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Mechanism and Inhibition of Peptidylglycine α-Hydroxylating Monooxygenase

by

Hengmiao Cheng

A THESIS

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To my father and mother

Abstract

Many neural peptides and hormones have a primary amide functionality at the carboxyl terminus. These compounds arise from corresponding *C*-terminal glycine-extended precursors by a two-step process catalyzed by two enzymes: peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL).

The mechanism of PHM oxidation was investigated. Using ^{14}C and ^{3}H labeled substrates, it was demonstrated that the *pro*-S hydrogen of their glycine residue is stereospecifically removed in the PHM oxidation. To distinguish between dehydrogenation or direct *C*-hydroxylation mechanisms, a substrate analogue *N*-Ac-D-Phe-L-Phe-glycolate (**32**), which cannot undergo dehydrogenation, was prepared and tested with PHM. Small turnovers suggest a direct *C*-oxidation. An isotope dilution experiment shows that only one diastereomer of *N*-Ac-D-Phe-L-Phe-α-hydroxyGly (**42**) is produced by PHM. Determination of the absolute stereochemistry of this intermediate by both independent synthesis and indirect methods were attempted. Labeling studies using $^{18}O_2$ demonstrated that the oxygen of the hydroxylated intermediate originates from molecular oxygen, thereby supporting a direct *C*-hydroxylation mechanism.

PHM shares many characteristics with dopamine β-hydroxylase (DBH) which has been shown to catalyze a radical hydroxylation mechanism. Inhibitors were designed which would capitalize on the stabilization and relocation of a transient radical. A number of such compounds were synthesized and tested with PHM using a new radiochemical assay based on the production of radioactive glyoxylate. D-Phe-L-Phe-D-vinylGly (**78**) displays potent, irreversible, time-dependent and mechanism-based inhibition, whereas D-Phe-L-Phe-L-vinylGly (**80**) shows only moderate competitive inhibition. *N*-Ac-D-Phe-L-Phe-NCHO (**91**) was found to be an irreversible, mechanism-based inactivator. These data support a radical mechanism for PHM oxidation. The weak competitive inhibition of

D-Phe-L-Phe-D-cyclopropylGly (**85**) suggests that the enzyme active site is not large enough to bind this compound. *N*-Ac-D-Phe-L-Phe-2-D-benzylthioGly (**90A**) shows potent competitive inhibition, and its diastereoisomer *N*-Ac-D-Phe-L-Phe-2-L-benzylthioGly (**90B**) exhibits only moderate competitive inhibition. *N*-Ac-D-Phe-L-Phe-2-D-methylthioGly (**89A**) displays slow binding inhibition. PHM does not bind a peptide analogue D-phenylalanyl hydrazinosuccinic acid (**101**). Product inhibition by *N*-Ac-D-Phe-L-Phe-2-"L"-hydroxyGly (**42A**) is potent, but only competitive.

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Table of Contents

Chapter		Page
1	Introduction	1
	General Background	1
	Biosynthesis and Formation of Peptide Amides	2
	Isolation and Purification of Amidating Enzyme	4
	Cofactors and pH Optimum of Amidating Enzyme	7
	Substrate Specificity of Amidating Enzyme	8
	Enzyme Assays	10
	Mechanism of α-Amidation	11
	Inhibition of PHM	18
2	Studies on the Mechanism of Peptidylglycine α-Hydroxylating Monooxygenase	20
2.1	Syntheses of the Model Substrates	20
	Synthesis of D-Tyr-L-Val-Gly	20
	Synthesis of D-Phe-L-Phe-Gly and *N*-Ac-D-Phe-L-Phe-Gly	24
2.2	Enzyme Isolation and Assay	31
2.3	Synthesis of a Glycolate Peptide Analogue and Studies of Oxidation Pathways	34
	D-Tyrosine-L-Valyl-Glycolic Acid	34
	Synthesis and Testing of *N*-Ac-D-Phe-L-Phe-Glycolic acid	42
2.4	Studies to Determine the Structure and Stereochemistry of the PHM Reaction Product	49
	Synthesis of the α-Hydroxyglycine Peptides and Identification of the Enzyme Reaction Intermediate	49

Studies to Determine the Structure of α-Hydroxyglycine Diastereomers by Independent Synthesis 56
Studies to Determine the Absolute Configuration of PHM-Produced Peptidyl-α-Hydroxyglycine by Indirect Methods 69
2.5 Determination of the Oxygen Source in α-Hydroxyglycine Intermediate 75
3. Inhibition Studies of the Peptidylglycine α-Hydroxylating Monooxygenase 79
3.1 The Design and Synthesis of Mechanism-based Inactivators of PHM 79
Inhibitor design 79
Synthesis of Potential PHM Inhibitors 80
Syntheses of D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine and D-Phenylalanyl-L-Phenylalanyl-L-Vinylglycine 80
Preparation of D-Phenylalanyl-L-Phenylalanyl-D-Cyclopropylgycine 87
Synthesis of *trans*-2-(2-Phenylcyclopropyl)-ethanoic Acid 91
Methylthio and Benzylthioglycine Derivatives 92
Preparation of *N*-Acetyl-D-Phenylalanyl-L-Phenylalanyl-*N*-Formylamide 95
Preparation of *N*-Acetyl-L-Prolyl-*N*-Nitroso-Glycine 97
Preparation of D-Phenylalanyl Hydrazinosuccinic Acid 102
3.2 Kinetic Studies of Inhibitors with PHM 104
Development of a New PHM Assay Using Cation Ion-exchange Resin 104
Inhibition Studies of PHM 105
Inhibition Studies with D-Phenylalanyl-L-Phenylalanyl-D-Cyclopropylglycine (**85**) 107

Inhibition Studies with *trans*-2-(2-Phenylcyclopropyl)-ethanoic Acid (**88**) 108

Inhibition of PHM by Peptides Containing a *C*-Terminal Glycine Bearing Sulfur Sidechain **89A**, **89B**, **90A**, **90B** 108

Determination of Stereochemistry of **89A**, **89B**, **90A**, **90B** by an Independent Method 109

Inhibition Studies with *N*-Acetyl-D-Phenylalanyl-L-Phenylalanyl-*N*-Formylamide (**91**) 111

Inhibition Studies with *N*-Acetyl-L-Prolyl-*N*-Nitroso-Glycine (**96**) 112

Inhibition Studies with D-Phenylalanyl Hydrazinosuccinic Acid (**101**) 113

Inhibition by D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine (**78**) and D-Phenylalanyl-L-Phenylalanyl-L-Vinylglycine (**80**)..... 113

Summary of PHM Inhibition Studies 115

3.3 Protein Labeling Studies with *N*-Dansyl-D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine 116

Synthesis of *N*-Dansyl-D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine 117

Interaction of PHM with Fluorescent Inhibitor 122

Experimental 123

References 198

List of Tables

Table		Page
1.	Partial Amino Acid Sequences of Peptide Amide Precursors	4
2.	Comparison of Properties of PAM, PHM, and PAL	18
3.	Enzyme Isolations	33
4.	PHM Turnovers of **7**, **14**, and **22**	34
5.	PHM Assay With Glycolate Peptide Using the Nitrosobenzene Procedure	48
6.	PHM Assay of *N*-Ac-D-Phe-L-Phe-[1,2-^{14}C]-Gly (**23**) Using the Nitrosobenzene Procedure	54
7.	HPLC Separation of *N*-Ac-D-Phe-L-Phe-[1,2-^{14}C]-Gly (**30**) and *N*-Ac-D-Phe-L-Phe-α-[1,2-^{14}C]-hydroxyGly (**42A** and **42B**)	54
8.	Comparison of the Physical Appearance, α-H Chemical Shift, and Inhibition Properties of **89A**, **89B**, **90A**, **90B**, **78** and **80**	110
9.	Summary of Inhibition Studies	116
10.	Comparison of the ^{1}H NMR Data, Relative HPLC Retention Times, and Inhibition Properties of **78**, **107**, **80**, and **106**	121

List of Figures

Figure		Page
1.	Other Monooxygenase Reactions Catalyzed by PAM	10
2.	Possible Mechanisms of PAM Oxidation	11
3.	PAM Catalyzed Oxidation of Hydrazone	12
4.	The Capture of Glyoxylic Acid by Nitrosobenzene	13
5.	The *pro-S* Hydrogen Is Stereospecifically Removed During PAM Oxidation	13
6.	Copper-mediated α-Hydroxylation of *N*-Salicyloylglycine	14
7.	Proposed Mechanism of Sulfoxidation Catalyzed by PAM	15
8.	Two Step Reaction Mechanism of PAM	18
9.	Synthesis of *p*-Bromophenacyl Glycolate	38
10.	Synthesis of *N*-BOC-*O*-TBDMS-D-Tyr-L-Val-Glycolic Acid *p*-Bromophenylacyl Ester	38
11.	Synthesis of *N*-Dansyl-Tyr-Val-α-HydroxyGly	50
12.	HPLC Separation of the Two Isomers of **42** by Using Two Aminopropyl Columns	53
13.	HPLC Separation of the Enzyme Assay Solution after Dilution by *N*-Acetyl-D-Phenylalanyl-L-Phenylalanyl-α-Hydroxyglycine	55
14.	Seebach's Method for β-Hydroxy Amino Acids	59
15.	The Formation of *N*-Salicyloyl-α-MethoxyGly *via* *N*-Acyl Imine Intermediate	66
16.	Proposed Inactivation Mechanism of PHM by *trans*-4-Phenyl-3-Butenoic Acid	70
17.	Kinetic Resolution of Racemic Ethyl 2-Hydroxy-4-Phenylbutenoate by lipase from *P. Fluorescens*	71

18. Positive Ion FAB Mass Spectra of α-HydroxyGly Intermediate 77
19. The Formation of a Radical During Direct Hydroxylation 79
20. Mechanism of Ester Deprotection by Trimethylsilyl Iodide 83
21. Reverse-phase HPLC Separation of **78** and **80** 88
22. Mechanism for the Formation of Thioglycine Derivatives 93
23. Mechanism for Nitrosation of the Amide Nitrogen 99
24. A Typical Plot of Competitive Inhibition Assay 106
25. A Typical Plot of Preincubation Studies 107

List of Abbreviations

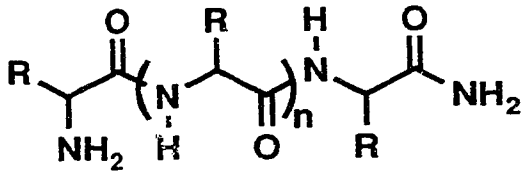
Ac	acetyl
Bn	benzyl
BOC	*tert*-butyloxycarbonyl
n-BuLi	*n*-butyl lithium
CI	chemical ionization
Cbz	benzyloxycarbonyl
dansyl	5-dimethylamino-1-naphthalene-sulfonyl
DBH	dopamine β-hydroxylase
DCC	1,3-dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
dpm	decompositions per minute
DPPA	diphenyl phosphoryl azide
EDTA	ethylenediaminetetraacetic acid
EI	electron impact ionization
Enz	enzyme
Et	ethyl
FAB	fast atom bombardment
HPLC	high performance liquid chromatography
HONB	*N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide
IC_{50}	concentration at 50% inhibition
IR	infrared spectroscopy
K_M	Michaelis constant

LDA	lithium diisopropylamide
Me	methyl
MeOH	methanol
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
NHF	*N*-hydroxyformanilide
NMR	nuclear magnetic resonance
n. O. e.	nuclear Overhauser enhancement
PBP	*p*-bromophenacyl
PAL	peptidyl-α-hydroxyglycine α-amidating lyase
PAM	peptidylglycine α-amidating monooxygenase
PHM	peptidylglycine α-hydroxylating monooxygenase
Ph	phenyl
R_t	retention time
TBDMS	*tert*-butyldimethylsilyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
Ts	*p*-toluenesulfonyl
V_{max}	maximal velocity

CHAPTER 1 INTRODUCTION

General Background

A diverse group of naturally occurring peptides have carboxyl termini with a primary amide functionality ("peptide amides"). They are widely distributed in the animal kingdom, from invertebrates to mammals, but have not been discovered in unicellular organisms (such as bacteria or algae), nor in higher plants.[1] Peptide amides occur primarily among neural and hormonal peptides.[2] Some examples of the numerous peptide amides in mammalian species are gastrin,[3] α-melanocyte stimulating hormone (MSH),[4] galanin,[5] calcitonin,[6] substance P,[7] vasoactive intestinal peptide,[8] and thyrotropin-releasing hormone.[4] Peptide amides such as melittin[9] and apamin[10] from bee venom, eledoisin from an octopus,[11] cecropins from moths,[12] toxin II from scorpion venom,[13] conotoxins from a marine snail,[14] and pheromone biosynthesis activating neuropeptide (PBAN) from the corn earworm[15] and the silkmoth[16] are produced by invertebrates. The sizes of these peptides vary from three amino acids for thyrotropin-releasing hormone[4] to 64 amino acids for scorpion toxin II.[13]



a peptide amide

These peptides have quite different physiological roles. Calcitonin is employed in calcium regulation.[6] The gastrins have potent stimulation of gastric acid secretion and may be implicated in certain types of stomach ulcers.[3] Oxytocin is used to induce uterine contractions and to stimulate lactation in clinic.[17] Substance P is a neurotransmitter which

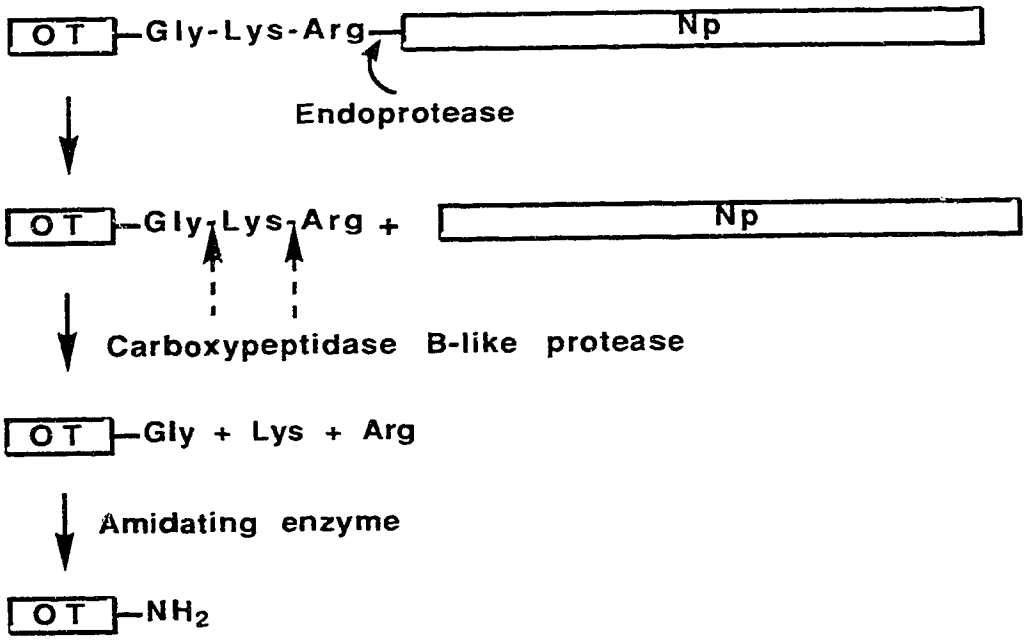
may be involved in the transmission of pain[7] and vasopressin is used as an antidiuretic.[17] Galanin demonstrates strong stimulation of food intake in a dose-dependent manner.[18] PBAN stimulates the production of insect sex pheromones which serve as attractants to allure mates.[19] A few isolated peptide amides appear to have more than one physiological role.[20]

Biosynthesis and Formation of Peptide Amides

Like most biologically active peptides, peptide amides are biosynthesized from large precursor proteins which are cleaved to proproteins immediately after synthesis in the rough endoplasmic reticulum.[21,22] The smaller, biologically active peptides are contained in these proteins or prohormones which are themselves not active.[23] The prohormones are transported to the Golgi apparatus and then packaged in secretory granules.[21] These granules contain the necessary enzymatic machinery for cleavage of the prohormone to the active peptides and for any further processing of the peptides such as acetylation, amidation, sulfation, methylation, phosphorylation, or glycosylation. The cleavage of the prohormone occurs almost invariably at pairs of basic amino acids, usually lysine and arginine, although not all pairs of these amino acids are cleaved.[23] This is strong evidence that the three dimensional structure of the prohormone is critical in the recognition of cleavage sites.[22] The processing of precursors at single arginine residues has been discovered in a few cases.[24]

A specific example is given to illustrate the formation of the nonapeptide oxytocin (OT) from its precursor.[25] OT is produced both in the hypothalamus and in the ovarian corpus luteum. It is derived from an 11 kDa common precursor with neurophysin I (Np) (in the cow, the oxytocin associated neurophysin). In this proform the nonapeptide sequence is at the NH_2-terminus and a "restriction sequence" $Gly^{10}Lys^{11}Arg^{12}$ separates the OT domain from the carboxyl-terminal neurophysin. An endoprotease produces a single cleavage at the peptide bond situated immediately after the $Lys^{11}Arg^{12}$ doublet of a

synthetic substrate, pro OT/Np, which reproduces the entire NH_2-terminal domain of the oxytocin precursor. A carboxypeptidase B-like activity removes the extra LysArg doublet on the carboxyl terminus of C-terminally extended OT, i.e., OTGlyLysArg, to give glycine extended OT. An amidating enzyme then converts this OTGly to the peptide amide OT-NH_2.



In all the cases studied so far the peptide amide is produced from the oxidative cleavage of a terminal glycine residue.[23] Table 1 lists some selected peptide amides and the amino acid sequence of part of their precursor proteins. Of particular interest is the common characteristic of a glycine (bold) followed by two basic amino acids (underlined) used for selective cleavage and amidation. The presence of a primary amide at the carboxyl terminus is essential for full biological activity.[23] For example, the free acid form of gastrin and cholecystokinin are completely inactive,[26] and the amide form of lutenizing hormone-releasing factor is 1000 times more active than the acid.[27] This single transformation is therefore of great importance to produce active peptide hormones. The

recognition of the primary amide group of oxytocin and vasopressin[28,29,30] was probably the first example of posttranslational modification of peptides to be discovered.[1]

Table 1. Partial Amino Acid Sequences of Peptide Amide Precursors.

Peptide	Precursor
Hinge peptide	-Pro-Arg-**Gly**-Lys-Arg-Ser-Tyr-
Calcitonin	-Gly-Ala-Pro-**Gly**-Lys-Lys-Arg-Asp-
Vasopressin	-Pro-Arg-Gly-**Gly**-Lys-Arg-Ala-Met-
α-MSH	-Lys-Pro-Val-**Gly**-Lys-Lys-Arg-Arg-
Gastrin	-Met-Asp-Phe-**Gly**-Arg-Arg-Ser-Ala-

Isolation and Purification of Amidating Enzyme

In 1982 Bradbury *et al.*[31] demonstrated the presence of enzymatic activity in porcine pituitary capable of converting a synthetic tripeptide to the corresponding dipeptide amide. The pituitary was chosen because the amidated hormones oxytocin, vasopressin,

and α-MSH occur in this tissue.[32] The authors chose D-tyrosyl-L-valyl-glycine (D-Tyr-L-Val-Gly) as the peptide for three reasons: first, the unnatural D configuration of the tyrosine should prevent enzymatic hydrolysis; second, the aromatic tyrosyl residue is suitable for radioiodination; and third, the L-valyl-glycine (L-Val-Gly) was thought to be the minimal structural requirement to mimic the precursor to α-MSH which ends L-Val-Gly. Therefore, by radioiodinating the tripeptide and incubating this with tissue preparations they were able to separate a product dipeptide amide and confirm that the

structure was D-tyrosyl-L-valinamide. They also demonstrated that the nitrogen atom of the amide is derived from the nitrogen of a mandatory carboxyl-terminal glycine residue. It does not originate from ammonia as might have been expected, either by direct addition which occurs in the formation of glutamine from glutamic acid,[33] or by ammonolysis of the peptide bond that links the glycine to the reminder of the peptide chain.[34] This surprising finding was demonstrated by observing that when a peptide with [^{15}N]-glycine at its carboxyl terminus was converted to the corresponding peptide amide residue, the product retained the ^{15}N-label. Though no experimental details were given, the other product of the reaction was claimed to be glyoxylic acid.

Since this initial work, amidating activity has been detected and purified in a variety of tissues from many organisms. In humans it has been found in plasma, serum,[35] cerebrospinal fluid and central nervous system tissue.[36] It has been detected and purified from neurointermediate pituitaries in cows[37] and pigs,[38] and also from heart antrum in pigs.[39] In sheep the enzymatic activity has been detected in the brain and pituitary.[40] In rats the enzymatic activity has been found in tissue and serum,[41] in the pancreas of neonates,[42] in the hypothalamus,[43] in the anterior pituitary,[44] in sciatic nerves,[45] in medullary thyroid carcinomas,[46] in the brain and small intestine,[47] and in exocrine[48] and pituitary[49] secretion granules. The tissue distribution of the enzyme has been studied for the rat; of the 24 different tissues examined, only the thymus and liver had no detectable activity, whereas the heart atrium and the pituitary showed the highest activity.[50] Amidating enzyme has been detected and purified from the skin of the frog, *Xenopus laevis*.[51, 52] It has also been observed and purified in several cell lines grown in culture.[53,54,55,56,57,58]

Several procedures have been employed to purify amidating enzymes to homogeneity from different organisms. The amidating enzymes have molecular weights varying from as small as 36 kDa[47] to as large as 113 kDa.[59] The amidating enzyme having a molecular mass of 36 kDa exists in soluble form, and the one with a molecular mass of

113 kDa exists in a membrane-bound form. Depending on the source, molecular mass of the amidating enzyme is found either in the ~40 kDa region, ~70 kDa region or ~100 kDa region. In the case of the enzyme obtained from *Xenopus*,[52] a complete amino acid sequence was subsequently derived from the nucleotide sequence which allowed the molecular weight (40.5 kDa) to be assigned precisely.[62]

Amidating enzyme from porcine pituitary was reported to have a MW of 64 kDa and the enzyme appears to be glycosylated since it is retained on a Concanavalin-Sepharose column.[38] However, after extensive purification from porcine[60] or bovine[37] pituitary, the enzyme exhibited a MW in the region of 40 kDa and was found to be devoid of carbohydrate. When amidating activity is extracted from porcine pituitary by aqueous buffers and the extracts are examined for the presence of glycosylated enzyme by chromatography on Concanavalin-Sepharose, 80 percent of the activity is not retained on the column; the 20 percent that is retained can be associated with a glycosylated molecule but the bulk of the activity is present in molecules that lack carbohydrate.[60] Both the carbohydrate-free and carbohydrate-containing forms of the amidating enzyme appear to exist in multiple molecular species. These different forms can be resolved by gel filtration chromatography, exhibiting apparent molecular weights which range from 40 kDa to approximately 70 kDa.[60]

Complementary DNAs encoding amidating enzymes have been cloned from bovine pituitary,[61] frog skin,[62] human thyroid carcinoma,[63] and rat atrium,[64,65] and most code for an approximately 100-kDa protein. It has therefore been suggested that the amidating enzyme is transcribed as a 100-kDa protein and the smaller, soluble forms of the enzyme arise from posttranslational proteolysis.[61] The different types of amidating enzyme found in human and rat tissues and the analysis of the genomic DNA confirm the hypothesis that different forms of amidating enzyme are generated by alternative splicing. It is intriguing to speculate that multiple forms of amidating enzymes are necessary for the recognition of the different peptide substrates or for the regulation of this process.

Cofactors and pH Optimum of Amidating Enzyme

The amidating reaction is blocked by removal of oxygen and stimulated by pure O_2.[66] The reaction is also stimulated by the presence of a reduced cofactor, and ascorbic acid has usually been found to be the optimal reductant. As for other ascorbate-catalyzed reactions, catalase must be present during the assay to prevent enzyme inactivation by hydrogen peroxide produced during the reaction.[37,65,67] Even with purified enzyme, the reaction is not absolutely dependent on exogenous cofactors.[37,49,51,52,68] For the purified bovine pituitary enzyme, the amount of ascorbate consumed in the reaction is nearly equimolar with the amount of amidated peptide produced;[68] the ascorbate consumed may be oxidized to dehydroascorbate or semidehydroascorbate, as is the case for dopamine β-hydroxylase (DBH).[69,70] The properties outlined above lead to the designation of this enzyme as peptidyl glycine α-amidating monooxygenase or PAM.[71]

The reaction is blocked by divalent metal ion chelators such as EDTA, 1,10-phenanthroline and diethyldithiocarbamate. Many studies show that only cupric ion restores full enzymatic activity, but there are conflicting reports on the same tissues that Ni^{2+}, Co^{2+}, Mn^{2+}, or Fe^{3+} enhance activity.[71] A possible explanation is that impure enzyme preparations may contain copper associated with the contaminating proteins, and displacement of this by other metal ions may provide sufficient copper for amidation. Copper concentrations in excess of an optimum value lead to a decrease in amidating activity, and this can cause difficulty in assaying the enzyme at different stages of purification.[60]

The study of the pH optimum of the α-amidating enzyme has aroused a certain amount of controversy. For example, early reports on the crude enzymes from porcine[72] and bovine[73] pituitaries indicated values of 6.5-7.5 for their pH optima, but a highly purified preparation from bovine pituitary (PAM B) exhibited maximum activity at pH 9.0-9.5 while another component (PAM A) was most active at pH 8.5.[37] For purified porcine pituitary enzyme a maximum activity at pH 7.0 was reported, but a similar level of activity

was also seen at pH 8.0.[38] Matsuo's group reported a nearly neutral pH optimum (6.5-7.5) for the amidating enzymes from a various rat tissues.[50] On the other hand, a high level of α-amidating activity at an alkaline pH (8.0-9.5), often seen as another pH optimum peak in addition to the neutral one, has been observed with enzymes from rat hypothalamus, submaxillary gland, and serum.[41,43] A similar pH dependence has recently been reported for sheep brain and pituitary enzymes.[40] Noguchi and co-workers found that the enzymes from rat brain, pituitary, and small intestine show such a pH profile with one optimum at pH 6.5-7.0 and another at alkaline pH (8.5-9.0) with any one of the three substrates, D-Tyr-L-Leu-Asn-Gly, D-Tyr-Asn-Gly or D-Try-Val-Gly.[47] With the combination of ion-exchange and gel filtration chromatography, they obtained two fractions from these enzymes, designated S-1 and S-2. The S-1 fraction contained an α-amidating enzyme with an apparent molecular weight of 36 kDa that exhibited a single pH optimum at 8.5. The S-2 fraction contained a protein with a molecular weight of 41 kDa which apparently showed almost no or only marginal activity at either pH 7 or pH 8.5, but when S-2 was combined with S-1, a neutral pH optimum at 7 could be elicited.

Substrate Specificity of Amidating Enzyme

The most characteristic feature of the specificity of PAM is the mandatory requirement for a C-terminal glycine residue.[60] The reaction does not proceed when the C-terminal Gly is replaced by L-Lys, L-Glu, L-Leu, L-Ala, D-Ser, or D-Leu. The reaction proceeds at a reduced rate with D-Ala at the C-terminus,[74] indicating that the orientation of the substituent attached to the α-carbon of the terminal residue is important and that only one of the two α-carbon hydrogen bonds is involved in the amidation reaction. The reaction does not proceed with D-Tyr-Val-Gly-Lys or D-Tyr-Val-*N*-methyl-Gly as substrates.[31]

The nature of the residue in the penultimate position of a substrate also has considerable influence on the rate of amidation.[72] For example, hydrophobic residues,

such as phenylalanine or valine, provide active substrates for amidating enzyme from pig pituitaries whereas charged residues, such as aspartic acid or arginine, are much less reactive. Although glycine and proline amides occur at the C-terminus of several hormones, precursors terminating in Gly-Gly or Pro-Gly are not among the best substrates for PAM.

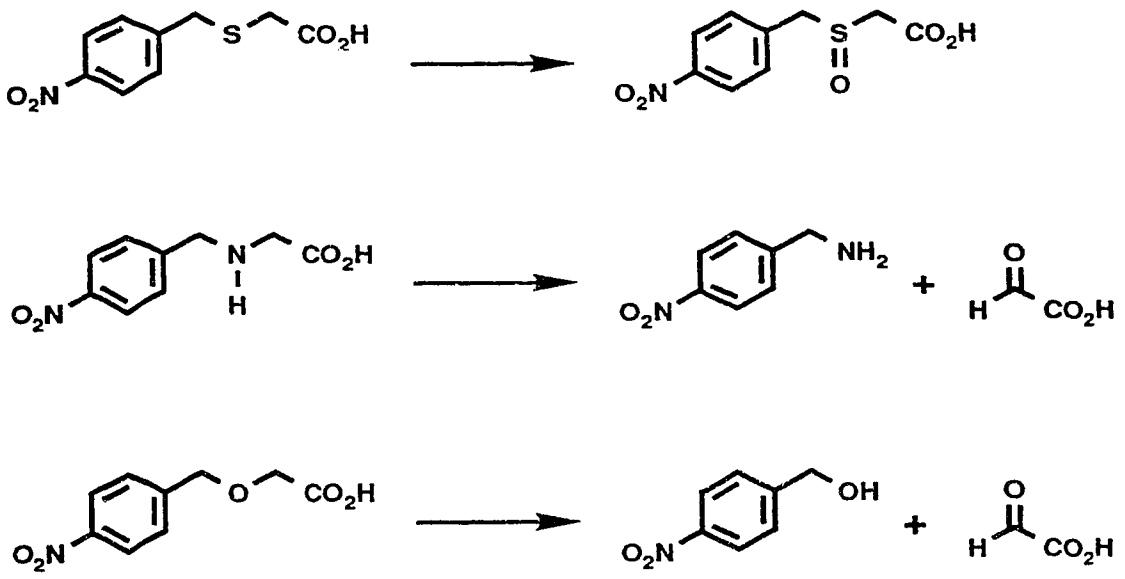
Recently, a more detailed study was carried out by Tamburini and co-workers.[75,76] A peptidyl α-amidating enzyme was partially purified from conditioned medium derived from cultured medullary thyroid CA-77 cells. The interaction of this enzyme with a series of tripeptides, pentapeptides, and mature glycine-extended prohormones was studied. If the amino acid at the penultimate position of the tripeptide is substituted with each of the 20 common L-amino acids, the binding affinity decreases through the following side chain groupings: sulfur containing > aromatic > histidine > nonpolar > glycine > charged. The structural elements important for enzyme recognition are located entirely within the C-terminal pentapeptide segment since the affinities for selected prohormones and their pentapeptide mimics are equal.

PAM can also accept a variety of non-peptides as substrates.[77] Several phenylhydrazones and semicarbazones of glyoxylic acid exhibit greater binding affinities than the tripeptide D-Tyr-Val-Gly, suggesting that the disposition of the carbon, hydrogen, and nitrogen atoms in the imino structure of the hydrazone can be more acceptable to the amidating enzyme than the relatively flexible arrangement of these atoms in a C-terminal glycine residue. This is supported by the strong affinity of *trans*-4-phenyl-3-butenoic acid (K_I = 1 μM).[78,79] PAM also catalyzes the transformation of hippuric acid and several ring-substituted derivatives to the corresponding benzamides and glyoxylic acid, with the best substrate of this class being 4-nitrohippuric acid.[79] These compounds are the smallest amide substrates yet reported for PAM, and thus show that the minimal structure of an acylglycine is required for amidation.

PAM also catalyzes three other monooxygenase reactions: sulfoxidation, amine

N-dealkylation and *O*-dealkylation.[79] Thus, (4-nitrobenzyl)thioacetic acid was converted to the analogous sulfoxide, *N*-(4-nitrobenzyl)glycine was converted to 4-nitrobenzylamine and glyoxylate, and [(4-nitrobenzyl)oxy]acetic acid was converted to 4-nitrobenzyl alcohol and glyoxylate (Figure 1). These reactions display the characteristics expected for the normal PAM-catalyzed pathway.

Figure 1. Other Monooxygenase Reactions Catalyzed by PAM



Enzyme Assays

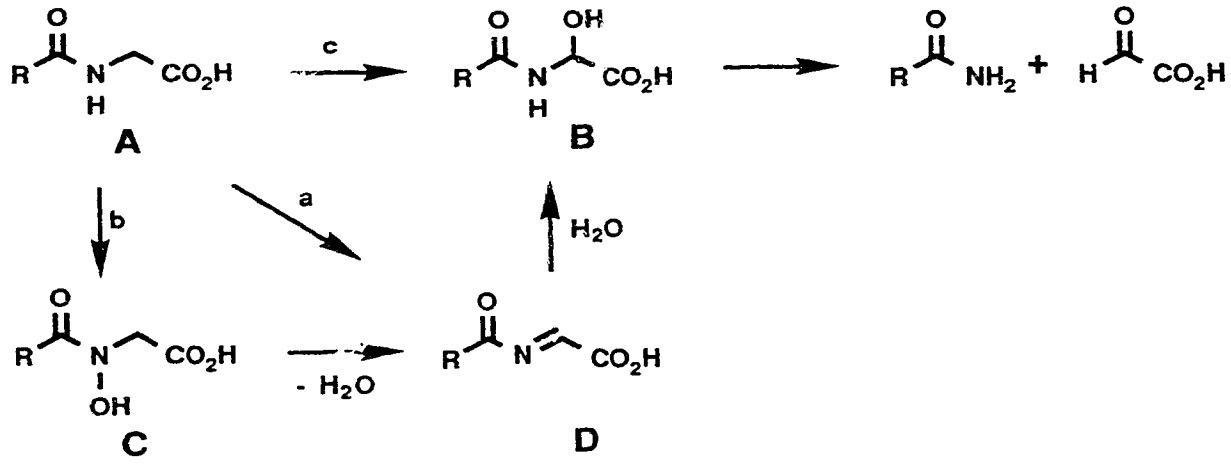
There are several published assays for this enzyme. The first assay published by Bradbury *et al.*[31] employing radioiodinated D-Tyr-Val-Gly has been widely used. A modification of this assay uses radioiodinated *N*-Ac-D-Tyr-Phe-Gly which facilitates separation of the starting peptides from the product amide.[52] Another method uses a radioimmunoassay to selectively detect the D-Tyr-Val-NH_2.[80] A reverse-phase high-performance liquid chromatographic separation and fluorometric detection assay using

N-dansyl-Tyr-Val-Gly was also reported.[81] These assays all have the limitation that they are suitable only for a specific amide product. Two radioactive assays have been developed in our group which are based upon the detection of radioactive glyoxylate derived from D-Tyr-L-Val-[1,2-^{14}C]-Gly[82] and D-Phe-L-Phe-[1,2-^{14}C]-Gly.[83] These assays should be readily adaptable to analogous systems since they are based on the common product of all peptide amidating enzymes, glyoxylate.

Mechanism of α-Amidation

The exact mechanism of the reaction is still not fully understood. The first mechanism suggested for PAM begins with dehydrogenation of the glycine-bearing peptide **A** to an *N*-acylimine **D** (pathway a in Figure 2).[31] This would spontaneously add water to form **B**, which would cleave to the peptide amide and glyoxylate. A second possibility

Figure 2. Possible Mechanisms of PAM Oxidation



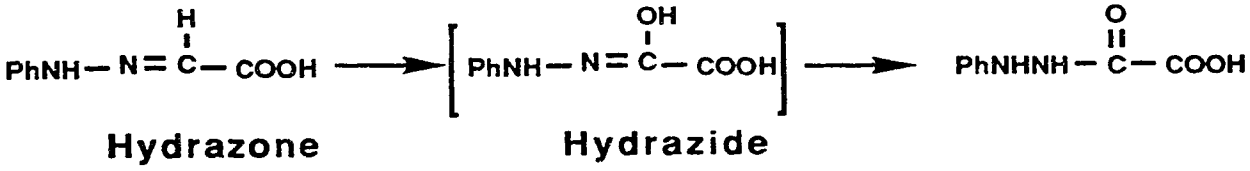
involves hydroxylation of carbon to give **B** directly (c in Figure 2), in analogy to dopamine β-hydroxylase,[66,77] which also requires copper, oxygen, and ascorbate. Although perhaps less likely, a third route may be *N*-hydroxylation to generate **C** followed by

transformation to **C** and **B** (b in Figure 2). A chemical precedent for this sequence exists in the oxidation of *N*-aroylglycines with lead tetraacetate.[84]

Kizer and co-workers demonstrated the non-enzymatic conversion of peptides to amides under conditions which closely resemble those required by PAM.[85] They found that certain peptides, when treated with copper and ascorbic acid at pH 6.0, are oxidatively cleaved to the amides and a carbonyl compound. However, there are several differences between the enzymatic and chemical oxidation. In the cases of non-enzymatic conversion the terminal residue can be glycine, alanine, γ-aminobutyric acid, butylamine, or δ-aminovaleric acid, while the amidating enzyme only accepts peptides with a glycine terminus. The tripeptide D-Tyr-Val-Gly was not converted to the corresponding amide under the model conditions. Similar primary α-isotope effects of deuterium substitution on the methylene carbon of the terminal glycine were observed for the model and enzymatic systems (k_H/k_D = 1.9 and 2.2, respectively). On the basis of the non-enzymatic oxidation these authors favored the direct cleavage of a carbon hydroxylated intermediate (path c in Figure 2) without the formation of an imine (path a or b in Figure 2), and proposed a radical based mechanism for the oxidation.[85]

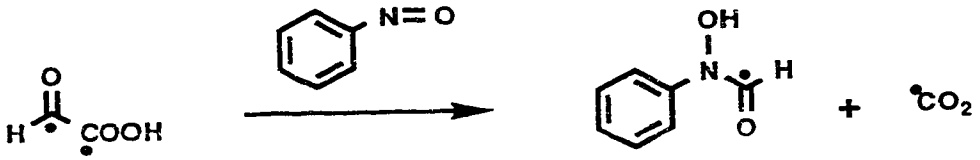
In 1987, Bradbury and Smyth reported that PAM could accept the phenylhydrazone of glyoxylic acid as a substrate, catalyzing its oxidation to oxalic acid phenylhydrazide (Figure 3).[77] The hydrazide differs from the hydrazone only in the replacement of a hydrogen by a hydroxyl group, which suggests that the reaction occurs by oxygenation and not by dehydrogenation.

Figure 3. PAM Catalyzed Oxidation of Hydrazone



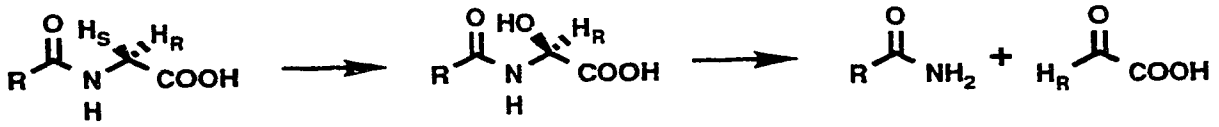
In order to determine the stereochemistry of hydrogen removal from the glycine residue of substrate, samples of glycines bearing stereospecific tritium labels in the *pro-R* and *pro-S* positions were synthesized by Dr. S. E. Ramer (University of Alberta), and (*RS*)-[2-$^{3}H_1$]-glycine was prepared by sodium boro-[^{3}H]-hydride reduction of glyoxylate in aqueous ammonia.[82] The (*R*)-, (*S*)-, and (*RS*)-[2-$^{3}H_1$]-glycines were individually mixed with [1,2-$^{14}C_2$]-glycine and then transformed to the corresponding D-tyrosyl-L-valyl-glycines respectively.[82] These doubly labeled tripeptides were exposed to the purified PAM enzyme from porcine pituitary, the resulting radioactive glyoxylates were captured by the nitrosobenzene as illustrated in Figure 4, and the $^{3}H/^{14}C$ ratios of the *N*-hydroxyformanilides (NHF) were compared to those of the starting tripeptides.

Figure 4. The Capture of Glyoxylic Acid by Nitrosobenzene



The results clearly demonstrate that PAM stereospecifically removes the *pro-S* hydrogen of the glycine residue during catalysis (Figure 5). Only when the *pro-R* hydrogen was stereospecifically labeled with ^{3}H was the label retained in glyoxylate.

Figure 5. The *pro-S* Hydrogen Is Stereospecifically Removed During PAM Oxidation

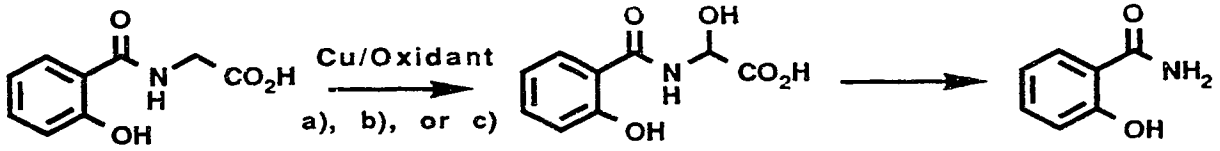


Recently, evidence for formation of the proposed hydroxylated intermediate (B in Figure 2) has been provided by Young and Tamburini[86] and the structure (without stereochemistry) was established by Tajima and co-workers.[87]

Young and Tamburini[86] also prepared *N*-dansyl-Tyr-Val-Gly and its *N*-hydroxyglycine derivative. They found that *N*-dansyl-Tyr-Val-Gly is a good substrate, but that the *N*-hydroxyglycine derivative is not converted by the α-amidating enzyme. This result disfavors the *N*-hydroxylation pathway.

The direct hydroxylation is also supported by a recent model study with α-hydroxylation of *N*-salicyloyl-glycine[88] to *N*-salicyloyl-α-hydroxyglycine in non-aqueous systems using three different copper-containing oxidant systems (Figure 6). The product carbinolamide appears to be the formed alone at the beginning of the reaction, and the corresponding amide results from its slow subsequent cleavage.

Figure 6. Copper-mediated α-Hydroxylation of *N*-Salicyloylglycine

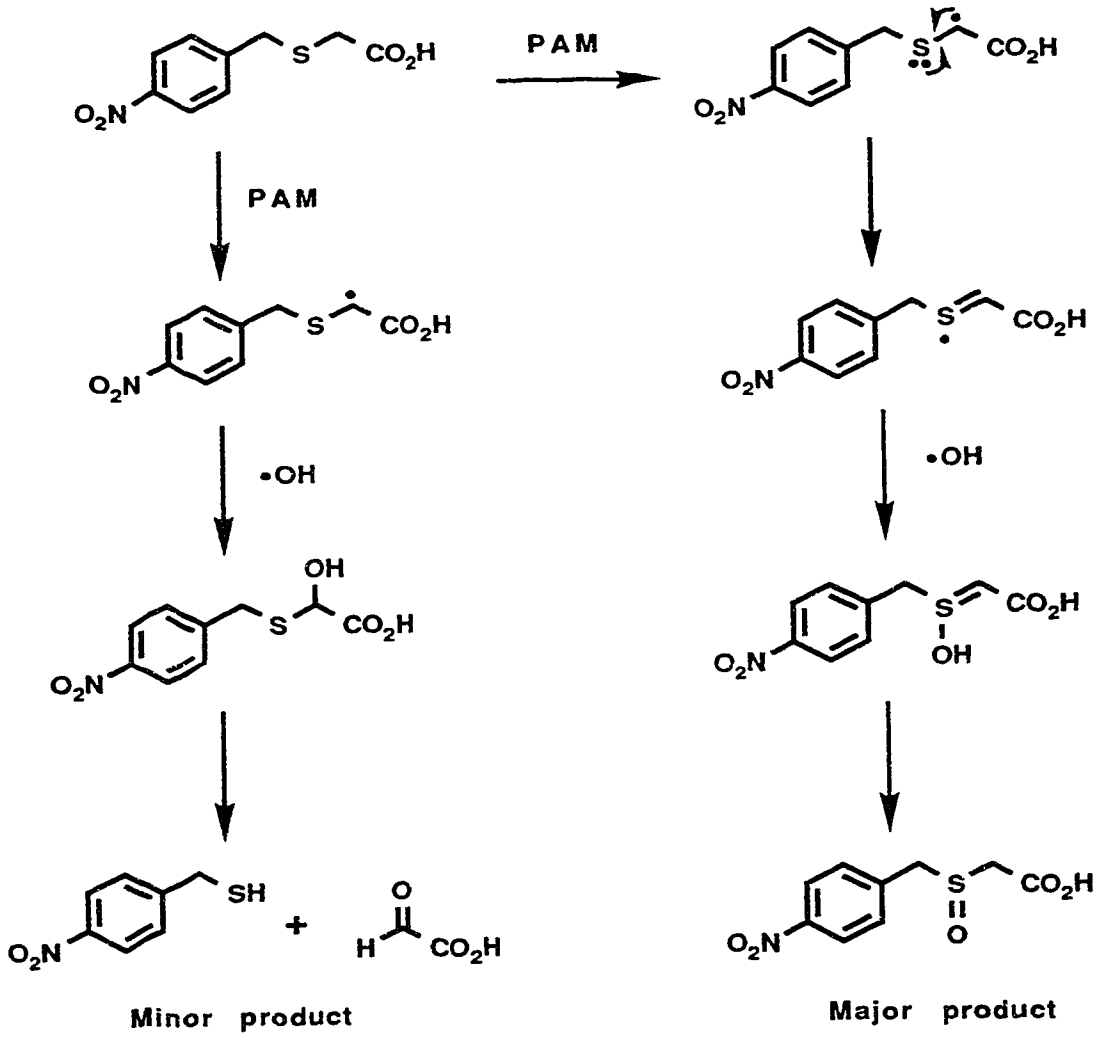


a). Copper turnings, O_2, pyridine, 65 °C, 4 h. b). Copper peroxide, pyridine, 60 °C, 2 h. c). Cu(II) salt of *N*-salicyloyl-glycine, trimethylamine oxide, CH_3CN, 65 °C, 20 h.

Sulfoxidation is another facile monooxygenase reaction which has been demonstrated with DBH,[89] *Pseudomonas oleovorans* monooxygenase,[90] P_{450} enzyme,[91] and flavin monooxygenase.[92] As described before, the (4-nitrobenzyl)thioacetic acid is an excellent substrate for PAM, and the V_{max} for sulfoxidation is comparable to that of D-Tyr-Val-Gly.[79] Since the sulfur cation radical has a strong tendency toward sulfoxidation,[93,94] it seems probable that the competing pathway for S-dealkylation entails initial oxygenative

attack at the α-carbon leading to an α-hydroxy sulfide, which then breaks down to the thiol and glyoxylate (Figure 7).[79]

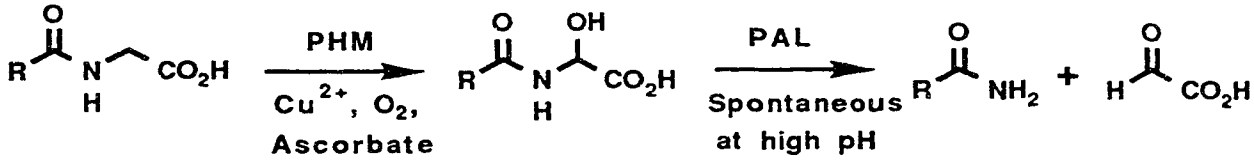
Figure 7. Proposed Mechanism of Sulfoxidation Catalyzed by PAM



The reactions of PAM with amine and ether analogues are directly related to the amidation reaction.[79] The obvious difference between amine and amide PAM substrates is that, at physiological pH, alkylamines are protonated and this may lead to poor binding at the active site and poor reactivity toward the electrophilic copper-oxygen-activated species. Consistent with this notion, the pH optimum of amine substrate is higher than other substrates.

The second stage in the amidation reaction involves conversion of the hydroxyglycine intermediate to the peptide amide and glyoxylic acid. This takes place without enzymic assistance at acid or alkaline pH, but appears to occur relatively slowly at physiological pH. May[95] and Noguchi[96] independently discovered that a second co-occurring enzyme from bovine neurointermediate pituitary[95] and rat brain[96] catalyzes dealkylation of α-hydroxyglycine derivatives. This enzyme has subsequently been found and purified from several other tissues.[97,98,99] Hence the production of α-amidated peptides from their glycine extended precursors can be a two-step process catalyzed by two enzymes, peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) (Figure 8),[97] respectively.

Figure 8. Two Step Reaction Mechanism of PAM



It was reported that the cDNA encoding a 108 kDa amidating enzyme precursor protein in the bovine neurointermediate pituitary is endoproteolytically cleaved to form PHM (38 kDa) and PAL (43 kDa).[97] The primary sequence data of PAL from bovine pituitaries established that it is part of the putative precursor which also contains PHM,[100]

and a soluble 75-kDa enzyme purified from MTC cells appears to retain both PHM and PAL activities since it catalyzes the conversion of glycine-extended peptides to peptide amides.[86,101] Therefore the intact precursor continues to be described as peptidylglycine α-amidating monooxygenase (PAM).

The reports of unusual pH optima for peptide α-amidation probably reflect the peptide substrate used, the tissue selected, and the degree of enzyme purity achieved. Experimental results may represent a 'compromise' between the optimum pH of PHM (6.5-7.0) and the higher pH necessary for non-enzymic breakdown of the hydroxylated intermediate. The non-enzymic breakdown may also be accelerated by copper ion or at higher temperature.[86] The 41-kDa protein that co-occurs with the amidating enzyme in rat brain but is devoid of amidating activity is in fact the PAL.[74,96] The purpose of PAL may be not only to catalyze formation of the amide product, but also to ensure that the release of glyoxylate occurs at a site in the cell where such a reactive molecule would be relatively harmless.[32]

The PAL enzyme purified by May *et al.* has an apparent molecular mass of 45 kDa, a K_M value of 0.44 mM for α-hydroxybenzoylglycine and a pH optimum at 6.6.[95] Very recently, Eipper and co-workers identified a 50-kDa protein as the major form of PAL in bovine neurointermediate pituitary granules.[102] With *N*-acetyl-Tyr-Val-α-hydroxyglycine as the substrate, PAL exhibits a pH optimum of 5.0; enzymatic activity is inhibited by high concentrations of salt but is relatively resistant to thiol reagents and urea. PAL activity is inhibited by EDTA and restored by a number of divalent metals, including Cd^{2+}, Cu^{2+}, Zn^{2+}, and Ca^{2+}. Kinetic studies using *N*-acetyl-Tyr-Val-α-hydroxyglycine indicate that PAL has a K_M of 38 μM and a turnover number of 220/s. In general, these properties of PAL are well suited to its functioning in the secretory granule environment, which is acidic and relatively low ionic strength.[103,104]

Table 2 gives a comparison of the properties for PAM, PHM, and PAL.

Table 2. Comparison of Properties of PAM, PHM, and PAL

	PAM	PHM	PAL
MW	~100 kDa	~40 kDa	~50 kDa
pH Optimum	6.5-7.0	8.5-9.0	5.0, 6.6
Cofactors	Copper, O_2, Ascorbate	Copper, O_2, Ascorbate	Copper
Inhibited	By divalent metal ion chelators	By divalent metal ion chelators	By high salt concentrations

Inhibition of PHM

Carboxylic acids such as *trans*-4-phenyl-3-butenoic acid[78, 79] and *trans*-benzoylacrylic acid[79] are mechanism-based inactivators of PHM, with inhibition constants (K_I) of 1 μM and 0.16 mM, respectively. With *trans*-4-phenyl-3-[2-^{14}C]-butenoic acid, the radioactivity is incorporated into protein that co-chromatographs with active enzyme, thereby suggesting the covalent reaction with PHM.[78] Inactivation may involve the removal of the α-hydrogen of the carboxylic acid by PHM and formation of a conjugated radical. By comparing the inhibition ability of various carboxylic acids such as pentanoate, 3-butenoate, *trans*-4-phenyl-3-butenoic acid, it is clear that the inhibitory properties of a carboxylic acid are increased if the molecule contains a phenyl group.[78] This agrees with the apparent preference for hydrophobic substrates, and requirement for a carboxylic acid.

Incubation of rat thyroid carcinoma CA77 cells in the presence of *trans*-4-phenyl-3-butenoic acid leads to a decrease in the levels of intracellular amidating activity and of thyrotropin-releasing hormone, an amidated peptide produced by these cells. The inhibitory effects reach a maximum at approximately 15 h, after which the enzyme levels return to the control values even though the concentration of *trans*-4-phenyl-3-butenoic acid

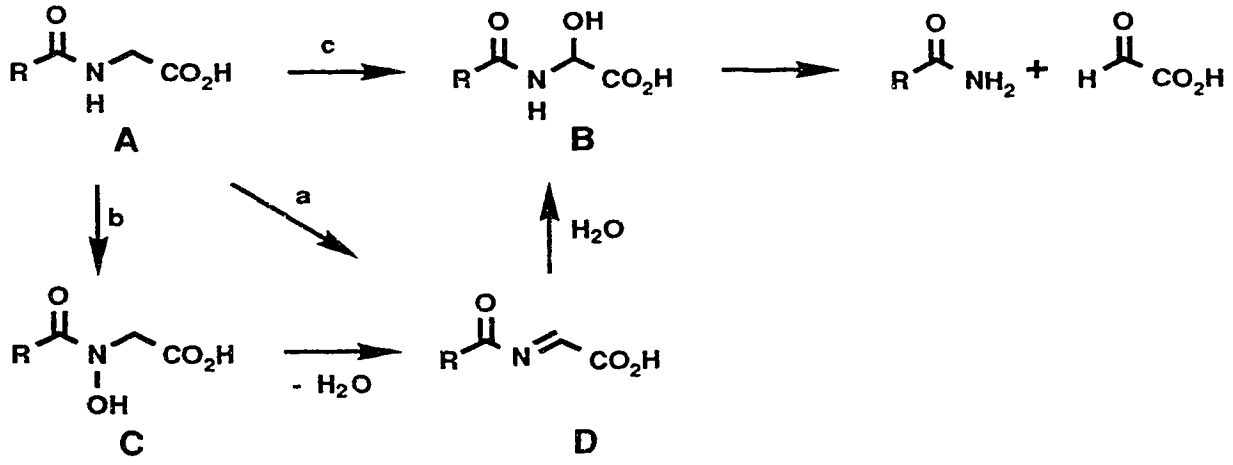
in the cells remained unchanged. The results indicate that a mechanism exists in these cells for regulation of amidating activity.[78]

The major goals of this project are to determine the exact chemical mechanism of PHM oxidation and to design and construct suicide substrates and potent competitive inhibitors which may be useful tools for biological studies in endocrinology and may have medicinal applications.

CHAPTER 2 STUDIES ON THE MECHANISM OF PEPTIDYL-GLYCINE α-HYDROXYLATING MONOOXYGENASE

One of the primary goals of the present research presented is to define the exact chemical mechanism of this enzyme catalyzed reaction. This involves determing the detailed structure of reaction intermediates, the oxygen source for hydroxyglycine peptide and ultimately, among the possibilities of *N*-hydroxylation (pathway b), dehydrogenation (pathway a) and direct *C*-hydroxylation (pathway c), which is the enzyme oxidation pathway (Figure 2). First of all, the syntheses of substrates are described.

Figure 2. Possible Mechanisms of PHM Oxidation



2.1 SYNTHESES OF THE MODEL SUBSTRATES

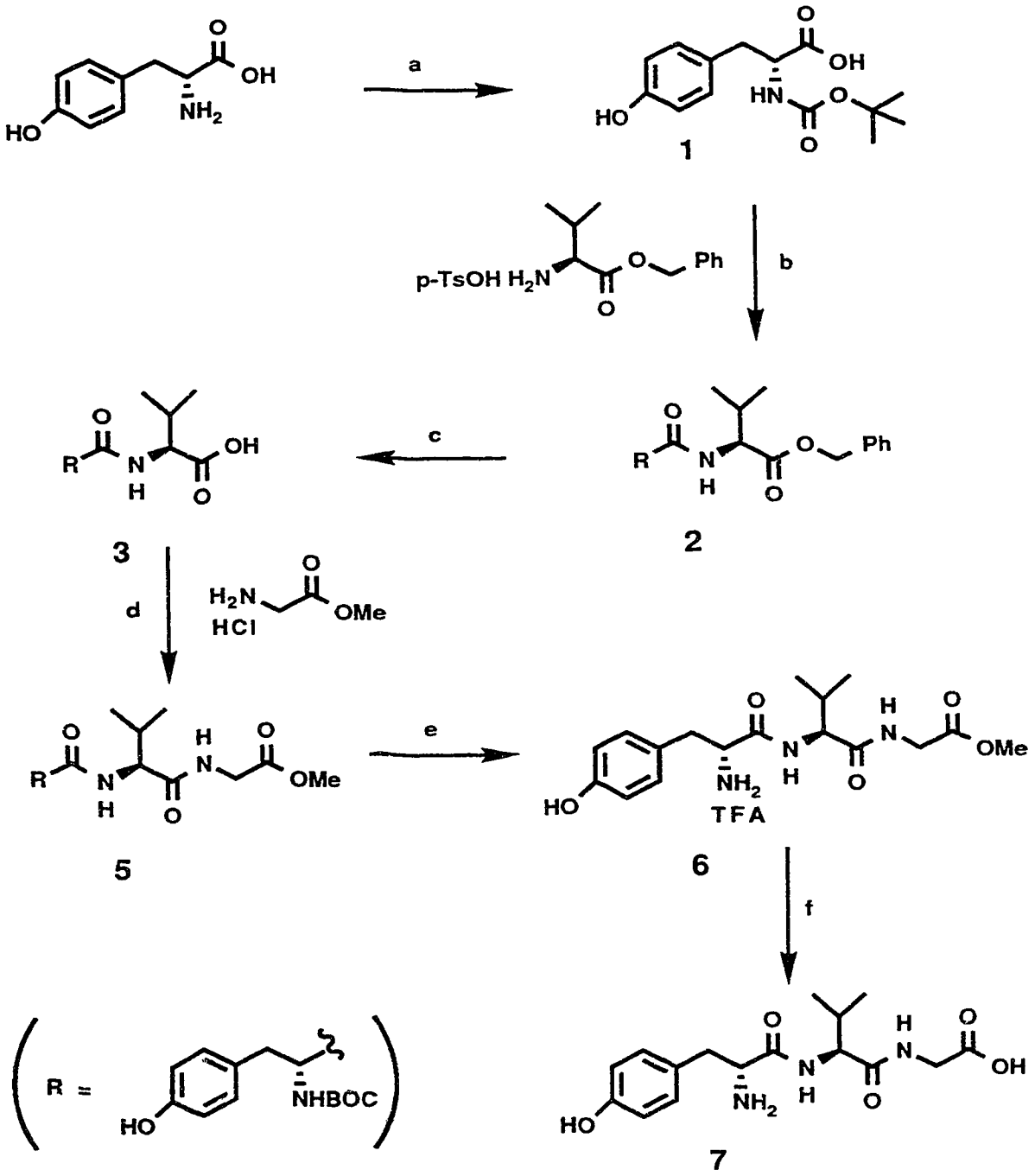
Synthesis of D-Tyr-L-Val-Gly

Since the model substrate, D-Tyr-L-Val-Gly, chosen by Bradbury *et al.*[31] and also used by Dr. S. E. Ramer,[82] gave good results during their studies, this model substrate was initially used in the present research before switching to the better substrates,

D-Phe-L-Phe-Gly and *N*-Ac-D-Phe-L-Phe-Gly. Both D-Tyr-L-Val-Gly, used for preparing the affinity column, and the corresponding ^{14}C labeled substrate, D-Tyr-L-Val-[1,2-^{14}C]-Gly, needed for the enzyme activity assay,[82] were prepared by standard solution-phase peptide chemistry. Diphenylphosphorylazide (DPPA)[105] was used to couple the nitrogen protected amino acids with the carboxyl protected amino acids. The DPPA procedure converts carboxylic acids to acyl azides which then react with amines to form the amides. *tert*-Butyloxycarbonyl (BOC) protecting groups are used to protect the amino group and are then removed with trifluoroacetic acid (TFA), and the carboxyl groups are protected as the methyl or benzyl esters and then deprotected by alkaline hydrolysis or catalytic hydrogenolysis, respectively.[106,107]

Treatment of D-tyrosine with di-*tert*-butylpyrocarbonate under basic conditions gives crude *N*-BOC-D-Tyr (**1**) (Scheme 1).[108] Conversion to its dicyclohexylamine salt, crystallization and acidification of this salt produces pure **1** in 93% yield. Coupling of the *N*-protected D-Tyr **1** with the *p*-toluenesulfonic acid salt of L-valine benzyl ester gives *N*-BOC-D-Tyr-L-Val benzyl ester (**2**) in 62% yield. Hydrogenolysis of the benzyl ester **2** using 10% palladium on carbon affords *N*-BOC-D-Tyr-L-Val (**3**) in 99% yield. Coupling of the *N*-protected peptide **3** with glycine methyl ester hydrochloride salt (**4**), which was prepared quantitatively from glycine by bubbling HCl gas into its methanolic solution, generates the diprotected tripeptide **5** in 91% yield. Hydrolysis of the methyl ester with sodium hydroxide in aqueous methanol, aqueous workup, and treatment with neat TFA at room temperature to remove the *N*-BOC group gives the free peptide **7** in an overall 62% yield from **5**.

Scheme 1



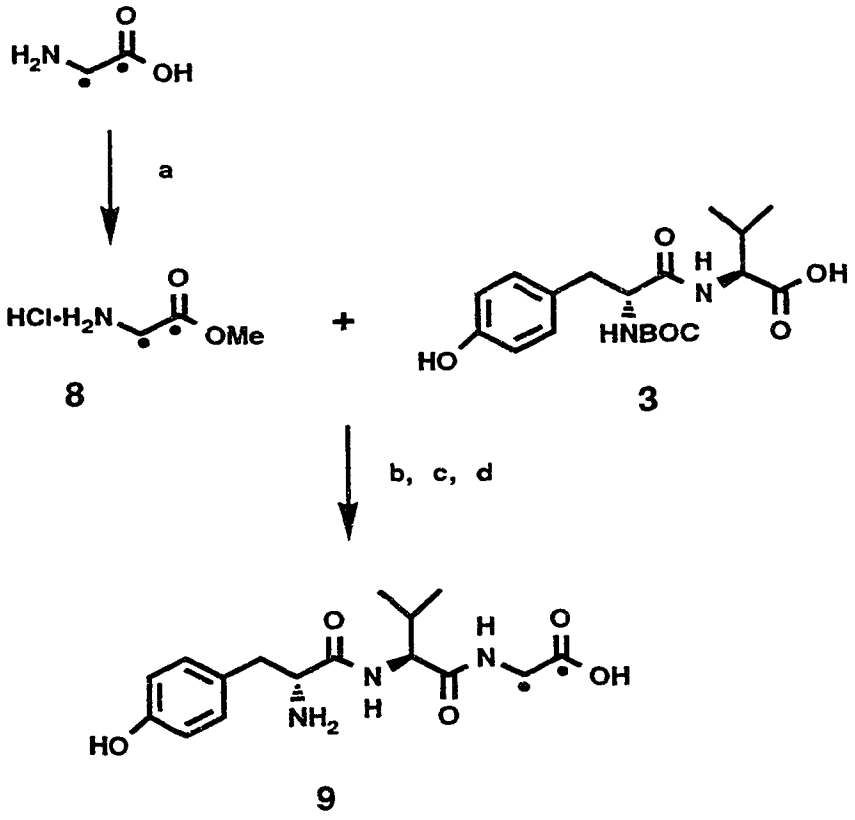
a. BOC_2O, 0.5 N NaOH, 2:1 Dioxane:H_2O; Dicyclohexylamine; $KHSO_4$

b. DPPA, DMF, Et_3N c. H_2, Pd/C, EtOAc d. DPPA, Et_3N, DMF

e. CF_3CO_2H f. NaOH, MeOH/H_2O

To synthesize the tripeptides incorporating a ^{14}C labeled glycine, *N*-BOC-D-tyrosyl-L-valine (**3**) is coupled with labeled glycine methyl ester hydrochloride (This was performed by Dr. S. E. Ramer.) (Scheme 2). Thus reaction of [1,2-^{14}C]-glycine (259 μCi, Amersham, 113 μCi/μmol, 96% ^{14}C) with methanol saturated with dry HCl forms the methyl ester **8**. This is coupled to the protected dipeptide **3** using the DPPA procedure. Deprotection as described for the unlabeled material gives D-Tyr-L-Val-[1,2-^{14}C]-Gly (**9**), which is isolated as its trifluoroacetate salt in 47% overall yield (118 μCi). This material shows a single radioactive spot on TLC and co-chromatographs with unlabeled peptide **7** in two different solvent systems. This tripeptide is then purified by reverse-phase HPLC before its use in enzyme assay.

