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Mechanism of Formation of Serine β-Lactones; Studies on Peptidylglycine α-Amidating Monooxygenase; Biosynthesis of Multicolosic Acid, Cladosporin, and Averufin.

by

Shawn Elliot Ramer

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Department of Chemistry

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Mechanism of Formation of Serine β-Lactones; Studies on Peptidylglycine α-Amidating Monooxygenase; Biosynthesis of Multicolosic Acid, Cladosporin, and Averufin submitted by Shawn E. Ramer in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Supervisor

Brian D Sykes

External Examiner

Date January 25, 1988.

To my mother and my father

Abstract

The ring closure of *N*-benzyloxycarbonyl-L-serine under Mitsunobu conditions (Ph_3P, dimethyl azodicarboxylate, -78°C) to give the corresponding β-lactone was shown by deuterium and oxygen-18 labelling studies to proceed by hydroxy group activation. (2*S*, 3*R*)-[2,3-$^2H_2$]-*N*-benzyloxycarbonylserine was prepared by reduction of *Z*-2-acetamido-3-methoxyacrylic acid with deuterium, followed by resolution, deprotection, and acylation. Mitsunobu cyclizations of this 3*R* deuterated *N*-acyl serine, of the [hydroxy-^{18}O] analog, and of the [carboxy-^{18}O] derivative (prepared by treatment of the β-lactone with potassium [$^{18}O_2$]acetate and with $Na^{18}OH$, respectively) showed that lactonization occurs with inversion of configuration at C-3, loss of the hydroxy oxygen, and retention of the carboxy oxygens. Similar labelling experiments demonstrated that aqueous sodium hydroxide opens the β-lactone ring by exclusive attack at the carbonyl to regenerate *N*-benzyloxycarbonylserine, whereas acidic hydrolysis proceeds primarily by attack of water at the methylene group of the β-lactone.

A new assay has been developed for peptidylglycine α-amidating monooxygenase (PAM) and the stereochemistry of the enzymatic oxidation of D-tyrosyl-L-valylglycine to D-tyrosyl-L-valinamide and glyoxylic acid was investigated using stereospecifically tritiated D-tyrosyl-L-valyl-[2-^3H]glycine. The assay is based on reaction of ^{14}C-glyoxylate, formed during the enzymatic oxidation of D-tyrosyl-L-valyl-[1,2-$^{14}C_2$]glycine, with nitrosobenzene to give ^{14}C-*N*-hydroxyformanilide. *R*- and *S*-[2-^3H]glycine were prepared by reduction of (3*R*, 5*R*, 6*S*)- and (3*S*, 5*S*, 6*R*)-4-benzyloxycarbonyl-3-bromo-5,6-diphenyl-2,3,5,6-tetrahydro-1,4-oxazine-2-one with tritium in [^3H]-water, and were analyzed for stereochemical purity with D-amino acid oxidase and by ^3H nmr of the (1*S*)-(-)-camphanamide derivatives. The [2-^3H]glycines were independently transformed to D-tyrosyl-L-valyl-[2-^3H]glycine; subsequent PAM oxidation demonstrated that the pro-*S* hydrogen of the glycine residue is removed.

Biosynthesis of multicolosic acid, cladosporin, and averufin was studied. Incorporation of $^{18}O_2$ and ^{13}C nmr analysis showed that five of the oxygens of multicolosic acid isolated from *Penicillium multicolor* are derived from oxygen gas. For cladosporin from *Cladosporium cladosporioides* and averufin from *Aspergillus parasiticus*, the stereochemistry of carbons bearing a deuterium and a hydrogen from incorporation of [2-^{13}C, 2-^{3}H]acetate was determined using ^{1}H-^{13}C chemical shift correlation nmr spectra and compared to analogous stereochemistry of oleic acid isolated from these organisms. For cladosporin the stereochemistries correspond to opposite configurations on the growing polyketide and fatty acid chains during biosynthesis, whereas for averufin the stereochemistries were the same.

Acknowledgements

I would like to thank my supervisor, Dr. John C. Vederas, for his endless enthusiasm, encouragement, and support. I am grateful to Dr. Monica Palcic for her collaboration and assistance. I would like to acknowledge the enthusiastic assistance from Sven E. Aippersbach and Dean E. Frohlich as summer research assistants and especially from my fellow graduate student, Hengmiao Cheng. I am also grateful to my collaborators on the various projects, Dr. Miyuki Kaneda, Dr. Bernard Rawlings, Dr. Paul Reese, and Susan Brobst and Dr. Craig Townsend (Johns Hopkins University). My sincere thanks goes to all of the co-workers in the research group for helpful and insightful discussions. The expert staff of the National Tritium Labeling Facility are gratefully acknowledged, especially Dr. Hiromi Morimoto for his help in operating the tritiation apparatus and Dr. Philip G. Williams for recording the ^{3}H nmr spectra. I would especially like to thank Dr. Robert M. Williams (Colorado State University) for the generous gift of starting materials and for sharing technical information for his synthesis of labelled glycines. The staff of the spectral and analytical services in the Chemistry Department of the University of Alberta are thanked for their assistance in characterizing compounds. Finally, I would like to thank Victoria Myer for her support and encouragement during all stages of this Ph. D. work.

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List of Abbreviations

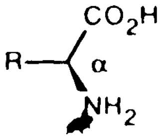
Ac	CH_3CO
Bu	butyl
BOC	*tert*-butoxycarbonyl
ci	chemical ionization
CoA	coenzyme A
DAAO	D-amino acid oxidase
DABCO	1,4-diazabicyclo[2.2.2]octane
DCC	1,3-dicyclohexylcarbodiimide
DMAD	Dimethyl azodicarboxylate
DMAP	4-dimethylaminopyridine
DMF	*N*, *N*-dimethylformamide
dpm	decompositions per minute
DPPA	diphenylphosphorylazide
ei	electron impact ionization
Enz	enzyme
Et	ethyl
FAB	fast atom bombardment
hplc	high performance liquid chromatography
ir	infrared spectroscopy
K_m	Michaelis constant
Me	methyl
ms	mass spectrometry
NBS	*N*-bromosuccinimide
NHF	*N*-hydroxyformanilide

nmr	nuclear magnetic resonance
PAM	peptidylglycine α-amidating monooxygenase
PBP	*p*-bromophenacyl
Ph	phenyl
POSFAB	positive ion fast atom bombardment
p.p.b.	parts per billion
Pr	propyl
R_f	retardation factor
TBDMS	*tert*-butyldimethylsilyl
THF	tetrahydrofuran
tlc	thin layer chromatography
TMS	tetramethylsilane
Ts	*p*-toluenesulfonyl
V_{max}	maximal velocity
Z	benzyloxycarbonyl

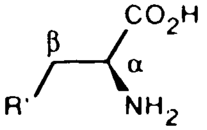
Chapter 1. Mechanism of Formation of Serine β-Lactones

INTRODUCTION

The α-amino acids are a large and diverse group of biologically important molecules. While only 20 such compounds are found as constituents of proteins, hundreds of others occur in Nature in either free form or as parts of larger molecules, and many more have been produced synthetically.[1,2,3] Many amino acids can be further classified as β-substituted alanines and most have at least one asymmetric carbon. The synthesis of these compounds has been of interest to chemists for more than a century,[1,4] and much work has gone into their preparation as either mixtures of enantiomers[1,5-8] or in stereochemically pure form.[1,4,9-25] The synthesis of isotopically labelled amino acids for studying biochemical processes has also been of interest.[26,27]



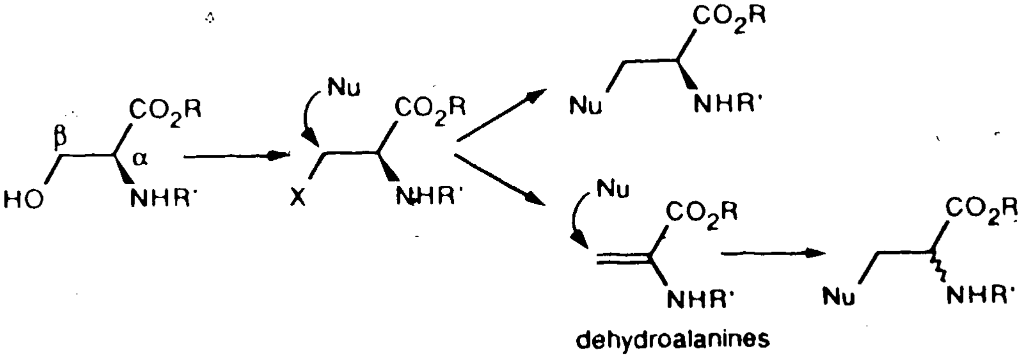
α-amino acid



β-substituted alanine
alanine R' = H
serine R' = OH

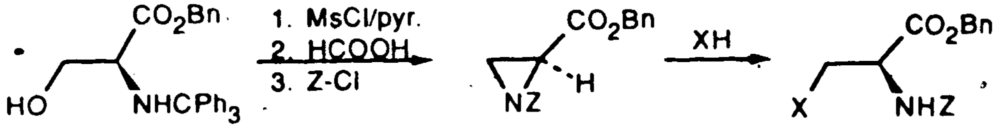
One general and versatile approach has been to use a readily accessible and optically pure amino acid as the starting material in the synthesis of other stereochemically pure amino acids.[1,28] In the synthesis of β-substituted alanines, the amino acid serine (R' = OH) is an attractive starting material since it is commercially available in enantiomerically pure form, and the hydroxyl function at the β-position provides a chemical handle for further transformations. Approaches involving activation of the hydroxyl group to a leaving group often suffer from low yields and the facile elimination to dehydroalanine derivatives (Scheme 1).[29-33] Although nucleophiles readily add to this α-β unsaturated carbonyl system, the stereochemistry of the α-position is lost.

Scheme 1.



A recent approach to the formation of stereochemically pure β-substituted alanines has been to cyclize serine to substituted aziridines which are attacked by nucleophiles at the C-3 position (Scheme 2).[34,35] The synthesis of the aziridine requires several steps since the triphenylmethyl protecting group on nitrogen required for the cyclization must be replaced by an acyl moiety (eg. Z = benzyloxycarbonyl) for successful ring openings. Simple nucleophiles such as thiols and alcohols react well, however $BF_3{\bullet}Et_2O$ catalysis is often required.[34,35] Amines appear to be unsuitable for ring opening.[34,35]

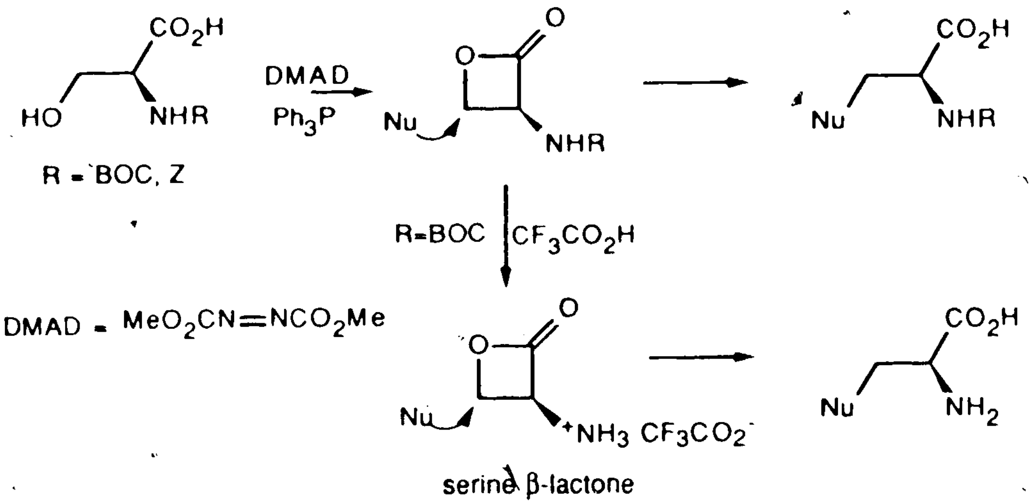
Scheme 2.



A somewhat similar, more recent approach to the synthesis of stereochemically pure β-substituted alanines from serine involves the formation of serine β-lactones.[9] Nitrogen acylated serines undergo cyclization with modified Mitsunobu conditions (Ph_3P, dimethylazodicarboxylate, THF, -78 °C) to give good yields of stereochemically pure β-lactones (Scheme 3).[9,36] These strained lactones may be attacked by a variety of heteroatom and carbon based nucleophiles to give optically pure β-substituted alanines.[9,36] The approach is particularly attractive since only a single protection and deprotection is required. Furthermore, when the nitrogen protecting group is *tert*-butyloxycarbonyl

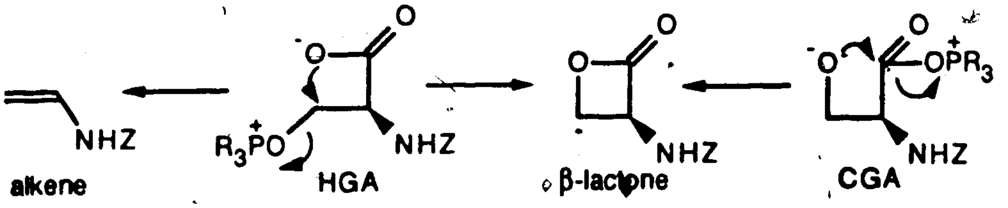
(BOC), a salt of free amino β-propiolactone can be obtained.[37] Nucleophilic attack on this compound generates directly the unprotected β-substituted alanine.[37]

Scheme 3.



The Mitsunobu[38] reaction of β-hydroxy carboxylic acids can generate either β-lactones or alkenes depending on substrate and solvent polarity.[39-42] In the reaction of serine derivatives judicious choice of solvent and reaction temperature favors the formation of β-lactones over the the undesired alkene.[9] The initially formed triphenylphosphine-azodicarboxylate adduct[43,44] in the Mitsunobu reaction can activate alcohols or carboxylic acids (Scheme 4).[39,41] In *intermolecular* esterification hydroxyl group activation (HGA) is the primary pathway[38,43] but for β-hydroxy acids this type of activation usually leads to olefinic products.[39-42] In previously studied cases, β-lactone products arose principally by carboxyl group activation (CGA).[39,41]

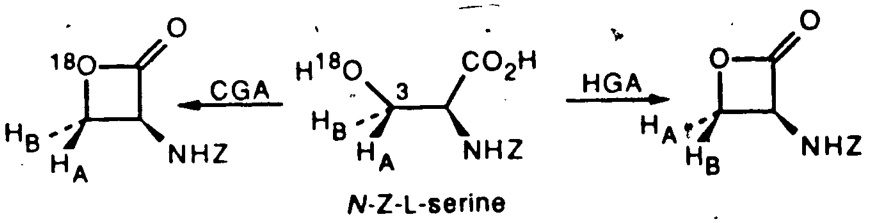
Scheme 4.



Since serine β-lactones are important intermediates in the synthesis of β-substituted alanines,[9,36,37] and the availability of these compounds stereospecifically labelled with hydrogen isotopes at the β-position would be useful for studies of enzyme mechanisms,[26,27] the stereochemistry of formation of serine β-lactones was investigated.

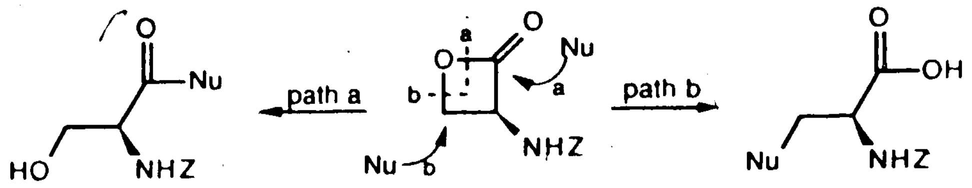
Our approach was to cyclize *N*-Z-L-serines labelled with ^{18}O and with ^{2}H at the C-3 position. It was well established that HGA proceeds with inversion of configuration at the carbon bearing the hydroxyl group, whereas CGA results in retention of configuration.[38,39,41] Labelling of the oxygens also allows discrimination between these two pathways since the activated oxygen is lost during the reaction as Ph_3PO (Scheme 5).

Scheme 5.



Another important feature of β-lactone chemistry is that nucleophiles can attack at either the carbonyl carbon with acyl-oxygen cleavage (path a in Scheme 6), or at the methylene carbon with alkyl-oxygen cleavage (path b in Scheme 6). Studies on the parent heterocycle, β-propiolactone,[45-50] as well as on serine β-lactones[9,36,37] show that the position of attack is largely dependent on the nucleophile. Heteroatom[9,45,47] and organocuprate[36,50] nucleophiles favor attack at the β-methylene position (alkyl-oxygen cleavage, path b), while alkoxide[48] and organolithium[47] reagents predominantly attack at the carbonyl carbon (acyl-oxygen cleavage, path a).[51-53]

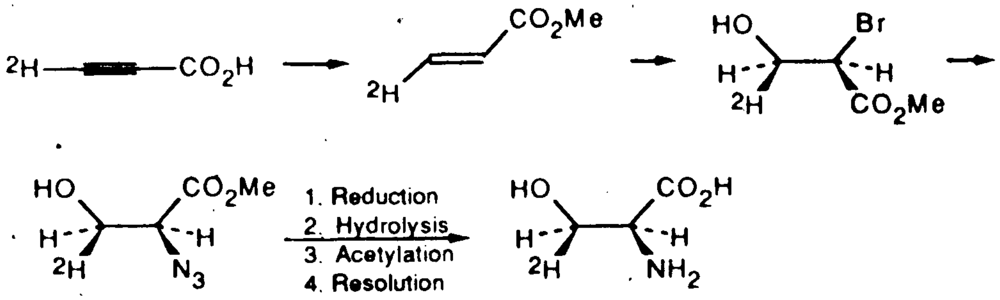
Scheme 6.



When the nucleophile is water or hydroxide (Nu = OH) isotopic labelling is necessary to distinguish between the two pathways. Early studies on the reaction of β-butyrolactone[54] and β-propiolactone[55] with water indicated that alkyl-oxygen cleavage occurs at neutral pH, whereas acyl-oxygen cleavage predominates under strongly acidic or basic conditions. Since attack of hydroxide at the β-carbon (path b, Scheme 6) inverts the stereochemistry of the C-3 position and is a method for interconverting 3*S* and 3*R*-[3-^{2}H] serines we have determined the position of attack of hydroxide by opening the serine β-lactones with ^{18}O enriched water and trapping the resulting serine derivatives as methyl esters.

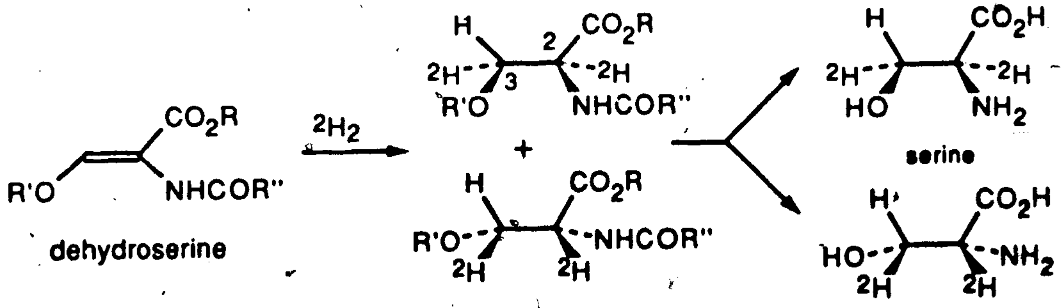
Studies on the stereochemistry of the C-3 position during cyclization required the availability of serine stereospecifically labelled with deuterium at this position. A number of syntheses of this compound have appeared in the literature.[56-62] Several involve microbial transformations which limit the quantity of serine which may be produced and are therefore better suited for radioactive isotopes. The procedure of Walsh and coworkers[57] as modified by Slieker and Benkovic[60] (Scheme 7) has been used by several groups of researchers to prepare [3-^{2}H]serines. The stereochemical purity of the resulting serines is reasonable (88% enantiomeric excess).

Scheme 7.



In the present work an alternative synthesis of stereospecifically labelled serine was developed as a continuation of studies initiated by Dr. Richard N. Moore[63] in our laboratories. The general approach involved the catalytic reduction with deuterium gas of a suitably protected dehydroserine (Scheme 8). The syn addition of deuterium across the double bond fixes the relative stereochemistries of the C-2 and C-3 positions. Resolution of the C-2 stereochemistry therefore resolves the C-3 position. The studies by Dr. Moore had indicated that reduction of the *N*-acetyl methyl ether methyl ester of dehydroserine (R=R'=R''=Me), or the corresponding *N*-acetyl methyl ether (R'=R''=Me, R=H) would be reasonable intermediates for the synthesis of labelled serine.[63]

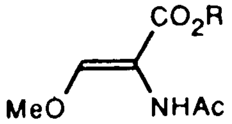
Scheme 8.



Near the completion of this work a synthesis was published[56] which is very similar to the one described in this thesis. The reduction of a dehydroserine with different protecting groups (R=Et, R'=PhCO, R''=Ph) afforded the stereospecifically labelled serine after deprotection and resolution. This synthesis suffers from some of the same problems encountered in our own work. The material presented in this chapter has been published.[64]

Synthesis of Serine Stereospecifically Labelled at C-3 with Deuterium

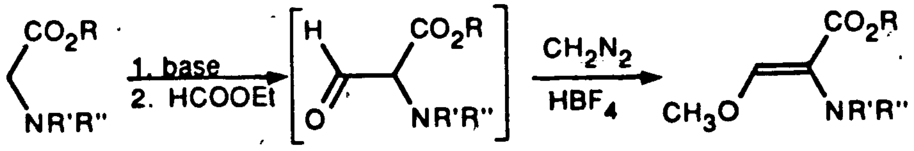
A suitable dehydroserine for catalytic reduction required nitrogen and oxygen protecting groups which were stable to the reaction conditions and which could be easily removed to produce the free amino acid without epimerisation of C-2 or C-3. Dr. Moore's work prior to these investigations indicated that the *N*-acetyl methyl ether 1 (R'=R''=Me, R=H in Scheme 8) could be reduced with deuterium to give a single diastereomer.[63] The *N*-acetyl group is required for subsequent enzymatic resolution of the C-2 position[65] and the methyl ether is cleaved by BBr_3 without epimerisation.[66]



1 R = H
2 R = Me

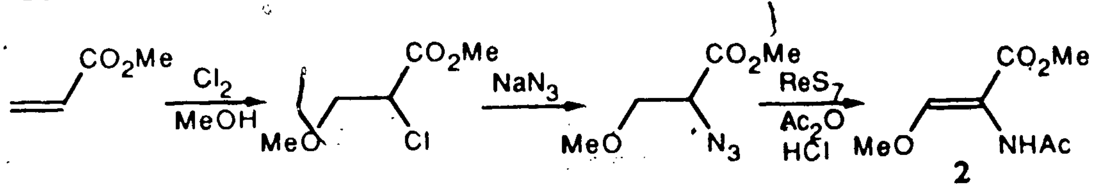
Previous syntheses of dehydroserines involved condensation of anions of protected glycines with formate esters (Scheme 9).[56,63,67] These reactions proceed in low (<20%) yield unless the nitrogen is fully substituted and lacks an acidic proton.[63] Furthermore the oxoalanine products are generally unstable and must be immediately converted to enol ethers.[56,63]

Scheme 9.



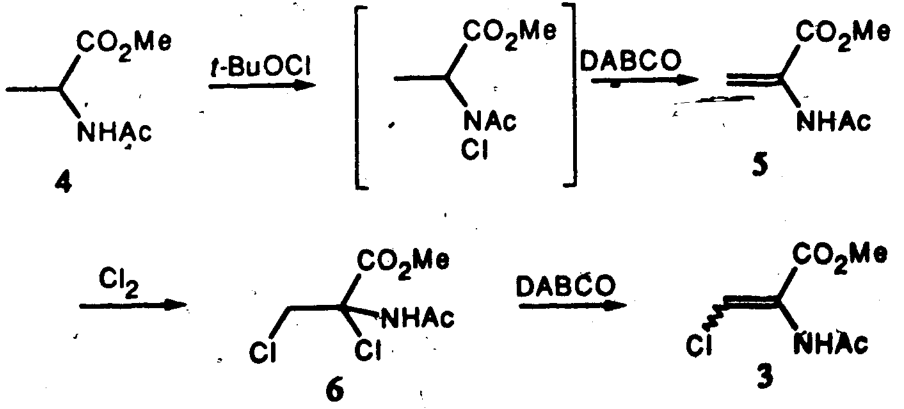
A more recent report[68] describes the synthesis of *N*-acetyl dehydroserine methyl ether methyl ester 2 by the novel reaction of α-azido esters with acetic anhydride in the presence of catalytic amounts of rhenium heptasulfide and hydrochloric acid (Scheme 10). The *N*-acetyl product **2** was obtained in 33% yield as a single diastereomer.[68] The α-azido precursor was prepared via the α-chloro derivative which was obtained as one of 5 compounds in the chlorination of methyl acrylate in methanol.[69]

Scheme 10.



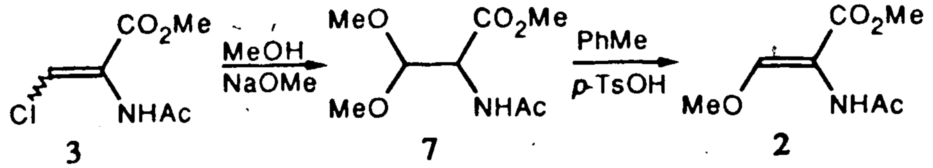
An alternative route to the dehydroserine **1** was developed in an attempt to overcome the low yields obtained in the condensation of glycine anions with formate esters. The 3-chloro ester **3** is available on large scale as a mixture of *Z* and *E* isomers (Scheme 11).[70] Treatment of *N*-acetylalanine methyl ester (**4**)[71] with *tert*-butyl hypochlorite[72] produces the *N*-chloro compound which was not isolated but directly converted to dehydroalanine **5** in 80% yield with 1,4-diazabicyclo[2.2.2]octane (DABCO).[71] Chlorination of **5** with chlorine gas gave the dichloro material **6** which was transformed to the vinyl chloride **3** in 57% yield by treatment with DABCO.[70]

Scheme 11.

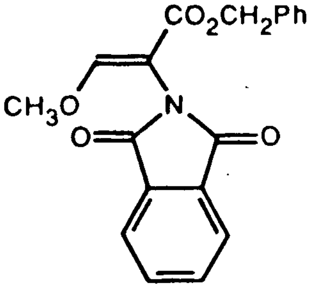


Attempts to obtain the 3-methoxy compound **2** from addition of methanol to **3** with one equivalent of sodium methoxide gave a mixture of two materials which were identified as **2** and **7** (Scheme 12). Compound **7** arises from the addition of methanol to **2** and could be obtained in high yield (96%) by refluxing **3** with one equivalent of sodium methoxide. The nmr spectrum of the dimethoxy material **7** is characteristic in the region of 4.6 to 4.8 ppm. The acetal proton is a doublet (δ 4.61, J=3.5 Hz) and the α-proton is a doublet of doublets (δ 4.88, J=3.5, 8.0 Hz) from coupling with the acetal and amide protons, respectively.

Scheme 12.



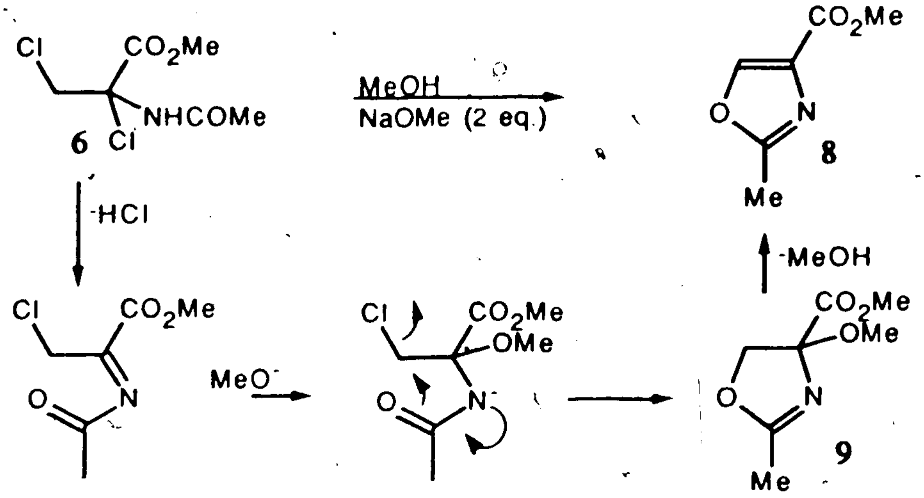
Elimination of methanol from **7** in refluxing toluene and catalytic *p*-toluenesulfonic acid produced the vinyl methoxy compound **2**. The methanol formed during the reaction was removed by attaching a Soxhlet extractor with a thimble of calcium hydride or, for large scale reactions, by azeotropic distillation. In this way the equilibrium can be forced to favor **2**. Nmr analysis of **2** showed only a single vinyl proton peak at 7.23 ppm, indicating only the *Z* isomer had formed.[68] In a single experiment the *E* diastereomer was obtained in < 5% yield from treatment of the dichloro compound **6** at room temperature with one equivalent of sodium methoxide. This material showed similar spectral characteristics to **2** except the vinyl hydrogen had a ^{1}H nmr chemical shift of 6.26 ppm, 0.97 ppm upfield from the *Z* isomer. This upfield shift for the vinyl proton in the *E* isomer is consistent with what had been observed for these types of compounds, including the *N*-phthalimido derivative for which the geometry of the double bond had been confirmed by x-ray crystallography.[63]



Interestingly, treatment of the dichloro compound **6** with refluxing methanol and 2 equivalents of sodium methoxide gave a mixture of compounds from which 13% of the oxazole **8**[73] was isolated and characterized. A possible mechanism for the formation of **8** is illustrated in Scheme 13. The acidic amide proton of **6** is removed under the basic reaction conditions and chloride is eliminated to generate the imine. If methanol adds to the electrophilic imine followed by cyclization as shown (Scheme 13), the oxazoline **9** will be formed. This would be expected to eliminate methanol to form the oxazole **8**. In a single experiment when the reaction was stopped early, a small amount of material was isolated which had a ^{1}H nmr spectrum ($CDCl_3$, 80 MHz) consistent with the intermediate oxazoline **9**: δ 2.09 (s, 3H, $COCH_3$), 3.39 (s, 3H, OCH_3), 3.81 (s, 3H, CO_2CH_3), 4.23 (d, 10, 1H, CHH), 4.48 (d, 10, 1H, CHH). Unfortunately, due to the limited quantity isolated and chemical instability, further characterization could not be completed. When the reaction was repeated only the oxazole **8** was isolated.

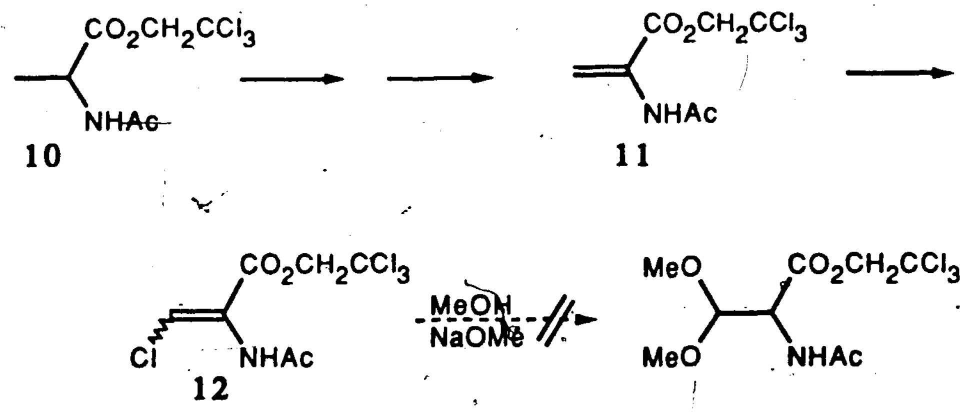
Attempted hydrolysis of the unsaturated methyl ester **2** to the free acid **1** with NaOH in alcohol and water gave a complex mixture of unidentified products, presumably from the facile Michael addition of hydroxide to the α,β-unsaturated double bond of **2**. To avoid these problems a protecting group for the carboxylic acid which could be cleaved under non-hydrolytic conditions was investigated.[74] The 2,2,2-trichloroethyl ester was chosen because it is easily formed[75,76] and is removed by treatment with zinc in acetic acid under mild conditions[76,77] in which the unsaturated product should be stable.

Scheme 13.



