °C and the disappearance of the β-lactone carbonyl stretch (1830 cm^{-1}) was monitored over a 1 h period.

Mass Spectrometry of HAV 3C-13a Inhibitor Complex.

HAV 3C proteinase was dialyzed against a solution containing 2 mM EDTA and 100 mM K_3PO_4 at pH 7.5 to remove DTT using a Centriprep-10 (Amicon) centricon ultrafiltration unit. Dialyzed HAV 3C proteinase (0.3 mM) was incubated with (10 equivalents) of **13a** and 1% DMF at 25 °C for 1 h with mixing. The HAV 3C-**13a** complex was then dialyzed against H_2O for 1 h using a Centriprep-10 (Amicon) centricon

ultrafiltration unit, to a volume of approximately 300 μL. In addition, a control parallel experiment was performed on the enzyme alone without inhibitor **13a**. Mass spectrometric analysis was performed by positive mode electrospray ionization on a Micromass ZabSpec Hybrid Sector-TOF. The liquid carrier used was a 0.5% solution of formic acid in acetonitrile : water (1 : 1), infused into the electrospray source by means of a Harvard syringe pump at a flow rate of 10 μL / min. An aliquot of the sample to be analyzed was dissolved in 0.5% solution of formic acid in acetonitrile : water (1 : 1) and introduced *via* a 1 μL-loop-injector. Prepurified nitrogen was used as a spray pneumatic aid and filtered air as the bath gas, heated at *ca.* 60 °C. The low resolution mass spectra were acquired by full scan with the magnet from 300 to 3000 daltons, at a rate of 5 sec / decade. The obtained data, corresponding to a series of multiple charged ions, were processed (smoothed, peak detection, production of centroid spectra, series calculation and data transformation) to produce average molecular weights. Data acquisition and processing was achieved by using the OPUS software package on a Digital Alpha station with VMS operating system.

^{1}H / ^{13}C HMQC Spectroscopy of Model Compounds, 13a(β-^{13}C), 102a(β-^{13}C), 112, 113, 118, and 120; HAV 3C and HAV 3C-13a(β-^{13}C) Inhibitor Complex.

Solutions of individual compounds **13a**(β-^{13}C), **102a**(β-^{13}C) and **112**, **113**, **118**, and **120** contained 1.2 mM of the model compound, 6% DMSO-d_6 in 20 mM K_3PO_4 / D_2O at pD 7.5 to give a total volume of 700 μL. Due to significant hydrolysis in buffer solution at pD 7.5 during the HMQC NMR acquisition of **13a**(β-^{13}C), the NMR sample solution was altered to contain 1.2 mM of **13a**(β-^{13}C), 6% DMSO-d_6 in D_2O at pD 5.0 (adjusted with 20% solution of DCl; D = 99.5%) to give a total volume of 700 μL. Prior to use, DTT was removed from the enzyme preparation by dialysis with a Centriprep-10 (Amicon) centricon ultrafiltration unit with 20 mM Na_3PO_4 / D_2O at pD 7.5. The resulting enzyme solution (1.2 mM) was inactivated with inhibitor **13a**(β-^{13}C) (1.2 mM) and 1%

THF-d_8. Model compounds **13a**(β-^{13}C), **102a**(β-^{13}C), **112**, **113**, **118**, and **120**, HAV 3C alone and the HAV 3C-**13a**(β-^{13}C) enzyme inhibitor complex were analyzed by HMQC NMR using an Inova 600 Varian instrument. The parameters for model compound **13a**(β-^{13}C): temperature: 27 °C, solvent: D_2O, number of transients: 4, number of increments: 512, number of data point: 2368, acquisition time: 0.247 sec, sweep width in F2: 4801.9 Hz, sweep width in F1: 30172.3 Hz. The parameters for the HAV 3C-**13a**(β-^{13}C) enzyme inhibitor complex: temperature: 27 °C, solvent: D_2O, number of transients: 98, number of increments: 512, number of data point: 2368, acquisition time: 0.247 sec, sweep width in F2: 4801.9 Hz, sweep width in F1: 30172.3 Hz. Solvent presaturation was used for 1.2 sec, and ^{1}H, ^{13}C decoupling was applied. The chemical shifts were referenced to 1% external acetone.

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S are based on F_o^2; conventional R-factors R_I are based on F_o, with F_o set to zero for negative F_o^2. The observed criterion of $F_o^2 > 2\sigma(F_o^2)$ is used only for calculating R_I, and is not relevant to the choice of reflections for refinement. R-factors based on F_0^2 are statistically about twice as large as those based on F_o, and R-factors based on ALL data will be even larger.

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