Scheme 2.



a. MeOH, HCl b. DPPA, Et_3N, DMF c. NaOH, MeOH/H_2O d. TFA

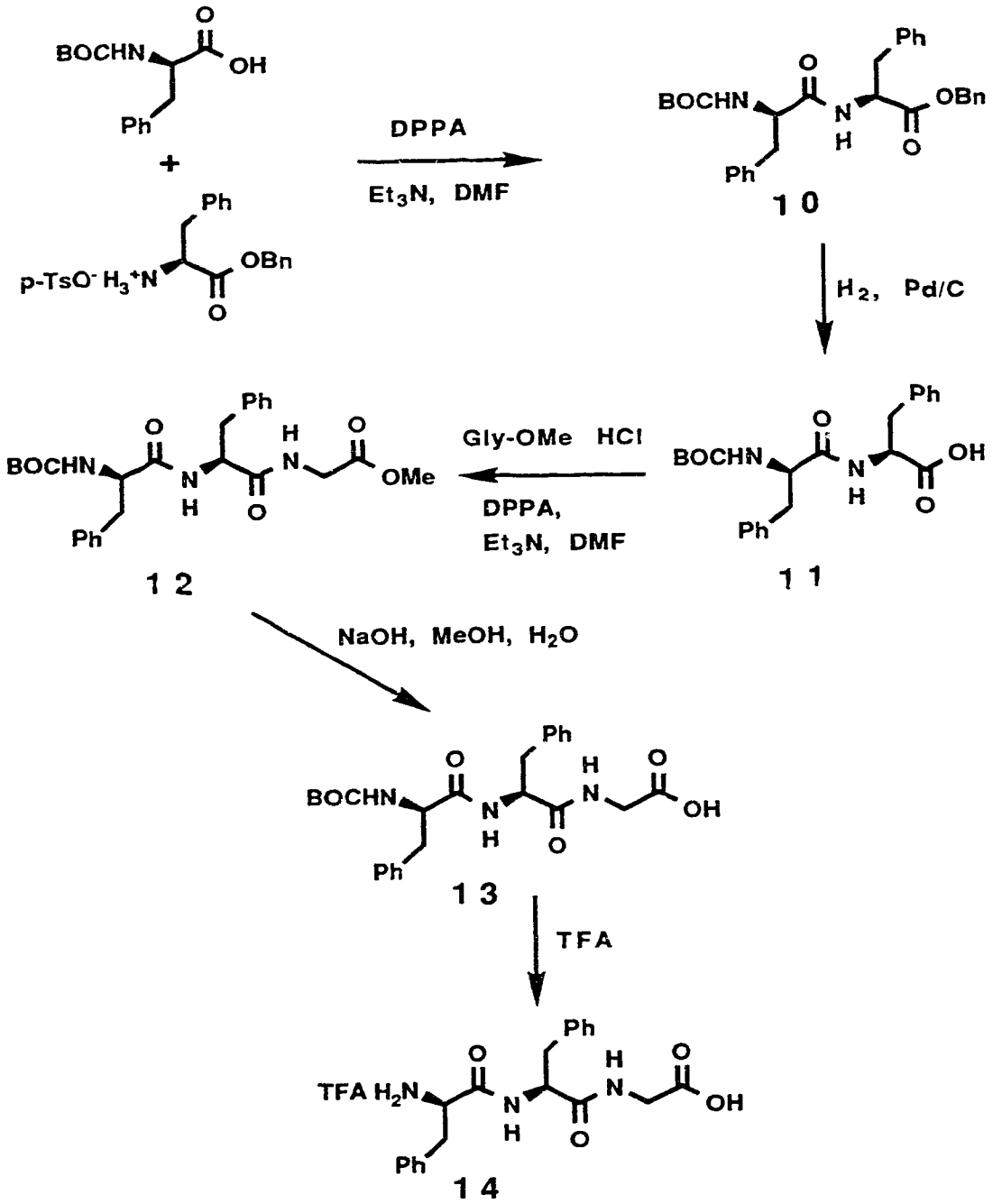
Synthesis of D-Phe-L-Phe-Gly and *N*-Ac-D-Phe-L-Phe-Gly

Substrate specificity studies of an amidating enzyme from porcine pituitaries by Bradbury and Smyth[72] show that a neutral amino acid such as phenylalanine at position two of the tripeptides D-Tyr-L-X-Gly-OH leads to facile reaction, whereas acidic or basic residues at this position give much slower conversions. If the first amino acid, tyrosine, is also replaced by phenylalanine, then the protection and deprotection of the aromatic hydroxyl group can be omitted. Recently, kinetic parameters of the substrates of the form *N*-dansyl-$(Gly)_4$-X-Gly-OH, in which the amino acid at position X was substituted with each of the 20 natural amino acids, were obtained against the α-amidating enzyme isolated from rat medullary thyroid CA-77 cells[75]. The enzyme used in these studies was a highly enriched preparation of α-amidating enzyme secreted by a clonal (CA-77) cell line which actively expresses mature α-amidated peptides. A 130-fold and 11-fold variation, respectively, in apparent K_M and V_{max} values was observed. The effect of the amino acid side chain at position X in stabilizing the enzyme-substrate complex decreased through the series X = planar aromatic or sulfur containing > neutral aliphatic > polar and basic > cyclic aliphatic or acidic. Furthermore, if the *N*-terminal amino group is further protected by an acetyl group, the tripeptide should still be a good substrate and the *N*-BOC deprotection can be omitted. Hence, both unlabeled and labeled D-Phe-L-Phe-Gly and *N*-Ac-D-Phe-L-Phe-Gly were synthesized and tested as substrates of the PHM enzyme.

The general procedure for preparation of D-Tyr-L-Val-Gly was adapted to synthesize both D-Phe-L-Phe-Gly and *N*-Ac-D-Phe-L-Phe-Gly. Thus, commercially available *N*-BOC-D-phenylalanine is coupled with L-phenylalanine benzyl ester *p*-toluenesulfonic acid salt by the DPPA procedure to generate *N*-BOC-D-Phe-L-Phe benzyl ester (**10**) in 80% yield (Scheme 3). Catalytic hydrogenolysis of the benzyl ester **10** gives the free acid **11** quantitatively. The dipeptide **11** couples with glycine methyl ester hydrochloride in the presence of DPPA and triethylamine to give *N*-BOC-D-Phe-L-Phe-Gly

methyl ester (**12**) in 48% yield. Hydrolysis of **12** with sodium hydroxide in water/methanol affords the *N*-protected tripeptide acid **13** in 62% yield. Finally,

Scheme 3.

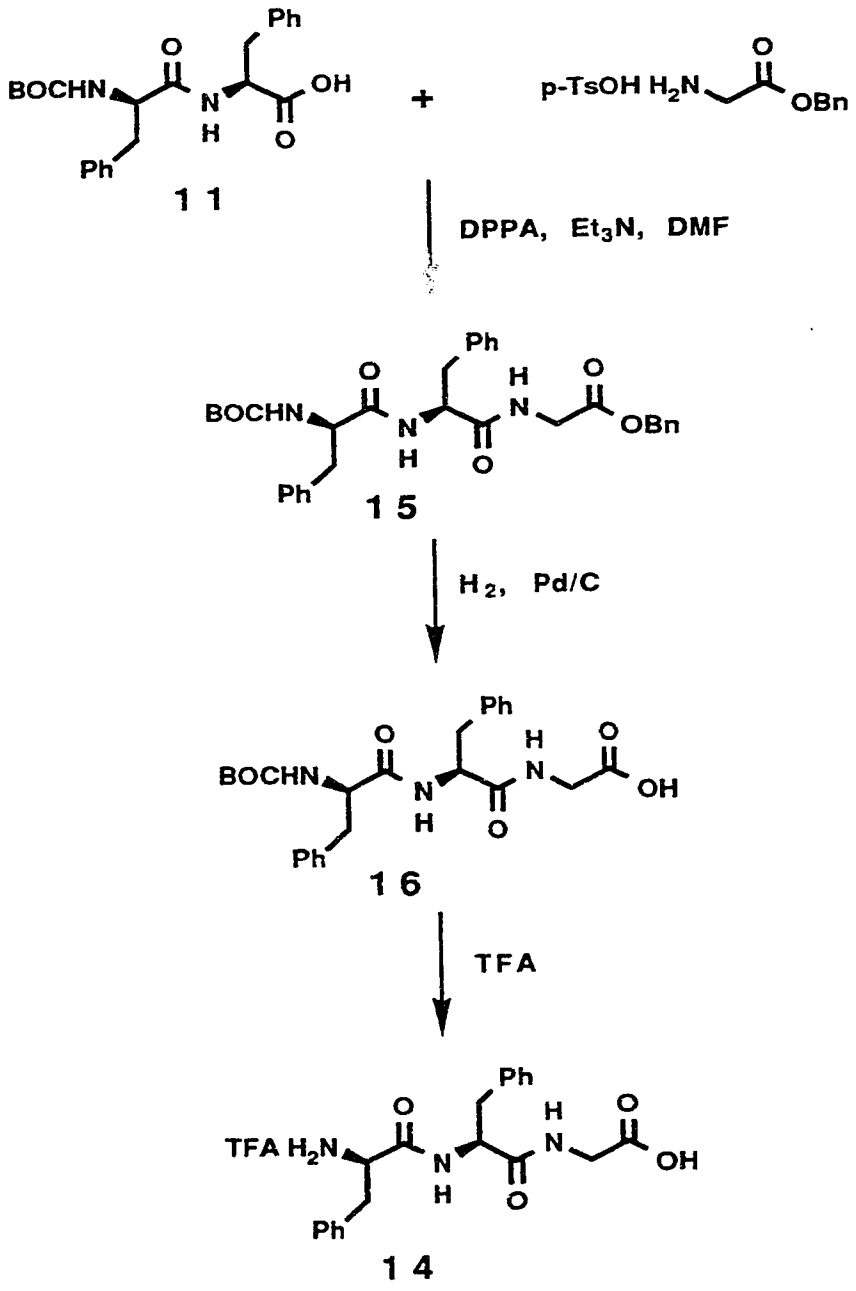


removal of the *N*-BOC group by treatment with neat TFA gives the desired tripeptide, D-Phe-L-Phe-Gly trifluoroacetate salt (**14**), in 97% yield.

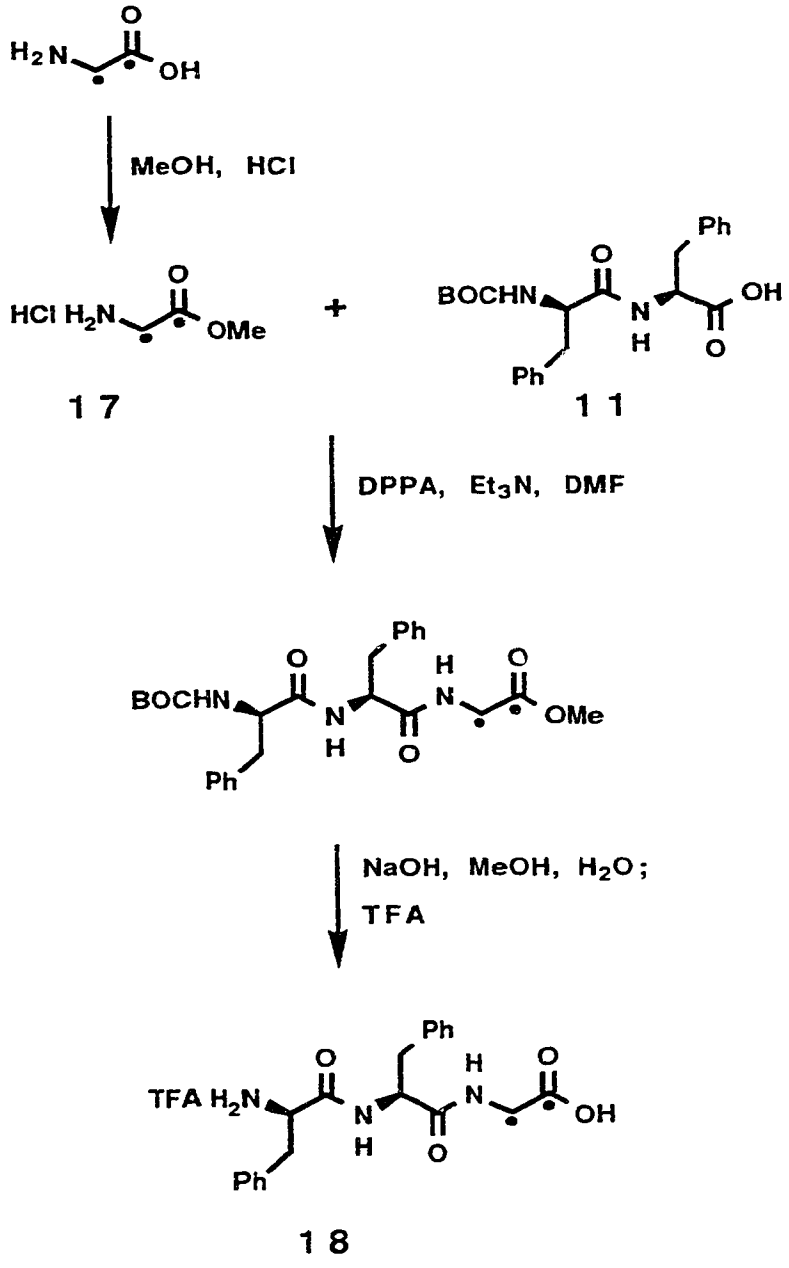
Peptide **14** was also prepared from commercially available benzyl glycinate *p*-toluenesulfonate salt (Scheme 4). Thus *N*-BOC-D-Phe-L-Phe (**11**) is coupled with commercially available glycine benzyl ester *p*-toluenesulfonic acid salt by the DPPA procedure to give the diprotected tripeptide **15** in 72% yield. This coupling step gives a much better yield than the corresponding reaction with glycine methyl ester. The benzyl ester is cleaved by catalytic hydrogenolysis; subsequent treatment with TFA removes the *N*-BOC group to give the tripeptide **14** in 97% yield from **15**.

The corresponding labeled tripeptide was synthesized from [1,2-^{14}C]-glycine methyl ester (**17**), prepared from [1,2-^{14}C]-glycine (Scheme 5). The reason for using the methyl ester is its facile preparation and deprotection on a small scale. Coupling of the dipeptide **11** with **17** using the DPPA procedure gives the diprotected radiolabeled tripeptide. Hydrolysis of the methyl ester by sodium hydroxide in aqueous methanol, and removal of the *N*-BOC group with TFA generates the labeled tripeptide, D-Phe-L-Phe-[1,2-^{14}C]-Gly (**18**) in 63% overall yield. This material shows a single radioactive spot on TLC which comigrates with unlabeled peptide **14** in two different solvent systems. It also has the same retention time as the unlabeled tripeptide **14** on reverse-phase HPLC.

Scheme 4.

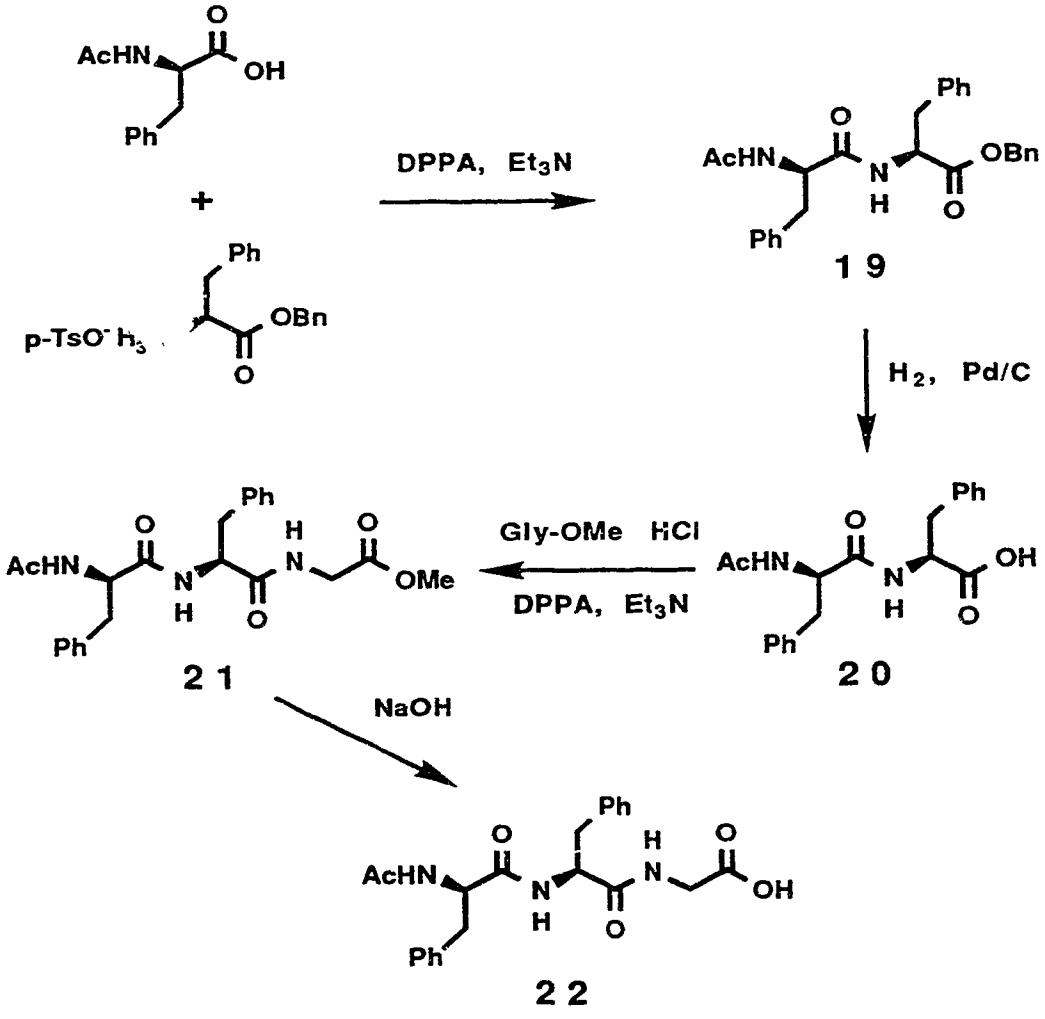

BOCHN
Ph
OH
Ph
11
+
p-TsOH H2N
OBn
DPPA, Et3N, DMF
15
H2, Pd/C
16
TFA
TFA H2N
14

Scheme 5.



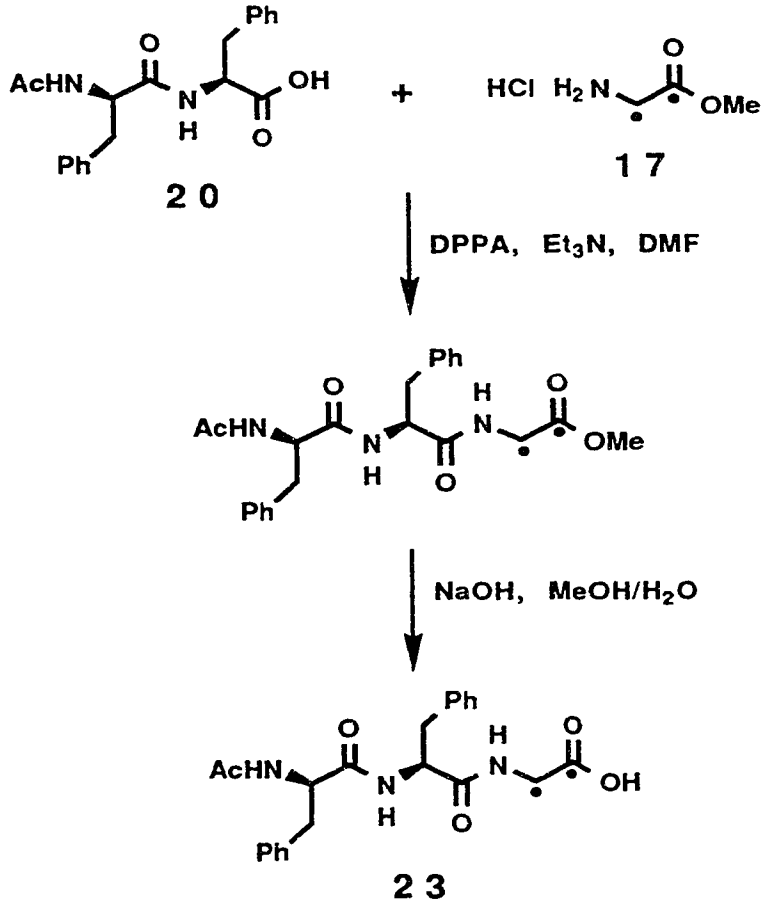
The *N*-acetyl peptide was synthesized similarly. Coupling of commercially available *N*-acetyl-D-phenylalanine with L-phenylalanine benzyl ester *p*-toluenesulfonic acid salt using the DPPA procedure gives the diprotected peptide **19** in 93% yield (Scheme 6). Catalytic hydrogenolysis cleaves the benzyl ester to generate *N*-Ac-D-Phe-L-Phe-OH (**20**) in 99% yield. This dipeptide then couples with glycine methyl ester hydrochloride to give the diprotected tripeptide **21** in high yield (92%). Finally, hydrolysis of the methyl ester affords the desired tripeptide, *N*-Ac-D-Phe-L-Phe-Gly (**22**), in 82% yield.

Scheme 6.



The corresponding labeled tripeptide was then synthesized. [1,2-^{14}C]-Glycine methyl ester (**17**) is coupled with the dipeptide acid **20** using the DPPA procedure to give the diprotected radiolabeled tripeptide in 46% yield (Scheme 7). The methyl ester is then hydrolyzed by sodium hydroxide in aqueous methanol to give *N*-Ac-D-Phe-L-Phe-[1,2-^{14}C]-Gly (**23**) in 69% yield. This material also shows only a single radioactive spot on TLC which cospots with unlabeled peptide **22** in two different solvent systems. It also gives the same retention time as the unlabeled tripeptide **22** by reverse-phase HPLC.

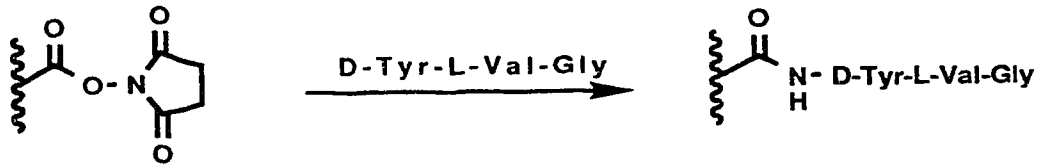
Scheme 7



2.2 ENZYME ISOLATION AND ASSAY

The method utilized for the isolation of PHM was that of Dr. S. E. Ramer,[82] which itself was an adaptation of the procedure of Youngblood and co-workers.[38] The reason for choosing this method is because it is straightforward with only two columns being used to yield enzyme of high purity (>95%). The key step of the rapid isolation, allowing many fold purification in a single step, is the use of a substrate affinity column which was prepared from Bio-Rad Affi-Gel 15 and D-Tyr-L-Val-Gly (7) as previously described.[38] The Affi-Gel resin contains *N*-hydroxysuccinimide activated esters which form amide bonds with the terminal amino group of the substrate (Scheme 8). The tripeptide is shaken with the Affi-Gel in buffer overnight followed by subsequent treatment with urea to block any unreacted *N*-hydroxysuccinimide esters on the resin. After a thorough wash with buffer, the column is ready for use. When dissolved enzyme is passed through the affinity column, it selectively binds to the substrate attached to the column while other proteins pass unhindered. PHM does not degrade the column since ascorbic acid and copper (II), necessary cofactors for the conversion of the substrate, are not available. The enzyme is

Scheme 8.



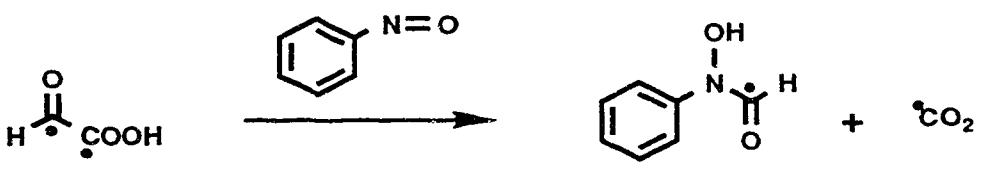
Affi-gel 15

removed from the column by elution with a concentrated buffer containing urea, *N*-acetylglycine, and glycylglycine. This probably slightly denatures the protein and distorts the active site geometry.

The enzyme was isolated from frozen pig pituitaries (Pel-Freez, Rogers, Arkansas) which had been stored at -60 °C for an indefinite period of time and were then homogenized and centrifuged. The supernatant was applied to a Sephadex G-100 column and the column was eluted with phosphate buffer. The active fractions were combined and applied to the affinity column. After removal of contaminating protein with phosphate buffer, the enzyme was eluted from the affinity column with urea buffer, and the resulting fractions were dialyzed. The entire isolation can be performed within a 60 h period. All operations are done at 4 °C and it appears to be critical to work as quickly as possible to obtain the highest enzyme activity.[109] The PHM enzyme is particularly sensitive to the affinity column eluent, and these fractions must be dialyzed immediately. The enzyme isolated this way can be stored at -20 °C or -60 °C for an indefinite period of time.

The assay for amidating activity, which employs nitrosobenzene to capture the liberated glyoxylic acid, and forms the *N*-hydroxyformanilide (Figure 4), was developed by Dr. S. E. Ramer.[110] To assay the enzyme activity, D-Tyr-L-Val-[1,2-^{14}C]-Gly (**9**) and a cocktail containing ascorbic acid, copper sulfate, and potassium iodide were added to the enzyme solution. After 2 h incubation at 37 °C, unlabeled glyoxylic acid and four equivalents of nitrosobenzene were added and the solution was heated for 1 h at 60 °C followed by extraction with ether. The ether extracts were passed through a Pasteur pipette filled with ~2 g of Na_2SO_4 which serves to dry the ether layer and to remove the residual

Figure 4. The Capture of Glyoxylic Acid by Nitrosobenzene



radioactive peptide dissolved in the wet ether layer. The ether washes were directly collected in scintillation vials and the radioactivity was determined by liquid scintillation counting. In general, assays run in duplicate vary by less than 10% and the background was consistently low (~37 dpm), only 16 dpm above instrument background. The substrate D-Tyr-L-Val-Gly (**7**) has a K_M of 26 μM and V_{max} of 244 pmol/μg/h with this assay procedure.[111] Table 3 is a summary of the enzyme isolations. The amount of protein was determined by a modified procedure of Bradford[112] using bovine serum albumin as the standard. The variation between isolations is probably due to a combination of factors, including both experience and the length of time the pituitaries were stored and the quantity of pituitaries that were used.

Table 3. Enzyme Isolations

Combined Sephadex Fractions			Affinity Column Fractions[a]		
mg/mL	total mg	nmol/mg/h[b]	μg/mL	total μg	nmol/mg/h[b]
2.04	245	0.089	15	84	14
1.95	332	0.129	18	127	17
2.23	446	3.20	21	283	28

[a]These numbers are for the affinity column fraction showing the highest activity. In general, other fractions were less than half of the activity of the major fraction.
[b]These values are derived from dpm by the following conversion factors: 2.22×10^{12} dpm/Ci; 113 mCi/mmol; 0.5 carbons retained; 0.55 trapping efficiency.

After reverse-phase HPLC purification, both D-Phe-L-Phe-[1,2-^{14}C]-Gly (**18**) and *N*-Ac-D-Phe-L-Phe-[1,2-^{14}C]-Gly (**23**) were tested as a substrates of PHM enzyme using the nitrosobenzene assay. Table 4 lists the turnovers of D-Tyr-L-Val-Gly (**7**), *N*-Ac-D-Phe-L-Phe-Gly (**14**) and D-Phe-L-Phe-Gly (**22**) by PHM under the same conditions. A

K_M of 8.5 μM for D-Phe-L-Phe-[1,2-^{14}C]-Gly was obtained by Dr. T. M. Zabriskie of our group, using the assay procedure with ion-exchange resin. This is three fold better than D-Tyr-L-Val-Gly which has a K_M of 26 μM.[82]

Table 4 PHM Turnovers of 7, 14, and 22.[a]

	Compound **7**	Compound **14**	Compound **22**
Assay Turnover	6116 dpm	9785 ± 0.133% dpm	9539±0.0159% dpm
Control	72 dpm	170 ± 0.100% dpm	540 ± 0.0315% dpm

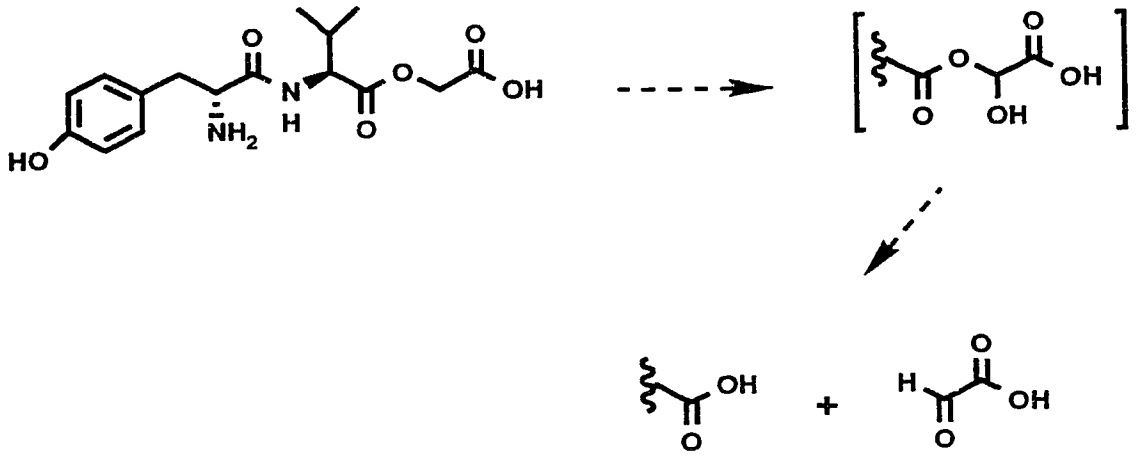
[a]These experiments were carried out in duplicate, the average results are reported.

2.3 SYNTHESIS OF A GLYCOLATE PEPTIDE ANALOGUE AND STUDIES OF OXIDATION PATHWAYS

D-Tyrosine-L-Valine-Glycolic Acid

To investigate the mechanism of the PHM oxidation, a modified substrate was synthesized. We reasoned that if PHM converted a modified peptide, in which the glycine residue had been replaced by a glycolic acid moiety, to glyoxylic acid and the dipeptide (Scheme 9), this would provide further evidence that the oxidation proceeds at the methylene carbon (c in Figure 2) and not by direct dehydrogenation or oxidation involving the amide nitrogen (a or b in Figure 2). At the time this research was initiated, the amine *N*-dealkylation and *O*-dealkylation had not been reported.[79]

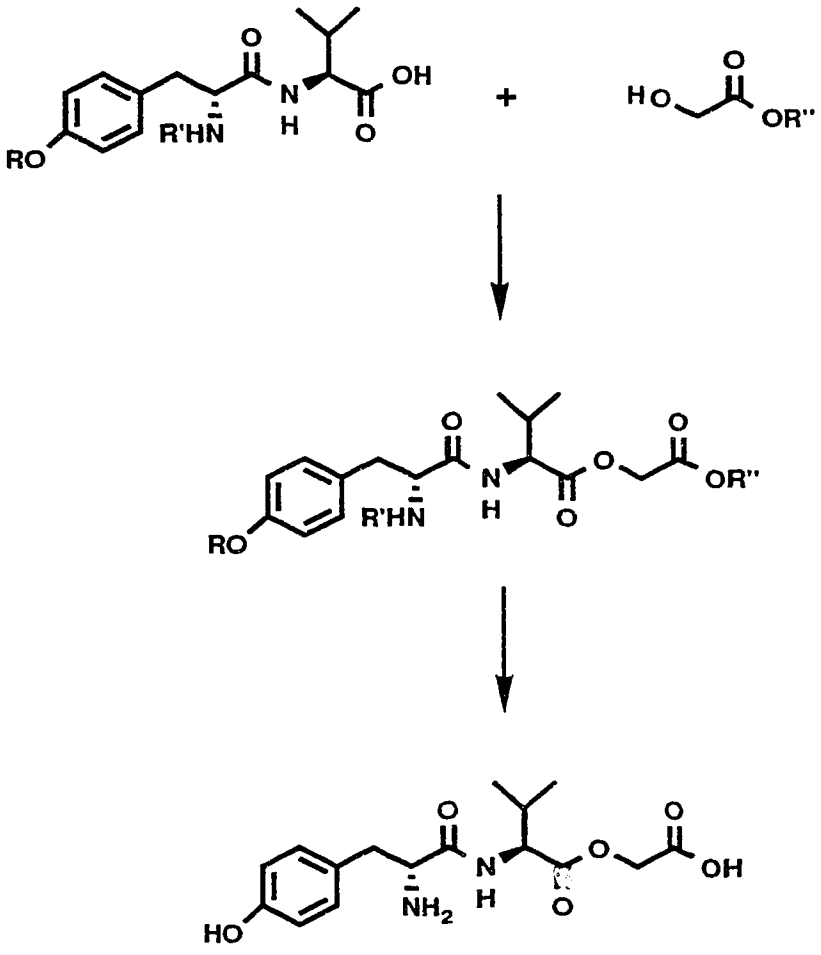
Scheme 9.



In the synthesis of the modified peptide, a strategy similar to that used in the preparation of D-Tyr-L-Val-Gly was employed. Coupling of a protected glycolate with a suitably protected derivative of D-Tyr-L-Val followed by the necessary deprotections should give the desired product (Scheme 10). However, several important differences must be considered. Since the coupling involves the formation of an ester bond instead of an amide bond, the tyrosyl hydroxyl group must be protected from competing for an activated carboxylic acid. In addition, the protecting groups must be removed under conditions which do not hydrolyze the ester linkage. Finally, the synthesis must be adaptable to the use of [^{14}C]-glycolic acid on small scale.

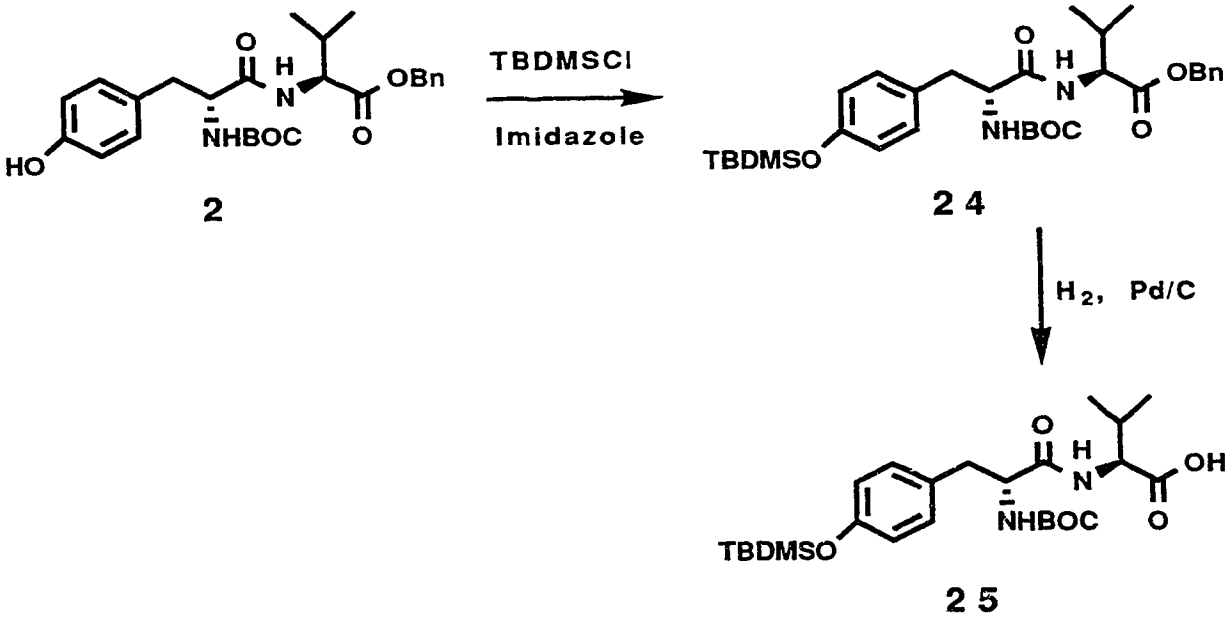
The choice of protecting groups was crucial. The BOC group was considered a satisfactory protecting group for the tyrosine amino group since cleavage by trifluoroacetic acid should leave the ester bond intact. The *tert*-butyldimethylsilyl (TBDMS) group was chosen to protect the hydroxyl group of the tyrosine since it can be selectively cleaved by

Scheme 10.



fluoride ion under non-hydrolytic conditions.[113,114] The *N*-BOC-*O*-TBDMS-D-Tyr-L-Val benzyl ester (**24**) is synthesized in 82% yield by treating *N*-BOC-D-Tyr-L-Val benzyl ester (**2**), previously prepared as an intermediate in the synthesis of the substrate **7**, with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) (Scheme 11).[113,115] The benzyl group is selectively removed by hydrogenolysis in 96% yield to give the free acid **25**.

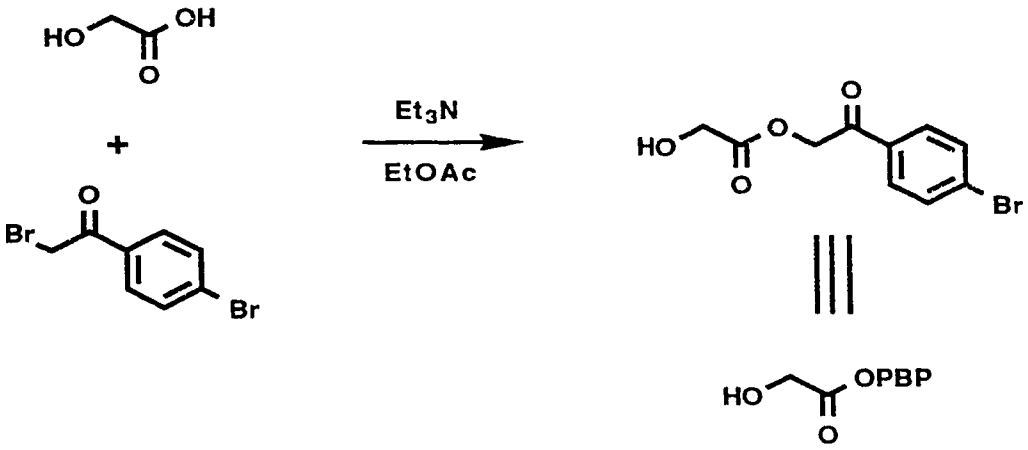
Scheme 11.



2 **24** **25**

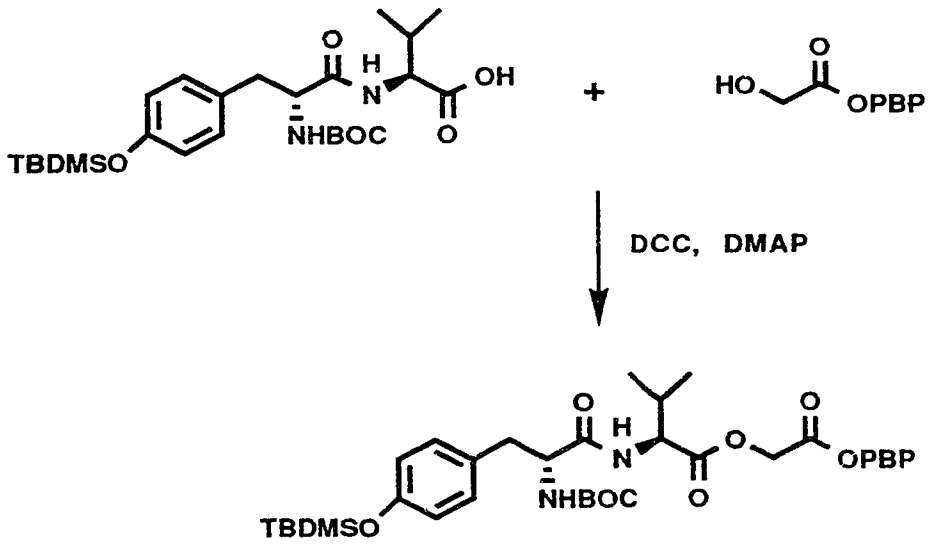
Dr. Ramer had attempted to use the *p*-bromophenacyl (PBP) group for carboxyl protection.[116] The desired PBP ester was prepared in high yield by reacting glycolic acid with 2,4'-dibromoacetophenone in ethyl acetate with triethylamine (Figure 9).[114]

Figure 9. Synthesis of *p*-Bromophenacyl Glycolate



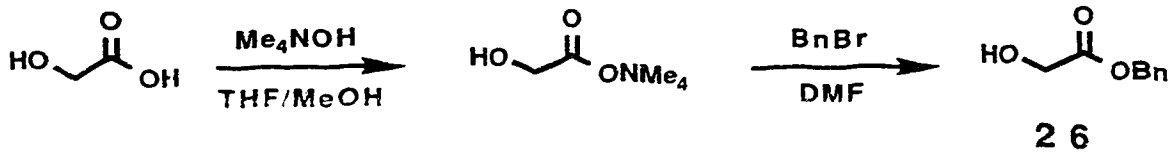
Although the formation of the ester bond between the PBP ester and **25** was conveniently achieved in 65% yield through the use of dicyclohexylcarbodiimide (DCC) in the presence of catalytic 4-dimethylaminopyridine (DMAP) (Figure 10), it proved impossible to cleave the PBP ester either by catalytic hydrogenolysis or by zinc in acetic acid.

Figure 10. Synthesis of *N*-BOC-*O*-TBDMS-D-Tyr-L-Val-Glycolic Acid *p*-Bromophenylacyl Ester



With this information available, the benzyl ester was then selected as the carboxyl protecting group. The well known method for the formation of carboxylate esters by the reaction of alkyl halides with metal salts of carboxylates was not compatible with our synthesis since it requires vigorous conditions and produces poor yields. However, replacement of the metal ion by a quaternary ammonium ion dramatically increases the rate of the reaction under mild conditions.[117] Thus glycolic acid is converted to its tetramethylammonium salt by reaction with tetramethylammonium hydroxide in THF and MeOH. This salt is dried under vacuum and then treated with benzyl bromide in DMF to give glycolic acid benzyl ester (**26**) in 99% yield (Scheme 12).

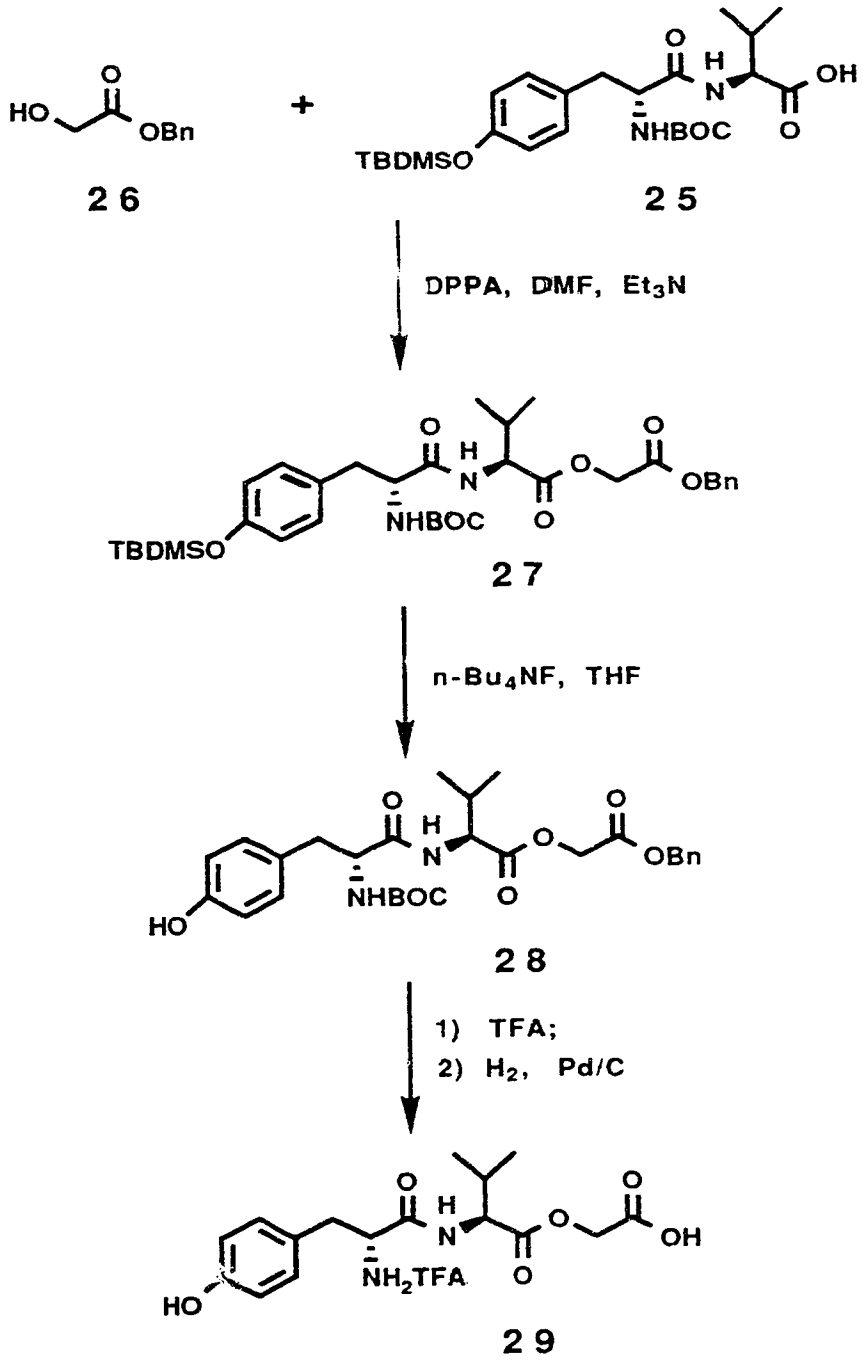
Scheme 12.



26

The benzyl ester **26** is coupled with *N*-BOC-*O*-TBDMS-D-Tyr-L-Val (**25**) to give benzyl *N*-BOC-*O*-TBDMS-D-Tyr-L-Val-glycoate (**27**) in 80% yield (Scheme 13) by using DCC in the presence of catalytic DMAP.[118] The TBDMS group is removed by room temperature treatment with tetrabutylammonium fluoride in THF to give *N*-BOC-D-Tyr-L-Val-glycolic acid benzyl ester (**28**) in quantitative yield.[119] The *N*-BOC group is cleaved by TFA at room temperature. Following evaporation of the TFA, the benzyl group is catalytically hydrogenolyzed to give the desired product, D-Tyr-L-Val-glycolic acid trifluoroacetate salt (**29**) in 85% yield.

Scheme 13.


HO
OBn
26
+
TBDMSO
NHBOC
OH
25
DPPA, DMF, Et3N
27
n-Bu4NF, THF
28
1) TFA;
2) H2, Pd/C
NH2TFA
29

As the preparation of the unlabeled glycolate peptide was successful, i.e. 67% from glycolic acid in five steps, the synthesis of the corresponding labeled compound was attempted. A sample of [1,2-^{14}C]-glycolic acid calcium salt (25 μCi, 30-50 mCi/mmol), commercially available from ICN Radiochemicals, is treated with tetramethylammonium hydroxide and then with benzyl bromide. However, the labeled glycolic acid benzyl ester (**30**) forms obtained in only 5% yield (Scheme 14). Due to the low concentrations of labeled material, it may have been difficult to convert most of the glycolic acid calcium salt to the corresponding tetramethylammonium salt, thereby possibly accounting for the low yield. With such a small amount of benzyl ester available as the starting material, not enough final product could be synthesized in the following four steps. Therefore, the strategy for preparing the glycolate peptide was reconsidered. One way was to reduce the numbers of steps involving the labeled material, i.e. choosing a different substrate analogue which only involves coupling with labeled glycolate ester and then deprotection. *N*-Ac-D-Phe-L-Phe-glycolate is an ideal substrate analogue for this purpose because only two steps would be required to prepare *N*-Ac-D-Phe-L-Phe-[1,2-^{14}C]-glycolate from the labeled benzyl glycolate. Therefore, this new substrate was synthesized and tested with PHM.

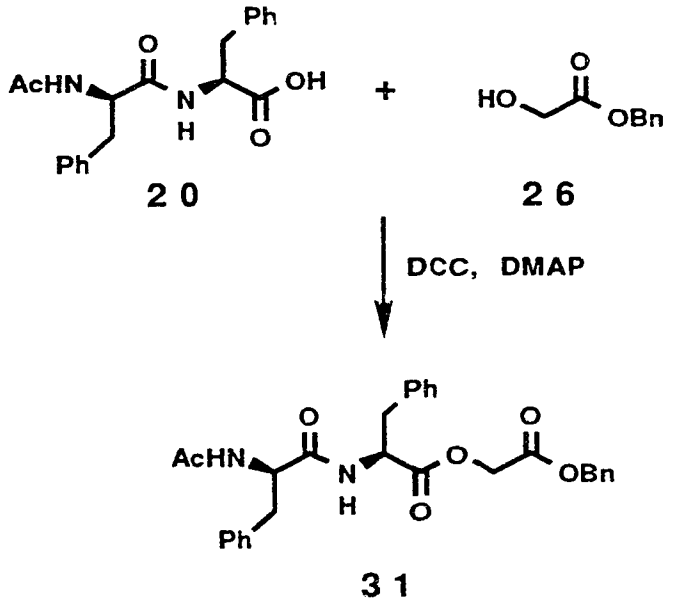
Scheme 14.

30

Synthesis and Testing of *N*-Ac-D-Phe-L-Phe-Glycolic Acid

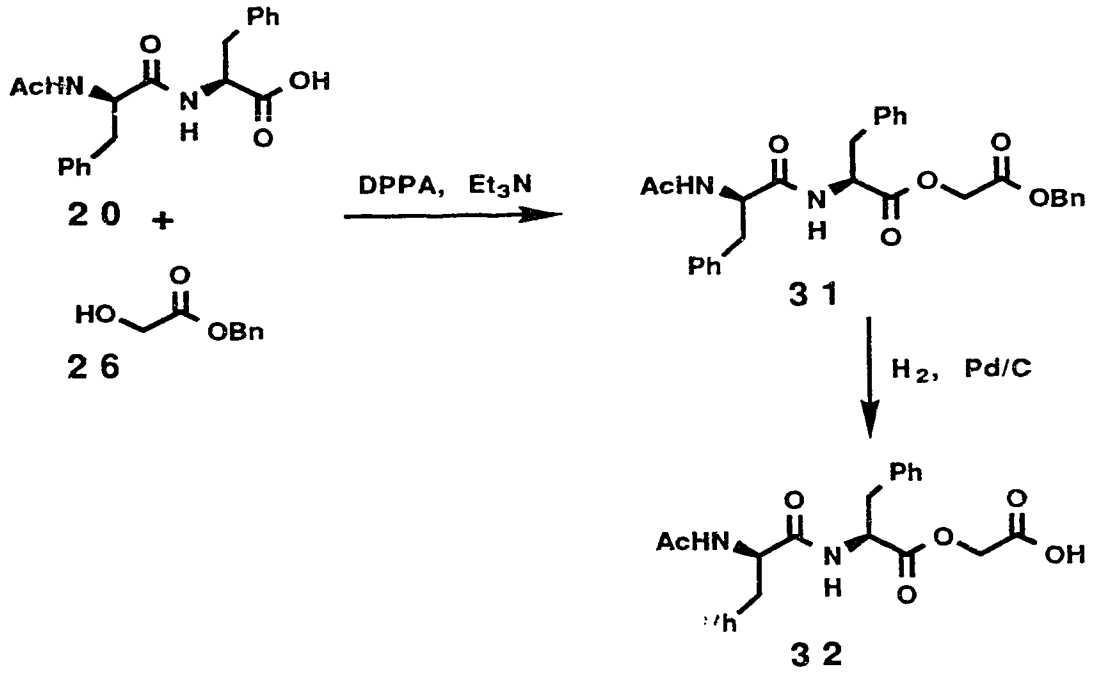
To make the unlabeled target molecule, the same method was attempted as for the preparation of *N*-BOC-*O*-TBDMS-D-Tyr-L-Val-glycolic acid benzyl ester (**27**). Thus benzyl glycolate (**26**) was coupled with the dipeptide acid **20** using DCC in the presence of catalytic DMAP (Scheme 15). Although the expected coupling did occur, it proved very difficult to separate the product from dicyclohexylurea.

Scheme 15.



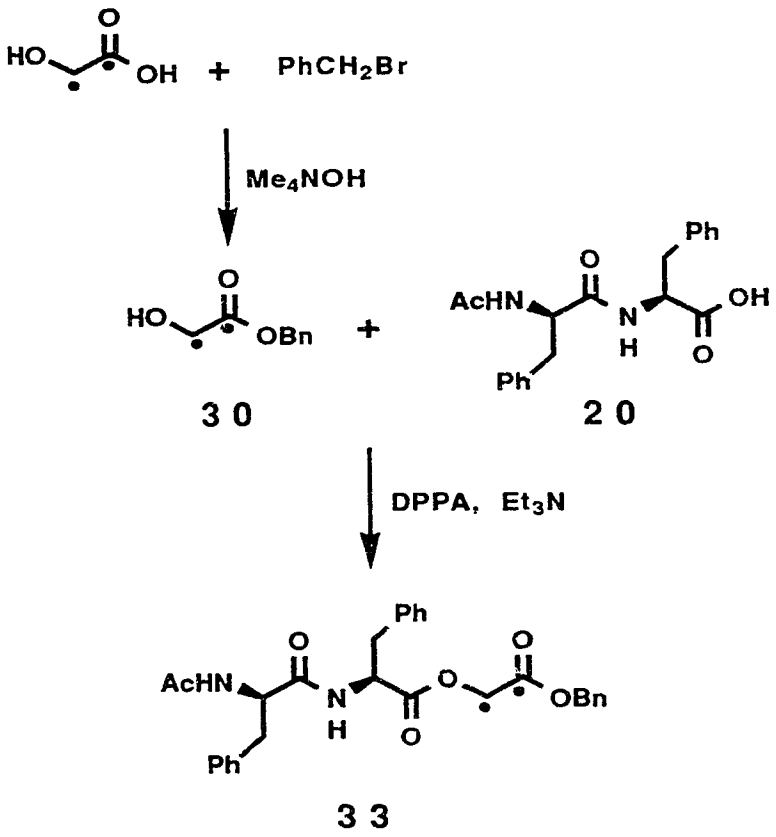
To minimize the purification problems, diisopropylcarbodiimide and DPPA were tried; of these DPPA was found to be most effective. It produced good yields and the purification of the product was relatively easy. Hence, the *N*-protected dipeptide **20** was coupled with benzyl glycolate (**26**) using the DPPA procedure to give the protected peptide analog **31** in 77% yield (Scheme 16). Hydrogenolysis cleaved the benzyl ester to give the desired product **32** in 86% yield after purification by flash chromatography.

Scheme 16.



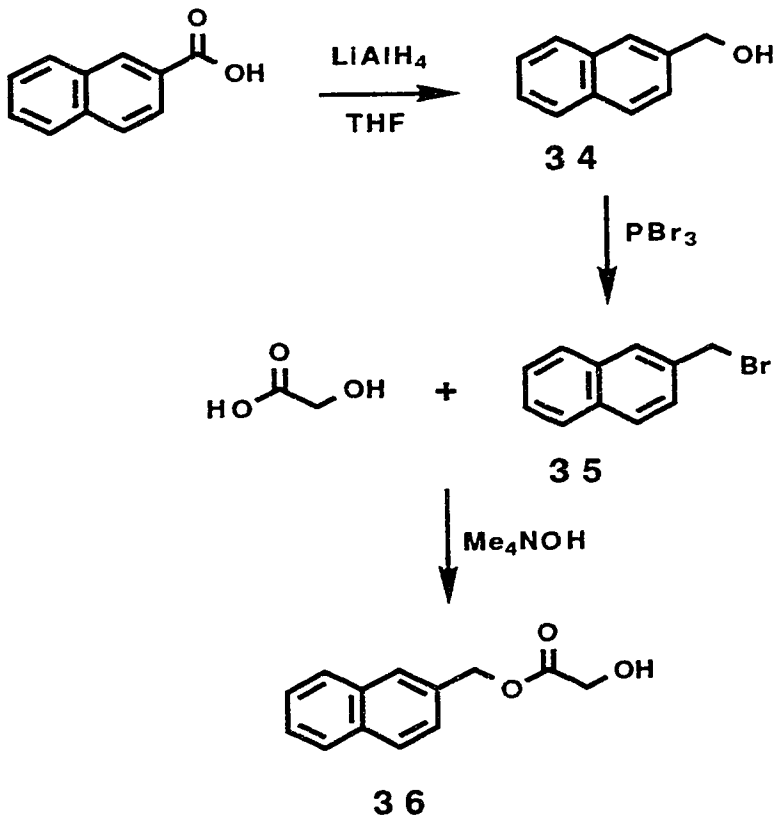
After the synthesis of the unlabeled **32**, the preparation of the labeled material was attempted. The ^{14}C-labeled benzyl glycolate was resynthesized as described previously. Benzyl [1,2-^{14}C]-glycolate was obtained in 22% yield, and this was then coupled with **20** using the DPPA procedure (Scheme 17). Unfortunately, only an 8% yield of the desired product **33** could be isolated from the reaction mixture. This could be possibly due to the volatility of **30** and the small amounts employed. When the starting materials were dried under vacuum, it was determined that some of the radioactive material was lost. To confirm this, 265 mg of benzyl glycolate was placed under high vacuum (0.10 mm Hg) for 24 hours. Reweighing the sample revealed a loss of 25 mg during this period.

Scheme 17.



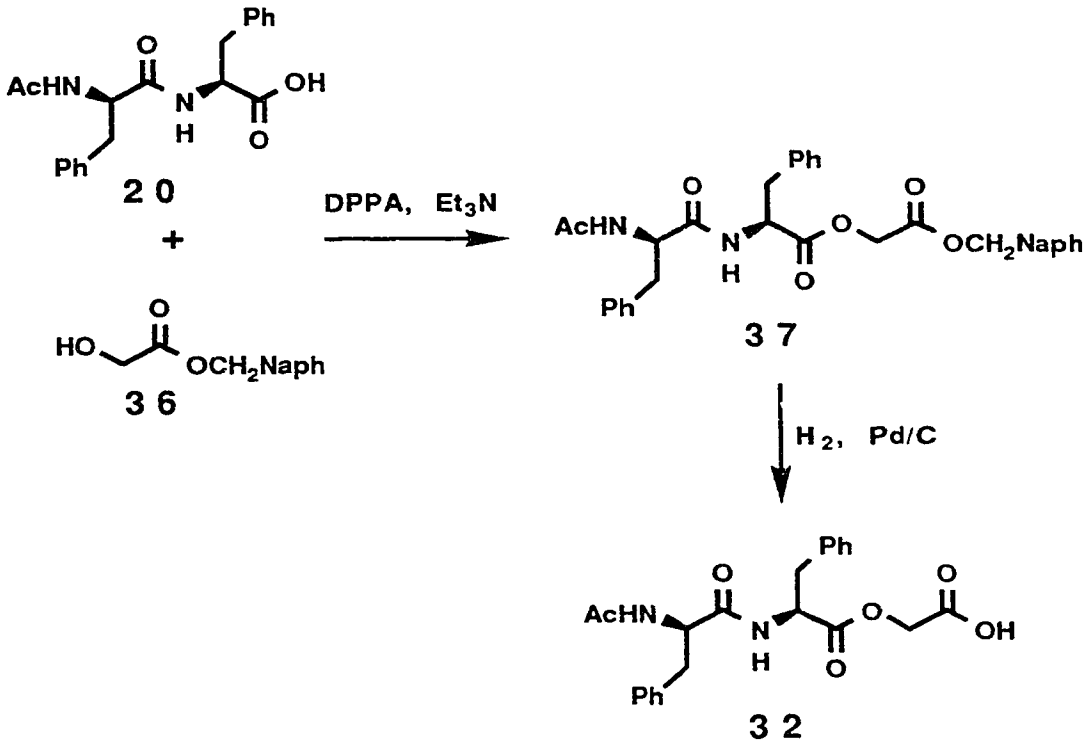
To overcome this difficulty, 2-naphthalenemethyl glycolate (**36**) was used instead of the benzyl ester; the increased molecular weight should render **36** much less volatile. To prepare **36**, commercially available 2-naphthoic acid was reduced by $LiAlH_4$ to give the corresponding alcohol **34** quantitatively (Scheme 18). Alcohol **34** was converted to the 2-naphthalenemethyl bromide (**35**) by treatment with PBr_3. Finally, **35** was allowed to react with glycolic acid tetramethylammonium salt to give the desired ester **36** in 81% yield.

Scheme 18.



The 2-naphthalenemethyl ester **36** was coupled with the *N*-acetyl dipeptide **20** using the DPPA procedure to give *N*-Ac-D-Phe-L-Phe-glycolic acid 2-naphthalenemethyl ester (**37**) in 45% yield (Scheme 19). The 2-naphthalenemethyl group was subsequently removed by hydrogenolysis to afford *N*-Ac-D-Phe-L-Phe-glycolic acid (**32**) in 92% yield after purification.

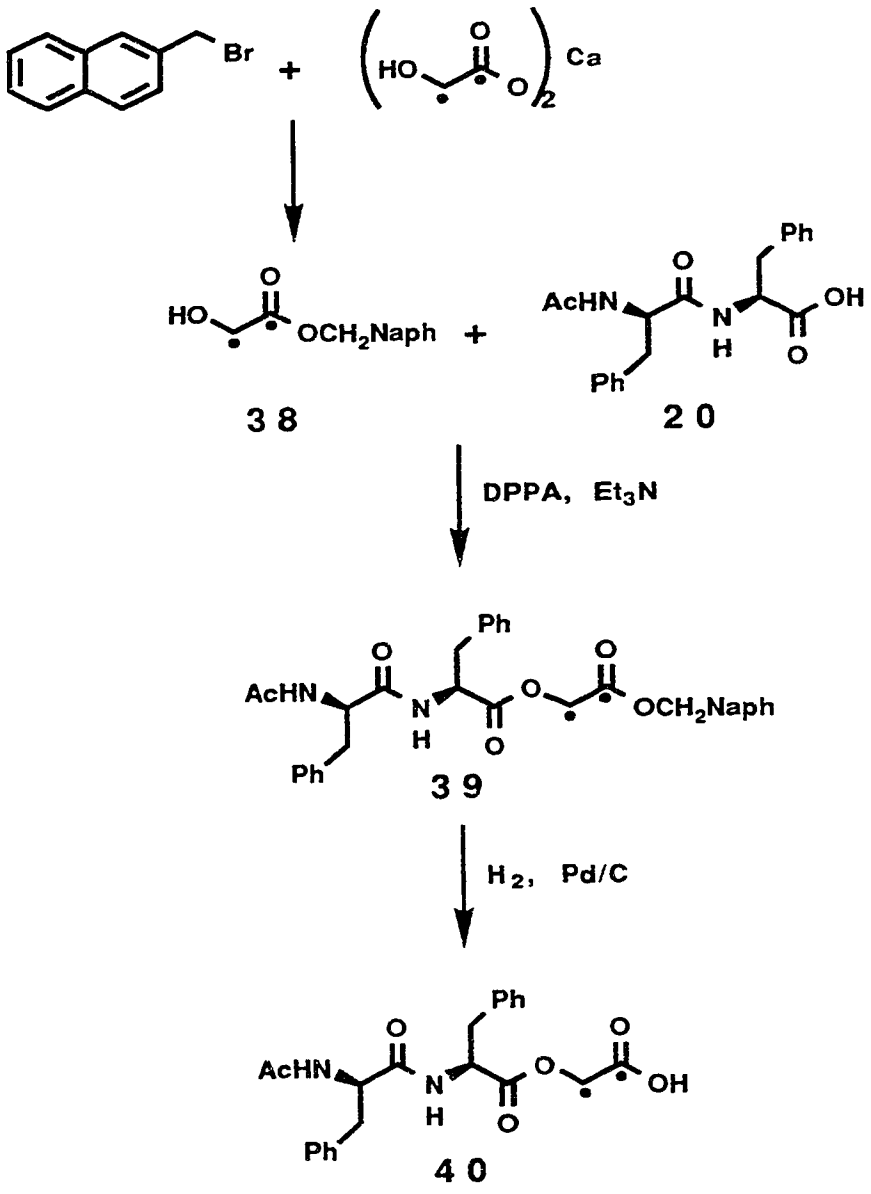
Scheme 19.



The labeled material was synthesized in an analogous manner. Since the tiny amount of the radioactive material always created problems during the synthesis, the specific activity of the commercially available [1,2-^{14}C]-glycolic acid was diluted to 10 μCi/μmol to obtain a larger amount of material for the synthesis. The diluted [1,2-^{14}C]-glycolic acid (68.0 μCi, 6.8 μmol) was converted to the ester **38** in 14% yield (Scheme 20). Coupling of the labeled ester **38** with the dipeptide **20** using the DPPA procedure gave the protected peptide analog **39** in 25% yield. Hydrogenation of **39** gave the desired product **40** in 87% yield. This material was further purified by reverse-phase HPLC, and was then assayed against the PHM enzyme using the nitrosobenzene assay procedure.