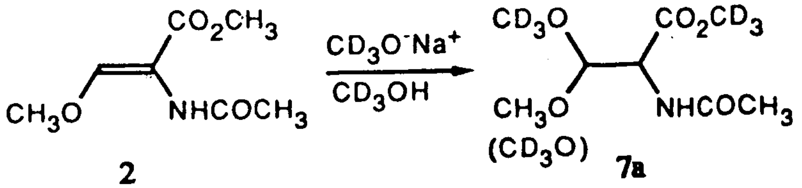
N-Acetyl alanine was converted to the trichloroethyl ester **10** in high yield.[75] This was treated with *tert*-butyl hypochlorite[72] followed by DABCO (as for methyl ester **5**[71]) to give the protected dehydroalanine **11** in 84% yield (Scheme 14). Treatment with chlorine gas and subsequent elimination[70] formed the chloro compound **12** in moderate (52%) yield as a mixture of isomers. All attempts to form the dimethoxy material as with the methyl ester gave a mixture of products arising from transesterification to the methyl ester under the reaction conditions.

Scheme 14.



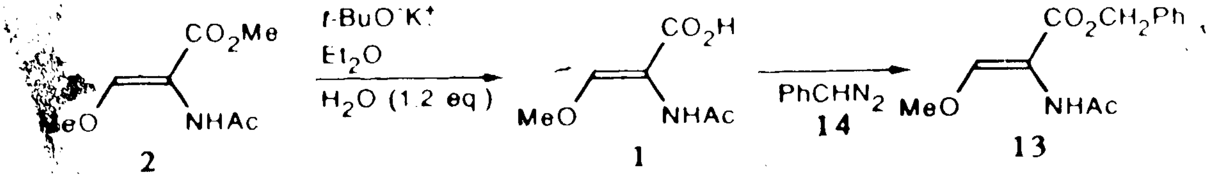
To examine this further, compound **2** was treated with CD_3O^-/CD_3OD; after aqueous workup the dimethoxy compound **7a** was isolated (Scheme 15). The ^1H nmr spectrum ($CDCl_3$) of this substance had signals at δ 2.00 (s, 3H, COC*H*$_3$), 3.43 (s, 1.5H, C*H*$_3$O), 3.73 (s, 0.1H, CO$_2$C*H*$_3$), 4.55 (s, 1H, C*H*), and 6.20 (br s, 1H, N*H*). The very low integration for the methyl ester resonance demonstrates the facile exchange of that group with the deuterated solvent, in accord with the results observed with the trichloroethyl ester. The 50% lower than expected integration of the methyl ether peaks is indicative of the reversible nature of the formation/elimination of the dimethyl acetal under the reaction conditions.

Scheme 15.



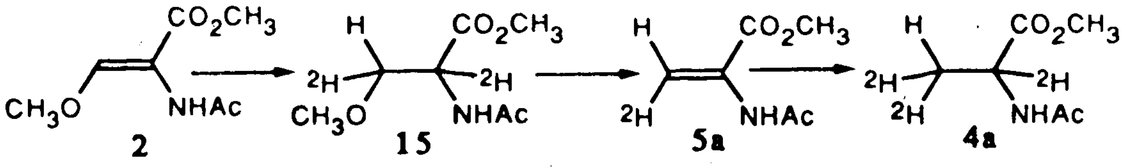
Upon further investigation it was found that the methyl ester **2** could be hydrolyzed using the "anhydrous" hydroxide procedure of Gassman and coworkers[78] to give the analytically pure free acid **1** in 96% yield (Scheme 16). This procedure generates equimolar amounts of hydroxide in anhydrous ether from potassium *tert*-butoxide and water.[78] Presumably, under these conditions the ester is hydrolyzed before Michael addition can take place. The *Z* stereochemistry of the double bond was further confirmed by conversion of **1** to the known[79] benzyl ester **13** with phenyldiazomethane (**14**) and comparison of the ^1H nmr spectra.

Scheme 16.



With both the methyl ester **2** and the free acid **1** available, studies were initiated to find suitable reaction conditions for the reduction. This proved to be more problematic than anticipated.[63] Initial attempts to reduce the methyl ester **2** produced significant amounts of the alanine derivative **4a** (Scheme 17), even after addition of varying amounts of triethylamine (Table 1).[63] This product presumably arises from the elimination of methanol from the serine derivative **15** and subsequent reduction of dehydroalanine ester **5a** to alanine ester **4a**. Also of concern was the appearance of a proton at the α-position. Table 1 outlines the results obtained from reduction of the methyl ester **2** under varying conditions. The reactions were performed as follows. The catalyst (5% Rh(C), 30-40% by weight of the starting ester 2) was predeuterated for 3 h in solvent (diethyl ether unless otherwise indicated) under 1 atm of D_2 gas. The ester **2** (0.5 mmol), dissolved in ether (unless otherwise indicated), was added to the catalyst solution, and the mixture was stirred for 3 h under 1 atm of D_2. The catalyst was removed by filtration and the solvent was removed *in vacuo*. In all cases the recovery of material was 90-99%.

Scheme 17.



The formation of alanine ester **4a** was determined from the ^1H nmr spectrum by comparison of the integration of the methyl ether resonance at 3.40 ppm of **15** to the combined integration of the signals at 2.0-2.1 ppm from the *N*-acetyl methyl resonances of **4a** and **15**. The residual α-proton of **4** and **15** was determined by comparing the integration of the signals in the 4.5-4.8 ppm region to the combined *N*-acetyl methyl resonances. The serine β-proton was calculated from comparison of the integration of the combined methyl ester resonances and serine β-proton resonances to the combined *N*-acetyl methyl resonances and the methyl ether signal. In cases where peaks from unidentified contaminating materials were present the α and β protons were not determined.

Table 1. Yields of 4a from Catalytic Reduction of Ester 2.

Et_3N(eq.)	**4a** (%)	β-^{1}H	α-^{1}H	Notes
0.61	21	0.7	0.5	
0.50	19	0.7	0.6	a
0.25	19	0.3	0.4	b
0.35	13	1.0	0.3	b,c
0.25	25	0.8	0.5	c
0.37	27	0.7	0.2	b
0.39	35	0.2	0.2	c,d
0.35	27	0.7	0.1	c,d,e
0.37	39	0.4	0.3	c,d
0.47	54	nd	0.3	c,d
0.00	71	0.7	0.4	c
0.00	6	nd	nd	f,g

a. When **15** was purified by prep. tlc ^{1}H nmr spectrum indicated α-H 0.4H, β-H δ 3.61 (0.6H), 3.85 (0.4H).

b. When **15** was purified by prep. tlc ^{1}H nmr spectrum the relative integrations for the α and β protons were as reported in the table.

c. Starting material **2** was pre-exchanged with D_2O.

d. Ether was washed with D_2O.

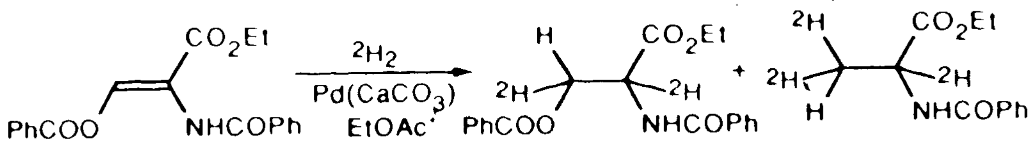
e. Ether was dried ($MgSO_4$) after washing with D_2O.

f. 71% of starting material **2** remained, 23% of serine derivative **15** was formed.

g. Anhydrous methanol was the solvent, Pd(C) was used as catalyst.

From the results in Table 1 several observations can be made. The addition of triethylamine to the reaction appears to inhibit, but not abolish, the formation of the alanine derivative. The exact reasons for this remain unclear. Amines are known catalyst poisons and Aberhart and Russell[56] also observed that the use of a less active catalyst, Pd($CaCO_3$), instead of Pd/C reduced the amount of alanine ester formed in the reduction of a dehydroserine derivative (Scheme 18).[56]

Scheme 18.



Alternatively, the triethylamine may serve to remove or hydrogen bond the amide proton of 2 and therefore decreases the removal of the α-proton necessary to eliminate methanol.[63] The presence of water from saturation of the ether with D_2O also increased the amount of alanine ester **4a** formed. The low integration for the β-proton, and the persistent presence of an α-proton even though the starting material and solvent were extensively exchanged with D_2O, has interesting implications and may indicate some type of transfer of the proton from the β-position to the α-position during the reduction. This was not investigated further and we turned our attention to the reduction of the free acid **1**.

The reduction of the free acid **1** proved as troublesome. The general experimental conditions were as described for the methyl ester **2** above except that **1** (0.2 mmol) was added in ethyl acetate instead of ether (unless otherwise indicated). The yield of *N*-acetylalanine **17** (Scheme 19) was determined as described above for the methyl esters and in cases where starting material **1** was present, the amount remaining was determined from integration of the resonance for the vinyl hydrogen at 7.4 ppm.

Scheme 19.

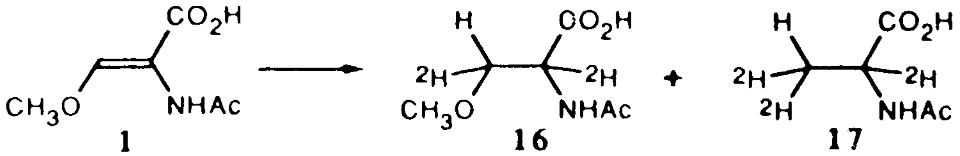


Table 2. Yields of Products from the Catalytic Reduction of Free Acid 1.

Et_3N(eq.)	16 %	17 %	1 %	notes
—	27	27	44	
—	19	81	0	a
—	25	40	35	
—	32	20	48	b
—	5	95	0	c
0.08	32	58	10	
1.08	35	52	13	d
0.90	35	45	20	
1.50	40	60	0	e
1.50	0	0	100	f
1.50	20	44	36	g,h

a. D_2O was the solvent.

b. Toluene (1 eq.) was added.

c. Solvent was washed with D_2O.

d. After AG50x8 ion exchange column 16 had the following ^1H nmr spectrum (400 MHz, D_2O). δ 2.01 (s, 3H, $COCH_3$), 3.38 (s, 3H, OCH_3), 3.73 (br s, 0.3H, CHD), 3.84 (br s, 0.6H, CHD).

e. Partial ^1H nmr spectrum of 16 (D_2O): 3.82 (0.6H), 3.66 (0.4H).

f. Pyridine was used instead of Et_3N.

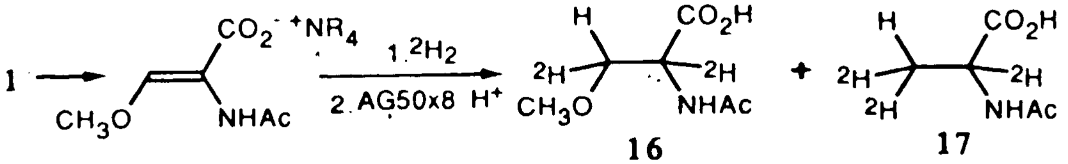
g. 2,4,6-collidine was used instead of Et_3N.

h. Partial ^1H nmr spectrum of 16 (D_2O): 3.83 (0.8H), 3.66 (0.2H).

Several observations can be made concerning the reduction of the free acid 1 (Table 2). The use of D_2O as the solvent or the addition of water by saturation of the reduction solvent (ether/EtOAc) with D_2O increases the rate of the reaction but favors the formation of the *N*-acetylalanine. Interestingly, in contrast to the results obtained in the reduction of the methyl ester **2**, the addition of varying amounts (0.08-1.5 eq.) of triethylamine, or of pyridine or collidine, appears to have little effect on the ratio of alanine to serine derivatives.

In an attempt to decrease the amount of *N*-acetylalanine formed during the reduction of the free acid **1**, tetraalkylammonium salts were prepared and reduced as described above for the free acid, except that the salts were dissolved in methanol and added to the predeuterated catalyst in ethyl acetate (Scheme 20). After removal of the catalyst by filtration and the solvent *in vacuo* the free acid **16** was obtained by treating the residual salt with AG50x8 H^+ ion exchange resin. Products were determined from the ^1H nmr resonances as before.

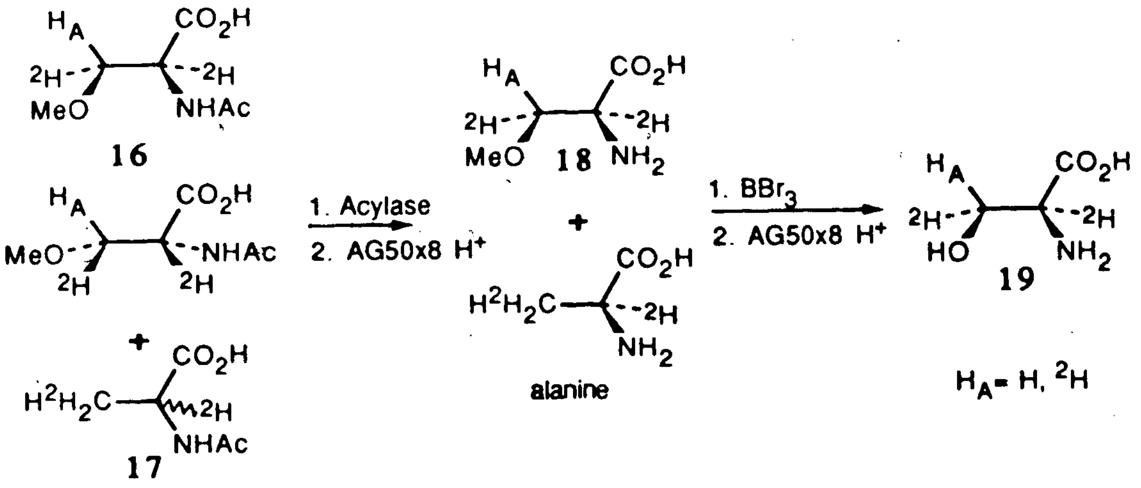
Scheme 20.



16 **17**

A few generalizations can be made from the data in Table 3. Changing the alkyl group from methyl to ethyl or to *n*-butyl has little, if any, influence on the amount of *N*-acetylalanine **17** formed. Addition of 10 mol% ethanedithiol or diaminoethane also has no effect. Increasing the temperature did appear to slightly decrease the amount of **17** produced, but this experiment was not repeated. The most *N*-acetylalanine **17** was formed in the preparative (12 mmol) scale reaction, but the cause of this is not known since this reaction was an exact scale-up of the 3 mmol reaction. The amount of alanine derivative obtained in these experiments is the same as Aberhart and Russell[56] observed in their system (Scheme 18). With the publication of their analogous synthesis[56] and lack of significant improvement in reducing the amount of *N*-acetylalanine formed despite considerable time and effort, the material from the 12 mmol reduction (a mixture of *N*-acetylalanine **17** and serine **16**) was isolated and the free acids were treated with hog kidney acylase I as previously described (Scheme 21).[65] This commercially available enzyme provides a convenient means for the selective hydrolysis of 2*S* *N*-acetyl amino acids in the presence of the 2*R* isomers. The free 2*S* amino compounds are easily separated from the unreacted 2*R* amides with AG50x8 H+ ion exchange resin.

Scheme 21.



This mixture of L-alanine and (2*S*, 3*R*)-[2,3-$^2H_2$]-*O*-methylserine **18** was treated for three days with BBr_3 in CH_2Cl_2[66] to give L-alanine and (2*S*, 3*R*)-[2,3-$^2H_2$]serine **19**. These could be separated by careful ion-exchange chromatography (AG50x8 H+) with a slow gradient elution from water to 1N HCl. The serine **19** was isolated as the hydrochloride salt in 7% overall yield from **1**.

Table 3. Yields of 17 from the Reduction of Tetraalkylammonium Salts of 1.

R	17 %	notes
Me	34	
Me	29	
Me	14	a,b
Me	29	c
Et	35	
Et	30	
Et	33	d,e
Bu	35	
Bu	39	d
Bu	37	d,f,g
Bu	32	g
Bu	32	d,g,h
Bu	20	d,f,g,i
Bu	49	d,j

a. Reaction incomplete, 58% 1 remained.
b. 10mol% $HSCH_2CH_2SH$ added.
c. 10mol% $H_2NCH_2CH_2NH_2$ added.
d. Salt of 1 exchanged with D_2O prior to hydrogenation, CH_3OD used instead of MeOH.
e. Partial ^1H nmr spectrum ($CDCl_3$) of 16, δ 3.86 (0.8H), 3.38 (3H).
f. Partial ^1H nmr spectrum ($CDCl_3$) of 16, δ 3.92 (0.5H), 3.38 (3H).
g. Reaction was on 3 mmol scale.
h. Reaction was at -5 °C, 1 remained.
i. Reaction was at 50 °C.
j. Reaction was on 12 mmol scale. Partial ^1H nmr spectrum ($CDCl_3$) of 16, δ 3.85 (0.5H), 3.37 (3H).

The ^1H nmr spectrum of serine in strongly basic medium[80] (10%NaOD/D_2O) has two sets of signals for the pro-*S* and pro-*R* protons at the β-position. For unlabelled serine these are doublets of doublets from coupling to each other and to the α-proton. In the [2,3-

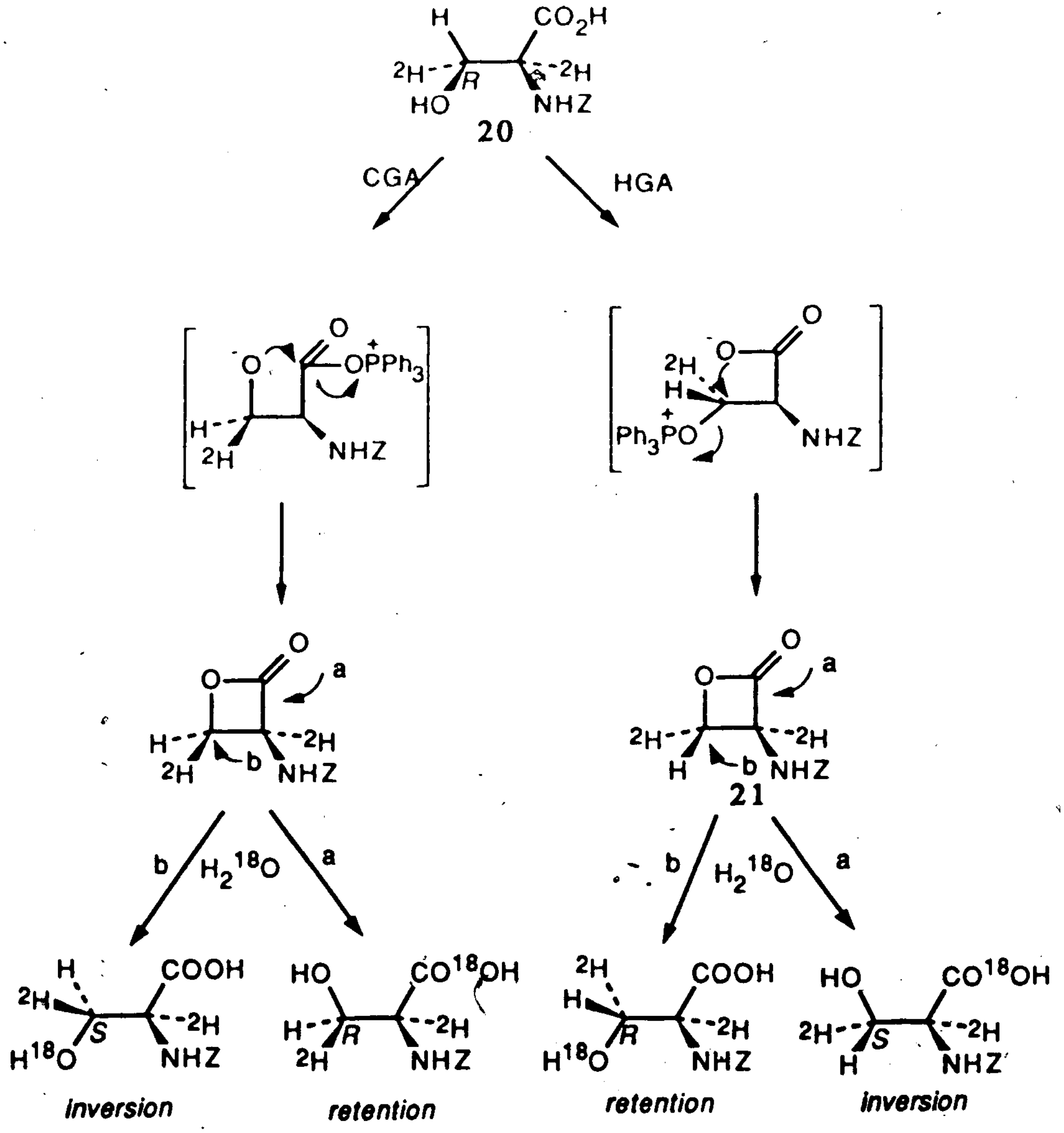
$^2H_2$]serine the corresponding peaks are singlets.[56,58] For the labelled serine **19** prepared as described above, ^{1}H nmr clearly showed only a single peak at 3.58 ppm indicating a 3*R* stereochemistry.[56,58] The ^{2}H nmr spectrum (10%NaOH/H_2O) showed broad but separated signals at 3.31 ppm (1D, C*D*N), 3.59 ppm (0.6D, C*D*H), and 3.72 ppm (1D, C*DD*). These signals arise from the mixture of dideuterio (40%) and trideuterio (60%) serines synthesized. These results correspond to those obtained from the ^{1}H nmr spectrum and show that **19** was a mixture of (2*S*, 3*R*)-[2,3-$^2H_2$]serine and (2*S*)-[2,3,3-$^2H_3$]serine. This material, however, was suitable for investigations of the stereochemistry of β-lactone formation.

The mechanism for the formation of the trideutero serine during the reduction of **1** must involve either an exchange of the vinyl proton of **1**, presumably mediated by the rhodium catalyst, or a dehydrogenation of the serine derivative **16** after reduction to regenerate **1** with the vinyl proton replaced by deuterium. A recent investigation involving partial reduction of alkenes with $^3H_2$ demonstrates isotope exchange of the vinyl hydrogens during reduction with Wilkinson's catalyst ($[Ph_3P]_3RhCl$).[81] For example, partial reduction of styrene ($PhCH=CH_2$) and isolation of the unreduced starting material showed that a significant amount of PhCH=CHT was produced but no $PhCT=CH_2$ or PhCT=CTH as might be expected from a hydrogenation-dehydrogenation mechanism.[81] Whether this is the case in the reduction of **1** remains speculative since unreduced **1** was not isolated from the reactions.

THE MECHANISM OF SERINE β-LACTONE FORMATION

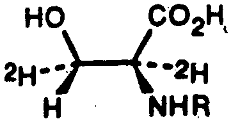
Scheme 22 illustrates the four possible outcomes of formation of β-lactones from stereospecifically labelled *N*-benzyloxycarbonylserine (*N*-Z-serine) followed by opening with ^{18}O labelled hydroxide. To distinguish between the two pathways (CGA and HGA) requires two experiments; one to determine the final stereochemistry of the serine and another to ascertain the position of attack of hydroxide as being either at the carbonyl carbon (a in Scheme 22) or at the methylene carbon (b in Scheme 22).[54,55] Opening of the β-lactone with ^{18}O hydroxide and determining the position of label in the resulting protected serine allows discrimination between these two sites of attack. Regeneration of the unprotected serine after cyclization and opening of *N*-Z-(2*S*, 3*R*)-[2,3-$^2H_2$]serine 20 allows the stereochemistry of C-3 to be determined by ^1H and ^2H nmr analysis.

Scheme 22.



The mixture of (2*S*)-[2,3,3-$^2H_3$]serine and (2*S*, 3*R*)-[2,3-$^2H_2$]serine (**19**) was converted[82] in 47% yield to the *N*-Z derivative **20** necessary for formation of the β-lactone. Cyclization using the modified Mitsunobu conditions (PPh_3, $MeO_2CN{=}NCO_2Me$, THF, -78 °C) produced a mixture of the trideuterated and dideuterated serine β-lactones **21** in 32% yield.[9] The low yield of this reaction is probably due to temperature control problems encountered when working on small scale. While the phosphorous betaine is presumably formed at -78 °C,[43] cyclization probably occurs during warming to room temperature.[9,36] If the rate of warming is too fast, as is the case in small volume reactions, the betaine remains at room temperature where decarboxylative elimination is favored over cyclization.[39-42]

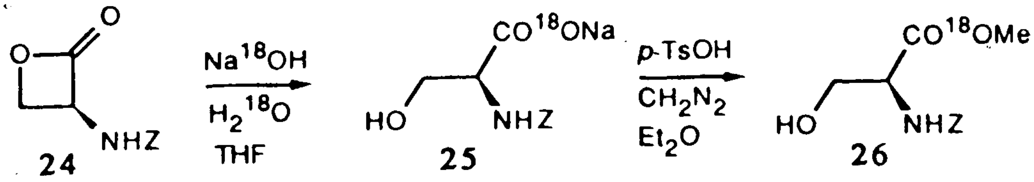
Opening of the deuterium labelled β-lactone **21** with NaOH in water/THF followed by acidic workup gave the *N*-Z-serine **22** in 95% yield. Hydrogenolysis of the Z group under standard conditions (Pd(C), H_2, 1N HCl) gave a quantitative yield of the serine hydrocholride salt **23**. Nmr analysis of this compound clearly showed *inversion* of the C-3 position had taken place. The ^1H nmr spectrum in strong base[80] (10% NaOD/D_2O) showed a single peak at δ 3.74 ppm, corresponding to a 3*S* configuration.[56,58] The ^2H nmr spectrum had signals at δ 3.40 (1D), 3.79 (1D), 3.93 (0.6D). While the chemical shifts are downfield compared to previous spectra (δ 3.31, 3.59, 3.72) the relative positions of the peak and corresponding integrations clearly indicated that inversion had taken place. The change in chemical shifts of the signals may be due to a variation in the concentration of the sodium hydroxide solutions which were freshly prepared for each nmr sample.[80]



22 R = Z
23 R = H

This single experiment does not allow determination of the activation pathway (Scheme 22) without knowledge of the position of attack of hydroxide. Treatment of the unlabelled β-lactone **24** with $Na^{18}OH$ in $H_2{}^{18}O$/THF gave the sodium salt of the *N*-Z-serine **25** (Scheme 23). Since aqueous acidic workup of this material has the potential for isotope exchange at the carboxyl oxygens,[83] the sodium salt was treated directly with *p*-toluenesulfonic acid in ether followed by diazomethane to generate the methyl ester **26**.

Scheme 23.



The *N*-Z-serine methyl ester **26** was analyzed for ^{18}O content by ^{13}C nmr. Signals from carbons bearing ^{18}O isotopes are shifted upfield relative to carbons with ^{16}O. This effect is well documented.[83-86] The magnitude of the isotope shift is dependent on the carbon chemical environment.[85] For example, carbonyl carbons usually show a larger isotope shift than carbons bearing a hydroxyl group.[85,86] The ^{13}C nmr spectrum of the methyl ester showed ^{18}O-induced α-isotope shifts of the expected magnitude at the carbonyl carbon ($\Delta\delta$ 15 p.p.b. and 38 p.p.b.) and at the methyl carbon ($\Delta\delta$ 26 p.p.b.). No shift was detected at the hydroxyl carbon. These results indicate that the attack of hydroxide occurs at the carbonyl carbon (a in Scheme 22) with acyl-oxygen cleavage and, together with the observation of inversion of stereochemistry at C-3, suggest that cyclization occurs via hydroxyl group activation (HGA).[54,55]

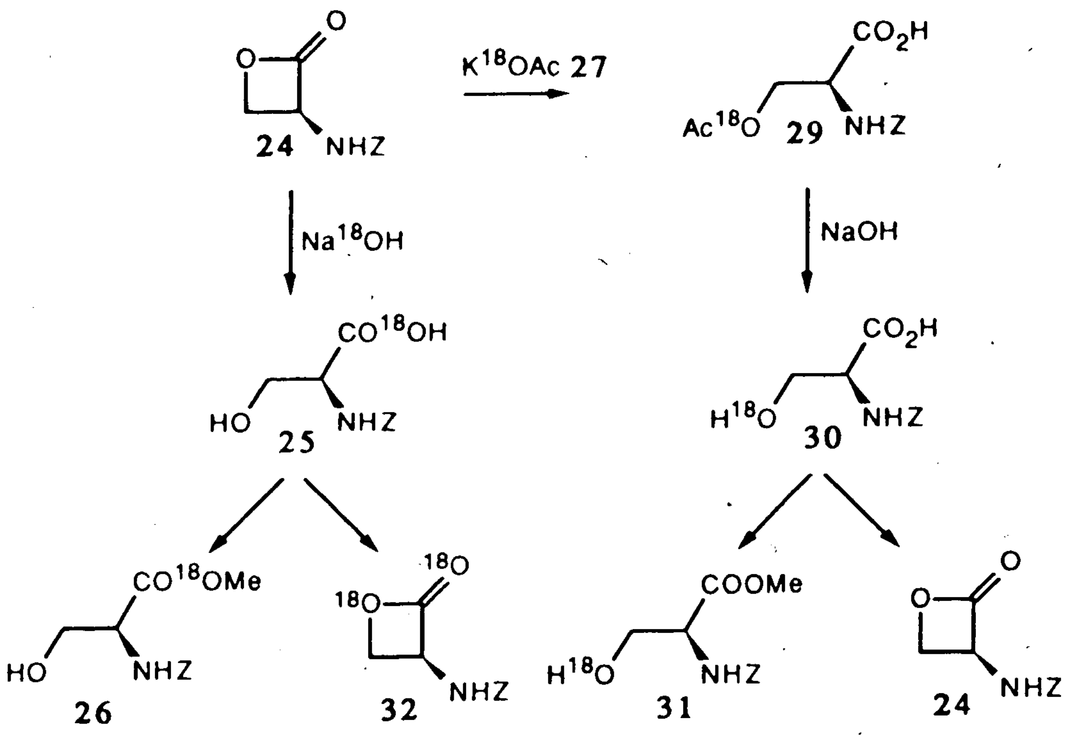
Since the isotope shift at the hydroxyl carbon might be too small to be detected and there was the possibility, though unlikely,[83] that the ^{18}O in the carboxyl oxygens was a result of exchange with the $H_2{}^{18}O$ of the sodium salt of the *N*-Z-serine **25**, further ^{18}O

labelling experiments were conducted to confirm these observations. These experiments are outlined in Scheme 24.

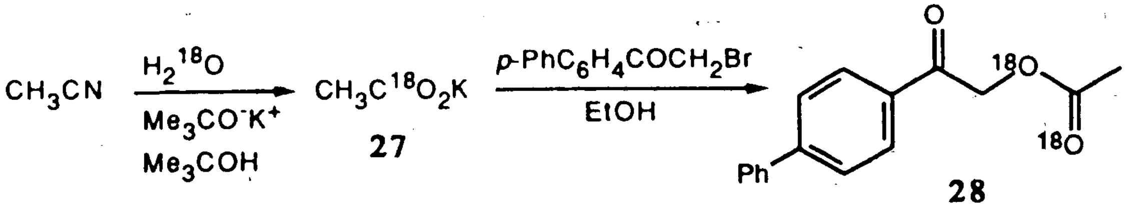
Potassium [$^{18}O_2$]acetate (**27**) was prepared from hydrolysis of acetonitrile with $H_2{}^{18}O$ and potassium *tert*-butoxide.[87] To analyze for ^{18}O content this was converted to the *p*-phenylphenacyl ester **28**[83,88] in 74% yield (Scheme 25). The chemical ionization mass spectrum (ci-ms) indicated an isotopic content (±0.5%) of 21.6% $^{18}O_1$ and 76.9% $^{18}O_2$. The β-lactone **24** was opened with the labelled potassium acetate **27** in DMF with a small amount of water.[9] This gave 99% yield of the *O*-[$^{18}O_2$]acetyl-*N*-Z-serine (**29**) with an isotopic content (±0.5%) of 21.4% $^{18}O_1$ and 77.1% $^{18}O_2$. Hydrolysis of the ester with NaOH in water/THF gave 82% of the [hydroxy-^{18}O]-*N*-Z-serine (**30**) with an isotopic content of 76.2% $^{18}O_1$. This isotopic ratio corresponds to an approximately 10% loss of ^{18}O in the hydrolysis of **29** and may be due to an elimination of **29** followed by the addition of water or to the loss of ^{18}O in the carboxyl group which may arise during the formation of **29** from opening of the β-lactone **24** at the carbonyl carbon to give a mixed anhydride which undergoes an intramolecular acetate transfer. The hydroxy labelled serine **30** was converted to the methyl ester **31** with ethereal diazomethane.

The ^{18}O-induced α-isotope shift in the ^{13}C nmr spectrum of **31** could not be resolved. The electron impact mass spectrum (Ei-ms), however, gave peaks for both the molecular ion and fission of a $COOCH_3$ fragment (Scheme 26). For the hydroxy labelled serine **31** this results in retention of ^{18}O label ($C_{10}H_{12}NO_2{}^{18}O$ = 196.0684) but for the carboxyl labelled **26** (derived from the opening of the β-lactone **24** with ^{18}O hydroxide) the result is loss of ^{18}O label ($C_{10}H_{12}NO_3$ = 194.0810). This confirms that the label was in the carboxyl oxygens of **26**.

Scheme 24.

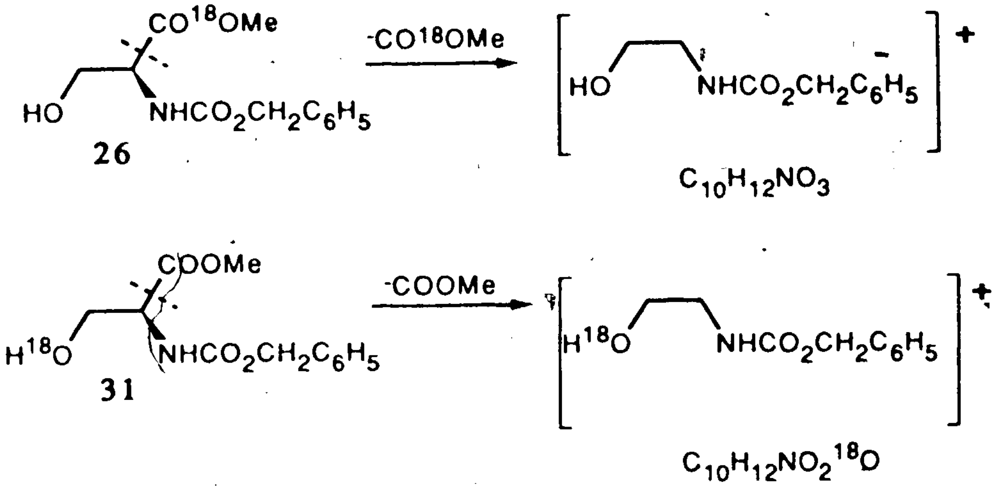


Scheme 25.

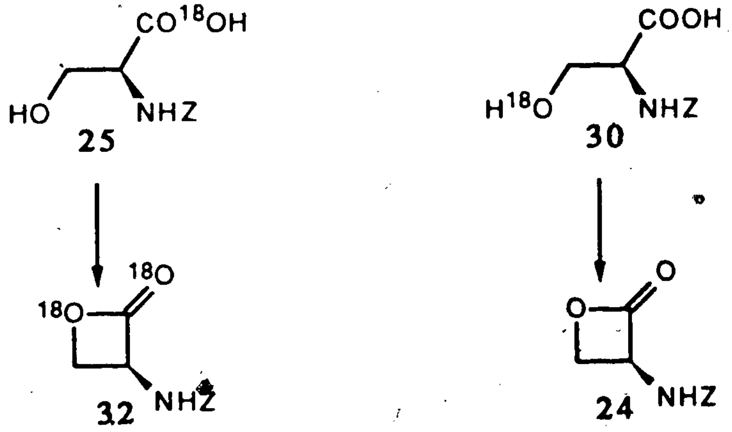


As a final confirmation of these observations, *N*-Z-serines bearing ^{18}O in the hydroxyl (**30**) and in the carboxyl oxygens (**25**) were independently cyclized to the β-lactones (Scheme 27). The carboxyl-^{18}O serine **25** was prepared by opening of the β-lactone **24** with ^{18}O hydroxide and acidic workup to give the free acid of **25**. The carboxyl labelled serine (**25**, isotopic content 71±1% ^{18}O) was cyclized to give the β-lactone **32** with an isotopic content of 73±1% ^{18}O, indicating complete *retention* of label. Conversely, when serine having the hydroxy labelled with ^{18}O (**30**, isotopic content 76%) was cyclized, the β-lactone **24** showed no evidence of ^{18}O in the ci-ms.

Scheme 26.

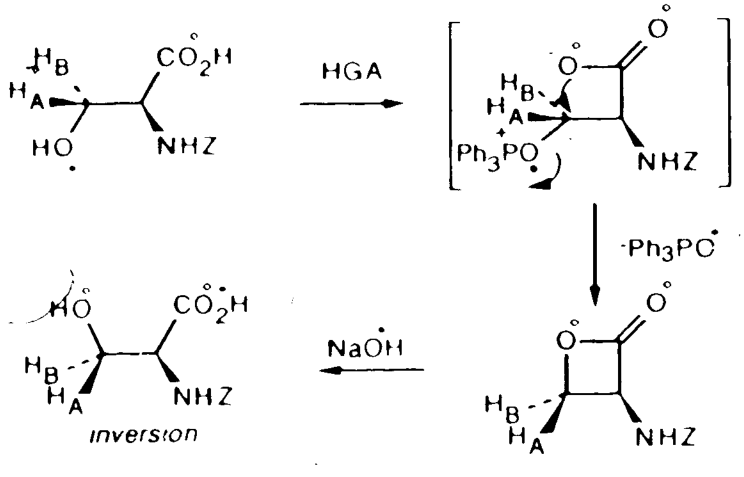


Scheme 27.



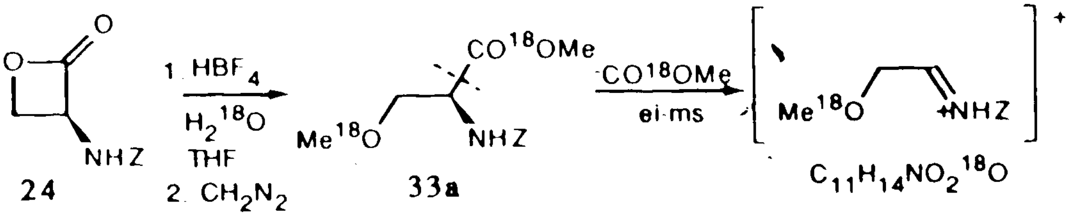
These experiments show that the formation of serine β-lactones occurs by initial activation of the hydroxyl group by the triphenylphosphine azodicarboxylate adduct (Scheme 28).[38-44] Cyclization proceeds with inversion of stereochemistry at the C-3 position.[38,39,41] Subsequent opening of the β-lactone under aqueous basic conditions involves attack of hydroxide at the carbonyl carbon with acyl oxygen cleavage and retention of configuration at the C-3 position.[54,55] The overall inversion at C-3 provides a simple route for the interconversion of 3*S* and 3*R* deuterated serines.

Scheme 28.



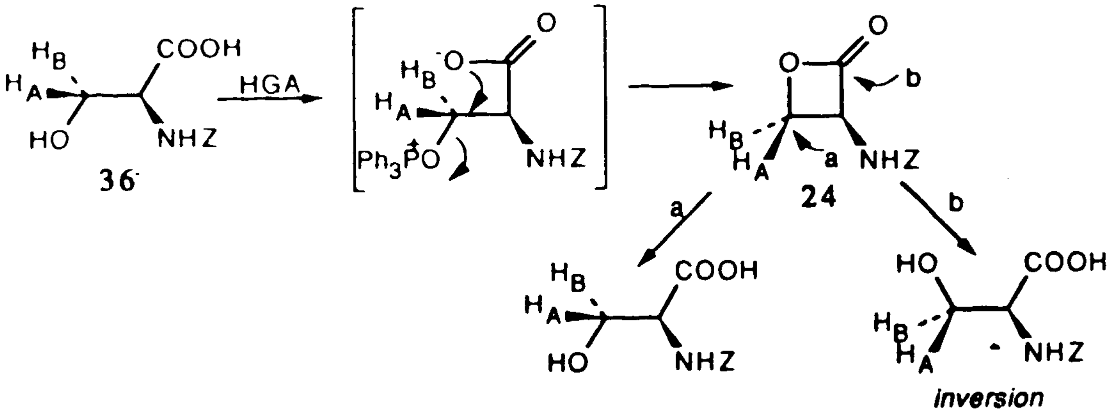
The opening of β-lactone **24** under acidic conditions was also investigated. Initial attempts to treat the β-lactone with dilute HCl produced only β-chloroalanine.[9] Use of an acid with a non-nucleophilic counterion, HBF_4, in $H_2{}^{18}O$ at 20 °C gave, after 11 days, labelled *N*-Z-serine which was derivatized by direct treatment with diazomethane to give the *O*-methyl-*N*-Z-serine methyl ester (**33a**) (Scheme 29). The position of label was determined by comparison with the labelled *O*-methyl-*N*-Z-serine methyl esters **33** and **34** formed from the previously prepared methyl esters **31** and **26**, respectively, by treatment with diazomethane and catalytic HBF_4. Again the electron impact mass spectra displayed expected molecular ion peaks as well as peaks corresponding to loss of $COOCH_3$. The fission gives mass peaks $C_{11}H_{14}NO_3$ (208.0980) for the methyl ethers from unlabelled (**35**) and carboxy labelled (**34**) serine derivatives. For the ether of the hydroxy labelled material from ^{18}O-acetate opening **33** and for the derivatized product of acidic opening **33a** the fission peaks were $C_{11}H_{14}NO_2{}^{18}O$ (210.1020), indicating that at least some of the label is in the hydroxyl oxygen after acidic opening. This demonstrates that opening of the β-lactone **24** with water under acidic conditions occurs, at least in part, by attack at the methylene position (Scheme 29).[54,55]

Scheme 29.



Our experiments show that Mitsunobu ring closure of *N*-Z-serine (36) proceeds by hydroxy group activation (HGA, Scheme 30) with subsequent loss of the oxygen and inversion of configuration at C-3 to give β-lactone **24**. Opening of **24** with aqueous hydroxide involves exclusive attack at the carbonyl (b in Scheme 30). In contrast, under acidic conditions with water as the nucleophile, displacement at C-3 (a in Scheme 30) is the major (if not exclusive) pathway of ring cleavage.

Scheme 30.



The vast majority of reported β-lactone formations by Mitsunobu coupling of β-hydroxy carboxylic acids occur by carboxyl group activation (CGA).[39,41] In a few cases where the α-carbon (C-2) has much greater steric bulk than the β-carbon (C-3), a minor amount (12%-44%) of β-lactone formation by the HGA mechanism has been detected.[41] Generally the HGA pathway with β-hydroxy acids has been described to give primarily decarboxylative elimination,[39-42] presumably because of antiperiplanar alignment of the breaking bonds. However, these transformations were usually done at about 20 °C, and it has been shown[9,89] that at such temperatures the primary product of Mitsunobu reaction of *N*-Z-serine (**36**) is the olefin (Scheme 4). If the rate of additi[illegible] of **36** to the triphenylphosphine/azodicarboxylate adduct is carefully controlled and the temperature is kept at -78 °C, the olefin becomes a minor product and 60% isolated yields of β-lactone **24** can be consistently achieved.[9] Since thermal elimination of carbon dioxide from β-lactones once they are formed requires high temperatures ($\geq$ 100°C),[42] it is the partitioning of the initial HGA adduct which accounts for formation of olefin and **24**. Although increase of solvent polarity should promote charge separation and has been shown to affect the ratio of olefin to CGA β-lactone formation in other systems,[39] indications are that a change from THF to acetonitrile has little effect on cyclization of *N*-acyl serines.[9,89] Apparently steric interactions favor the HGA intermediate regardless of solvent polarity, and the partitioning to olefin or β-lactone **24** is influenced primarily by temperature. Possible explanations for the temperature effect may include the degree of ionization of the C-3 to oxygen bond or the participation of pentacoordinate phosphorus intermediates.[43]

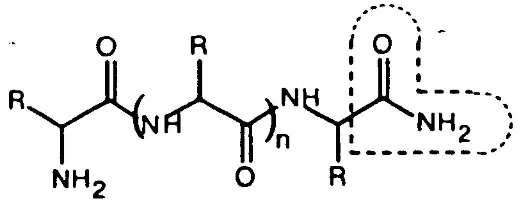
In the cleavage reactions, attack by hydroxide at the carbonyl to give **36** (b in Scheme 30) is in accord with earlier studies[54,55] and with behavior of β-lactones with other "hard" nucleophiles like methoxide.[9,45] However hydroxide, unlike methoxide,[9] does not cause epimerization at the α-carbon (C-2). Most weaker nucleophiles generally displace at C-3 (a in Scheme 30),[9] in agreement with the observed mode of ring opening by [^{18}O]water under acidic conditions. Use of an acid with a non-nucleophilic counter ion (e.g., HBF_4) is essential to obtain attack by water; use of dilute aqueous HCl results in rapid formation of β-chloroalanine.[9,90] Cleavage by nucleophiles other than water is probably a complicating factor in the early studies of acidic β-lactone hydrolysis.[54,55] However, the general observation of alkyl oxygen fission at neutral to moderately low pH agrees with our results. The previously reported acyl oxygen cleavage in very strong acid (8N H_2SO_4) was not examined with serine β-lactone **24**.[54,55]

Knowledge of the stereochemical outcome of formation of serine β-lactones and their cleavage mechanisms permits synthesis of a large variety of β-substituted alanines[9] which are stereospecifically labelled at C-3. Such compounds find continuing use in work on enzyme mechanisms.[26,27,91,92] In addition, Mitsunobu cyclization and hydroxide opening of *N*-acyl serines stereospecifically labelled with hydrogen isotopes at C-3 permits facile interconversion of the 3*R* and 3*S* isomers. These compounds are widely employed in biochemical studies, but generally each isomer has required an independent multistep synthesis. At present, however, an efficient synthesis of stereospecifically labelled serines of high (> 98%) optical purity is still not available.

Chapter 2. Studies on Peptidylglycine α-Amidating Monooxygenase

INTRODUCTION

Peptide amides are a diverse group of naturally occurring compounds in which the carboxyl terminus of a peptide is a primary amide. They are widespread in the animal kingdom from invertebrates to mammals but have not been detected in unicellular organisms (such as bacteria or algae) nor in higher plants.[93] A few examples of the numerous peptide amides in mammalian species are gastrin,[94] α-melanocyte stimulating hormone,[95] calcitonin,[96] cholecystokinin,[94] substance P,[97] vasoactive intestinal peptide,[98] and thyrotropin-releasing hormone.[95] Caerulein,[99] bombesin,[99] sauvagine,[100] and dermorphin[101] are found in frog skins. Invertebrates have yielded peptide amides such as melittin[102] and apamin[103] from bee venom, adipokinetic hormone from locusts,[93] red pigment concentrating hormone from prawns,[93] eledosin from an octopus,[104] cecropins from moths,[105] toxin II from scorpion venom,[106] and conotoxins from a marine snail.[107] These peptides vary in size from 3 amino acids for thyrotropin-releasing hormone[95] to 64 amino acids for scorpion toxin II.[106]

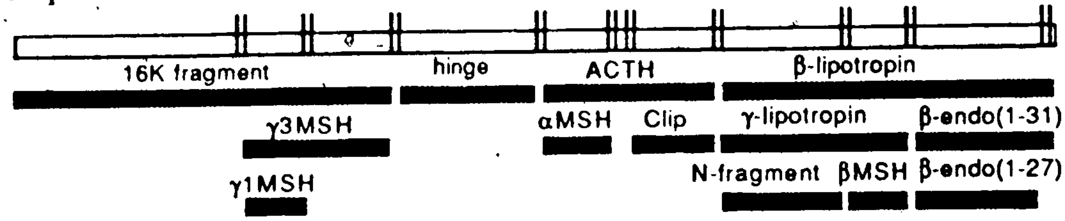


a peptide amide

The physiological roles of these peptides are also varied. Calcitonin is involved in calcium regulation.[96] The gastrins are potent stimulants of gastric acid secretion and may be implicated in certain types of stomach ulcers.[94] Oxytocin is used clinically to induce uterine contraction and to stimulate lactation.[108] Substance P is a neurotransmittor which may be involved in the transmission of pain.[97] Vasopressin is used as an antidiuretic.[108] Several of the isolated peptide amides appear to have more than one physiological role.[109]

Methods for the detection of peptide amides in biological systems have been developed.[93] The general method of Tatemoto and Mutt[110] involves proteolytic hydrolysis of a peptide or mixture of peptides to the amino acids. Treatment of the crude hydrolysate with dansyl chloride allows selective extraction into ethyl acetate of the derivatized amino amides (from the carboxy terminus of the peptide amide) while the derivatized amino acids (from the chain of the peptide amide or from other peptides) remain in the aqueous layer. The dansyl amides are identified by 2-D polyamide chromatography and comparison against known standards. This method has been used to detect several new peptide amides.[111,112]

Peptide amides, like most biologically active peptides, are biosynthesized from large precursor proteins which are cleaved to proproteins immediately after synthesis in the rough endoplasmic reticulum.[113,114] These proproteins or prohormones contain the amino acid sequences of the smaller, biologically active peptides, but are themselves not active.[115] The prohormones are transported to the golgi apparatus where they are packaged in secretory granules.[113] These granules contain the prohormone and the necessary enzymatic machinery for cleavage to the active peptides and for any further processing of the peptides such as acetylation, amidation, phosphorylation, or glycosidation. The cleavage of the prohormone has been shown to almost invariably occur at pairs of basic amino acids, usually lysine and arginine, though not all pairs of these amino acids are sites of cleavage.[115] This is strong indication that the three dimensional structure of the prohormone is important in the recognition of cleavage sites.[114] The diagram below schematically illustrates the cleavage of proopiomelanocotropin to smaller bioactive hormones.[115,116] The double lines indicates pairs of basic amino acids where cleavage may, but does not always, occur. The abbreviations used here are ACTH for corticotropin, MSH for melanotropin, Clip for corticotropin-like intermediate lobe peptide, and endo for endorphin.



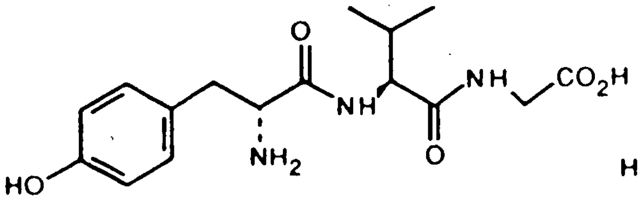
The processing of precursor proteins to bioactive peptides has been shown to be influenced by external stimuli.[117-119] Furthermore, since one prohormone often has the amino acid sequence for several different peptides, the selective processing of the prohormones may alter which peptides are produced.[114] For example proopiomelanocortin is processed to ACTH in the anterior lobe of the pituitary and to β-melanocyte stimulating hormone in the middle lobe of the pituitary.[120] Many of the peptide hormones are found in varying concentrations throughout the body and are believed to have different, location dependent biological functions.[121] Not surprisingly, the direct injection of peptide hormones, especially neuropeptides, into the central nervous system exerts dramatic effects on behavior.[109] For example neuropeptide Y, when injected into the hypothalamus of rats, is the most potent chemical stimulant of feeding behavior known.[122] Peptide amides occur primarily among neural and hormonal peptides.[123]

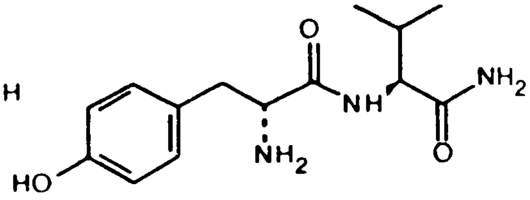
In the cases studied so far the peptide amide arises from the cleavage of a terminal glycine residue.[115] Table 4 illustrates some selected peptide amides and the amino acid sequence of part of their precursor proteins. Notice the common feature of a glycine (bold) followed by two basic amino acids (underlined) used for selective cleavage and amidation. The presence of a primary amide at the carboxyl terminus is essential for full biological activity.[115] For example, the free acid forms of gastrin and cholecystokinin are completely inactive,[124] and the amide form of lutenizing hormone-releasing factor is 1000 times as active as the acid.[125] This single transformation is therefore of paramount importance in the production of active peptide hormones. The recognition of the primary amide group of oxytocin and vasopressin[126-128] was probably the first[93] example of posttranslational modification of peptides to be discovered.

Table 4. 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-Aps-
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-

In 1982 Bradbury *et al.*[129] demonstrated the presence of enzymatic activity in porcine pituitary capable of converting a synthetic tripeptide to a dipeptide amide. They chose D-tyrosyl-L-valylglycine (D-TyrValGly, 37) as the peptide for three reasons. The unnatural D configuration of the tyrosine should prevent enzymatic hydrolysis, the aromatic tyrosyl residue is suitable for radioiodination, and the L-valylglycine (L-ValGly) was thought to be the minimal structural requirement to mimic the precursor to α-MSH which ends L-ValGly. Therefore, by iodinating the tripeptide with ^{125}I 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 (D-TyrValNH$_2$, **38**). They also demonstrated, by incorporation of ^{15}N into the valine-glycine amide bond, that this nitrogen remained as the primary amide nitrogen in the product peptide amide. Though no experimental details were given, the other product of the reaction was claimed to be glyoxylic acid.


D-TyrValGly 37


D-TyrValNH_2 38

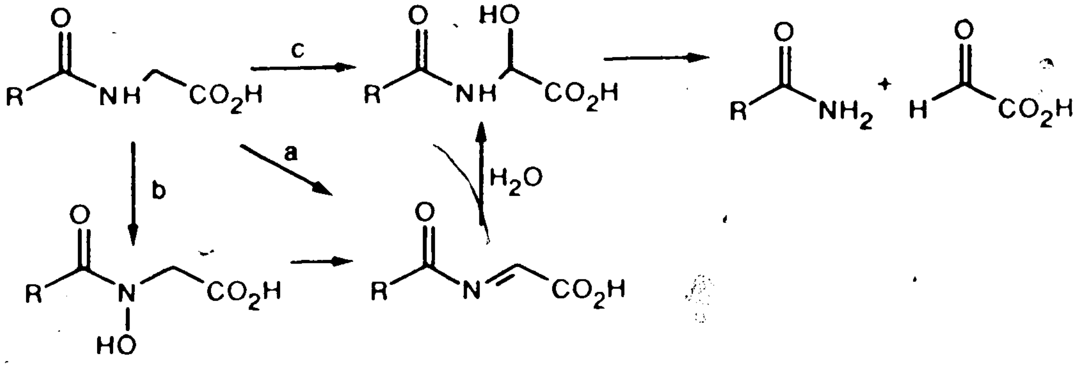
Since this initial work, amidating activity has been detected and partially purified in a variety of tissues from several organisms. In humans it has been found in plasma and serum,[130] cerebrospinal fluid and central nervous system tissue.[131] In cows[132] and pigs[133] it has been detected and purified from neurointermediate pituitaries. In rats the enzymatic activity has been found in tissue and serum,[134] in the pancreas of neonates,[135] in the hypothalamus,[136] in the anterior pituitary,[137] and in exocrine[138] and pituitary[139] secretion granules. Recently 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.[140] Amidating enzyme has also been detected and purified from the skin of the amphibian *Xenopus laevis.*[141,142] It has also been observed in several cell lines grown in culture.[143-145]

Although amidation activity has been detected in a variety of tissues it is still not clear, due in part to the small amount of enzyme present, if a single enzyme or group of similar enzymes is responsible. Eipper and coworkers[132] isolated 10 nanograms (ng) of highly purified enzyme from 300 bovine pituitaries. They calculate that in the pituitary only a few molecules of enzyme are present per secretory granule.[132] Even at these low concentrations, however, enough enzyme is present to produce sufficient peptide amide hormones. They,[132] and other workers,[146] have also reported finding more than one form of the enzyme apparently differing in size and specificity, and the enzyme from *Xenopus laevis* appears to have significantly different characteristics.[141] To date no amino acid sequence information has been published. A few generalizations may be made however. The molecular weight has been reported to be about 50k-70k daltons.[133,139,146] It has been shown that the enzyme requires ascorbate,[147] oxygen, and copper for maximal activity.[148] The enzyme has now been called peptidylglycine α-amidating monooxygenase (PAM).[134,137]

There are three published assays for this enzyme. The assay reported by Bradbury[129] using radioiodinated D-TyrValGly has been widely utilized. A recent modification of this assay uses radioiodinated *N*-acetyl-D-TyrPheGly which facilitates separation of the starting peptide from the product amide.[142] A third method involves the use of radioimmunoassay to selectively detect the D-TyrValNH_2.[149] These assays all have in common the limitation that they are suitable only for a specific amide product. While radioimmunoassay has the potential for application to a variety of amides, each one requires the development of specific antibodies. One of the major goals of the research presented in this thesis was to develop an assay based on the glycine extension of the peptide precursors which would therefore be generally applicable since this is the common feature of all the PAM enzyme substrates.

The mechanism of the reaction is still speculative. The enzyme accepts only glycine as the terminal amino acid and has a strong preference for a neutral amino acid in the position adjacent to the glycine.[148] Kizer *et. al.* have reported what appears to be a second form of the enzyme isolated from rat brain which is capable of converting peptides which end in amino acids other than glycine to their corresponding amide.[146] Bradbury proposed the substrate is dehydrogenated to form an imine which is subsequently hydrolysed (a in Scheme 31).[129,148] Presumably this imine could also form by hydroxylation of the nitrogen followed by elimination of water (b in Scheme 31). Alternatively, oxidation of the methylene carbon and cleavage of the hydroxy amide also forms glyoxylic acid and the amide (c in Scheme 31). The cofactor requirements for PAM (copper, oxygen, ascorbic acid) are the same as for the enzyme dopamine β-hydroxylase which oxidizes a benzylic methylene carbon in the conversion of dopamine to norepinephrine.[150]

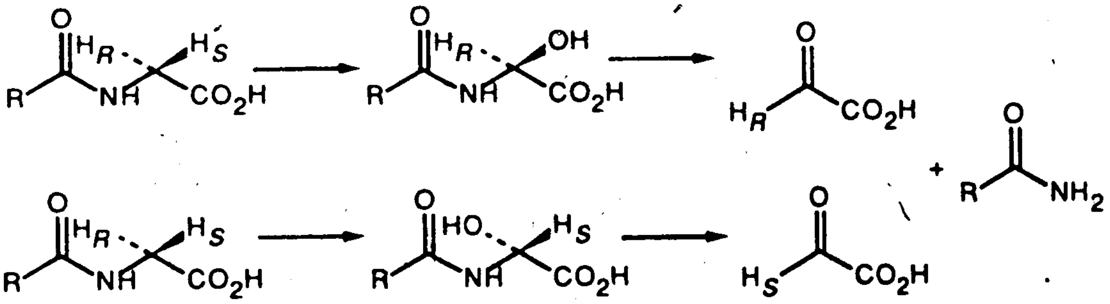
Scheme 31.



A recent paper by Kizer and coworkers demonstrates the non-enzymatic conversion of peptides to amides under conditions which closely resemble those required by PAM.[151] They found that certain peptides, when treated with copper and ascorbic acid at pH 6.0, are oxidativly cleaved to the amides and a carbonyl compound. However, several differences between the enzymatic and chemical oxidations exist. The terminal residue can be glycine, alanine, γ-aminobutyric acid, butylamine, or δ-aminovaleric acid as opposed to the complete specificity for glycine exhibited by the enzyme[148,151] (except in the case of one of the enzymes isolated from rat brain).[146] The tripeptide D-TyrValGly was not converted to the corresponding amide under the chemical conditions.[151] A similar primary α-isotope effect of deuterium substitution on the methylene carbon of the terminal glycine was observed for the chemical and enzymatic systems ($k_H/k_D = 1.9$ and 2.2, respectively).[133,151] This system may prove valuable in the determination of the mechanism of oxidation of PAM.[151] On the basis of the non-enzymatic oxidation these authors favored the direct cleavage of a carbon hydroxylated intermediate (path c in Scheme 31) without the formation of an imine (path a or b in Scheme 31), and they proposed a radical based mechanism for the oxidation.[151]

A primary goal of our studies with PAM was to determine which of the two diastereotopic methylene protons of the glycine is removed during oxidation (Scheme 32). This information can be useful in the design of modified peptide substrates as potential inhibitors of the enzyme, a long term goal of the research project. Inhibition of PAM may be an effective method for regulating the production of peptide amides in the body. The design of inhibitors based on the amino acid sequence of a target substrate peptide or precursor could allow for selective control of the amount of a specific peptide amide formed. Of particular interest would be the design of substrates which might act as mechanism based inactivators (suicide substrates)[152] for PAM. These are characterized as inhibitors from which the enzyme, during the course of normal processing of this modified substrate, unmasks a highly reactive intermediate that attacks the enzyme and irreversibly destroys its catalytic activity.[152] For the design of this type of inhibitor a thorough knowledge of the stereochemistry, substrate specificity, and mechanism of the enzyme is required.[152]

Scheme 32.

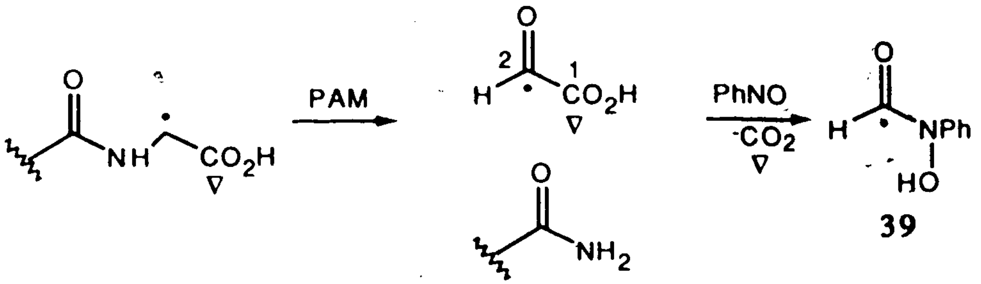


DEVELOPMENT OF A NEW ASSAY FOR PAM.

Glyoxylic acid is the common by-product in the formation of peptide amides,[129] and measuring the amount produced would be a general method of assaying PAM activity. The glyoxylic acid also retains chemical information of the stereochemistry of the oxidation since the methylene proton of glycine is retained as the aldehyde proton of glyoxylic acid (see Scheme 32). Although glyoxylic acid is a common metabolite there are few methods for quantitatively measuring its presence.[153-158] None of the published procedures were adaptable to the PAM system which requires sensitivity and specificity because of the small amount of product formed.[129,133,137] The most direct method of determining the amount of glyoxylic acid is by monitoring ultraviolet (uv) absorption caused by the formation of a phenylhydrazone when phenylhydrazine is added.[153] The sensitivity, however, is insufficient for PAM and the procedure is not specific for glyoxylic acid since most α-keto acids show a comparable reaction with phenylhydrazine.[153] α-Keto acids in general have been monitored by gas liquid chromatography[156,158] and hplc[154,155,157] but the sensitivity is too low and the required sample preparation and derivatization is tedious, especially for an enzyme assay.

Recently, however, a procedure was published for the formation of *N*-hydroxyformanilide (39, NHF) from the reaction of glyoxylic acid with nitrosoaromatic compounds.[159] The procedure was promising as a method to detect glyoxylic acid because the reaction is specific for glyoxylic acid, occurs in aqueous media, in high yields, and the product is easily extracted with ether (Scheme 33).[159] It appeared that the glyoxylic acid formed by PAM could be trapped by reaction with nitrosobenzene (PhNO) with isolation of the resulting NHF by extraction with ether. According to Corbett and Corbett,[159] in the formation of NHF the carboxylic acid carbon (C-1) of the glyoxylic acid is lost as CO_2 and the aldehyde carbon (C-2) is the source of the formyl carbon in the NHF.[159] Therefore, if the glyoxylic acid was produced from a peptide with ^{14}C-glycine terminus, the NHF could be analyzed for radioactivity. If reaction of glyoxylic acid with PhNO in D_2O does not exchange the aldehyde proton of glyoxylic acid or NHF with the solvent, the procedure would be suitable for tracing the fate of the glycine hydrogens.

Scheme 33.



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Studies on the Determination of Glyoxylate

To develop this reaction as a method of analyzing for glyoxylic acid in solution the time and temperature required for maximum formation and extraction of NHF must be determined. In initial investigations we monitored the amount of NHF produced by observing the uv absorbance at 250 nm.[159] The general procedure for this type of experiment was to add a known amount of glyoxylic acid to a buffer solution (50 mM sodium phosphate, 0.2M NaCl, pH 6.2) followed by the addition of PhNO (1-3 eq., 0.3 M in 95% ethanol). After a period of time the product was extracted with ether, the ether removed by evaporation with a gentle stream of argon, the residue dissolved in a known amount of 95% ethanol, and the uv absorbance recorded. The maximum absorbance for NHF occurs at 250 nm (ε 11626), and for PhNO at 280 nm (ε 9254). The molar absorptivity (ε) was determined by independently measuring the absorbance at the desired wavelength of at least three different concentrations of NHF and PhNO over the range of concentrations to be studied. The slope of the linear plot of these data is the molar absorptivity. Since in this type of experiment the excess PhNO would be expected to be extracted with the NHF, the molar absorptivity of NHF at 280 nm (ε 2618) and of PhNO at 250 nm (ε 1300) was determined. In this way the equations can be solved to determine the concentration of NHF and the concentration of PhNO of a mixture by measuring the absorbances at 250 nm and 280 nm. Any analysis using these equations assumes that the absorptions at these two wavelengths are only from NHF and PhNO but this limitation was reasonable for the resulting experimental simplicity.

$$A_{250} = 11626[\mathrm{NHF}] + 1300[\mathrm{PhNO}]$$
$$A_{280} = 2618[\mathrm{NHF}] + 9254[\mathrm{PhNO}]$$
$$[\mathrm{NHF}] = 8.88\times10^{-5}A_{250} - 1.25\times10^{-5}A_{280}$$
$$[\mathrm{PhNO}] = 1.12\times10^{-4}A_{280} - 2.51\times10^{-5}A_{250}$$

We first investigated the time and temperature necessary to produce maximum yields of NHF from a known amount of glyoxylic acid and PhNO (Table 5). After the indicated reaction time the solutions were cooled on ice, extracted with ether (3x0.5 mL), the ether evaporated, the residue dissolved in 10.0 mL of 95% ethanol, and the uv absorption measured at 250 nm and 280 nm.