While the PHM assays with D-Tyr-L-Val-[1,2-^{14}C]-Gly gave high turnovers, the assays with labeled glycolate peptide **40** gave turnovers only slightly above background. These assays were repeated with solutions which were diluted with unlabeled

Scheme 20.



N-hydroxyformanilide (NHF) and glycolate tripeptide, and then purified by reverse-phase HPLC. The peaks for NHF and glycolate tripeptide were collected directly into scintillation vials and counted. The results were very similar to those obtained with the nitrosobenzene procedure (Table 5).

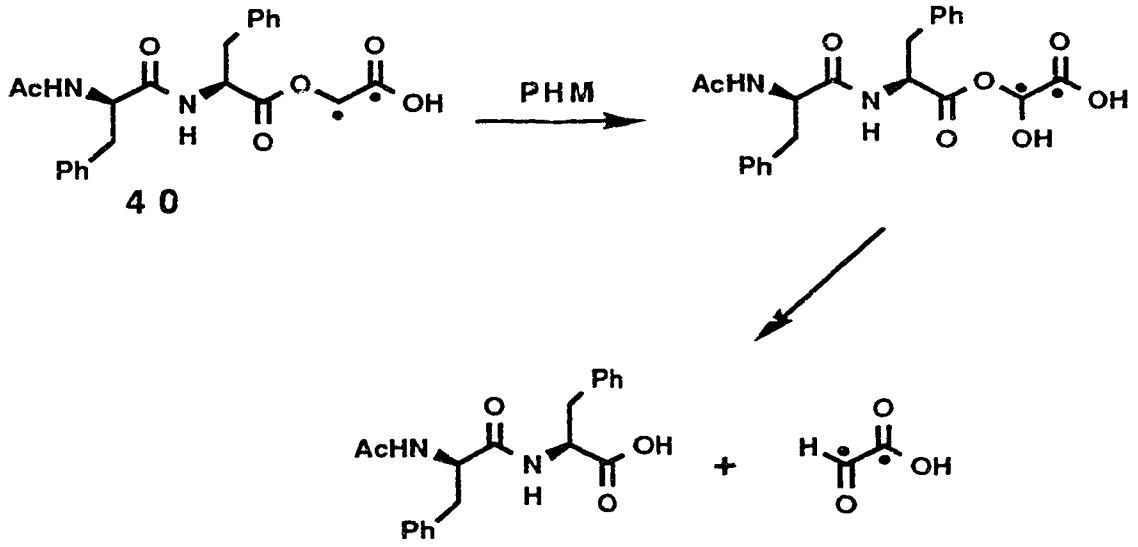
Table 5[a] PHM Assay with Glycolate Peptide Using the Nitrosobenzene Procedure

	40	**40[b]**	**9**
Assay	239 ± 0.0377% dpm	117 ± 0.128% dpm	4866±0.0169% dpm
Background	139 ± 0.0692% dpm	63 ± 0.137% dpm	50 dpm

[a]These experiments were performed in duplicate, the average result is reported.
[b]The assay solutions were separated by reverse-phase HPLC.

Although the turnover of the labeled glycolate peptide is above background, it is very small compared to the turnover for the D-Tyr-L-Val-[1,2-^{14}C]-Gly tripeptide **9**. The fact that **40** is oxidized by PHM suggests the mechanism proceeds by direct carbon hydroxylation (Scheme 21). However this is not conclusive evidence for the mechanistic pathway because of the small difference between the background and the trapped glyoxylates. It is possible that **40** is also a strong inhibitor of PHM, thus resulting in

Scheme 21.



40

the lower turnover. Attempts were made to prepare more labeled peptide analogues, but these were unsuccessful.

Recently, May and Katopodis[79] reported that the ester [(4-methoxybenzoyl)oxy] acetic acid is not a PHM substrate and shows competitive inhibition of the enzyme, while the amide 4-methoxyhippuric acid (K_M = 1.0 mM) is a substrate for PHM. In contrast the ether analog [(4-nitrobenzyl)oxy]acetic acid (K_M = 0.16 mM) is an excellent substrate compared to the amide. They argued that the difference between the ester and the ether analogues may reflect small differences in binding, allowing an α-hydroxylation pathway to be operative in the case of the ether, but resulting in the α-protons of the ester being inaccessible to the copper-oxygen species.

In another mechanistic study, Young and Tamburini[86] prepared *N*-dansyl-Tyr-Val-Gly and the *N*-hydroxyglycine derivative, and found that *N*-dansyl-Tyr-Val-Gly was a good substrate, but that the *N*-hydroxyglycine derivative was not converted by the α-amidating enzyme. This result strongly disfavors the *N*-hydroxylation pathway (b in Figure 2), since the corresponding intermediate is not accepted by the enzyme.

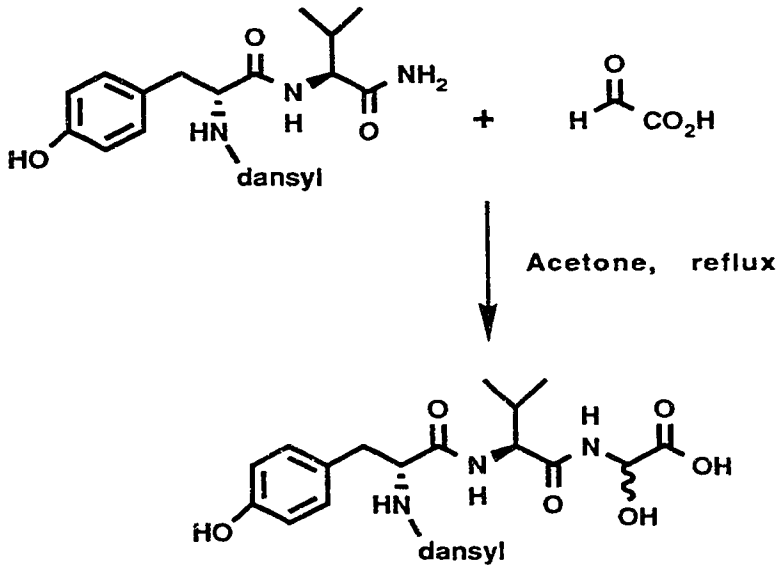
2.4 STUDIES TO DETERMINE THE STRUCTURE AND STEREOCHEMISTRY OF THE PHM REACTION PRODUCT

Synthesis of α-Hydroxyglycine Peptides and Indentification of the Enzyme Reaction Intermediate

In 1989, Young and Tamburini[86] reported that only one isomer of the α-hydroxyglycine derivatives is an intermediate in the pathway of enzymatic α-amidation of *N*-dansyl-Tyr-Val-Gly. This was demonstrated by synthesizing α-hydroxyglycine derivatives of *N*-dansyl-Tyr-Val-Gly by condensing *N*-dansyl-Tyr-Val-NH_2 with glyoxylic

acid in refluxing acetone (Figure 11). The resulting diastereomers were separated by reverse-phase HPLC.

Figure 11. Synthesis of *N*-Dansyl-Tyr-Val-α-HydroxyGly

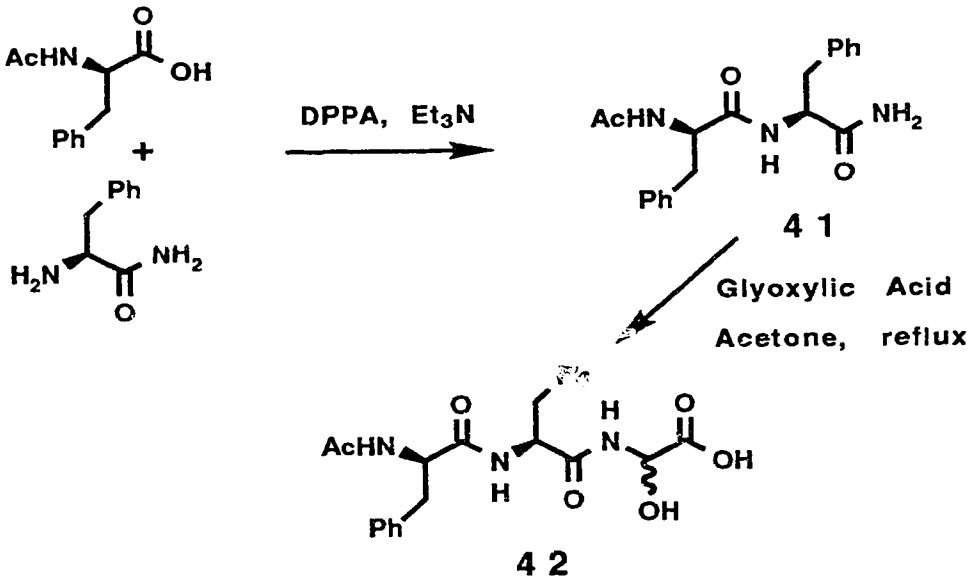


The ability of a homogeneous α-amidating enzyme to convert the α-hydroxyglycine analogues was then studied. They discovered that only one of the α-hydroxyglycine peptides, which eluted later on a reverse-phase C_{18} HPLC column, was converted to the peptide amide. The conversion of this single isomer occurred at rates substantially greater than those observed for the conversion of *N*-dansyl-D-Tyr-L-Val-Gly, and this process was independent of L-ascorbate.

Although they proposed that this is the enzyme reaction intermediate, they did not detect the intermediate produced directly from the normal substrate after enzyme oxidation. Hence, it appeared desirable to investigate the production of a hydroxyglycine intermediate using a radioactive dilution method and reverse-phase HPLC.

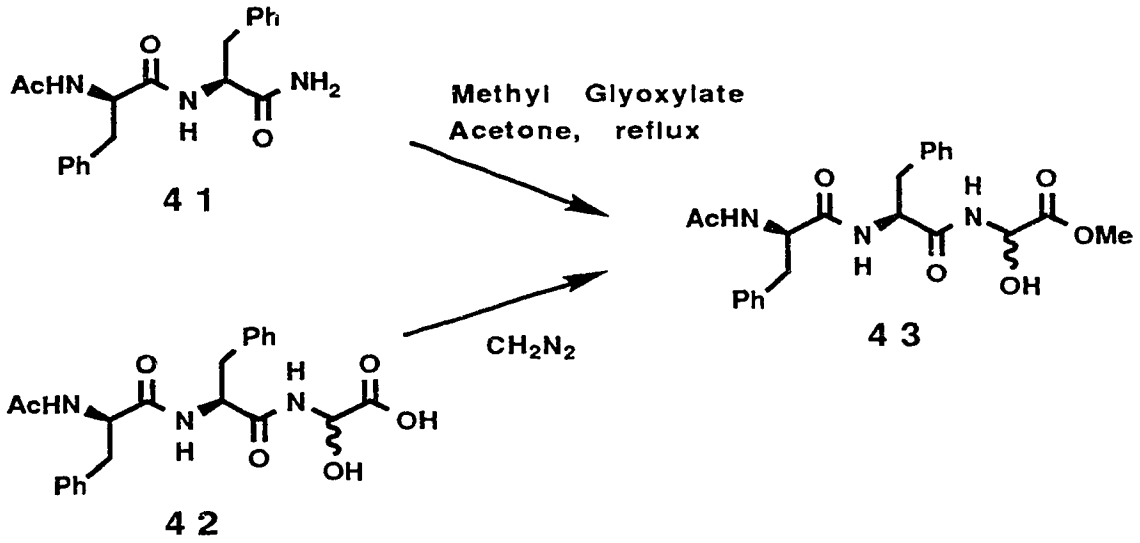
A mixture of isomers of *N*-Ac-D-Phe-L-Phe-(α-hydroxyGly) could be prepared as illustrated in Scheme 22. Coupling of commercially available *N*-acetyl-D-phenylalanine with L-phenylalaninamide using the DPPA procedure gives **41** in 75% yield. Condensation of peptide amide **41** with glyoxylic acid in refluxing acetone produces the desired α-hydroxyglycine peptide diastereomers **42** in 60% yield.[120]

Scheme 22.



The corresponding methyl ester **43** is prepared from the condensation of **41** and methyl glyoxylate (prepared by Dr. L. K. Lam of our group) (Scheme 23) in 64% yield. The free acid **42** is also easily esterified by diazomethane to give **43** in quantitative yield.

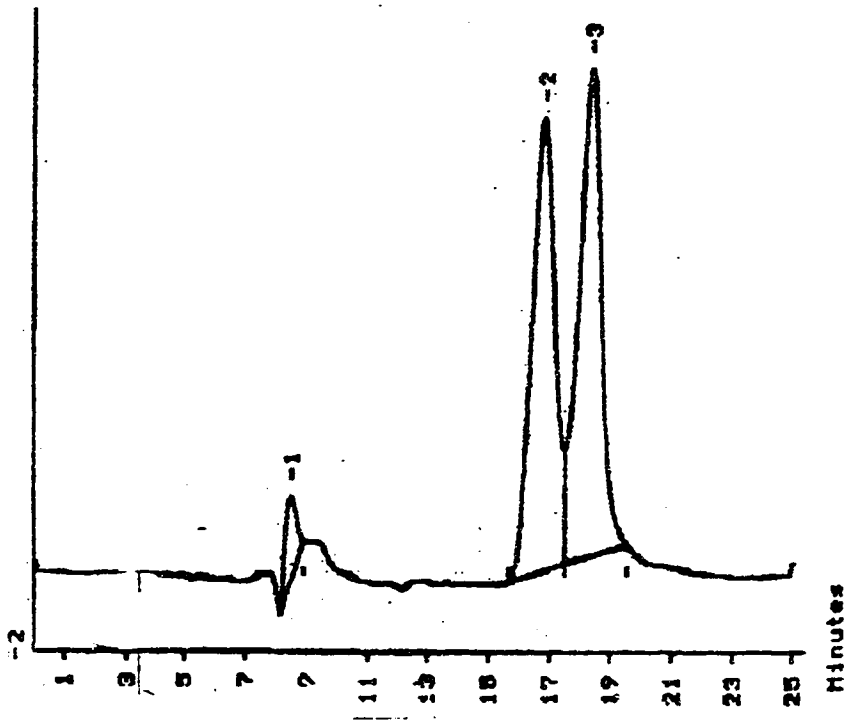
Scheme 23.



Although the isomers of **42** could not be separated by reverse-phase HPLC on either C_{18} or C_8 columns, they were easily separated by reverse-phase HPLC on two aminopropyl columns (Figure 12).

To identify which isomer is the enzyme oxidation product, a radioactive dilution experiment was performed. Assays using *N*-Ac-D-Phe-L-Phe-[1,2-^{14}C]-Gly (**23**) (205,000 dpm, 0.953 nmol, 384 ng) and a cocktail containing ascorbic acid, copper sulfate, and potassium iodide were added to the enzyme solution, and incubated at 37 °C for 2 h. Identical control assays with boiled enzyme (inactive) were done simultaneously. In some cases, unlabeled glyoxylic acid and four equivalents of nitrosobenzene were added to the solutions, heated for 1 h at 60 °C, and then assayed as usual. The results from this nitrosobenzene assay procedure are listed in Table 6. To the other solutions, 750. μg of **42** and 800. μg of *N*-Ac-D-Phe-L-Phe-Gly (**22**) were added and these were then separated directly by reverse-phase HPLC. The HPLC conditions were the same as described above for the separation of **42** using one aminopropyl column, the retention

Figure 12. HPLC Separation of the Two Isomers of 42 by Using Two Aminopropyl Columns



The conditions for reverse-phase HPLC separation are: Waters aminopropyl μ-Bondapak Radial Pak 8 mm x 10 cm cartridge, 10 μm particle size; A: 0.1% TFA in water, B: 0.1% TFA in 80:20 acetonitrile-water; isocratic elution at 35% B; flow rate 1.00 mL/min; detection at 214 nm. Peak 2 is for **42A**, peak 3 is for **42B**.

Table 6[a] Assay of *N*-Ac-D-Phe-L-Phe-[1,2-^{14}C]-Gly (23) Using the Nitrosobenzene Procedure

	Compound 23
Assay	7051 ± 0.0962% dpm
Control	900 ± 0.0411% dpm

[a]These experiments were performed in duplicate, the average results are reported.

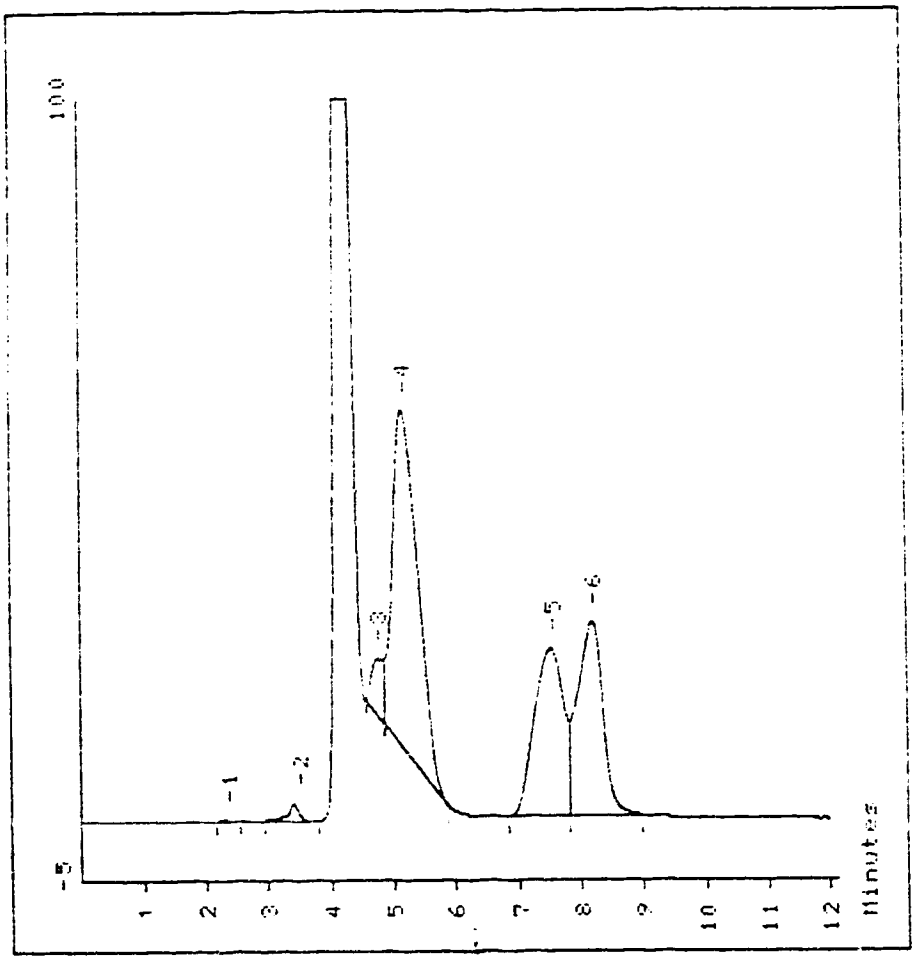
time for the substrate **22** is 5.20 min. Figure 13 shows a chromatogram of the HPLC separation of the enzyme assay solution after dilution by cold authentic materials. The peaks corresponding to substrate and the two diastereomers A and B were collected separately, and the radioactivity for each fraction was determined. The results from the radioactive dilution and reverse-phase HPLC separation are listed in Table 7.

Table 7[a] HPLC Separation of *N*-Ac-D-Phe-L-Phe-[1,2-^{14}C]-Gly (23) and *N*-Ac-D-Phe-L-Phe-(α-HydroxyGly) (42A and 42B)

	Assay	Control
Compound 23	24,263 ± 0.0869% dpm	99,514 ± 0.0331% dpm
Isomer A	103,780 ± 0.0489% dpm	845 ± 0.108% dpm
Isomer B	4,537 ± 0.0148% dpm	791 ± 0.0354% dpm

[a]These experiments were performed in duplicate, the average results are reported.

Figure 13. HPLC Separation of the Enzyme Assay Solution After Dilution by *N*-Acetyl-D-Phenylalanyl-L-Phenylalanyl-α-Hydroxyglycine



The conditions for reverse-phase HPLC separation are: Waters aminopropyl μ-Bondapak Radial Pak 8 mm x 10 cm cartridge, 10 μm particle size; A: 0.1% TFA in water, B: 0.1% TFA in 80:20 acetonitrile-water; isocratic elution at 35% B; flow rate 1.00 mL/min; detection at 214 nm. Peak 4 is for substrate **22**, peak 5 is for **42A**, peak 6 is for **42B**.

From the HPLC assay, it is clear that only isomer A is radioactive, indicating that it is the product of PHM oxidation. The small radioactivity, less than 5% of that for isomer A, found in isomer B is probably due to contamination by isomer A since the separation of the two isomers is not complete under these conditions. While the result here shows that the first isomer of *N*-Ac-D-Phe-L-Phe-(α-hydroxyGly) eluting from HPLC is the PHM reaction product, Young and Tamburini reported that the second isomer of *N*-dansyl-D-Tyr-Val-α-hydroxyGly eluting from HPLC is the PHM reaction product. The discrepancy can be explained by the change of the HPLC conditions. In fact, one would expect the change of retention times for both isomers after the hydrophobic C_{18} column was replaced by the hydrophilic aminopropyl column even though the solvents were the same in both cases.

While the HPLC assay also shows that more than 50% of the substrate was converted to the α-hydroxyglycine peptide, the nitrosobenzene assay only detected 3.4% conversion of the starting substrate. This indicates that most of the α-hydroxyglycine peptide does not cleave to product under our assay conditions and that the enzyme isolated by our procedure is mostly PHM with little, if any PAL.

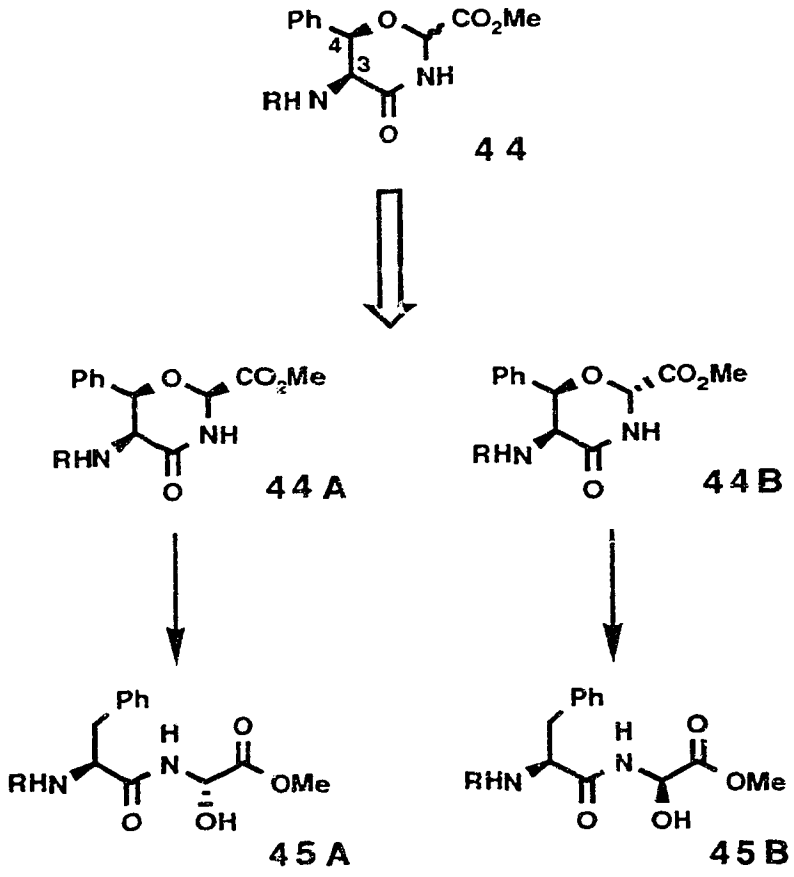
The formation of α-hydroxyglycine in PHM oxidation was further confirmed by Tajima and co-workers.[87] They used phenylalanyl-glycyl-phenylalanyl-glycine as a substrate for the amidating enzyme purified from equine serum. The PHM reaction product, phenylalanyl-glycyl-phenylalanyl-α-hydroxyglycine, was isolated by reverse-phase HPLC from the enzyme reaction mixture and the structure of the α-hydroxyl derivative was determined by fast atom bombardment mass spectrometry and proton NMR.

Studies to Determine the Structure of α-Hydroxyglycine Diastereomers by Independent Synthesis

Although it was shown that only one α-hydroxyglycine peptide derivative was the product of PHM oxidation, the absolute stereochemistry was not known. To assign the

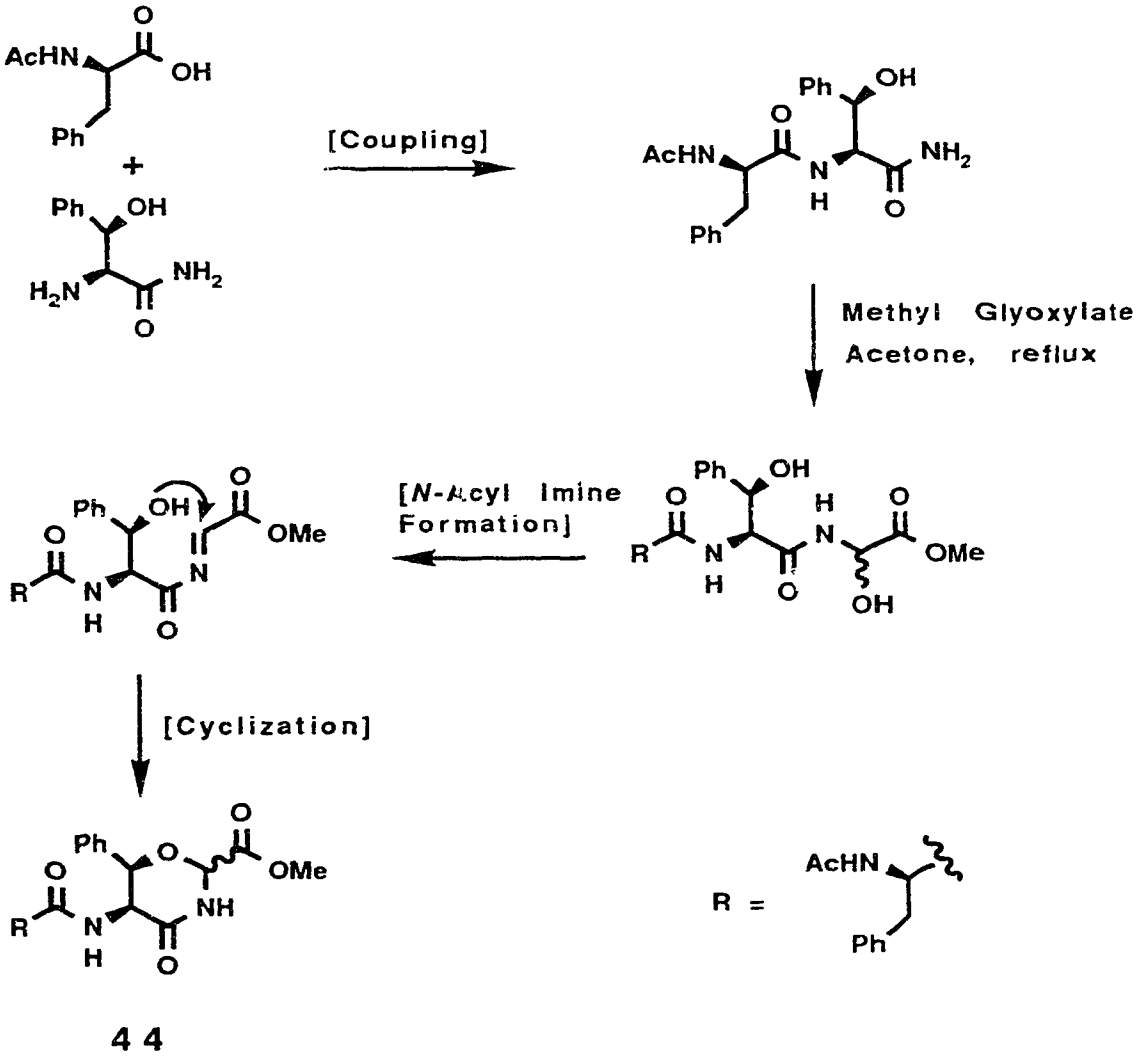
stereochemistry, an independent synthesis was designed. If the six-membered ring peptide analogue **44** could be prepared, the two isomers **44A** and **44B** of this compound should be separable by HPLC (Scheme 24). With known absolute stereochemistry at positions 3 and 4, the configuration at C-6 of each isomer could then be determined by n. O. e. measurements. Catalytic hydrogenolysis should then open the ring to give the corresponding peptides as the *S*-isomer **45A** and the *R*-isomer **45B**. HPLC comparison of the two isomers with the product from the PHM oxidation after methylation would define the stereochemistry of the enzyme product.

Scheme 24.



The proposed synthesis of compound **44** from L-β-phenylserinamide is shown in Scheme 25. Commercially available *N*-acetyl-D-phenylalanine could be coupled with L-β-phenylserinamide to give the dipeptide amide. This compound would then be condensed with methyl glyoxylate to give the α-hydroxyglycine derivative. Intramolecular cyclization via an *N*-acylimine should give the desired product **44**.

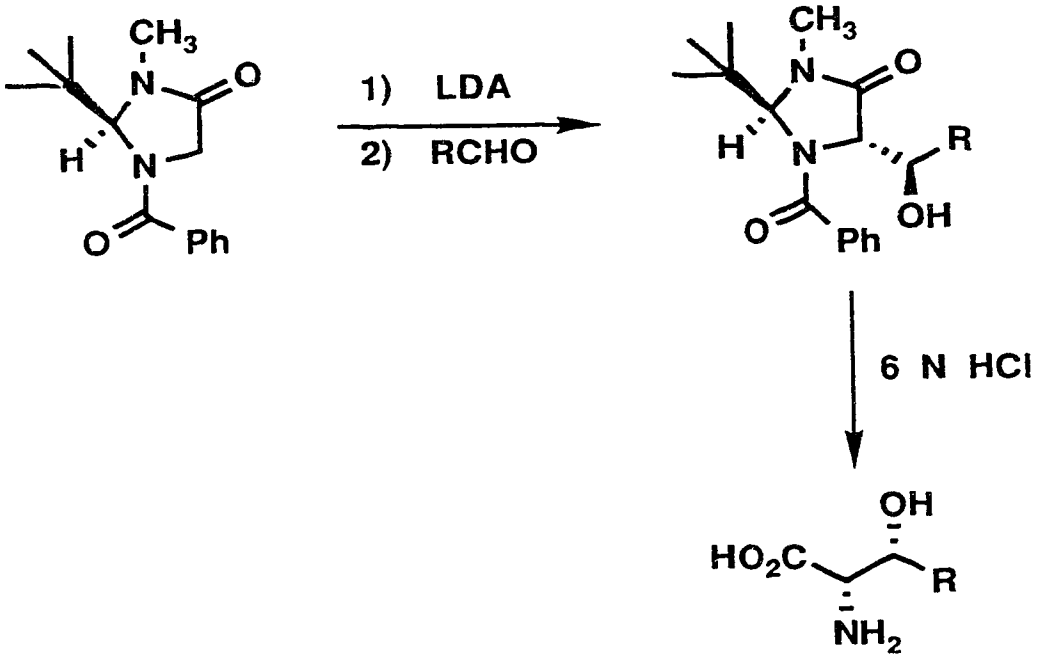
Scheme 25.



44

To implement Scheme 25, a highly stereoselective method to prepare β-phenylserinamide is necessary. During the last decade, a large number of synthetic methods have been established to stereoselectively synthesize β-hydroxy amino acids.[121-133] An attractive approach is Seebach's[124] method, which uses a chiral imidazolidinone enolate to serve as a glycine-equivalent which can undergo condensations with a variety of aldehydes to yield the corresponding aldol adducts (Figure 14). These readily hydrolyze in aqueous HCl to β-hydroxy amino acids. The high stereoselectivity of this aldol condensation can be rationalized as follows. When the electrophilic carbonyl compound approaches the chiral enolate, the entry *cis* to the bulky *tert*-butyl group is sterically encumbered; therefore the attack of an electrophile *anti* to the *tert*-butyl group proceeds predominantly to generate the *anti* diastereomer. Another significant advantage of this process, is control of the stereochemistry at the C-3 hydroxy center (diastereomeric

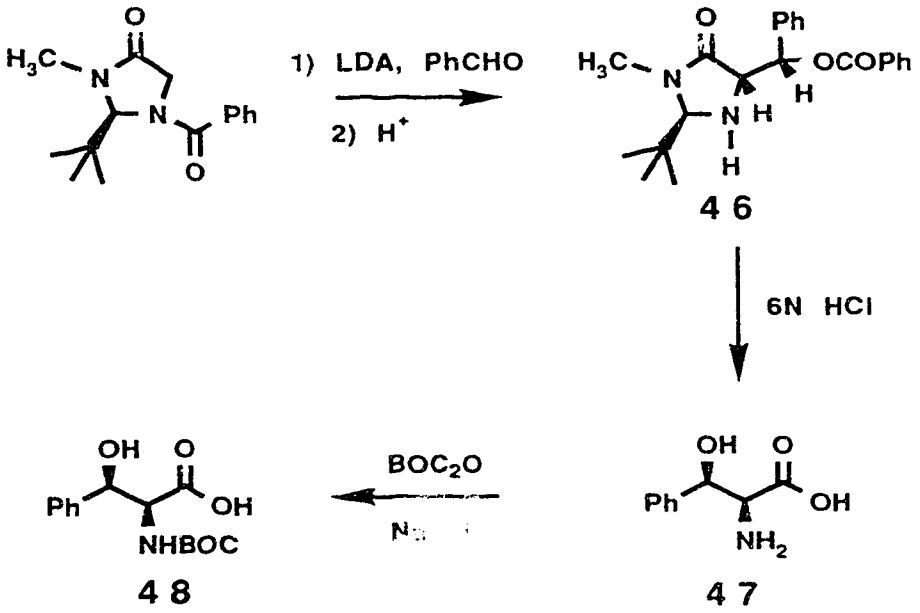
Figure 14. Seebach's Method for β-Hydroxy Amino Acids



excess (d.e.) >95%). This may be due to a delicate conformational interaction between the chiral enolate and the trigonal carbonyl group of the aldehyde during the condensation, as proposed by Seebach and co-workers.[124]

Hence, the L-β-phenylserine needed for the preparation of L-β-phenylserinamide was synthesized by Seebach's method. The enolate of commercially available (2*S*)-1-benzoyl-2-(1,1)-dimethylethyl-3-methyl-4-imidazolidinone, generated with LDA, reacts with benzaldehyde to produce the rearranged compound **46** in 84% yield after chromatographic purification (Scheme 26). The rearrangement to produce **46** involves migration of the benzoyl group from nitrogen as is reported by Seebach and co-workers for the condensation of benzaldehyde with (2*S*)-1-benzoyl-2-(1,1)-dimethylethyl-3-methyl-4-imidazolidinone. Acidic hydrolysis of **46** gave L-β-phenylserine (**47**) in 95% yield. The amino group was then protected with a BOC group using di-*tert*-butyl pyrocarbonate under basic conditions to produce **48** in 73% yield.

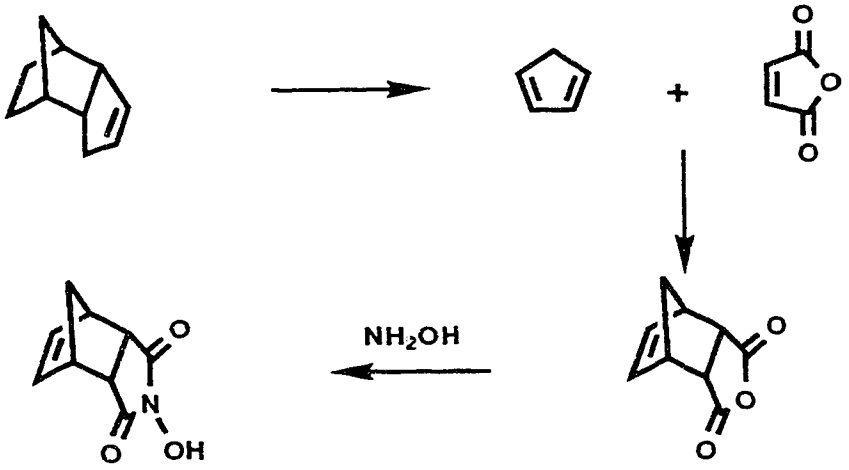
Scheme 26.



To convert the acid **48** to the corresponding amide, a reagent which selectively activates the carboxyl group was required. *N*-Hydroxy-5-norbornene-endo-2,3-dicarboximide (HONB) (**50**) was reported by Fujino *et al.*[134] to activate the serine carboxyl group for their peptide synthesis, without affecting the hydroxyl group. This method is basically a "one step reaction", which allows peptides to be prepared with little or no racemization.

HONB (**50**) was easily prepared in two steps by the literature procedures[135,136] (Scheme 27). Fractional distillation of cyclopentadiene dimer gives the monomer which undergoes a Diels-Alder reaction with maleic anhydride to give **49** quantitatively.[135] Treatment of the anhydride **49** with hydroxylamine at 65 °C for 1 h affords **50** in 96% yield.[136]

Scheme 27.

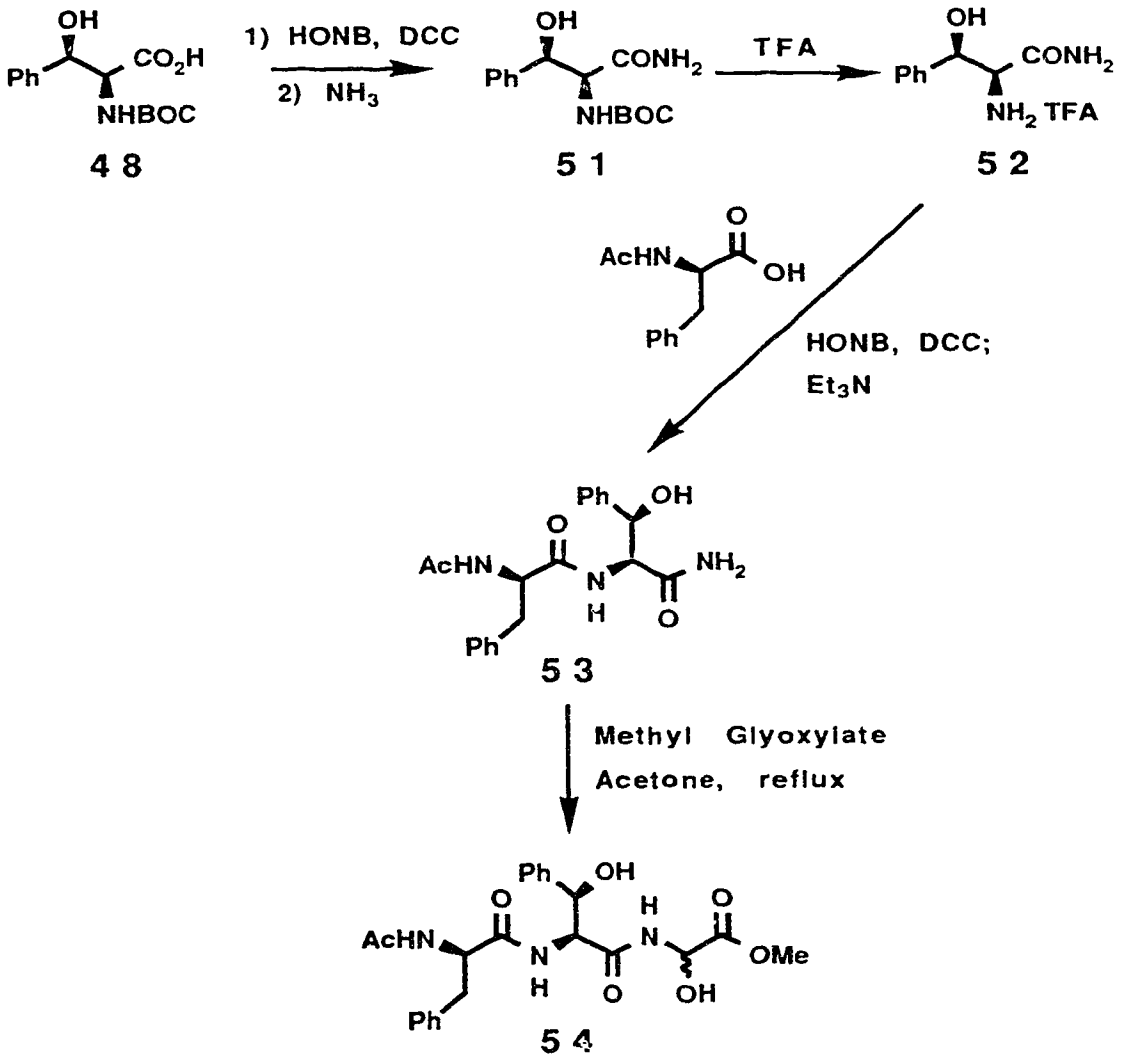


50 **49**

N-BOC-L-β-Phenylserine (**48**) was activated with HONB (**50**) and DCC; subsequent treatment with aqueous ammonia solution gave the amide **51** in 82% yield (Scheme 28). The *N*-BOC group was then removed by treatment with neat TFA to give

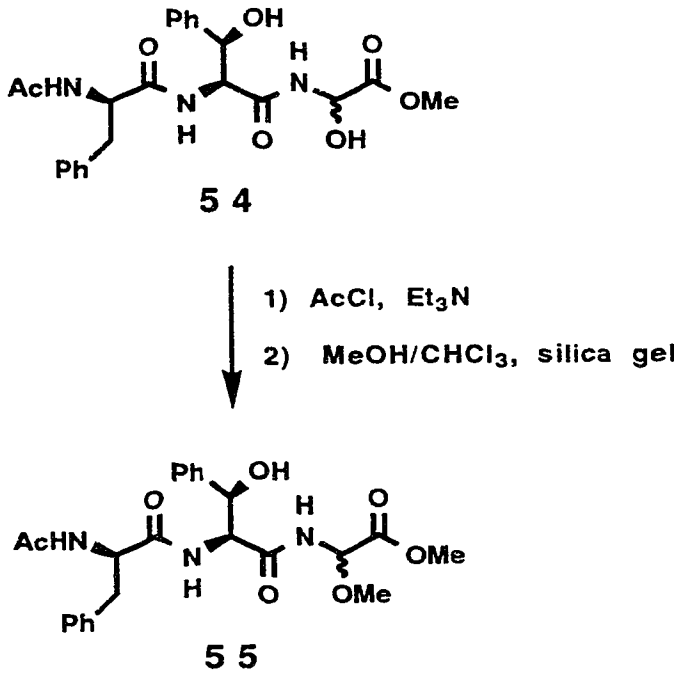
L-β-phenylserinamide trifluoroacetate salt (**52**) in 97% yield. Compound **52** was coupled with commercially available *N*-acetyl-D-phenylalanine using the HONB/DCC procedure to generate **53** in 83% yield. This amide was condensed with excess methyl glyoxylate in refluxing acetone to give the desired product **54** in 79% yield as a 1:1 mixture of diastereomers (by ^{1}H NMR).

Scheme 28.



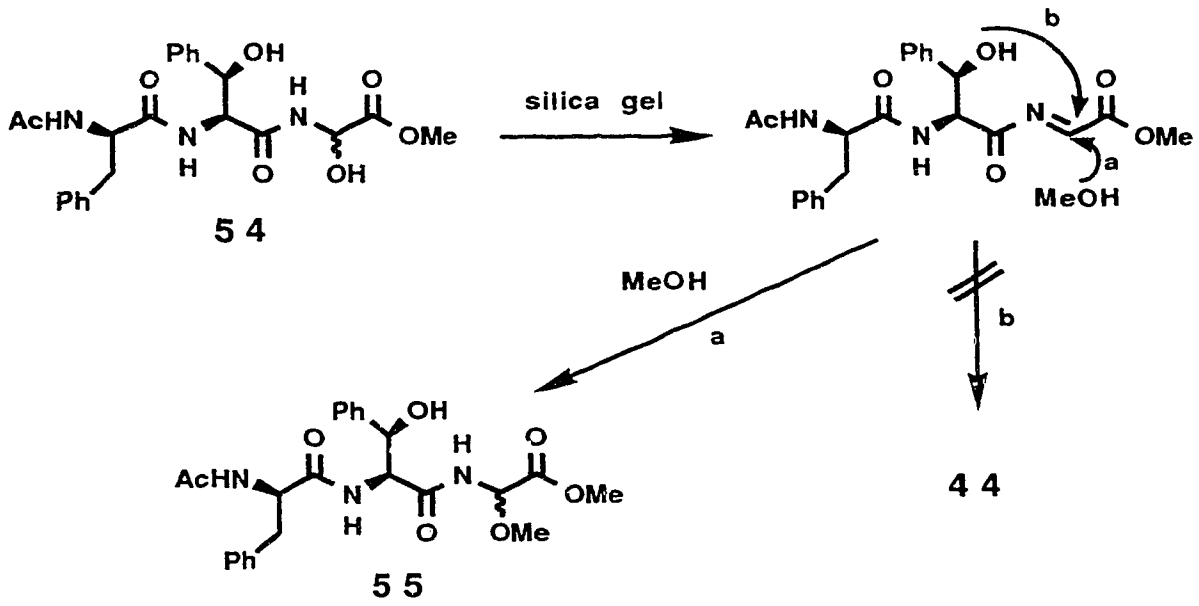
In order to generate the desired *N*-acyl imine from compound **54**, a variety of reagents, which are reported to catalyze the formation of *N*-acyl imines such as $BF_3{\cdot}Et_2O$,[137] TFA,[138] formic acid, *p*-toluenesulfonyl chloride and methanesulfonic acid,[139] were tried. However, the desired cyclized product **44** could not be isolated. An attempt was then made to acetylate the α-hydroxyl group using acetyl chloride and triethylamine (Scheme 29). After silica gel purification using 6% MeOH in $CHCl_3$ as eluent, the methoxy ether **55** was obtained. The product produced from the acetylation

Scheme 29



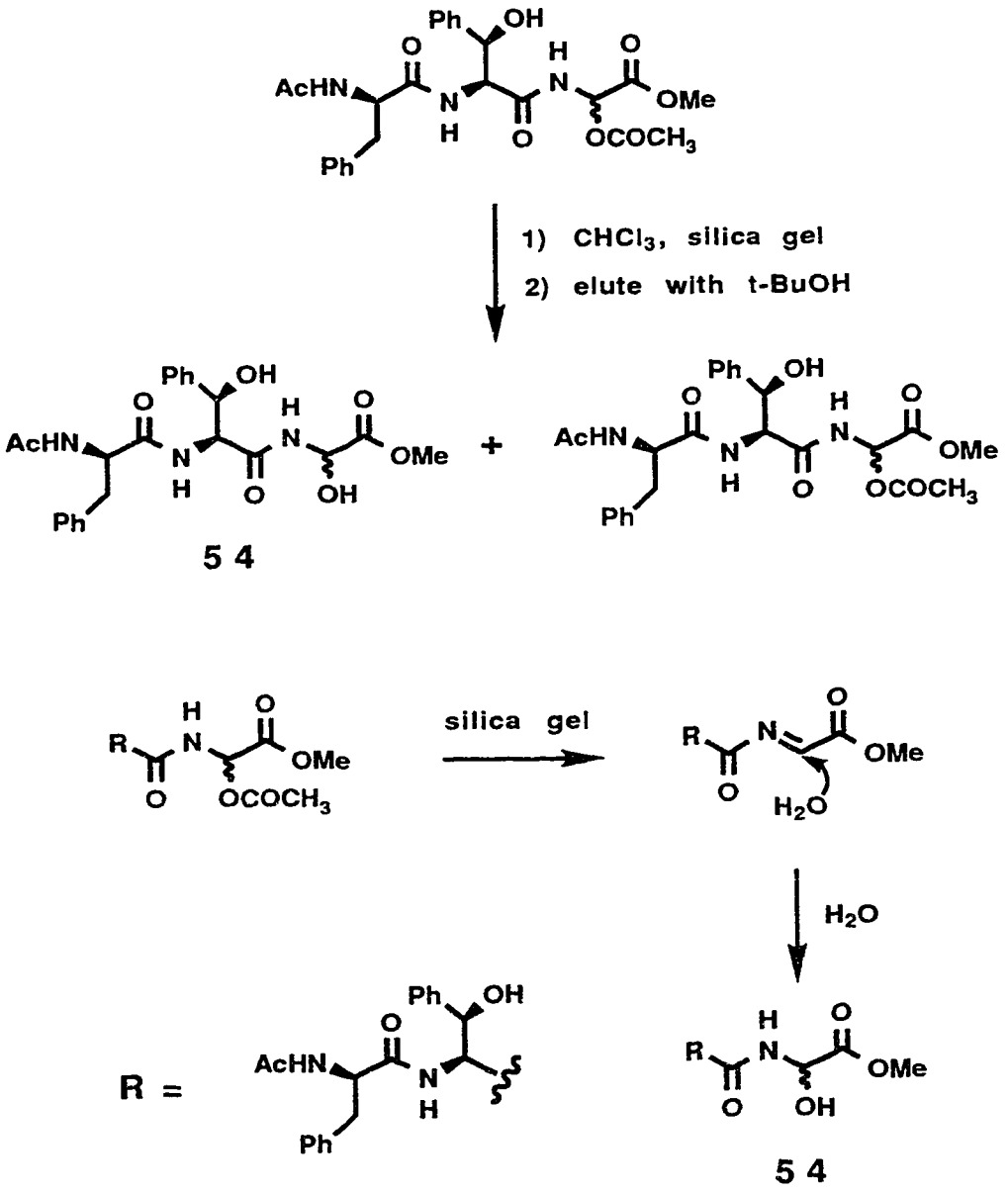
presumably forms the *N*-acyl imine on silica gel under mild acidic conditions (Scheme 30). The formation of the six-membered ring compound **44** via intramolecular cyclization (path b) must be disfavored in this case; the intermolecular addition by methanol (path a) proceeds to give **55**.

Scheme 30.



The above hypothesis was confirmed by the following experiment (Scheme 31). Approximately 7 mg of acetylated **54** (without purification by silica gel) was dissolved in 3 mL of $CHCl_3$ followed by the addition of 200 mg of silica gel. After 60 min of stirring at room temperature, this mixture was passed through a pipette and eluted with *tert*-butyl alcohol. TLC showed spots corresponding to acetylated product and starting material **54**. This suggests that some of the acetylated peptide forms the *N*-acyl imine and subsequent water addition to this highly reactive acyl imine affords the starting α-hydroxyglycine derivative. When the column was eluted with *tert*-butyl alcohol, the unreacted acetylated peptide and the α-hydroxyglycine derivative were washed off the column. Due to steric hindrance, *tert*-butyl alcohol cannot react in the same manner as methanol to give the ether product.

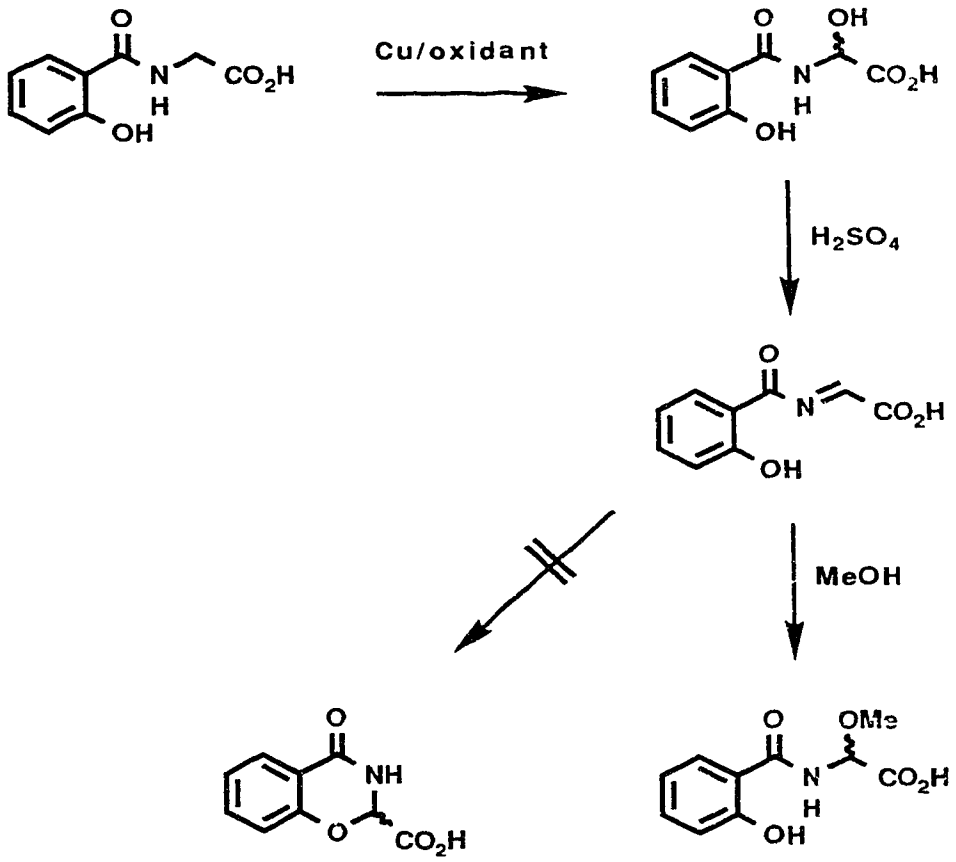
Scheme 31.



The fact that the intermolecular reaction is preferred over the six-membered ring formation is further confirmed by a recent report by Capdevielle and Maumy[88] on the chemical oxidation of *N*-salicyloyl-glycine (Figure 15). The product *N*-salicyloyl-α-hydroxyglycine from α-hydroxylation forms the α-methoxy compound under strong acid

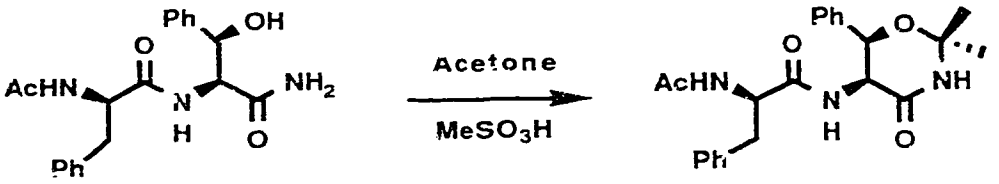
(H_2SO_4) catalysis in MeOH. The generally favored formation of an *endo* six-membered ring compound does not occur in this case.

Figure 15. The formation of *N*-Salicyloyl-α-MethoxyGly *via* *N*-Acyl Imine Intermediate



However, when **53** and one equivalent of glyoxylic acid were heated to reflux in acetone with two equivalents of methanesulfonic acid in an attempt to form the cyclized product, a six-membered ring compound **56** was isolated in 56% yield (Scheme 32).

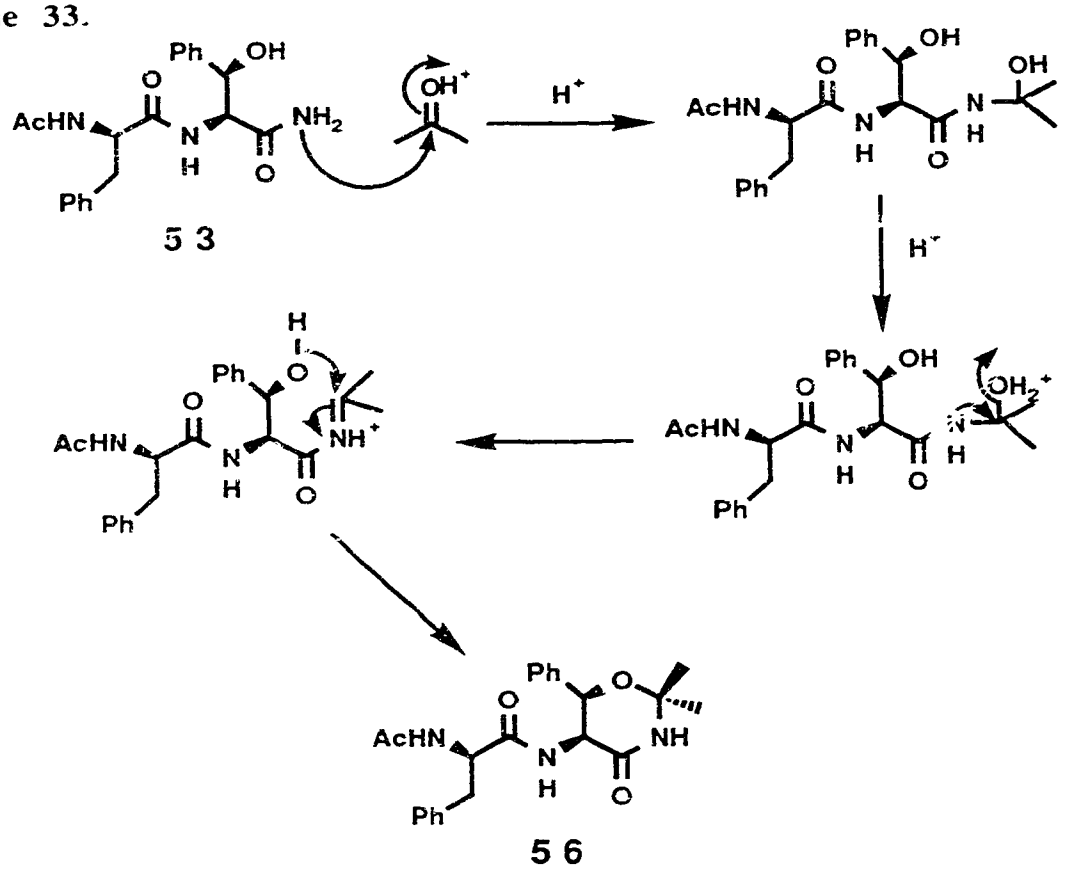
Scheme 32.



53 56

The formation of **56**, may proceed by attack of the primary amide on the carbonyl of acetone to give an aminal intermediate (Scheme 33). Under acidic conditions, the aminal intermediate may lose water to form an acyl imine intermediate which undergoes subsequent intramolecular cyclization to produce **56**.

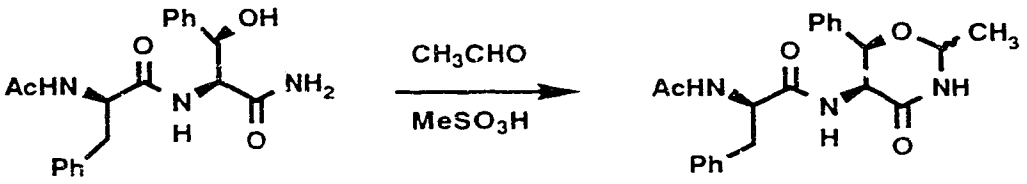
Scheme 33.



53 56

With this information, condensations of **53** with aldehydes such as cinnamaldehyde and dimethylacrolein under reflux were attempted, but the desired products could not be isolated. To test whether an aldehyde can react similarly to acetone, acetaldehyde was used. Thus, **53** was dissolved in acetaldehyde followed by the addition of methanesulfonic acid (two equivalents). The reaction mixture was stirred at room temperature for 6 h. Indeed, the desired product **57** was detected in the reaction mixture (Scheme 34). With dimethylacrolein, the expected reaction does not occur, probably because dimethylacrolein is too reactive and undergoes polymerization.

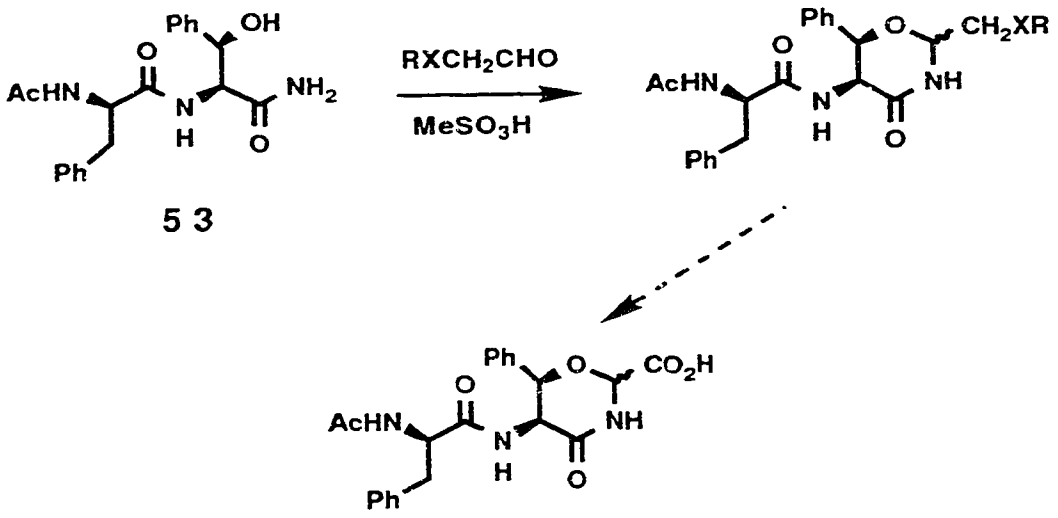
Scheme 34.



53 **57**

Presumably an appropriate aldehyde which allows formation of the six membered-ring, and whose side chain could be transformed to a carboxylic acid group, would provide access to the target compound (Scheme 35). Unfortunately, this could not yet be achieved.

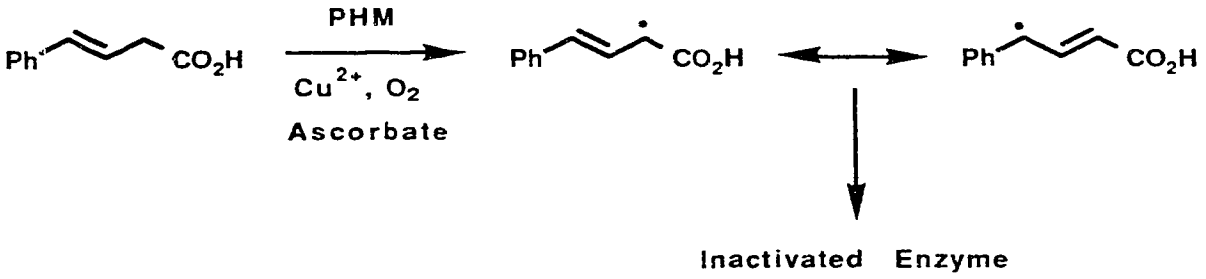
Scheme 35.



Studies to Determine the Absolute Configuration of PHM-Produced Peptidyl-α-HydroxyGlycine by Indirect Methods

Since attempts to assign the stereochemistry of the enzymatically formed peptidyl-α-hydroxyglycine were unsuccessful, a new strategy was designed to address the problem by indirect means. It has been reported that the known DBH inhibitor *trans*-4-phenyl-3-butenoic acid is also a potent mechanism-based inactivator of PHM (K_I = 1 μM).[78,79] It was proposed, as in the inactivation of DBH by this compound, that a free radical species inactivates the enzyme at the active site (Figure 16).[78,79]

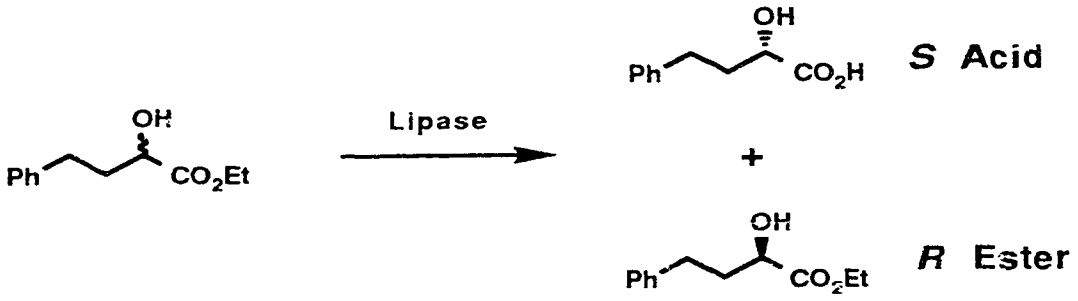
Figure 16. Proposed Inactivation Mechanism of PHM by *trans*-4-Phenyl-3-Butenoic Acid



Since it is unlikely that each catalytic event results in enzyme inactivation, it is reasonable to assume that a finite amount of the corresponding α-hydroxy acid would be formed. Because it is well established that the enzymic reaction is stereospecific, only one of the α-hydroxy acid isomers should be produced by PHM oxidation. If one could synthesize the two enantiomers of this acid separately, with known stereochemistry, the enantiomer having the same configuration as the enzymic product should show a much stronger "product" inhibition of the enzyme, thereby indirectly disclosing the stereochemistry of the PHM product.