Table 5. Optimization of Reaction Time and Temperature for NHF Formation.[a]

	temp. (°C)	time (min)	A_{250}	A_{280}	NHF (µmol)	PhNO (µmol)	yield %
1[b]	94	1	0.128	0.103	0.26	0.205	15
2[b]	94	5	0.335	0.112	0.71	0.105	42
3[b]	94	10	0.449	0.112	0.96	0.033	56
4	40	15	0.411	0.161	0.35	0.078	21
5	40	30	0.748	0.199	0.64	0.035	38
6	40	60	1.11	0.345	0.94	0.108	55
7	40	120	1.35	0.408	1.14	0.119	69
8[c]	60	45	1.75	0.437	1.50	0.051	88
9[c,d]	60	45	1.58	0.385	1.35	0.053	80

[a] Reactions contained 1.7 µmol of glyoxylic acid (3.4mM) and 2 eq. of PhNO in 500 µl of buffer (50mM sodium phosphate, 0.2M NaCl, pH 6.2).

[b] After a single 0.5 mL extraction, 1/5th of the product was diluted with 5.0 mL of ethanol.

[c] The values are the average of three assays. The yield of NHF varied by less than ±1%.

[d] These results were obtained one year after the previous results; see Table 6.

These results indicated that it was possible to trap almost 90% of the glyoxylic acid formed by adding 2 eq. of nitrosobenzene and incubating at 60 °C for 45 min (entry 8, Table 5). With these promising preliminary results the enzyme was isolated and assayed. However, we returned to these experiments a year later (entry 9, Table 5) to quantitatively determine the amount of glyoxylic acid trapped under conditions identical to the PAM assay which had been slightly modified from the above preliminary studies. Table 6 outlines the differences in the conditions of the PAM assays and the experiments in Table 5.

Table 6. Comparison Between Optimized and PAM Assay Conditions.

	Table 5	PAM assay
PhNO (eq.)	2	4
[glyoxylate]	3.4 mM	1.3 mM
total volume	500 μL	300 μL
pH	6.2	6.8
[ascorbate]	—	0.7 mM
[Cu^{++}]	—	3 μM
[KI]	—	17 mM

Table 7 shows the results of systematically changing some of the variables listed in Table 6. Increasing the amount of PhNO from 2 to 4 eq. somewhat (5-7%) improved the yield of NHF (entries 1 and 2, Table 7). Decreasing the glyoxylate concentration from 3.6 mM to 1.3 mM had little if any effect (entries 2 and 3, Table 7). Decreasing the reaction volume from 500 to 300 μL caused a 10% drop in the yield of NHF (entries 3 and 4, Table 7). Increasing the pH from 6.2 to 6.8 had no effect on the amount of glyoxylic acid trapped (entries 4 and 5, Table 7). The reasons for the lower yields upon decreasing the reaction volume are uncertain, but may be due to changes associated with the extraction.

Table 7. **Effects of Varying Volume, Concentration, and pH on Yield of NHF.**[a]

	volume (µL)	[glyoxylate] (mM)	PhNO (eq.)	pH	A_{250}	A_{280}	NHF (µmol)	PhNO (µmol)	yield (%)
1	500	3.6	2	6.2	1.65	0.407	1.41	0.042	84
2	500	3.6	4	6.2	1.86	0.494	1.59	0.086	91
3	500	1.3	4	6.2	1.34	0.373	0.58	0.040	88
4	300	1.3	4	6.2	0.759	0.318	0.32	0.094	79
5	300	1.3	4	6.8	0.729	0.339	0.31	0.098	77

[a]These entries are the average of at least two experiments. The yield of NHF varied by less than ±3%.

Table 8 shows the results obtained when copper, ascorbic acid, and potassium iodide are included in the experiment. For these results the experimental conditions were the same as in Table 7 except that the reaction time was increased to 60 min. These results clearly show that the combination of reagents in the PAM assay causes an inhibition in the amount of glyoxylic acid trapped. In particular, ascorbic acid alone appears to inhibit the reaction, but not to the extent as all of the reagents combined. The slightly higher absorbances at 280 nm when ascorbate is added may be a result of either an inhibition of the reaction and extraction of unreacted nitrosobenzene, or interference from a contaminating reagent or by-product. The exact causes of this inhibition and the reaction between nitrosobenzene and ascorbic acid are being investigated. These results indicate that 55% of the glyoxylic acid is trapped under the conditions of the PAM assay and this factor was taken into account when determining the amount of glyoxylic acid produced by the enzyme.

Table 8. Effects of Copper, Ascorbic Acid, and KI on the Yield of NHF.[a]

Reagents added[b]	A_{250}	A_{280}	NHF (μmol)	PhNO (μmol)	yield %
none	0.799	0.201	0.34	0.012	85
Cu, KI, Ascorbate	0.519	0.155	0.22	0.022	55
KI	0.760	0.186	0.33	0.009	87
Cu	0.771	0.196	0.33	0.013	82
Ascorbate	0.651	0.216	0.28	0.039	70
Ascorbate[c]	0.005	0.002			

[a]These values are the average of at least two experiments. The yield of NHF varied by less than ±2%.

[b]The concentrations of added reagents were $CuSO_4$, 3 μM; ascorbic acid, 0.7 mM; KI, 17 mM.

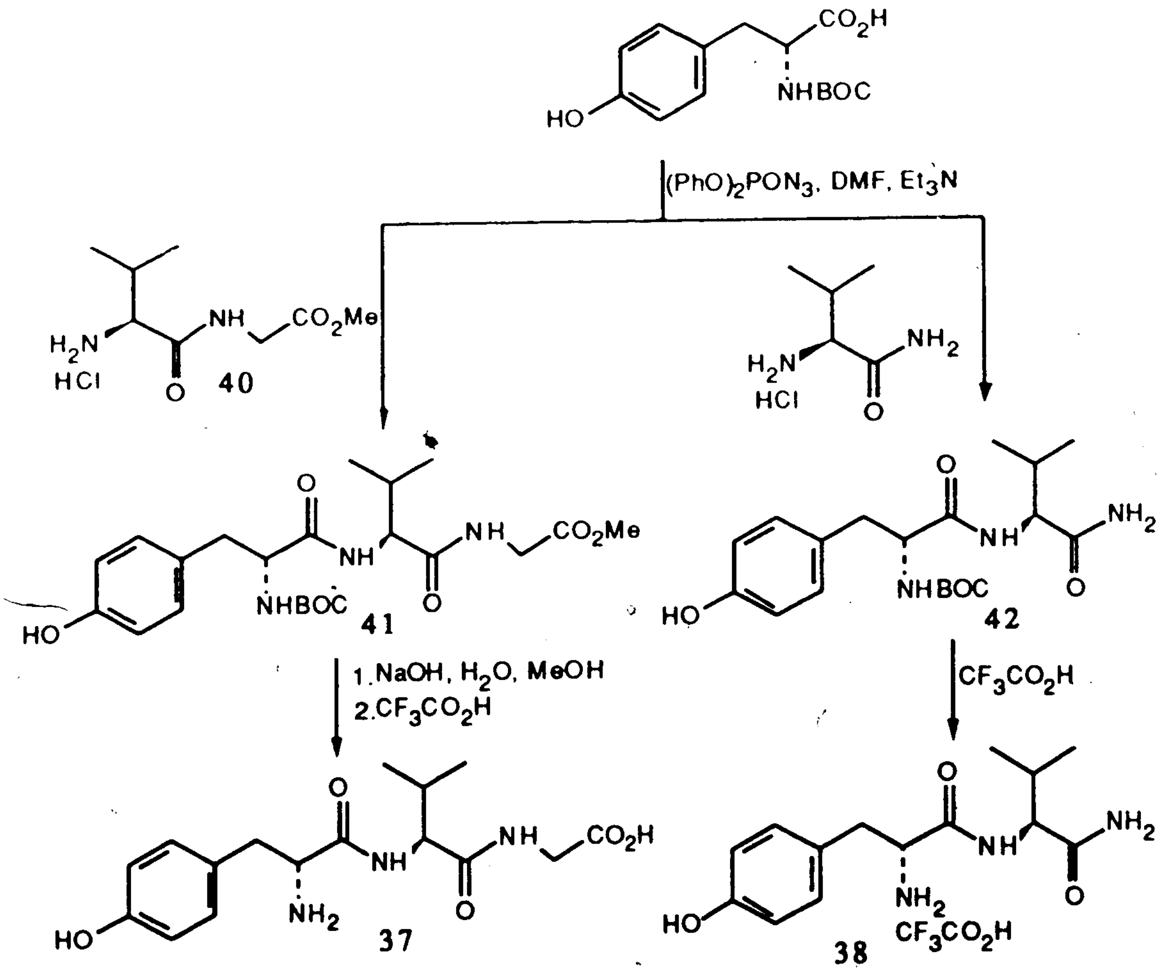
[c]Nitrosobenzene was excluded to determine any absorbance from ascorbate.

Synthesis of Radiolabelled Tripeptide

To form radioactive glyoxylic acid during the PAM assay the carbons of the glycine residue of D-TyrValGly must be labelled with ^{14}C. Uniformly labelled [1,2-^{14}C]glycine is commercially available in high specific activity (113 mCi/mmol, 96% ^{14}C). The syntheses of the peptides required for these studies, both labelled and unlabelled, employed standard solution phase peptide chemistry. Diphenylphosphorylazide (DPPA)[160] was used to couple the nitrogen protected amino acids with the carboxyl protected amino acids. This reagent converts carboxylic acids to acyl azides which react with amines to form the amides.[160] *tert*-Butyloxycarbonyl (BOC) protecting groups were used for nitrogen protection and removed with CF_3CO_2H, and methyl or phenylmethyl esters were used for carboxyl protection and removed by alkaline hydrolysis or catalytic hydrogenolysis, respectively.[74,161]

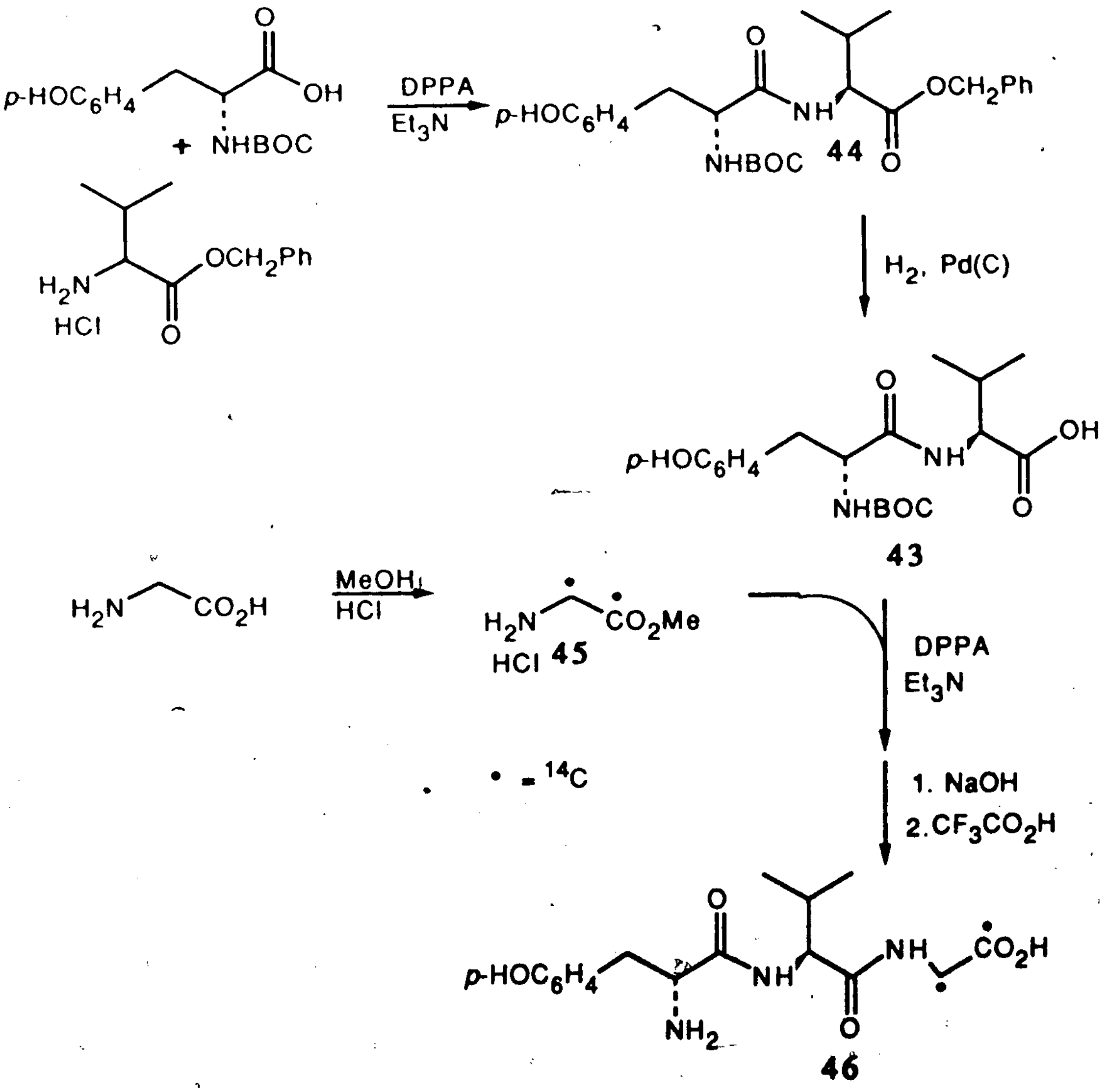
To prepare unlabelled D-TyrValGly **37**, commercially available BOC-D-tyrosine was coupled with L-valylglycine methyl ester hydrochloride **40**, prepared from commercially available L-valylglycine, to give the diprotected tripeptide **41** in 66% isolated yield (Scheme 34). The methyl ester was hydrolysed with sodium hydroxide in water/methanol and, after aqueous acidic workup, the *N*-BOC group was removed by room temperature treatment with neat CF_3COOH. The product **37** was isolated as the free peptide by neutralization with NH_4OH and recrystalization from water/ethanol in 65% yield from **41**. Authentic D-tyrosyl-L-valinamide **38** was prepared using the same strategy by coupling *N*-BOC-D-tyrosine with commercially available L-valine amide hydrochloride to give *N*-BOC-D-TyrValNH$_2$ **42** in 48% yield. The BOC group was removed to give D-tyrosyl-L-valinamide hydrochloride **38** in 70% recrystalized yield.

Scheme 34.



For the synthesis of tripeptides in which the glycine is labelled, *N*-BOC-D-tyrosyl-L-valine **43** was prepared by first coupling *N*-BOC-D-tyrosine with L-valine phenylmethyl ester hydrochloride to give **44** in 55% yield after recrystalization (Scheme 35). The phenylmethyl (benzyl) protecting group was removed by catalytic hydrogenolysis to give 43. This material is suitable for coupling to labelled glycine methyl ester hydrochloride.

Scheme 35.



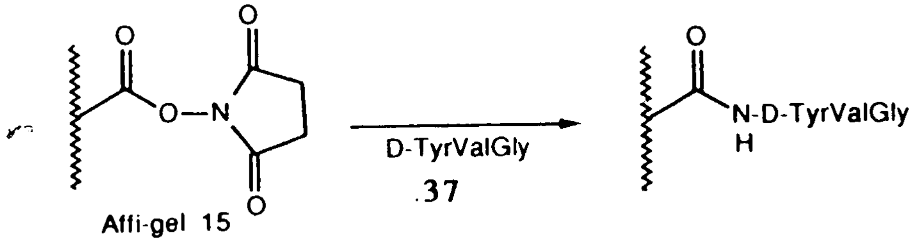
[1,2-^{14}C]Glycine (250 µCi, Amersham, 113 mCi/mmol, 96% ^{14}C) was converted to the methyl ester 45 in a small volume of methanol with dry HCl$_{(g)}$ and this was coupled to the

N-BOC protected dipeptide **43** using the DPPA procedure. Deprotection as for the unlabelled material gave D-TyrVal-[1,2-^{14}C]Gly (**46**) isolated as the CF_3COOH salt in 47% overall yield (118 μCi). This material shows only a single radioactive spot which co-spots with unlabelled peptide **37** in two different solvent systems. For most of the assays described **46** was used without further purification. D-TyrValGly can be purified by hplc as described in the general experimental.[137]

Enzyme Isolation

The isolation of PAM was an adaptation of the procedure of Youngblood and co-workers.[133] This method was chosen because it is straightforward with only two columns being required to give enzyme of high purity (> 95%).[133] The use of a substrate affinity column is the key to the isolation allowing many fold purification in a single step. The affinity column is prepared from Bio-Rad Affi-Gel 15 and D-TyrValGly **37** as described.[133] The Affi-Gel resin contains *N*-hydroxysuccinimide activated esters which form amide bonds with the nitrogen terminus amino group of the substrate (Scheme 36). 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 the enzyme active site selectively binds to the substrate attached to the column while other proteins pass unhindered. PAM does not degrade the column since ascorbic acid and copper, necessary cofactors for the degradation of the substrate, are not available. The enzyme is removed from the column by eluting with a concentrated buffer containing urea, *N*-acetylglycine, and glycylglycine. This combination of reagents probably slightly distorts the geometry of the active site of the enzyme as well as displacing the column bound substrate from the active site.

Scheme 36.



37

To isolate the enzyme, frozen pig pituitaries (Pel-Freez), stored at -60 °C for an indefinite period of time, were 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 washing the affinity column the enzyme was eluted with urea buffer, and the resulting fractions were dialyzed. The entire isolation can be done in 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 highest activities of enzyme. The PAM enzyme is particularly sensitive to the affinity column eluent and these fractions must be dialyzed immediately.

To assay for enzyme activity, D-TyrVal[1,2-^{14}C]Gly (46) and a cocktail containing ascorbic acid, copper sulfate, and potassium iodide were added to the enzyme solution. After a 2-3 h incubation at 37 °C unlabelled glyoxylic acid and 4 eq. of PhNO were added and the solution was heated for 1 h at 60 °C followed by extraction with ether. Initial attempts to assay the enzyme under these conditions produced results of several thousand decompositions per minute (dpm), even when the enzyme was denatured by heating. This high control was lowered by passing the ether extracts through a Pasteur pipette filled with ~2 g of Na_2SO_4 which serves to dry the ether layer and to remove the residual radioactive peptide dissolved in the wet ether layer. In this way the dpm for control assays were always below 200 (usually less than 150), and 500 to 3000 dpm were observed for fractions containing active enzyme. In general, assays run in duplicate vary by less than 10%. When 46 was purified by hplc, the background for the assay was consistently reduced to 37 dpm, only 16 dpm above instrument background. Table 9 is a summary of the enzyme isolations. The amount of protein was determined by a modification of the method of Bradford[162] using bovine serum albumin as the standard. There is significant variation between the isolations probably due to a combination of factors including experience and the length of time the pituitaries were stored.

Table 9. Isolations of PAM from Pig Pituiries.

centrifuge supernatant		combined Sephadex fractions			affinity column fraction[a]		
protein		protein		PAM activity	protein		PAM activity
mg/mL	total mg	mg/mL	total mg	nmol/mg/h[b]	µg/mL	total µg	nmol/mg/h[b]
9.1	146	0.66	53	0.088	1.3	5.2	10.
5.2	120	1.25	120	0.061	5.6	22.	14.
17.	340	2.14	257	0.063	7.5	30.	10.
					13.	53.	16.
					8.8	26.	21.
6.0	120	0.61	60	0.190	5.8	26.	38.

[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.

In order to confirm that the radioactivity measured was from NHF as proposed an isotope dilution experiment was performed. When the extract from a PAM assay was added to unlabelled NHF and this was purified by repeated recrystalizations, all of the radioactivity co-purified with the NHF. Also of concern was the possibility that D-TyrValGly was hydrolysed to glycine which was oxidized to glyoxylic acid either enzymatically or chemically. However, when [1,2-^{14}C]glycine (5.8×10^4 dpm, 0.23 nmol) was incubated with PAM under the same conditions as the peptide, the resulting radioactivity was not above background (77 dpm). Also tested as a substrate was *N*-acetylglycine. This compound has what might be considered the minimum structural requirements for PAM, an amide bond with a glycine extension. When *N*-acetyl-[1,2-^{14}C]glycine (**47**, 4.5x10^4 dpm, 0.18 nmol) was treated with the enzyme under standard assay conditions no radioactivity above that observed with denatured enzyme was detected in the ether extracts (76 dpm).

To investigate the effect of substrate concentration on the enzyme rate, varying amounts of D-TyrVal-[1,2-^{14}C]Gly (**46**) were added to the assay to give the concentrations indicated in Table 10. The volume of the reaction was 200 -210 μL and unlabelled D-TyrValGly (**37**) was added for the high concentrations. The reaction time was 2 h and each assay contained 0.56 μg of protein. Table 10 lists the results obtained and Figure 1 is the double reciprocal (Lineweaver-Burk) plot of the data from which the Michaelis-Menten constant (K_m) was determined to be 26 μM and the V_{max} was calculated as 244 pmol/μg/h at an ascorbic acid concentration of 1 mM. A wide range of numbers have been reported for these values.[133,137,163] Kizer *et al.*[133] reported a K_m of 0.3 mM and a V_{max} of 8100 pmol/μg/h at an ascorbic acid concentration of 1 mM for enzyme isolated by the same method and assayed by radioimmunoassay.[149] Eipper *et al.*[163] determined the K_m to be 42 μM and the V_{max} to be 39 pmol/μg/h at an ascorbic acid concentration of 0.5 mM for the enzyme isolated from rat pituitary and assayed using the Bradbury[129] procedure. They also reported a K_m of 37 μM and V_{max} of 2.9 pmol/g/h with an unspecified ascorbic acid concentration for the partially purified enzyme from rat anterior pituitary.[137] The range of values observed probably reflect variations in enzyme purity and in the methods for assaying the enzyme rather than actual physical differences. Kizer *et al.*[133] demonstrated that the kinetics of the enzyme show competitive inhibition for both ascorbic acid and D-TyrValGly, thereby indicating a two-step or "ping-pong" mechanism[164] with both of these substrates competing for the active site. We were unable to analyze the results of varying the concentration of ascorbic acid (Table 11 and Figure 2) because of complications from the inhibition of the formation of NHF by ascorbate, but it is significant that the enzyme was stimulated by the addition of ascorbic acid as has been reported in all cases to date.[119,134-139,144,149,163]

Table 10. PAM Activity With Varying D-TyrValGly Concentrations.

[D-TyrValGly]	dpm	pmol/µg/h
0.62 µM	468	6.1
	420	5.4
3.1 µM	2107	28
	1678	21
16 µM	5421	71
	5292	69
50 µM	624[a]	263
	740[a]	312
100 µM	522[b]	441
	443[b]	374

[a]The specific activity of D-TyrValGly was 3.5 mCi/mmol
[b]The specific activity of D-TyrValGly was 1.7 mCi/mmol

Figure 1. Lineweaver-Burk Plot of Enzyme Velocity.

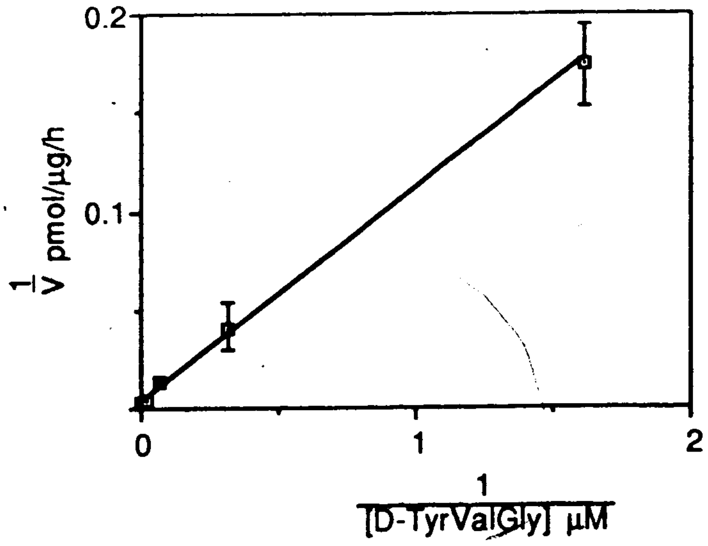


Table 11. PAM Activity With Varying Ascorbic Acid Concentrations.

[Ascorbate]	dpm	V (pmol/μg/h)
0 mM	532	14.
	584	15.
0.5 mM	932	24.
	994	26.
1.0 mM	1041	27.
	1020	26.
5.0 mM	74	1.9
	0	0
10. mM	14	0.4
	0	0

Figure 2. Plot of PAM Activity vs. Ascorbic Acid Concentration.

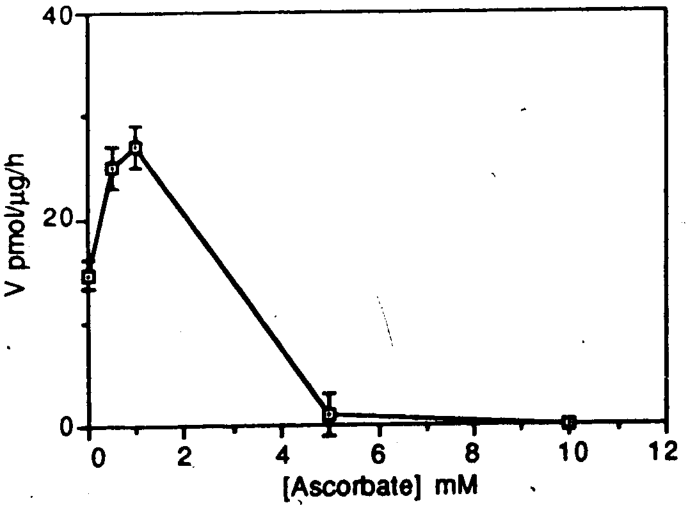
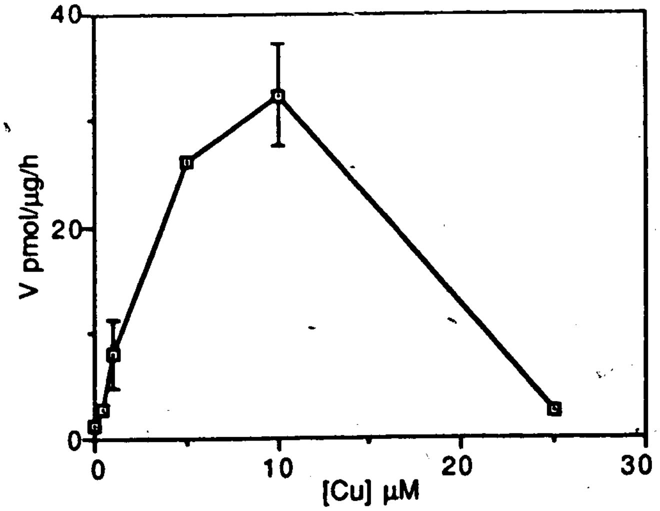


Table 12 and Figure 3 show the effect on enzyme activity of varying the concentration of added copper. The enzyme was completely inactive in the absence of added copper with a concentration of 10 μM giving maximal stimulation and higher concentrations inhibiting the enzyme. Kizer *et al.* reported that the enzyme isolated by the same procedure was active without added copper, with a maximum increase in activity observed with an added copper concentration of 1 μM.[133] Interestingly, they also observed an decrease of activity with higher copper concentrations,[133] as have other groups.[131,137,144]

Table 12. PAM Activity With Varying Copper Concentrations.

[Cu]	dpm	V (pmol/μg/h)
0 μM	0	0
	0	0
0.5 μM	100	2.6
	110	2.8
1.0 μM	373	9.7
	250	6.5
5.0 μM	1033	27.
	1025	27.
10. μM	1158	30.
	1345	35.
25. μM	105	2.7
	85	2.2

Figure 3. Plot of PAM Activity vs. Copper Concentration.



The addition of diethyldithiocarbamic (DDC) acid had been reported to inhibit PAM,[133,139,163,165] presumably by complexing the copper. When various concentrations of DDC were added to the assay containing 5 μM Cu^{++} a partial inhibition of the enzyme activity was observed. These results are shown in Table 13.

Table 13. Effect of Varying Concentrations of DDC on PAM Activity.

[DDC] μM	dpm	V (pmol/μg/h)	%inhibition
0	968	25.	
2	1124	29.	0
4	764	20.	20
8	339	8.8	65

In summary the enzyme isolated by us from pig pituitaries shows characteristics similar to those reported for PAM from the same tissue[133] and from other sources.[119,134-139,144,149,163] In addition, our assay based on the formation of NHF from the glyoxylic acid produced by PAM is a convenient, sensitive, and reproducible method for assaying this enzyme but is subject to chemical interference such as that observed with ascorbic acid.

Recently a modification of the Bradbury[129] assay was published.[135] When PAM is incubated with radioiodinated *N*-acetyl-D-TyrPheGly, the product amide *N*-acetyl-D-TyrPheNH$_2$ is extracted into ethyl acetate while the unreacted starting material remains in the aqueous layer. This method has the advantage over previous assays of experimental simplicity in the separation of the product amide from the starting peptide. To determine if this assay could be adapted to use carbon-14, *N*-[1-^{14}C]acetyl-D-TyrValGly (**48**) was synthesized from D-TyrValGly (**37**) and [1-^{14}C]acetic anhydride. The assay was performed by a modification of the literature procedure[135] using **48**. As with the NHF assay it was necessary to dry the organic layer by passing through a Pasteur Pipette filled with Na_2SO_4 to decrease the control assays to < 500 dpm with active fractions giving measurements of 2000-4000 dpm, depending on the activity of the enzyme.

The amount of radioactivity in assays without enzyme was further reduced following hplc purification of **48** on a C_{18} column as described in the general experimental.[137] The *N*-acetyl peptide (**48**) has a retention time of 15.04 min. As observed with D-TyrVal-[1,2-^{14}C]Gly (**46**), hplc purification of **48** reduces the radioactivity from control experiments. The results obtained using this modified assay correspond to an enzyme activity of 19 pmol/μg/h for the *N*-acetyl-D-TyrValGly (4.2 μM), compared to an activity of 48 pmol/μg/h with D-TyrValGly (1.3 μM) as substrate. These observations are preliminary however as experiments were not performed to determine the amount of product extracted under these conditions, although it is repo[illegible] to be > 95% for the *N*-acetyl-D-TyrPheNH$_2$.[135]

The *N*-acetyl assay[135] has several advantages over the NHF assay for detecting the presence of enzyme. It is faster, not requiring the 1 h incubation at 60 °C, and is a direct measure of enzyme activity not dependent on a second, chemical reaction which may be inhibited by cofactors present during the assay. Also, by the use of high specific activity [^{3}H]acetyl tripeptide, the dpm for an assay can be increased with better sensitivity than the carbon-14 based assays. A major disadvantage, in contrast to the NHF assay, is that it is specific for the particular tripeptide and is difficult to extend to other peptide precursors. Since the results obtained thus far are preliminary it remains to be determined if this modified assay is reproducible and generally applicable.[135,140]

Assay of Glycolate Oxidase

Glyoxylic acid is a common metabolite and it was of interest to determine if the NHF procedure for detection was applicable to other enzyme systems. Three commercially available enzymes were known to catalyze the formation of glyoxylic acid. Two, L-amino acid oxidase (EC 1.4.3.2) and D-amino acid oxidase (EC 1.4.3.3),[166] form glyoxylate from glycine, while the third glycolate oxidase (EC 1.1.3.15) produces glyoxylate from glycolic acid.[153] Since glycolate oxidase was known to form significant quantities of glyoxylate and a convenient assay was available for comparison,[153] this enzyme was investigated further.

The published assay for glycolate oxidase monitored the formation of glyoxylate by uv detection at 324 nm of the hydrazone formed between glyoxylic acid and phenylhydrazine.[153] The radioactive NHF assay was modified and calcium [1,2-^{14}C]glycolate was incubated with glycolate oxidase but no radioactivity was observed in the ether extracts although the phenylhydrazine assay clearly showed the enzyme was active under the assay conditions. Further experiments demonstrated that the addition of catalase increased the absorbance of the hydrazone in the uv assay, presumably due to prevention of the oxidation of glyoxylate by hydrogen peroxide,[153] but had no effect on the failure to extract radioactivity in the NHF procedure. To investigate why the radioactive assay failed, a series of uv experiments was performed to determine the amount of NHF formed from reaction of the glyoxylate with nitrosobenzene. The results summarized in Table 14 clearly show that the addition of cysteine significantly interferes with the trapping of glyoxylate by nitrosobenzene. The cysteine was subsequently omitted from the glycolate oxidase assays. Using the phenylhydrazone assay this was determined to have only a small effect on the enzymatic reaction. Recent work by Hamilton and co-workers suggests that the cysteine reacts with the glyoxylic acid to form a thiohemiacetal which can cyclize to the thiazolidine-2,4-dicarboxylic acid (Scheme 37),[167,168] effectively competing with PhNO for the glyoxylic acid. When glycolate oxidase is present the thiohemiacetal is converted to an oxalyl thiolester.[167,168]

Table 14. Yield of NHF in the Presence of Glycolic Acid and Cysteine.[a]

Reagent added	A_{250}[b]	A_{280}[b]	NHF(μmol)	PhNO(μmol)	%yield
none[c]	0.518	0.159	0.132	0.014	44
none[d]	0.516	0.142	0.132	0.009	44
none[d]	0.357	0.117	0.091	0.004	30
glycolic acid[c]	0.558	0.176	0.142	0.006	47
glycolic acid[c]	0.613	0.183	0.156	0.005	52
glycolic acid[d]	0.594	0.166	0.152	0.004	51
cysteine[c]	0.050	0.039	0.012	0.003	4
cysteine[c]	0.067	0.045	0.016	0.003	5
cysteine[d]	0.070	0.054	0.017	0.004	6
cysteine[d]	0.160	0.096	0.039	0.007	13

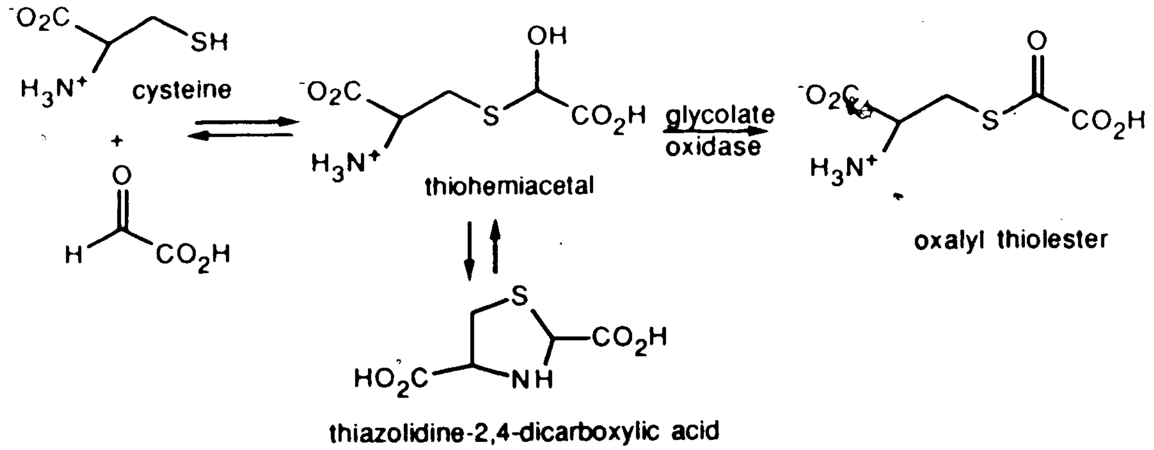
[a] These experiments were performed by Sven Aippersbach.

[b] After evaporation of the ether, the residue was dissolved in 3.0 mL of 95% EtOH.

[c] Total volume was 2.3 mL. Concentration of reagents was 0.13 mM glyoxlic acid, 8.7 mM glycolic acid (if added), and 4.3 mM cysteine (if added).

[d] Total volume was 0.5 mL. Concentration of reagents was 0.60 mM glyoxlic acid, 40 mM glycolic acid (if added), and 20 mM cysteine (if added).

Scheme 37.



For accurate comparison of the phenylhydrazone and NHF assays it was necessary to be able to irreversibly stop the enzymatic reaction at a given time. Addition of 50 μL of

4 M HCl stopped the reaction when monitored by the phenylhydrazone assay. Subsequent raising of the pH by the addition of 200 μL of 2 M potassium phosphate buffer (pH 6.5) did not increase the absorbance even after 30 min. This indicated that pH shock is an effective method of stopping the reaction.

To determine if the concentration of glyoxylate affects the yield of NHF formed varying amounts of glyoxylic acid were added. Table 15 lists the results which show that a considerably higher yield of the NHF is obtained when the glyoxylate concentration is increased and that 76% of the glyoxylic acid can be trapped under the assay conditions if the concentration of glyoxylic acid is 1.2 mM.

Table 15. Yield of NHF With 1.2 mM and 0.086 mM Glyoxylic Acid.[a]

[glyoxylate]	250 nm	280 nm	NHF(μmol)	PhNO(μmol)	%yield
1.2 mM[b]	1.28	0.366	1.9	0.16	76
0.086 mM[c]	0.139	0.059	0.058	0.016	32

[a] The total volume was 2.1 mL, 4 eq. of PhNO were added.

[b] After evaporation of the ether, the residue was dissolved in 5.0 mL of 95% EtOH and 2.0 mL of this was diluted with 5.0 mL of 95% EtOH. Average of 7 assays.

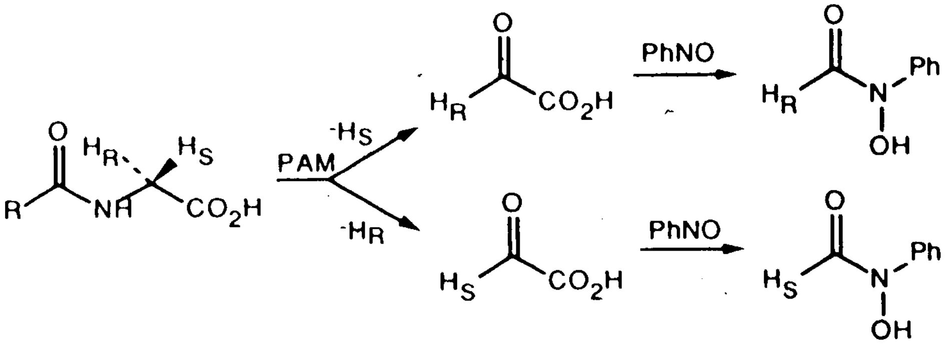
[c] After evaporation of the ether, the residue was dissolved in 5.0 mL of 95% EtOH. Average of 2 assays

The radioactive and phenylhydrazone assays were compared as described in the general experimental. After a 20 min incubation the phenylhydrazone assay gave a conversion of 4.6% of the glycolic acid. After taking into account the 75% trapping the results from the radioactive NHF assay correspond to a conversion of 3.0% and 3.8%. The comparison was repeated with a 25 min incubation time. The phenylhydrazone assay gave conversions of 5.0% and 4.6% whereas the radioactive assay gave 2.8% and 3.4%. The radioactive NHF assay gives results of approximately 65% of those obtained with the phenylhydrazone assay. There are several possible explanations for this descrepancy. The phenylhydrazone is not specific for glyoxylic acid and the higher conversion may be an artifact of the presence of other keto acids in the enzymatic preparation. Alternatively, the efficiency of trapping the glyoxylate with NHF may be lower than indicated in the studies where enzyme is excluded. These experiments indicate that the NHF procedure can be used to determine the presence of glyoxylic acid and to assay for its formation, but that the interference from reagents such as cysteine and the varying yields of the NHF make it difficult to obtain quantitative results.

STEREOCHEMISTRY OF PAM OXIDATION OF D-TYROSYL-L-VALYLGLYCINE

Determination of which of the two diastereotopic protons of the glycine methylene group in D-TyrValGly is removed during the oxidation by PAM would provide valuable information about possible sites of substitution for potential inhibitors of PAM and would contribute to knowledge of the steric requirements of the active site. This required the synthesis of the tripeptide from glycine stereospecifically labelled with tritium. The loss or retention of tritium from D-TyrVal-[2-^{3}H]Gly can be monitored by reaction of the glyoxylic acid produced with PhNO and isolation of the NHF formed (Scheme 38), provided the aldehyde proton of glyoxylic acid or NHF does not exchange with the solvent. To confirm this, glyoxylic acid was reacted with PhNO in D_2O and the resulting NHF was analyzed by ^{1}H nmr and ci-ms. This clearly showed that no deuterium was incorporated in the aldehyde position and indicated that this approach would be suitable for tracing the fate of the glycine hydrogens.

Scheme 38.



Enzymatic Preparation of Stereospecifically Labelled Glycine

There have been several syntheses of stereospecifically tritiated glycine reported in the literature.[169-177] The chemical syntheses are several steps and are complicated by incorporation of tritium early in the synthesis.[173,174-177] Two enzymes, serine hydroxymethyl transferase and glutamic-pyruvic transaminase (EC 2.6.1.2), have been used to stereospecifically exchange one of the methylene protons of glycine with water.[169,171,172] Glutamic-pyruvic transaminase is available commercially and was chosen to prepare the glycine. This enzyme catalyzes the stereospecific exchange of the pro-*R* hydrogen with water.[169,172] Incubation of *RS*-[2-^{3}H]glycine (**49a**) with enzyme in H_2O therefore produces *S*-[2-^{3}H]glycine (**50a**), while treatment of unlabelled glycine with enzyme in ^3H_2O forms *R*-[2-^{3}H]glycine (**51a**).[169,172] Since this preparation is straightforward it was the first approach attempted.

Of concern was the resulting specific activity of the [2-^{3}H]glycine since it was known that PAM only produces small amounts (~100 pmol) of glyoxylic acid. From the results in Table 10 it was estimated that a specific activity of 5 mCi/mmol was the minimum necessary to produce significant results. *RS*-[2-^{3}H]glycine (**49a**) is commercially available at high specific activity (20 Ci/mmol) and exchange of one of these tritiums with a proton should produce *S*-[2-^{3}H]glycine (**50a**) with a specific activity of 10 Ci/mmol.

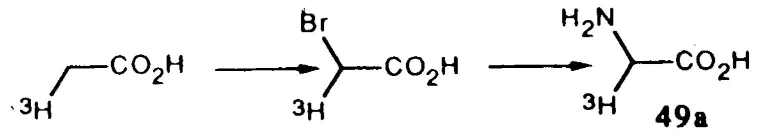
Commercially available [^{3}H]-H_2O is 18 mCi/mmol (1 Ci/mL), and since this activity is for two hydrogens the highest specific activity attainable in the *R*-[2-^{3}H]glycine (**51a**) is 9 mCi/mmol, close to the estimated minimum required.

The glycines were prepared by a modification of the procedures described.[169,172] In this way 41 µCi of *R*-[2-^{3}H]glycine (**51a**) was prepared from an incubation of 10 µmol of glycine with glutamic-pyruvic transaminase in [^{3}H]-H_2O for 3 days at 37 °C. If 100% of the glycine is recovered the specific activity is 4.1 mCi/mmol, and if the specific activity is the highest theoretically possible (9 mCi/mmol) the yield of the glycine is 46%. For the preparation of *S*-[2-^{3}H]glycine (**50a**) the tritium of *RS*-[2-^{3}H]glycine must be exchanged with H_2O. To allow determination of the complete exchange of the tritium, the *RS*-[2-^{3}H]glycine (**49a**) was combined with [1,2-^{14}C]glycine and the ^{3}H/^{14}C ratio (dpm ^{3}H/dpm ^{14}C), which is expected to decrease to half of the starting value, was monitored. The initial ratio was 24, after 2 h of incubation the ratio had dropped to 23, after 6 h to 19, after 19 h to 13, and after 21 h to 12. The reaction was stopped at this point and the glycine was isolated in 62% yield. This glycine was determined to have a ^{3}H/^{14}C ratio of 12. Further treatment of this glycine with glutamic-pyruvic transaminase lowered the ratio to 10 after 10 h and to 9.5 after 14 h, at which point the *S*-[2-^{3}H]glycine (**50a**) was isolated in 54% yield.

The excessive, continual loss of tritium observed may be due to a slow, non-enzymatic pyridoxal 5'-phosphate catalyzed exchange of the glycine.[178] In the case of the *R* isomer, where the tritium is exchanged into the glycine from the water, this non-enzymatic exchange would be insignificant since the tritium compromises less than 0.05% of the available hydrogens from the water and therefore only 1 out of every 2000 exchanges would be observable as an increase in the amount of tritium in the glycine. However, in the preparation of the *S* isomer, where the tritium is being exchanged with a protium from the water, every exchange removing a tritium from the glycine is recorded, resulting in an apparent non-enzymatic rate 2000 times that observed in the preparation of the *R* isomer.

The possibility that the *RS*-[2-^{3}H]glycine (**49a**) supplied is a non-racemic mixture (with an excess of the *R* isomer) was excluded upon communication with the supplier (ICN Radiochemicals) who outlined a synthetic route illustrated in Scheme 39.

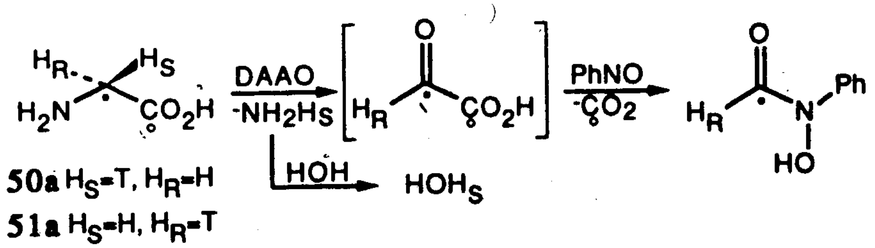
Scheme 39.



Determination of the Stereochemical Purity of [2-^{3}H]Glycines

The literature method for determining the stereochemical purity of [2-^{3}H]glycines is based upon treatment with commercially available D-amino acid oxidase (DAAO) which catalyzes the formation of glyoxylic acid by oxidation of glycine with stereospecific removal of the pro-*S* hydrogen.[171,179] The hydrogen or tritium is released into the water which is isolated and analyzed for tritium content. Two major problems with this method are that *quantitative* trapping of the water by lyophilization is required, and that the glycine must be entirely oxidized by the DAAO.[179] This latter problem is complicated by the low activity of DAAO with glycine as a substrate.[171,179,180]

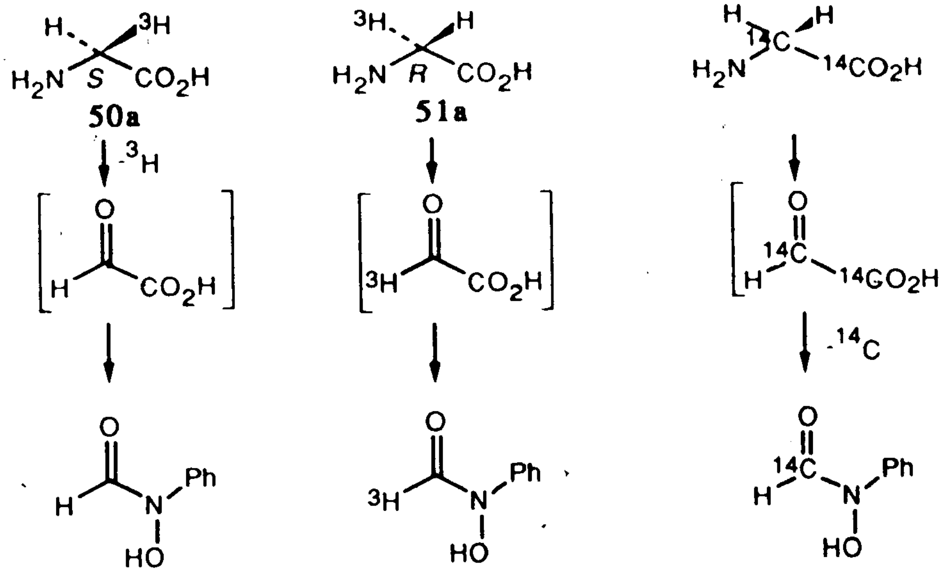
Scheme 40.



Both of these problems can be solved by analyzing the glyoxylic acid formed. If the tritiated glycine is treated with DAAO in the presence of ^{14}C-glycine, and the glyoxylate produced is isolated, the ^{14}C serves as an internal standard which eliminates both the problem of requiring complete oxidation of the glycine and difficulties associated with quantitatively isolating the ^{3}H-water. Of course this assumes that any isotope effects can be determined. Trapping of the glyoxylic acid with nitrosobenzene provides a convenient method for isolating the hydrogen and carbon from C-2 of the glyoxylic acid formed from oxidation of glycine. The details for the adaptation of the DAAO assay[179] for trapping the glyoxylate with nitrosobenzene are provided in the experimental section.

The analysis of the ^{3}H/^{14}C ratios obtained is not at first obvious. Scheme 41 illustrates the results of treatment of the *R*- and *S*-[2-^{3}H]glycines **51a** and **50a** with DAAO and trapping the glyoxylic acid with nitrosobenzene. For the *R*-[2-^{3}H]glycine (**51a**), the carbon-proton bond is cleaved and the resulting NHF retains the ^{3}H. For the *S*-[2-^{3}H]glycine (**50a**), however, the carbon-tritium bond is cleaved and the resulting NHF is not radioactive. This is crucial since it means that the tritium remaining in the NHF from the analysis of a mixture of **51a** and **50a** is *entirely* from the *R*-[2-^{3}H]glycine (**51a**), the amount of *S*-[2-^{3}H]glycine (**50a**) being reflected only in the initial ^{3}H/^{14}C ratio. For the ^{14}C-glycine, initially a ^{14}C-proton bond is cleaved to give the ^{14}C glyoxylate which during reaction with the nitrosobenzene loses one of two ^{14}C atoms. Therefore, in the analysis of a mixture of *R*-[2-^{3}H]glycine (**51a**) and ^{14}C-glycine, all of the initial tritium is retained in the NHF but half of the ^{14}C is lost causing the final ^{3}H/^{14}C ratio to be *twice* the initial ratio. For the case of pure *S*-[2-^{3}H]glycine (**50a**), however, none of the tritium is carried through in the NHF, half of the ^{14}C is lost, and the final ratio is therefore zero. In the analysis of a mixture of all three labelled glycines, the tritium from the *S* isomer is lost, the tritium from the *R* isomer is retained, and half of the ^{14}C is lost. For a mixture of racemic *RS*-[2-^{3}H]glycine (**49a**) and ^{14}C-glycine, the final ^{3}H/^{14}C ratio will be *equal* to the starting ratio since half the tritium (from the *S* isomer) and half of the ^{14}C is lost. For calculating the %*R* isomer, the final ^{3}H/^{14}C ratio is therefore divided by twice the initial ratio. It should be noted that the primary tritium isotope effect is transparent to the analysis because the rate of oxidation of the *S* isomer, in which a carbon-tritium bond is cleaved, does not affect the final $^{3}H/^{14}C$ ratio since the product of this reaction is not radioactive.

Scheme 41.



The results of the analysis with DAAO of stereospecifically tritiated glycine prepared from enzymatic exchange are summarized in Table 16 and indicate that the *R* isomer **51a** is obtained in higher stereochemical purity than the *S* isomer **50a**. This is expected for incomplete exchange with solvent in the enzymatic preparation of these materials. Of more concern was the decrease in the ratio observed in the analysis of the *RS* isomer **49a**. Again the synthetic method used to prepare this material provided insight into these results. Communication with the supplier (ICN Radiochemicals) outlined the synthetic route as illustrated in Scheme 42. From this, and the high specific activity of **49a** (20 Ci/mmol, ~29 Ci/mmol is equivalent to one tritium atom per molecule), it can be concluded that the commercial **49a** is a mixture of mono-tritiated [illegible]S-[2-$^{3}H_{1}$]glycine and di-tritiated [2-$^{3}H_{2}$]glycine. This would be expected to alter the analysis with DAAO because the [2-$^{3}H_{2}$]glycine material would count as two tritiums in the initial ratio and would be slow to be oxidized because of a primary isotope effect, resulting in a decrease in the final $^{3}H/^{14}C$ ratio.

Table 16. Stereochemical Purity of Enzymatically Prepared [2-^{3}H]Glycines.[a]

	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$	% s.p.[b]
S-glycine[c] **50a**				
initial	338971	27263	12.4±0.12	
product	5568	1535	3.62±0.10	85
control	131	37		
S-glycine[d] **50a**				
initial	255274	27179	9.39±0.14	
product	4801	1772	2.71±0.03	86
control	135	42		
R-glycine **51a**				
initial	683471	84809	8.06±0.11	
product	32434	2099	15.5±0.25	96
control	79	55		
RS-glycine **49a**				
initial	319160	21095	15.2±0.05	
product	15121	1329	11.4±0.09	
control	431	38		
initial	235604	24140	9.76±0.05	
product	20445	2476	8.26±0.06	
control	206	40		

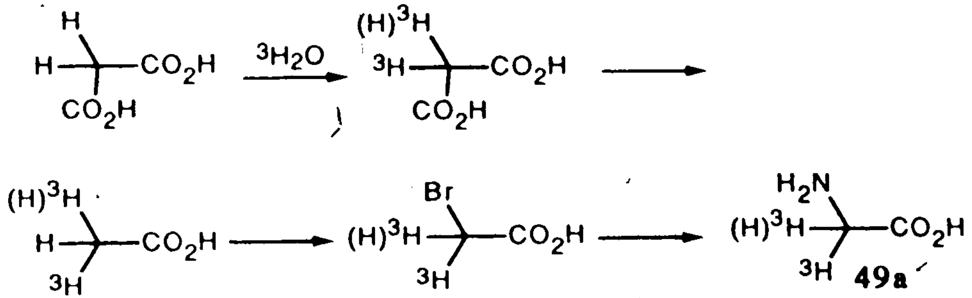
[a] These experiments were performed in triplicate, average values are reported.

[b] The % stereochemical purity (s.p.) is the percent of the major isomer.

[c] After first incubation with glutamic-pyruvic transaminase.

[d] After second incubation with glutamic-pyruvic transaminase.

Scheme 42.



Preparation of D-TyrVal[2-^{3}H]Gly from Enzymatically Prepared [2-^{3}H]Glycines

The *RS*-[2-^{3}H]glycine **49a**, *S*-[2-^{3}H]glycine **50a**, and *R*-[2-^{3}H]glycine **51a** were transformed to the corresponding tripeptides **52a**, **53a**, and **54a** as described for the preparation of ^{14}C labelled tripeptide (Scheme 43). The results of the synthesis are summarized in Table 17. The overall yields during the synthesis are 16% for the *R* and *S* isomers and 23% for the *RS* isomer. For the *S* and *RS* isomers the ^{3}H/^{14}C ratio was followed and a significant decrease in the amount of tritium was observed in each case. The *R* isomer was synthesized without the addition of ^{14}C label.

Scheme 43.

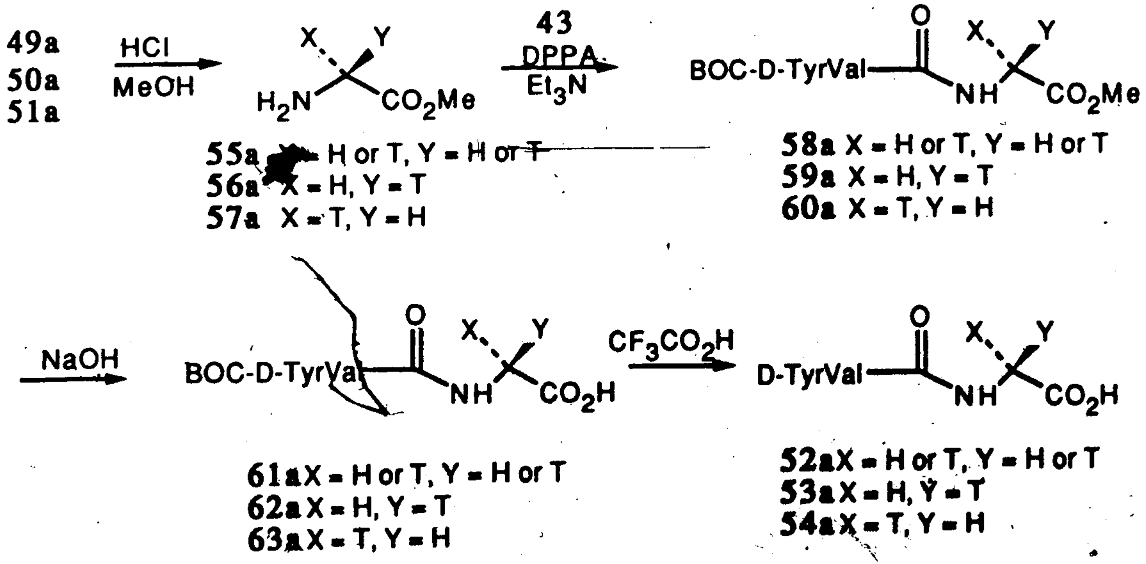


Table 17. Synthesis of D-TyrVal-[2-^{3}H]Gly from [2-^{3}H]Glycines 49a, 50a, and 51a.

	^{3}H(μCi)	^{14}C(μCi)	yield(%)^{a}	isotope ratio	δ isotope ratio(%)
D-TyrVal-*R*-[2-^{3}H]Gly **54a**					
51a	36				
60a	22		60		
54a	5.8		26		
D-TyrVal-*S*-[2-^{3}H]Gly **53a**					
50a	11.6	1.2		9.39±0.14	
56a	11.0	1.2	>99	9.4	
59a	2.1	0.23	19	9.2	
62a	1.7	0.19	83	8.9	
53a	1.6	0.19	>99	7.55±0.11	-20
D-TyrVal-*RS*-[2-^{3}H]Gly **52a**					
49a	50.	3.8		13.	
58a	11.5	1.1	29	10.8	
52a	9.7	0.87	79	9.55±0.16	-25

^{a}This is the yield from the previous indicated intermediate.

The peptides obtained were not purified further but were treated directly with PAM. The assay was exactly as for the ^{14}C tripeptide except that the respective radioactive solutions (20-90 μL) were placed into the assay tubes and lyophilized before the enzyme and cocktail were added. The results summarized in Table 18 were disappointing. The ^{14}C dpm clearly show that the *RS* isomer **52a** was oxidized in reasonable quantities, the *S* isomer **53a** was converted in low quantities (but higher than control experiments), while the *R* isomer **54a** failed to produce radioactivity above that observed in control experiments. There are several possible explanations for this outcome. The specific activity of the *R* isomer **54a** might be too low, but that of the *S* isomer **53a** should have been approximately 40% of the *RS* isomer **52a** (based on the change in $^3H/^{14}C$ ratio during the preparation of **50a**) or double the values observed with PAM for **53a**. Alternatively, the *R*- and *S*-[2-^{3}H]glycines **51a** and **50a** may have been contaminated with non-radioactive amino acids from the enzymatic reaction and these amino acids would be converted to tripeptides which display an inhibitory effect on PAM.[163] Both of these problems can be solved by a purely chemical synthesis of [2-^{3}H]glycines **51a** and **50a** (i.e. no enzymatic transformations) and this was undertaken.

Table 18. Incubation of PAM with D-TyrVal-[2-^{3}H]Gly Derived from 49a, 50a, and 51a.[a]

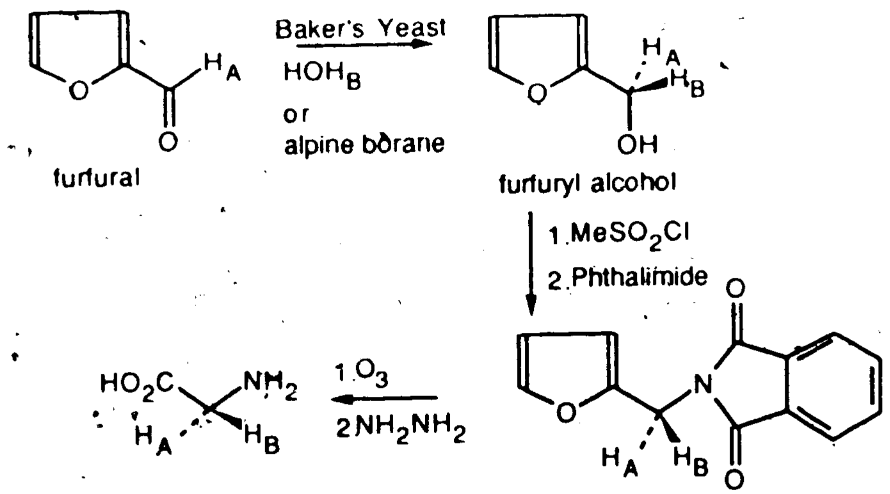
	^{3}H (dpm)	^{14}C (dpm)	$^3H/^{14}C$
D-TyrVal-*RS*-[2-^{3}H]Gly **52a**			
initial	781779	81907	9.55±0.16
product	13888±960	2051±136	6.77±0.04
control	3187	450	7.08
D-TyrVal-*S*-[2-^{3}H]Gly **53a**			
initial	610998	80952	7.5[illegible]±0.11
product	2895±131	622±41	4.67±0.30
control	1476	282	5.23
D-TyrVal-*R*-[2-^{3}H]Gly **54a**			
initial	894989	82619	10.8±0.24
product	1347±101	187±10	7.47±0.21
control	890	147	6.07

[a]The experiments were performed in triplicate, average results are reported.

Chemical Synthesis of Stereospecifically Labelled Glycine

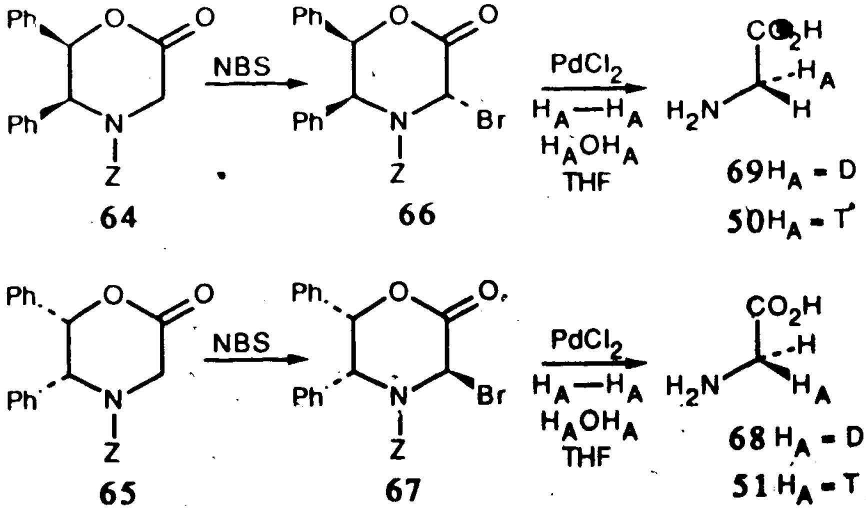
A purely chemical synthesis of tritiated glycine was sought which would avoid possible contamination from other amino acids and would give glycine of high specific activity. At the onset of this work the most direct and shortest synthesis published started from furfural (Scheme 44).[177] However, the tritium is introduced in the first step, in the case of the *R* isomer the label is introduced from water limiting the specific activity obtainable, and the use of yeast has the potential of introducing contaminating amino acids.[177] Furthermore, although it had been used to prepare deuterated glycines, there were no reports of its use in the preparation of tritiated glycines. A modification suggested to us by Dr. Ronald Woodard (University of Michigan, personal communication) used Midland's alpine borane (1*R*- or 1*S*-α-pinene and 9BBN) method[181] for the stereospecific reduction of furfural. This has the advantages of potentially producing both enantiomers of furfuryl alcohol in high specific activity and of avoiding the use of yeast. However Dr. Heinz G. Floss (Ohio State University, personal communication) reported difficulties in the preparation and use of tritiated alpine borane. As a result the preparation of tritiated furfural would be necessary thereby adding several steps to the synthesis.

Scheme 44.



Williams *et al.* have recently reported a synthesis of stereospecifically deuterated glycines which introduces the label in a final catalytic reduction (Scheme 45).[170] The preparation of each of the starting materials **64** and **65** requires several steps,[10] but a generous gift from Dr. Robert M. Williams (Colorado State University) made available gram quantities of each enantiomer. The use of tritium gas in reasonable quantities requires special facilities. The National Tritium Labeling Facility at the Lawrence Berkeley Laboratories is available for the use of pure (100%) tritium gas under safe, monitored conditions. Researchers can travel to the facilities to conduct experiments using pure (100% $^3H_2$) tritium gas. This results in the production of materials of high specific activity (~29 Ci/mol/tritium incorporated). The published procedure[170] for the preparation of [2-^{2}H]glycines was not optimized for use with tritium gas for several reasons. These facilities are not equipped for reductions above atmospheric pressure, and the length of the reaction cannot exceed 5 h for safety monitoring reasons. Thus the 40 psi, 40 h reaction conditions needed to be modified.[170]

Scheme 45.