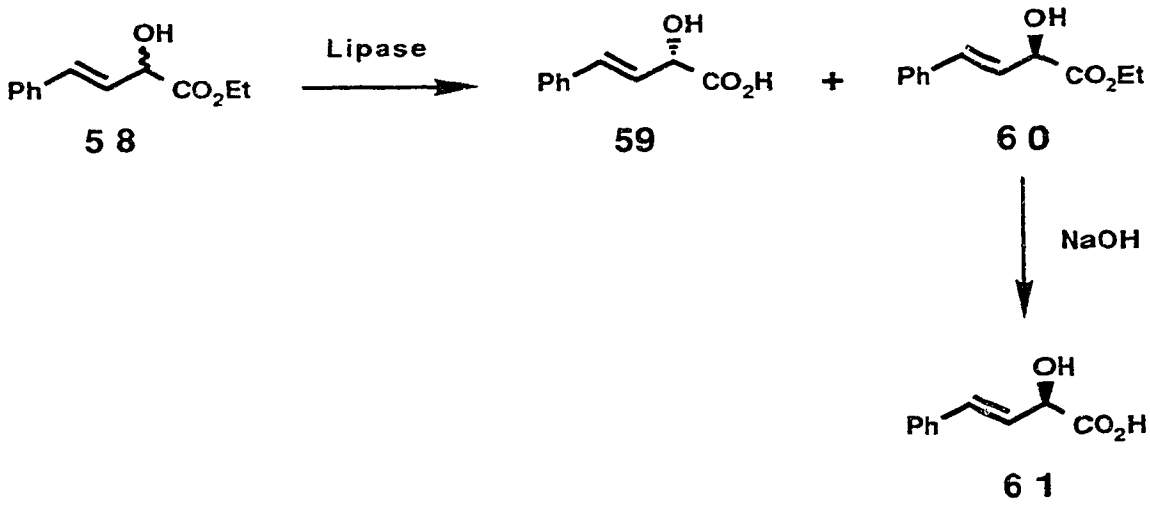
While the independent synthesis of both enantiomers has not been reported, the enzyme-catalyzed kinetic resolutions of substituted racemic esters to prepare enantiomercially pure substituted esters and acids are well documented.[145 -153] One closely related example is the kinetic resolution of racemic ethyl 2-hydroxy-4-phenylbutanoate (**58**) by a bacterial lipase from *Pseudomonas fluorescens* (Figure 17).[153]

Figure 17. Kinetic Resolution of Racemic Ethyl 2-Hydroxy-4-Phenylbutenoate by lipase from *P. Fluorescens*



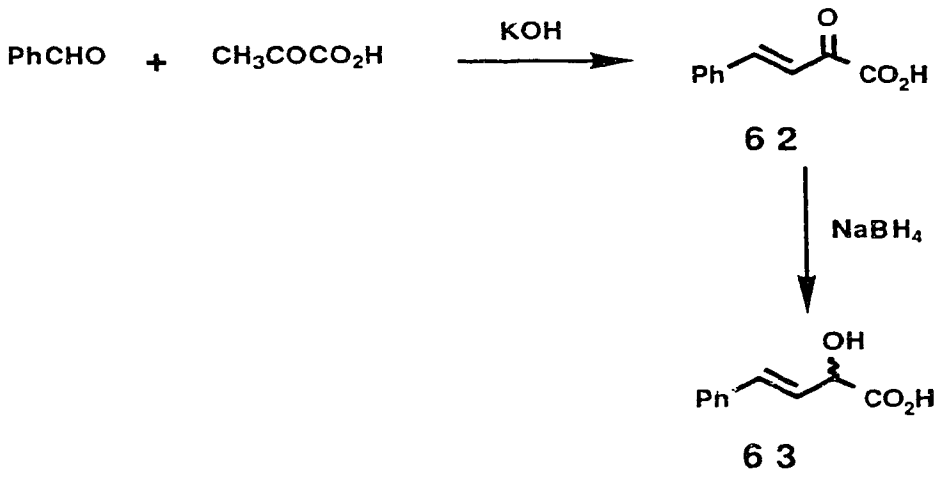
Preparation of the corresponding unsaturated racemic ester **58**, which only differs from the above compound by the presence of a double bond should provide a substrate for this lipase. The lipase should selectively hydrolyze the *S*-ester to the corresponding acid **59** and leave the *R*-ester **60** unhydrolyzed (Scheme 36). Compound **60** could be subsequently hydrolyzed under basic conditions to give the corresponding acid **61**.

Scheme 36.



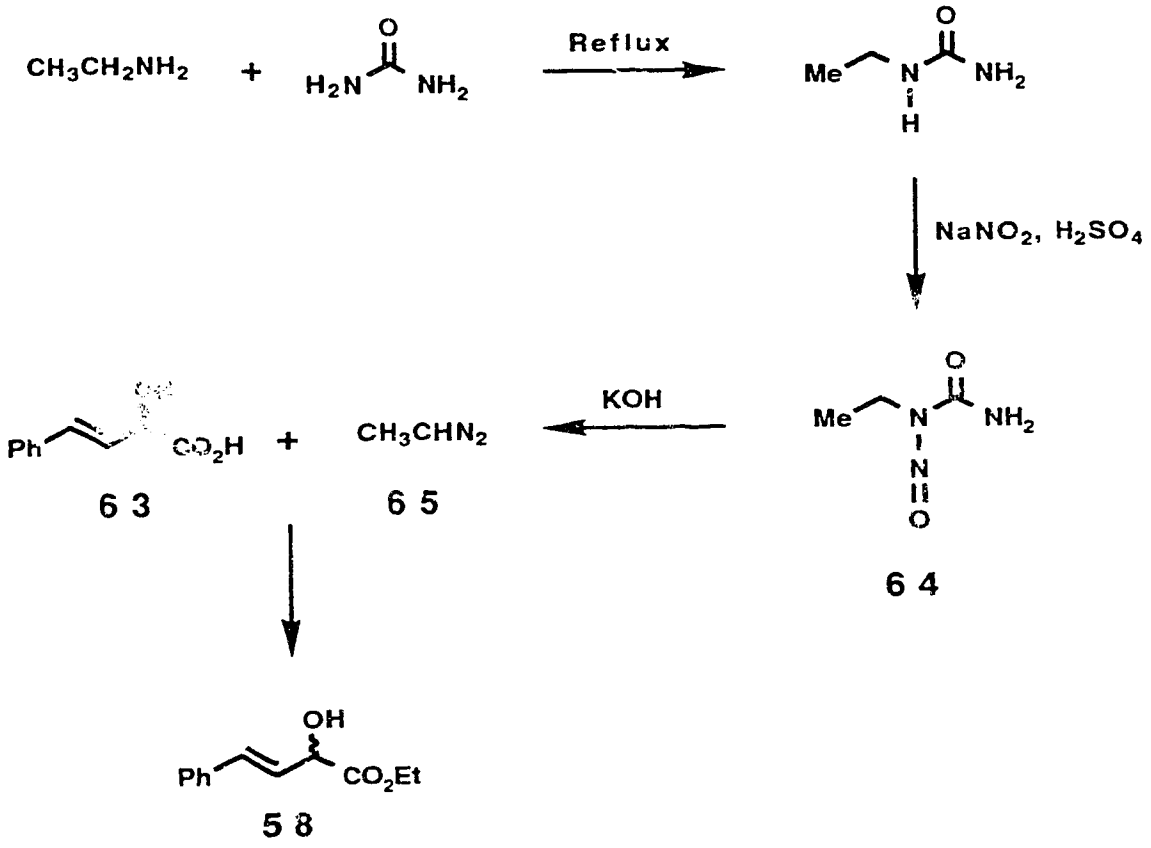
The racemic *trans*-2-hydroxy-4-phenyl-3-butenoic acid (**63**) was synthesized using a modification of the procedure of Coffen *et al.*[154] Benzaldehyde was condensed with pyruvic acid under basic conditions to give *trans*-4-phenyl-2-oxo-3-butenoic acid (**62**) in 96% yield (Scheme 37). Sodium borohydride reduction of **62** gave racemic α-hydroxy acid **63** in 89% yield.

Scheme 37.



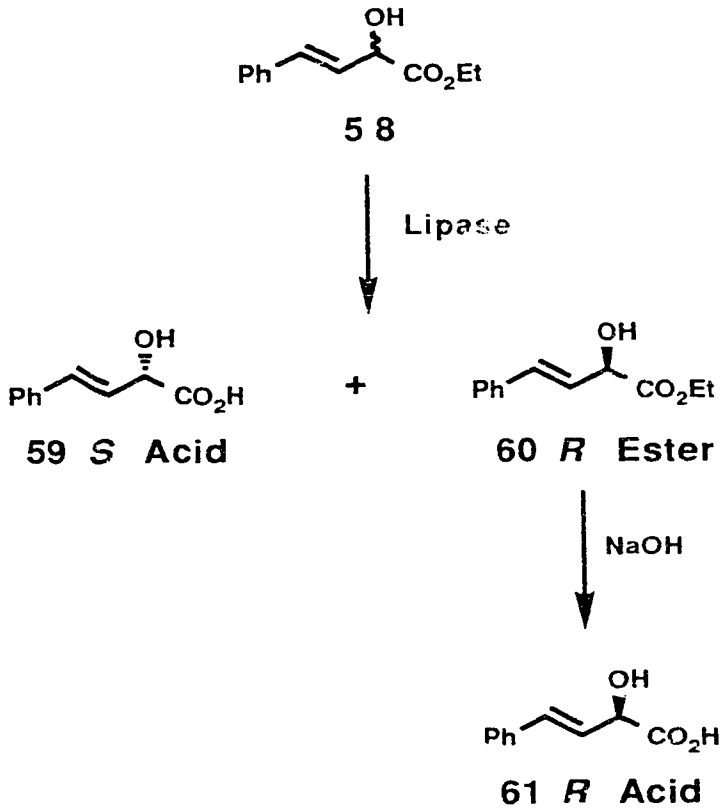
The diazoethane (**65**) needed to esterify the acid **63** was prepared as outlined in Scheme 38.[155] Ethylamine was treated with urea in boiling water to give ethylurea which was then treated with nitrous acid to give nitrosoethylurea (**64**) in 69% yield. Compound **64** reacts with potassium hydroxide to produce diazoethane (**65**), which was directly used for the esterification of **63**.

Scheme 38.



The resulting racemic ester **58** is subjected to enzyme catalyzed hydrolysis using commercially available lipase from *P. fluorescens* (Scheme 39). The *S*-acid **59** is obtained by stopping the hydrolysis at 41% conversion, and the *R*-ester **60** is obtained by stopping the reaction at 50% conversion. Ester **60** is then hydrolyzed by 1 N NaOH to give the *R*-acid **61** in 94% yield.

Scheme 39.



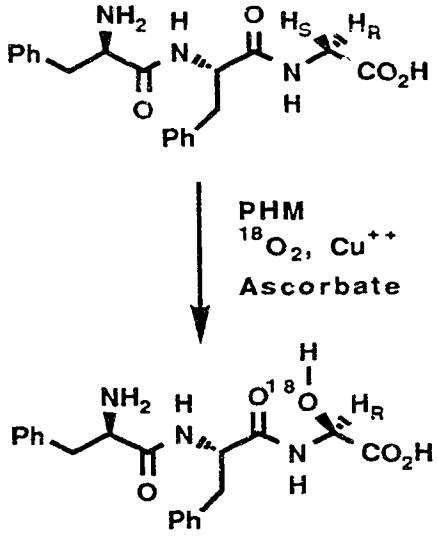
The enzyme inhibition studies were performed by Dr. T. M. Zabriskie. Although neither acid is a strong inhibitor for PHM, the *S*-acid **59** at 10 mM concentration showed 50% inhibition after 1 h incubation and the *R*-acid **61** showed 57% inhibition under the same conditions; at 1 mM concentration, neither acid showed any inhibition at all. Experiments were also done by Dr. T. M. Zabriskie to observe the formation of the expected product, *trans*-2-hydroxy-4-phenyl-3-butenoic acid. The incubated solution of *trans*-4-phenyl-3-butenoic acid (**62**) and the PHM enzyme was separated by reverse-phase HPLC, but the expected product was not detected.

The two isomers of *N*-Ac-D-Phe-L-Phe-α-hydroxyGly **42** were also separated and tested against PHM by Dr. T. M. Zabriskie. The isomer eluted first from reverse-phase HPLC was defined as **42A**, and the isomer eluted second was defined as **42B**. Testing

The results show that the hydroxyl oxygen of the α-hydroxyglycine peptide obtained by PHM oxidation contains 30% ^{18}O and also demonstrate that aerobic oxygen is the primary source of the hydroxyl group. Although a mechanism involving the generation of "sequestered" [^{18}O]water in the enzyme active site which then adds to an enzyme bound *N*-acyl imine intermediate cannot be rigorously excluded, the highly reactive nature of the glyoxylate-derived *N*-acyl imine, and the large amounts of unlabeled water present in the mixture strongly suggest a direct carbon hydroxylation mechanism.

In conclusion, these studies suggest that a direct *C*-hydroxylation takes place in the PHM oxidation (Scheme 42). The addition of the aerobic oxygen occurs with loss of the *pro-S* hydrogen in the substrate, to give one stereoisomer of the α-hydroxyglycine peptide as the reaction product of PHM. The *R*-configuration at the hydroxylated carbon is tentatively fa[illegible] product inhibition studies with **42A** and **42B**, **59** and **61**. However, the stereochemistry of the hydroxyl-bearing carbon is still not conclusively proven.

Scheme 42.



CHAPTER 3 INHIBITION STUDIES OF PEPTIDYLGLYCINE α-HYDROXYLATING MONOOXYGENASE

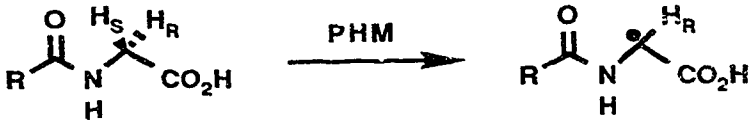
Another major goal of this project was to design and construct suicide substrates and potent competitive inhibitors which may be useful tools for biological studies in endocrinology and may have medicinal applications. Of particular interest was the design of mechanism-based inactivators (suicide substrates). These are characterized as inhibitors from which the enzyme, during the normal course of processing a modified substrate, unmasks a highly reactive intermediate which attacks the enzyme and irreversibly destroys its catalytic ability.[156,157] This kind of inhibition also provides clues which help understand the enzyme reaction mechanism.

3.1 THE DESIGN AND SYNTHESIS OF MECHANISM-BASED INACTIVATORS OF PHM

Inhibitor Design

The design of potential mechanism-based inactivators was based on several factors, including the stereospecific abstraction of the glycine *pro*-S hydrogen during the oxidative process (Figure 5),[82] the ability of the enzyme to convert peptides terminating in D-alanine instead of glycine,[74] and the possibility that a transient radical may form at the glycine α-carbon during direct hydroxylation (Figure 19).[83]

Figure 19. The Formation of a Radical During Direct Hydroxylation



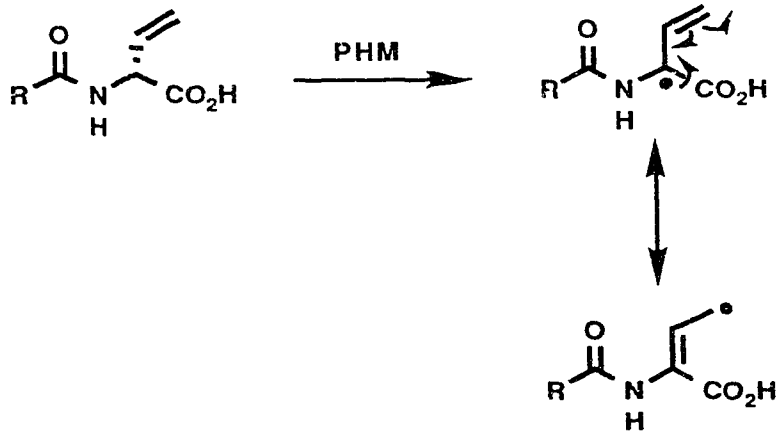
In mammalian systems, peptides having a C-terminal D-amino acid should have increased metabolic stability toward cleavage by proteases but would still be capable of being converted by PHM to natural, biologically active peptides. The possibility of having a substituent at the terminal glycine carbon provides a site for the attachment of moieties for mechanism-based inhibition, since aberrant reactions could result if the radical character of the intermediate could be stabilized or relocated to another site. Alternatively, the hydroxylation of the α-carbon of a modified D-amino acid could also generate a reactive intermediate after further transformation.

Synthesis of Potential PHM Inhibitors

Syntheses of D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine and D-Phenylalanyl-L-Phenylalanyl-L-Vinylglycine

Based on the above discussion, if the terminal glycine is replaced by D-vinylglycine, upon removal of the α-hydrogen by PHM, the developing radical character at the α-carbon could then be transmitted to a distal site where it could attack the enzyme (Scheme 43).

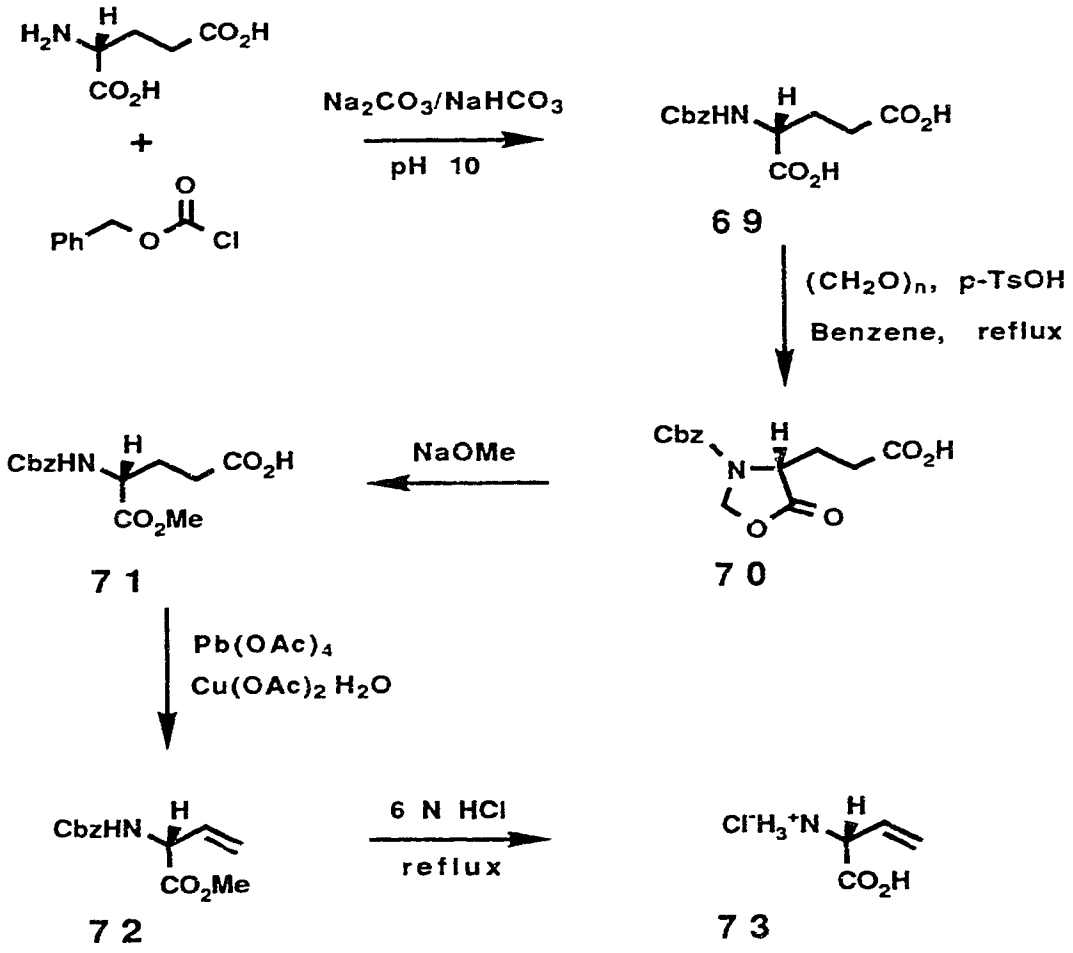
Scheme 43.



To prepare the D-vinylglycine tripeptide, optically pure D-vinylglycine was needed. Several methods existed for making optically pure L-vinylglycine, namely those of Rapoport,[158] Hanessian[159] and Castelhano.[160] Rapoport's procedure makes L-vinylglycine by the thermal degradation of an L-methionine derivative. Castelhano synthesized L-vinylglycine by treatment of a glycine cation equivalent with a vinyl Grignard reagent, and Hanessian employed the oxidation of L-glutamic acid to prepare pure L-vinylglycine. We elected to use Hanessian's procedure since it uses inexpensive, readily available starting materials, the reaction is carried out under relatively mild conditions, and the product is easily purified.

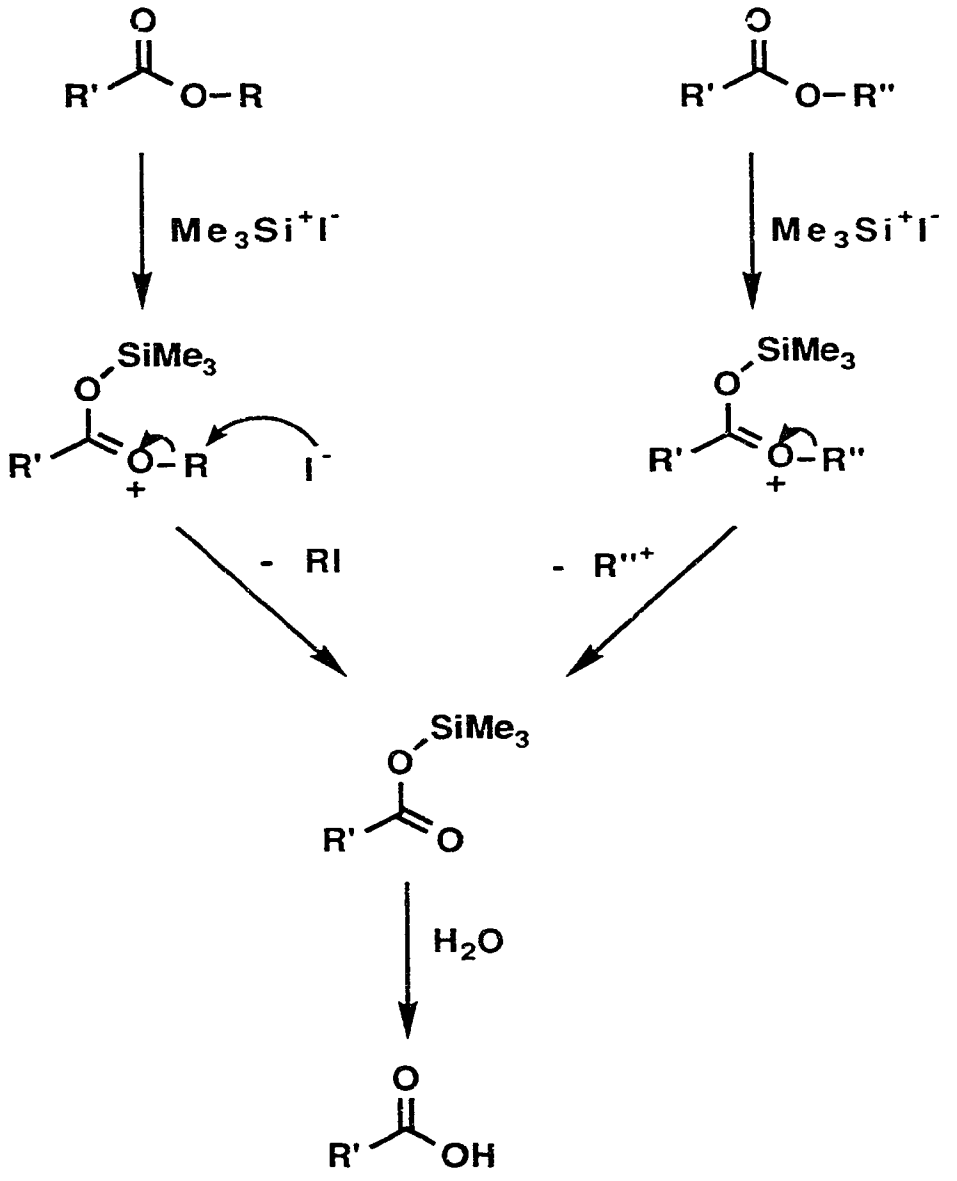
Thus, commercially available D-glutamic acid was treated with benzyl chloroformate at pH 10 to give **69** in 96% yield (Scheme 44).[159] Compound **69** was then heated to reflux with paraformaldehyde in benzene in the presence of a catalytic amount of *p*-toluenesulfonic acid to produce the oxazolidinone **70** in 73% yield.[161] This was opened by sodium methoxide to give *N*-Cbz-α-methyl-D-glutamate (**71**) in 99% yield. Compound **71** was then oxidized by lead tetraacetate and cupric acetate to give the protected vinylglycine derivative **72** in 40% yield. D-Vinylglycine hydrochloride (**73**) was subsequently obtained in good yield (82%) by hydrolyzing **72** in refluxing 6 N HCl.[182]

Scheme 44.



Prior to coupling **73** with the *N*-protected dipeptide **11**, the carboxyl group of **73** had to be protected. A protecting group removable under mild neutral or acidic conditions had to be chosen, since the compound is prone to isomerization under basic conditions. A benzyl ester was selected since it is easily removed under mild neutral conditions by trimethylsilyl iodide[162] (Figure 20). The mechanism proceeds as follows: the trimethylsilyl cation complexes to the carbonyl group of the ester, the iodide anion then displaces the R group via an S_N2 process if R is methyl, ethyl, *etc.*. If R is benzyl or *tert*-butyl, an S_N1 process occurs. Hydrolysis of the trimethylsilyl ester then gives the desired acid.[162]

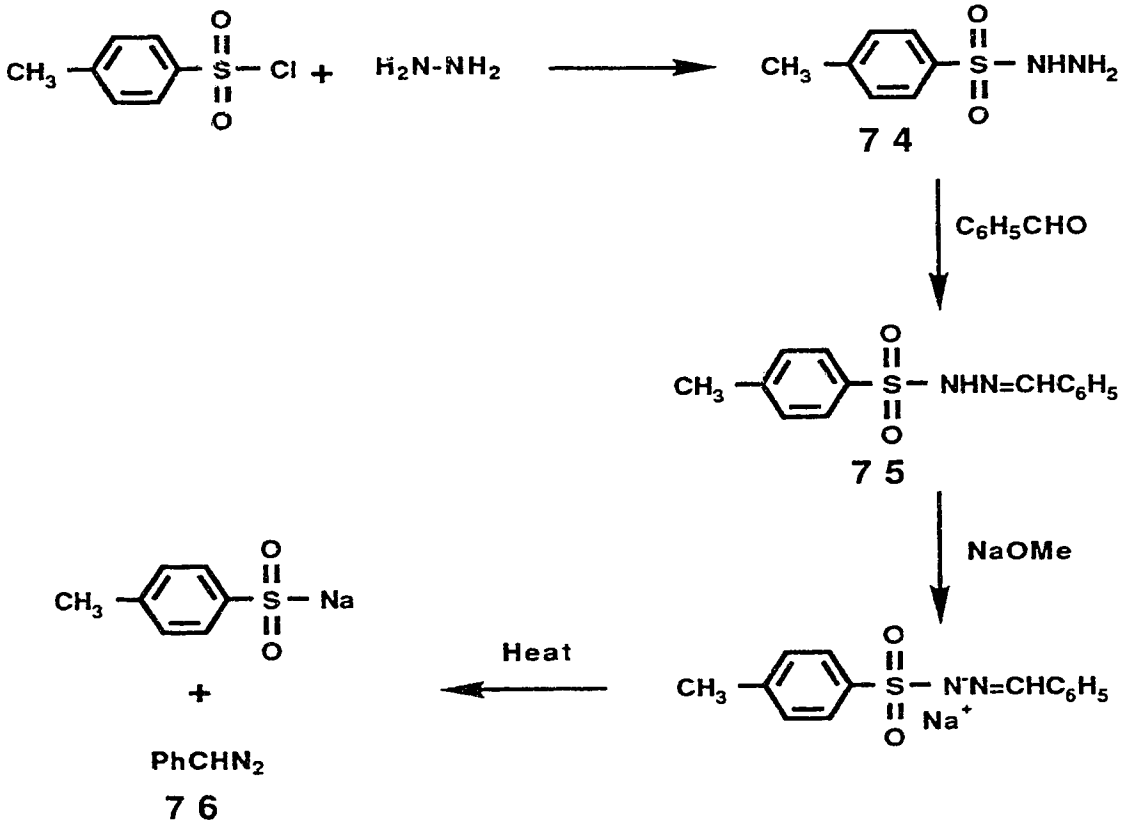
Figure 20. Mechanism of Ester Deprotection by Trimethylsilyl Iodide



An attempt was made to esterify the D-vinylglycine (**73**) by heating it to reflux in benzyl alcohol with acidic catalysis. This failed due to instability of **73** under these conditions. Direct esterification using phenyldiazomethane was then used to protect **73**.

Pure phenyldiazomethane was prepared using Creary's method.[163] Thus, tosyl chloride reacts with hydrazine to produce **74** in 92% yield (Scheme 45).[164] Treatment of this with benzaldehyde gives the tosylhydrazone **75** in 30% yield. After treating **75** with sodium methoxide, thermal pyrolysis and purification by reduced pressure distillation affords **76** in 85% yield.

Scheme 45.



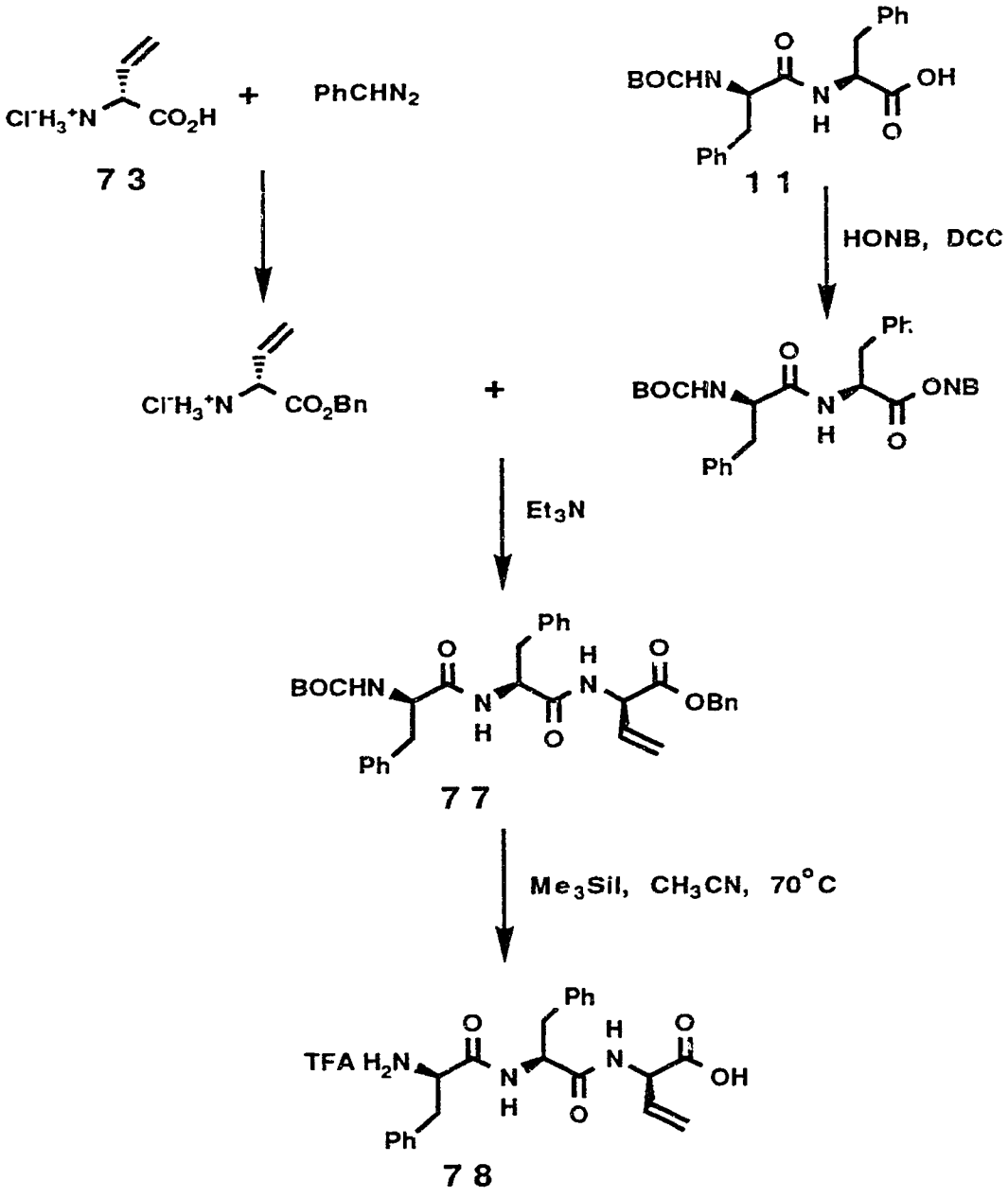
D-Vinylglycine (**73**) was then treated with phenyldiazomethane (**76**) to give the corresponding benzyl ester (Scheme 46). However, the D-vinylglycine benzyl ester prepared this way is unstable because the double bond is prone to isomerization even under

neutral conditions at -20 °C after purification by flash chromatography. Therefore the crude benzyl ester was used immediately in the coupling step without purification.

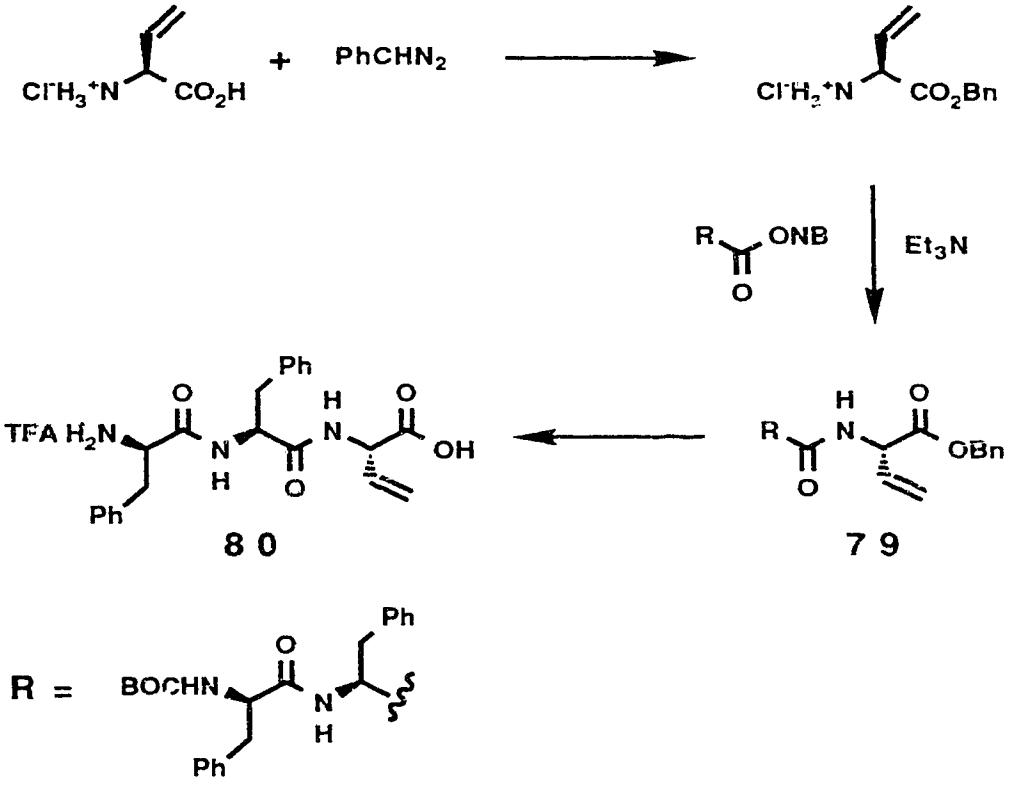
The carboxyl group of **11** was activated by the HONB/DCC procedure and then allowed to react with D-vinylglycine benzyl ester hydrochloride in the presence of 0.75 equivalent triethylamine. After purification, the diprotected tripeptide **77** was obtained in 63% yield over two steps. Both the *N*-BOC group and the benzyl ester were removed by the action of iodotrimethylsilane in acetonitrile. TLC (5% methanol in chloroform) showed that the *N*-BOC group was removed in 15 min at room temperature, but the benzyl ester was not as easily cleaved. The reaction mixture was heated to 70 °C for 3 h to complete the removal of the benzyl ester. A 47% yield of the desired tripeptide **78** was obtained after reverse-phase HPLC purification.

Very recently, Townsend reported that Hanessian's procedure causes racemization to varying extents during the oxidative decarboxylation to form the protected vinylglycines.[165] To confirm the purity of **78** and determine the extent of racemization, D-Phe-L-Phe-L-vinylglycine (**80**) was also synthesized from commercially available L-vinylglycine using the above method (Scheme 47).

Scheme 46.


Cl⁻H₃⁺N CO₂H
PhCHN₂
73
BOCHN
Ph
OH
11
HONB, DCC
Cl⁻H₃⁺N CO₂Bn
ONB
Et₃N
OBn
77
Me₃SiI, CH₃CN, 70°C
TFA H₂N
78

Scheme 47.

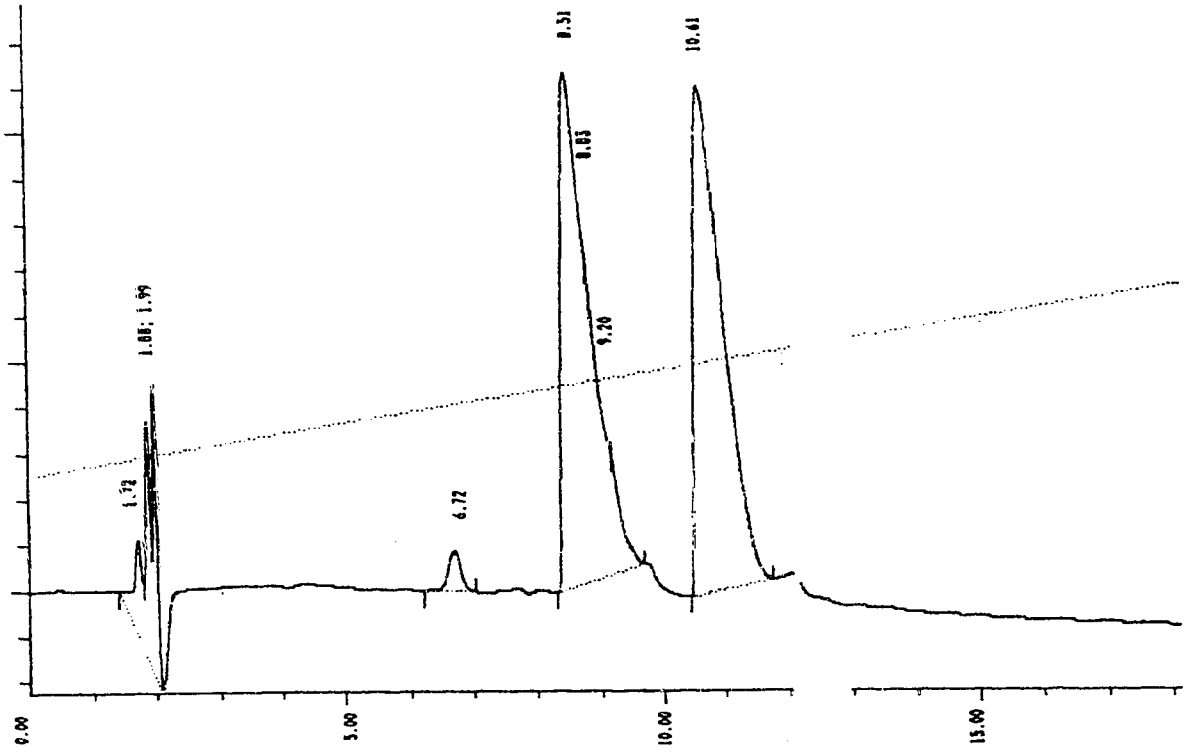


HPLC analysis of **78** and **80** demonstrated that they are easily separable (Figure 21). The retention time for **78** was 10.61 min, the retention time for **80** was 8.51 min. Analysis of the HPLC chromatogram of crude **78** showed that 19% racemization occurred during the preparation of D-vinylglycine by Hanessian's procedure or during subsequent deprotection. However, with the excellent separation afforded by the HPLC conditions, the D-vinylglycine tripeptide **78** used for further studies is essentially free of any contaminating L-vinylglycine diastereomer.

Preparation of D-Pheylalanyl-L-Phenylalanyl-D-Cyclopropylglycine

Because the ring opening reactions of cyclopropyl-substituted radicals provide another means of relocating radical character as well as a clock for estimating lifetimes of

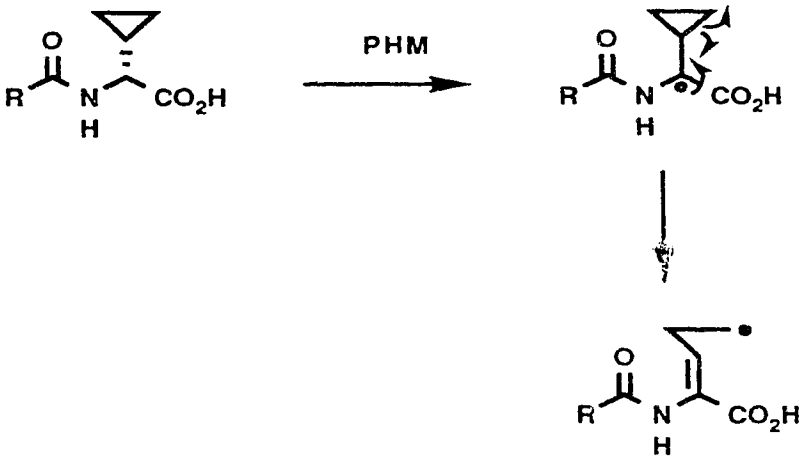
Figure 21. Reverse-phase HPLC Separation of 78 and 80



The conditions for reverse-phase HPLC separation are: Waters μ-Bondapak Radial Pak, 8 mm x 10 cm, 4 μm C_{18} reverse phase cartridge; A: 0.1% TFA in water; B: 0.1% TFA in 80:20 acetonitrile-water; 30 to 60% B linear gradient over 20 min; flow rate 1.50 mL/min; UV detection at 214 nm. The numbers in the chromatogram are retention times. The retention times for **78** is 10.61 min, and for **80** is 8.51 min.

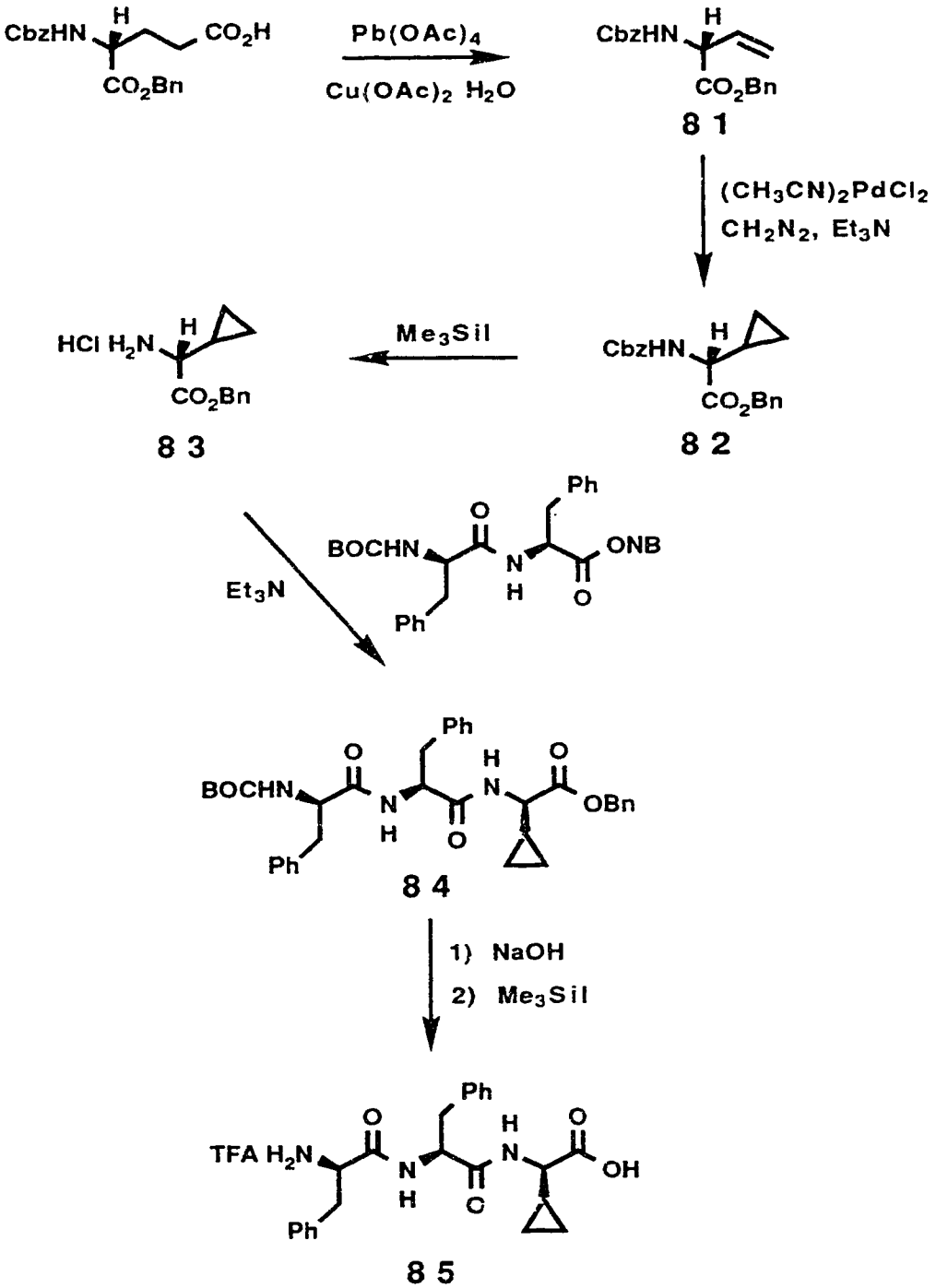
radical intermediates in biochemical processes,[166] a cyclopropyl-bearing tripeptide was also an attractive target as a PHM inhibitor (Scheme 48). Hence, D-Phe-L-Phe-D-cyclopropylglycine (**85**) was synthesized.

Scheme 48.



To prepare **85**, palladium-catalyzed cyclopropanation of the terminal double bond of vinylglycine using diazomethane was employed as a key step.[167,168] *N*-Cbz-D-Vinylglycine benzyl ester (**81**) was prepared from commercially available *N*-Cbz-D-glutamic acid benzyl ester by Hanessian's method[159] in 44% yield (Scheme 49). Palladium-catalyzed addition of diazomethane[167,168] to **81** produced the corresponding protected D-cyclopropylglycine derivative **82** in 76% yield. The *N*-BOC group was then removed by iodotrimethylsilane at room temperature to give **83** in 81% yield. Benzyl D-cyclopropylglycine (**83**) was coupled to **11** using the HONB/DCC procedure to yield the diprotected tripeptide **84** in 92% yield. The benzyl ester was subsequently hydrolyzed by sodium hydroxide/methanol and the *N*-BOC group was removed by iodotrimethylsilane to generate the desired tripeptide **85** in 80% yield after reverse-phase HPLC purification.

Scheme 49.

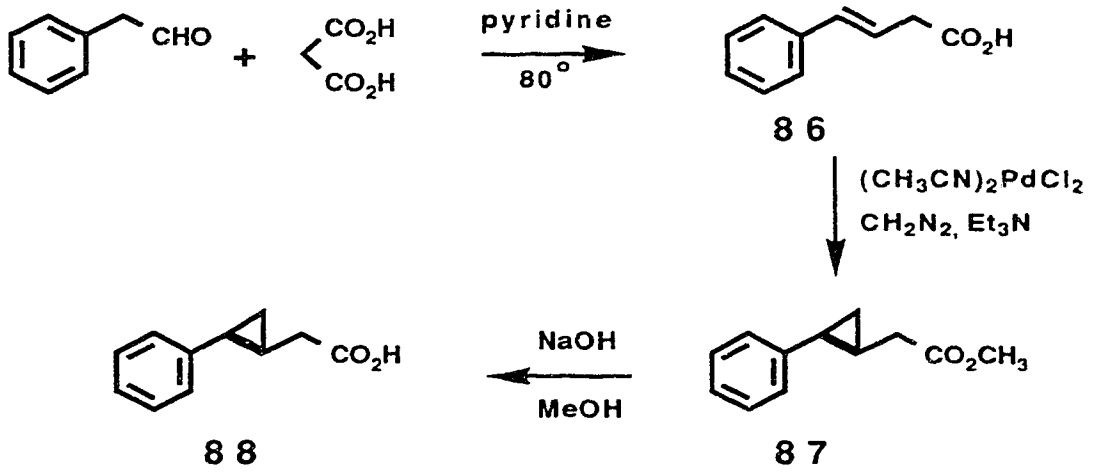

CbzHN
H
CO2H
CO2Bn
Pb(OAc)4
Cu(OAc)2 H2O
81
(CH3CN)2PdCl2
CH2N2, Et3N
HCl H2N
Me3SiI
82
83
Et3N
BOCHN
ONB
Ph
84
OBn
1) NaOH
2) Me3SiI
TFA H2N
OH
85

Synthesis of trans-2-(2-Phenylcyclopropyl)ethanoic Acid

Bradbury *et al.* recently reported that *trans*-4-phenyl-3-butenoic acid is a potent mechanism-based inactivator for PHM.[78] May and Katopodis subsequently published a similar result.[79] Because the cyclopropyl group can also transmit the radical character at the α-carbon to the distal carbon, the *trans*-2-(2-phenylcyclopropyl)ethanoic Acid (**88**) could potentially also be a mechanism-based inactivator of PHM. Thus **88** was prepared using palladium-catalyzed addition of diazomethane to *trans*-4-phenyl-3-butenoic acid.

The *trans*-4-phenyl-3-butenoic acid (**86**) was conveniently prepared in 85% yield using Fichter's method[169] for the condensation of phenylacetaldehyde with malonic acid (Scheme 50). Compound **86** then reacted with diazomethane and the palladium catalyst to give methyl *trans*-2-(2-phenylcyclopropyl)ethanoate (**87**) in 84% yield. The side product, methyl *trans*-4-phenyl-3-butenoate, was difficult to separate from the desired product by flash chromatography. However, it could be oxidized to the easily separable epoxy compound by *meta*-chloroperbenzoic acid. Finally, **87** was hydrolyzed by sodium hydroxide to give **88** in quantitative yield.

Scheme 50.

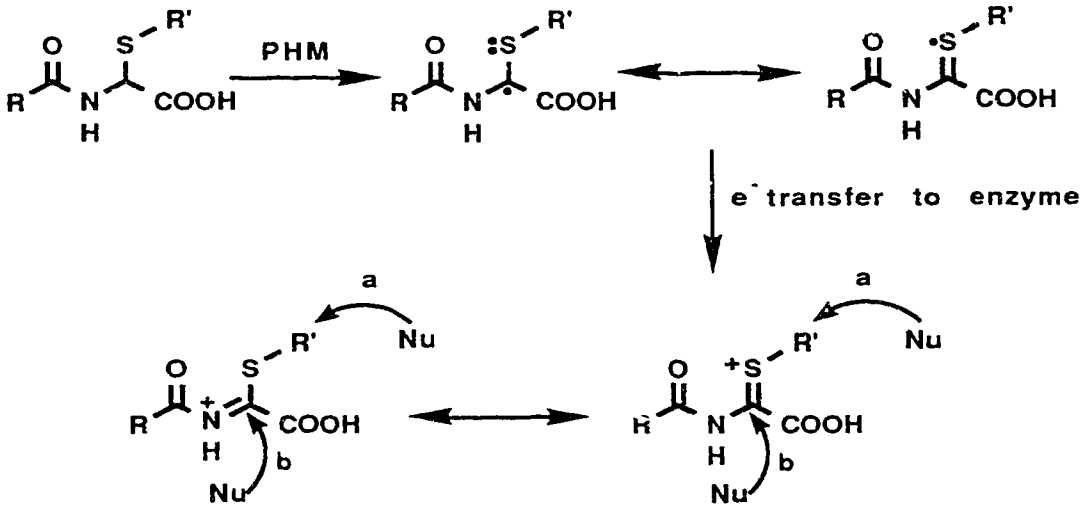


Methylthio and Benzylthioglycine Derivatives

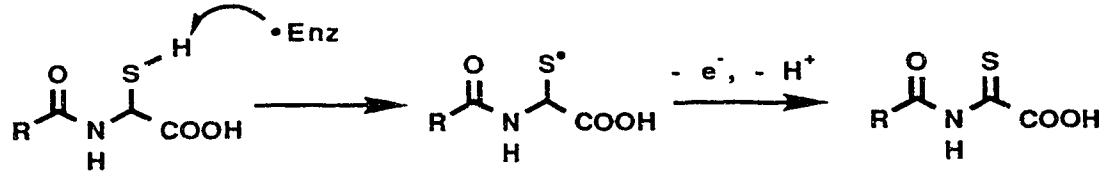
Other possible mechanism-based inactivators of PHM could contain sulfur substituted glycines such as thiolglycine, methylthioglycine, and benzylthioglycine. The radical generated at the α-carbon of these compounds could interact with the sulfur to a reactive species which subsequently inactivates the enzyme (Scheme 51).

Scheme 51.

General: R' = H, Me, Bn

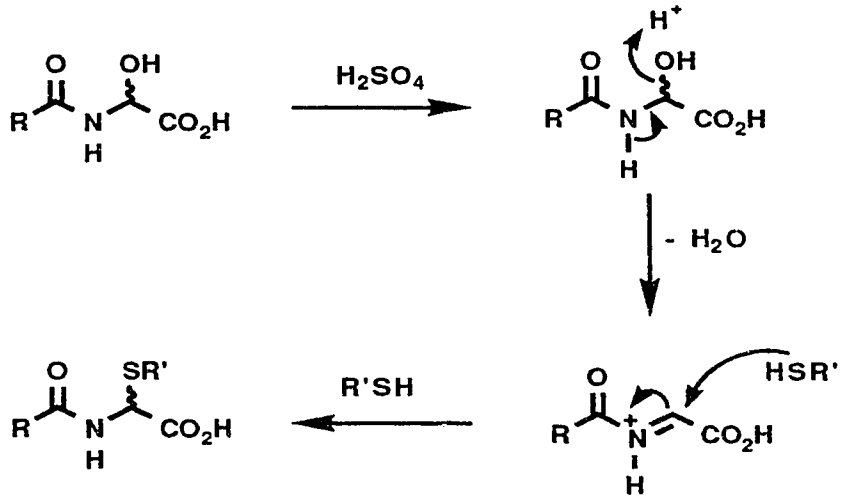


Special case: R' = H



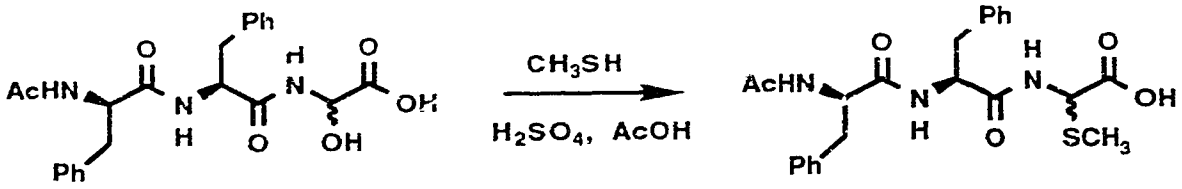
A general procedure utilizing a strong acid as catalyst[120] was employed to prepare this type of compound. Treatment of an α-hydroxyglycine derivative with RSH (R = H, CH_3, $PhCH_2$) in glacial acetic acid in the presence of a catalytic amount of concentrated sulfuric acid, generates the corresponding α-thioglycine derivatives via an acyl imine intermediate (Figure 22).

Figure 22. Mechanism for the Formation of Thioglycine Derivatives



Thus, *N*-Ac-D-Phe-L-Phe-methylthioGly (**89**) was obtained in 95% yield by treating **42** with methanethiol in glacial acetic acid together with a catalytic amount of concentrated sulfuric acid (Scheme 52). The two diastereomers were conveniently separated by reverse-phase HPLC, but the absolute stereochemistry was not assigned.

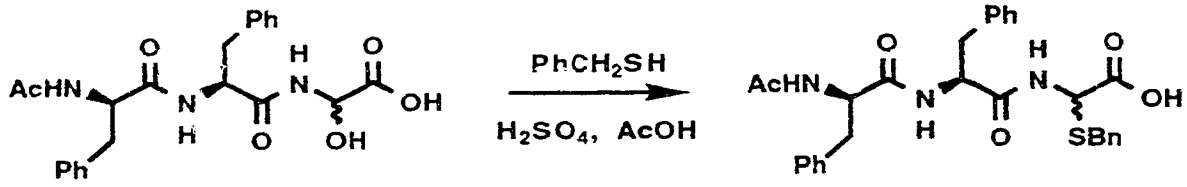
Scheme 52.



42 89

Similarly, *N*-Ac-D-Phe-L-Phe-benzylthioGly (**90**) was obtained in 85% yield (Scheme 53). The two diastereomers were also easily separated by reverse-phase HPLC, but again the absolute stereochemistry was unknown.

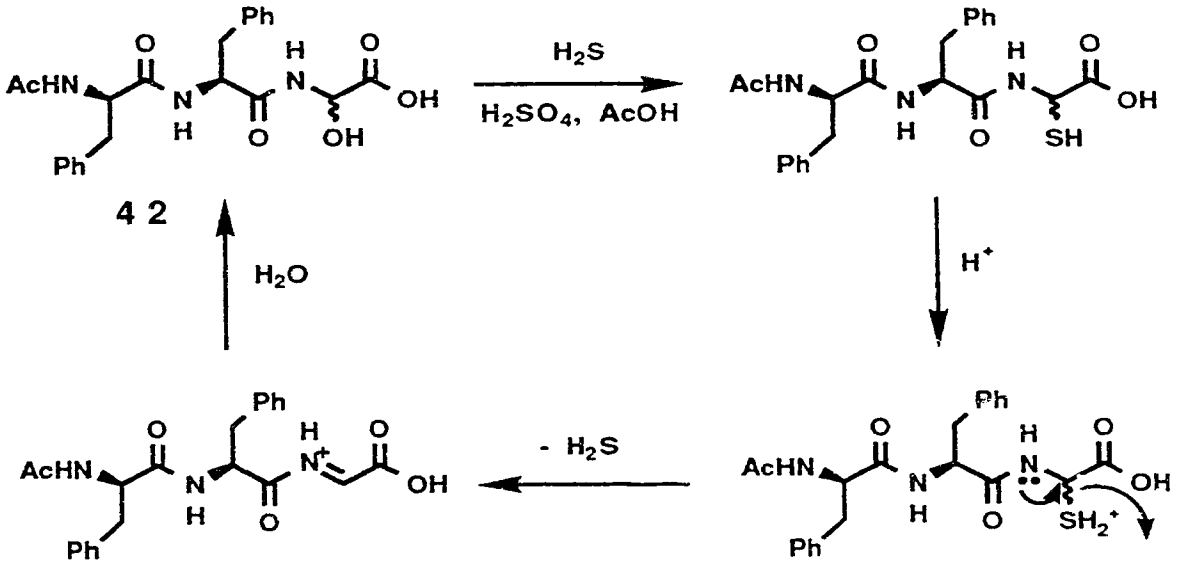
Scheme 53.



42 90

Although *N*-Ac-D-Phe-L-Phe-thiolglycine could be detected by FAB-MS and ^{1}H NMR in the crude product from the reaction of **42** with hydrogen sulfide, it proved to be unstable under reverse-phase HPLC conditions (acidic aqueous solution). It is likely that the product releases hydrogen sulfide and is degraded to starting material under these conditions (Scheme 54); this was supported by the odor of hydrogen sulfide present in the HPLC eluent.

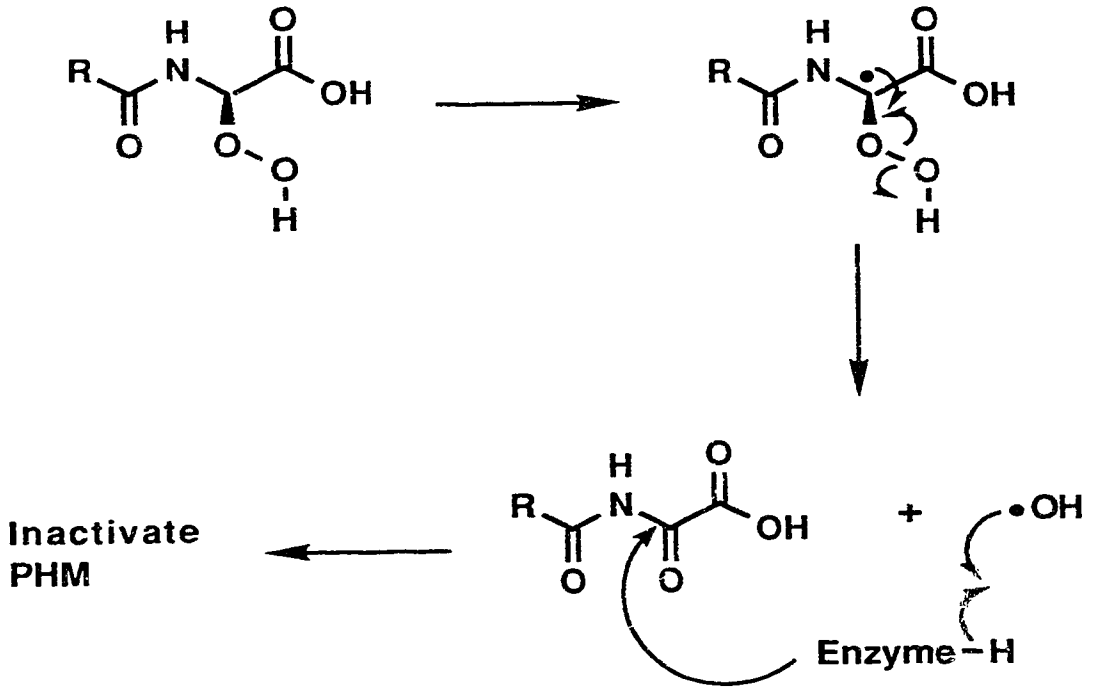
Scheme 54.



Preparation of N-Acetyl-D-Phenylalanyl-L-Phenylalanyl-N-Formylamide

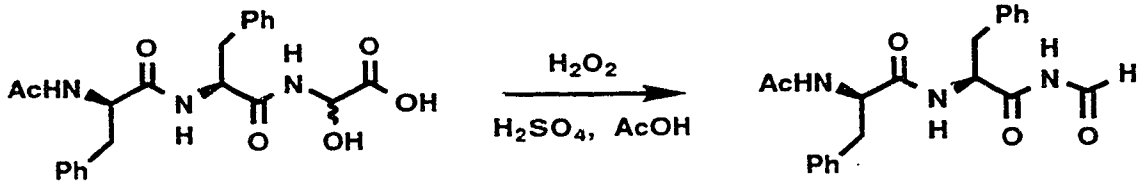
Another compound which could act as mechanism-based inactivator is hydroperoxyglycine tripeptide. If the hydroperoxyglycine derivative is stable, it should be easily prepared using the same procedure employed for the preparation of thioglycine compounds. PHM oxidation to form the carbon radical could cleave the peroxide bond and generate a reactive hydroxyl radical. Such radicals could attack the enzyme (Scheme 55).

Scheme 55.



Thus, compound **42** was treated with hydrogen peroxide in glacial acetic acid with a catalytic amount of concentrated sulfuric acid. The major product from this reaction, in 45% yield after reverse-phase HPLC purification, was characterized as *N*-acetyl-D-phenylalanyl-L-phenylalanyl-*N*-formylamide (**91**) instead of the expected hydroperoxide derivative
(Scheme 56).

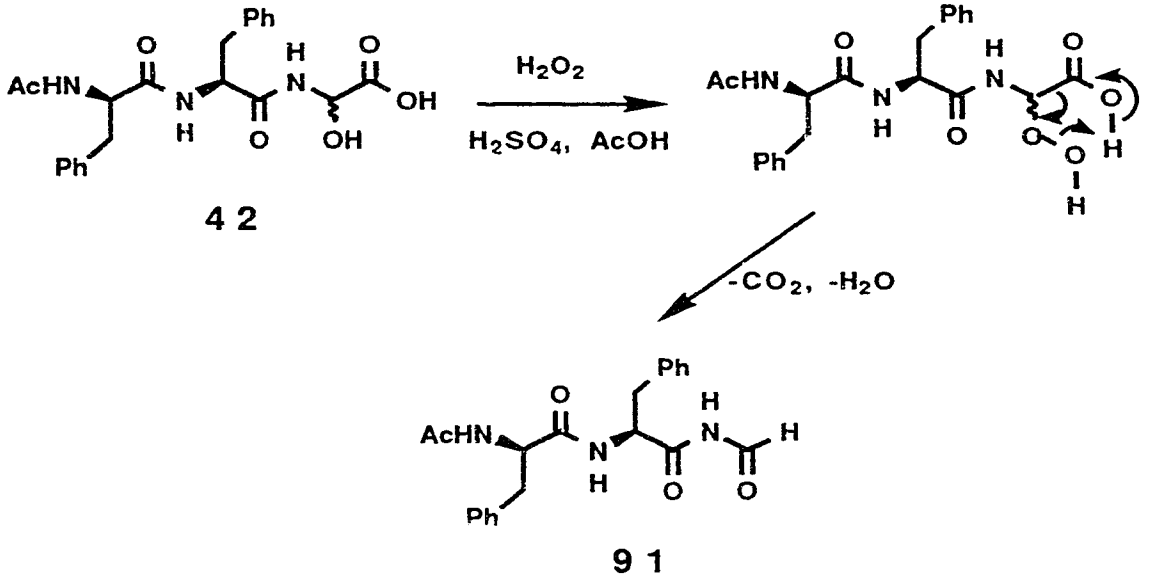
Scheme 56.



42 **91**

This result suggests the hydroperoxide derivative initially forms, but is unstable and decomposes to give the isolated product (**91**) with the concomitant release of carbon dioxide (Scheme 57).

Scheme 57.

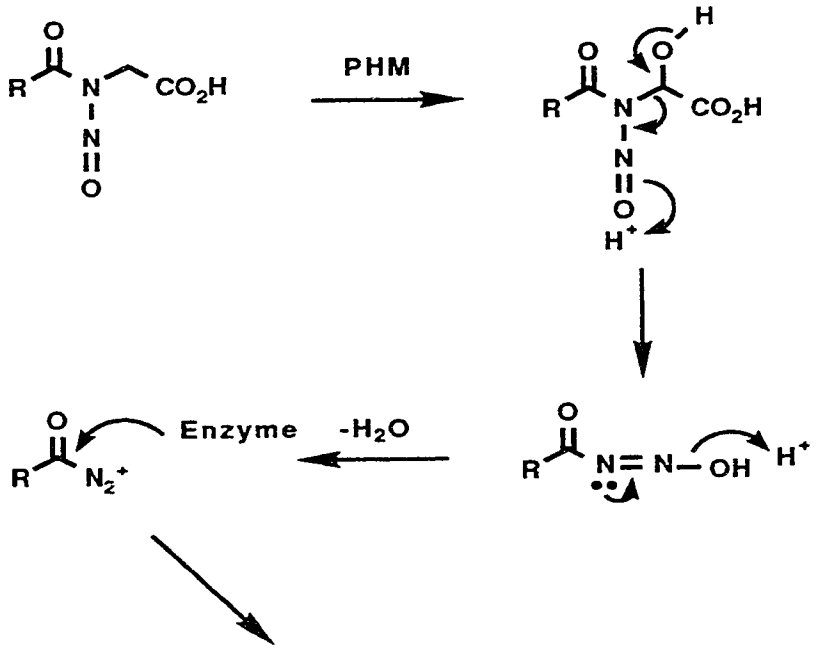


42 **91**

Preparation of N-Acetyl-L-Prolyl-(N-Nitroso)-Glycine

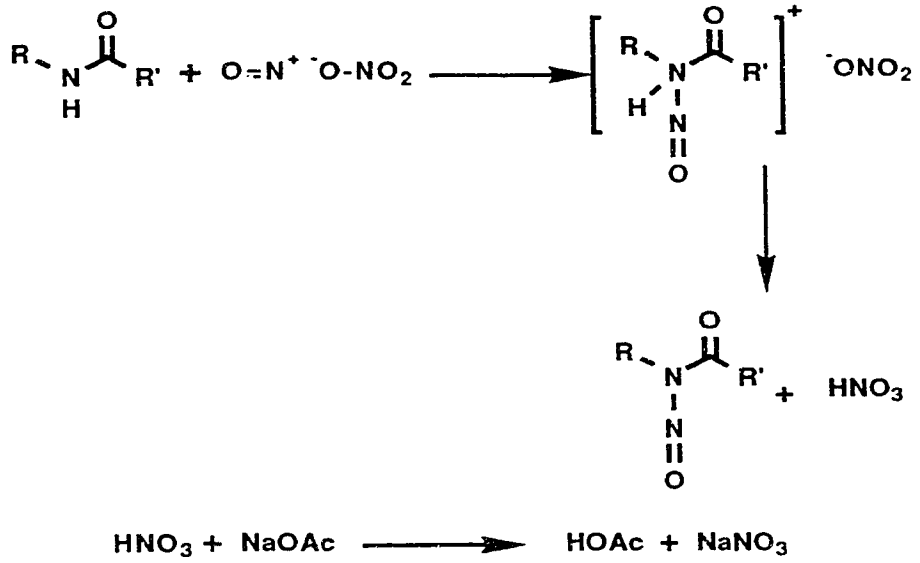
A modified glycine analog possessing an *N*-nitroso group offers another possibility for the inhibition of PHM, since it may be transformed by PHM to a hydroxy derivative which would spontaneously decompose to glyoxylic acid and a very reactive acyl diazonium species. The latter could then acylate the enzyme (Scheme 58).

Scheme 58.



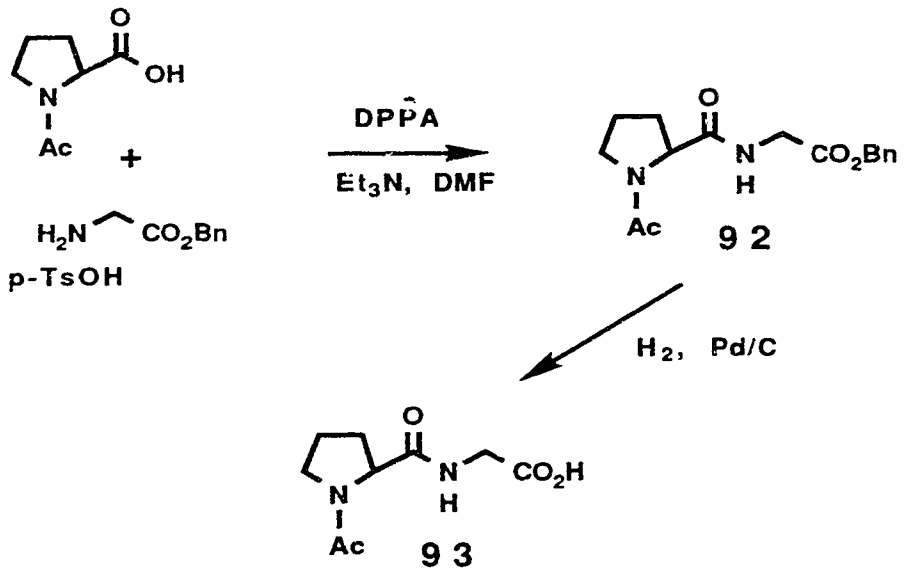
Precedent suggests that such nitrosation can be accomplished on amide nitrogens using dinitrogen tetraoxide.[170] It has been proposed that nitrogen tetraoxide is in equilibrium with a small amount of $ON^+ONO_2^-$, and that the reaction can be represented as shown in Figure 23. The nitrogen tetraoxide is used with an excess of anhydrous sodium acetate to remove the nitric acid side product, since in the absence of base the reverse reaction of denitrosation by the nitric acid occurs.

Figure 23. Mechanism for Nitrosation of the Amide Nitrogen



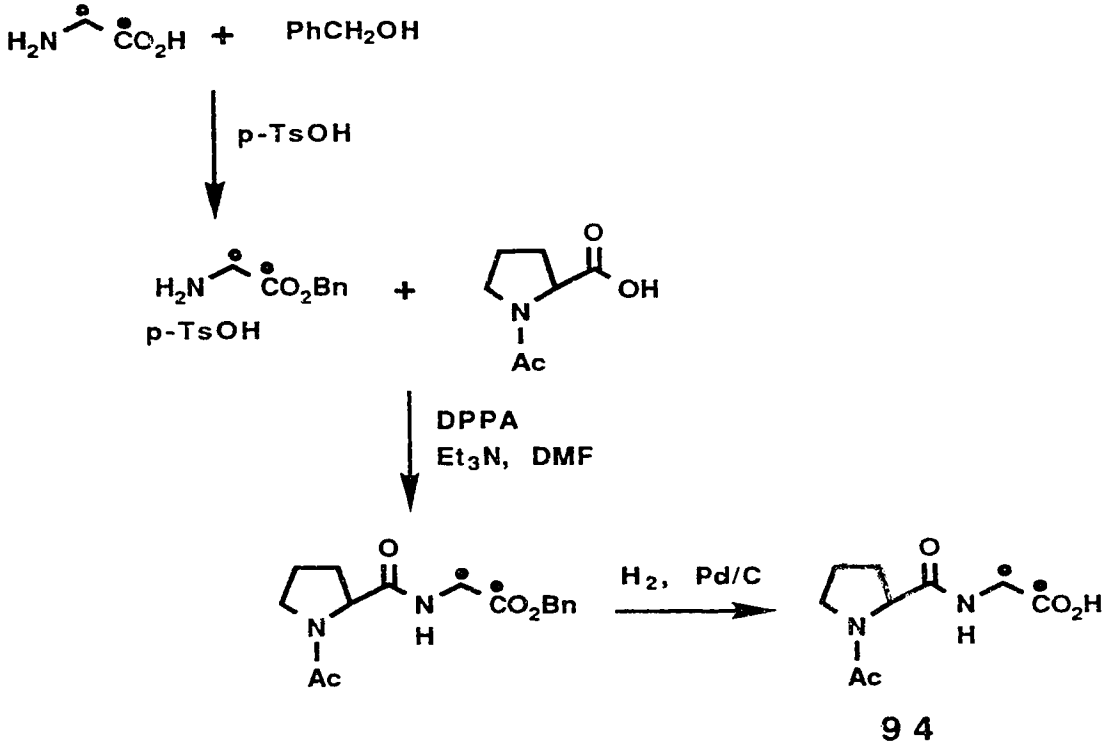
Since this procedure nitrosates all amide nitrogens bearing a hydrogen, a new substrate, *N*-acetyl-L-prolyl-glycine, was chosen to avoid the necessity of blocking other nitrogens in the peptide. The protected dipeptide **92** was easily prepared from commercially available glycine benzyl ester and *N*-acetyl-L-proline using the DPPA procedure (Scheme 59). Catalytic hydrogenolysis of **92** gave the desired product **93** in 53% yield over the two steps.

Scheme 59.



The corresponding labeled material, *N*-acetyl-L-prolyl-[1,2-^{14}C]-glycine, was also synthesized using the same strategy (Scheme 60). Commercially available [1,2-^{14}C]-glycine was esterified in refluxing benzyl alcohol in the presence of *p*-toluenesulfonic acid. Water was removed azeotropically and trapped using calcium hydride in a Soxhlet thimble. The resulting [1,2-^{14}C]-glycine benzyl ester was coupled with *N*-acetyl-L-proline by the DPPA procedure to generate the protected dipeptide. The benzyl ester of this dipeptide was

Scheme 60.

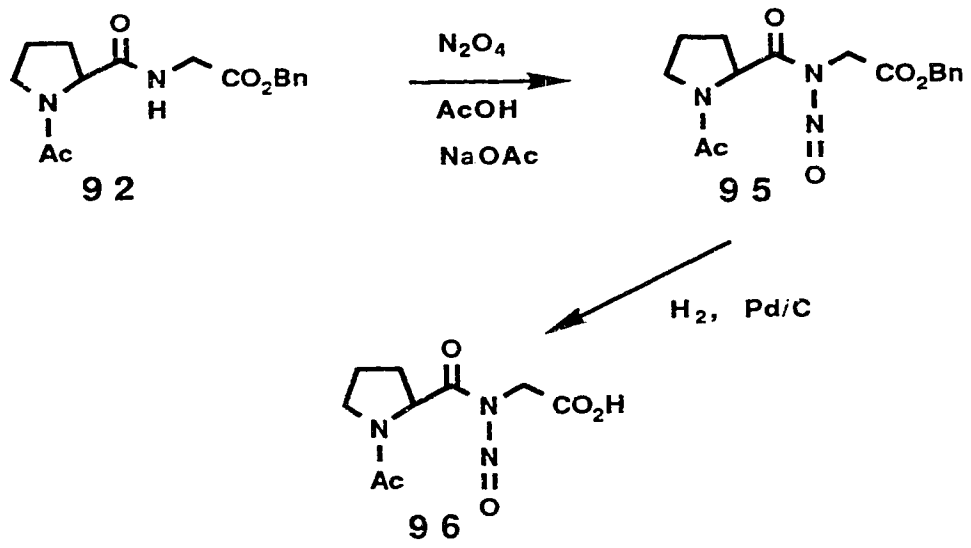


94

cleaved by catalytic hydrogenolysis to give the desired product **94** in 16% overall yield. This material shows only a single radioactive spot on TLC which cospots with the unlabeled peptide **93** in two different solvent systems. It also has the same retention time as the unlabeled peptide **93** by reverse-phase HPLC.

The protected dipeptide **92** was nitrosated as described above to give **95** in 91% yield (Scheme 61). The benzyl group in **95** was removed by catalytic hydrogenolysis at ambient temperature and pressure.[171] Although this compound was easily overreduced under these conditions, the desired product could be purified by reverse-phase HPLC to afford **96** in 35% overall yield.

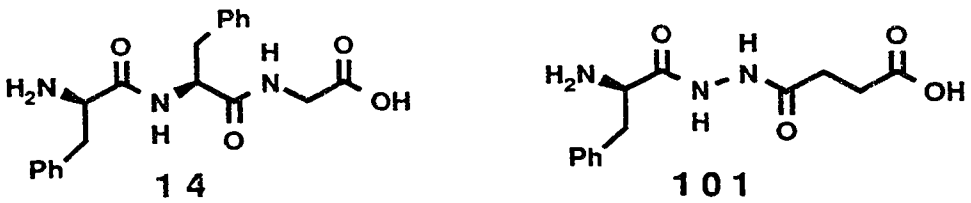
Scheme 61.



Preparation of D-Phenylalanyl Hydrazinosuccinic Acid

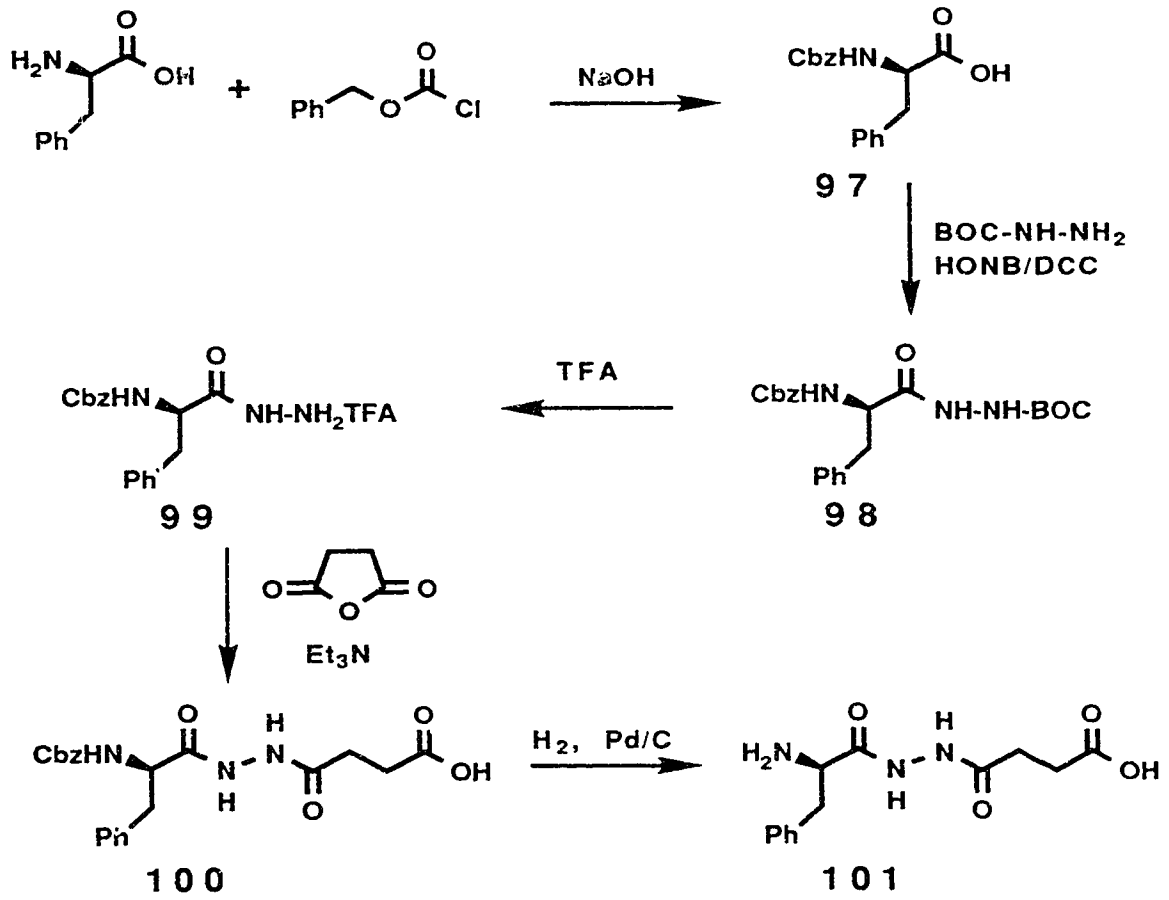
In addition to the potential mechanism-based inhibitors described above, potent competitive inhibitors are also attractive targets. D-Phenylalanyl hydrazinosuccinic acid (**101**), has a skeleton similar to the substrate **14** (Scheme 62) both in length and positioning of the carbonyl groups. If PHM recognizes and binds it similarly to its natural substrate, it may be a potent competitive inhibitor. If the oxidation of D-Phenylalanyl hydrazinosuccinic acid occurs, the product would provide valuable mechanistic insight and could have its absolute stereochemistry assigned. Such a result would help confirm the stereochemistry of the hydroxyl bearing carbon in the normal PHM product.

Scheme 62.



The synthesis of compound **101** was achieved as outlined in Scheme 63. D-Phenylalanine was treated with benzyl chloroformate under basic conditions to generate *N*-Cbz-D-Phe (**97**) in 99% yield.[159] This was then coupled with BOC-hydrazine using the HONB/DCC procedure to give **98** in 78% yield. The BOC group was removed by treatment with neat trifluoroacetic acid at room temperature to generate **99** in quantitative yield. Reaction of **99** with succinic anhydride gave the *N*-protected peptide analog **100** in 78% yield. Finally, the *N*-Cbz group was removed by catalytic hydrogenolysis to give the desired product (**101**) in 85% yield after reverse-phase MPLC purification.

Scheme 63.



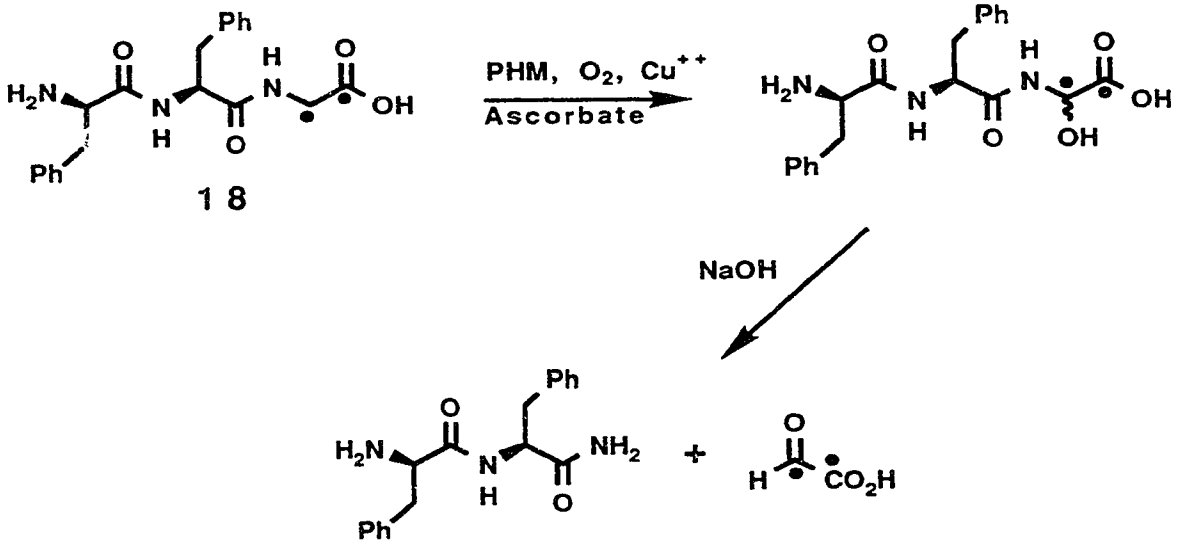
3.2 KINETIC STUDIES OF INHIBITORS WITH PHM

Development of A New PHM Assay Using Cation Ion-exchange Resin

Although the PAM assay with nitrosobenzene provides a sensitive method for analysis of the amount of glyoxylate produced, it is not convenient for kinetic studies and can be subject to errors when used under control conditions with pure PHM which generates the hydroxyglycine intermediate.

To overcome this, a new assay was developed by Dr. T. M. Zabriskie.[83] This assay is based, like the previous assay, on the detection of glyoxylate, the common product of peptide α-amidation, rather than on the variable peptide amide product. The transformation of D-phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycine (**18**) by PHM is monitored by the detection of [1,2-^{14}C]-glyoxylate liberated upon treatment with base (Scheme 64). For a typical time course study, a 25 μL aliquot was removed from

Scheme 64.



a 125 μL incubation mixture containing 1 mM ascorbate, 5 μM copper sulfate, 25 mM potassium iodide, 0.125 mg/mL catalase, 1.25 μM radioactive substrate, and PHM in 50 mM sodium phosphate buffer at pH 6.8. [illegible]quot was mixed in a microcentrifuge tube with 2 N NaOH solution (10 μL) and incubated at 37 °C for 5 min to cleave the α-hydroxyglycine peptide. The sample was acidified with 2 N HCl (12 μL) and applied to a column of cation exchange resin in a pipette (Bio-Rad AG 50W-X8, 200-400 mesh, H^+ form, 1 mL). Glyoxylate was washed from the column with water (3 mL) and collected in a scintillation vial. Unconverted substrate was eluted with 15% NH_4OH (4.5 mL) and collected separately; the amount of ^{14}C in both samples was determined by liquid scintillation counting. For single point assays the entire incubation (125 μL) was quenched with 30 μL of 2 N NaOH, incubated for 5 min and acidified with 32.5 μL of 2 N HCl. The entire contents were placed on the ion-exchange column and eluted as described.

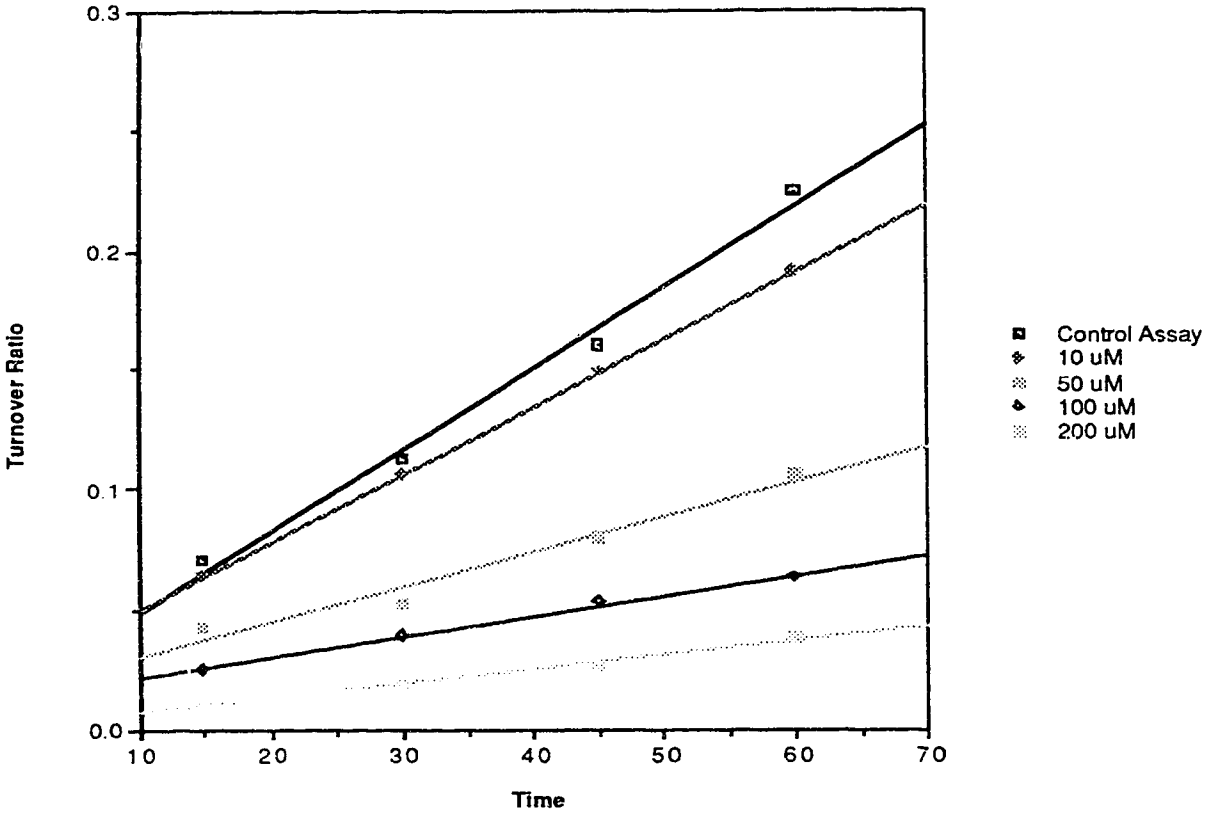
By dividing the disintegrations per minutes (dpm) for glyoxylate by the dpms for the unconverted substrate, a percentage of the substrate turned over by the enzyme is obtained. By generating the percent conversion in this manner, rather than just the amount of glyoxylate generated and the amount of substrate added, measuring and transferring errors are minimized.

Inhibition Studies of PHM

The details of assay for inhibition studies are described in the experimental section. The results will be discussed below. For competitive inhibition studies, a typical example is given in Figure 24 which shows the plot of PHM turnover of labeled substrate D-Phe-L-Phe-[1,2-^{14}C]-Gly vs incubation time (min) in the presence of various concentrations of *N*-Ac-D-Phe-L-Phe-α-D-benzylthioGly (**90A**). The IC_{50} value, the inhibitor at which concentration causes 50% inhibition of enzyme activity, was determined based on this plot

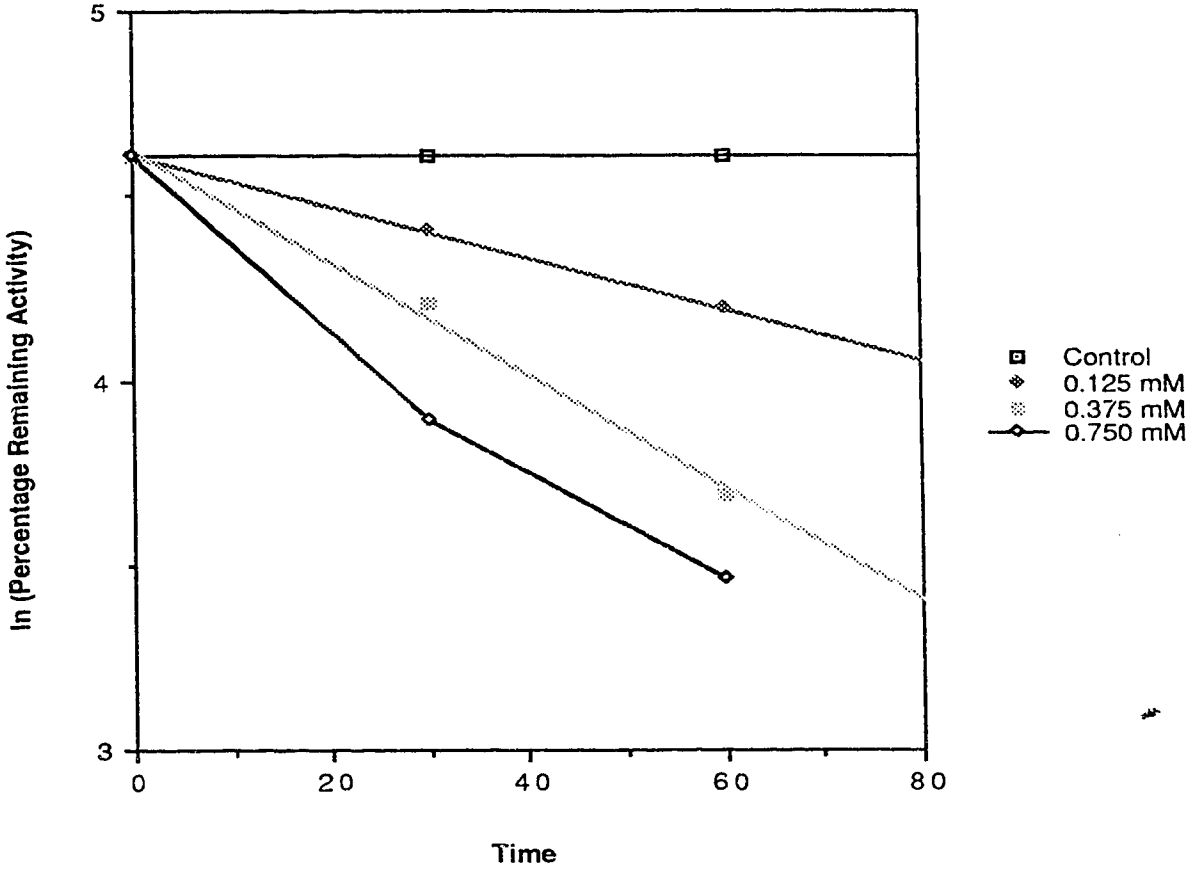
while the substrate concentration is 0.72 μM in all the cases. The competitive inhibition studies for all other compounds were done similarly (See experimental section for details).

Figure 24. A Typical Plot of Competitive Inhibition Assay



For the preincubation studies, a typical example is given in Figure 25 which shows the plot of natural log of percentage remaining enzyme activity vs incubation time (min) in the presence of various concentrations of *N*-Ac-D-Phe-L-Phe-NCHO (**91**). The slopes of this plot were the K_{obsd}s. The plot of $1/K_{obsd}$ vs 1/[I] was linear (where [I] is the concentration of inhibitors), and the apparent inhibition constant K_I was the -1/(X intercept).[79,156] The preincubation studies for other mechanism-based inhibitors were done similarly (See experimental section for details).

Figure 25. A Typical Plot of Preincubation Studies



Inhibition Studies with D-Phenylalanyl-L-Phenylalanyl-D-Cyclopropylglycine (85)

Although the cyclopropyl moiety can interact with the radical electron on the α-carbon, the cyclopropyl peptide **85** showed only weak inhibition. Inhibition tests suggested that the K_I value is greater than 5 mM. The plot of 'remaining activity vs time' shows a gradual loss of activity indicating a possible time-dependent inhibition. Thus the substitution of the glycine *pro-R* hydrogen in a substrate with a group that is much more sterically demanding than methyl (as in a D-alanine terminus) creates a species that does not

fit well in the enzyme active site. It is likely that poor binding of **85** to the enzyme active site results in the weak inhibition; however, once **85** is bound to the active site, hydrogen abstraction can occur (Scheme 49). More detailed studies on this compound will be done in future studies

Inhibition Studies with trans-2-(2-Phenylcyclopropyl)ethanoic Acid (88)

The inhibition studies of PHM by *trans*-2-(2-phenylcyclopropyl)ethanoic acid (**88**) were performed by Dr. T. M. Zabriskie. Although *trans*-4-phenyl-3-butenoic acid is a potent mechanism-based inactivator, we found that when the double bond was changed to a more bulky cyclopropyl functional group (as in **88**) no inhibition was observed at a concentration of 1 mM. It may be that the presence of double bond is important for the inactivation of PHM by these non-peptide inhibitors.

*Inhibition of PHM by Peptides Containing a C-Terminal Glycine Bearing a Sulfur Sidechain, **89A**, **89B**, **90A**, **90B**.*

The inhibition studies of PHM by the two isomers of **89** were also performed by Dr. T. M. Zabriskie. The isomer eluted first from reverse-phase HPLC was defined as **89A**, and the isomer which eluted second was defined as **89B**. Both isomers of **89** do exhibit PHM inhibition. Compound **89B** inhibits the enzyme activity by 5% after 1 h incubation at a 1 mM concentration, whereas **89A** inhibits the enzyme activity by 55% under the same conditions. Although initial studies of **89A** suggested that the enzyme activity was lost over time, further studies actually demonstrated that the inhibition is neither time-dependent nor irreversible. Compound **89A** is classified as a competitive slow binding inhibitor with an IC_{50} of 1.0 mM (Substrate concentration is 0.72 μM).

When the methylthioglycine (as in **89**) was replaced by the benzylthioglycine unit of **90**, more potent inhibition was observed. Similarly, the isomer eluted first from reverse-phase HPLC was defined as **90A**, and the isomer which eluted second was

defined as **90B**. Assays on PHM showed no loss of activity with time, suggesting that the inhibition is neither time-dependent nor mechanism-based. This suggests that the expected process proposed in Scheme 51, hydrogen abstraction by PHM followed by electron transfer, probably does not occur in this case. The IC_{50} value for **90A** was calculated to be 50 μM; the IC_{50} value for **90B** was much weaker and was determined as 400 μM (Substrate concentration is 0.72 μM).

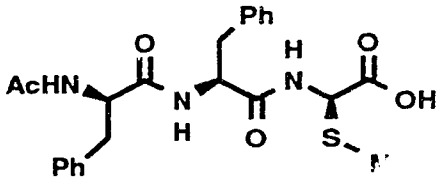
*Determination of Stereochemistry of **89A**, **89B**, **90A**, **90B** by an Independent Method*

It is likely that the inhibitory properties, physical appearance and glycine α-H chemical shifts of various peptides with substituted glycine moiety depend on stereochemistry of the substituent. Table 8 lists the physical appearance of the isomers of potential inhibitors after lyophilization from water/acetonitrile, as well as the chemical shift of the glycine α-hydrogens. The inhibition properties of both isomers of **89**, **90** and D- and L-vinylglycine peptides **78** and **80** are also shown. It is interesting to note that **89A**, **90A**, and **78** are flocculent after lyophilization, the α-hydrogen chemical shifts in the ^1H NMR of the glycine derivatives are at higher field, and the inhibitions of PHM are greater than those of **89B**, **90B**, and **80**. Since the glycine derivative of compound **78** has the R-configuration and the glycine derivative of compound **80** has the S-configuration, we propose that **89A** and **90A** possess the R configuration, and that **89B** and **90B** have the S configuration at the α-carbon of the glycine moiety.

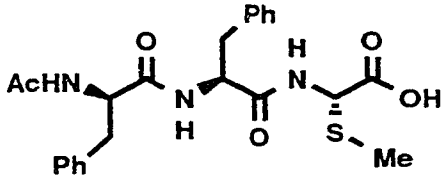
Table 8 **Comparison of the Physical Appearance, α-H Chemical Shift, and Inhibitory Properties of 89A, 89B, 90A, 90B, 78 and 80.**

	Physical Appearance	α-H Chemical Shift	Inhibition (IC_{50})
89A	Flocculent	5.33	Weak (1 mM)
89B	Granular	5.38	Very weak(>10 mM)
90A	Flocculent	5.25	Potent (50 μM)
90B	Granular	5.31	Moderate (400 μM)
78[b]	Flocculent	5.89	Potent ([a]20 μM)
80[b]	Granular	5.98	Moderate (350 μM)

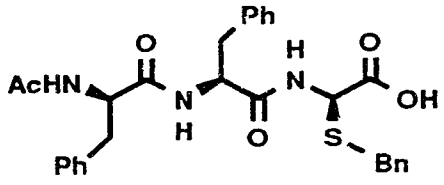
[a]This is K_I value. [b]The inhibition studies will be discussed later.



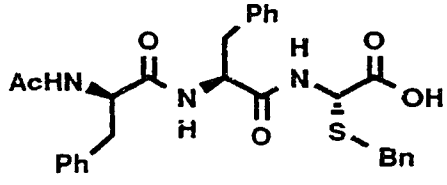
89A



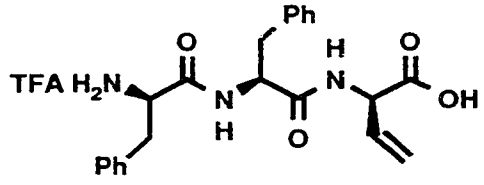
89B



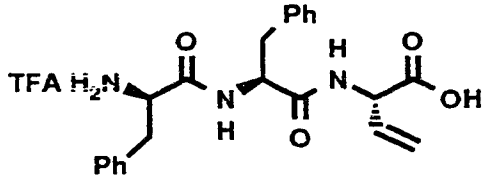
90A



90B



78



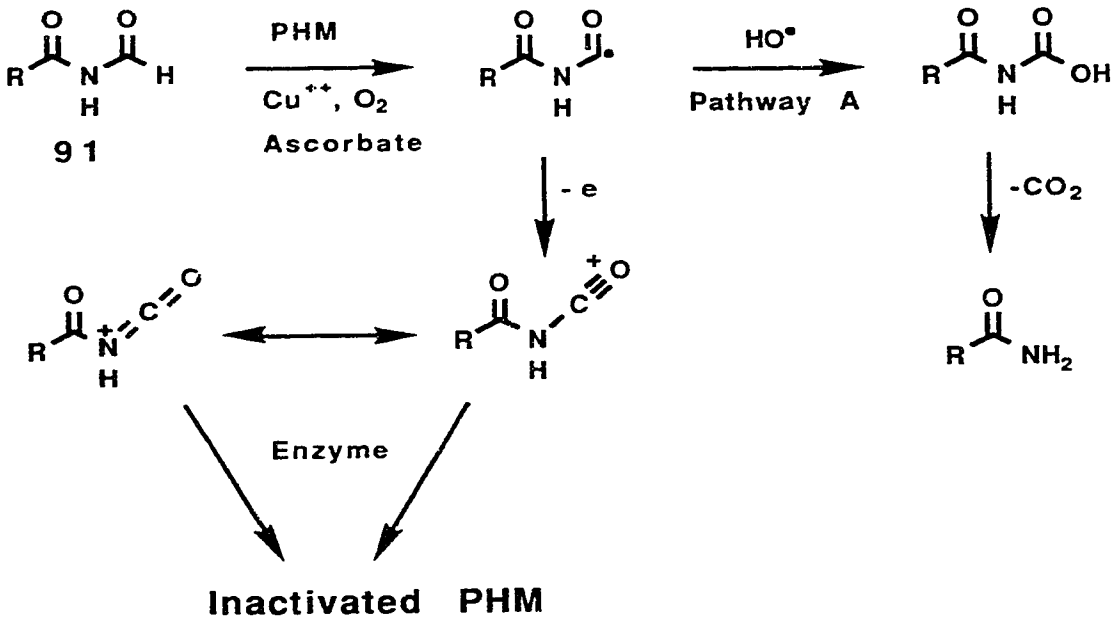
80

Inhibition Studies with N-Acetyl-D-Phenylalanyl-L-Phenylalanyl-N-Formylamide (91)

Surprisingly, *N*-acetyl-D-phenylalanyl-L-phenylalanyl-*N*-formylamide (**91**) exhibited moderate time-dependent inhibition of PHM. The kinetic parameters for the inactivation reaction were determined by the dilution assay method; inactivation by **91** is first order and concentration dependent, and the double-reciprocal plot of 1/rate vs 1/[I] gives the value of $K_I = 0.40$ mM. Incubation of the enzyme with **91** (3.00 mM) for 60 minutes, followed by removal of the inhibitor by repetitive concentration/dilution steps using a micro ultrafiltration device, gave no recovery of activity, thereby demonstrating that the inactivation is irreversible. Preincubation of PHM with the substrate D-Phe-L-Phe-Gly (**21**) and **91** indicated protection of the enzyme in proportion to the amount of added substrate, indicating that **91** acts at the active site of the enzyme. Treatment of PHM with **91** in the absence of Cu(II) and ascorbate for one hour followed by removal of the inhibitor resulted in no significant loss of activity. The requirement that all cofactors be present for inactivation to occur illustrates that **91** is a mechanism-based inhibitor.

Possible mechanisms for action of **91** are depicted in Scheme 65. Once the enzyme binds to the analogue **91**, hydrogen abstraction may generate a reactive carbonyl radical. This carbonyl radical may be oxidized by the copper-oxo species to form the unstable acid which subsequently undergoes decarboxylation to give the dipeptide amide (path A). Alternatively it could lose an electron to copper in the enzyme active site to form the acylium ion which is stabilized by the isocyanate resonance form. Attack by a nucleophilic residue of PHM on this highly reactive species could lead to the irreversible inactivation. The free radical abstraction of hydrogen atoms from aldehydes is well documented.[172,173] However, this type of substrate and inactivation are novel for the PHM enzyme, and will require additional investigation to confirm the mechanism.

Scheme 65.



Inhibition Studies with N-Acetyl-L-Prolyl-N-Nitroso-Glycine (96)

Interaction of *N*-Ac-L-Pro-[1,2-^{14}C]-Gly (**94**) with PHM followed by nitrosobenzene assay demonstrated that this was not a good substrate for the enzyme. Under the same conditions the turnover for **94** was only 1/10 of the turnover of D-Tyr-L-Val-Gly (**7**). Even though mechanism-based inhibition for *N*-Ac-L-Pro-*N*-nitroso-Gly (**96**) may be expected, only very weak competitive inhibition of PHM was seen; the IC_{50} was calculated to be 0.80 mM (Substrate concentration is 0.72 μM). Apparently an additional nitroso group on the amide nitrogen makes the binding of **96** to the enzyme active site more difficult than for **94**. Like the *N*-hydroxy[86] and *N*-methyl analogues,[31] the *N*-nitroso compound is not recognized by PHM as a substrate and only weak competitive inhibition is observed, rather than mechanism-based inhibition.

*Inhibition Studies with D-Phenylalanyl Hydrazinosuccinic Acid (**101**)*

The inhibition studies of D-phenylalanyl hydrazinosuccinic acid (**101**) on PHM were performed by Dr. T. M. Zabriskie. No inhibition of the PHM enzyme could be observed at concentrations as high as 1 mM. The PHM active site will not recognize this non-peptide compound as a possible substrate.

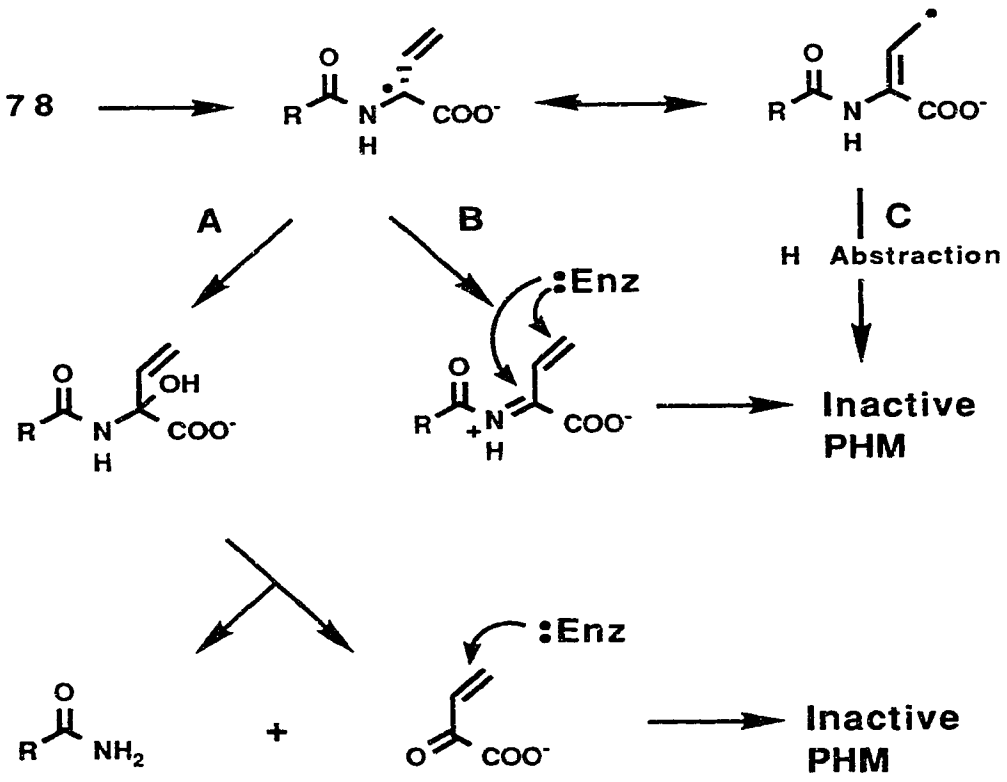
*Inhibition by D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine (**78**) and D-Phenylalanyl-L-Phenylalanyl-L-Vinylglycine (**80**)*

The inhibition of PHM was first studied with D-phenylalanyl-L-phenylalanyl-D-vinylglycine (**78**). Preliminary tests showed that **78** causes strong time-dependent inhibition of PHM. These were followed by detailed studies of the inhibitory effect of **78** on the PHM enzyme by Dr. T. M. Zabriskie. An apparent K_I of 20 μM was established by subsequent preincubation studies. Incubation of the enzyme with a 250 μM solution of **78** for 45 minutes, followed by removal of the inhibitor by repetitive concentration/dilution steps using a micro ultrafiltration device, gave no recovery of activity. Furthermore, treatment with 4 M urea and removal of inhibitor also failed to restore activity, thereby demonstrating that the inactivation is irreversible and probably not due to a slow-binding inhibitor with a high dissociation constant. Preincubation of PHM with the substrate D-Phe-L-Phe-Gly (**14**) and **78** revealed protection of the enzyme in proportion to the amount of added substrate, indicating that **78** acts at the active site of the enzyme. Treatment of PHM with **78** in the absence of Cu(II) and ascorbate for one hour followed by removal of the inhibitor resulted in no significant loss of activity. The requirement that all cofactors be present for inactivation to occur illustrates that **78** is a mechanism-based inhibitor.

Possible mechanisms of action for **78** are depicted in Scheme 66. Abstraction of the α-hydrogen from the D-vinylglycine residue produces a conjugated radical at the

α-carbon. Direct hydroxylation (path A) would generate a vinylglycine carbinolamide that can cleave to give the peptide amide and the α-keto acid, a potential alkylating species. Oxidative electron transfer (path B) could form a very reactive *N*-acyl iminium species that could result in the covalent attachment of the inhibitor to the enzyme. Path C involves interaction of the resonance-stabilized radical at the distal primary carbon with amino acid residues in the enzyme active site (e.g. hydrogen abstraction). Further studies on this inactivation mechanism will be discussed later in the protein labeling section.

Scheme 66.



The PHM enzyme was also tested against compound **80**, the L-vinylglycine analogue, to provide a comparison with the inhibiting properties of **78**. An IC_{50} of 350 μM was obtained from the competitive inhibition assay (Substrate concentration is 0.72

μM). The preincubation studies of **80** showed no time-dependent loss of activity. This result suggests that PHM will bind **80** but cannot abstract hydrogen due to the stereochemistry of the terminal vinyl group of **80**; hence only moderate competitive inhibition is observed for **80**.

Summary of PHM Inhibition Studies

Table 9 gives a summary of the inhibition properties of the inhibitors discussed above. The mechanism-based inactivation of PHM by D-Phe-L-Phe-D-vinylGly (**78**) supports the free radical, direct hydroxylation mechanism for PHM oxidation, and the moderate competitive inhibition of PHM by D-Phe-L-Phe-L-vinylGly (**80**) agrees with the stereospecific abstraction of the glycine *pro-S* hydrogen during the oxidative process[82] and the ability of the enzyme to convert peptides terminating in D-alanine instead of glycine.[74] The weak inhibition by both D-Phe-L-Phe-D-cyclopropylGly (**85**) and *N*-Ac-L-Pro-*N* - nitroso-Gly (**96**) suggests that the enzyme active site is not large enough to bind these compounds. Although *N*-Ac-D-Phe-L-Phe-D-methylthioGly (**89A**) shows weak slow binding inhibition, *N*-Ac-D-Phe-L-Phe-D-benzylthioGly (**90A**) displays potent competitive inhibition of PHM. However, mechanism-based inactivation of PHM does not occur with either of these compounds. Although *trans*-4-phenyl-3-butenoic acid is a potent mechanism-based inactivator of PHM, *trans*-2-(2-phenylcyclopropyl)acetic acid (**88**) shows no inhibition of PHM. When the peptide substrate is modified as in the case of D-phenylalanyl-hydrazinosuccinic acid (**101**), PHM does not recognize it at all. The potent competitive inhibition of PHM by *N*-Ac-D-Phe-L-Phe-α-"L"-hydroxyGly (**42A**) indicates the strong binding between PHM and its product and demonstrates the important role the PAL enzyme plays during the amidating process. Finally a novel suicide substrate of PHM, *N*-Ac-D-Phe-L-Phe-NCHO (**91**), has been discovered.

Table 9. Summary of Inhibition Studies

	IC_{50} (μM)	K_I (μM)	Type of Inhibition
78		20	Mechanism-based
80	350		Competitive
91		400	Mechanism-based
85		>5,000	"Competitive"
88	none		N/A
89A	1000		Slow Binding
89B	>10, 000		Competitive
90A	50		Competitive
90B	400		Competitive
42A[a]	10		Competitive
42B[a]	750		Competitive
96	800		Competitive
101	none		N/A

[a]The inhibition was discussed in Chapter 2.

3.3 PROTEIN LABELING STUDIES WITH *N*-DANSYL-D-PHENYLALANYL-L-PHENYLALANYL-D-VINYLGLYCINE

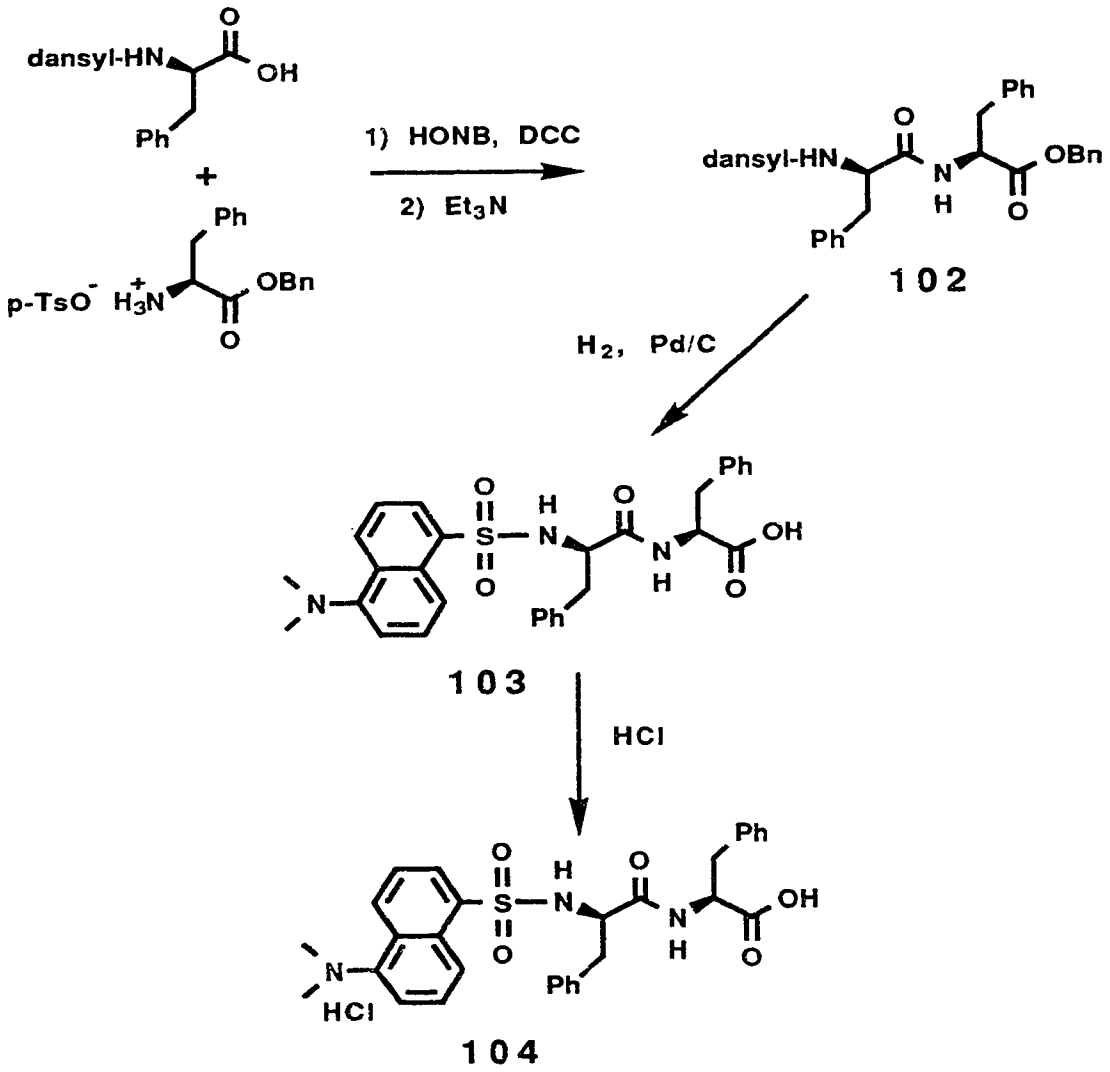
The previously discussed inhibition studies show that D-phenylalanyl-L-phenylalanyl-D-vinylglycine (**78**) is a potent mechanism-based inactivator of PHM. To gain further insight into the inactivation mechanism, an inhibitor was needed which

contained a label that would allow one to determine if it was covalently attached to the inactivated enzyme. Attachment of a radiolabeled moiety to the amino terminus of **78** appeared problematic becouse it could require conditions known to isomerize the vinylglycine, and would also require a lengthy synthesis of small amounts of highly radioactive material. Hence, a fluorescent label was used. The fluorescent dansyl group (5-dimethylamino-1-naphthalene-sulfonyl) was chosen since *N*-dansyl-D-Phe is inexpensive and readily available in large quantities. Fluorescence detection is also a very sensitive technique, and the synthesis of this compound could be easily achieved by the same methodology used for the synthesis of D-Phe-L-Phe-D-vinylGly (**78**).

Synthesis of *N*-Dansyl-D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine

Commercially available *N*-dansyl-D-phenylalanine was coupled with L-phenylalanine benzyl ester *p*-toluenesulfonic acid salt using the HONB/DCC procedure to generate **102** in 84% yield (Scheme 67). Catalytic hydrogenolysis cleaved the benzyl ester to produce the free acid **103** in 98% yield. This compound was thus converted to its hydrochloride salt **104** with 1 N HCl.

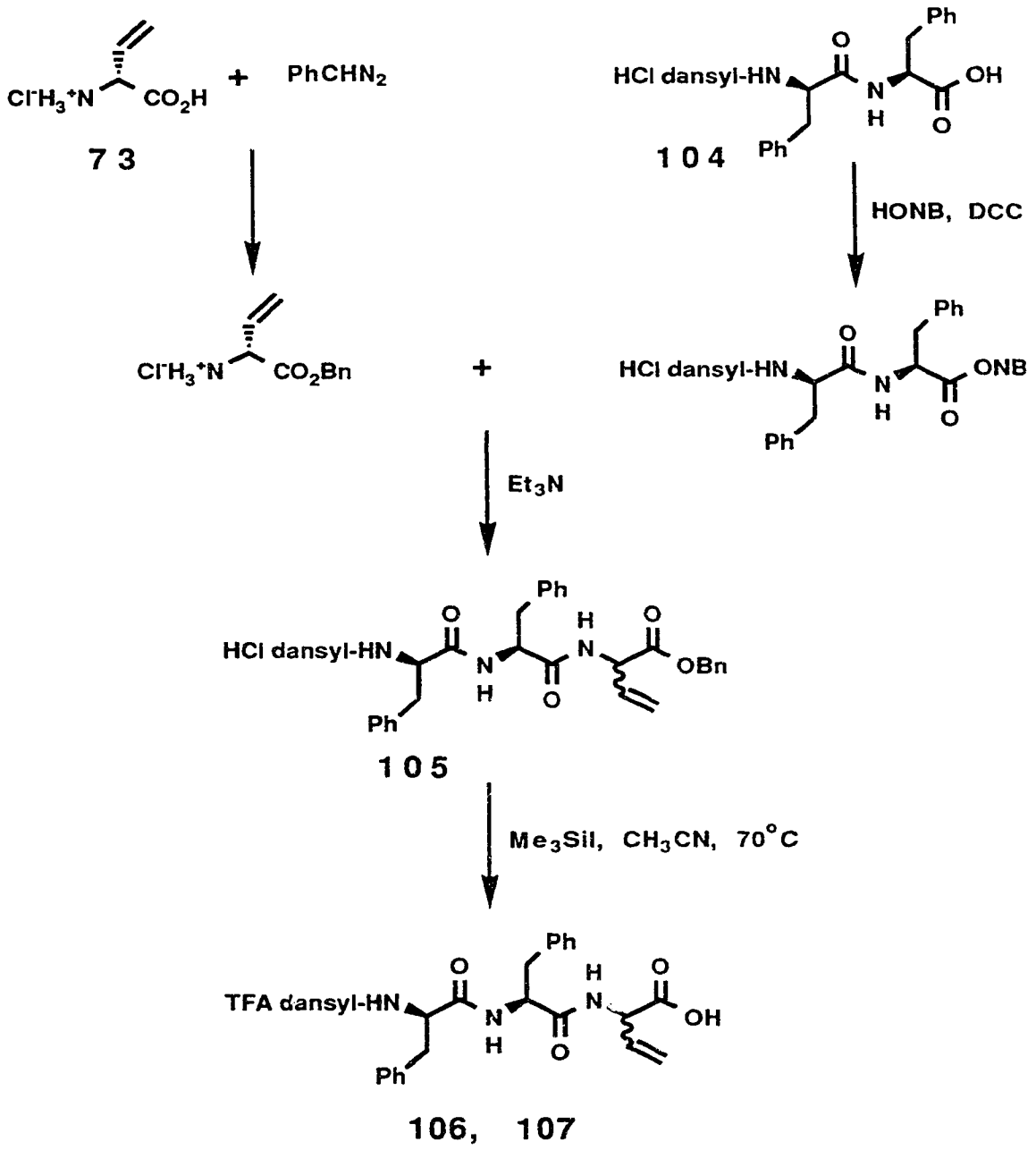
Scheme 67.



D-Vinylglycine hydrochloride was then treated with phenyldiazomethane to produce the benzyl ester, which was then coupled with **104** using the HONB/DCC procedure, to furnish the tripeptide **105** in 40% yield (Scheme 68). The low yield may be due in part to partially decomposed coupling reagent. The vinyl region of the ^{1}H NMR spectrum revealed the presence of two isomers for compound **105**, but the isomers were difficult to separate at this point and were thus used as a mixture. The benzyl group was

cleaved by iodotrimethylsilane and the product was purified by reverse-phase MPLC to give a mixture of diastereomers **106** and **107** in 63% isolated yield.

Scheme 68.



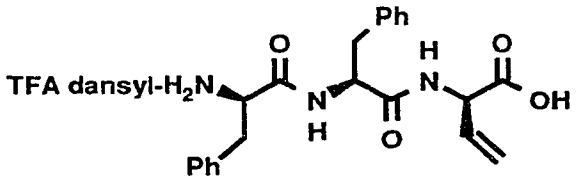
The two diastereomeric tripeptides were separated by reverse-phase HPLC (Waters C_{18} μ-Bondapak Radial Pak, 25 mm x 10 cm, 10 μm cartridge; A: 0.1% TFA in water, B: 0.1% TFA in 80:20 acetonitrile-water; isocratic system of 45% B; flow rate 4.5 mL/min; detection at 220 nm) to give **106** (retention time: 37.27 min) and **107** (retention time: 41.49 min).

Comparison of the chemical shifts, coupling patterns of the vinyl groups, and retention times on HPLC and the inhibition properties of these two isomers **106** and **107** to D-Phe-L-Phe-D-vinylGly (**78**) and D-Phe-L-Phe-L-vinylGly (**80**) peptides showed the same pattern of characteristics (Table 10). Both **106** and **80** elute earlier than **107** and **78** respectively, when purified by reverse-phase HPLC. The chemical shifts of the vinyl hydrogens (CH=C<u>H</u>) for **106** and **80** are higher than those of **107** and **78**. The chemical shift differences of the terminal vinyl hydrogens (C<u>H</u>$_2$=CH) of both **106** and **80** are greater than those of **107** and **78** respectively, and the coupling constants of these hydrogens for **106** and **80**, compared to **107** and **78** are also similar. Both **107** and **78** exhibited irreversible inhibition of PHM, while the inhibition of PHM by **106** and **80** was reversible. Therefore, compound **106** is similar to **80** and must be the L-vinylglycine diastereomer, and compound **107** is similar to **78** and is the D-vinylglycine diastereomer. In the synthesis, isomerization leading to a mixture of diastereomers probably occurred during the storage of the starting material (benzyl D-vinylglycinate hydrochloride salt) or during the coupling step due to the presence of the dimethylamino group in **104**.

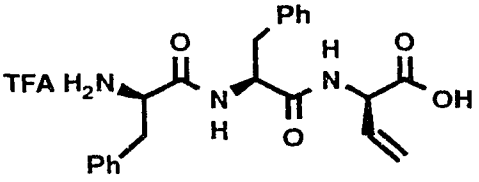
Table 10. Comparison of the ^1H NMR Data, Relative HPLC Retention Times, and Inhibition Properties of 78, 107, 80, and 106.

	78	**107**	**80**	**106**
Retention Time	Later than **80**	Later than **106**	Earlier than **78**	Earlier than **107**
CHH=CH	5.12 (d, 17.3[a])	5.18 (d, 2.9[a])	5.24 (d, 10.2[a])	5.28 (d, 10.5[a])
CHH=CH	5.18 (d, 10.8[a])	5.25 (d, 10.3[a])	5.37 (d, 16.9[a])	5.40 (d, 17.5[a])
CHH=CH	5.89 (m)	5.94 (m)	5.98 (m)	6.01 (m)
Inhibition	Irreversible	Irreversible	Reversible	Reversible

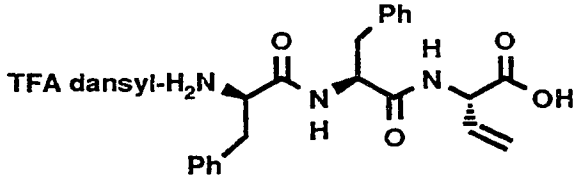
[a]Coupling constant in Hz.



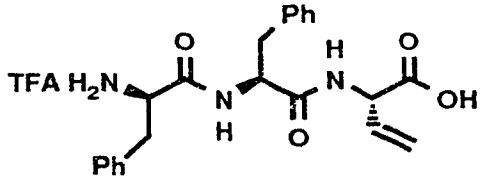
107



78



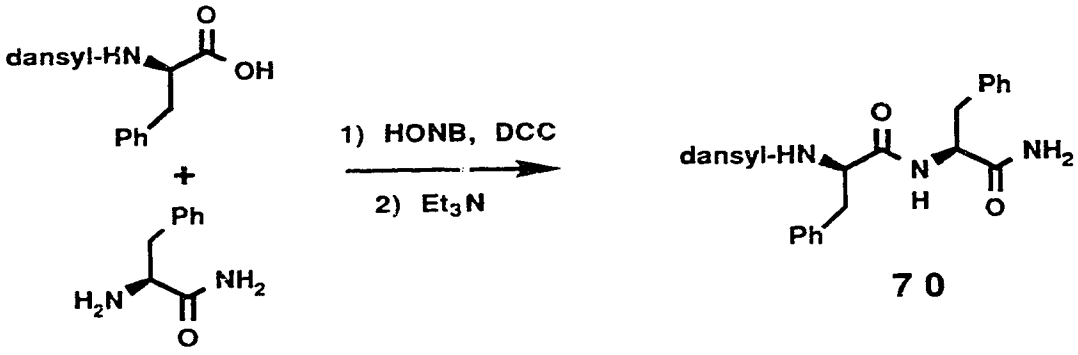
106



80

The dipeptide amide, *N*-dansyl-D-phenylalanyl-L-phenylalaninamide (**108**), was also synthesized for the enzyme studies. It was prepared in one step from *N*-dansyl-D-phenylalanine and L-phenylalaninamide using the HONB/DCC procedure (Scheme 69). Again the yield was low (48%), probably due to poor purity of coupling reagent.

Scheme 69.