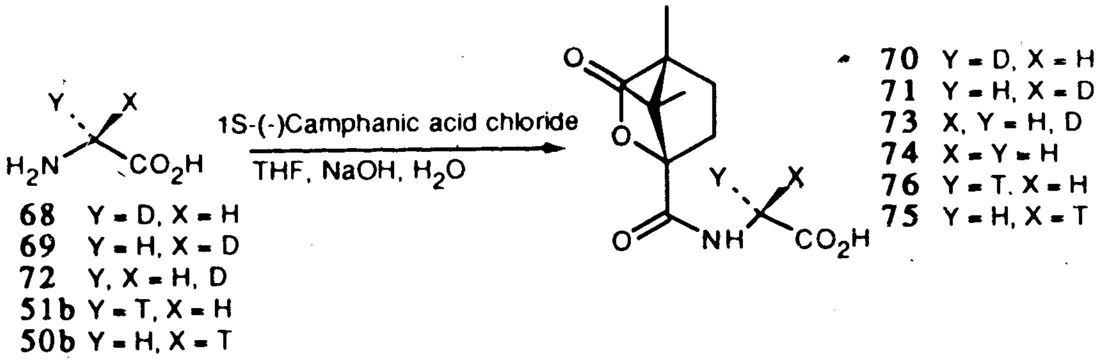


The formation of the bromides **66** and **67**,[10,170] which are unstable and used directly without purification, was difficult to monitor until a tlc system was found. Initially an

EtOAc/hexane mixture was used but this was unsuitable because of solubility problems. The use of 20:0.4 $CHCl_3$:MeOH gave better results. Also the *N*-bromosuccinimide (NBS) must be highly purified[182] and stored in the dark over P_2O_5 for reproducible results; even then the formation of **66** and **67** takes varying amounts of time from 15 min to 1.5 h. However, once these technical difficulties were overcome the reaction was very convenient, and the bromide could be prepared and stored at -20 °C if moisture was excluded.[10]

The formation of glycine was monitored by tlc and hplc.[183] The hplc system used a Water's μ-Bondapak NH_2 column and an acetonitrile/phosphate buffer gradient which allows for the detection of amino acids without derivatization (see general experimental).[183] The stereochemical purity of the *R*-[2-^{2}H]glycine (**68**) and *S*-[2-^{2}H]glycine (**69**) was determined by conversion to the corresponding 1*S*-(-)-camphanamide derivatives **70** and **71** (Scheme 46), which were analyzed by ^{1}H nmr and ci-ms.[170,173] *RS*-[2-^{2}H]glycine (**72**) was prepared from $NaBD_4$ reduction of glyoxylic acid in aqueous ammonia[184] and converted to the 1*S*-(-)-camphanamide derivative **73**. Figure 4 illustrates the ^{1}H nmr spectrum of **70**, **71**, **73**, and glycine 1*S*-(-)-camphanamide (**74**).[170,173]

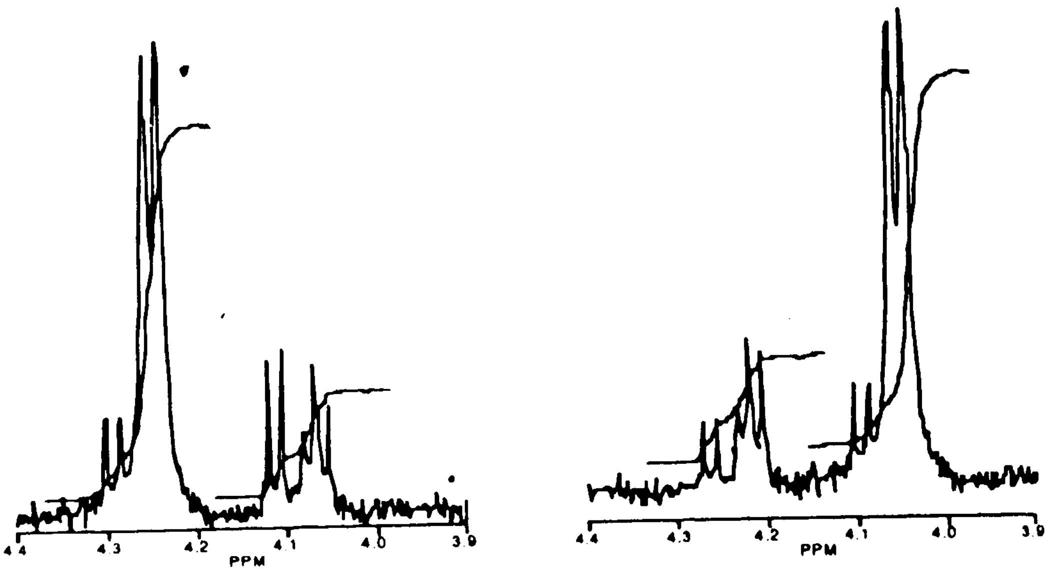
Scheme 46.



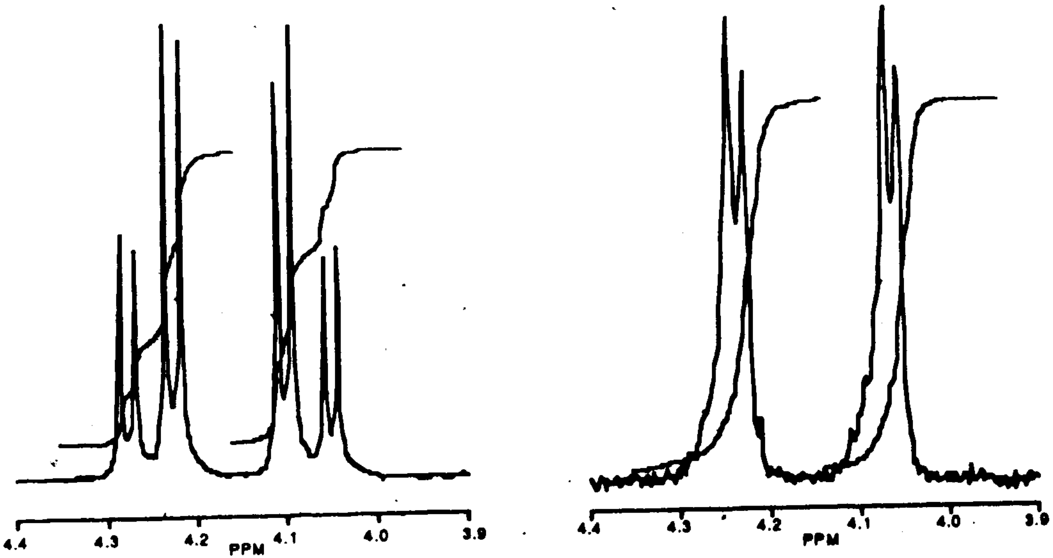
68 Y = D, X = H
69 Y = H, X = D
72 Y, X = H, D
51b Y = T, X = H
50b Y = H, X = T

70 Y = D, X = H
71 Y = H, X = D
73 X, Y = H, D
74 X = Y = H
76 Y = T, X = H
75 Y = H, X = T

Figure 4. 360 MHz ^{1}H nmr Spectra of [2-^{2}H]Glycine (1*S*)-(-)-Camphanamides.



R-[2-^{2}H]glycine 1*S*-(-)-camphanamide 70   *S*-[2-^{2}H]glycine 1*S*-(-)-camphanamide 71



glycine 1*S*-(-)-camphanamide 74   *RS*-[2-^{2}H]glycine 1*S*-(-)-camphanamide 73

The conditions for the reduction of bromides 66 and 67 were modified.[170] It was quickly established that increasing the amount of $PdCl_2$ from 0.3 eq. to 1.0 eq. produced good yields (40-60%) of glycine in 4 h under 1 atm of deuterium gas. Table 19 summarizes the results obtained from reduction of 66 and 67 under various conditions. The ratio of

THF to water is critical in obtaining reasonable yields of glycine (entries 1-4, Table 19), and in the absence of water the reaction fails (entry 1, Table 19). Of particular importance is the observation that the isotope incorporated into the glycine is derived from the water (entries 5-7, Table 19). Reduction with deuterium gas in H_2O produces glycine with only 11.7% deuterium (entry 5, Table 19), whereas reduction with hydrogen gas in ^2H_2O forms glycine with 81.4% deuterium (entry 7, Table 19). The stereochemical purity of the resulting glycine is not influenced by the change from deuterium to hydrogen gas (entries 6 and 7, Table 19). When the volume of the solvent was reduced, and the THF/water ratio held constant, the reaction either failed (entry 2, Table 19) or produced glycine of low stereochemical purity (entry 8, Table 19). The reasons for this are not known. All of the reductions produced less than 1% of dideuterated glycine except entry 8 (Table 19, 1.7%).

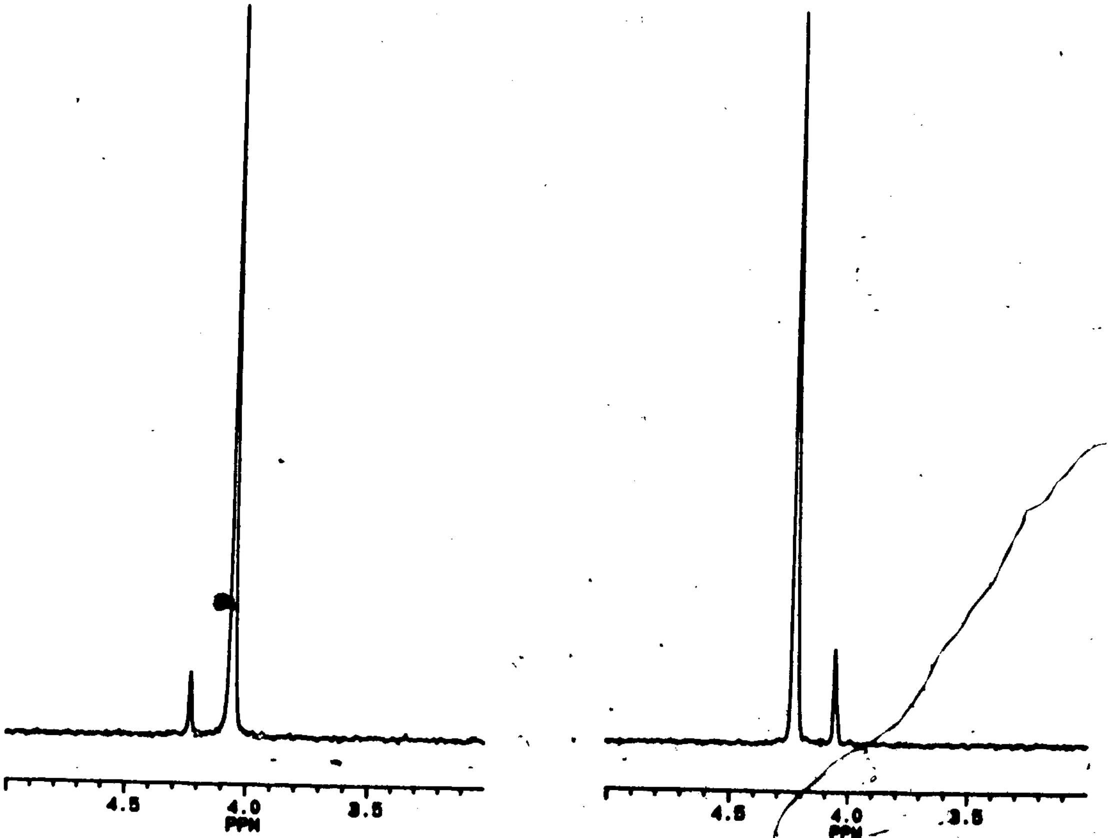
Table 19. Catalytic Reductions of 66 and 67.

	Reduction Conditions						Glycine Formed				
		μmol	THF (mL)	X_2O (mL)	X	Y_2	yield (%)	%H_2	%$^2H_1$	%s.p.[a]	Glycine
1	**67**	121	3	0	n.a.	$^2H_2$	<1				
2	**67**	79.0	0.4	0.07	^2H	$^2H_2$	<5				
3	**67**	118	3	0.10	^2H	$^2H_2$	<5				
4	**66**	94.9	2	0.07	^2H	$^2H_2$	<5				
5	**67**	139	3	0.50	H	$^2H_2$	49	88.2	11.7	n.d.	*R* **68**
6	**67**	106	2	0.35	^2H	$^2H_2$	49	12.8	87.1	89.2	*R* **68**
7	**67**	127	3	0.50	^2H	H_2	45	18.2	81.4	90.5	*R* **68**
8	**66**	95.9	0.4	0.07	^2H	$^2H_2$	42	16.6	81.6	72.7	*S* **69**
9	**66**	109	3	0.50	^2H	$^2H_2$	42	14.6	84.5	89.0	*S* **69**
10	**67**	114	3	0.55	^3H	$^3H_2$	31			92.7	*R* **51b**
11	**66**	111	3	0.55	^3H	$^3H_2$	38			88.2	*S* **50b**

[a] **Stereochemical purity (s.p.) is the percent of the major isomer as determined from integration of the nmr signals of the 1*S*-(-)-camphanamide derivatives.**

At this point the bromides **66** and **67** were brought to the National Tritium Labeling Facility where they were hydrogenated with the assistance and supervision of Dr. Hiromi Morimoto. When tritium gas (100% $^3H_2$) and [^{3}H]-H_2O (50 Ci/mL, 0.9 Ci/mmol) was used the [2-^{3}H]glycines **50b** and **51b** were obtained in good yield and stereochemical purity (entries 10 and 11, Table 19) after purification by hplc.[183] The stereochemical purities of the *R*- and *S*-[2-^{3}H]glycine **50b** and **51b** were determined by conversion to the corresponding 1*S*-(-)-camphanamides **75** and **76** (Scheme 46) and analyzed by ^{3}H nmr (Figure 5). The 320 MHz ^{1}H-decoupled ^{3}H nmr spectra were obtained by Dr. Philip G. Williams at the National Tritium Labeling Facility.

Figure 5. ^{1}H decoupled 320 MHz ^{3}H nmr Spectra of *S*- and *R*-[2-^{3}H]Glycine 1*S*-(-)-Camphanamides.



R-[2-^{3}H]glycine 1*S*-(-)-camphanamide **76**

S-[2-^{3}H]glycine 1*S*-(-)-camphanamide **75**

The stereochemical purities of tritiated glycines **50b** and **51b** were also analyzed by the D-amino acid oxidase procedure. Incubation with this enzyme and trapping of the glyoxylate as NHF gave the results summarized in Table 20. The [2-^{3}H]glycines **50b** and **51b** were mixed with [1,2-^{14}C]glycine and purified by hplc.[183] For the *S* isomer **50b** there is good agreement between the results obtained with DAAO and those from ^{3}H nmr (Table 19), however for the *R* isomer **51b** there is a difference of 8% when the ratios are automatically determined by the instrument. The reason for this difference appears to be due to instrument limitations in determining high ^{3}H/^{14}C ratios and therefore the *R* and *S* ^{3}H/^{14}C ratios were confirmed (Table 20, values in parentheses) by adding ^{3}H- and ^{14}C-toluene standards as described in the experimental section. When these values are used to determine the % stereochemical purity, the results agree within 5% of the those obtained from tritium nmr (Table 19). A future consideration in performing these experiments might be to keep the initial ratios at 5, especially for the *R* isomer **51b** .

Also included in Table 20 are the results from treating racemic [2-^{3}H]glycine with DAAO. For the commercially available material **49a** a 17% decrease in the ^{3}H/^{14}C ratio was observed as in previous experiments. However, for the mono-tritiated *RS*-[2-^{3}H$_1$]glycine **49b**, prepared by Hengmiao Cheng from [^{3}H]NaBH$_4$ reduction of glyoxylic acid in ammonia as for **72**, the ratio remains the same within experimental error (4% increase) in agreement with the hypothesis that the decrease in ^{3}H/^{14}C ratios observed with the commercial material is a result of the presence of di-tritiated [2-^{3}H$_2$]glycine. These results confirm that the DAAO procedure based on the trapping of glyoxylate as NHF is a useful method for determining the stereochemistry of [2-^{3}H]glycines and the results obtained are in close (5%) agreement with the stereochemical purity determined by ^{3}H nmr spectrometry.

Table 20. Stereochemical Purity of [2-^{3}H]Glycines as Determined with DAAO.[a]

	^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C[b]	% s.p.[b,c]
S-[2-^{3}H]Glycine[d] **50b**				
initial	1045597	106239	9.8422±0.037 (10.559±0.246)	
product	7811	3008	2.6652±0.048 (2.8070±0.065)	86.4±0.3 (85.7±0.3)
control	434	58		
R-[2-^{3}H]Glycine[d] **51b**				
initial	867146	101843	8.5970±0.183 (8.8020±0.077)	
product	41123	2841	14.534±0.122 (15.394±0.163)	84.5±0.7 (87.5±0.9)
control	238	39		
RS-[2-^{3}H]Glycine **49a**				
initial	308678	50943	6.0597±0.016	
product	16747	3301	5.0732±0.024	
control	581	76		
RS-[2-^{3}H]Glycine **49b**[d]				
initial	945461	116144	8.1399±0.011	
product	57253	6737	8.4973±0.062	
control	1082	97		

[a] These experiments were performed in triplicate and the average values are reported.

[b] Values in parentheses are those obtained by adding ^{14}C- and ^{3}H-toluene standards.

[c] % Stereochemical purity (s.p.) is the percent of the major isomer.

[d] These experiments were performed by Hengmiao Cheng.

The *RS*-[2-^{3}H]glycine (**49b**), *S*-[2-^{3}H]glycine (**50b**), and *R*-[2-^{3}H]glycine (**51b**) were used to synthesize the corresponding D-TyrVal-[2-^{3}H]Gly **52b**, **53b**, and **54b** as previously described for the glycines prepared from enzymatic exchange (Scheme 47). The results of these syntheses are summarized in Table 21. The overall yield for the synthesis of the *RS* isomer **49b** was 12%, for the *S* isomer **50b** was 8%, and for the *R* isomer **51b** was 1%. The ^{3}H/^{14}C ratio was followed in an attempt to determine if any tritium was lost during the synthesis as a possible indication of epimerisation. It is difficult to draw conclusions on the individual steps in the synthesis, but in each case the final ratio was lower than the ratio at the start of the synthesis. The overall decrease in the ^{3}H/^{14}C ratios during the synthesis is 3% for *RS*-[2-^{3}H]glycine (**49b**), 5% for *S*-[2-^{3}H]glycine (**50b**), and 19% for *R*-[2-^{3}H]glycine (**51b**). The slight increases in the ^{3}H/^{14}C ratio observed in the first step of the synthesis using the *R* and *S* isomers **51b** and **50b** is probably a result of ^{14}C impurities in the [1,2-^{14}C]glycine.

Scheme 47.

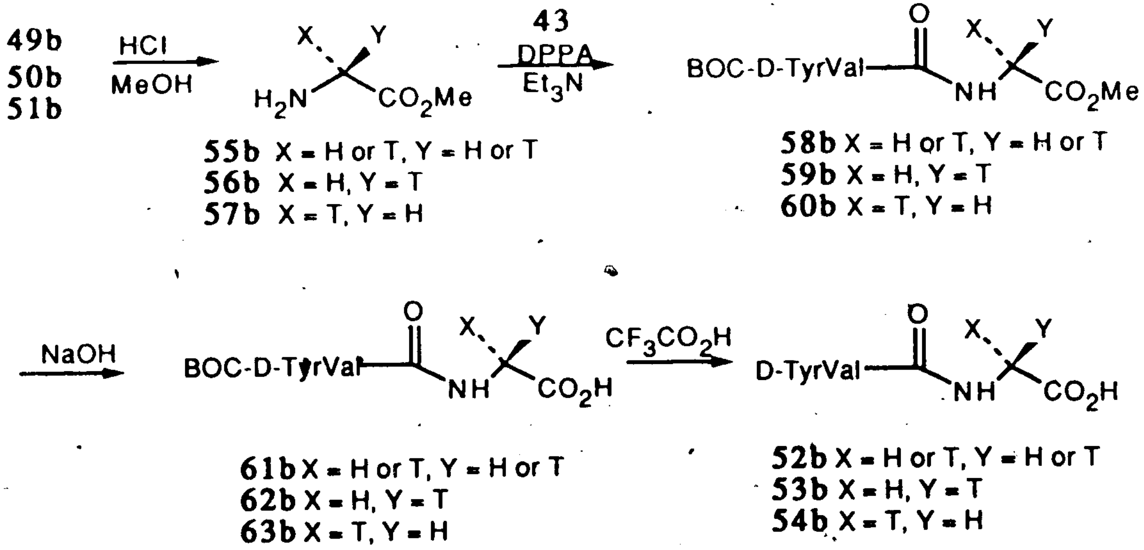


Table 21. **Syntheses of D-TyrVal-[2-^{3}H]Gly from [2-^{3}H]Glycines 49b, 50b, and 51b.**

	^{3}H(μCi)	^{14}C(μCi)	yield(%)	isotope ratio	δisotope ratio(%)
D-TyrVal-*R*-[2-^{3}H]Gly **54b**					
51b	923	49.8		18.629±0.056	
57b	513	26.9	54	19.061±0.105	+2.3
60b	17.7	0.93	3.5	19.212±0.116	+0.79
63b	9.1	0.56	60	16.179±0.200	-15.8
54b	7.9	0.52	93	15.215±0.154	-6.0
D-TyrVal-*S*-[2-^{3}H]Gly **53b**					
50b	848	50.8		16.702±0.148	
56b	647	37.0	73	17.521±0.301	+4.9
59b	83.8	4.9	13.2	16.935±0.098	-3.3
62b	69.4	4.3	88	16.159±0.106	-4.6
53	65.2	4.1	96	15.889±0.063	-1.7
D-TyrVal-*RS*-[2-^{3}H]Gly **52b**[a]					
49b	430	52.8		8.1399±0.011	
55b	296	36.8	70	8.0581±0.040	-1.0
58b	196	24.7	67	7.9301±0.021	-1.6
61b	163	21.4	87	7.6161±0.038	-4.0
52b	185	24.4	>99	7.6081±0.017[b]	-0.1

[a] These experiments were performed by Hengmiao Cheng.

[b] When this was purified by hplc the ratio was 7.9287±0.031

Peptides **53b** and **54b** were each combined with ^{14}C tripeptide **46** and the mixture was purified by hplc on a C$_{18}$ column (see general experimental).[137] These were treated with PAM. The results in Table 22 show that with the *S* isomer **53b** the ^{3}H/^{14}C ratio *decreases* and with the *R* isomer **54b** the ratio *increases*, but these do not go to the values expected based on the analysis of the [2-^{3}H]glycines **50b** and **51b** with DAAO (Table 20). The two possible explanations for this are that PAM is not completely stereospecific or that the glycine has epimerised during the synthesis of D-TyrVal-[2-^{3}H]Gly. Since it is unlikely that this enzyme would be only partially stereospecific,[185] the extent to which the glycine residue may have epimerised during the synthesis of the tripeptide was investigated.

Table 22. Incubation of D-TyrVal[2-^{3}H]Gly 52b, 53b, and 54b with PAM.[a]

		^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C
D-TyrVal-*S*-[2-^{3}H]Gly **53b**				
	initial	344315	76962	4.4750±0.026
	product	6716	1960	3.4270±0.068
	control	85	29	
D-TyrVal-*R*-[2-^{3}H]Gly **54b**				
	initial	203208	57383	3.5410±0.009
	product	5247	1157	4.5369±0.021
	control	90	28	
D-TyrVal-*RS*-[2-^{3}H]Gly **52b**[b]				
	initial	859860	108400	7.9287±0.031
	product	35224	4661	7.5467±0.143
	control	1601	210	

[a]These experiments were performed in triplicate, the average results are reported.
[b]These experiments were performed by Hengmiao Cheng.

The peptides were hydrolyzed to the amino acids by heating in constant boiling (5.7M) HCl in a sealed tube at 115 °C for 14 h.[1,186] The glycine was isolated by a two fold hplc purification (C_{18} column, retention time 1.8 min, followed by NH_2 column, see general experimental). The initial C_{18} column serves to separate the glycine from L-ValGly, a product of incomplete hydrolysis of D-TyrValGly, which co-elutes with glycine on the NH_2 column but is well separated on the C_{18} column. The isolated *S* and *R* glycines **50c** and **51c** were treated with DAAO and the results (Table 23) clearly show that the glycine has epimerised during the synthesis of D-TyrVal-[2-^{3}H]Gly. The possibility of racemization during the hydrolysis of the peptide cannot be excluded but the $^3H/^{14}C$ ratios of peptides **53b** and **54b** and the corresponding glycines **50c** and **51c** are similar (5-8% decrease on hydrolysis). Also the changes in the isotope ratio observed in the DAAO analysis of **50c** and **51c** obtained after hydrolysis of **53b** and **54b** are almost identical to those seen from the treatment of the peptides with PAM, thereby indicating that PAM and DAAO [illegible] stereospecifically remove the same (pro-*S*) methylene proton.

Table 23. Stereochemical Purity of [2-^{3}H]Glycines 50c and 51c Obtained After Hydrolysis of D-TyrVal-[2-^{3}H]Gly.[a]

	^{3}H (dpm)	^{14}C (dpm)	$^3H/^{14}C$	% s.p.[b]
S-[2-^{3}H]glycine **50c**				
initial	389824	92304	4.2234±0.0[illegible]4	
product	15178	5373	2.8270±0.028	66.5±0.3
control	512	117		
R-[2-^{3}H]glycine **51c**				
initial	188080	57744	3.2568±0.011	
product	17779	4023	4.4176±0.037	67.8±0.6
control	708	193		

[a] The experiments were performed in triplicate, average results are reported.
[b] % Stereochemical purity (s.p.) is the percent of the major isomer.

These incubations of PAM with stereochemically enriched D-TyrVal-*R*-[2-^{3}H]Gly (**54b**) and D-TyrVal-*S*-[2-^{3}H]Gly (**53b**) show that PAM stereospecifically removes the pro-*S* hydrogen from the glycine methylene group during oxidation. Enzymes generally display a high degree of stereospecificity.[185] Dopamine β-hydroxylase, to which PAM is closely related in terms of cofactor requirements (oxygen, ascorbic acid, copper) and probably mechanism (i.e. hydroxylation on a methylene carbon), also shows complete stereospecificity during the oxidation of dopamine to norepinephrine.[187,188]

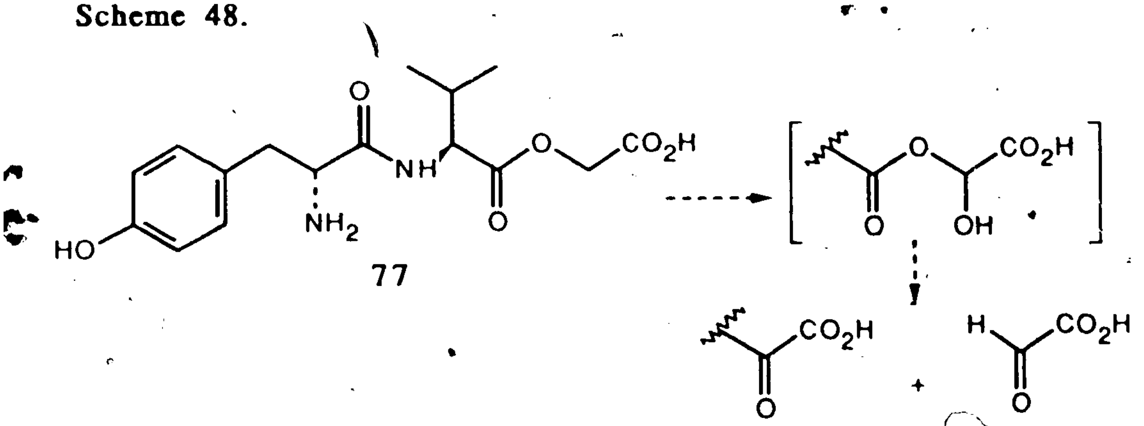
It is interesting to note that PAM removes the pro-*S* hydrogen which is only available in the non-proteinogenic D-amino acids, not in the L-amino acids. This could explain the specificity observed for glycine over other amino acids[148] since all other L-amino acids have a substituent at this position. No studies have been done with the carboxyl terminal glycine of a PAM substrate replaced by a D-amino acid which has available the "pro-*S*" hydrogen for oxidation. Since peptides containing D-amino acids are not readily hydrolyzed[1,89,129] in the body this may present a means of generating pro-drugs for treatment of peptide hormone imbalances. The extension of a target peptide amide hormone by a carboxy terminus D-amino acid would produce a peptide which may be a substrate for PAM and thereby converted to the desired hormone but which is stable against the normal degradative enzymes.[1] However, it remains to be determined if PAM oxidizes peptides which end in a D-amino acid residue.

SYNTHESIS OF GLYCOLATE PEPTIDE

To investigate further the mechanism of the PAM oxidation, the synthesis of a modified substrate was attempted. We reasoned that if PAM converted a modified peptide in which the glycine residue has been replaced by glycolic acid, to glyoxylic acid and the dipeptide (Scheme 48), this would be further evidence that the oxidation proceeds on the methylene carbon (Scheme 31, c) and not by direct dehydrogenation or oxidation involving the amide nitrogen (Scheme 31, a or b).

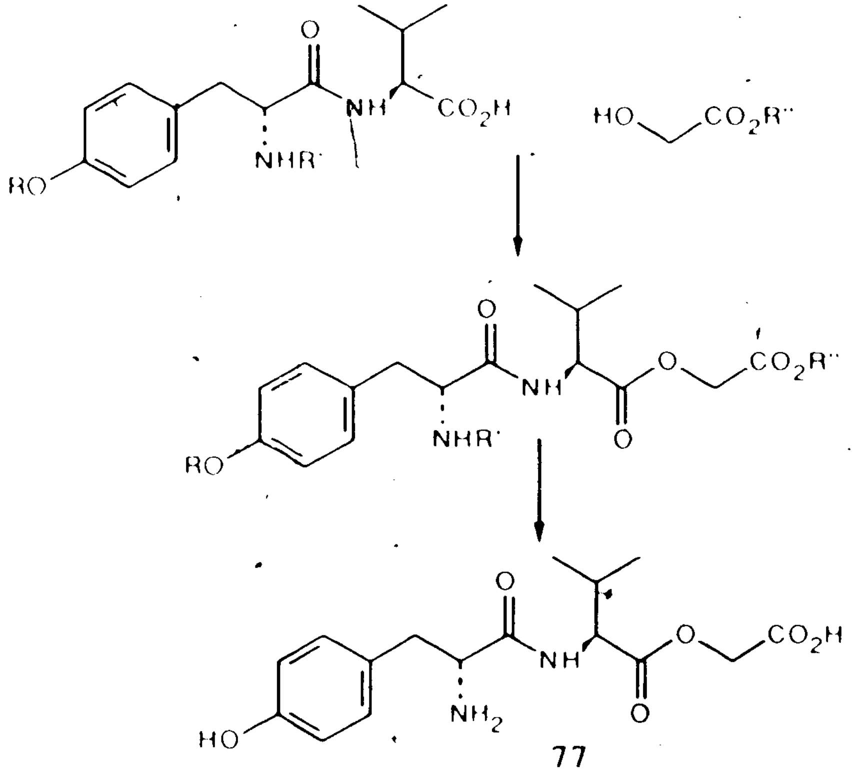
In the synthesis of this modified peptide 79 similar strategy was employed as was used in the preparation of the D-TyrValGly. Coupling of a protected glycolate ester with a suitably protected derivative of D-TyrVal followed by the necessary deprotections should give the desired product (Scheme 49). However 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. Furthermore, 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.

Scheme 48.

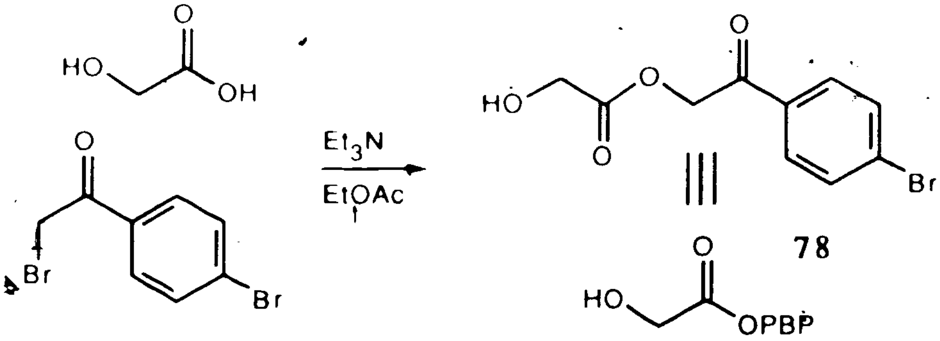


The choice of protecting groups was crucial. The BOC group was considered satisfactory since cleavage by trifluoroacetic acid should leave the ester bond intact. The *tert*-butyldimethylsilyl (TBDMS) group was chosen to protect the hydroxyl group of the tyrosine since it is selectively cleaved by fluoride under non-hydrolytic conditions.[189,190] The *p*-bromophenacyl (PBP) group[191,192] was chosen for the protection of the carboxyl rather than the benzyl ester because the latter was reported to be volatile, a disadvantage when working on small scale.[193] The PBP group seemed ideal since it was reported to be cleaved by catalytic hydrogenolysis[194] or by zinc in acetic acid.[195] Reaction of glycolic acid with 2,4'-dibromoacetophenone in ethyl acetate with triethylamine produc[illegible] desired PBP ester 78[191,196] in high yield (94%) (Scheme 50).[161]

Scheme 49.