Interaction of PHM with Fluorescent Inhibitor

The following experiments were performed by Dr. T. M. Zabriskie. Preliminary tests were first conducted to show that **107** irreversibly inhibits PHM. A large sample of the enzyme was then inactivated with the fluorescent peptide, a small amount of the active PHM was added to aid in isolation, and the inactivated enzyme was separated from **107** by gel filtration chromatography. Fractions containing amidating activity were pooled and concentrated, as were those which had a UV absorption at 250 nm (λ_{max} of **107**). Spectrofluorimetric analysis of these samples showed no fluorescence associated with the PHM fractions above the minimum level of detection (*ca.* 5 pmol). A single late-eluting fraction which did fluoresce was further analyzed by HPLC and found to contain the tripeptide **107**.

These results indicate that the substrate analogues **78** and **107** inactivate PHM by a mechanism that results in the cleavage of the terminal portion of the peptide substrate (Scheme 66). Current studies on elucidation of the actual mechanism involving the preparation of **78** with a radioactively labeled D-vinylglycine residue and determination of the mode of attachment of this moiety to PHM are being done by Dr. T. M. Zabriskie.

EXPERIMENTAL

General

All non-aqueous reactions were done in oven dried glassware under a slight positive pressure of dry argon. Solvents for anhydrous reactions were dried according to Perrin *et al.*[144] Specifically benzene, toluene, tetrahydrofuran (THF), and diethyl ether were distilled from sodium and benzophenone. Acetonitrile, triethylamine (Et_3N), diisopropylamine, and pyridine were distilled from CaH_2. Anhydrous ethanol (EtOH) and methanol (MeOH) were prepared by distillation from magnesium with catalytic iodine. Dimethylformamide (DMF) was stirred for 24 h over BaO, decanted, distilled under vacuum, and stored over 3 Å molecular sieves under 10 psi argon pressure. Trifluoroacetic acid (TFA) was dried over P_2O_5 before distillation. Solvents used for chromatography were distilled. Reagents for anhydrous reactions were dried overnight under vacuum over P_2O_5. The term *"removal of solvents in vacuo"* refers to rotatory evaporation under reduced pressure at <40 °C, followed by evacuation (<50 mtorr) to constant weight. Water was obtained from a Milli-Q reagent water system. Buffers were prepared with Milli-Q water and the pH was adjusted to the desired value at room temperature with NaOH and HCl solutions. Phosphate buffer for Sephadex columns was thoroughly degassed by stirring and heating under reduced pressure (30-60 min) and allowing to cool under argon. When indicated, water was degassed in the same way followed by passing a stream of argon through the solution for 15 min.

All reagents employed were ACS grade or finer. Air sensitive reagents were handled under an atmosphere of dry Ar. Commercial and organometallic reagents were obtained from Aldrich Chemical Co. unless otherwise specified. n-Butyllithium solution was periodically titrated against methanol/phenanthroline.[174] Amino acids and amino acid derivatives used as starting materials were obtained from Sigma Chemical Co.. Commercially available enzymes were obtained from Sigma and stored as indicated.

Catalase (from bovine liver) was purchased as an aseptically filtered aqueous solution from Sigma (cat. number C3155, ~15mg/mL, ~5000 units/mg). Sephadex G-100 was obtained from Pharmacia Labs. Inc. (Piscataway, New Jersey, U.S.A).

Whenever possible, reactions were followed by thin layer chromatography (TLC) and visualized using UV absorption by fluorescence quenching, I_2 staining, bromocresol green spray for acids, ninhydrin spray for amino acids and peptides, and dodecamolybdophosphoric acid spray. For monitoring reactions in DMF or water, the plate was kept under vacuum (<50 mtorr) for 10 min prior to developing.

Commercial thin layer and preparative layer chromatography plates were: normal silica, Merck 60 F-254; reverse-phase, Merck P-8F_{254} S and Macherey-Nagel and Co.; ion exchange resin, Polygram® Ionex-25 SA-Na. Silica gel for column chromatography was Merck type 60, 70-230 mesh or its equivalent from General Intermediates of Canada (Edmonton, AB). Flash chromatography was performed according to Still *et al.*[175] using Merck type 60, 230-420 mesh silica gel. Normal phase medium pressure liquid chromatography (MPLC) used a column of Merck Kieselgel 60 H (*ca.* 55 g, 2.5 x 30 cm). Reverse phase MPLC was performed on Merck Labor Lichroprep RP-8 columns. All solvent mixtures are listed as volume ratios, and all medium pressure liquid chromatography was performed using solvents which were previously degassed under vacuum. The cation exchange resin used was Bio-Rad AG50W-X 8 (H^+ form, 50-100 mesh).

All literature compounds had IR, ^1H NMR, and mass spectra consistent with the assigned structures. Melting points are uncorrected and determined on a Thomas Hoover or Buchi oil immersion apparatus using open capillary tubes. Optical rotations were measured on a Perkin Elmer 241 polarimeter with a microcell (10.00 cm, 0.9 mL) at ambient temperature. All specific rotations reported were measured at the sodium D line. Infrared spectra (IR) were recorded on a Nicolet 7199 FT-IR spectrometer. Mass spectra (MS) were recorded on Kratos AEI MS-50 (high resolution, electron impact ionization),

MS-12 (chemical (NH_3) ionization, CI-MS) and MS-9 (fast atom bombardment (FAB) with argon, POSFAB) instruments. The term 'Cleland's reagent' (as a matrix in FAB MS) refers to a 5:1 mixture of dithiothreitol and dithioerythritol. Microanalyses were obtained using a Perkin Elmer 240 CHN analyzer.

Nuclear magnetic resonance (NMR) spectra were measured on Bruker WP-80 (continuous wave), WH-200, AM-300, WM-360, or AM-400 instruments in the specified solvent with tetramethylsilane (TMS) or deuterated sodium 3-(trimethylsilyl)-1-propionate (TSP) in D_2O as internal standards in ^1H NMR. For ^{13}C NMR spectra, which were obtained on the WH-200 or AM-400, the deuterated solvent peak was used as the reference.

High performance liquid chromatography (HPLC) was performed on either a Hewlett Packard 1082B, Beckman System Gold, or Bio-Rad Gradient Module fitted with a variable wavelength detector. Columns were Waters μ-Bondapak Radial-Pak cartridges used with a Waters Z-module compression unit. Two different HPLC systems were employed. With reverse phase Radial-Pak C_{18} columns and the aminopropyl Radial-Pak column, a two solvent system was employed (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in 80:20 acetonitrile:water)(See each compound for details of flow rate and solvent ratios). HPLC grade acetonitrile (190 nm cutoff) was obtained from Terochem (Edmonton, AB). All HPLC solvents and buffers were freshly prepared and filtered with a Millipore filtration system under vacuum before use.

Radioactivity was determined using standard liquid scintillation procedures in plastic 10 mL scintillation vials (Terochem) with an Amersham ACS liquid scintillation cocktail, on Beckman LS100C and Beckman 1801 instruments. With the Beckman 1801 the automatic quench control was employed to directly determine decompositions per minute (dpm) in single and dual label samples by comparison against a quench curve prepared from Beckman ^3H and ^{14}C quenched standards. This automatically calculates $^3H/^{14}C$ ratios, but the results were confirmed by analyzing random samples with the

addition of standardized ^{14}C-toluene and ^{3}H-toluene solutions (ICN Radiochemicals). The values obtained always agreed within 5% of those calculated by the instrument. Radioactive TLC plates were analyzed with a Berthold LB2760 TLC-scanner.

Frozen pig pituitaries were obtained from Pel-Freez Biologicals (Rogers, Arkansas) and were stored frozen at -60 °C until needed. Homogenization was performed using a Ika-Werk Ultra-Turrax homogenizer. A Sorvall RC-5B refrigerated superspeed centrifuge with SS-34 rotor at 16.5 Krpm was used for the enzyme preparation. An LKB peristaltic pump was employed to control the flow rate on Sephadex and substrate affinity columns. Spectrapor membrane tubing (Fisher Scientific, 25 mm diameter, 12, 000-14, 000 molecular weight cutoff) was used for dialysis. Enzyme assays and extractions were conveniently carried out using 1.5 mL microcentrifuge tubes (Terochem).

_N_-(*tert*-Butyloxycarbonyl)-D-tyrosine (1)

The literature procedure was modified.[108] D-Tyrosine (4.00 g, 22.1 mmol) was added to a stirred solution of dioxane (50 mL), H_2O (25 mL) and 0.5 N NaOH (24 mL). This was cooled to 0 °C and di-*tert*-butylpyrocarbonate (5.50 g, 25.2 mmol) was added in a single portion. The solution was then stirred at room temperature for 2.5 h, during which time, the pH of the solution was kept around 9. About half of the solvent was removed *in vacuo* and EtOAc (100 mL) was added. The solution was acidified to pH 3 with 0.5 M $KHSO_4$ solution (80 ml). The organic layer was separated and the aqueous layer was extracted with EtOAc (2x100 mL). The organic layers were combined, washed with H_2O (15 mL) and saturated aqueous NaCl (2x15 mL), dried(Na_2SO_4), and concentrated *in vacuo* to give a gummy solid. This was isolated as the dicyclohexylamine salt by dissolving the residue in absolute ethanol (30 mL), adding dicyclohexylamine (6.8 mL, 34.0 mmol) and diluting to 200 mL with ether. The white precipitate was collected and dried under vacuum. This was then dissolved in a mixture of $KHSO_4$ (3.80 g, 1.5 equivalent.) in H_2O (170 mL) and EtOAc (170 mL). The aqueous layer was extracted with

EtOAc (2x30 mL) and the combined EtOAc extracts were washed with H_2O (2x40 mL) and saturated aqueous NaCl solution (40 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give **1**: 7.75 g (93%). IR ($CHCl_3$ cast) 3265, 2890, 1722, 1692, 1520 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.38 (s, 9 H, $(C\underline{H}_3)_3C$), 2.81 (dd, J = 13.3, 9.2 Hz, 1 H, aryl C$\underline{H}$), 3.03 (d, J = 13.7 Hz, 1 H, aryl C$\underline{H}$), 4.27 (br s, 1 H, NC$\underline{H}$CO), 6.69 (d, J = 7.8 Hz, 2 H, aryl $\underline{H}$), 7.03 (d, J = 7.7 Hz, 2 H, aryl $\underline{H}$); exact mass, 281.1266 (281.1263 calcd for $C_{14}H_{19}NO_5$).

_N_-(*tert*-Butyloxycarbonyl)-D-tyrosyl-L-valine Benzyl Ester (2)

The method of Shioiri and Yamada for peptide coupling with diphenylphosphoryl azide was used.[142] A dry flask containing *N*-BOC-D-tyrosine (1.47 g, 5.23 mmol) and L-valine benzyl ester *p*-toluenesulfonate salt (2.17 g, 5.72 mmol) was dried at 50 mTorr over P_2O_5 for 16 h. To this was added dry DMF (15 mL), and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (1.35 mL, 6.20 mmol) was added, followed by a solution of triethylamine (1.65 mL, 12.1 mmol) in DMF (10 mL), and stirring was continued at 0 °C for 10 h. The mixture was diluted with benzene (125 mL) and EtOAc (250 mL). This was washed successively with 1 N HCl (2x25 ml), H_2O (25 mL), saturated aqueous $NaHCO_3$ (2x25 mL), H_2O (25 mL), and saturated aqueous NaCl (2x25 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give an oil that was purified by recrystallization from toluene/Skelly B to give **2**: 1.52 g (62%). Mp 101-102 °C; IR ($CHCl_3$ cast) 3340, 2959, 2930, 1734, 1656, 1518 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.76, 0.82 (2 x d, J = 6.7 Hz, J = 6.7 Hz, 2 x 3 H, $(C\underline{H}_3)_2C$), 1.44 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.08 (m, 1 H, $(CH_3)_2C\underline{H}$), 2.99 (d, J = 6.9 Hz, 2 H, aryl C$\underline{H}_2$), 4.35 (m, 1 H, NC$\underline{H}$CO), 4.54 (dd, J = 8.7, 4.7 Hz, 1 H, NC$\underline{H}$CO), 5.06 (br s, 1 H, N$\underline{H}$), 5.12, 5.18 (ABq, J = 12.2 Hz, J = 12.2 Hz, 2 x 1 H, OC$\underline{H}_2$Ph), 6.04 (s, 1 H, O$\underline{H}$), 6.54 (d, J = 8.8 Hz, 1 H, N$\underline{H}$), 6.71 (d, J = 8.5 Hz, 2 H, aryl $\underline{H}$), 7.02 (d, J = 8.5 Hz, 2 H, aryl $\underline{H}$), 7.26 (m, 5 H, aryl $\underline{H}$); exact mass, 470.2414 (470.2417 calcd

for $C_{26}H_{34}N_2O_6$). Anal. Calcd for $C_{26}H_{34}N_2O_6$: C, 66.36; H, 7.28; N, 5.95. Found: C, 66.10; H, 7.17; N, 5.83.

N-(*tert*-Butyloxycarbonyl)-D-tyrosyl-L-valine (3)

A mixture of **2** (668 mg, 1.42 mmol) and 5% Pd/C (107 mg) in EtOAc (50 mL) was hydrogenated under hydrogen at 48 psi for 1.5 h. The reaction mixture was filtered through Celite, and the solvent was removed *in vacuo* to give **3**: 534 mg (99%). Mp 97-100 °C (lit.[82] mp 97-100 °C); IR ($CHCl_3$ cast) 3320, 1658, 1516 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 0.89 (m, 6 H, $(C\underline{H}_3)_2CH$), 1.40 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.10 (br, 1 H, $(CH_3)_2C\underline{H}$), 2.88 (br, 1 H, aryl $C\underline{H}H$), 3.10 (br, 1 H, aryl $CH\underline{H}$), 4.42 (br, 1 H, $NC\underline{H}CO$), 4.66 (br, 1 H, $NC\underline{H}CO$), 5.46 (br, 1 H, $N\underline{H}$), 6.69 (d, J = 8.5 Hz, 2 H, aryl $\underline{H}$), 6.88 (br, 1 H, $N\underline{H}$), 7.00 (br, 2 H, aryl $\underline{H}$); exact mass, 380.1949 (380.1947 calcd for $C_{19}H_{28}N_2O_6$).

Glycine Methyl Ester Hydrochloride (4)

Dry hydrogen chloride gas was bubbled for 0.5 h through a solution of glycine (5.00 g, 66.6 mmol) in dry MeOH (100 mL) cooled in an ice-water bath, with the temperature being maintained between 7 °C and 10 °C by controlling the rate of HCl bubbling. The reaction mixture was allowed to warm to room temperature and stirred for 18 h. After cooling to - 20 °C, the precipitate was collected and recrystallized from dry MeOH to give the pure methyl ester **4**: 2.58 g (31%). Mp 183 °C (lit.[199] mp 175 °C dec); IR (KBr) 1750 (br) cm^{-1}; ^1H NMR (80 MHz, D_2O) δ 3.70 (s, 3 H, $OC\underline{H}_3$), 3.79 (s, 2 H, $C\underline{H}_2$); exact mass, 89.0482 (89.0477 calcd for $C_3H_7NO_2$).

N-(*tert*-Butyloxycarbonyl)-D-tyrosyl-L-valyl-glycine Methyl Ester (5)

The literature procedure was modified.[142] A flask containing **3** (520 mg, 1.36 mmol) and **4** (200 mg, 1.59 mmol) was dried at 50 mTorr over P_2O_5 for 18 h. To this

was added dry DMF (10 mL) and the stirred solution was cooled to 0 °C. Diphenylphosphorylazide (0.31 mL, 1.46 mmol) was added followed by a solution of triethylamine (0.45 mL, 3.25 mmol) in DMF (10 mL), and stirring was continued at 0 °C for 6.5 h. The reaction was diluted with benzene (20 mL) and EtOAc (40 mL), and was then washed with 1 N HCl (2x15 mL), H_2O (15 mL), saturated aqueous $NaHCO_3$ (2x15 mL), H_2O (15 mL), and saturated aqueous NaCl (2x15 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give an oil which was purified by flash chromatography using 5% MeOH/$CHCl_3$ to give **5**: 561 mg (91%). Mp 87-89 °C (lit.[82] mp 87-89 °C); IR ($CHCl_3$ cast) 3306, 1688, 1649, 1516 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 0.83 (d, 6 H, $(C\underline{H}_3)_2CH$), 1.41 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.15 (m, 1 H, $(CH_3)_2C\underline{H}$), 2.99 (m, 2 H, aryl $C\underline{H}_2$), 3.72 (s, 3 H, $CO_2C\underline{H}_3$), 4.04 (br s, 2 H, $NC\underline{H}_2CO$), 4.40 (m, 2 H, NC<u>H</u>CO, NC<u>H</u>CO), 5.48 (d, 1 H, N<u>H</u>), 6.72 (d, J = 8.5 Hz, 2 H, aryl <u>H</u>), 6.98 (d, J = 8.5 Hz, 3 H, N<u>H</u>, 2 aryl <u>H</u>), 7.53 (br s, 1 H, N<u>H</u>), 7.75 (br s, 1 H, O<u>H</u>); exact mass, 451.2323 (451.2318 calcd for $C_{22}H_{33}N_3O_7$).

N-(*tert*-Butyloxycarbonyl)-D-tyrosyl-L-valyl-glycine (6)

The general procedure for peptide ester hydrolysis was modified.[141] To a stirred solution of the methyl ester **5** (550. mg, 1.22 mmol) in MeOH (3 mL) in a room temperature water bath was added 1 N NaOH (3.1 mL) solution and the stirring was continued at room temperature for 1.5 h. 1 N HCl (1 mL) was added and the MeOH was removed *in vacuo*. The residue was further acidified with 1 N HCl (2.1 mL) and this was extracted with EtOAc (2x10 mL). The combined organic layers were washed with H_2O (5 mL) and saturated aqueous NaCl (5 mL), dried (Na_2SO_4), and the solvent was removed *in vacuo* to give **6**: 459 mg (86%). IR ($CHCl_3$ cast) 3315, 1722, 1651 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$ + CD_3COD(1 drop)) δ 0.80 (d, J = 6.1 Hz, 6 H, $(C\underline{H}_3)_2CH$), 1.40 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.10 (m, 1 H, $(CH_3)_2C\underline{H}$), 2.94 (m, 2 H, aryl $C\underline{H}_2$), 3.88, 4.04 (2 x d, J = 18.3 Hz, 2 H, $NC\underline{H}_2CO$), 4.12 (dd, J = 15.3, 7.3 Hz, 1 H, aryl $CH_2C\underline{H}$), 4.26 (d, J =

6.5 Hz, 1 H, C<u>H</u>CH(CH_3)$_2$), 5.46 (d, J = 7.2 Hz, 1 H, N<u>H</u>), 6.70 (d, J = 7.9 Hz, 2 H, aryl <u>H</u>), 6.96 (d, J = 7.9 Hz, 2 H, aryl <u>H</u>), 7.02 (d, J = 7.9 Hz, 1 H, N<u>H</u>), 7.40 (br s, 1 H, O<u>H</u>); FAB-MS 438.32 (MH^+) (437.22 calcd for $C_{21}H_{31}N_3O_7$).

D-Tyrosyl-L-valyl-glycine (7)

The literature procedure was modified.[140] TFA (15 mL) was added to **6** (2.00 g, 4.58 mmol) and the mixture was allowed to stand at room temperature for 30 min. The TFA was removed *in vacuo* and the residue was triturated with ether. The off-white solid was collected, dissolved in a minimum amount of 90 °C water, and 2% NH_4OH solution was added until the pH was 6. Ethanol (95%, 2-3 volumes) was added and the solution was cooled. The crystals were collected by filtration and dried to give the pure peptide **7**: 1.16 g (75%). Mp 229-230 °C (dec.) (lit.[176] 229-230 °C); IR (KBr) 3280, 1620, 1518 cm^{-1}; ^1H NMR (360 MHz, CD_3COOD) δ 0.88 (dd, J = 13.4, 6.7 Hz 6 H, ($C\underline{H}_3$)$_2$CH), 3.22 (dd, J = 7.9, 2.4 Hz, 2 H, aryl $C\underline{H}_2$), 4.16 (d, J = 3.1 Hz, 2 H, $NC\underline{H}_2CO$), 4.50 (d, J = 6.7 Hz, 1 H, NC<u>H</u>CHMe$_2$), 4.55 (t, J = 7.9 Hz, 1 H, aryl $CH_2C\underline{H}$), 6.86 (d, J = 8.5 Hz, 2 H, aryl <u>H</u>), 7.20 (d, J = 8.5 Hz, 2 H, aryl <u>H</u>); FAB-MS 337.98 (337.16 calcd for $C_{16}H_{23}N_3O_5$). Anal. Calcd for $C_{16}H_{23}N_3O_5 \cdot H_2O$: C, 54.07; H, 7.09; N, 11.86. Found: C, 54.16; H, 6.77; N, 11.82.

***N*-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-phenylalanine Benzyl Ester (10)**

The literature procedure was modified.[142] In a dry flask were placed *N*-BOC-D-phenylalanine (1.10 g, 4.15 mmol) and L-phenylalanine benzyl ester p-toluenesulfonate salt (1.71 g, 4.00 mmol). This was dried at 50 mTorr over P_2O_5 for 16 h. Dry DMF (15 mL) was then added, and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (1.10 mL, 5.05 mmol) was added followed by a solution of triethylamine (1.50 mL, 11.0 mmol) in DMF (10 mL), and stirring was continued at 0 °C for 6 h. The mixture was

diluted with benzene (100 mL) and EtOAc (200 mL), and then was washed successively with 1 N HCl (2x25 ml), H_2O (25 mL), saturated aqueous $NaHCO_3$ (2x25 mL), H_2O (25 mL), and saturated aqueous NaCl (2x25 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 78:22 Skelly B:EtOAc to give **10**: 1.60 g (80%). Mp 125-126 °C; IR (KBr) 3326, 1738, 1687, 1656, 1525 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.35 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 3.00 (m, 4 H, aryl $C\underline{H}_2$), 4.34 (dd, J = 14.5, 6.8 Hz, 1 H, NC<u>H</u>CO), 4.82-4.92 (m, 2 H, NC<u>H</u>CO, N<u>H</u>), 5.07, 5.11 (ABq, J = 12.2 Hz, J = 12.2 Hz, 2 x 1 H, $OC\underline{H}_2Ph$), 6.38 (d, J = 7.6 Hz, 1 H, N<u>H</u>), 6.85-7.25 (m, 10 H, aryl <u>H</u>); exact mass, 502.2470 (502.2468 calcd for $C_{30}H_{34}N_2O_5$). Anal. calcd for $C_{30}H_{34}N_2O_5$: C, 71.69; H, 6.82; N, 5.57. Found: C, 71.25; H, 6.87; N, 5.83.

N-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-phenylalanine (11)

A mixture of **10** (1.50 g, 2.99 mmol) and 5% Pd/C (100 mg) in EtOAc (25 mL) and MeOH (25 mL) was hydrogenated under hydrogen at 48 psi for 2 h. After filtration through #2 filter paper, the solvent was removed *in vacuo* to give **11:** 1.31 g (>99%). Mp 61 °C (dec); IR (KBr) 3415, 3323, 1716, 1658, 1605, 1522 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.34 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.75-3.20 (m, 4 H, aryl $C\underline{H}_2$), 4.32 (dd, J = 9.1, 4.9 Hz, 1 H, NC<u>H</u>CO), 4.68 (t, J = 6.4 Hz, 1 H, NC<u>H</u>CO), 7.21 (m, 10 H, aryl <u>H</u>); FAB-MS 413.32 (MH^+) (412.20 calcd for $C_{23}H_{28}N_2O_5$).

N-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-phenylalanyl-glycine Methyl Ester (12)

The literature procedure was modified.[142] In a dry flask were placed **11** (501 mg, 1.21 mmol) and glycine methyl ester hydrochloride salt (170. mg, 1.35 mmol). This was dried at 50 mTorr over P_2O_5 for 18 h. To this was added dry DMF (10 mL), and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (0.33 mL, 1.52 mmol) was

added followed by a solution of triethylamine (0.46 mL, 3.38 mmol) in DMF (5 mL), and stirring was continued at 0 °C for 6 h. The mixture was diluted with 60 mL of benzene and 120 mL of EtOAc. This was washed successively with 1 N HCl (2x20 mL), H_2O (20 mL), saturated aqueous $NaHCO_3$ (2x20 mL), H_2O (20 mL), and saturated aqueous NaCl (2x20 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 8% MeOH in $CHCl_3$ to give **12**: 280. mg (48%). Mp 163-165 °C; IR ($CHCl_3$ cast) 3440, 3290, 1763, 1706, 1672, 1659, 1645 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.37 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.73-3.19 (m, 4 H, aryl $C\underline{H}_2$), 3.70 (s, 3 H, $OC\underline{H}_3$), 3.95 (m, 2 H, $NC\underline{H}_2CO$), 4.10 (m, 1 H, $NC\underline{H}CO$), 4.74 (m, 1 H, $NC\underline{H}CO$), 5.02 (d, J = 6.8 Hz, 1 H, $N\underline{H}$), 6.19 (br s, 1 H, $N\underline{H}$), 6.97 (br s, 1 H, $N\underline{H}$), 7.25 (m, 10 H, aryl $\underline{H}$); FAB-MS 484.55 (MH^+) (483.24 calcd for $C_{26}H_{33}N_3O_6$). Anal. Calcd for $C_{26}H_{33}N_3O_6$: C, 64.58; H, 6.88; N, 8.69. Found: C, 64.55; H, 6.71; N, 8.39.

***N*-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-phenylalanyl-glycine (13)**

The literature procedure was modified.[141] Aqueous 1 N NaOH (9.1 mL) was added to a stirred solution of **12** (250.0 mg, 0.518 mmol) in MeOH (10 mL) in a room temperature water bath, and the stirring was continued for 10 h. Then 1 N HCl (5 mL) was added and the MeOH was removed *in vacuo*. The remaining solution was acidified to pH 1 with 1 N HCl, and a heavy precipitate was formed. EtOAc (20 mL) was added, the aqueous phase was separated, and the aqueous layer was extracted with EtOAc (20 mL). The combined organic layers were washed with H_2O (10 mL) and saturated aqueous NaCl (10 mL), and then dried (Na_2SO_4). The solvent was removed to give **13**: 150. mg (62%). Mp 68 °C (dec); IR (KBr) 3405, 1748, 1717, 1684, 1646 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.34 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.57-3.33 (m, 4 H, aryl $C\underline{H}_2$), 3.84, 3.95 (ABq, J = 17.7 Hz, J = 17.7 Hz, 2 x 1 H, $NC\underline{H}_2CO$), 4.25 (dd, J = 9.1, 5.8 Hz, 1 H, $NC\underline{H}CO$), 4.72 (dd, J = 8.9, 4.8 Hz, 1 H, $NC\underline{H}CO$), 7.20 (m, 10 H, aryl $\underline{H}$); FAB-MS

470.54 (MH^+) (469.22 calcd for $C_{25}H_{31}N_3O_6$). Anal. Calcd for $C_{25}H_{31}N_3O_6$: C, 63.95; H, 6.66; N, 8.95. Found: C, 63.60; H, 6.63; N, 8.53.

D-Phenylalanyl-L-phenylalanyl-glycine Trifluoroacetic Acid Salt (14) from 13

The literature procedure was modified.[140] A solution of **13** (100.0 mg, 0.213 mmol) in TFA (5 mL) was stirred for 1.5 h at room temperature. The solvent was removed *in vacuo* and the residue was triturated with ether to produce an off white solid. The ether was evaporated *in vacuo* to give **14**: 100. mg (97%). This was further purified by reverse phase HPLC (Waters μ-Bondapak Radial Pak, 8 mm x 10 cm, 4 μm cartridge, retention time 22.47 min at 1.50 mL/min flow rate, gradient elution: 0 min, 10% B; 20 min, 50% B; 25 min, 50% B). Mp 173-174.5 °C; IR (KBr) 3385, 3280, 3086, 2826, 1666, 1546 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.70 (dd, J = 14.2, 8.2 Hz, 1 H, aryl CH), 2.82 (dd, J = 13.9, 9.9 Hz, 1 H, aryl CH), 2.91 (dd, J = 14.2, 5.4 Hz, 1 H, aryl CH), 3.18 (dd, J = 13.9, 5.0 Hz, 1 H, aryl CH), 3.90 (s, 2 H, NCH_2CO), 4.05 (dd, J = 7.5, 6.2 Hz, 1 H, NCHCO), 4.73 (dd, J = 9.7, 5.0 Hz, 1 H, NCHCO), 7.04-7.25 (m, 10 H, aryl H); FAB-MS 370.18 (MH^+) (369.17 calcd for $C_{20}H_{23}N_3O_4$). Anal. Calcd for $C_{20}H_{23}N_3O_4 \cdot CF_3CO_2H \cdot H_2O$: C, 52.69; H, 5.23; N, 8.31. Found: C, 52.52; H, 4.67; N, 8.31.

***N*-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-phenylalanyl-glycine Benzyl Ester (15)**

The literature procedure was modified.[142] A flask containing **11** (510. mg, 1.24 mmol) and glycine benzyl ester *p*-toluenesulfonic acid salt (470. mg, 1.39 mmol) was dried at 50 mTorr over P_2O_5 for 18 h. Dry DMF (10 mL) was then added, and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (0.33 mL, 1.52 mmol) was added followed by a solution of triethylamine (0.46 mL, 3.38 mmol) in DMF (5 mL), and stirring

was continued at 0 °C for 6 h. The mixture was diluted with benzene (60 mL) and EtOAc (120 mL). This was washed successively with 1 N HCl (2x15 ml), H_2O (15 mL), saturated aqueous $NaHCO_3$ (2x15 mL), H_2O (15 mL), and saturated aqueous NaCl (2x15 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 70/30 toluene/EtOAc to produce **15**: 500. mg (72%). Mp 164-165 °C; IR (KBr) 3430, 3290, 1757, 1719, 1643, 1551 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.35 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 3.00 (m, 4 H, aryl $C\underline{H}_2$), 3.84 (dd, J = 18.0, 4.8 Hz, 1 H, NC$\underline{H}$CO), 4.11 (m, 2 H, NC$\underline{H}_2$CO), 4.74 (dd, J = 14.5, 6.5 Hz, 1 H, NC$\underline{H}$CO), 5.04 (d, J = 5.9 Hz, 1 H, N$\underline{H}$), 5.14 (s, 2 H, OC$\underline{H}_2$Ph), 6.17 (d, J = 5.9 Hz, 1 H, N$\underline{H}$), 6.88 (br s, 1 H, N$\underline{H}$), 7.26 (m, 10 H, aryl $\underline{H}$); FAB-MS 560.24 (MH^+) (559.27 calcd for $C_{32}H_{37}N_3O_6$). Anal. Calcd for $C_{32}H_{37}N_3O_6$: C, 68.68; H, 6.66; N, 7.51. Found: C, 68.52; H, 6.61; N, 7.45.

D-Phenylalanyl-L-phenylalanyl-glycine Benzyl Ester Hydrochloride Salt (16)

The literature procedure was modified.[140] A solution of **15** (210. mg, 0.375 mmol) in TFA (5 mL) was stirred for 1 h. The solvent was removed *in vacuo* and the residue was triturated with ether to give an off white solid. The ether was removed *in vacuo* to give the crude product. This was purified by dissolving the crude product in 5% aqueous $NaHCO_3$ (5 mL), and extracting with CH_2Cl_2 (3x15 mL). The combined organic layers were washed with H_2O (5 mL), and 1 N HCl (5 mL) and then dried (Na_2SO_4). The solvent was removed to give **16**: 184 mg (99%). Mp 233 °C; IR (KBr) 3330, 3250, 1740, 1700, 1645, 1543 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 2.64-3.20 (m, 4 H, aryl $C\underline{H}_2$), 3.97, 4.01 (ABq, J = 17.9 Hz, J = 17.9 Hz, 2 x 1 H, NC$\underline{H}_2$CO), 4.07 (dd, J = 8.1, 5.7 Hz, 1 H, NC$\underline{H}$CO), 4.72 (dd, J = 10.1, 5.1 Hz, 1 H, NC$\underline{H}$CO), 5.18 (s, 2 H, OC$\underline{H}_2$Ph), 6.95-7.38 (m, 15 H, aryl $\underline{H}$); FAB-MS 460.27 (MH^+) (459.22 calcd for

$C_{27}H_{29}N_3O_4$). Anal. Calcd for $C_{27}H_{29}N_3O_4 \cdot HCl$: C, 65.38; H, 6.10; N, 8.47. Found: C, 65.11; H, 6.12; N, 8.35.

D-Phenylalanyl-L-phenylalanyl-glycine Trifluoroacetic Acid Salt (14) from 16

A mixture of **16** (151 mg, 0.263 mmol) and 5% Pd/C (35 mg) in EtOAc (5 mL) and MeOH (25 mL) was hydrogenated under 1 atmosphere of hydrogen for 1 h. After filtration through # 2 filter paper, the solvent was removed *in vacuo* to give **14**: 124 mg (98%). Mp. 173-174.5 °C; IR (KBr) 3385, 3280, 3086, 2826, 1666, 1546 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.70 (dd, *J* = 14.2, 8.2 Hz, 1 H, aryl CH), 2.82 (dd, *J* = 13.9, 9.9 Hz, 1 H, aryl CH), 2.91 (dd, *J* = 14.2, 5.4 Hz, 1 H, aryl CH), 3.18 (dd, *J* = 13.9, 5.0 Hz, 1 H, aryl CH), 3.90 (s, 2 H, NCH_2CO), 4.05 (dd, *J* = 7.5, 6.2 Hz, 1 H, NCHCO), 4.73 (dd, *J* = 9.7, 5.0 Hz, 1 H, NCHCO), 7.04-7.25 (m, 10 H, aryl H); FAB-MS 370.18 (MH^+) (369.17 calcd for $C_{20}H_{23}N_3O_4$). Anal. Calcd for $C_{20}H_{23}N_3O_4 \cdot TFA \cdot H_2O$: C, 52.69; H, 5.23; N, 8.38. Found: C, 52.52; H, 4.67; N, 8.31.

[1,2-^{14}C]-Glycine Methyl Ester Hydrochloride (17)

A solution of [1,2-^{14}C]-glycine (250 μCi, 2.21 μmol) in 0.1 N HCl (2.5 mL) was lyophilized. The residue was then dissolved in dry MeOH (5 mL). Hydrogen chloride gas was passed over the cooled (0 °C) solution for 60 min and the resulting solution was stirred at room temperature for 8.5 h. Then the solvent was removed to give **17**: 248.3 μCi (99%). This material shows a single radioactive spot on TLC and co-migrates with unlabeled compound **4** in two different solvent systems.

D-Phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycine Trifluoroacetic Acid Salt (18)

The methods used to prepare unlabeled peptide **14** were adapted for radioactive synthesis. A flask containing **11** (7.07 mg, 14.6 μmol) and **17** (74.4 μCi, 0.744 μmol) was dried at 50 mTorr over P_2O_5 for 18 h. Dry DMF (3 mL) was then added, and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (4.3 μL, 19.8 μmol) was added followed by a solution of triethylamine (8.0 μL, 58.8 μmol) in DMF (1 mL), and the stirring was continued at 0 °C for 6 h. The mixture was diluted with benzene (3 mL) and EtOAc (6 mL), and the mixture was washed successively with 1 N HCl (2x1 mL), H_2O (1 mL), saturated aqueous $NaHCO_3$ (2x1 mL), H_2O (1 mL), and saturated aqueous NaCl (2x1 mL). The organic layer was concentrated *in vacuo* to give *N*-(*tert*-butyloxycarbonyl)-D-phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycine methyl ester: 53.9 μCi (72%). This methyl ester was then dissolved in MeOH (2 mL) and the solution was stirred in a room temperature water bath. 1 N NaOH (0.5 mL) was added and the stirring was continued for 3 h. Then 1 N HCl (0.51 mL) was added and the MeOH was removed *in vacuo*. To the residue was added H_2O (1 mL) and this was extracted with EtOAc (4x3.5 mL). The organic layers were concentrated *in vacuo* to give *N*-(*tert*-butyloxycarbonyl)-D-phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycine: 51.8 μCi (96%). To the above residue was added TFA (2 mL) and the solution was stirred for 1 h. Then TFA was removed *in vacuo* to give **18**: 48.0 μCi (92%). The total yield was 63% in 4 steps. This material shows a single radioactive spot on TLC and co-migrates with unlabeled peptide **14** in two different solvent systems. This was further purified by reverse phase HPLC on a C18 column.

***N*-Acetyl-D-phenylalanyl-L-phenylalanine Benzyl Ester (19)**

The literature procedure was modified.[142] A dry flask containing *N*-acetyl-D-phenylalanine (1.87 g, 9.00 mmol) and L-phenylalanine benzyl ester *p*-toluenesulfonate salt

(3.85 g, 9.00 mmol) was dried at 50 mTorr over P_2O_5 for 16 h. Dry DMF (25 mL) was then added, and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (2.30 mL, 10.6 mmol) was added followed by a solution of triethylamine (2.90 mL, 21.3 mmol) in DMF (10 mL), and the stirring was continued at 0 °C for 6 h. The mixture was diluted with benzene (200 mL) and EtOAc (400 mL), and the mixture was washed successively with 1 N HCl (2x40 mL), H_2O (40 mL), saturated aqueous $NaHCO_3$ (2x40 mL), H_2O (40 mL), and saturated aqueous NaCl (2x40 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by recrystallization from EtOAc/hexane to produce **19**: 4.49 g (93%). Mp 169.5-170.5 °C; IR (KBr) 3298, 3272, 1745, 1663, 1645, 1543 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.90 (s, 3 H, $C\underline{H}_3CO$), 2.83-3.06 (m, 4 H, aryl $C\underline{H}_2$), 4.66 (dd, J = 15.0, 7.2 Hz, 1 H, NC<u>H</u>CO), 4.83 (m, 1 H, NC<u>H</u>CO), 5.06, 5.11 (ABq, J = 12.1 Hz, J = 12.1 Hz, 2 x H, $OC\underline{H}_2Ph$), 6.06 (d, J = 7.7 Hz, 1 H, N<u>H</u>), 6.33 (d, J = 8.3 Hz, 1 H, N<u>H</u>), 7.20 (m, 10 H, aryl <u>H</u>); exact mass, 444.2054 (444.2049 calcd for $C_{27}H_{28}N_2O_4$). Anal. Calcd for $C_{27}H_{28}N_2O_4$: C, 72.95; H, 6.35; N, 6.30. Found: C, 73.10; H, 6.27; N, 6.12.

_N_-Acetyl-D-phenylalanyl-L-phenylalanine (20)

A mixture of **19** (3.64 g, 8.19 mmol) and 5% Pd/C (410 mg) in EtOAc (50 mL) and MeOH (50 mL) was hydrogenated under hydrogen at 48 psi for 4 h. After filtration through # 2 filter paper, the solvent was removed *in vacuo* to give **20**: 2.87 g (99%). Mp 196-198 °C; IR (KBr) 3407, 3271, 2920, 1750, 1650, 1629, 1561, 1521 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.85 (s, 3 H, $C\underline{H}_3CO$), 2.63-3.19 (m, 4 H, aryl $C\underline{H}_2$), 4.58-4.68 (m, 2 H, 2 x NC<u>H</u>CO), 7.18 (m, 10 H, aryl <u>H</u>); exact mass, 354.1575 (354.1579 calcd for $C_{20}H_{22}N_2O_4$). Anal. Calcd for $C_{20}H_{22}N_2O_4$: C, 67.78; H, 6.26; N, 7.90. Found: C, 67.59; H, 6.30; N, 7.84.

***N*-Acetyl-D-phenylalanyl-L-phenylalanyl Glycine Methyl Ester (21)**

The literature procedure was modified.[142] A dry flask containing **20** (501 mg, 1.41 mmol) and glycine methyl ester hydrochloride salt (200. mg, 1.59 mmol) was dried at 50 mTorr over P_2O_5 for 18 h. Dry DMF (10 mL) was then added, and the stirred solution was cooled to 0 °C in an ice-water bath. Diphenylphosphoryl azide (0.35 mL, 1.61 mmol) was added followed by a solution of triethylamine (0.5 mL, 3.67 mmol) in DMF (5 mL), and the stirring was continued at 0 °C for 6 h. The mixture was diluted with benzene (70 mL) and EtOAc (140 mL), and was then washed successively with 1 N HCl (2x20 mL), H_2O (20 mL), saturated aqueous $NaHCO_3$ (2x20 mL), H_2O (20 mL), and saturated aqueous NaCl (2x20 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 8% MeOH in $CHCl_3$ to produce **21**: 550. mg (92%). Mp 200-202 °C; IR (KBr) 3280, 1754, 1642, 1547 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.86 (s, 3 H, $C\underline{H}_3CO$), 2.60-3.21 (m, 4 H, aryl $C\underline{H}_2$), 3.71 (s, 3 H, $OC\underline{H}_3$), 3.92, 3.96 (ABq, J = 17.5 Hz, J = 17.5 Hz, 2 x 1 H, $NC\underline{H}_2CO$), 4.47 (dd, J = 8.5, 6.1 Hz, 1 H, $NC\underline{H}CO$), 4.65 (dd, J = 9.6, 5.1 Hz, 1 H, $NC\underline{H}CO$), 7.20 (m, 10 H, aryl $\underline{H}$); FAB-MS 426.59 (MH^+) (425.20 calcd for $C_{23}H_{27}N_3O_5$). Anal. Calcd for $C_{23}H_{27}N_3O_5$: C, 64.93; H, 6.40; N, 9.88. Found: C, 64.60; H, 6.29; N, 9.58.

***N*-Acetyl-D-phenylalanyl-L-phenylalanyl-glycine (22)**

The literature procedure was modified.[141] Aqueous 1 N NaOH (7 mL) was added to a stirred solution of **21** (117 mg, 0.275 mmol) in MeOH (5 mL) in a room temperature water bath, and the stirring was continued for 10 h. Then 1 N HCl (4 mL) was added and the MeOH was removed *in vacuo*. The remaining solution was acidified to pH 1 with 1 N HCl; a white precipitate formed slowly. This was filtered by suction to give **22**: 93.0 mg (82%). This was further purified by reverse phase HPLC (Waters C_{18} μ-Bondapak Radial Pak, 8 mm x 10 cm, 10 μm cartridge, retention time 14.83 min at 2.00 mL/min flow rate,

gradient elution: 0 min, 20% B; 20 min, 50% B). Mp 204.5-205.5 °C; IR (KBr) 3298, 1674, 1667, 1644, 1605, 1537 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.86 (s, 3 H, $C\underline{H}_3CO$), 2.60-3.22 (m, 4 H, aryl $C\underline{H}_2$), 3.88, 3.94 (ABq, J = 17.7 Hz, J = 17.7 Hz, 2 x 1 H, $NC\underline{H}_2CO$), 4.47 (dd, J = 8.6, 6.0 Hz, 1 H, $NC\underline{H}CO$), 4.68 (dd, J = 9.4, 4.8 Hz, 1 H, $NC\underline{H}CO$), 7.25 (m, 10 H, aryl $\underline{H}$); FAB-MS 411.87 (MH^+) (411.18 calcd for $C_{22}H_{25}N_3O_5$). Anal. Calcd for $C_{22}H_{25}N_3O_5 \cdot H_2O$: C, 61.53; H, 6.34; N, 9.78. Found: C, 61.63; H, 6.24; N, 9.89.

_N_-Acetyl-D-phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycine (23)

The methods used to prepare unlabeled peptide **22** were adapted for radioactive synthesis. A dry flask containing **20** (5.0 mg, 14.1 μmol) and **17** (79.8 μCi, 0.798 μmol) was dried at 50 mTorr over P_2O_5 for 18 h. Dry DMF (3 mL) was then added, and the stirred solution was cooled to 0 °C in an ice-water bath. Diphenylphosphoryl azide (5.00 μL, 23.0 μmol) was added followed by a solution of triethylamine (10.0 μL, 73.4 μmol) in DMF (1 mL), and the stirring was continued at 0 °C for 16 h. The mixture was diluted with benzene (5 mL) and EtOAc (10 mL) and was washed successively with 1 N HCl (2x1 mL), H_2O (1 mL), saturated aqueous $NaHCO_3$ (2x1 mL), H_2O (1 mL), and saturated aqueous NaCl (2x1 mL). The organic layer was concentrated *in vacuo* to give _N_-acetyl-D-phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycine methyl ester: 36.8 μCi (46%). To this residue was added MeOH (1 mL), and the solution was stirred in a room temperature water bath. A solution of 1 N KOH (200 μL) was then added, and the stirring was continued for 1 h. Then 1 N HCl (220 μL) was added and The MeOH was removed *in vacuo*. To the residue was added H_2O (1 mL), and the resulting aqueous phase was extracted with EtOAc (3x4 mL). The combined organic layers were concentrated *in vacuo* to give **23**: 25.0 μCi (69%). The total yield was 32% in 2 steps. This material shows a single radioactive spot on TLC and co-chromatographs with unlabeled peptide **22** in two

different solvent systems. This compound was purified by reverse phase HPLC on a C_{18} column prior to enzyme assay.

N-(*tert*-Butyloxycarbonyl)-*O*-(*tert*-butyldimethylsilyl)-D-tyrosyl-L-valine Benzyl Ester (24)

The literature procedure was modified.[113, 115] A solution of *tert*-butyldimethylsilyl chloride (99.1 mg, 0.657 mmol), **6** (211 mg, 0.448 mmol) and imidazole (93.6 mg, 1.37 mmol) in dry DMF (3 mL) was stirred at room temperature for 6 h. Then 5% aqueous $NaHCO_3$ (20 mL) was added and the aqueous layer was extracted with a mixture of EtOAc:benzene (2:1, 3x30 mL). The combined organic layers were washed with H_2O (2x10 mL), saturated aqueous NaCl (10 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give crude product which was purified by flash chromatography using 7:2 Skelly B:EtOAc to give **24**: 215 mg (82%). IR (CH_2Cl_2 cast) 3340, 2959, 2930, 1734, 1705, 1690, 1650, 1500 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.15 (s, 6 H, $(C\underline{H}_3)_2Si$), 0.83 (d, J = 7.5 Hz, 6 H, $(C\underline{H}_3)_2C\underline{H}$), 0.94 (s, 9 H, $(C\underline{H}_3)_3CSi$), 1.39 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.08 (m, 1 H, $(CH_3)_2C\underline{H}$), 2.95 (m, 2 H, aryl $C\underline{H}_2$), 4.32 (m, 1 H, $NC\underline{H}CO$), 4.52 (dd, J = 8.7, 4.7 Hz, 1 H, $NC\underline{H}CO$), 4.92 (br s, 1 H, $N\underline{H}$), 5.10, 5.18 (ABq, J = 12.0 Hz, J = 12.0 Hz, 2 x 1 H, $OC\underline{H}_2Ph$), 6.39 (d, J = 8 Hz, 1 H, $N\underline{H}$), 6.85 (d, J = 8.5 Hz, 2 H, aryl $\underline{H}$), 7.05 (d, J = 8.5 Hz, 2 H, aryl $\underline{H}$), 7.34 (m, 5 H, aryl $\underline{H}$); exact mass, 584.3301 (584.3201 calcd for $C_{23}H_{48}N_2O_6Si$). Anal. Calcd for $C_{32}H_{48}N_2O_6Si$: C, 65.72; H, 8.27; N, 4.79. Found: C, 65.65; H, 8.29; N, 4.65.

N-(*tert*-Butyloxycarbonyl)-*O*-(*tert*-butyldimethylsilyl)-D-tyrosyl-L-valine (25)

A solution of **24** (901 mg, 1.54 mmol) and 5% Pd/C (200 mg) in EtOAc (30 mL) in a Parr flask was shaken under hydrogen at 50 psi for 1 h. The solution was suction filtered through an E sinter glass and the solvent was removed *in vacuo* to give **25**: 737

mg (96%). IR (KBr) 3330, 2959, 2930, 1745, 1660, 1510 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.15 (s, 6 H, $(C\underline{H}_3)_2Si$), 0.83 (d, J = 7.6 Hz, 6 H, $(C\underline{H}_3)_2CH$), 0.94 (s, 9 H, $(C\underline{H}_3)_3CSi$), 1.39 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.08 (m, 1 H, $(CH_3)_2C\underline{H}$), 2.95 (m, 2 H, aryl $C\underline{H}_2$), 4.50 (m, 1 H, NC<u>H</u>CO), 4.65 (m, 1 H, NC<u>H</u>CO), 6.75 (d, J = 8.3 Hz, 2 H, aryl <u>H</u>), 8.05 (d, J = 8.4 Hz, 2 H, aryl <u>H</u>); exact mass, 494.2850 (494.2812 calcd for $C_{25}H_{42}N_2O_6Si$). Anal. Calcd for $C_{25}H_{42}N_2O_6Si$: C, 60.70; H, 8.56; N, 5.66. Found: C, 60.33; H, 8.44; N, 5.38.

Benzyl Glycolate (26)

The literature procedure was modified.[117] To a stirred solution of glycolic acid (986 mg, 13.0 mmol) in MeOH (20 mL) was added 20% tetramethylammonium hydroxide solution (6.71 mL, 13.0 mmol) and stirring was continued for 20 min. THF (20 mL) was added, the solution was stirred for approximately 10 min, and the solvent was removed *in vacuo*. A further portion of THF (20 mL) was added, stirring was continued for a further 10 min, and then the solvent was removed *in vacuo*. The residue was dried under high vacuum overnight. The dry salt was dissolved in DMF (20 mL) and benzyl bromide (1.85 mL, 15.5 mmol) was added; the resulting solution was stirred vigorously for 12 h. The reaction mixture was poured into water (20 mL) and extracted with ether (3x50 mL). The combined ethereal layers were washed with water (15 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give a liquid which was purified by flash chromatography using 7:3 Skelly B:EtOAc to yield **26**: 2.15g (99%). IR ($CHCl_3$ cast) 3440 (br), 1745 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 2.88 (s, 1 H, O<u>H</u>), 4.22 (s, 2 H, $OC\underline{H}_2Ph$), 5.23 (s, 2 H, $COC\underline{H}_2$), 7.40 (s, 5 H, aryl <u>H</u>) ; exact mass, 166.0632 (166.0630 calcd for $C_9H_{10}O_3$).

_N_-(_tert_-Butyloxycarbonyl)-_O_-(_tert_-butyldimethylsilyl)-D-tyrosyl-L-valyl-glycolic Acid Benzyl Ester (27)

The literature procedure was modified.[118] A few small crystals of DMAP, **26** (120 mg, 0.723 mmol) and DCC (200. mg, 0.971 mmol) were added to a solution of **25** (325 mg, 0.693 mmol) in dry CH_2Cl_2 (8 mL) at -10°. The reaction mixture was stirred at -10° for 5 min, and then warmed to room temperature; stirring was continued for several hours. The solution was suction filtered and the precipitate was washed with CH_2Cl_2 (2x8 mL). The filtrate was evaporated *in vacuo* and the crude product was purified by flash chromatography using 3/1 Skelly B-EtOAc to yield **27**: 353 mg (80%). IR ($CHCl_3$ cast) 3340, 2959, 2930, 1718, 1665, 1510 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 0.17 (s, 6 H, $(C\underline{H}_3)_2Si$), 0.87, 0.92 (2 x d, J = 7.0 Hz, 7.3 Hz, 2 x 3 H, $(C\underline{H}_3)_2CH$), 0.97 (s, 9 H, $(C\underline{H}_3)_3CSi$), 1.42 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.28 (m, 1 H, $(CH_3)_2C\underline{H}$), 2.98, 3.01 (2 x d, J = 2.2 Hz, J = 2.2 Hz, 2 x 1 H, aryl $C\underline{H}_2$), 4.31 (m, 1 H, $NC\underline{H}CO$), 4.56 (m, 2 H, $OC\underline{H}_2CO$), 4.77 (dd, J = 15.9, 4.1 Hz, 1 H, $NC\underline{H}CO$), 4.96 (br s, 1 H, $N\underline{H}$), 5.18 (s, 2 H, $OC\underline{H}_2Ph$), 6.34 (d, J = 8.2 Hz, 1 H, $N\underline{H}$), 6.76 (dd, J = 8.4, 2.0 Hz, 2 H, aryl $\underline{H}$), 7.07 (d, J = 8.3 Hz, 2 H, aryl $\underline{H}$), 7.36 (s, 5 H, aryl $\underline{H}$); FAB-MS 643.26 (MH^+) (643.22 calcd for $C_{34}H_{50}N_2O_8Si$). Anal. Calcd for $C_{34}H_{50}N_2O_8Si$: C, 63.52; H, 7.84; N, 4.36. Found: C, 63.30, H, 8.08; N, 4.25.

_N_-(_tert_-Butyloxycarbonyl)-D-tyrosyl-L-valyl-glycolic Acid Benzyl Ester (28)

The literature procedure was modified.[119] Commercially available tetrabutylammonium fluoride (1 M, 0.6 mL) was added to a solution of **27** (170. mg, 0.264 mmol) in THF (25 mL) with stirring. The reaction mixture was stirred at room temperature under argon for 2.5 h and then concentrated *in vacuo*. To the residue was added H_2O (20 mL) and the resulting aqueous phase was extracted with EtOAc (3x20 mL). The combined organic layers were washed with H_2O (20 mL) and the solvent was removed

in vacuo to give **28**: 154 mg (100%). IR (CH_2Cl_2 cast) 3320, 2959, 2930, 1750, 1660, 1517 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.87, 0.93 (2 x d, J = 7 Hz, J =7 Hz, 2 x 3 H, $(C\underline{H}_3)_2CH$), 1.42 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.18 (m, 1 H, $(CH_3)_2C\underline{H}$), 2.98 (m, 2 H, aryl $C\underline{H}_2$), 4.31 (m, 1 H, $NC\underline{H}CO$), 4.55 (m, 2 H, $OC\underline{H}_2CO$), 4.74 (dd, J = 16, 4 Hz, 1 H, $NC\underline{H}CO$), 5.02 (br s, 1 H, $N\underline{H}$), 5.18 (s, 2 H, $OC\underline{H}_2Ph$), 6.44 (d, J = 8 Hz, 1 H, $N\underline{H}$), 6.74 (dd, J = 8.4, 2.0 Hz, 2 H, aryl $\underline{H}$), 7.04 (d, J = 8.0 Hz, 2 H, aryl $\underline{H}$), 7.36 (s, 5 H, aryl $\underline{H}$); FAB-Ms 529.13 (MH^+) (528.25 calcd for $C_{28}H_{36}N_2O_8$).

D-Tyrosyl-L-valyl-glycolic Acid (29)

The literature procedure was modified.[140] A solution of **28** (34.0 mg, 0.0643 mmol) in TFA (2 mL) was stirred at room temperature for 1 h. The solvent was removed *in vacuo* to give the *N*-deprotected product. This was then dissolved in EtOAc (10 mL) and MeOH (10 mL). The 5% Pd/C catalyst (15 mg) was added and the resulting solution was shaken under H_2 at 50 psi for 1 h. The solution was filtered through a Celite pad and the solvent was removed *in vacuo* to give **29**: 24.7 mg (85%). This was further purified by reverse phase HPLC (Waters μ-Bondapak Radial Pak, 25 mm x 10 cm, 10 μm cartridge, retention time 12.84 min at 3.00 mL/min flow rate, 50% B isocratic elution). Mp 125-127 °C; IR (KBr) 3500-3200, 2970, 2940, 1740, 1676, 1614, 1516 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) (^1H NMR of **29** in CD_3OD showed it exists as two conformational isomers under these conditions.) δ 0.89 (d, J = 6.9 Hz, 3 H, $C\underline{H}_3CH$), 1.03 (m, 3 H, $C\underline{H}_3CH$), 2.18, 2.30 (2 x m, 1 H, $(CH_3)_2C\underline{H}$), 2.95-3.24 (m, 2 H, aryl $C\underline{H}_2$), 4.16 (m, 1 H, $NC\underline{H}CO$), 4.46, 4.53 (2 x d, J = 5.3 Hz, J = 5.8 Hz, 1 H, $NC\underline{H}CO$), 4.64 (s, 2 H, $OC\underline{H}_2CO$), 6.81, 6.83 (2 x d, J = 4.3 Hz, J = 3.4 Hz, 2 x 1 H, aryl $\underline{H}$), 7.16, 7.23 (2 x d, J = 2.2 Hz, J = 2.0 Hz, 2 x 1 H, aryl $\underline{H}$); FAB-MS 339.17 (MH^+) (338.15 calcd for $C_{16}H_{22}N_2O_6$). Anal. Calcd for $C_{16}H_{22}N_2O_6 \cdot CF_3CO_2H$: C, 47.79; H, 5.12; N, 6.19. Found: C, 47.81; H, 5.26; N, 6.26.

[1,2-^{14}C]-Glycolic Acid Benzyl Ester (30)

To a stirred solution of [1,2-^{14}C]-glycolic acid (20 μCi, specific activity 30-50 μCi/μmol) in MeOH (2 mL) was added 20% tetramethylammonium hydroxide solution (1.63 μL) and stirring was continued for 20 min. THF (2 mL) was added and the solution was stirred for approximately 10 min and the solvent was removed *in vacuo*. A further portion of THF (2 mL) was added, stirring was continued for a further 10 min, and then the solvent was removed *in vacuo*. The residue was dried under high vacuum overnight. The dry salt was dissolved in DMF (2 mL) and benzyl bromide (0.352 μL) was added; the resulting solution was stirred vigorously for 12 h. The reaction mixture was poured into water (2 mL) and extracted with ether (3x5 mL). The combined ethereal layers were washed with water (1 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give the product **30**: 1.01 μCi (5%). This material shows a single radioactive spot on TLC and co-migrates with unlabeled compound **26** in two different solvent systems.

***N*-Acetyl-D-phenylalanyl-L-phenyalanyl-(glycolic Acid) Benzyl Ester (31)**

The literature procedure was modified.[142] A dry flask containing **20** (60.0 mg, 0.169 mmol) and **26** (56.3 mg, 0.339 mmol) was dried at 50 mTorr over P_2O_5 for 16 h. Dry DMF (5 mL) was then added, and the stirred solution was cooled to 0 °C in an ice-water bath. Diphenylphosphoryl azide (100. μL, 0.458 mmol) was added followed by a solution of triethylamine (400. μL, 2.93 mmol) in DMF (10 mL), and the stirring was continued at 0 °C for 24 h. The mixture was diluted with benzene (40 mL) and EtOAc (80 mL) and was then washed successively with 1 N HCl (2x5 ml), H_2O (5 mL), saturated aqueous $NaHCO_3$ (2x5 mL), H_2O (5 mL), and saturated aqueous NaCl (2x5 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 50:49:1 of toluene:EtOAc:HCO_2H to produce **31**: 66.2 mg (77%). Mp 133 °C (dec.); IR (KBr) 3329, 1769, 1748, 1644, 1531 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.93(s, 3 H,

$C\underline{H}_3CO$), 3.04 (m, 4 H, aryl $C\underline{H}_2$), 4.62-4.95 (m, 4 H, 2 x $NC\underline{H}CO$, $OC\underline{H}_2CO$), 5.24 (s, 2 H, $OC\underline{H}_2Ph$), 6.09 (d, J = 8.0 Hz, 1 H, $N\underline{H}$), 6.45 (d, J = 8.0 Hz, 1 H, $N\underline{H}$), 7.25 (m, 15 H, aryl $\underline{H}$); CI-MS 503 (MH^+) (502.21 calcd for $C_{29}H_{30}N_2O_6$).

N-Acetyl-D-phenylalanyl-L-phenylalanyl-glycolic Acid (32) from 31

A solution of **31** (104. mg, 0.207 mmol) and 50.0 mg of 5% Pd/C in MeOH (10 mL) and EtOAc (10 mL) was hydrogenated under hydrogen at 50 psi for 1 h. After filtration through #2 filter paper, the solvent was removed to give the crude product which was purified by flash chromatography using 90:9:1 of $CHCl_3$:MeOH:HCO_2H to yield **32**: 73.4 mg (86%). Mp 71-73 °C; IR (KBr) 3300, 3086, 3064, 3029, 1748, 1651, 1544 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.84 (s, 3 H, $C\underline{H}_3CO$), 2.64 (dd, J = 13.9, 8.9 Hz, 1 H, aryl $C\underline{H}$), 2.89 (dd, J = 14.9, 5.5 Hz, 1 H, aryl $\underline{H}$), 2.95 (dd, J = 14.2, 9.3 Hz, 1 H, aryl $C\underline{H}$), 3.25 (dd, J = 14.1, 4.9 Hz, 1 H, aryl $C\underline{H}$), 4.61 (dd, J = 9.0, 5.5 Hz, 1 H, $NC\underline{H}CO$), 4.66 (d, J = 15.2 Hz, 2 H, $OC\underline{H}_2CO$), 4.77 (dd, J = 9.4, 5.0 Hz, 1 H, $NC\underline{H}CO$), 7.03-7.31 (m, 10 H, aryl $\underline{H}$); ^{13}C NMR (100 MHz, CD_3OD) δ 173.44, 172.96, 172.15, 170.76, 138.30, 138.06, 130.42, 130.24, 129.59, 129.35, 127.97, 127.70, 62.10, 55.68, 54.61, 38.96, 38.22, 22.37; FAB-MS 413.06 (MH^+) (412.16 calcd for $C_{22}H_{24}N_2O_6$). Anal. Calcd for $C_{22}H_{24}N_2O_6 \cdot 2H_2O$: C, 58.92; H, 6.29; N, 6.25. Found: C, 58.99; H, 5.30; N, 6.05.

N-Acetyl-D-phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycolic Acid Benzyl Ester (33)

The methods used to prepare unlabeled peptide **31** were adapted for radioactive synthesis. To a stirred solution of [1,2-^{14}C]-glycolic acid (50 μCi, specific activity 30-50 μCi/μmol) in methanol (5 mL) was added 20% tetramethylammonium hydroxide solution (2.00 mL) and the stirring was continued for 20 min. THF (5 mL) was added, and the solution was stirred for approximately 10 min and the solvent was removed *in vacuo*. A

further portion of THF (5 mL) was added, stirring was continued for a further 10 min, and then the solvent was removed *in vacuo*. The residue was dried under high vacuum overnight. The dry salt was dissolved in DMF (3 mL), benzyl bromide (0.450 mL) was added, the resulting solution was stirred vigorously for 20 h. Then the reaction mixture was poured into water (2 mL) and extracted with ether (4x3 mL). The combined ethereal layers were washed with water (1 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give the crude product: 23.8 μCi (48%). This was purified by Prep-TLC using 65:35 hexane:EtOAc to give [1,2-^{14}C]-glycolic acid benzyl ester: 11.0 μCi (22%). This ester and **20** (5.00 mg) were placed in a dry flask and dried at 50 mTorr over P_2O_5 for 6 h. Dry DMF (3 mL) was then added, and the stirred solution was cooled to 0 °C in an ice-water bath. Diphenylphosphoryl azide (4.0 μL) was added followed by a solution of triethylamine (8.0 μL) in DMF (1 mL), and the stirring was continued at 0 °C for 24 h. The mixture was diluted with benzene (4 mL) and EtOAc (8 mL) and was washed successively with 1 N HCl (2x1 ml), H_2O (1 mL), saturated aqueous $NaHCO_3$ (2x1 mL), H_2O (1 mL), and saturated aqueous NaCl (2x1 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give **33**: 0.864 μCi (8%). This material shows a single radioactive spot on TLC and co-spots with unlabeled compound **31** in two different solvent systems.

2-Naphthalenemethanol (34)

$LiAlH_4$ (4.00 g, 105. mmol) was added to THF (200 mL) in a three-neck round bottom flask. This was heated to reflux in an oil bath for 30 min. The mixture was then cooled to 0 °C and 2-naphthoic acid (10.0 g, 58.1 mmol) was added in small portions. The resulting solution was heated to reflux for 3 h, Celite (6 g) was added followed by water (20 mL) and 0.10 M NaOH (20 mL). The mixture was filtered and the residue was washed with EtOAc. The solvent was then removed *in vacuo* and the product was extracted from the aqueous phase with EtOAc (3x200 mL). The combined organic layers

were washed with H_2O (20 mL), and saturated aqueous NaCl (20 mL), and the solvent was removed to give **34**: 9.66 g (99%). Mp 78-79 °C (lit.[143] mp 78-81 °C); IR (KBr cast) 3230, 1041 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 4.77 (s, 2 H, aryl $C\underline{H}_2$), 7.28 (m, 4 H, aryl $\underline{H}$), 7.45 (m, 3 H, aryl $\underline{H}$); exact mass, 158.0732 (158.0732 calcd for $C_{11}H_{10}O$).

2-Naphthalenemethyl Bromide (35)

A solution of **34** (2.00 g, 12.6 mmol) and PBr_3 (4 mL) in THF (100 mL) was stirred at room temperature for 1 h. Water (10 mL) was added and the solvent was removed *in vacuo* to give the crude product. The crude product was dissolved in ether (200 mL), and the ethereal solution was washed successively with saturated aqueous $NaHCO_3$ (2x15 mL), H_2O (15 mL), and saturated aqueous NaCl (2x15 mL). It was then dried (Na_2SO_4). The solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 8% EtOAc in hexane to give **35**: 2.23 g (80%). Mp 53-55 °C (lit.[143] mp 51-54 °C); IR (KBr) 3440, 1598, 1507 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 4.73 (s, 2 H, $C\underline{H}_2$), 7.55 (m, 3 H, aryl $\underline{H}$), 7.85 (m, 4 H, aryl $\underline{H}$); exact mass, 221.9868 (219.9888 calcd for $C_{11}H_9Br$).