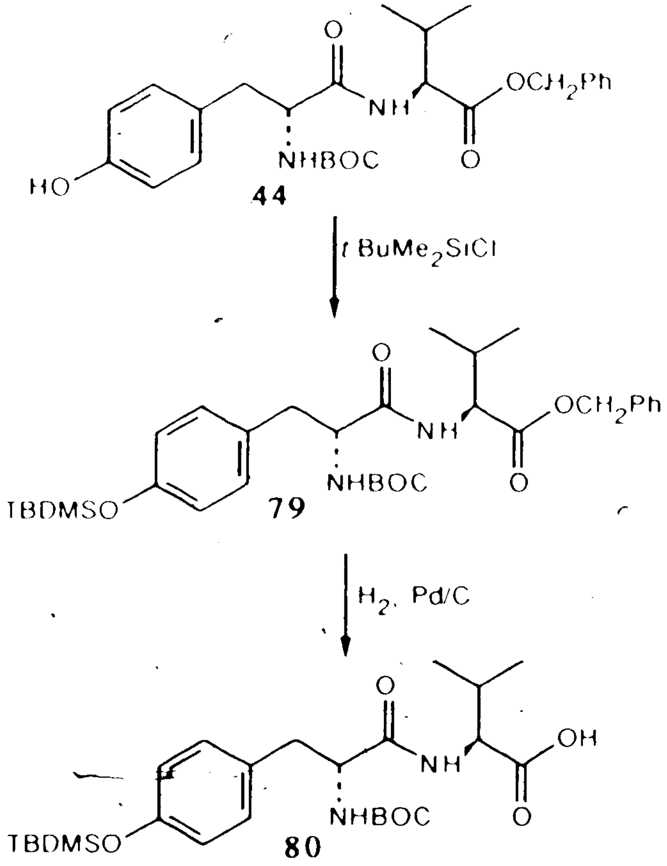


Scheme 50.



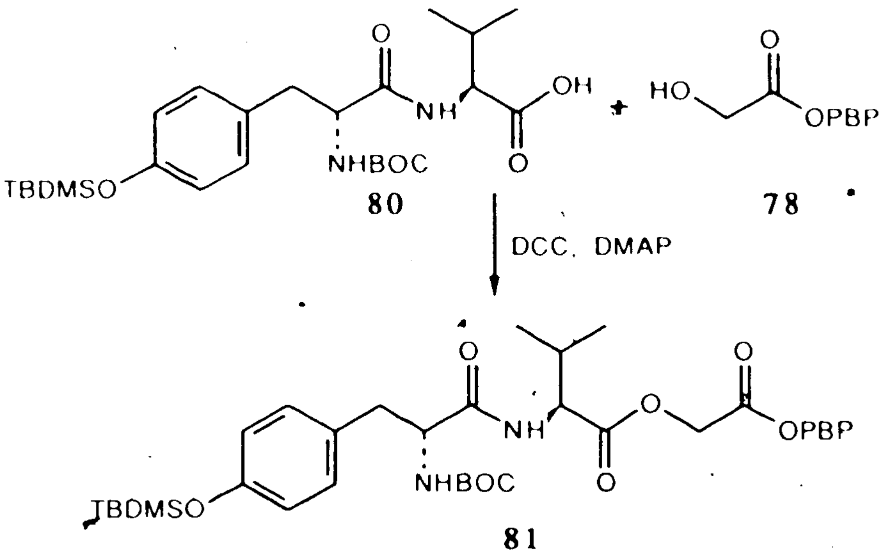
The *N*-BOC-*O*-TBDMS-D-TyrVal benzyl ester (**79**) was synthesized in 65% yield by treating *N*-BOC-D-TyrVal benzyl ester (**44**), previously prepared as an intermediate in the synthesis of the radiolabelled tripeptides, with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) (Scheme 51).[189] The benzyl group was selectively removed by hydrogenolysis in 97% yield to give the free acid **80**.

Scheme 51.

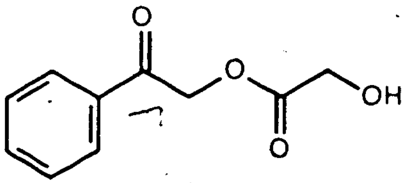


The formation of the ester bond between **78** and **80** was conveniently achieved in 65% yield through the use of dicyclohexylcarbodiimide in the presence of catalytic 4-dimethylaminopyridine (DMAP) to give **81** (Scheme 52). Deprotection of this compound proved to be very problematic. Repeated attempts to cleave the PBP ester of **81** by hydrogenolysis failed and in most cases starting material was recovered.[194] Treatment with zinc in acetic acid formed a mixture of products which was determined from the nmr spectrum to be starting material and material resulting from cleavage of the BOC group.[195] Attempts to deprotect glycolic acid PBP ester (**78**) by catalytic hydrogenolysis in water/methanol, or in ethyl acetate gave near quantitative recovery of unreacted starting material. Treatment of **78** with sodium thiophenoxide in DMF for 24 h gave recovered starting material.[192] It was apparent that despite the literature claims,[191,194,195] the PBP ester was not a good choice for protecting glycolic acid. To investigate whether the *p*-bromosubstituent was hindering deprotection, glycolic acid phenacyl ester (**82**) was prepared[161] but attempts to cleave this by hydrogenolysis also failed.[194]

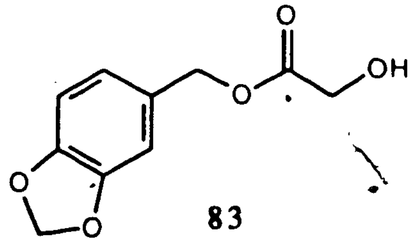
Scheme 52.



The 3,4-methylenedioxybenzyl ester of glycolic acid (**83**) was also synthesized because this protecting group is cleaved by treatment with CF_3CO_2H,[197] but this route was not pursued. This project is being continued by Hengmiao Cheng who, by the use of the benzyl ester protecting group, was able to synthesize **77** in both unlabelled and labelled forms, but has not yet produced sufficient quantities of the ^{14}C-labelled material for enzymatic testing.



82



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In summary, PAM has been isolated from pig pituitaries and a new assay has been developed which should be readily adaptable to analogous peptide amide systems since it is based on the glycine residue of the hormone precursors. The assay was also modified to detect glyoxylic acid formed from the oxidation of glycolic acid with glycolate oxidase. Stereospecifically tritiated glycine has been synthesized by a modification of literature methods for the preparation of deuterated glycines,[170] and this material was transformed to the D-TyrVal-[2-^{3}H]Gly. Treatment of these stereospecifically labelled peptides with PAM demonstrated that this enzyme removes the pro-*S* hydrogen from the glycine methylene group. These results have important implications in the design of potential drugs for the regulation of peptide amide hormones in biological systems.

Chapter 3. Biosynthetic Studies

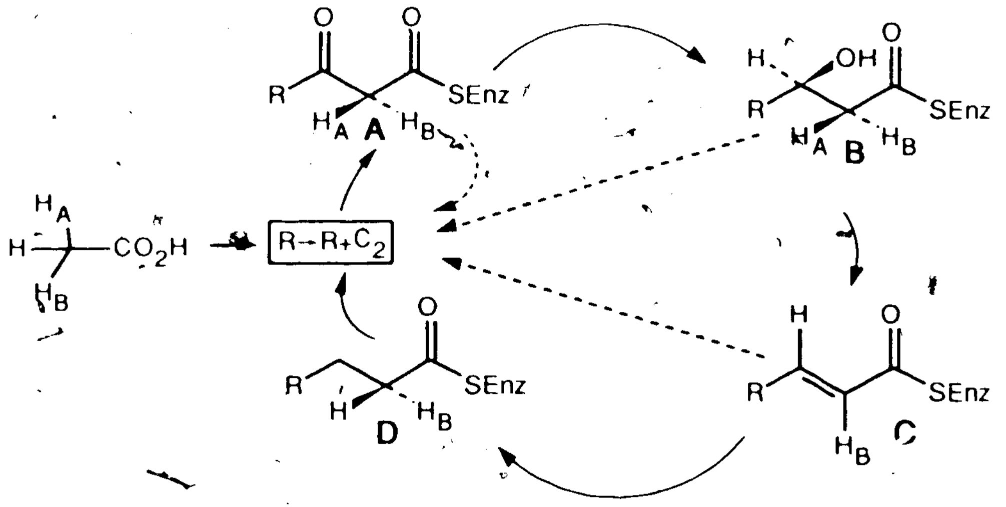
INTRODUCTION

In recent years there has been an explosive growth in the use of stable isotopes in studies of the biosynthesis of natural products.[198] These have been facilitated by the availability of ^{2}H, ^{13}C, ^{18}O, and ^{15}N compounds along with the advent of new nmr techniques and increased instrument sensitivity. Investigations of polyketide biosynthesis in particular have drawn considerable attention because of the incredibly diverse range of biologically important natural products that are assembled from acetic, propanoic, and/or butyric acids.[199,200] The availability of acetate singly and multiply labelled with ^{2}H, ^{13}C, and ^{18}O often allows determination of biosynthetic pathways with only a few incorporation experiments.[198]

Since the complete ^{1}H and ^{13}C nmr assignments of metabolites is usually a prerequisite for biosynthetic studies, new nmr techniques for spectral assignments have been of considerable importance.[201,202] To make these assignments, experiments such as homonuclear ^{1}H correlation to assign the proton-proton coupling scheme, heteronuclear ^{1}H-^{13}C correlation to determine the proton-carbon connectivity, and ^{13}C homonuclear correlation on biosynthetically ^{13}C enriched samples to provide carbon-carbon connectivities have been of great value.[198]

Polyketides are assembled by a mechanism which closely resembles fatty acid biosynthesis.[199,200] Fatty acids are synthesized by a multifunctional enzyme complex, the fatty acid synthase. In the first step an acetyl-CoA starter unit, derived from acetic acid, condenses with malonyl-CoA derived from carboxylation of acetyl-CoA. The β-keto ester formed (A in Scheme 53) is reduced with β-nicotinamide adenine dinucleotide phosphate (NADPH) to **B**, dehydrated to **C**, and further reduced to the two carbon homolog **D** of the starter unit. The cycle is then repeated to elongate the chain by two carbons for each repetition. The stereochemistry of these steps has been studied.[200] The β-keto ester (**A** in Scheme 53) is reduced to the *R*-alcohol (**B** in Scheme 53) and subsequent *syn* elimination gives the *E*-double bond (**C** in Scheme 53). The final reduction in the sequence by enol thiol ester reductase to give **D** can form either the *R* (as shown in Scheme 53) or the *S* stereochemistry at the α-carbon, depending on the organism, when one of the hydrogens (H_B) is derived from [2-$^2H_3$]acetate.[203-208]

Scheme 53.



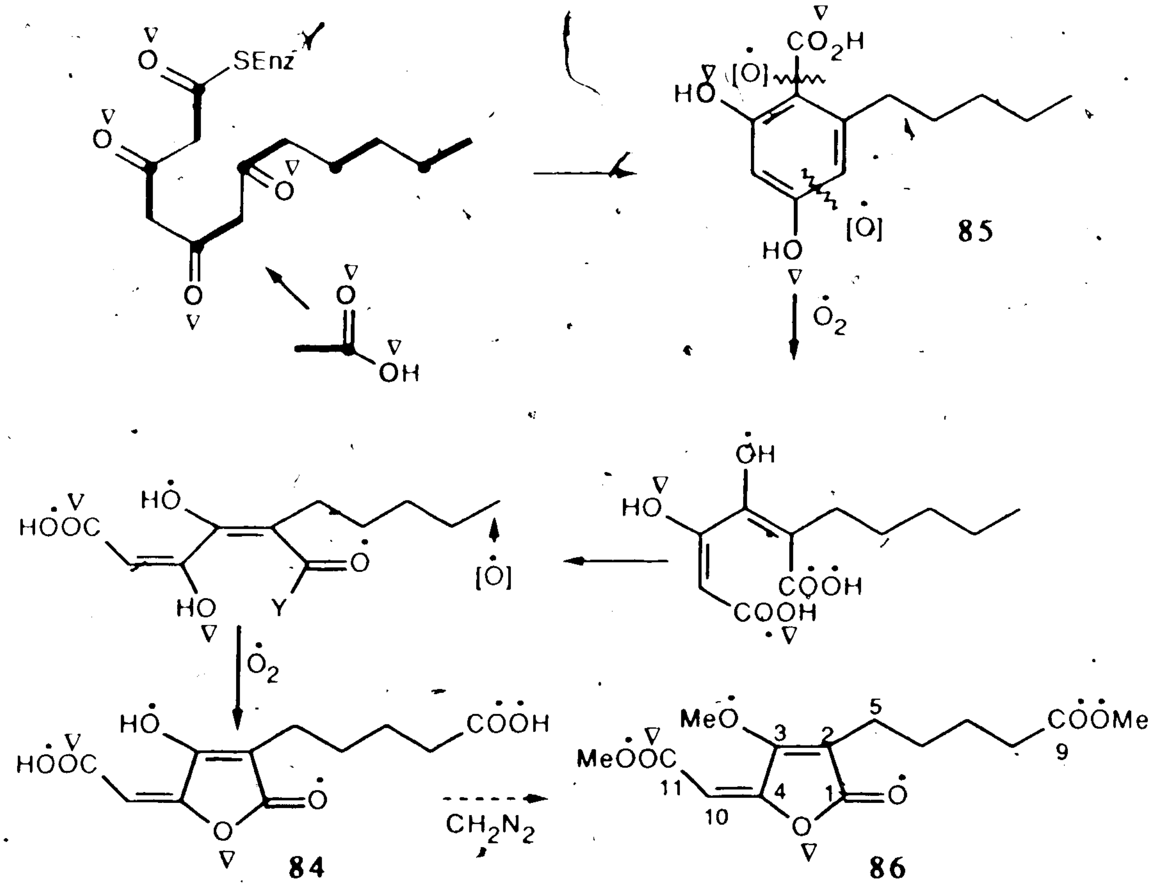
Polyketide formation proceeds by a very similar pathway but intermediates (**A**, **B**, or **C**) are intercepted by the addition of the next building block (eg. an acetate unit) before the reductive process is complete.[199,200] This leads to incorporation of carbonyl, hydroxyl, or double bond functionality into the growing polyketide chain as indicated by the dashed arrows in Scheme 53. These groups can initiate further transformations such as cyclizations or provide sites for attack by other enzymes once assembly is complete and the product has been released from the polyketide synthase. Despite the commercial and biological significance of these metabolites, only a few polyketide synthases have been purified and these produce only simple aromatic compounds such as 6-methylsalicylic acid.[199] Current biosynthetic studies often focus on establishing which carbon-hydrogen, carbon-carbon, and carbon-oxygen bonds are derived from acetate (or other building blocks) during the assembly process and which are derived from modifications occuring after release of the polyketide chain. They also often attempt to derive information on the mechanism of polyketide assembly and its exact relationship to fatty acid synthesis.[198,199,200]

BIOSYNTHETIC STUDIES ON MULTICOLOSIC ACID

Multicolosic acid (**84**) is one of several related metabolites isolated from *Penicillium multicolor*[209,210] which belong to the general class of ylidene tetronic acids and which show interesting antifungal and antibiotic properties.[211-213] The biosynthesis of multicolosic acid (**84**) has been studied and incorporation experiments with sodium [1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]-, [1-^{13}C, $^{18}O_2$]-acetate and ethyl [2-^{14}C]-6-pentylresorcylate (**85**) have indicated that **84** is a polyketide metabolite derived from oxidative cleavage of an aromatic precursor (Scheme 54).[210,214]

The occurrence of ^{18}O-induced α-isotope shifts in the ^{13}C nmr spectrum has proven to be a predictable and general method for detecting the incorporation of ^{18}O in biosynthetic studies.[85,198] The shift, when observable, is invariably upfield, facilitating interpretation of the spectra.[85] For the study of polyketide metabolites sodium [1-^{13}C,$^{18}O_2$]acetate and $^{18}O_2$ gas can be incorporated to distinguish between the oxygens derived from acetate and those derived from the atmosphere. In the case of multicolosic acid (**84**) it had already been shown that the lactone oxygen and one of the C-11 carboxyl oxygens are derived from acetate.[214] In particular, labelling studies with [1-^{13}C, $^{18}O_2$]acetate demonstrated that the bond between C-4 and the lactone oxygen remains intact during biosynthesis of **84** and that such an intact bond can also be found at the C-11 carboxyl group. This work describes the origin of the remaining oxygens as being from oxygen gas.

Scheme 54.



Multicolosic acid (**84**) was isolated as the dimethyl ester methyl ether **86** by Dr. Miyuki Kaneda from cultures of *P. multicolor* grown as previously described[210] except that the fermentation was done in a closed system[215] in which normal oxygen was gradually replaced by $^{18}O_2$ (50% isotopic purity). The resulting mixture of metabolites was methylated with diazomethane and separated in the usual way to afford pure dimethyl *O*-methylmulticolosate (**86**).[210]

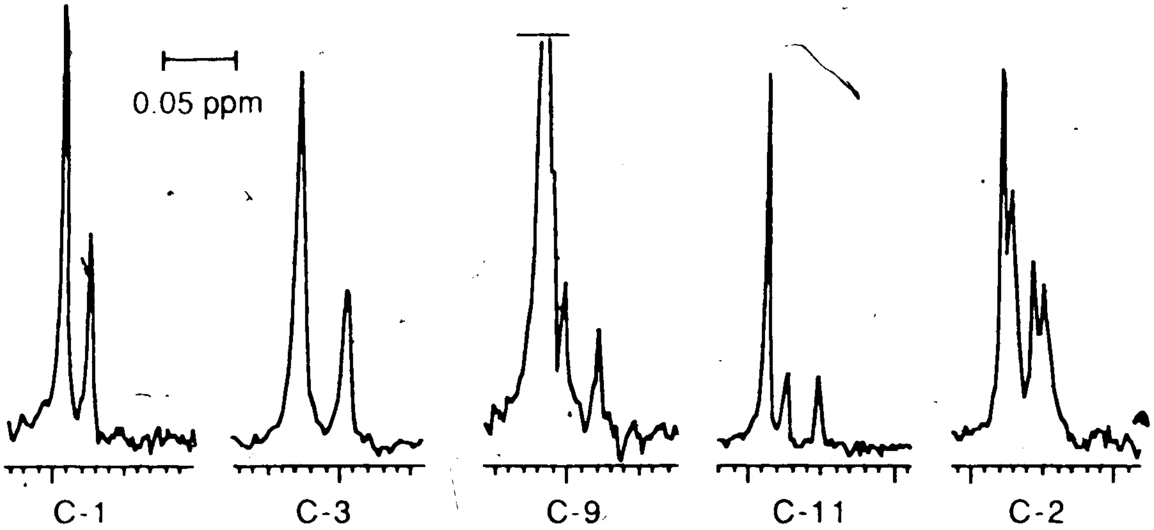
To determine the location of the ^{18}O atoms the broadband proton decoupled ^{13}C nmr spectrum was obtained on a Bruker WH400 spectrometer at 100.6 MHz in $CDCl_3$ solution (~0.05M) with Me_4Si as an internal standard. Clear isotope shifts could be observed at all carbons bearing oxygen except C-4 (Table 24 and Figure 6). The lactone carbonyl (C-1) displays a single ^{18}O-shifted resonance ($\Delta\delta$ = 18.0 p.p.b.) which, together with the absence of an isotope shift at C-4, indicates that only the doubly-bonded oxygen is labelled. The same $^{16}O:^{18}O$ ratio (70:30) is observed at C-3. As expected on the basis of [1-^{13}C,$^{18}O_2$]acetate labelling studies,[214] the C-11 carbon exhibits two upfield isotope-shifted resonances ($\Delta\delta$ = 12.1, 35.3 p.p.b.). The ratio of unlabelled to labelled peaks is 69:16:15 since $^{18}O_2$ and acetate each contribute one oxygen to this position, and these are randomized in the free carboxyl group of **84**. Although little if any exchange with the medium occurs at C-1, C-3, or C-11, the 90:5:5 ratio at C-9 suggests that a considerable amount of the oxygen at the side chain carboxy terminus originates from water. Possibly this is due to the intermediacy of an aldehyde functionality (which can exchange oxygen readily with the medium) during oxidation of the side chain *en route* to multicolosic acid (**84**).

Table 24. ^{13}C Nmr Spectrum of Dimethyl *O*-Methylmulticolosate 86.

Carbon	δ	$\Delta\delta$ p.p.b	$^{16}O:^{18}O$
1	161.4	18.0	72:28
2	110.1	7.2,22.3,29.9	42:24:19:15
3	168.5	34.5	70:30
4	150.7	—	—
9	173.5	13.7,37.8	90:5:5
11	164.3	12.1,35.3	69:16:15
(3-OMe)	59.7	31.1	70:30
(9-OMe)	51.4	25.3	94:6
(11-OMe)	52.0	26.5	84:16

Unexpected large β-isotope shifts (7.2, 22.3, and 29.9 p.p.b.) were observed at C-2 which bears no oxygen. Generally ^{18}O β-isotope shifts are less than 10 p.p.b.,[85,216] but recently two other examples have been found in which an olefinic carbon two bonds away from an enolic oxygen displays a very large β-isotope shift.[215] Since such isotope shifts are directly additive,[216] the 29.9 p.p.b. shift is caused by molecules of **86** bearing ^{18}O at both C-1 and C-3. On this basis it appears that the 22.3 p.p.b. shift at C-2 results from molecules of **86** bearing ^{18}O at C-3 and only ^{16}O at C-1. Hence the 7.2 p.p.b. shift at C-2 is due to molecules bearing ^{16}O at C-3 and ^{18}O at the C-1 carbonyl oxygen. Neither C-10 nor C-8 displays visible β-isotope shifts. Examination of a large number of [^{18}O]carbonyl compounds has demonstrated that their β-isotope shifts are usually too small to be readily observed despite their large α-isotope shifts.[86] The situation at C-2 is obviously an exception.

Figure 6. Partial ^{13}C Nmr Spectrum of Dimethyl *O*-Methylmulticolosate 86

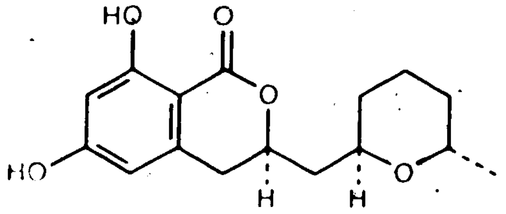


These results, which have been published,[217] support the biosynthetic pathway proposed in Scheme 54. The appearance of equal amounts of ^{18}O label from $^{18}O_2$ at both C-1 and C-11 demonstrates that the cleavage of the aromatic ring proceeds by a different mechanism than that observed in patulin formation.[218,219] It is interesting to note that a variety of pathways for oxidative aromatic ring cleavage are available in *Penicillium* species. The occurrence of unusually large β-isotope shifts in ^{13}C nmr spectra, whose magnitudes are similar to those of α-shifts, emphasizes the need for caution in using this technique for detection of ^{18}O labelling.

COMPARISON OF THE STEREOCHEMISTRY OF POLYKETIDE AND FATTY ACID BIOSYNTHESIS

One approach to examining the relationship of polyketide and fatty acid biosynthesis in fungi involves comparison of the cryptic stereochemistry of the acetate derived hydrogen in both types of metabolites in a single organism.[203,220] This may be especially useful since the stereochemistry of the last reduction enzyme of fatty-acid biosynthesis (enoyl thiol ester reductase) varies with its source.[203-208] To elucidate the stereochemistries requires determination of the chirality of a methylene carbon where one of the hydrogens is substituted by deuterium incorporated from deuterium labelled acetate.

Deuterium is a widely used isotope in determining both biological and chemical mechanisms and is routinely observed by changes induced in the ^1H nmr spectra, by direct ^2H nmr, or by differences in the ^{13}C nmr spectra of carbons that are directly attached to deuterium (α-isotope shifts) or two bonds away (β-isotope shifts).[198] Determination of the stereochemistry of a methylene carbon bearing deuterium and hydrogen is often difficult in biosynthetically derived samples because low incorporation precludes the use of ^1H nmr and the broad signals of ^2H nmr often fail to resolve the differences in the chemical shifts of the diastereotopic deuteriums.[221-224] Recently, however, a technique based on α-isotope shifts in the ^2H-decoupled ^1H, ^{13}C chemical shift correlation nmr spectra has been developed which allows direct determination of such stereochemistry.[225] The work presented here makes use of this technique to compare stereochemistries of acetate derived deuterium in two polyketide metabolites, cladosporin (**87**) from *Cladosporium cladosporioides*, and averufin (**88**) from *Aspergillus parasiticus* to the corresponding stereochemistry of the fatty acids isolated from the respective organisms. The work on the biosynthesis of averufin (**88**) was in collaboration with Dr. Craig Townsend and Susan Brobst of the Johns Hopkins University and is currently in press.[226] In the work on the biosynthesis of cladosporin (**87**) which is also in press,[227] the actual incorporations and isolations were done by Drs. Paul Reese and Bernard Rawlings.



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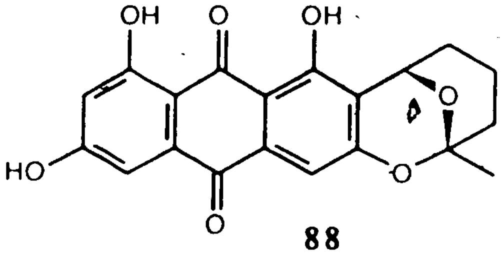
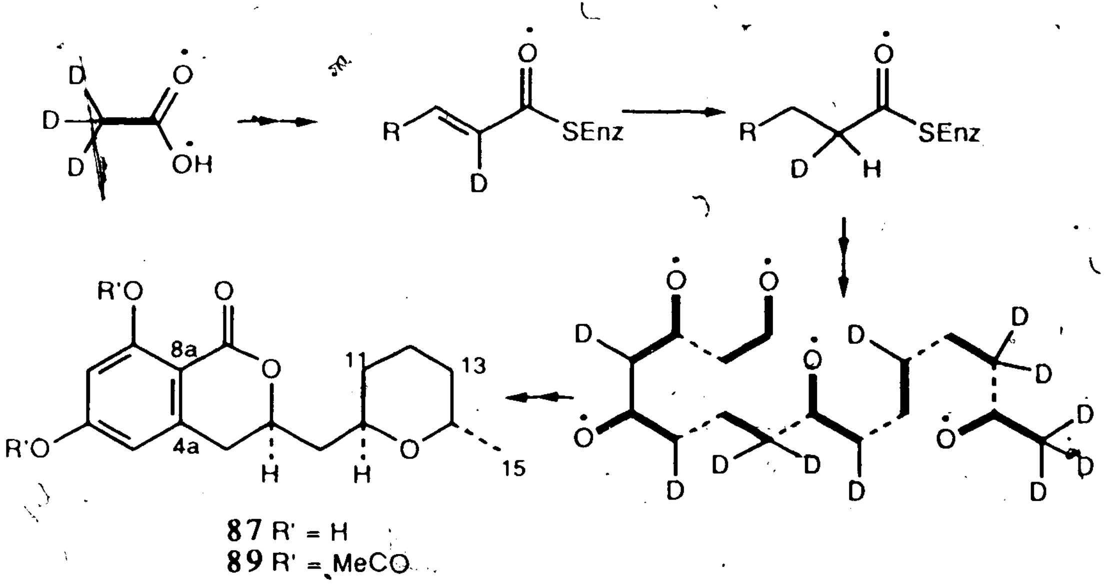


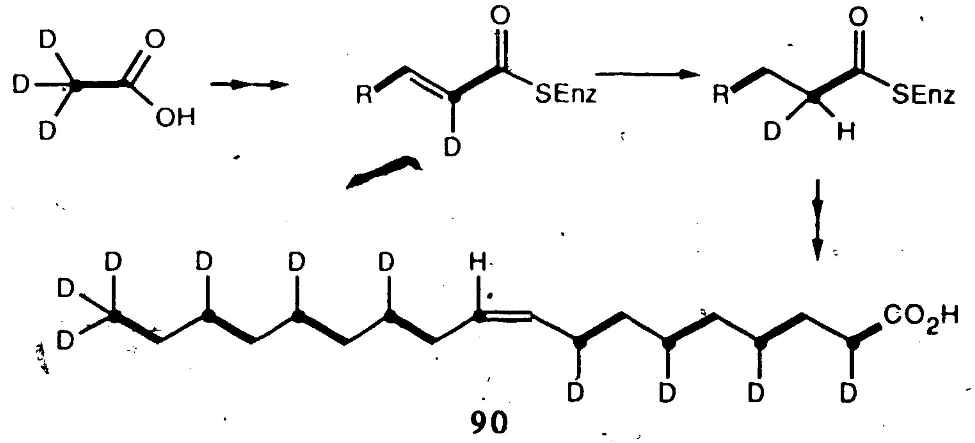
88

Cladosporin (**87**) is an antibiotic and plant growth regulator produced by various fungal sources.[228-231] The absolute stereochemistry has previously been reported[231] and the general features of its biosynthesis have been determined by administration of [1-^{13}C]acetate and 2-[^{14}C]malonate.[232] Experiments in our laboratories have identified the bonds derived intact from acetate as shown (Scheme 55) through incorporations of sodium [1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]-, [1-^{13}C-$^2H_3$]-, and [1-^{13}C, $^{18}O_2$]-acetate followed by nmr analysis of the isolated diacetate **89** resulting from labelled cladosporin (**87**). The number of acetate derived hydrogens at non-aromatic sites corresponds to that predicted during an assembly process akin to fatty acid biosynthesis. Thus the stereochemistry of the single acetate hydrogen at C-11 would be determined by enzymatic reduction by enol thiol ester reductase *en route* to cladosporin (**87**) (Scheme 55). This is in analogy to the construction of stearic acid and its dehydrogenation product,[203,233-235] oleic acid (**90**) (Scheme 56) which is also produced by *C. cladosporioides* from acetate.

Scheme 55.

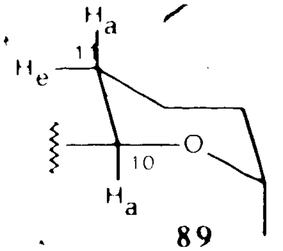


Scheme 56.



Cladosporin (87) was isolated as the diacetate 89 after incorporation of [2-$^2H_3$,^{13}C]acetate in the same experiment that the oleic acid (90) was isolated by Dr. P. Reese. This material was analyzed by broadband deuterium decoupled ^{1}H-^{13}C heteronuclear shift correlation nmr spectra.[225] In this method correlations between the carbon and the diastereotopic protons are observed. When the carbon bears two protons, two correlated signals are observed. However, when the carbon is stereospecifically labelled with deuterium, the upfield shifted ^{13}C signal from the ^{2}H-induced α-isotope effect correlates only to the chemical shift of the diastereotopic proton which remains on the carbon. If the assignment of the diastereotopic protons is known, or can be determined, unambiguous assignment of the stereochemistry of the substituted methylene group can be made.[225]

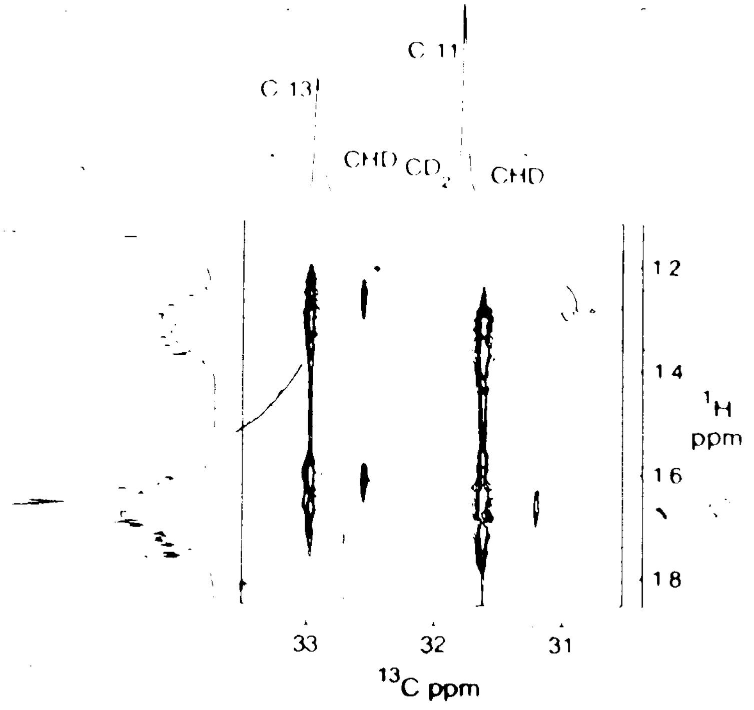
For cladosporin (**87**) the correlation of the diacetate **89** (Figure 7) shows that the hydrogen of the CHD group at C-11 occupies the downfield position. This has been assigned by Dr. B. Rawlings as the equatorial pro-*R* position based on the coupling constants of the two diastereotopic protons to the C-10 axial hydrogen. The axial C-11 methylene hydrogen has a 8.8 Hz coupling constant and the equatorial hydrogen has a 2.8 Hz coupling constant, both of which are consistent with values for oxygen containing rings.[236] The stereochemistry of C-11 is therefore *S* which corresponds to *S* on the growing polyketide chain.