2-Naphthalenemethyl Glycolate (36)

The literature procedure was modified.[117] To a stirred solution of glycolic acid (500. mg, 6.58 mmol) in MeOH (15 mL) was added 20% tetramethylammonium hydroxide solution (4.10 mL, 7.94 mmol), and the stirring was continued for 20 min. THF (20 mL) was added, the solution was stirred for approximately 10 min, and the solvent was removed *in vacuo*. A further portion of THF (20 mL) was added, stirring was continued for a further 10 min, and then the solvent was removed *in vacuo*. The residue was dried under high vacuum at 50 mTorr overnight. The dry salt was dissolved in DMF (20 mL) and 2-naphthalenemethyl bromide (1.45 g, 6.56 mmol) was added. The resulting

solution was stirred vigorously for 12 h. Then the reaction mixture was poured into water (20 mL) and extracted with ether (3x50 mL). The combined ethereal layers were washed with water (15 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 4% MeOH in $CHCl_3$ to yield **36**: 1.15 g (81%). This was further purified by recrystallization from EtOAc and hexane. IR (KBr) 3460, 3422, 1743 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 2.44 (t, J = 5.4 Hz, 1 H, O<u>H</u>), 4.24 (d, J = 5.4 Hz, 2 H, OC<u>H</u>$_2$CO), 5.48 (s, 2 H, aryl C<u>H</u>$_2$), 7.48 (m, 3 H, aryl <u>H</u>), 7.85 (m, 4 H, aryl <u>H</u>); exact mass, 216.0785 (216.0786 calcd for $C_{13}H_{12}O_3$).

_N_-Acetyl-D-phenylalanyl-L-phenylalanyl-glycolic Acid 2-Naphthalene-methyl Ester (37)

The literature procedure was modified.[142] A dry flask containing **20** (590. mg, 1.67 mmol) and **36** (360 mg, 1.67 mmol) was dried at 50 mTorr over P_2O_5 for 8 h. Dry DMF (20 mL) was then added, and the stirred solution was cooled to 0 °C in an ice-water bath. Diphenylphosphoryl azide (600. μL, 2.75 mmol) was added followed by a solution of triethylamine (900. μL, 6.59 mmol), and the stirring was continued at 0 °C for 12 h. The mixture was diluted with benzene (120 mL) and EtOAc (240 mL). The resulting mixture was washed successively with 1 N HCl (2x20 ml), H_2O (20 mL), saturated aqueous $NaHCO_3$ (2x20 mL), H_2O (20 mL), and saturated aqueous NaCl (2x20 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 60:40:1 of EtOAc:toluene:HCO_2H to give **37**: 413 mg (45%). Mp 145 °C (dec.); IR (KBr) 3400, 3305, 1770, 1744, 1647, 1527 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.94 (s, 3 H, C<u>H</u>$_3$CO), 3.20 (m, 4 H, aryl C<u>H</u>$_2$), 4.60-5.00 (m, 4 H, 2 x NC<u>H</u>CO, OC<u>H</u>$_2$CO), 5.38 (s, 2 H, OC<u>H</u>$_2$Ph), 5.97 (d, J = 8 Hz, 1 H, N<u>H</u>), 6.27 (d, J = 8 Hz, 1 H, N<u>H</u>), 7.20 (m, 10 H, aryl <u>H</u>), 7.50 (m,3 H, aryl <u>H</u>), 7.85 (m, 4 H, aryl <u>H</u>); FAB-MS 553.27 (MH^+) (552.23 calcd for $C_{33}H_{34}N_2O_6$).

_N_-Acetyl-D-phenylalanyl-L-phenylalanyl-glycolic Acid (32) from 37

A solution of **37** (234 mg, 0.424 mmol) and 5% Pd/C (120. mg) in MeOH (15 mL) and EtOAc (15 mL) was hydrogenated under hydrogen at 50 psi for 1.5 h. After filtration through #2 filter paper, the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 90:9:1 of $CHCl_3$:MeOH:HCO_2H to yield **32**: 160. mg (92%). Mp 71-73 °C; IR (KBr) 3300, 3086, 3064, 3029, 1748, 1651, 1544 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.84 (s, 3 H, CH_3CO), 2.64 (dd, J = 13.9, 8.9 Hz, 1 H, aryl CH), 2.89 (dd, J = 14.9, 5.5 Hz, 1 H, aryl H), 2.95 (dd, J = 14.2, 9.3 Hz, 1 H, aryl CH), 3.25 (dd, J = 14.1, 4.9 Hz, 1 H, aryl CH), 4.61 (dd, J = 9.0, 5.5 Hz, 1 H, NCHCO), 4.66 (dd, J = 20.4, 15.2 Hz, 2 H, OCH_2CO), 4.77 (dd, J = 9.4, 5.0 Hz, 1 H, NCHCO), 7.03-7.31 (m, 10 H, aryl H); ^{13}C NMR (100 MHz, CD_3OD) δ 173.44, 172.96, 172.15, 170.76, 138.30, 138.06, 130.42, 130.24, 129.59, 129.35, 127.97, 127.70, 62.10, 55.68, 54.61, 38.96, 38.22, 22.37; FAB-MS 413.06 (MH^+) (412.16 calcd for $C_{22}H_{24}N_2O_6$). Anal. Calcd for $C_{22}H_{24}N_2O_6 \cdot 2H_2O$: C, 58.92; H, 6.29; N, 6.25. Found: C, 58.99; H, 5.30; N, 6.05.

2-Naphthalenemethyl [1,2-^{14}C]-Glycolate (38)

The method used to prepare unlabeled compound **36** was adapted for radioactive synthesis. To a stirred solution of [1,2-^{14}C]-glycolic acid calcium salt (55.0 μCi, specific activity 10 μCi/μmol) in MeOH (0.5 mL) was added 20% tetramethylammonium hydroxide solution (15.0 μL). Stirring was continued for 20 min. THF (1 mL) was added, the solution was stirred for approximately 10 min, and the solvent was removed *in vacuo*. A further portion of THF (1 mL) was added, stirring was continued for a further 10 min, and then the solvent was removed *in vacuo*. The residue was dried under high vacuum at 50 mTorr over night. The dry salt was dissolved in DMF (1 mL) and 2-naphthalenemethyl bromide (14 mg) was added. The solution was stirred vigorously for 24 h. Then the reaction mixture was poured into water (2 mL) and extracted with ether (3x5 mL). The

combined ethereal layers were washed with water (1 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give the crude product which was purified by Prep-TLC using 5% MeOH in $CHCl_3$ to give **38**: 7.40 μCi (13.5%). This material shows a single radioactive spot on TLC and co-migrates with unlabeled compound **36** in two different solvent systems.

N-Acetyl-D-phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycolic Acid 2-Naphthalenemethyl Ester (39)

The methods used to prepare unlabeled peptide **37** were adapted for radioactive synthesis. A dry flask containing **20** (20.0 mg) and **38** was dried at 50 mTorr over P_2O_5 over night. Dry DMF (1 mL) was then added, and the stirred solution was cooled to 0 °C in an ice-water bath. Diphenylphosphoryl azide (30.0 μL) was added followed by a solution of triethylamine (40.0 μL) in DMF (5 mL), and the stirring was continued at 0 °C for 12 h. The mixture was diluted with benzene (7 mL) and EtOAc (14 mL). This was washed successively with 1 N HCl (2x2 ml), H_2O (2 mL), saturated aqueous $NaHCO_3$ (2x2 mL), H_2O (2 mL), and saturated aqueous NaCl (2x2 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by Prep-TLC using 60:40 EtOAc:toluene to give **39**: 1.87 μCi (25%). This material shows a single radioactive spot on TLC and co-chromatographs with unlabeled compound **37** in two different solvent systems.

N-Acetyl-D-phenylalanyl-L-phenylalanyl-[1,2-^{14}C]-glycolic Acid (40)

The methods used to prepare unlabeled compound **32** were adapted for radioactive synthesis. A solution of **39** and 5% Pd/C (8.0 mg) in MeOH (3 mL) and EtOAc (1.5 mL) was hydrogenated at 1 atmosphere overnight. After filtration through Celite, the solvent was removed *in vacuo* to give **40**: 1.63 μCi (87%). This material shows a single

radioactive spot on TLC and co-migrates with unlabeled compound **32** in two different solvent systems. This was purified by reverse-phase HPLC prior to enzyme assay.

N-Acetyl-D-phenylalanyl-L-phenylalaninamide (41)

The literature procedure was modified.[142] A dry flask containing L-phenylalaninamide (2.50 g, 15.2 mmol) and *N*-acetyl-D-phenylalanine (3.30 g, 15.9 mmol) was dried over P_2O_5 under vacuum (0.05 mmHg) for 10 h. Dry DMF (30 mL) was then added, and the mixture was stirred in an ice-water bath. Diphenylphosphoryl azide (3.70 mL, 16.99 mmol) was added followed by the addition, over 15 min, of triethylamine (4.60 mL, 33.7 mmol) and stirring was continued at 0 °C for 12 h. The mixture was taken up into benzene (200 mL) and EtOAc (400 mL), and was washed with 1 M HCl (2x25 mL), H_2O (25 mL), and saturated aqueous $NaHCO_3$ (2x25 mL). Then triethylamine (2 mL) was added and the mixture was washed with 1 M HCl (25 mL) and H_2O (2x25 mL). White crystals appeared in the organic layer. This was allowed to stand at room temperature for 1 h and the crystals were collected by vacuum filtration to yield **41**: 4.00 g (75%). Mp 232.5-234.5 °C; IR (KBr) 3420, 3278, 3200, 1672, 1630, 1550 cm^{-1}; ^1H NMR (200 MHz, CD_3SOCD_3) δ 1.72 (s, 3 H, $C\underline{H}_3CO$), 2.40-3.09 (m, 4 H, aryl $C\underline{H}_2$), 4.45 (m, 2 H, 2 x NC$\underline{H}$CO), 7.06-7.25 (m, 10 H, aryl $\underline{H}$), 7.42 (s, 2 H, $N\underline{H}_2$), 8.03 (d, J = 7.9 Hz, 1 H, N$\underline{H}$), 8.36 (d, J = 8.5 Hz, 1 H, N$\underline{H}$); ^{13}C NMR (100 MHz, CD_3OD) 173.02, 171.19, 169.32, 138.07, 137.89, 129.25, 129.09, 127.99, 127.95, 126.24, 126.15, 54.25, 53.70, 37.56, 37.43, 22.35; FAB-MS 354.08 (MH^+) (353.17 calcd for $C_{20}H_{23}N_3O_3$). Anal. Calcd for $C_{20}H_{23}N_3O_3$: C, 67.97; H, 6.56; N, 11.89. Found: C, 67.47; H, 6.42; N, 11.77.

N-Acetyl-D-phenylalanyl-L-phenylalanyl-α-hydroxyglycine (42)

The literature procedure was modified.[120] A solution of **41** (520. mg, 1.47 mmol) and glyoxylic acid monohydrate (1.45 g, 15.6 mmol) in acetone (180 mL) was heated to

reflux overnight. The solvent was removed *in vacuo* to give the crude product which was then purified by reverse phase MPLC (C_8 column, 33% CH_3CN in H_2O) to yield **42**: 360. mg (60%). The two isomers were separated by reverse phase HPLC (Waters NH_2 Radial-Pak, 8 mm x 10 cm 10 μm cartridge, retention times 7.48 min for isomer A and 8.15 min for isomer B at 1.00 ml/min flow rate, 35% B isocratic elution). Mp 139-141 °C; IR (KBr) 3283, 3080, 1702, 1645, 1559 cm^{-1}; ^1H NMR (400 MHz, CD_3SOCD_3) δ 1.68 (s, 3 H, CH_3CO), 2.35 (m, 1 H, aryl CH), 2.54 (m, 1 H, aryl CH), 2.73 (m, 1 H, aryl CH), 3.03 (m, 1 H, aryl CH), 4.48 (m, 1 H, NCHCO), 4.66(m, 1 H, NCHCO), 5.45 (dd, J = 8.5, 3.7 Hz, 1 H, NCH(OH)CO), 6.98-7.34 (m, 10 H, aryl H), 7.91 (dd, J = 9.1, 8.9 Hz, 1 H, NH), 8.47 (dd, J = 12.6, 8.8 Hz, 1 H, NH), 8.94 (dd, J = 8.4, 4.0 Hz, 1 H, NH); ^{13}C NMR (100 MHz, CD_3OD) δ 22.31, 38.54, 38.60, 55.65, 56.28, 72.61, 127.76, 127.83, 129.42, 129.50, 130.17, 130.40, 138.23, 172.51, 172.60, 173.20, 173.31, 173.62, 173.68; FAB-MS 428.07 (MH^+) (427.17 calcd for $C_{22}H_{25}N_3O_6$). Anal. calcd for $C_{22}H_{25}N_3O_6 \cdot H_2O$: C, 59.32; H, 6.11; N, 9.43. Found: C, 59.39; H, 5.84; N, 9.30.

_N_-Acetyl-D-phenylalanyl-L-phenylalanyl-α-hydroxyglycine Methyl Ester (43) from 41

The literature procedure was modified.[120] A solution of **41** (744 mg, 2.00 mmol) and methyl glyoxylate hydrate (800. mg, 7.55 mmol) in acetone (100 mL) was heated to reflux overnight. The solvent was removed *in vacuo* to give a residue which was purified by flash chromatography using 3% MeOH in $CHCl_3$ to give **43**: 570. mg (64%). Mp 147-149 °C; IR (KBr) 3286, 2600, 1880, 1755, 1644, 1541 cm^{-1}; ^1H NMR (200 MHz, CD_3SOCD_3) δ 1.71 (s, 3 H, CH_3CO), 2.27-3.07 (m, 4 H, aryl CH_2), 3.67 (d, J = 3.5 Hz, 3 H, OCH_3), 4.48 (m, 1 H, NCHCO), 4.62 (m, 1 H, NCHCO), 5.52 (m, 1 H, NCHCO), 6.71 (dd, J = 10.2, 6.6 Hz, 1 H, OH), 7.00-7.33 (m, 10 H, aryl H), 7.92 (dd,

J = 8.4, 4.9 Hz, 1 H, NH), 8.41 (dd, J = 8.1, 7.8 Hz, 1 H, NH), 9.03 (dd, J = 8.3, 6.3 Hz, 1 H, NH); FAB-MS 442.20 (MH^+) (441.19 calcd for $C_{23}H_{27}N_3O_6$).

_N_-Acetyl-D-phenylalanyl-L-phenylalanyl-α-hydroxyglycine Methyl Ester (43)

To a solution of **42** (10.0 mg, 0.0234 mmol) in ether (1 mL) and MeOH (1 mL) in an ice-water bath was added ethereal CH_2N_2 until a yellow coloration persisted. Stirring was continued at room temperature for 30 min. Acetic acid was added dropwise until the solution became clear. The solvent was removed to give **43** in quantitative yield. Spectrum data are as those of **43** prepared from **41** above.

(2*R*, 5*S*, α*R*)-4-(α-Benzoyloxybenzyl)-2-(*tert*-butyl)-1-methyl-imidazolidin-4-one (46)

The literature procedure was modified.[124] A solution of dry diisopropylamine (3.50 mL, 24.9 mmol) in THF (40 mL) was cooled to -78 °C. 1.6 M n-BuLi in hexanes (13.0 mL, 20.8 mmol) was added, the mixture was warmed to 0 °C, and the stirring was continued for 10 min. This LDA solution was slowly added to a stirred solution of (*S*)-1-benzoyl-2-*tert*-butyl-3-methylimidazolidin-4-one (4.07 g, 15.6 mmol) in THF (100 mL) at -78 °C, and the stirring was continued for 15 min. The temperature was then lowered to -100 °C for 30 min, and benzaldehyde (3.30 mL, 33.0 mmol) was added. After 5 min, the reaction was quenched with half saturated NH_4Cl solution (150 mL) and ether (150 mL). After warming to room temperature, the phases were separated and the aqueous layer was extracted with ether (3x150 mL). The combined organic layers were washed with H_2O (60 mL), dried ($MgSO_4$), and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 70:30 ether:petroleum ether to give **46**: 4.80 g (84%). Mp 153-154 °C (lit.[124] mp 153.2-153.8 °C); $[\alpha]_D$=+24.1° (c=1.05, $CHCl_3$) (lit.[124] $[\alpha]_D$=+23.4 °C); IR (KBr) 3440, 3350, 1723, 1688 cm^{-1}; ^1H NMR (200

MHz, $CDCl_3$) δ 0.96 (s, 9 H, $(C\underline{H}_3)_3C$), 2.08 (m, 1 H, N<u>H</u>), 2.92 (s, 3 H, $C\underline{H}_3N$), 4.11 (d, J = 2.3 Hz, 1 H, <u>H</u>-C(5)), 4.19 (d, J = 2.3, 1 H, <u>H</u>-C(2)), 6.36 (d, J = 2.9 Hz, 1 H, <u>H</u>-C(α)), 7.25-7.65, 8.05-8.10 (2 x m, 10 H, aryl <u>H</u>); CI-MS 367 (MH^+) (366.19 calcd for $C_{22}H_{26}N_2O_3$). Anal. Calcd for $C_{22}H_{26}N_2O_3$: C, 72.11; H, 7.15; N, 7.64. Found: C, 72.32; H, 7.33; N, 7.72.

(-)-(2*S*, 3*R*)-3-Phenylserine (47)

The literature procedure was modified.[124] A mixture of **46** (3.49 g, 9.50 mmol) and 6 N HCl (100 mL) was heated to reflux for 8 h. The cooled solution was extracted with ether (3x100 mL) and evaporated *in vacuo*. The residue was applied to an ion exchange AG50W-X8 column. The column was washed with water until neutrality. Elution with 1.5 N NH_3 solution (1.5 L) and evaporation gave **47**: 1.80 g (95%). This was crystallized from $EtOH/H_2O$. Mp 177-178 °C (dec.) (lit.[124] mp 181-182 °C (dec.)); $[\alpha]_D$=-29.5 ° (c=1.00, H_2O) (lit.[124] $[\alpha]_D$=-32 °C); IR (KBr) 3440, 3368, 3341, 3272, 1629, 1594 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 3.90 (d, J = 4.4 Hz, 1 H, NC<u>H</u>CO), 5.30 (d, J = 4.4 Hz, 1 H, <u>H</u>-C(3)), 7.45 (m, 5 H, aryl <u>H</u>); FAB-MS 182.23 (MH^+) (181.07 calcd for $C_9H_{11}NO_3$).

***N*-(*tert*-Butyloxycarbonyl)-(-)-(2*S*, 3*R*)-3-phenylserine (48)**

The literature procedure was modified.[108] A solution of **47** (540. mg, 2.98 mmol) in dioxane (6 mL), water (3 mL), and 1 N NaOH (6 mL) was stirred in an ice-water bath. Di-*tert*-butyl pyrocarbonate (800. mg, 3.67 mmol) was added and the stirring was continued at room temperature for 30 min. Then the solution was concentrated *in vacuo* to 8 mL, covered with EtOAc (15 mL), and acidified with 0.1 N $KHSO_4$ to pH 2-3. The aqueous solution was extracted with EtOAc (2x15 mL) and the extracts were pooled and washed with H_2O (2x10 mL), and saturated aqueous NaCl (10 mL). The solution was dried (Na_2SO_4) and the solvent was evaporated to give the crude product. This was

dissolved in saturated aqueous $NaHCO_3$ (30 mL), and the resulting solution was washed with hexane (2x30 mL), and then acidified with 0.5 N $KHSO_4$. The aqueous layer was extracted with EtOAc (3x40 mL). The organic layers were washed with H_2O (15 mL), saturated aqueous NaCl (15 mL) and dried (Na_2SO_4). Then the solvent was removed *in vacuo* to give **48**: 620. mg (73%). This was further purified by MPLC on an RP-C_8 column using 40% CH_3CN in H_2O. Mp 121-123 °C (dec.); IR (KBr) 3411, 3000, 1742, 1669 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.27 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 4.35 (d, J = 2.9 Hz, 1 H, NC<u>H</u>CO), 5.22 (d, J = 2.9 Hz, 1 H, C<u>H</u>(OH)), 7.18-7.37 (m, 5 H, aryl <u>H</u>); FAB-MS 282.07 (MH^+) (281.13 calcd for $C_{14}H_{19}NO_5$). Anal. Calcd for $C_{14}H_{19}NO_5$: C, 59.78; H, 6.81; N, 4.98. Found: C, 59.55; H, 6.88; N, 4.81.

5-Norbornene-*endo*-2,3-dicarboxylic Anhydride (49)

The literature procedure was modified.[135] Maleic anhydride (60.0 g, 612 mmol) was suspended in benzene (270 mL), and this was cooled to 0 °C in an ice-water bath. Then cyclopentadiene (6.00 mL, 72.4 mmol) was added and the flask was swirled, causing an exothermic reaction. The product was isolated by vacuum filtration to give **49** in quantitative yield. Mp 161-163 °C (lit.[135] mp 164-165 °C); IR (KBr) 1855, 1842, 1772 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.57, 1.80 (2 x d, J = 9.6 Hz, J = 9.6 Hz, 2 x 1 H, $C\underline{H}_2$), 3.55 (m, 4 H, 4 x C<u>H</u>), 6.35 (2 x d, J = 1.5 Hz, J = 1.5 Hz, C<u>H</u>=C<u>H</u>); exact mass, 164.0477 (164.0473 calcd for $C_9H_8O_3$). Anal. Calcd for $C_9H_8O_3$: C, 65.85; H, 4.91. Found: C, 65.63; H, 5.15.

***N*-Hydroxy-5-norbornene-*endo*-2,3-dicarboximide (50)**

The literature procedure was modified.[136] A solution was prepared by adding sodium carbonate (20.5 g) to a solution of hydroxylamine (26.3 g) in H_2O (60 mL). To this aqueous solution was added **49** (50.0 g, 0.305 mol), and the mixture was heated to 65 °C for 1 h. The clear solution was cooled in refrigerator overnight. The crystals were

collected and washed with ice-cold 5 N HCl (2x20 mL) to give **50**: 52.5 g (96%). Mp 161-164 °C (lit.[136] mp 165-166 °C); IR (KBr) 3107, 2947, 2854, 1767, 1713, 1664, 1514 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.56 (dd, J = 8.0, 0.6 Hz, 1 H, CHH), 1.79 (m, 1 H, CHH), 3.35 (m, 4 H, 4 x CH), 6.13 (2 x d, J = 1.5 Hz, J = 1.5 Hz, 2 H, CH=CH); exact mass, 179.0582 (179.0582 calcd for $C_9H_9NO_3$). Anal. Calcd for $C_9H_9NO_3$: C, 60.33; H, 5.07; N, 7.82. Found: C, 59.91; H, 5.17; N, 7.55.

***N*-(*tert*-Butyloxycarbonyl)-(-)-(2*S*,3*R*)-3-phenylserinamide (51)**

The literature procedure was modified.[134] To a stirred solution of **48** (610. mg, 2.17 mmol) and **50** (458 mg, 2.56 mmol) in a mixture of EtOAc (15 mL) and THF (15 mL) in an ice-water bath was added DCC (630. mg, 3.06 mmol). Stirring was continued at 0 °C for 30 min and then at room temperature for 1 h. The DCU thus formed was filtered, and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in dioxane (15 mL) and the resulting solution was cooled to 0 °C. To this was added concentrated ammonia (0.5 mL), the mixture was stirred at room temperature for 4 h and then evaporated to dryness. EtOAc (200 mL) was then added, and the solution was washed successively with 4% aqueous $NaHCO_3$ (2x25 mL), H_2O (25 mL), 0.1 N HCl (2x25 mL), H_2O (25 mL), and saturated aqueous NaCl (2x25 mL). The solution was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give the crude product, which was purified by flash chromatography using 5% MeOH in $CHCl_3$ to yield **51**: 500. mg (82%). Mp 55 °C (dec.); IR (KBr) 3380, 2976, 1675, 1600 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.22, 1.30 (s, 9 H, $CO_2C(CH_3)_3$), 4.26 (d, J = 2.8 Hz, 1 H, NCHCO), 5.18 (d, J = 2.8 Hz, 1 H, CH(OH)), 7.30 (m, 5 H, aryl H); FAB-MS 281.00 (MH^+) (280.14 calcd for $C_{14}H_{20}N_2O_4$). Anal. Calcd for $C_{14}H_{20}N_2O_4$: C, 59.99; H, 7.19; N, 9.99. Found: C, 59.90; H, 7.21; N, 9.99.

(-)-(2*S*,3*R*)-3-Phenylserinamide Trifluoroacetate Salt (52)

The literature procedure was modified.[140] A solution of **51** (450. mg, 1.61 mmol) in TFA (15 mL) was stirred at room temperature for 30 min. Then the solvent was removed *in vacuo* to give **52**: 460. mg (97%). Mp 58-60 °C; IR (MeOH) 2900-3300, 1675, 1203, 1137 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 3.85 (d, J = 2.8 Hz, 1 H, NC<u>H</u>CO), 4.02 (d, J = 2.8 Hz, 1 H, C<u>H</u>(OH)), 7.35-7.54 (m, 5 H, aryl <u>H</u>); FAB-MS 181.76 (MH^+) (180.09 calcd for $C_9H_{12}N_2O_2$). Anal. Calcd for $C_9H_{12}N_2O_2 \cdot CF_3CO_2H \cdot H_2O$: C, 42.31; H, 4.84; N, 8.97. Found: C, 42.29; H, 4.25; N, 8.65.

***N*-Acetyl-D-phenylalanyl-L-β-phenylserinamide (53)**

The literature procedure was modified.[134] A dry flask containing *N*-acetyl-D-phenylalanine (360. mg, 1.74 mmol) and **50** (310. mg, 1.73 mmol) was dried under vacuum for a few hours. Then the mixture was dissolved in EtOAc (5 mL) and THF (10 mL) and then cooled in an ice-water bath. DCC (394. mg, 1.91 mmol) was added with stirring, the resulting solution was stirred at 0 °C for 30 min, then kept at room temperature for 30 min. The DCU thus formed was filtered and the solvent was removed *in vacuo*. To the residue was added **52** (430. mg, 1.46 mmol) and the mixture was dissolved in THF (20 mL). Triethylamine (0.225 mL, 1.49 mmol) was added with stirring and the reaction mixture was stirred at room temperature for 4 h. Then the solvent was removed in *vacuo* to give a residue which was dissolved in n-BuOH (400 mL). The resulting solution was then washed with 4% aqueous $NaHCO_3$ (2 x 25 mL), 1 N HCl (2 x 25 mL), and saturated aqueous NaCl (25 mL), then the n-BuOH was removed *in vacuo* to give the crude product. The residue was taken up in EtOH, and precipitate was filtered off. The crude product was further purified by MPLC on RP C_8 column with 35% CH_3CN in H_2O to give **53**: 450. mg (83%). Mp 196.6-198.6 °C; IR (KBr) 3348, 3299, 1686, 1657, 1633, 1594 cm^{-1}; ^1H NMR (200 MHz, CD_3SOCD_3) δ 1.71 (s, 3 H, C<u>H</u>$_3$CO), 2.42 (dd, J = 14.0, 10.1 Hz, 1

H, aryl CH), 2.55 (dd, J = 14.0, 3.6 Hz, 1 H, aryl CH), 4.33 (dd, J = 9.0, 2.4 Hz, 1 H, NCHCO), 4.53 (m, 1 H, NCHCO), 5.19 (dd, J = 4.9, 2.4 Hz, 1 H, CH(OH)), 5.73 (d, J = 4.9 Hz, 1 H, OH), 7.07-7.31 (m, 10 H, aryl H), 7.39 (d, J = 6.7 Hz, 2 H, NH_2), 8.01 (d, J = 7.6 Hz, 1 H, NH), 8.15 (d, J = 9.1 Hz, 1 H, NH); FAB-MS 370.06 (MH^+) (369.17 calcd for $C_{20}H_{23}N_3O_4$).

_N_-Acetyl-D-phenylalanyl-L-β-phenylserine-α-hydroxyglycine Methyl Ester (54)

The literature procedure was modified.[120] A solution of **53** (146 mg, 0.396 mmol) and methyl glyoxylate (200. mg, 2.27 mmol) in acetone (60 mL) was refluxed for 6 h and then cooled to room temperature. Acetone was removed to give a crude product which was purified by MPLC on an RP C_8 column using 0.1% TFA in 65/35 H_2O/CH_3CN to yield **54**: 142 mg (79%). Mp 88.5-90.5 °C; IR (KBr) 3350, 1750, 1655, 1531 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.76 (s, 3 H, CH_3CO), 2.5-2.9 (m, 2 H, aryl CH_2), 3.76 (d, J = 6.7 Hz, 3 H, OCH_3), 4.55-4.68 (m, 2 H, NCHCO, NCHCO), 5.25, 5.30 (2 x d, J = 3.3 Hz, J = 2.5 Hz, 1 H, NCHCO), 5.61 (m, 1 H, CH(OH)), 7.00-7.40 (m, 10 H, aryl H); FAB-MS 458.10 (MH^+) (457.18 calcd for $C_{23}H_{27}N_3O_7$).

_N_-Acetyl-D-phenylalanyl-L-β-phenylserine-α-methoxyglycine Methyl Ester (55)

To a stirred solution of **54** (12.0 mg, 26.2 μmol) in CH_2Cl_2 (5 mL) in an ice-water bath was added acetyl chloride (2.04 μL, 28.8 μmol) in CH_2Cl_2 (2 mL) and triethylamine (4.02 μL, 28.8 μmol) in CH_2Cl_2 (2 mL), the resulting mixture was stirred for 30 min. Then the solvent was removed *in vacuo* to give the crude product (R_f = 0.61 in 10% MeOH in $CHCl_3$, R_f (starting material) = 0.39). This was purified by flash chromatography using 6% MeOH in $CHCl_3$. The purified product was characterized as the methoxy ether **55**: 7.20 mg (58%). ^1H NMR (200 MHz, $CDCl_3$) δ 1.93 (s, 3 H, CH_3CO), 2.73-3.02 (m, 2

H, aryl $C\underline{H}_2$), 3.38 (s, 3 H, $CO_2C\underline{H}_3$), 3.75 (d, J = 7 Hz, 1 H, N$\underline{H}$), 3.78 (d, J = 2.5 Hz, 3 H, $OC\underline{H}_3$), 4.57 (m, 1 H, NC$\underline{H}$CO), 4.77 (dd, J = 9, 4 Hz, 1 H, NC$\underline{H}$CO), 5.34 (m, 1 H, aryl C$\underline{H}$(OH)), 5.49 (dd, J = 12.6, 9.1 Hz, 1 H, NC$\underline{H}$(OH)CO), 6.07 (d, J = 7.3 Hz, 1 H, O$\underline{H}$), 7.03 (d, J = 8 Hz, 1 H, N$\underline{H}$), 6.92, 7.26 (2 x m, 10 H, aryl $\underline{H}$), 7.85 (dd, J = 8.5, 4.5 Hz, 1 H, N$\underline{H}$); FAB-MS 472.1 (MH^+) (471.2 calcd for $C_{24}H_{29}N_3O_7$).

3*S*-(*N*-Acetyl-D-phenylalanyl)-4*R*-phenyl-5-oxa-(6,6)-dimethyl-2-piperidone (56)

The literature procedure was modified.[120] A solution of **53** (15.0 mg, 0.0404 mmol), glyoxylic acid (3.72 mg, 0.0404 mmol), and methanesulfonic acid (2 equivalent.) in acetone (5 mL) was refluxed overnight. The acetone was then removed *in vacuo* and the resulting residue was dissolved in H_2O (10 mL). This was extracted with EtOAc (3x15 mL) and the organic layers were washed with H_2O (10 mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* to give the crude product which was purified by MPLC with an RP C_8 column using 60/40 CH_3CN/H_2O to give **56**: 9.50 mg (56%). IR ($CHCl_3$) 3284, 2988, 1741, 1709, 1660, 1540 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.35, 1.45 (2 x s, 6 H, $C(C\underline{H}_3)_2$), 1.86 (s, 3 H, $C\underline{H}_3CO$), 3.00-3.20 (m, 2 H, aryl $C\underline{H}_2$), 4.00 (d, 1 H, NC$\underline{H}$CO), 4.95 (m, 1 H, NC$\underline{H}$CO), 5.83, 5.89 (2 x d, 2 H, 2 x N$\underline{H}$), 6.17 (d, 1 H, PhC$\underline{H}$), 6.34 (br s, 1 H, N$\underline{H}$), 7.10-7.40 (m, 10 H, aryl $\underline{H}$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.01, 30.12, 30.44, 37.74, 52.94, 63.50, 72.27, 74.85, 126.38, 127.07, 128.41, 128.58, 128.64, 129.54, 136.90, 169.57, 170.34; FAB-MS 410.20 (MH^+) (409.20 calcd for $C_{23}H_{27}N_3O_4$).

3*S*-(*N*-Acetyl-D-phenylalanyl)-4*R*-phenyl-5-oxa-6(*R*,*S*)-formyl-2-piperidone (57)

To a stirred solution of **53** (10.0 mg, 27.1 μmol) in acetaldehyde (6 mL) at room temperature was added methanesulfonic acid (3.50 μL), and the stirring was continued for

90 min. The excess acetaldehyde was removed *in vacuo* and the residue was dissolved in H_2O (4 mL). The resulting aqueous layer was extracted with $CHCl_3$ (2x10 mL). The organic layers were dried (Na_2SO_4) and the solvent was removed *in vacuo* to give the crude product. The expected product was detected by mass spectrometry. FAB-MS 396.18 (MH^+) (395.18 calcd for $C_{22}H_{25}N_3O_4$).

Ethyl *trans*-4-Phenyl-2-hydroxy-3-butenoate (58)

To a stirred solution of **63** (7.00 g, 39.3 mmol) in EtOH (70 mL) in an ice-water bath was added ethereal diazoethane until a yellow coloration persisted. Acetic acid was added until the yellow color disappeared. The solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 10% EtOAc in hexane to give **58**: 7.00 g (86%). Mp 52-53 °C; IR (KBr) 3420, 2981, 1756, 1648 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.32 (t, J = 7.2 Hz, 3 H, $CH_2\underline{CH_3}$), 2.95 (br s, 1 H, O<u>H</u>), 4.30 (m, 2 H, $\underline{CH_2}CH_3$), 4.82 (dd, J = 5.5, 1.6 Hz, 1 H, C<u>H</u>(OH)), 6.25 (dd, J = 15.8, 5.6 Hz, <u>H</u>-C(3)), 6.28 (dd, J = 15.8, 1.6 Hz, 1 H, aryl C<u>H</u>), 7.33 (m, 5 H, aryl <u>H</u>); exact mass, 206.0952 (206.0943 calcd for $C_{12}H_{14}O_3$). Anal. Calcd for $C_{12}H_{14}O_3$: C, 69.89; H, 6.84. Found: C, 69.86; H, 6.61.

***trans*-4-Phenyl-2-(*S*)-hydroxy-3-butenoic Acid (59) and Ethyl *trans*-4-Phenyl-2-(*R*)-hydroxy-3-butenoate (60)**

The literature procedure was modified.[153] To a 250 mL 3-necked round bottom flask equipped with a mechanical stirrer and pH-stat was added **58** (2.00 g, 9.71 mmol). THF (7 mL), water (60 mL) and 0.05 M sodium phosphate buffer (pH 7.0, 15 mL) were added to the flask and the pH was adjusted to 8 with 1 N NaOH. Lipase from *Pseudomonas fluorescens* (80.0 mg) was added, and the mixture was vigorously stirred at 40 °C. The solution was maintained at pH.8 by automatic addition of 0.1N NaOH, and THF (14 mL) was added periodically. At 50% conversion, the reaction was stopped.

THF was removed *in vacuo*, and the reaction mixture was extracted with EtOAc (3x100 mL). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (50 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give the *R*-ester: 1.18 g. The aqueous layer was acidified to pH 1.5 with concentrated HCl and extracted with EtOAc (3x100 mL). The combined EtOAc layers were dried (Na_2SO_4) and the solvent was removed *in vacuo* to give **59**: 580. mg. $[\alpha]_D$ = -2.8 ° (C = 1.00, MeOH). Its chromatographic and spectral properties were identical to those of the racemic material **62** prepared above.

From the above results, only 41% of the ester was hydrolyzed. The S-acid obtained this way was relatively pure. To obtain a more optically pure R-ester, the following experiment was performed. Compound **58** (0.600 g, 2.91 mmol) was dissolved in THF (5 mL). Water (25 mL) and 0.05 M sodium phosphate buffer (pH 7.0, 5 mL) were added and the pH was adjusted to 8 with 1 N NaOH. Lipase from *Pseudomonas fluorescens* (110. mg)was added and the mixture was stirred vigorously at 40 °C. The solution was maintained at pH.8 by automatic addition of 0.1N NaOH, and THF was added periodically. When 17 mL NaOH solution was consumed, the reaction was stopped. THF was removed *in vacuo*, and the reaction mixture was extracted with EtOAc (3x80 mL). The combined organic layers were washed with H_2O (40 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give a residue which was purified by flash chromatography using 10% EtOAc in hexane to give **60**: 300. mg. $[\alpha]_D$ = +2.5 ° (C = 1.00, $CHCl_3$). Its chromatographic and spectral properties were identical to those of the racemic material **65** prepared above.

trans-4-Phenyl-2-(*R*)-hydroxy-3-butenoic Acid (61)

The literature procedure was modified.[141] 1 N NaOH (0.4 mL) was added to a solution of **60** (38.0 mg, 0.146 mmol) in EtOH (3 mL) with stirring at room temperature. After 4 min, the reaction was quenched with 1 N HCl (0.4 mL). The EtOH was removed

in vacuo and the aqueous layer was extracted with EtOAc (3x20 mL). The combined organic layers were washed with saturated aqueous NaCl (5 mL), dried (Na_2SO_4) and the solvent was removed *in vacuo* to give **61**: 31.0 mg (94%). Its chromatographic and spectral properties were identical to those of the racemic material **65** prepared above.

trans-4-Phenyl-2-oxo-3-butenoic Acid (62)

The literature procedure was modified.[154] To a 250 mL 3-neck flask equipped with a mechanical stirrer, a thermometer, an addition funnel and a gas adaptor was added, under Ar, benzaldehyde (30.4 g, 287 mmol) and MeOH (10 mL). The resulting solution was cooled to 5 °C and kept at 5-10 °C in an ice-water bath while pyruvic acid (21.5 mL) was added over a 20 min period. A cold solution of KOH (25.5 g) dissolved in MeOH (80 mL) was then added *via* an addition funnel at a steady rate to maintain the temperature of the reaction at 5 - 10 °C. Shortly after all the KOH was added, a yellow precipitate was formed. This was diluted with MeOH (70 mL), and stirred overnight. The precipitate was isolated by filtration, resuspended in MeOH (70 mL) and filtered again to give **62**: 59.0 g (96%). Mp 253 °C; IR (KBr) 3420, 3040, 1681, 1629, 1605, 1575 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 6.69 (d, J = 16.5 Hz, 1 H, aryl C<u>H</u>), 7.33 (m, 5 H, aryl <u>H</u>), 7.51 (d, J = 16.5 Hz, 1 H, C<u>H</u>CO); exact mass, 176.0472 (176.0473 calcd for $C_{10}H_8O_3$).

trans-4-Phenyl-2-hydroxy-3-butenoic Acid (63)

The literature procedure was modified.[154] To a 500 mL Erlenmeyer flask was added water (125 mL) and ice (40 g). To the resulting slurry was added **62** (19.0 g, 88.8 mmol) in one portion. $NaBH_4$ (2.89 g) was added in small portions over a 10 min period, keeping the temperature below 10 °C. After the addition was complete, the reaction mixture was allowed to slowly warm to room temperature and the stirring was continued overnight. Ice (35 g) was added and the mixture was cooled to 2 °C. Concentrated HCl (25 mL) was added in portions, and the stirring was continued for 10 min. EtOAc (2x200

mL) was used to extract the mixture and the combined organic layers were washed with saturated aqueous NaCl (200 mL), dried ($MgSO_4$) and the solvent was removed to give **63**: 14.0 g (89%). Mp 103-104 °C; IR (KBr) 3449, 2900, 2640, 1734 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 4.76 (d, J = 5.9 Hz, 1 H, C<u>H</u>CO), 6.31 (dd, J = 15.9, 5.9 Hz, 1 H, <u>H</u>-C(3)), 6.72 (d, J = 15.9 Hz, 1 H, aryl C<u>H</u>), 7.25 (5 H, m, aryl <u>H</u>); exact mass, 178.0630 (178.0630 calcd for $C_{10}H_{10}O_3$).

Nitrosoethylurea (64)

The literature procedure was modified.[155] Ethylamine hydrochloride salt (122.3 g, 1.50 mol) was dissolved in H_2O (200 mL), then further water was added to bring the total mass to 500 g. Urea (300. g, 5.00 mol) was added and the solution was heated gently reflux for 2.75 h and then vigorous reflux for 0.25 h. The solution was cooled to room temperature, 95% $NaNO_2$ (110. g, 1.50 mol) was added to it, and the whole solution was cooled to 0 °C. A mixture of ice (600 g) and concentrated sulfuric acid (100 g) in a 3 L beaker was surrounded by an efficient ice-water bath, and the cool ethylurea-nitrite solution was added slowly with mechanical stirring at such a rate that the temperature did not rise above 0 °C. The nitrosoethylurea, which rose to the surface as a foamy precipitate, was filtered at once with suction and pressed well on the filter. The crystals were stirred to a paste with about 50 mL of cold water, sucked as dry as possible, and dried in a vacuum desiccator to constant weight to yield **64**: 110. g (69%). ^1H NMR (200 MHz, CD_3OD) δ 0.92 (t, J = 7.1 Hz, 3 H, C<u>H</u>$_3$), 3.82 (q, J = 7.1 Hz, 2 H, C<u>H</u>$_2$).

Diazoethane (65)

The literature procedure was modified.[155] 50% aqueous KOH (60 mL) and ether (200 mL) were introduced into a 500 mL round bottom flask. The mixture was cooled to 5 °C and nitrosoethylurea (22.6 g, 0.193 mol) was added with continued cooling and shaking. The deep yellow ether layer was decanted and used in the next step.

***N*-Benzyloxycarbonyl-D-glutamic Acid (69)**

The literature procedure was modified.[159] To a stirred solution of D-glutamic acid (30.0 g, 204 mmol) in pH 10 sodium carbonate buffer (900 mL) cooled in an ice-water bath was added benzylchloroformate (54.0 g, 317 mmol) over 15 min. The ice-water bath was then allowed to warm to room temperature over 2.5 h. After stirring for 7 h, the reaction mixture was extracted with ether (700 mL), and the aqueous layer was acidified with concentrated HCl until the evolution of CO_2 ceased and the solution remained a cloudy white. The aqueous layer was then extracted with EtOAc (800 mL, 600 mL, 400 mL), and the organic layers were dried ($MgSO_4$). The solvent was removed *in vacuo* to give **69**: 55.3 g (96%). Mp 118-120 °C; IR (KBr) 3305, 3032, 2960, 1703, 1690, 1550, 1527 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 1.92, 2.15 (2 x m, 2 x 1 H, NCC$\underline{H}_2$), 2.41 (t, J = 7.6 Hz, 2 H, C$\underline{H}_2$CO), 4.21 (dd, J = 9.3, 4.9 Hz, 1 H, NC$\underline{H}$CO), 5.09 (s 2 H, OC$\underline{H}_2$Ph), 7.34 (m, 5 H, aryl $\underline{H}$); FAB-MS 281.89 (MH^+) (281.09 calcd for $C_{13}H_{15}NO_6$). Anal. Calcd for $C_{13}H_{15}NO_6$: C, 55.51; H, 5.38; N, 4.98. Found: C, 55.19; H, 5.23; N, 5.25.

(*R*)-3-3(Benzyloxycarbonyl-5-oxazolidinon-4-yl)-propanoic Acid (70)

The literature procedure was modified.[161] Compound **69** (30.9 g, 110. mmol), paraformaldehyde (6.00 g, 143 mmol), and *p*-toluenesulfonic acid (1.24 g, 7.20 mmol) were dissolved in freshly distilled benzene (400 mL). After a brief period of stirring, the flask was fitted with a Dean Stark trap and a condenser, and the solution was refluxed for 3.5 h. Then the reaction mixture was cooled to room temperature, and washed with H_2O (2x80 mL) and saturated aqueous $NaHCO_3$ (2x100 mL). The aqueous solution was acidified by concentrated HCl and then extracted with EtOAc (3x500 mL). The combined organic layers were dried ($MgSO_4$) and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 0.5% AcOH in EtOAc and hexane (1:1) to give **70**: 23.5 g (73%). IR (MeOH cast) 3600, 3200, 1801, 1714 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 2.06-2.35 (m, 2 H, NCC$\underline{H}_2$), 2.40 (m, 2 H, C$\underline{H}_2$CO),

4.41 (m, 1 H, NCHCO), 5.16 (s, 2 H, OCH_2Ph), 5.25 (d, J = 4.3 Hz, 1 H, NCHOC 5.50 (d, 1 H, NCHOCO), 7.35 (m, 5 H, aryl H); FAB-MS 293.87 (MH^+) (293.09 calcd for $C_{14}H_{15}NO_6$). Anal. Calcd for $C_{14}H_{15}NO_6$: C, 57.34; H, 5.16; N, 4.78. Found: C, 57.08; H, 5.21; N, 4.62.

α-Methyl-*N*-Benzyloxycarbonyl-D-glutamate (71)

The literature procedure was modified.[159] Freshly cut sodium metal (1.07 g, 46.5 mmol) was placed in a 1 L round bottom flask and dissolved in dry MeOH (200 mL) at 0 °C under Ar. The resulting solution was slowly added to a solution of **70** (6.47 g, 22.1 mmol) in freshly distilled MeOH (230 mL) via syringe over a period of 30 min. Additional MeOH (30 mL) was used to rinse the flask and the solution was added to the reaction vessel. After 1.5 h stirring, the solution was warmed to room temperature. Formic acid (88%, 5 mL) and water (250 mL) were added followed by extraction with EtOAc (400 mL, 300 mL, 200 mL). The combined organic layers were dried (Na_2SO_4), and the solvent was removed *in vacuo* to give **71**: 6.44 g (99%). Mp 65-66 °C; IR (KBr) 3323, 3063, 2952, 1740, 1706, 1693, 1659, 1543 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.98, 2.19 (2 x m, 2 x 1 H, $NCCH_2$), 2.44 (m, 2 H, CH_2CO), 3.74 (s, 3 H, OCH_3), 4.46 (dd, J = 13.3, 8.2 Hz, 1 H, NCHCO), 5.11 (s, 2 H, OCH_2Ph), 5.54 (d, J = 7.8 Hz, 1 H, NH), 7.38 (m, 5 H, aryl H); FAB-MS 295.84 (MH^+) (295.11 calcd for $C_{14}H_{17}NO_6$). Anal. Calcd for $C_{14}H_{17}NO_6$: C, 56.95; H, 5.80; N, 4.74. Found: C 57.00; H, 5.83; N, 4.73.

Methyl *N*-Benzyloxycarbonyl-D-vinylglycinate (72)

The literature procedure was modified.[159] To a solution of **71** (3.20 g, 10.8 mmol) in benzene (120 mL) was added cupric acetate monohydrate (541 mg, 2.70 mmol) and the suspension was stirred under argon for 1 h. Lead tetraacetate (9.59 g, 21.6 mmol), which had been previously dried over P_2O_5 and KOH at 0.05 mmHg for 48 h, was added to the mixture. The suspension was then stirred under argon for 1 h followed by stirring at

reflux for 15 h. After filtration through a Celite pad, the benzene solution was diluted with EtOAc (100 mL) and washed with H_2O (3x15 mL), brine (15 mL), dried ($MgSO_4$). The solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 9:1 hexane:EtOAc to give **72**: 1.11 g (41%). IR ($CHCl_3$ cast) 3340, 1747, 1724, 1621, 1213 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 3.80 (s, 3 H, $OC\underline{H}_3$), 4.96 (m, 1 H, $NC\underline{H}CO$), 5.15 (s, 2 H, $OC\underline{H}_2Ph$), 5.35 (m, 2 H, vinyl $C\underline{H}_2$), 5.47 (br s, 1 H, $N\underline{H}$), 5.92 (m, 1 H, vinyl $C\underline{H}$), 7.40 (s, 5 H, aryl $\underline{H}$); FAB-MS 249.95 (MH^+) (249.10 calcd for $C_{13}H_{15}NO_4$).

D-Vinylglycine Hydrochloride Salt (73)

The literature procedure was modified.[158] A solution of **72** (1.06 g, 4.26 mmol) in 6 N HCl (20 mL) was refluxed for 1 h. The solution was then cocled to room temperature, washed with $CHCl_3$ (2x10 mL), and evaporated to dryness to give a white residue which was recrystallized from acetone to give **73**: 480. mg (82%). Mp 167-169 °C (dec.); IR (KBr) 3400, 3000, 1740, 1644, 1619, 1500 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 4.55 (d, J = 7.0 Hz, 1 H, $NC\underline{H}CO$), 5.55 (m, 2 H, vinyl $C\underline{H}_2$), 6.00 (m, 1 H, vinyl $C\underline{H}$); FAB-MS 102.09 (MH^+) (101.05 calcd for $C_4H_7NO_2$). Anal. Calcd for $C_4H_7NO_2 \cdot HCl$: C, 34.92; H, 5.86; N, 10.18. Found: C, 34.61; H, 6.06; N, 10.36.

***p*-Toluenesulfonylhydrazide (74)**

The literature procedure was modified.[164] To a solution of tosyl chloride (95.0 g, 0.498 mol) in THF (200 mL) was added hydrazine at such a rate as to maintain a temperature of 10-15 °C. After 15 min, more of the lower layer was drawn off and the upper layer was washed with saturated aqueous NaCl solution (2x50 mL). The organic layer was dried ($MgSO_4$), and solvent was removed *in vacuo* to give a residue which was treated with petroleum ether. The resulting solid was isolated by vacuum filtration to yield **74**: 85.0 g (92%). Mp 104 °C (lit. mp 101-104 °C); IR (KBr) 3389, 3259, 3232, 3206,

1600, 1551, 1544, 1507 cm^{-1}; ^1H NMR (200 MHz, CD_3SOCD_3) δ 2.40 (s, 3 H, $C\underline{H}_3$), 7.40 (dd, J = 7.8, 7.6 Hz, 2 H, aryl $\underline{H}$), 7.67 (m, 2 H, aryl $\underline{H}$), 8.75 (br s, 2 H, $N\underline{H}_2$), 9.58 (s, 1 H, $N\underline{H}$); exact mass, 186.0462 (186.0463 calcd for $C_7H_{10}N_2O_2S$).

Benzaldehyde Tosylhydrazone (75)

The literature procedure was modified.[163] Absolute MeOH (50 mL) was added to **74** (29.2 g, 0.156 mol) in a 250 mL Erlenmeyer flask. The resulting slurry was swirled as freshly distilled benzaldehyde (15.0 g, 0.142 mol) was added rapidly. A mildly exothermic reaction ensued and the *p*-toluenesulfonylhydrazide dissolved. After 15 min, some solid was filtered and the product crystallized. This was collected by vacuum filtration to give **75**: 12.0 g (30%). Mp 124-126 °C (lit.[163] mp 124-125 °C); IR (KBr) 3440, 3226, 1596 cm^{-1}; ^1H NMR (200 MHz, CD_3SOCD_3) δ 2.35 (s, 3 H, $C\underline{H}_3$), 7.38 (m, 5 H, aryl $\underline{H}$), 7.54 (m, 2 H, aryl $\underline{H}$), 7.76 (dd, J = 8.3, 1.2 Hz, 2 H, aryl $\underline{H}$), 7.90 (d, J = 1.1 Hz, 1 H, $N\underline{H}$), 11.43 (s, 1 H, $C\underline{H}$); exact mass, 274.0776 (274.0776 calcd for $C_{14}H_{14}N_2O_2S$).

Phenyldiazomethane (76)

The literature procedure was modified.[163] Benzaldehyde tosylhydrazone (**75**) (6.70 g, 24.5 mmol) was introduced into a 200 mL, single-necked, round-bottomed flask. A solution of NaOMe in MeOH (1.0 M, 27 mL) was added via syringe and the mixture was swirled until dissolution was complete. The methanol was then removed by rotary evaporator. The last traces of of methanol were removed by evacuation of the flask at 0.1 mm for 2 h. The solid tosylhydrazone salt was broken up with a spatula and the flask was fitted with a vacuum take-off adaptor and a 25 mL receiver flask. The system was evacuated at 0.1 mm and the receiver flask was cooled in a dry ice-acetone bath to about -50 °C. The flask containing the salt was immersed in an oil bath and the temperature was raised to 90 °C. At this temperature, red phenyldiazomethane began to collect in the

receiver flask. The temperature was raised to 160 °C over an 1.5 h period, after which the reaction had terminated. The product was purified by reduced pressure distillation at 0.2 mm Hg at room temperature to give **76**: 2.46 g (85%).

_N_-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-phenylalanyl-D-vinylglycine Benzyl Ester (77)

The literature procedure was modified.[134] A dry flask containing **11** (229 mg, 0.556 mmol) and HONB (110. mg, 0.614 mmol) were dried over P_2O_5 under vacuum for 14 h. The mixture was dissolved in DMF (10 mL) and EtOAc (10 mL) and the resulting solution was cooled to 0 °C in an ice-water bath. DCC (125. mg, 0.610 mmol) was then added with stirring. The stirring was continued at 0 °C for 30 min and then at room temperature for 2 h. The DCU thus formed was filtered and the solvent was removed *in vacuo* to give the activated dipeptide which was directly used in the next coupling step. To a stirred solution of **73** (68.8 mg, 0.500 mmol) in MeOH (2 mL) in an ice-water bath was added phenyldiazomethane until the solution retained a red coloration. Stirring was continued at room temperature until the red color disappeared (~10 min) and the solvent was then removed *in vacuo*. To the residue was added the solution of *N*-BOC-D-Phe-L-Phe-ONB made above in DMF (10 mL). Et_3N (50.0 μL, 0.367 mmol) in DMF (1.2 mL) was slowly added over 10 min with stirring at 0 °C, and the solution was stirred at room temperature for a further 8 h. Benzene (30 mL) and EtOAc (60 mL) were then added and the mixture was washed with H_2O (2x15 mL) and saturated aqueous NaCl (15 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give a yellow residue which was purified by flash chromatography with $CHCl_3$ to yield **77**: 185. mg (63%). This was further purified by normal phase MPLC using 5% EtOAc in $CHCl_3$. Mp 76 °C (dec.); IR (KBr) 3416, 3288, 1746, 1716, 1695, 1646, 1538 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.35 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.77-3.10 (m, 4 H, aryl $C\underline{H}_2$), 4.16 (dd, *J* = 14.1, 7.0 Hz, 1 H, NC<u>H</u>CO), 4.74 (dd, *J* = 13.8, 7.5 Hz, 1 H, NC<u>H</u>CO), 5.03 (dd, *J*

= 7.5, 1.6 Hz, 1 H, NCHCO), 5.07 (dd, J = 12.4, 2.7 Hz, 1 H, NH), 5.15 (s, 2 H, OCH_2Ph), 5.16-5.23 (m, 2 H, $CH=CH_2$), 5.74-5.87 (m, 1 H, $CH=CH_2$), 6.39 (dd, J = 13.8, 1.5 Hz, 1 H, NH), 6.86 (d, J = 11.1 Hz, 1 H, NH), 7.12-7.36 (m, 15 H, aryl H); FAB-MS 586.15 (MH^+) (585.28 calcd for $C_{34}H_{39}N_3O_6$). Anal. Calcd for $C_{34}H_{39}N_3O_6$: C, 69.72; H, 6.71; N, 7.17. Found: C, 69.67; H, 6.91; N, 7.15.

D-Phenylalanyl-L-phenylalanyl-D-vinylglycine (78)

The literature procedure was modified.[162] To a stirred solution of **77** (30.0 mg, 0.0513 mmol) in CH_3CN (5 mL) was added Me_3SiI (18.2 μL, 0.128 mmol) and the resulting solution was stirred at 70 °C for 3 h. The reaction mixture was then cooled to room temperature. Water (15 mL) and 1 M HCl (1 mL) were added and the solution was washed with ether (3x10 mL). The aqueous layer was lyophilized to give the crude product which was purified by reverse phase HPLC (Waters C18 μ-Bondapak Radial Pak, 8 mm x 10 cm 4 μm cartridge, retention time 10.61 min at 1.50 mL/min flow rate, gradient solution: 0 min, 30% B, 20 min, 60% B) to give **78**: 12.3 mg (47%). IR (KBr) 3440, 3280, 3089, 1668, 1550 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.73-3.10 (m, 4 H, aryl CH), 4.05 (dd, J = 8.1, 6.1 Hz, 1 H, NCHCO), 4.80 (m, 2 H, NCHCO, NCHCO), 5.12 (d, J = 17.3 Hz, 1 H, CH=CHH), 5.18 (d, J = 10.8 Hz, 1 H, CH=CHH), 5.89 (m, 1 H, $CH=CH_2$), 7.16-7.35 (m, 10 H, aryl H); ^{13}C NMR (100 MHz, CD_3OD) δ 38.48, 39.42, 55.54, 55.69, 118.14, 128.04, 128.88, 129.63, 130.11, 130.44, 133.29, 135.36, 138.00, 169.29, 172.67; FAB-MS 396.44 (MH^+) (395.18 calcd for $C_{22}H_{25}N_3O_4$).

***N*-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-phenylalanyl-L-vinylglycine Benzyl Ester (79)**

The literature procedure was modified.[134] A dry flask containing **11** (180. mg, 0.437 mmol) and HONB (87.1 mg, 0.487 mmol) was dried over P_2O_5 under vacuum for 14 h. The mixture was then dissolved in DMF (10 mL) and EtOAc (10 mL) and the

solution was cooled to 0 °C in an ice-water bath. DCC (99.0 mg, 0.480 mmol) was added to the solution with stirring, and the reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 2 h. The formed DCU was filtered off and the solvent was removed *in vacuo* to give the activated dipeptide which was directly used in the next coupling step. Commercially available L vinylglycine hydrochloride salt (40.0 mg, 0.390 mmol) was dissolved in MeOH (2 mL) and the solution was cooled to 0 °C. Phenyldiazomethane was added with stirring until the solution stayed red in color. Stirring was continued at room temperature until the red color disappeared (~10 min) and the solvent was then removed *in vacuo*. To the residue was added a solution of *N*-BOC-D-Phe-L-Phe-ONP made above in DMF (10 mL). Et_3N (46.5 μL) in DMF (1 mL) was slowly added over 10 min with stirring at 0 °C and the solution was stirred at room temperature for 8 h. Benzene (30 mL) and EtOAc (60 mL) were added and the resulting mixture was washed with H_2O (2x10 mL) and saturated aqueous NaCl (10 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give a yellow residue which was purified by flash chromatography eluting with $CHCl_3$ to yield **79**: 101. mg (44%). This was further purified by normal phase MPLC using 5% EtOAc in $CHCl_3$. Mp 67-69 °C; IR (KBr) 3286, 3064, 3030, 2977, 2929, 1745, 1716, 1696, 1647, 1540 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.35 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.95 (m, 4 H, aryl $C\underline{H}_2$), 4.23 (dd, J = 14.4, 7.1 Hz, 1 H, NC<u>H</u>CO), 4.72 (dd, J = 14.4, 6.7 Hz, 1 H, NC<u>H</u>CO), 4.98 (br s, 1 H, N<u>H</u>), 5.05 (m, 1 H, NC<u>H</u>CO), 5.14 (s, 2 H, $OC\underline{H}_2Ph$), 5.22 (m, 2 H, $CH=C\underline{H}_2$), 5.84 (m, 1 H, $C\underline{H}=CH_2$), 6.43 (br d, J = 6.8 Hz, 1 H, N<u>H</u>), 6.76 (br s, 1 H, N<u>H</u>), 7.25 (m, 15 H, aryl <u>H</u>); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28 19, 37.51, 38.16, 54.09, 54.60, 56.32, 66.67, 67.31, 80.36, 118.21, 127.06, 128.13, 129.27, 129.33, 131.49, 135.22, 136.08, 136.44, 155.44, 169.51, 170.03, 171.25; FAB-MS 585.64 (MH^+) (585.28 calcd for $C_{34}H_{39}N_3O_6$). Anal. Calcd for $C_{34}H_{39}N_3O_6$: C, 69.72; H, 6.71; N, 7.17. Found: C, 69.71; H, 6.93; N, 7.10.

D-Phenylalanyl-L-phenylalanyl-L-vinylglycine (80)

The literature procedure was modified.[162] To a solution of **79** (45.0 mg, 76.9 μmol) in CH_3CN (15 mL) was added Me_3SiI (100. μL, 0.703 mmol) and the resulting solution was heated to reflux for 1 h. The reaction mixture was cooled to room temperature, water (15 mL) and 1 M HCl (1 mL) were added and the resulting solution was washed with ether (3x15 mL). The aqueous layer was lyophilized to give the crude product which was purified by reverse phase HPLC (Waters C18 μ-Bondapak Radial Pak, 8 mm x 10 cm 4 μm cartridge, retention time 8.51 min at 1.50 mL/min flow rate, gradient elution: 0 min, 30% B; 20 min, 60% B) to give **80**: 9.47 mg (24%). IR (KBr) 3430, 1676, 1207, 1144 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.67-3.24 (m, 4 H, aryl $C\underline{H}_2$), 4.04 (dd, J = 7.6, 5.9 Hz, 1 H, NC<u>H</u>CO), 4.75 (dd, J = 4.8, 4.8 Hz, 1 H, NC<u>H</u>CO), 4.96 (d, J = 5.7 Hz, 1 H, NC<u>H</u>CO), 5.24 (d, J = 10.2 Hz, 1 H, CH=C<u>H</u>H), 5.37 (d, J = 16.9 Hz, 1 H, CH=CH<u>H</u>), 5.98 (m, 1 H, C<u>H</u>=CH_2), 6.98 and 7.24 (2 x m, 10 H, aryl <u>H</u>); FAB-MS 396.27 (MH^+) (395.18 calcd for $C_{22}H_{25}N_3O_4$).

Benzyl *N*-Benzyloxycarbonyl-D-vinylglycinate (81)

The literature procedure was modified.[159] To a solution of commercially available *N*-benzyloxycarbonyl-D-glutamic acid α-benzyl ester (2.19 g, 5.90 mmol) in benzene (100 mL) was added cupric acetate monohydrate (295 mg, 1.48 mmol), and the suspension was stirred under argon for 1 h. Lead tetraacetate (5.245 g, 11.8 mmol), which had been dried over P_2O_5 and KOH at 0.05 mmHg for 48 h, was added and the suspension was stirred for 1 h under argon, then stirred at reflux for 20 h. After filtration through a Celite pad, the filtrate was diluted with EtOAc (100 mL). The organic solution was washed with H_2O (3x25 mL), saturated aqueous NaCl (25 mL) and dried ($MgSO_4$). The solvent was removed to give the crude product which was purified by flash chromatography using 90:10 hexane: ethyl acetate to give **81**: 840. mg (44%). Mp 71-73 °C; IR (KBr) 3400, 3303, 1745, 1689, 1538 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 4.96 (m, 1 H, NC<u>H</u>CO),

5.12 (s, 2 H, $C\underline{H}_2Ph$), 5.19 (s, 2 H, $C\underline{H}_2Ph$), 5.30 (m, 2 H, $CH{=}C\underline{H}_2$), 5.45 (d, J = 7.6 Hz, 1 H, N$\underline{H}$), 5.90 (m, 1 H, $C\underline{H}{=}CH_2$), 7.33 (s, 10 H, aryl $\underline{H}$); FAB-MS 325.69 (MH^+) (325.13 calcd for $C_{19}H_{19}NO_4$). Anal. Calcd for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.30. Found: C, 69.95; H, 5.66; N, 4.10.

Benzyl *N*-Benzyloxycarbonyl-D-cyclopropylglycinate (82)

The literature procedure was modified.[167, 168] To a stirred solution of **81** (163. mg, 0.502 mmol) and $Pd(CH_3CN)_2Cl_2$ (6 mg) in ether (20 mL) at 0 °C in an ice-water bath was added ethereal CH_2N_2 until the color stayed yellow; a large excess of CH_2N_2 was then added. After stirring at room temperature for 20 min, the solution was filtered through a Celite pad, and the ether was removed *in vacuo* to give the crude product which was purified by flash chromatography using 10% EtOAc in hexane to give **82**: 129 mg (76%). Mp 51-52 °C; IR (KBr) 3350, 3315, 1744, 1705, 1688, 1554 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.55 (m, 4 H, cyclopropyl $C\underline{H}_2C\underline{H}_2$), 1.08 (m, 1 H, cyclopropyl C$\underline{H}$), 3.90 (dd, J = 8.1, 7.7 Hz, 1 H, NC$\underline{H}$CO), 5.19 (s, 2 H, $C\underline{H}_2Ph$), 5.22 (s, 2 H, $C\underline{H}_2Ph$), 5.35 (d, J = 7.0 Hz, 1 H, N$\underline{H}$), 7.35 (s, 10 H, aryl $\underline{H}$); FAB-MS 339.63 (MH^+) (339.15 calcd for $C_{20}H_{21}NO_4$). Anal. Calcd for $C_{20}H_{21}NO_4$: C, 70.78; H, 6.24; N, 4.13. Found: C, 70.91; H, 6.40; N, 4.08.

Benzyl D-Cyclopropylglycinate Hydrochloride Salt (83)

The literature procedure was modified.[162] To a stirred solution of **82** (228 mg, 0.672 mmol) in chloroform (20 mL) was added $(CH_3)_3SiI$ (0.956 mL, 6.72 mmol) and the stirring was continued for 10 min. Water (15 mL) and 1 N HCl (4 mL) were added, and the aqueous layer was separated. The organic layer was washed with H_2O (10 mL), and the combined aqueous layers were washed with $CHCl_3$ (10 mL) and then lyophilized to give the crude product. This was purified by flash chromatography using 6% methanol in chloroform to give **83**: 132 mg (81%). IR (MeOH cast) 3340, 3280, 3000, 1736 cm^{-1};

^{1}H NMR (200 MHz, CD_3OD) δ 0.28-0.57 (m, 4 H, cyclopropyl $C\underline{H}_2C\underline{H}_2$), 0.98 (m, 1 H, cyclopropyl C<u>H</u>), 2.82 (d, *J* = 8.4 Hz, 1 H, NC<u>H</u>CO), 5.18 (dd, *J* = 17.6, 12.8 Hz, 2 H, $C\underline{H}_2Ph$), 7.34 (m, 5 H, aryl <u>H</u>); FAB-MS 206.05 (MH^+) (205.10 calcd for $C_{12}H_{15}NO_2$).

_N_-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-phenylalanyl-D-cyclopropylglycine Benzyl Ester (84)

The literature procedure was modified.[134] A dry flask containing **11** (120. mg, 0.291 mmol) and HONB (58.0 mg, 0.324 mmol) was dried under vacuum at 50 mTorr for a few hours. The mixture was dissolved in THF (5 mL) and EtOAc (5 mL). DCC (67.0 mg, 0.325 mmol) was added and the mixture was stirred at 0 °C in an ice-water bath for 30 min, then at room temperature for 5 h. The formed DCU was filtered off and the solvent was removed to yield the activated dipeptide. To a solution of **83** (45.0 mg, 0.220 mmol) in DMF (5 mL) was added the above prepared *N*-BOC-D-Phe-L-Phe-ONB in DMF (10 mL). Et_3N (0.90 equivalent.) was slowly added and stirring was continued at room temperature for 22 h. Benzene (30 mL) and EtOAc (60 mL) were added, and the resulting mixture was washed with 1 N HCl (2x10 mL), H_2O (10 mL), saturated aqueous $NaHCO_3$ (2x10 mL), H_2O (10 mL) and saturated aqueous NaCl (2x10 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 2% MeOH in $CHCl_3$ to give **84**: 121 mg (92%). Mp 73.5-75.5 °C; IR (KBr) 3400, 3000, 1746, 1715, 1695, 1646, 1587 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.20-0.51 (m, 4 H, cyclopropyl $C\underline{H}_2C\underline{H}_2$), 0.90 (m, 1 H, cyclopropyl C<u>H</u>), 1.36 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 2.70-3.09 (m, 4 H, aryl $C\underline{H}_2$), 3.96 (dd, *J* = 8.2, 7.9 Hz, 1 H, NC<u>H</u>CO), 4.20 (dd, *J* =14.2, 7.2 Hz, 1 H, NC<u>H</u>CO), 4.66 (m, 1 H, NC<u>H</u>CO), 4.97 (d, *J* = 7.8 Hz, 1 H, N<u>H</u>), 5.08, 5.16 (ABq, *J* = 13 Hz, *J* = 13 Hz, 2 x 1 H, $OC\underline{H}_2Ph$), 6.40 (d, *J* = 8.0 Hz, 1 H, N<u>H</u>), 6.50 (br s, 1 H, N<u>H</u>), 7.00-7.36 (m, 15 H,

aryl H); FAB-MS 600.14 (MH^+) (599.30 calcd for $C_{35}H_{41}N_3O_6$). Anal. Calcd for $C_{35}H_{41}N_3O_6$: C, 70.10; H, 6.89; N, 7.01. Found: C, 69.82; H, 6.81; N, 6.93.

D-Phenylalanyl-L-phenylalanyl-D-cyclopropylglycine (85)

The literature procedure was modified.[162] To a solution of **84** (50.0 mg, 83.4 μmol) in MeOH (4 mL) was added 1 N NaOH (4 mL), and the reaction mixture was stirred at room temperature for 30 min. The solution was then acidified with 1 N HCl (4.1 mL), and MeOH was evaporated. The aqueous layer was extracted with EtOAc (3x10 mL) and the organic layers were dried (Na_2SO_4). The solvent was removed to yield *N*-BOC-D-Phe-L-Phe-D-cyclopropylglycine: 40.0 mg. This was dissolved in dry CH_3CN (15 mL), followed by the addition of $(CH_3)_3SiI$ (100. μl, 0.703 mmol) and the solution was stirred at room temperature for 15 min. Water (30 mL) and 1 N HCl (1 mL) were added, the mixture was then washed with ether (2x20 mL). The aqueous layer was evaporated to give the crude product which was purified by reverse phase HPLC (Waters C18 μ-Bondapak Radial Pak, 8 mm x 10 cm 10 μm cartridge, retention time 12.75 min at 1.20 mL/min flow rate, gradient elution: 0 min, 55% B; 20 min, 100% B) to give **85**: 25.0 mg (80%). Mp 119-121 °C; IR (KBr) 3440, 3280, 3070, 1670 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 0.15-0.64 (m, 4 H, cyclopropyl CH_2CH_2), 1.04 (m, 1 H, cyclopropyl CH), 2.72-3.06 (m, 4 H, aryl CH_2), 3.60 (d, J = 8.9 Hz, 1 H, NCHCO), 4.05 (dd, J = 7.8, 6.4 Hz, 1 H, NCHCO), 4.76 (dd, J = 8.5, 6.8 Hz, 1 H, NCHCO), 7.06-7.33 (m, 10 H, aryl H); FAB-MS 410.12 (MH^+) (409.20 calcd for $C_{23}H_{27}N_3O_4$).

***trans*-4-Phenyl-3-butenoic Acid (86)**

The literature procedure was modified.[169] Phenylacetaldehyde (13.1 g, 0.109 mol), malonic acid (11.3 g, 0.109 mol) and pyridine (8.50 g, 0.107 mol) were mixed in a 100 mL flask and heated in a water bath to 80 °C. Evolution of CO_2 was observed and lasted for 30 min. Heating was continued for a further hour. Then the mixture was cooled

in an ice-water bath, and acidified with concentrated H_2SO_4. Water (15 mL) was added, and the resulting yellow precipitate was filtered off to give **86**: 15.0 g (85%). The product was recrystallized from hot water to give bright, fluffy needle crystals. Mp 84-86 °C (lit. 85 °C); IR (KBr) 3430, 3059, 1703 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 3.30 (dd, J = 6.9, 1.3 Hz, 2 H, C<u>H</u>$_2$), 6.29 (m, 1 H, <u>H</u>-C(3)), 6.53 (d, J = 15.9 Hz, 1 H, <u>H</u>-C(4)), 7.35 (m, 5 H, aryl <u>H</u>), 10.1 (br s, 1 H, CO$_2$<u>H</u>); exact mass, 162.0675 (162.0681 calcd for $C_{10}H_{10}O_2$). Anal. Calcd for $C_{10}H_{10}O_2$: C, 74.06; H, 6.21. Found: C, 74.02; H, 6.14.

Methyl *trans*-2-(2-Phenylcyclopropyl)ethanoate (87)

The literature procedure was modified.[167, 168] To a stirred solution of **86** (162. mg, 1.00 mmol) and $Pd(CH_3CN)_2Cl_2$ (12 mg) in ether (25 mL) cooled in an ice-water bath was added ethereal CH_2N_2. After a persistent yellow color was observed, a large excess of CH_2N_2 was then added, and the solution was stirred at room temperature for 20 min. After filtration through a Celite pad, the solvent was removed to give the crude product: 188 mg (>99%). To separate out the side product, 4-phenyl-3-butenoic acid methyl ester, *m*-chloroperbenzoic acid (173 mg, 1.00 mmol) was added and the mixture was dissolved in CH_2Cl_2 (25 mL). After 2 h, the solvent was removed *in vacuo* and the product was purified by flash chromatography using 8% EtOAc in hexane to yield the pure **87**: 160. mg (84%). IR (KBr) 3000, 2960, 1760, 1605, 1510 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.87, 1.01 (2 x m, 2 x 1 H, cyclopropyl C<u>H</u>$_2$), 1.39 (m, 1 H, <u>H</u>-C(3)), 1.77 (m, 1 H, aryl C<u>H</u>), 2.35, 2.48 (2 x dd, J = 15.8, 7.1 Hz, J = 15.8, 7.0 Hz, 2 x 1 H, C<u>H</u>$_2$CO), 3.70 (s, 3 H, C<u>H</u>$_3$), 7.20 (m, 5 H, aryl <u>H</u>); exact mass, 190.0991(190.0994 calcd for $C_{12}H_{14}O_2$).

***trans*-2-(2-Phenylcyclopropyl)ethanoic Acid (88)**

The literature procedure was modified.[141] To a stirred solution of **87** (106. mg, 0.558 mmol) in MeOH (10 mL) in a room temperature water bath was added 1 N NaOH (2 mL) and the stirring was continued for 8 h. The solution was acidified with 1 N HCl to pH 2 and most of the solvent was removed *in vacuo*. The aqueous solution was extracted with EtOAc (3 x 20 mL), and the combined organic layers were dried (Na_2SO_4) and solvent was removed *in vacuo* to give **88** in quantitative yield. Mp 47-48 °C; IR (KBr) 3420, 3023, 1705, 1605 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.89, 1.03 (2 x m, 2 x 1 H, cyclopropyl C$\underline{H}_2$), 1.40 (m, 1 H, $\underline{H}$-C(3)), 1.79 (m, 1 H, aryl C$\underline{H}$), 2.40, 2.52 (2 x dd, J = 16.2, 7.1 Hz, J = 16.2, 7.0 Hz, 2 x 1 H, C$\underline{H}_2$CO), 7.18 (m, 5 H, aryl $\underline{H}$); exact mass, 176.0840 (176.0837 calcd for $C_{11}H_{12}O_2$). Anal. Calcd for $C_{11}H_{12}O_2$: C, 74.98; H, 6.86. Found: C, 74.48; H, 6.61.

***N*-Acetyl-D-phenylalanyl-L-phenylalanyl-2-(D,L)-methylthioglycine Isomers (89A, 89B)**

The literature procedure was modified.[120] A dry flask containing **42** (23.6 mg, 0.0552 mmol) was dried under vacuum at 50 mTorr for 12 h. It was then cooled to 0 °C in an ice-water bath, and methane thiol (4 drops) and glacial AcOH (1 mL) were added. Concentrated H_2SO_4 (1 drop) was added and the mixture was stirred at room temperature for 48 h. Then ice (2 g) was added and the organic material was extracted with EtOAc (3x5 mL). The combined organic layers were washed with water (3 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give the mixture of isomers **89A** and **89B**: 24 mg (95%). These isomers were separated by reverse phase HPLC (Waters C18 μ-Bondapak Radial Pak, 8 mm x 10 cm 10 μm cartridge, flow rate 1.50 mL/min, 40% B isocratic elution). Retention time was 10.95 min for **89A**, and 12.43 min for **89B**. Data for **89A**: Mp 171-173 °C; IR (KBr) 3400, 1660, 1541 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.85 (s, 3 H, C$\underline{H}_3$CO), 2.19 (br s, 3 H, SC$\underline{H}_3$), 2.50-3.15 (m, 4 H, aryl C$\underline{H}_2$), 4.60-4.64 (m,

2 H, NCHCO, NCHCO), 5.33 (br s, 1 H, NCHSCH$_3$); FAB-MS 457.91 (MH$^+$) (457.20 calcd for $C_{23}H_{27}N_3O_5S$). Data for **89B**: Mp 167-169 °C; IR (KBr) 3420, 1670, 1547 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 1.86 (s, 3 H, CH_3CO), 2.04 (br s, 3 H, SCH_3), 2.65-3.15 (m, 4 H, aryl CH_2), 4.52-4.67 (m, 2 H, NCHCO, NCHCO), 5.38 (br s, 1 H, NCHSCH$_3$); FAB-MS 457.91 (MH$^+$) (457.20 calcd for $C_{23}H_{27}N_3O_5S$).

_N_-Acetyl-D-phenylalanyl-L-phenylalanyl-(D, L)-benzylthioglycine Isomers (90A, 90B)

The literature procedure was modified.[120] A dry flask containing **42** (10.0 mg, 23.4 μmol) was dried under vacuum at 50 mTorr for 12 h. Then it was cooled to 0 °C in an ice-water bath, and benzyl mercaptan (8 drops) and glacial AcOH (1 mL) were added. Concentrated H_2SO_4 (1 drop) was added and the mixture was stirred at room temperature for 48 h. Ice (2 g) was added and the organic material was extracted with EtOAc (3x5 mL). The combined organic layers were washed with water (3 mL) and dried (Na_2SO_4). The solvent and unreacted benzyl mercaptan were removed by blowing argon through the solution, yielding a mixture of the isomers **90A** and **90B**: 10.6 mg (85%). These isomers were separated by reverse phase HPLC (Waters C_{18} μ-Bondapak Radial Pak, 25 mm x 10 cm 10 μm cartridge, flow rate 3.00 mL/min, 50% B isocratic elution). Retention time was 53.33 min for **90A**, and 60.19 min for **90B**. Data for **90A**: IR (KBr) 3425, 3280, 1675, 1642, 1545 cm^{-1}; ^{1}H NMR (200 MHz, CD_3SOCD_3) δ 1.66 (s, 3 H, CH_3CO), 2.35-3.10 (m, 4 H, aryl CH_2), 3.81, 3.87 (ABq, J = 12.9 Hz, J = 12.9 Hz, 2 x 1 H, SCH_2Ph), 4.55 (m, 1 H, NCHCO), 4.70 (m, 1 H, NCHCO), 5.25 (d, J = 8.3 Hz, 1 H, NCH(SBn)CO), 7.26 (m, 15 H, aryl H), 7.95 (d, J = 8.5 Hz, 1 H, NH), 8.43 (d, J = 8.4 Hz, 1 H, NH), 8.95 (d, J = 8.4 Hz, 1 H, NH); FAB-MS 533.77 (MH$^+$) (533.23 calcd for $C_{30}H_{31}N_3O_5S$). Data for **90B**: IR (KBr) 3410, 3284, 1641, 1552 cm^{-1}; ^{1}H NMR (200 MHz, CD_3SOCD_3) δ 1.66 (s, 3 H, CH_3CO), 2.35-3.10 (m, 4 H, aryl CH_2), 3.81 (s, 2 H, SCH_2Ph), 4.48 (m, 1 H, NCHCO), 4.71 (m, 1 H, NCHCO), 5.31 (d, J = 8.5 Hz, 1 H,

NCH(SBn)CO), 7.20 (m, 15 H, aryl H), 7.93 (d, J = 8.5 Hz, 1 H, NH), 8.47 (d, J = 8.5 Hz, 1 H, NH), 9.05 (d, J = 8.47 Hz 1 H, NH); FAB-MS 533.77 (MH^+) (533.23 calcd for $C_{29}H_{31}N_3O_5S$).

_N_-Acetyl-D-phenylalanyl-L-phenylalanyl-_N_-formamide (91)

The literature procedure was modified.[120] A dry flask containing **42** (40.0 mg, 0.0937 mmol) was dried under vacuum at 50 mTorr overnight. Then this was cooled to 0 °C, and 90% H_2O_2 (15 drops, was added, followed by glacial AcOH (2 mL) and concentrated H_2SO_4 (2 drops). The reaction mixture was stirred at room temperature for two days. Ice (4 g) was added, and the mixture was extracted with EtOAc (2x5 ml). The combined organic layers were dried (Na_2SO_4) and the solvent was removed *in vacuo* to give the crude product which was purified by reverse phase HPLC (Waters C18 μ-Bondapak Radial Pak, 25 mm x 10 cm 10 μm cartridge, retention time 32.03 min at 2.00 mL/min flow rate, 50% B isocratic elution) to give **91**: 16.0 mg (45%). IR (KBr) 3420, 1748, 1684, 1546 cm^{-1}; ^1H NMR (400 MHz, CD_3SOCD_3) δ 1.70 (s, 3 H, CH_3CO), 2.40 (t, J = 13.3 Hz, 1 H, aryl CH), 2.56 (d, J = 13.8 Hz, 1 H, aryl CH), 2.74 (t, J = 11.4 Hz, 1 H, aryl CH), 3.05 (d, J = 11.4 Hz, 1 H, aryl CH), 4.53 (m, 2 H, NCHCO, NCHCO), 7.20 (m, 10 H, aryl H), 7.96 (d, J = 7.3 Hz, 1 H, NH), 8.58 (d, J = 7.2 Hz, 1 H, NH), 9.04 (d, J = 8.6 Hz, 1 H, NH), 11.42 (d, J = 7.7 Hz, 1 H, NCHO); ^{13}C NMR (100 MHz, CD_3SOCD_3) δ 22.30, 36.36, 37.68, 53.58, 54.33, 126.09, 126.54, 127.86, 128.07, 128.99, 129.26, 136.97, 137.66, 163.15, 168.95, 171.48, 173.31; FAB-MS 382.17 (MH^+) (381.17 calcd for $C_{21}H_{23}N_3O_4$).

Benzyl _N_-Acetyl-L-prolyl-glycine (92)

The literature procedure was modified.[142] A dry flask containing *N*-acetyl-L-proline (2.36 g, 15.0 mmol) and glycine benzyl ester *p*-toluenesulfonate salt (5.10 g, 15.1 mmol) was dried at 50 mTorr over P_2O_5 for 16 h. Dry DMF (50 mL) was then added, and

the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (3.75 mL, 17.2 mmol) was added followed by a solution of triethylamine (4.50 mL, 33.0 mmol) in DMF (15 mL), and the stirring was continued at 0 °C for 10 h. The mixture was diluted with benzene (270 mL) and EtOAc (540 mL), and was washed successively with 1 N HCl (2x50 mL), H_2O (50 mL), saturated aqueous $NaHCO_3$ (2x50 mL), H_2O (50 mL), and saturated aqueous NaCl (2x50 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give a gummy solid which was purified by flash chromatography using 85:15 EtOAc:EtOH to give **92**: 3.37 g (74%). IR ($CHCl_3$ cast) 3300, 2980, 1749, 1648, 1634, 1530 cm^{-1} ; ^1H NMR (200 MHz, $CDCl_3$) δ 1.7-2.4 (m, 4 H, $C\underline{H}_2C\underline{H}_2CHCO$), 2.00 (s, 3 H, $C\underline{H}_3CO$), 3.22-3.54 (m, 2 H, $C\underline{H}_2NCO$), 3.90-4.06 (m, 2 H, $NC\underline{H}_2CO$), 4.50-4.60 (m, 1 H, $NC\underline{H}CO$), 5.08 (s, 2 H, aryl $C\underline{H}_2$), 7.26 (s, 5 H, aryl $\underline{H}$), 7.54 (br s, 1 H, $N\underline{H}$); exact mass, 304.1428 (304.1423 calcd for $C_{16}H_{20}N_2O_4$). Anal. Calcd for $C_{16}H_{20}N_2O_4 \cdot H_2O$: C, 59.62; H, 6.88; N, 8.69. Found: C, 59.38; H, 6.18; N, 8.63.

***N*-Acetyl-L-prolyl-glycine (93)**

A mixture of **92** (200. mg, 0.658 mmol) and 5% Pd/C (20 mg) in MeOH (5 mL) and water (5 mL) was hydrogenated at 48 psi for 2 h. After filtration through E sinter glass, the solvent was removed *in vacuo* to give **93:** 100. mg (71%). This was further purified by reverse phase HPLC (Waters C_{18} μ-Bondapak Radial Pak, 8 mm x 10 cm 10 μm cartridge, retention time 2.75 min at 2.00 mL/min flow rate, gradient elution: 0 min, 0% B; 10 min, 10% B). IR (KBr) 3329, 1737, 1654, 1602, 1533 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 1.90-2.35 (m, 4 H, $C\underline{H}_2C\underline{H}_2CCO$), 2.10 (s, 3 H, $C\underline{H}_3CO$), 3.50-3.70 (m, 2 H, $C\underline{H}_2NCO$), 3.94 (s 2 H, $NC\underline{H}_2CO$), 4.42-4.56 (m, 1 H, $NC\underline{H}CO$); exact mass, 214.0957 (214.0953 calcd for $C_9H_{14}N_2O_6$).

N-Acetyl-L-prolyl-[1,2-^{14}C]-glycine (94)

The methods used to prepare unlabeled peptide **93** were adapted for radioactive synthesis. A solution of glycine (150. μCi, 1.5 μmol), *p*-toluenesulfonic acid monohydrate (2..00 mg, 11.0 μmol) in distilled benzyl alcohol (4 mL) and benzene (8 mL) was refluxed for 14 h, with the liberated water being removed azeotropically and trapped with the aid of CaH_2 in the soxhlet thimber. The mixture was cooled to room temperature, and the solvent was removed *in vacuo* to give the crude product which was purified by ion-exchange column with AG50WX8 resin to give [1,2-^{14}C]-glycine benzyl ester: 120. μCi (80%). This and *N*-acetyl-L-proline (20.0 mg, 127 μmol) were dried at 50 mTorr over P_2O_5 for 16 h. Dry DMF (2 mL) was then added, and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (33.0 μL, 152 μmol) was added followed by a solution of triethylamine (0.2 mL, 1.47 mmol) in DMF (2 mL), and the stirring was continued at 0 °C for 12 h. The mixture was diluted with benzene (5 mL) and EtOAc (10 mL) and was washed with 1 N HCl (2x2 ml), H_2O (2 mL), saturated aqueous $NaHCO_3$ (2x2 mL), H_2O (2 mL), and saturated aqueous NaCl (2x2 mL). The organic solvent was removed to give *N*-acetyl-L-prolyl-[1,2-^{14}C]-glycine benzyl ester: 34.0 μCi (28%). To this was added 5% Pd/C (2.5 mg), EtOH (1 mL) and water (1 mL) and the mixture was hydrogenated at 48 psi for 1.5 h. The solution was filtered, and the solvent was removed *in vacuo* to give **94**: 11.8 μCi (72%). This material shows a single radioactive spot on TLC and co-migrates with unlabeled peptide **93** in two different solvent systems. This was further purified by reverse phase HPLC prior to the enzyme assay.

Benzyl *N*-Acetyl-L-prolyl-*N*-nitroso-glycinate (95)

The literature procedure was modified.[170] A modified version of White's procedure was utilized. To a solution of **92** (330. mg, 1.09 mmol) in CH_2Cl_2 (4 mL) was added NaOAc (287 mg, 3.50 mmol) and the resulting mixture was cooled to 0 °C. Nitrogen dioxide gas was bubbled through the stirred solution for 10 min, and the stirring

was continued at 0 °C for 10 min. Argon was bubbled through the solution followed by warming to room temperature. The mixture was poured into a slurry of ice and water (20 g), the nitrosoamide was extracted with ether (50 mL) and the organic phase was washed H_2O (10 mL), saturated aqueous Na_2CO_3 (10 mL), H_2O (10 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give **95**: 330. mg (91%). IR ($CHCl_3$ cast) 2960, 1747, 1651, 1515 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.90-2.30 (m, 4 H, $C\underline{H}_2C\underline{H}_2CCO$), 2.10 (s, 3 H, $C\underline{H}_3CO$), 3.50-3.80 (m, 2 H, $NC\underline{H}_2C$), 4.52 (s, 2 H, $NC\underline{H}_2CO$), 5.02, 5.08 (ABq, J = 12.0, J = 12.0 Hz, 2 x 1 H, $OC\underline{H}_2Ph$), 5.50-5.76 (m, 1 H, $NC\underline{H}CO$), 7.32 (m, 10 H, aryl H); FAB-MS 333.26 (MH^+) (333.13 calcd for $C_{16}H_{19}N_3O_5$).

N-Acetyl-L-prolyl-*N*-nitroso-glycine (96)

The literature procedure was modified.[171] A solution of **95** (95.0 mg, 0.285 mmol) and 5% Pd/C (40.0 mg) in EtOH (10 mL) in a Parr flask was shaken under H_2 at 20 psi for 45 min. After filtration through #2 filter paper, the solvent was removed *in vacuo* to give the crude product. ^1H NMR analysis revealed that this compound was partially over-reduced. Purification by reverse phase HPLC (Waters C_{18} μ-Bondapak Radial Pak, 8 mm x 10 cm 4 μm cartridge, retention time 3.62 min at 2.00 mL/min flow rate, gradient elution: 0 min, 10% B; 20 min, 35% B) afforded **96**: 24.2 mg (35 %). Mp 108-110 °C (lit. 109 °C); IR (KBr) 3200, 1800, 1780, 1737, 1610, 1515 cm^{-1}; ^1H NMR (200 MHz, CD_3COCD_3) δ 2.10 (s, 3 H, $C\underline{H}_3CO$), 1.85-2.50 (m, 4 H, $C\underline{H}_2C\underline{H}_2CCO$), 3.50-3.90 (m, 2 H, $C\underline{H}_2NCO$), 4.40-4.60 (m, 2 H, $NC\underline{H}_2CO$), 5.60-5.85 (m, 1 H, $NC\underline{H}CO$); FAB-MS 244.07 (MH^+) (243.08 calcd for $C_9H_{13}N_3O_5$).

N-Benzyloxycarbonyl-D-phenylalanine (97)

The literature procedure was modified.[159] D-Phenylalanine (10.0 g, 60.5 mmol) was dissolved in aqueous sodium carbonate buffer (pH 10, 400 mL) and the solution was

cooled to 0 °C in an ice-water bath. Benzyl chloroformate (15.5 g, 90.8 mmol) was added with stirring over 10 min, and the ice-water bath was allowed to warm to room temperature over 2.5 h . After 2 h stirring at room temperature, the mixture was extracted with ether (300 mL). The aqueous layer was acidified with concentrated HCl until CO_2 ceased to evolve and the solution remained a cloudy white in nature. The aqueous layer was then extracted with EtOAc (3 x 250 mL). The combined organic layers were dried (Na_2SO_4) and the solvent was removed *in vacuo* to give **97**: 18.0 g (99%). Mp 82-83 °C; IR (KBr) 3420, 3325, 1695, 1528 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 2.88 (dd, J = 13.9, 9.2 Hz, 1 H, aryl CH), 3.19 (dd, J = 13.9, 4.9 Hz, 1 H, aryl CH), 4.41 (dd, J = 9.2, 4.9 Hz, 1 H, NCHCO), 5.02 (s, 2 H, OCH_2Ph), 7.22 (s, 5 H, aryl H), 7.28 (s, 5 H, aryl H); FAB-MS 300.05 (MH^+) (299.12 calcd for $C_{17}H_{17}NO_4$). Anal. Calcd for $C_{17}H_{17}NO_4$: C, 68.22; H, 5.72; N, 4.68. Found: C, 68.50; H, 5.53; N, 4.88.

_N_-Benzyloxycarbonyl-D-phenylalanyl-_N_-(*tert*-butyloxycarbonyl)-hydrazide (98)

The literature procedure was modified.[134] A dry flask containing **97** (5.00 g, 16.7 mmol) and HONB (3.20 g, 18.4 mmol) was dried under vacuum at 50 mTorr for a few hours. The mixture was dissolved in THF (100 mL) and EtOAc (100 mL), and the solution was cooled to 0 °C. To the stirred solution was added DCC (3.80 g, 18.5 mmol) and the stirring was continued at 0 °C for 30 min then at room temperature for 4 h. The formed DCU was filtered off and the solvent was removed *in vacuo*. To the residue was added BOC-$NHNH_2$ (2.50 g, 18.9 mmol), the mixture was dissolved in THF (150 mL) and EtOAc (150 mL), and the solution was stirred at room temperature for 12 h. The solvent was removed *in vacuo* and the residue was redissolved in EtOAc (200 mL). The solution was washed with 1 N HCl (2x20 mL), H_2O (20 mL), saturated aqueous $NaHCO_3$ (2x20 mL), H_2O (20 mL), and saturated aqueous NaCl (2x20 mL), then dried (Na_2SO_4) and the solvent was removed *in vacuo* to give the crude product. Purification by

flash chromatography with 35% EtOAc in hexane yielded **98**: 5.30 g (78%). Mp 56 °C (dec.); IR (KBr) 3300, 3298, 3000, 1700, 1525 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.45 (s, 9 H, $CO_2C(C\underline{H}_3)_3$), 3.03, 3.17 (2 x dd, J = 13.9, 7.5 Hz, J = 13.9, 6.4 Hz, 2 x 1 H, aryl $C\underline{H}_2$), 4.51 (m, 1 H, $NC\underline{H}CO$), 5.05 (d, J = 1.5 Hz, 2 H, $OC\underline{H}_2Ph$), 5.37 (d, J = 7.5 Hz, 1 H, $N\underline{H}$), 6.46 (br s, 1 H, $N\underline{H}$), 7.26 (m, 10 H, aryl $\underline{H}$), 7.97 (br s, 1 H, $N\underline{H}$); FAB-MS 414.20 (MH^+) (413.20 calcd for $C_{22}H_{27}N_3O_5$). Anal. Calcd for $C_{22}H_{27}N_3O_5$: C, 63.91; H, 6.58; N, 10.16. Found: C, 64.02; H, 6.46; N, 10.23.

N-Benzyloxycarbonyl-D-phenylalanyl-hydrazide Trifluoroacetate Salt (99)

The literature procedure was modified.[140] A solution of **98** (1.50 g, 3.63 mmol) in TFA (10 mL) was stirred at room temperature for 30 min. Then TFA was removed *in vacuo* to give **99** in quantitative yield. Mp 115-117 °C; IR (KBr) 3400, 3293, 3220, 3600, 3450, 1687, 1546 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 2.93, 3.12 (2 x dd, J = 13.8, 8.5 Hz, J = 13.8, 6.3 Hz, 2 x 1 H, aryl $C\underline{H}_2$), 4.39 (dd, J = 8.5, 6.3 Hz, 1 H, $NC\underline{H}CO$), 5.02 (s, 2 H, $OC\underline{H}_2Ph$), 7.25 (m, 10 H, aryl $\underline{H}$); FAB-MS 313.97 (MH^+) (313.14 calcd for $C_{17}H_{19}N_3O_3$).

N-Benzyloxycarbonyl-D-phenylalanyl-hydrazinosuccinic Acid (100)

A dry flask containing **99** (490. mg, 1.15 mmol) and succinic anhydride (150. mg, 1.50 mmol) was dried under vacuum at 50 mTorr for a few hours. Dry DMF (40 mL) was then added with stirring, followed by the addition of triethylamine (208.5 µl ,1.50 mmol)) in DMF (2 mL) and the stirring was continued for 12 h. Benzene (100 mL) and EtOAc (200 mL) were added and the mixture was washed with 1 N HCl (2 x 25 mL), saturated aqueous NaCl (2 x 25 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give **100**: 370. mg (78 %). Mp 165-167 °(dec.); IR (KBr) 3282, 3199, 3088-3034, 1791, 1719, 1692, 1671 cm^{-1}; ^1H NMR (200 MHz, CD_3SOCD_3) δ 2.42 (m, 4 H, $C\underline{H}_2C\underline{H}_2$), 2.75, 3.00 (2 x dd, J = 13.6, 11.3 Hz, J = 13.6, 3.7 Hz, 2 x 1

H, aryl C$\underline{H}_2$), 4.31 (m, 1 H, NC$\underline{H}$CO), 4.91 (s, 2 H, OC$\underline{H}_2$Ph), 7.24 (m, 10 H, aryl $\underline{H}$), 7.57 (d, J = 9.1 Hz, 1 H, N$\underline{H}$), 9.91 (s, 1 H, N$\underline{H}$), 10.10 (s, 1 H, N$\underline{H}$), 12.14 (br s, 1 H, $CO_2\underline{H}$); FAB-MS 414.34 (MH^+) (413.16 calcd for $C_{21}H_{23}N_3O_6$). Anal. Calcd for $C_{21}H_{23}N_3O_6$: C, 61.01; H, 5.61; N, 10.16. Found: C, 61.14; H, 5.44; N, 10.27.

D-Phenylalanyl Hydrazinosuccinic Acid (101)

A mixture of **100** (105 mg, 0.254 mmol) and 5% Pd/C (25 mg) in MeOH (80 mL) was stirred under H_2 at 1 atm. for 1 h. After filtration through a Celite pad, the solvent was removed to give the crude product which was purified by MPLC using RP C_8 column utilizing 0.1% TFA in 25% CH_3CN/H_2O to give **101**: 60.0 mg (85%). Mp 69-71 °C; IR (KBr) 3500-2900, 1711, 1671, 1516 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 2.58 (m, 4 H, C$\underline{H}_2$C$\underline{H}_2$CO), 3.06, 3.25 (2 x dd, J = 14.1, 7.1 Hz, J = 14.1, 6.5 Hz, 2 x 1 H, aryl C$\underline{H}_2$), 4.07 (dd, J = 7.6, 6.5 Hz, 1 H, NC$\underline{H}$CO), 7.34 (m, 5 H, aryl $\underline{H}$); FAB-MS 280.06 (279.12 calcd for $C_{13}H_{17}N_3O_4$).

Benzyl *N*-Dansyl-D-phenylalanyl-L-phenylalaninate (102)

The literature procedure was modified.[134] A dry flask containing *N*-dansyl-D-phenylalanine (1.05 g, 2.51 mmol) and HONB (0.500 g, 2.79 mmol) was dried at 50 mTorr for 16 h. This mixture was dissolved in THF (15 mL) and EtOAc (15 mL). DCC (0.580 g, 2.81 mmol) was added and the solution was stirred at room temperature for 12 h. The formed DCU was filtered off and the solvent was removed *in vacuo* to give *N*-dansyl-D-Phe-ONB, to which was added L-phenylalanine benzyl ester *p*-toluenesulfonic acid (1.20 g, 2.81 mmol) and the mixture was dried under vacuum for 8 h. Dry DMF (20 mL) was added with stirring, followed by a solution of triethylamine (0.400 mL, 2.94 mmol) and the stirring was continued at room temperature overnight. Benzene (150 mL) and EtOAc (300 mL) were added and the solution was washed with 1 N HCl (2x30 mL), H_2O 30 mL), saturated aqueous $NaHCO_3$ (2x30 mL), H_2O (30 mL), saturated aqueous NaCl

(2x30 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 2.5% EtOAc in hexane to give **102**: 1.34 g (84%). Mp 60-62 °C; IR (KBr) 3280, 1738, 1659, 1520 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 2.68-2.99 (m, 4 H, aryl C$\underline{H}_2$), 3.85 (dd, *J* = 13.5, 6.6 Hz, 1 H, NC$\underline{H}$CO), 4.77 (m, 1 H, NC$\underline{H}$CO), 4.96 (d, *J* = 6.4 Hz, 1 H, N$\underline{H}$), 5.07, 5.11 (ABq, *J* = 12.2 Hz, *J* = 12.2 Hz, 2 x 1 H, OC$\underline{H}_2$Ph), 6.66 (dd, *J* = 8.3, 1.5 Hz, 1 H, aryl $\underline{H}$), 6.78 (d, *J* = 8.4 Hz, 1 H, N$\underline{H}$), 6.90-7.46 (m, 18 H, aryl $\underline{H}$), 8.03 (d, *J* = 8.6 Hz, 1 H, aryl $\underline{H}$), 8.16 (dd, *J* = 7.8, 1.3 Hz, 1 H, aryl $\underline{H}$), 8.56 (br s, 1 H, aryl $\underline{H}$); FAB-MS 635.75 (MH^+) (635.27 calcd for $C_{37}H_{37}N_3O_5S$). Anal. Calcd for $C_{37}H_{37}N_3O_5S$: C, 69.90; H, 5.87; N, 6.61. Found: C, 69.64; H, 5.74; N, 6.85.

N-Dansyl-D-phenylalanyl-L-phenylalanine (103) and Hydrochloride Salt (104)

A mixture of **102** (800. mg, 1.26 mmol) and 5% Pd/C (250 mg) in EtOAc (30 mL) and MeOH (30 mL) in a Parr flask was shaken under H_2 at 45 psi at room temperature for 1.5 h. After filtration through #2 filter paper, the solvent was removed *in vacuo* to give **103**: 690 mg (98%). Mp. 97-99 °C; IR (KBr) 3300, 1734, 1660, 1525 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 2.64-2.95 (m, 4 H, aryl C$\underline{H}_2$), 2.96 (s, 6 H, (C$\underline{H}_3$)$_2$N), 3.87 (dd, *J* = 14.5, 6.7 Hz, 1 H, NC$\underline{H}$CO), 4.60 (m, 1 H, NC$\underline{H}$CO), 5.46 (d, *J* = 8 Hz, 1 H, N$\underline{H}$), 6.98 (d, *J* = 8 Hz, 1 H, N$\underline{H}$), 6.66-7.53 (m, 13 H, aryl $\underline{H}$), 8.08 (d, *J* = 8 Hz, 1 H, aryl H), 8.16 (d, *J* = 8 Hz, 1 H, aryl $\underline{H}$), 8.55 (d, *J* = 8 Hz, 1 H, aryl $\underline{H}$); FAB-MS 545.82 (545.23 calcd for $C_{30}H_{31}N_3O_5S$). Anal. Calcd for $C_{30}H_{31}N_3O_5S$: C, 66.04; H, 5.73; N, 7.70. Found: C, 65.73; H, 5.90; N, 7.71. Some of the product was converted to its hydrochloride salt by adding 1 N HCl and then extracting with EtOAc. The organic layer was dried ($NaSO_4$) and the solvent was removed *in vacuo* to give **104**. ^1H NMR (200 MHz, $CDCl_3$) δ 2.64-2.95 (m, 4 H, aryl C$\underline{H}_2$), 2.96 (s, 6 H, (C$\underline{H}_3$)$_2$N), 3.87 (dd, *J* = 14.3, 7.0 Hz, 1 H, NC$\underline{H}$CO), 4.68 (m, 1 H, NC$\underline{H}$CO), 5.28 (d, *J* = 7 Hz, 1 H, N$\underline{H}$),

6.81 (d, J = 8 Hz, 1 H, NH), 6.66-7.53 (m, 13 H, aryl H), 8.07 (d, J = 8 Hz, 1 H, aryl H), 8.14 (d, J = 8 Hz, 1 H, aryl H), 8.56 (d, J = 8 Hz, 1 H, aryl H); FAB-MS 545.82 (545.23 calcd for $C_{30}H_{31}N_3O_5S$). Anal. Calcd for $C_{30}H_{31}N_3O_5S \cdot HCl$: C, 61.90; H, 5.54; N, 7.22. Found: C, 61.89; H, 5.40; N, 7.35.

N-Dansyl-D-phenylalanyl-L-phenylalanyl-D-vinylglycine Benzyl Ester (105)

The literature procedure was modified.[134] A dry flask containing **104** (307 mg, 0.528 mmol) and HONB (105 mg, 0.587 mmol) was dried under vacuum at 50 mTorr for 16 h. The mixture was dissolved in THF (5 mL) and EtOAc (5 mL) and cooled to 0 °C in an ice-water bath. To the stirred solution was added DCC (121 mg, 0.587 mmol) and the stirring was continued at 0 °C for 30 min, then at room temperature overnight. The formed DCU was filtered off, and the solvent was removed *in vacuo* to give N-dansyl-D-Phe-L-Phe-ONB hydrochloride salt. A solution of **73** (72.9 mg, 0.530 mmol) in MeOH (5 mL) was cooled to 0 °C in an ice-water bath. Phenyldiazomethane was added with stirring until the solution turned red in color, and the solution was stirred at room temperature until the red color disappeared. The solvent was removed *in vacuo* to give D-vinylglycine benzyl ester hydrochloride salt. N-Dansyl-D-Phe-L-Phe-ONB hydrochloride salt prepared above was added to D-vinylglycine benzyl ester hydrochloride salt and the mixture was dissolved in dry DMF (10 mL) and cooled to 0 °C in an ice-water bath. With stirring a solution of triethylamine (63.4 µL, 45.1 µmol, 0.85 equivalent.) in DMF (1.2 mL) was slowly added and the solution was stirred at 0 °C for 1 h then at room temperature for 7 h. Benzene (50 mL) and EtOAc (100 mL) were added, and the resulting solution was washed with H_2O (2x10 mL) and saturated aqueous NaCl (10 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 15% EtOAc in $CHCl_3$ to give **105**: 160 mg (40%). This material was further purified by MPLC on a silica column using 10% EtOAc in $CHCl_3$. Mp 69-71 °C; IR (KBr) 3280, 3050, 2936, 1744, 1647, 1594 cm^{-1}; ^1H NMR (200 MHz

$CDCl_3$) δ 2.85 (m, 4 H, aryl $C\underline{H}_2$), 2.95 (s, 6 H, $(C\underline{H}_3)_3N$), 3.84 (m, 1 H, NC<u>H</u>CO), 4.55 (dd, *J* = 14.0, 7.2 Hz, 1 H, NC<u>H</u>CO), 5.02 (m, 2 H, N<u>H</u>, NC<u>H</u>CO), 5.14 (s, 2 H, $OC\underline{H}_2Ph$), 5.25 (m, 2 H, $C\underline{H}_2$=CH), 5.79 (m, 1 H, C<u>H</u>=CH_2), 6.55 (d, *J* = 7.8 Hz, 1 H, N<u>H</u>), 6.79 (d, 1 H, N<u>H</u>), 6.65-7.50 (m, 18 H, aryl <u>H</u>), 8.10 (m, 2 H, aryl <u>H</u>), 8.54 (d, *J* = 8.0 Hz, 1 H, aryl <u>H</u>); FAB-MS 719.40 (MH^+) (718.31 calcd for $C_{41}H_{42}N_4O_6S$). Anal. Calcd for $C_{41}H_{42}N_4O_6S$: C, 68.50; H, 5.89; N, 7.79. Found: C, 68.67; H, 5.95; N, 7.45.

_N_-Dansyl-D-phenylalanyl-L-phenylalanyl-D-vinylglycine Trifluoroacetate salt (106), _N_-Dansyl-D-phenylalanyl-L-phenylalanyl-D-vinylglycine trifluoroacetate (107)

The literature procedure was modified.[162] A flask containing **105** (40.0 mg, 0.0557 mmol) was dried under vacuum at 50 mTorr for 12 h. This was dissolved in dry CH_3CN (10 mL), followed by the addition of $(CH_3)_3SiI$ (150 μL), and the resulting solution was heated to reflux for 1 h. After cooling to room temperature, water (20 mL) and 1 N HCl (1 mL) were added and the mixture was extracted with ether (3 x 30 mL). The organic solvent was removed *in vacuo* to give the crude product which was purified by reverse phase MPLC on a C_8 column with 0.1% TFA in 50% CH_3CN/H_2O to give the product: 25 mg (63%). ^1H NMR (200 MHz) showed two isomers. They were separated by reverse phase HPLC (Waters C18 μ-Bondapak Radial Pak, 25 mm x 10 cm 10 μm cartridge, retention times 37.27 min (**106**) and 41.49 min (**107**) at 4.50 mL/min flow rate, 45% B of isocratic elution). By comparing the retention time on HPLC, the inhibition properties on PHM and ^1H NMR spectra of these isomers with D-Phe-L-Phe-D-vinylGly (**78**) and D-Phe-L-Phe-vinylGly (**80**), the isomer (**106**) was identified as the L-vinylGly peptide, the isomer (**107**) was identified as the D-vinylGly peptide. Data for **106**: IR (KBr) 3430, 1675, 1205, 1149 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.43 (dd, *J* = 14.2, 10.1 Hz, 1 H, aryl CH), 2.58 (dd, *J* = 14.1, 4.8 Hz, 1 H, aryl CH), 2.78 (dd, *J* = 13.9

8.8 Hz, 1 H, aryl CH), 3.00 (s, 6 H, $(CH_3)_2N$), 3.10 (dd, J = 13.9, 5.5 Hz, 1 H, aryl CH), 3.88 (dd, J = 9.7, 4.7 Hz, 1 H, NCHCO), 4.67 (dd, J = 8.7, 5.5 Hz, 1 H, NCHCO), 4.96 (d, J = 5.4 Hz, 1 H, NCHCO), 5.28, 5.40 (2 x d, J = 10.5 Hz, J = 17.5 Hz, 2 x 1 H, $CH=CH_2$), 6.01 (m, 1 H, $CH=CH_2$), 6.74 (m, 5 H, aryl H), 7.19 (m, 5 H, aryl H), 7.38 (d, J = 7.5 Hz, 1 H, aryl H), 7.50 (m, 2 H, aryl H), 8.01 (d, J = 6.3 Hz, 1 H, aryl H), 8.21 (d, J = 8.0 Hz, 1 H, aryl H), 8.40 (d, J = 8.7 Hz, 1 H, aryl H); FAB-MS 629.19 (MH^+) (628.26 calcd for $C_{34}H_{36}N_4O_6S$). Data for **107**: IR (KBr) 3430, 1680, 1205, 1149 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.46 (dd, J = 13.9, 7.3 Hz, 1 H, aryl CH), 2.53 (dd, J = 14.1, 4.9 Hz, 1 H, aryl CH), 2.71 (dd, J = 13.8, 9.0 Hz, 1 H, aryl CH), 2.94 (s, 6 H, $(CH_3)_2N$), 3.07 (dd, J = 13.8, 5.7 Hz, 1 H, aryl CH), 3.83 (dd, J = 9.5, 5.0 Hz, 1 H, NCHCO), 4.65 (dd, J = 8.5, 5.9 Hz, 1 H, NCHCO), 4.99 (d, J = 5.8 Hz, 1 H, NCHCO), 5.18, 5.25 (2 x d, J = 2.9 Hz, J = 10.3 Hz, 2 x 1 H, $CH=CH_2$), 5.94 (m, 1 H, $CH=CH_2$), 6.74 (m, 5 H, aryl H), 7.19 (m, 5 H, aryl H), 7.30 (d, J = 7.5 Hz, 1 H, aryl H), 7.47 (dd, J = 8.1, 7.6 Hz, 2 H, aryl H), 8.06 (d, J = 6.9 Hz, 1 H, aryl H), 8.17 (d, J = 8.7 Hz, 1 H, aryl H), 8.40 (d, J = 8.5 Hz, 1 H, aryl H); FAB-MS 629.31 (MH^+) (628.26 calcd for $C_{34}H_{36}N_4O_6S$).

_N_-Dansyl-D-phenylalanyl-L-phenylalaninamide (108)

The literature procedure was modified.[134] A flask containing _N_-dansyl-D-phenylalanine (200. mg, 0.502 mmol) and HONB (99.0 mg, 0.553 mmol) was dried under vacuum for 12 h. The mixture was dissolved in EtOAc (5 mL) and THF (5 mL) and the solution was cooled to 0 °C. To the stirred solution was added DCC (120. mg, 0.583 mmol), and the stirring was continued at 0 °C for 30 min then at room temperature for 3 h. The DCU thus formed was filtered off and the solvent was removed *in vacuo* to give _N_-dansyl-D-Phe-ONB. To this was added L-Phenylalaninamide (90.0 mg, 0.548 mmol) and the mixture was dried under vacuum for 6 h. Dry DMF (10 mL) was added, and the

were added and the mixture was washed successively with saturated aqueous $NaHCO_3$ (2x15 mL), H_2O (15 mL), 1 N HCl (2x15 mL), H_2O (15 mL), saturated aqueous NaCl (2x15 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed *in vacuo* to give the crude product which was purified by flash chromatography using 5% MeOH in $CHCl_3$ to give **108**: 140. mg (48%). Mp 70-72 °C; IR (KBr) 3400, 3300, 3200, 1661 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.45 (dd, *J* = 14.0, 9.5 Hz, 1 H, aryl CH), 2.52 (dd, *J* = 14.0, 5.0 Hz, 1 H, aryl CH), 2.72 (dd, *J* = 14.0, 9.1 Hz, 1 H, aryl CH), 2.86 (s, 6 H, $(CH_3)_2N$), 3.10 (dd, *J* = 14.0, 5.4 Hz, 1 H, aryl CH), 3.83 (dd, *J* = 9.4, 5.0 Hz, 1 H, NCHCO), 4.54 (dd, *J* = 9.1, 5.4 Hz, 1 H, NCHCO), 6.76 (m, 5 H, aryl H), 7.18 (m, 6 H, aryl H), 7.42 (dd, *J* = 8.6, 7.4 Hz, 1 H, aryl H), 7.45 (dd, *J* = 8.7, 7.5 Hz, 1 H, aryl H), 7.98 (dd, *J* = 7.3, 1.1 Hz, 1 H, aryl H), 8.11 (d, *J* = 8.6 Hz, 1 H, aryl H), 8.46 (dd, *J* = 7.5, 0.9 Hz, 1 H, aryl H); FAB-MS 544.29 (544.21 calcd for $C_{30}H_{32}N_4O_4S$).

Gel-filtration Column Preparation.

Sephadex G-100 (35 g, 40-120 μ particle size) was slurried in degassed buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) (700 mL) then heated at 94 °C for 5 h. This was then kept overnight at 4 °C, poured into a 2.5 cm x 100 cm column and packed with a flow of 1.0 mL/min of buffer. For storage for periods longer than 4 weeks, a solution of 0.05% NaN_3 in buffer (300 mL) was passed through the column.

Affinity Column Preparation.

As described previously, Bio-Rad Affigel-15 was allowed to settle at -20 °C in a graduated cylinder. Approximately 5 mL of settled resin was transferred to a Buchner funnel and immediately washed with isopropyl alcohol (15 mL) and 4 °C water (15 mL). The dry resin was added to a solution of D-tyrosyl-L-valyl-glycine (50.7 mg) in buffer (2-(*N*-morpholino)-ethanesulfonic acid, 0.1 M, pH 6.5) (3 mL) in a test tube, and this was

disposable syringe, the resulting column was washed with 6 M urea (25 mL), then with 3 cycles of alternating pH 4.0 buffer (0.1 M sodium acetate, 1 M NaCl, 25 mL/cycle) and pH 8.0 buffer (0.1 M sodium borate, 1 M NaCl, 25 mL/cycle). The column was then equilibrated with buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8).

Isolation of Peptidylglycine α-Amidating Monoxygenase (PAM).

The isolation of PAM followed the procedure used by Dr. S. E. Ramer.[103] The entire procedure is carried out at 4 °C. Frozen pig pituitaries (Pel-Freez, 10 g) were chopped with a razor blade and suspended in 4 °C buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.2) (20 mL). This was placed in an ice-water bath and homogenized with an IKA homogenizer for four, 40 s intervals with a 20 s delay between each homogenization. This thick solution was frozen in a -78 °C bath, thawed in a 4 °C water bath, and frozen and thawed again. After this had completely thawed, the homogenate was centrifuged for 30 min at 4 °C and 25000 g. The clear, red centrifuge supernatant was applied to the Sephadex column at a rate of 1 mL/min and eluted with buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8). 4 mL fractions were collected. The red band which elutes, presumably hemoglobin, was found to be a good marker for the PAM enzyme. Every fifth fraction was assayed, usually spanning a range of 40 fractions in which the red protein was centered.

Active fractions were combined to give a total volume of 150-200 mL and the resulting solution was applied to the affinity column at 1 mL/min. After application the column was washed with buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) (40 mL) and then eluted at 1 mL/min with a pH 4.0 buffer (3 M urea, 1 M glycylglycine, 0.1 M N-acetylglycine). 4 mL fractions were collected starting exactly 1 min after the elution buffer entered the column. These fractions were immediately transferred to dialysis tubing and dialyzed against buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) (4 °C,

the buffer being changed after 1 h, 3 h, and 7 h total time. The final dialysis was allowed to proceed overnight. Most of the enzyme activity was detected in fractions 2 and 3.

PAM Assay With Nitrosobenzene.[82]

Solutions of $CuSO_4$ (1.0 mM), KI (5.0 M), and ascorbic acid (0.40 M) were prepared in fully degassed water. These solutions were stored tightly stoppered at 4 °C for up to 1 week. A freshly prepared cocktail was used for each set of assays by adding 50 μL of the copper and KI solutions, 25 μL of the ascorbate solution, and catalase (1mg) to the buffer (50 mM sodium phosphate, then diluting to 5.0 mL with more buffer. To the enzyme solution (100 μL) to be assayed in a 1.5 mL microcentrifuge tube was added the cocktail (100 μL) and D-Tyr-Val-[1,2-^{14}C]Gly (~80 000 dpm, in 5 μL). These conditions give as a final concentration of reagents: 1 mM ascorbic acid, 25 mM KI, 5 μM $CuSO_4$, 0.1 mg/mL catalase, and 1.3 μM D-Tyr-L-Val-[1,2-^{14}C]-Gly. This mixture was shaken at 37 °C for 2 h and a glyoxylic acid solution (3.7 mmol in pH 6.8 buffer, 100 μL) and nitrosobenzene (0.30 M in 95% EtOH, 5 μL) were added. After shaking at 60 °C for 1 h, the solution was extracted three times with ether by adding ether (~0.5 mL), vortexing thoroughly, and removing the ether layer with a pipette. The ether extracts were passed down a Pasteur pipette containing Na_2SO_4 (~2.5 g) directly into a scintillation vial. After the third extraction, the pipette was rinsed with ether (0.5 mL) into the scintillation vial. The radioactivity was counted.

PHM Assay Using Cation Ion-exchange Resin.[83]

The assays using ion-exchange resin for PHM activity were as follows. Solutions of $CuSO_4$ (1.0 mM), KI (5.0 M), and ascorbic acid (0.40 M) were prepared in fully degassed water. A fresh cocktail was prepared for each set of assays by adding 50 μL of the copper and KI solutions, 25 uL of the ascorbate solution, and catalase (1 mg) to the buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) then diluted to 5.0 mL with more

buffer. To 10 μL (or 5 μL) of the enzyme solution to be assayed in a 1.5 mL microfuge tube were added 125 μL (or 62.5 μL) of the cocktail, 110 μL (or 55 μL) of buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) and D-Phe-L-Phe-[1, 2-^{14}C]-Gly (~45,000 dpm in 5 μL). These conditions give final concentrations of 1 mM ascorbic acid, 25 mM KI, 5 μM $CuSO_4$, 0.1 mg/mL catalase, and 0.72 μM D-Phe-L-Phe-[1, 2-^{14}C]-Gly. This solution was shaken at 37 °C for 2 h and quenched with 2 M NaOH (120 μL), shaken at 37 °C for 5 min, then acidified with 2 M HCl (125 μL). This was then applied to 1 mL ion-exchange AG5OWX-8 resin in a Pasteur pipette, the microfuge tube was washed with water (2 x 6 drops) and the washes were applied to the pipette column. Then the column was washed with 3 column volumes of water and the washes were counted to give the radioactivity of glyoxylate. The unconverted substrate was then washed off with 3 column volumes of 15% aqueous ammonia solution. The ion-exchange column was pushed dry by a rubber bulb and the washes were counted to give the radioactivity of unconverted substrate.

(Assays for all the inhibition studies were carried out in duplicate, the average results were used for further calculations.)

Competitive Inhibition Assay.

The PHM assay procedure using ion-exchange resin was used with the inclusion of inhibitors of various concentrations. At different times, aliquots of 25 μL were withdrawn, quenched with 2 M NaOH (10 μL), shaken at 37 °C for 5 min, then acidified with 2 M HCl (12 μL). This solution was applied to the ion-exchange column. The radioacitivties for glyoxylate and unconverted substrate were counted. By plotting the turnover ratio vs time, the IC_{50} was calculated.

Preincubation Dilution Assay.

To a 1.5 mL microfuge tube were added 125 μL of the cocktail, 13 μL of 20 μg/μL BSA solution, 50 μL enzyme, buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) and various concentrations of inhibitors to a total volume of 250 μL, which was then incubated at 37 °C. At different times, aliquots of 25 μL were withdrawn, delivered into a microfuge tube containing 250 μL of cocktail, 23.8 μL of 20 μg/μL BSA, 26.3 μL of 1.5 mM D-Phe-L-Phe-[1, 2-^{14}C]-Gly (~180,000 dpm) and buffer to a total volume of 500 μL. These conditions give final concentrations of 1 mM ascorbic acid, 25 mM KI, 5 μM $CuSO_4$, 0.1 mg/mL catalase, 1 mg/mL BSA, 0.08 mM D-Phe-L-Phe-Gly. This solution was shaken at 37 °C for 80 min and assayed as described above. The first-order inactivation constant was determined by linear regression analysis of the slopes of lines (K_{obsd}) resulting from the plot of ln(percent remaining activity) vs time. The plot of $1/K_{obsd}$ vs 1/[I] was linear, and K_I was the calculated -1/(X intercept).

Substrate Protection Assay.

To a 1.5 mL microfuge tube were added 125 μL of the cocktail, 13 μL of 20 μg/μL BSA, 50 μL enzyme, various concentrations of inhibitor and cold substrate. This was shaken at 37 °C for 1 h. Then aliquots of 25 μL were delivered to a 1.5 mL microfuge tube with 50 μL cocktail, ~45,000 dpm D-Phe-L-Phe-[1, 2-^{14}C]-Gly and buffer (50 mM sodium phosphate, 0.2M NaCl, pH 6.8) to a total volume of 125 μL. This was incubated at 37 °C for 80 min and then assayed as before. The inactivation rates of enzyme in the presence of different concentrations of cold substrate were compared.

Irreversible Test.

To a 1.5 mL microfuge tube were added 125 μL of the cocktail, 13 μL of 20 μg/μL BSA solution, 50 μL enzyme, 42 μL of buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) and 20 μL of various concentrations of inhibitors. This was incubated at 37 °C

for 1 h, then 25 μL aliquots were delivered to a 1.5 mL microfuge tube with 62.5 μL cocktail, ~45,000 dpm ^{14}C substrate and 34.5 μL buffer and the resulting solution was incubated at 37 °C for 80 min and then assayed as usual. The rest of the pre-incubated material was placed in a centricon-30, and this material was first washed with ~1 mL Milli-Q water, and centrifuged at 4, 000 xg for 15 min (All these were done at 4 °C). 500 μL buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) was added and concentrated until the volume was ~250 μL; and this was repeated twice more. Then 59.5 μL aliquots were delivered to a microfuge tube containing 62.5 μL cocktail and D-Phe-L-Phe-[1, 2-^{14}C]-Gly (~45,000 dpm) and assayed as before.

Mechanism-based Inactivation Assay.

To a 1.5 mL microfuge tube were added 125 μL of the cocktail (copper and ascorbate were not used in this cocktail.), 13 μL of 20 μg/μL BSA solution, 50 μL enzyme, 42 μL of buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) and 20 μL of various concentrations of inhibitors. This was incubated at 37 °C for 1 h, then 25 μL aliquots were delivered to a 1.5 mL microfuge tube with 62.5 μL cocktail, ~45,000 dpm ^{14}C Substrate, 34.5 μL buffer and the resulting solution was incubated at 37 °C for 80 min and then assayed as usual. The rest of the pre-incubated material was placed in a centricon-30, and this material was first washed with ~1 mL Milli-Q water, and centrifuged at 4, 000 xg for 15 min (All operations were carried out at 4 °C). 500 μL buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) was added and concentrated until the volume was again ~250 μL, and this was repeated twice more. Then 59.5 μL aliquots were delivered to a microfuge tube containing 62.5 μL cocktail and D-Phe-L-Phe-[1, 2-^{14}C]-Gly (~45,000 dpm) and assayed as before. As *N*-Ac-D-Phe-L-Phe-NCHO did not quite dissolve in 250 μL of the assay solution, 400 μL buffer and 100 μL CH_3CN were added to each tube and centrifuged. After repeating this two more times, 59.5 μL aliquot was withdrawn and assayed as above.

Determination of Protein Content.

The method of Bradford was used to determine protein concentration with a Bio-Rad protein assay dye reagent. A standard solution of bovine serum albumin (BSA) was used to prepare standard curves by diluting aliquots with water to obtain the desired concentrations. A standard curve was prepared from 5 different concentrations of protein for each of the normal and micro assays. For the homogenate, centrifuge supernatant and the combined Sephadex fractions, which contain relatively large amounts of protein (0.2-1.5 mg/mL), the standard assay was used. The solution to be analyzed (40 μL) was combined with 2.00 mL of dilute Bio-Rad protein assay reagent (1:4 concentrate:water). This was vortexed, allowed to stand 5 min, and the absorbance at 590 nm was measured against a blank of water (40 μL) and the dilute reagent (2.00 mL).

For the micro assay of solutions with a lower concentration (2.5-25 μg/μL) of protein the above procedure was modified. To 400 μL of the solution to be analyzed was added 100 μL of the concentrated reagent and 500 μL of water. The absorbance was measured after standing for 5 min.

Inhibition Studies for 78

Preliminary competitive inhibition studies were carried out with 2.00 mM, 1.00 mM, 0.500 mM, 0.100 mM and 0.0100 mM of **78**. After 60 min and 120 min, aliquots were withdrawn and assayed as described in competitive inhibition assay. Strong inhibition was observed.

Preliminary preincubation studies were performed with 2.40 mM, 1.20 mM, 0.240 mM, 0.120 mM and 0.0240 mM of **78**. After 30 min, 60 min and 120 min, aliquots were withdrawn and assayed as described in preincubation dilution assay. Potent time-dependent inhibition was observed.

Inhibition Studies for 80

Competitive inhibition studies were carried out with 500. μM, 200. μM and 50.0 μM of **80**. After 15 min, 30 min, 45 min and 60 min, aliquots were withdrawn and assayed as described in competitive inhibition assay.

Preliminary preincubation studies were performed with 1.00 mM of **80**. After 0 min, 5 min, 15 min, 30 min and 60 min, aliquots were withdrawn and assayed as described in preincubation dilution assay. There was no loss of enzyme activity vs time.

An IC_{50} of 350 μM was determined for **80**.

Inhibition Studies for 85

Competitive inhibition studies were carried out with 5.00 mM, 1.00 mM, 0.500 mM and 0.100 mM of **85**. After 60 min and 120 min, aliquots were withdrawn and assayed as described in competitive inhibition assay.

Inhibition Studies for 90A

Competitive inhibition studies were carried out with 200. μM, 100. μM, 50.0 μM and 10.0 μM of **90A**. After 15 min, 30 min, 45 min and 60 min, aliquots were withdrawn and assayed as described in competitive inhibition assay.

Preliminary preincubation studies were performed with 255 μM of **90A**. After 0 min, 5 min, 15 min and 30 min, aliquots were withdrawn and assayed as described in preincubation dilution assay. Time-dependent inhibition was not observed.

An IC_{50} of 50.0 μM was determined for **90A**.

Inhibition Studies for 90B

Competitive inhibition studies were carried out with 400. μM, 200. μM, 50.0 μM and 10.0 μM of **90B**. After 15 min, 30 min, 45 min and 60 min, aliquots were withdrawn and assayed as described in competitive inhibition assay.

An IC_{50} of 400. μM was determined for **90B**.

Inhibition Studies for 96

Competitive inhibition studies were carried out with 1.00 mM and 500. μM of **96**. After 20 min, 40 min and 90 min, aliquots were withdrawn and assayed as described in competitive inhibition assay.

Preliminary preincubation studies were performed with 2.00 mM of **96**. After 0 min, 5 min, 15 min, 30 min and 60 min, aliquots were withdrawn and assayed as described in preincubation dilution assay. There was no loss of enzyme activity vs time.

An IC_{50} of 800 μM was determined for **96**.

Inhibition Studies for 91

Competitive inhibition studies were carried out with 1.00 mm and 500. μM of **91**. After 15 min, 30 min, 45 min and 60 min, aliquots were withdrawn and assayed as described in competitive inhibition assay.

Preincubation dilution assays were performed with 750. μM, 375. μM and 125 μM of **91**. After 30 min, 60 min and 90 min, aliquots were withdrawn and assayed as described in preincubation assay. An K_I of 400. μM was determined for **91**.

Substrate protection studies were done with 3.00 mM of **91** and followed the procedure described in substrate protection assay.

Irreversible and mechanism-based inhibition studies were carried out with 1.50 mM of **91** and followed the procedures described in irreversible test and mechanism-based inactivation assay, respectively.

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