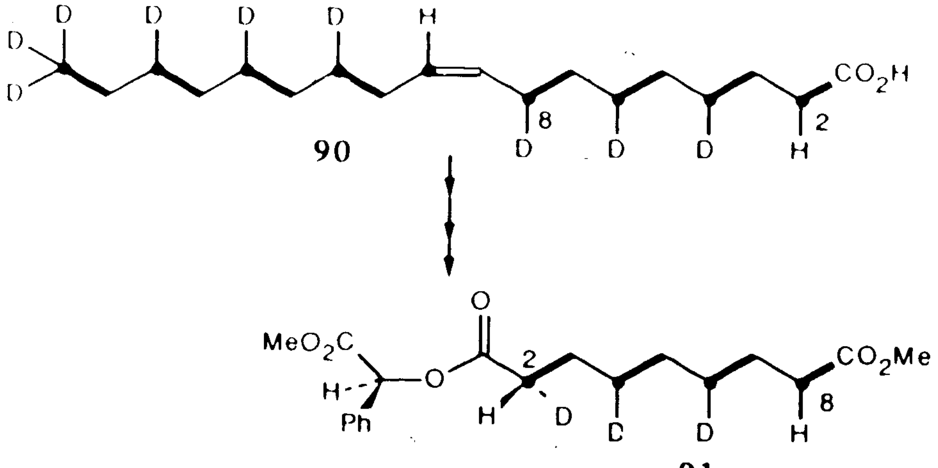
89

For determining the stereochemistry of the oleic acid formed by *C. cladosporioides*, the fats were isolated, hydrolysed, and the oleic acid (**90**) was purified as the methyl ester. Nmr analysis of this indicated that the deuterium at C-2 was lost, presumably due to exchange with the media. The oleic acid methyl ester was therefore degraded and converted to the *S*-(+)-methylmandelate derivative **91a** (Scheme 57) which was analyzed by nmr spectrometry.

Figure 7. ^1H ^{13}C Correlation of C-11 of Cladosporin (87).

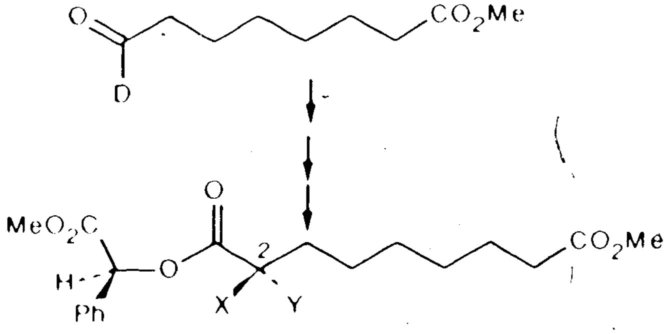


Scheme 57.



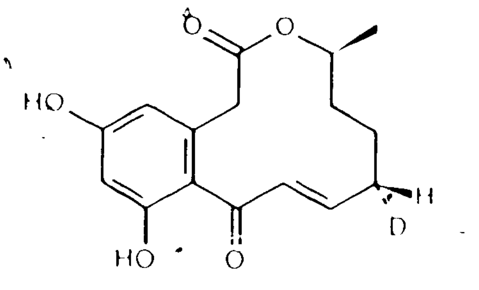
For *S*-(+)-methylmandelate esters of long chain acids the 2-pro-*R* hydrogens are known to appear downfield.[237,238] To confirm this for the functionalized ester **91a** obtained from the biosynthetic experiments, the deuterated diastereomers **91b** and **92** were independently synthesized by Dr. B. Rawlings (Scheme 58),[227] and analyzed using the ^{2}H decoupled ^1H-^{13}C correlation method (Figure 8). Comparison of the nmr spectra of **91a**, **91b**, and **92** clearly showed that the stereochemistry of the C-2 position of **91a** derived from degradation of oleic acid **90** was *S* which, because of a priority change, corresponds to *R* on the growing chain of the saturated fatty acid.

Scheme 58.

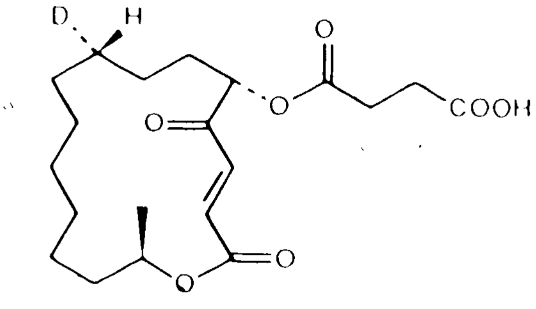


91b X = H, Y = D
92 X = D, Y = H

These results show that the stereochemistry of acetate derived deuterium on the growing chain during biosynthesis of cladosporin (**87**) is opposite the corresponding stereochemistry of the fatty acids produced in the same organism. Interestingly the absolute stereochemistry of single carbon-oxygen bonds of cladosporin (**87**) derived intact from acetate at C-14 and C-3 corresponds to *S* on the growing polyketide chain, again opposite to that expected (*R*) from fatty acid biosynthesis.[204-207] Two other cases studied in our laboratories, *Penicillium turbatum* and *Alternaria cinerariae* which produce antibiotic A26771B and dehydrocurvularin, respectively show an analogous opposite stereochemistry in fatty acid and polyketide biosynthesis.

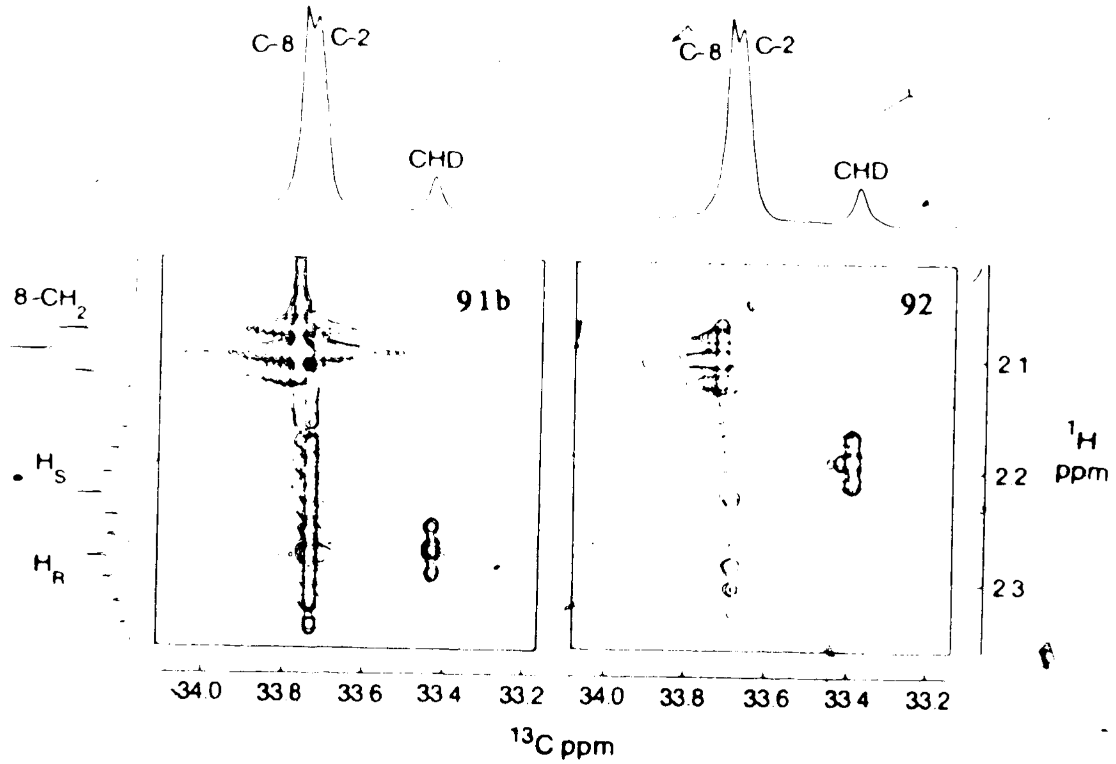


dehydrocurvularin



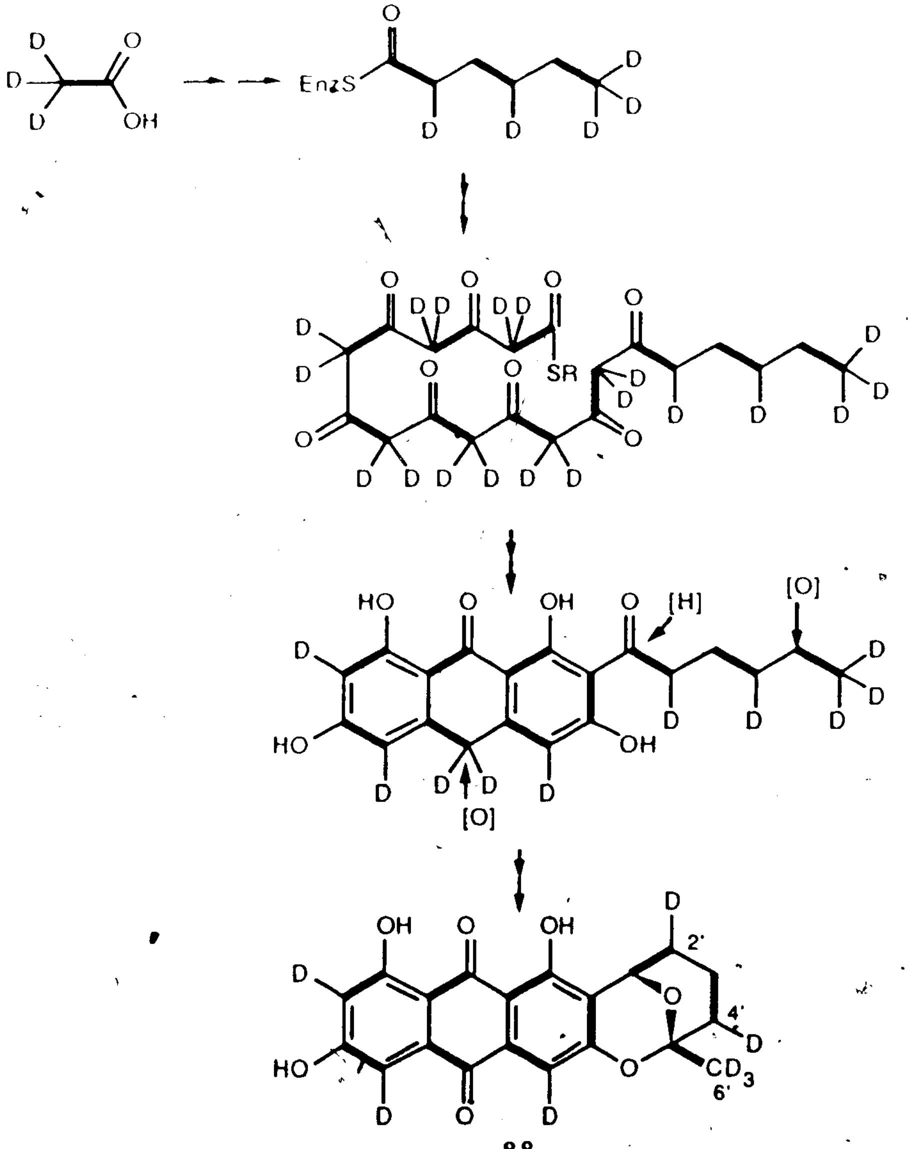
antibiotic A26771B

Figure 8. ^1H-^{13}C Correlation of Methyl Mandelates 91 and 92.



Averufin (**88**) is an intermediate metabolite in the biosynthesis of the highly toxic and carcinogenic aflatoxins.[239-243] The biosynthesis of **88** has been shown to follow a polyketide pathway, and incorporation of acetate units has outlined a biosynthesis as shown (Scheme 59).[239,244-246] Recent findings indicate that hexanoate (rather than acetate) may be incorporated as the starting unit for homologation to the polyketide which is eventually transformed to averufin (**88**).[247,248] This has been demonstrated, though not unambiguously,[249] through the incorporation of labelled hexanoic acid.[247,248] Further information concerning this observation can be obtained by comparing the stereochemistry of the [2-$^2H_3$]acetate derived CHD groups of averufin (**88**) (eg. C-4', C-2') and of the oleic acid (**90**) also produced by this organism.[233-235,250]

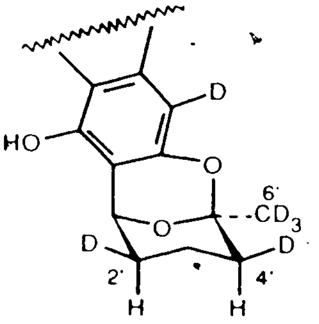
Scheme 59.



88

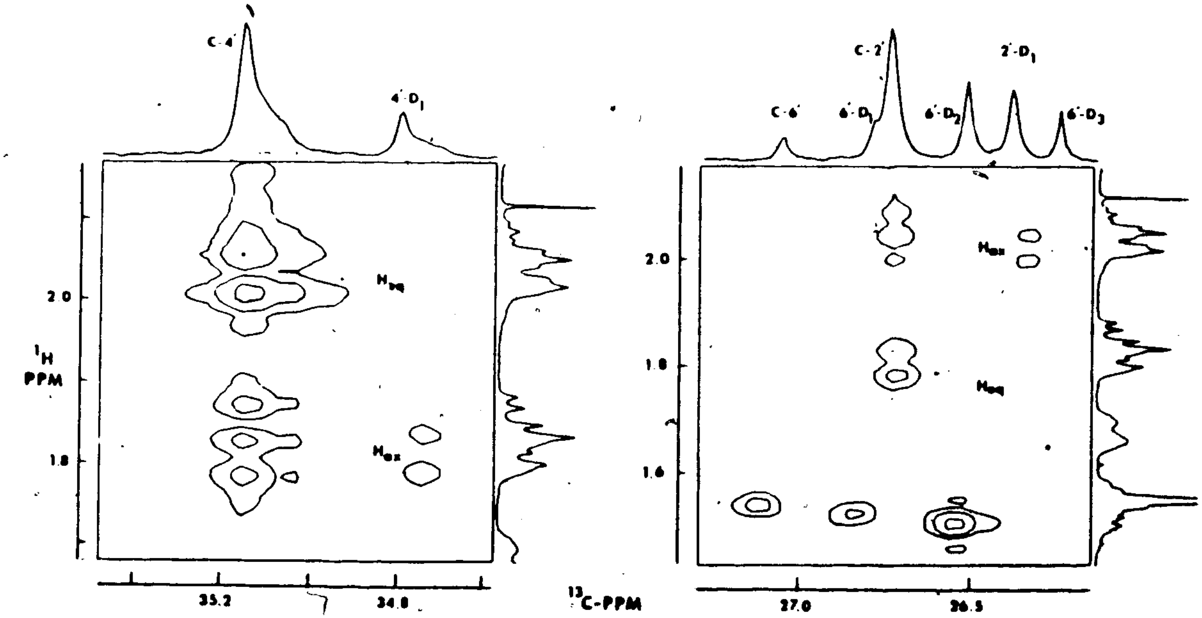
Averufin (**88**) was isolated[247,248] from an averufin accumulating mutant of *A. parasiticus* after incorporation of [2-$^2H_3$,^{13}C]acetate by S. Brobst and Dr. C. Townsend at

Johns Hopkins University. In the rigid bicyclic ketal system of **88** the H-2', H-3', and H-4' methylene hydrogens give rise to resonances separated by > 0.1 p.p.m. from their geminal partners but these signals have been unambiguously assigned.[251] The correlation experiment shows that the hydrogen introduced by the reductase at the 4' position in the CHD group is the upfield axial hydrogen (Figure 9). Since the absolute configuration is known to be 1'-*S* as drawn, the stereochemistry of the 4' position is *S*, which corresponds to *R* on the growing polyketide chain. Similarly, the hydrogen at the CHD-2' position is axial, hence this carbon has the *R* configuration, corresponding to *R* on the polyketide chain.



88

Figure 9. ^1H-^{13}C Correlation of C-4' and C-2' of Averufin (88).



To determine the corresponding stereochemistry of the fatty acids from *A. parasiticus*, oleic acid (**90**) was isolated and transformed to **91a**. The configuration of C-2 of **91a** was determined by ^{2}H decoupled ^{1}H-^{13}C correlation nmr to be *S*, which corresponds to *R* on the growing saturated fatty acid chain. Thus the results for averufin (**88**) show a common stereochemical course in the reductive formation of the six carbon side chain of **88** and in the oleic acid (**90**) isolated from the same organism. In this case, however, it appears that the starter unit for the formation of the polyketide may be a six carbon fatty acid made by a fatty acid synthase.[247,248] These results agree with this hypothesis,[247,248] especially when contrasted to what appears to be the emerging trend with cladosporin, dehydrocurvularin, and A26771B. Although the sample size is limited at this time in these cases polyketides and fatty acids synthesized by the same fungal organism have *opposite* stereochemistry of enoyl thiol ester reductase.

These experiments further demonstrate the utility of the ^{2}H decoupled ^{1}H-^{13}C correlation method[225] for determining the stereochemistry of CHD groups of polyketides labelled by incorporation of sodium [2-$^2H_3$, ^{13}C]acetate. A general trend appears to be emerging in fungi of opposite stereochemistry of enoyl thiol ester reductase in fatty acid and polyketide assembly. It will be interesting to see if this trend continues in the fungi and if analogous results will be found in other organisms.

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.*[182] Specifically benzene, toluene, tetrahydrofuran (THF), and diethyl ether were distilled from sodium and benzophenone. Acetonitrile, triethylamine (Et_3N), and pyridine were distilled from CaH_2. Anhydrous ethyl alcohol and methyl alcohol were prepared by distilling from Mg 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 was dried over P_2O_5 and distilled. Solvents used for chromatography were distilled. Reagents for anhydrous reactions were dried overnight under vacuum over P_2O_5. The term "*in vacuo*" refers to the removal of solvents 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 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 were obtained and used without further purification from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.) unless otherwise indicated. *N*-Bromosuccinimide (NBS) was recrystalized from water, dried under vacuum over P_2O_5, and stored in the dark .[182] Deuterium gas was from Matheson Gas Co. and was 99.5% D_2. Commercially available enzymes were obtained from Sigma and stored as indicated. Catalase (from bovine liver) was purchased as an aseptically filled aqueous solution from Sigma (cat.

number C3155, ~15mg/mL, ~50000 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) on Merck 60F-254 silica plates and visualized using UV absorption by fluorescence quenching and I_2 staining. Amino acids and peptides were detected with ninhydrin in acetone followed by heating. β-Lactones were visualized by spraying with a bromocresol green solution (0.04% in ethanol made blue with NaOH) and heating after which a yellow spot developed on a blue background. For monitoring reactions in DMF or water the plate was kept under vacuum (< 50 mtorr) for 10 min prior to developing. Silica gel for column chromatography was Merck type 60, 70-230 mesh. Flash chromatography was done according to Still *et al.*[252] using Merck type 60, 230-400 mesh silica gel. Ion exchange resin was purchased from Bio-Rad.

Melting points were determined on a Thomas Hoover oil-immersion apparatus in an open ended capillary tube and are reported uncorrected. Infrared spectra (ir) were recorded on a Nicolet 7199 FT-IR spectrometer, prominent peaks above 1500 cm^{-1} are reported. Mass spectra (ms) were recorded on a Kratos AEI MS-50 (high resolution, ei-ms), MS-12 (chemical NH_3 ionization, ci-ms), and MS-9 (fast atom bombardment with argon, POSFAB). 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 WH-400 instruments in the specified solvent with tetramethylsilane (TMS) or deuterated sodium 3-(trimethylsilyl)-1-propanesulfonate (TSP) in D_2O as internal standards in ^1H nmr. For ^{13}C nmr, which were obtained on the WH-400, the deuterated solvent peak was used as the reference. ^2H nmr spectra of serine were recorded on the WH-400 in protic solvent with an inner tube containing hexafluorobenzene (for fluorine lock) and $CDCl_3$ (for reference). ^3H nmr were recorded by Dr. P. G. Williams at the National Tritium Labeling Facility at the Lawrence

Berkeley Laboratory (California) at 320.1 MHz according to the method of Bloxsidge *et al.*[253] Approximately 6 mCi of material was used and 10000 scans were recorded.

High pressure liquid chromatography (hplc) was performed on a Hewlett Packard 1082B with a variable wavelength detector, Waters 660 with a LDC Spectrometer 1 variable wavelength detector, or Waters 510 with a Hewlett Packard 85/1040A UV diode array detector (National Tritium Labeling Facility). Columns were Waters µ-Bondapak Radial-Pak cartridges used with a Z-module compression unit. Two different hplc systems were employed. With a reverse phase Radial-Pak C_{18} column a two solvent system was employed (solvent A, 0.1% CF_3CO_2H in water; solvent B, 0.1% CF_3CO_2H in 80:20 acetonitrile:water) with a linear gradient from 0 to 25%B in 30 min and detection at 254 nm. The D-TyrValGly has a retention time of 16.78 ±0.173 min (6 runs). For the initial purification of glycine after peptide hydrolysis 0.1% phosphoric acid was used instead of 0.1% CF_3CO_2H and detection was at 200 nm. For the NH_2 Radial-Pak column a binary solvent system was used (solvent C, 500:70 acetonitrile:water; solvent D, 5 mM KH_2PO_4, pH 4.3) with detection at 200 nm and a gradient program of 0 min 5% D, 5 min 5% D, 20 min 30% D, 25 min 30% D. Glycine had a retention time from 17 to 20 min, depending in part on column condition. All separations used a flow rate of 2 mL/min. For purification of the tritiated glycines at the National Tritium Labeling Facility detection of the radioactive compounds was achieved using a Berthold LB5026 radiochemical detector with a Trace Northern TM7200 multichannel analyzer. Hplc grade acetonitrile (190 nm cutoff) was obtained from Terochem. All hplc solvents and buffers were prepared fresh daily 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 Amersham ACS liquid scintillation cocktail. The instruments used were a Beckman LS100C, Beckman 1801, or Packard Tricarb 1500 (National Tritium Labeling Facility). 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 ^{3}H and ^{14}C quenched standards. This automatically calculates ^{3}H/^{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 used 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. A LKB peristaltic pump was employed to control the flow rate on Sephadex and 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 in 1.5 mL MicroFuge tubes (Terochem).

Determination of the ^{3}H/^{14}C ratios. For the results obtained on the Beckman LS100C liquid scintillation counter, and to confirm the results from the Beckman 1801 instrument, the ^{3}H/^{14}C ratios were determined as follows. The samples were counted in a ^{14}C channel where the tritium spillover was 0, and in a ^{14}C and ^{3}H channel. To each sample was then added a ^{3}H-toluene standard (50 µL, 6.91x10^4 dpm) and these were recounted in the two channels. The cpm in the ^{14}C channel remained the same and the increase in the cpm of the ^{14}C and ^{3}H channel divided by the amount of standard added was the efficiency of tritium in the combined channel (~40%). To each sample was then added a ^{14}C-toluene standard (50 µL, 2.00x10^4 dpm) and these were again counted in the two channels. The increase in cpm in each channel divided by the amount of standard added gave the ^{14}C efficiencies (~47% in the ^{14}C only channel, ~95% in the dual channel). The dpm from ^{14}C in the original sample was determined by dividing the cpm in the ^{14}C channel obtained before the addition of standard by the calculated efficiency of that channel (eq. 1). The ^3H cpm was then determined by subtracting from the initial cpm of the combined ^3H and ^{14}C channel the dpm ^{14}C times the efficiency of ^{14}C in the combined channel. This was then divided by the efficiency of ^3H in the combined channel to give the ^3H dpm (eq. 2). The $^3H/^{14}C$ ratio is expressed as the ratio of the dpm of the two isotopes (eq. 3). For each set of triplicate assays the efficiencies for each sample were counted then averaged and used in equations 1-3 to determine the $^3H/^{14}C$ ratio. This reduces the effect from variations in the addition of radioactive standards in determining the efficiencies.

(eq. 1) $$D_C = \frac{C_{C1}}{E_{C1}}$$

(eq. 2) $$D_T = \frac{C_2 - D_C \cdot E_{C2}}{E_{T2}}$$

(eq. 3) $$\frac{^3H}{^{14}C} = \frac{D_T}{D_C}$$

D_C= dpm ^{14}C

C_{C1}=cpm ^{14}C in ^{14}C channel

E_{C1}=efficiency of ^{14}C in ^{14}C channel

E_{C2}=efficiency ^{14}C in ^{14}C and ^3H channel

D_T=dpm ^3H

C_2=cpm in ^{14}C and ^3H channel

E_{T2}=efficiency of ^3H in the ^{14}C and ^3H channel.

Z-2-Acetamido-3-methoxyacrylic acid (1). The procedure of Gassman *et al.*[78] was modified. To a suspension of potassium *tert*-butoxide (7.50 g, 66.8 mmol) in 150 mL anhydrous ether at 0 °C was added 0.195 mL (10.8 mmol) of H_2O. This was stirred for 5 min, methyl ester 2 (1.50 g, 8.66 mmol) was added, and stirring was continued at 20 °C for 3 h. Ice was added and the separated aqueous phase was run down a 20 mm x 150 mm AG 50 acid-washed ion exchange column. The product was eluted with 100 mL of H_2O and concentrated *in vacuo* to yield 1.32 g (96%) of the free acid 1 as a fluffy white powder. Mp 172-174 °C; ir (film) 3230, 3030, 1692, 1650 cm^{-1}; ^1H nmr (D_2O, 80 MHz) δ 2.05 (s, 3H, $COCH_3$), 3.88 (s, 3H, vinyl-OCH_3), 7.38 (s, 1H, vinyl-H); exact mass: 159.0532

(159.0531 calcd for $C_6H_9NO_4$). Anal. calcd for $C_6H_9NO_4$: C, 45.28; H, 5.70; N, 8.80. Found: C, 45.14; H, 5.60; N, 8.54.

Z-2-Acetamido-3-methoxyacrylic acid methyl ester (2). To a solution of 2-acetamido-3,3-dimethoxypropanoic acid methyl ester **7** (20.1 g, 98.0 mmol) in 300 mL of dry toluene was added 500 mg of *p*-toluenesulfonic acid and 130 mg of hydroquinone, and the solution was refluxed for 3.5 h with azeotropic distillation of methanol/toluene. Additional dry toluene was added as necessary. The toluene was removed *in vacuo*, the residue taken up in 225 mL of saturated NaCl solution and extracted with $CHCl_3$. The organic extracts were dried (Na_2SO_4) and concentrated *in vacuo* to yield a dark brown oil. This was repeatedly recrystallized from ethyl acetate to give 7.33 g (43%) of colourless vinyl methoxy compound **2**. Mp 91-92 °C (lit.[68] mp 93 °C); ir (film) 3160, 3020, 1720, 1660 cm^{-1}; ^1H nmr ($CDCl_3$, 80 MHz) δ 2.03 (s, 3H, $COCH_3$), 3.68 (s, 3H, CO_2CH_3), 3.83 (s, 3H, vinyl-OCH_3), 7.13 (s, 1H, vinyl-*H*), 7.38 (br s, 1H, N*H*); exact mass: 173.0690 (173.0688 calcd for $C_7H_{11}NO_4$). Anal. calcd for $C_7H_{11}NO_4$: C, 48.55; H, 6.40; N, 8.09. Found: C, 48.57; H, 6.34; N, 7.90.

2-Acetamido-3-chloroacrylic acid methyl ester (3). The method of Kolar and Olsen[70] was employed. In 500 mL of CCl_4 was dissolved 24.5 g (0.171 mol) of **5** and chlorine gas passed through the solution at room temperature until a yellow color persisted. This was stirred an additional 10 min and the solvent was removed *in vacuo*. The resulting oil **6** was taken up in 150 mL of CH_2Cl_2, filtered, and 19.2 g (0.171 mol) of DABCO was added with stirring. Stirring was continued for another 20 min at room temperature. The solvent was removed *in vacuo*, the residue was taken up in 100 mL of H_2O, acidified with 1N HCl, and the bottom organic layer was separated. The remaining aqueous layer was extracted with ether (3 x 50 mL), saturated with NaCl, extracted with another 50 mL ether and the combined organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo* to give **3** (17.5 g, 57%). Ir ($CHCl_3$ cast) 3250, 1733, 1673 cm^{-1}; ^1H nmr (80 MHz,

$CDCl_3$) δ 2.11, 2.15 (2s, 3H, CH_3CO), 3.80, 3.89 (2s, 3H, CH_3O), 6.98 (s, 0.65H, *Z*-vinyl-*H*), 7.62, 7.68 (br m, 1H, N*H*, *E*-vinyl-*H*), 7.98 (br, 0.35H, N*H*); exact mass: 177.0193 (177.0193 calcd for $C_6H_8ClNO_3$).

N-Acetylalanine methyl ester (4). The procedure of Kolar and Olsen[71] was followed. To 32.7 g (0.249 mol) of *N*-acetyl-DL-alanine (Sigma) dissolved in 225 mL dry methanol and cooled to 0 °C was slowly added 20 mL (0.28 mol) of acetyl chloride, and the resulting solution was stirred at room temperature overnight. The solvent was removed *in vacuo* and 50 mL of saturated $NaHCO_3$ added. Solid $NaHCO_3$ was added (vigorous evolution of CO_2) and the aqueous phase extracted with 2x50 mL of $CHCl_3$. The combined extracts were dried ($MgSO_4$) and the solvent was removed to give **4**, (31.0 g, 86%). This could be further purified by distillation at 103 °C (1.3 torr) (lit.[71] bp 78-85 °C/0.2 torr). Ir ($CHCl_3$ cast) 3290, 1750, 1660, 1540 cm^{-1}; ^1H nmr (80 MHz, $CDCl_3$) δ 1.43 (d, $J = 7.2$ Hz, 3H, $CHCH_3$), 2.03 (s, 3H, CH_3CO), 3.70 (s, 3H, CH_3O), 4.50 (m, 1H, C*H*), 7.35 (br, 1H, N*H*); exact mass: 145.0733 (145.0739 calcd for $C_6H_{11}NO_3$).

2-Acetamidoacrylic acid methyl ester (5). According to Kolar and Olsen,[71] *N*-acetylalanine methyl ester (4) (31.99 g, 0.2135 mol) was dissolved in 40 mL dry methanol and a few crystals of hydroquinone were added. This was stirred at 12 °C and *tert*-butyl hypochlorite (31.7 mL, 0.266 mol) was added in a single portion followed by 0.62 mL of a 1% (w/v) solution made from sodium metal and methanol. This was kept at 0 °C in the refrigerator overnight, the methanol was removed *in vacuo*, and the residue was taken up in CH_2Cl_2 (100 mL), washed with saturated NaCl (25 mL), dried ($MgSO_4$) and diluted to 500 mL with CH_2Cl_2. The flask was equipped with a reflux condensor and DABCO (23.83 g, 0.2124 mol) was added at a rate to just cause reflux. After addition was complete and the reflux had subsided, the solution was heated at 43 °C for 25 min then cooled at -20 °C for 2 h and filtered. The filtrate was washed with 50 mL portions of H_2O, 1N HCl, $NaHCO_3$, H_2O and dried ($MgSO_4$). The solvent was removed *in vacuo* to give **5**

(24.52 g, 80%). Mp 50-52 °C (lit.[71] mp 50-52 °C); ir (CHCl$_3$ cast) 3358, 1728, 1691, 1677, 1517 cm^{-1}; ^{1}H nmr (80 MHz, CDCl$_3$) δ 2.13 (s, 3H, C*H*$_3$CO), 3.83 (s, 3H, C*H*$_3$O), 5.78 (s, 1H, vinyl *H*), 6.43 (s, 1H, vinyl *H*), 7.98 (br, 1H, N*H*); exact mass 143.0584 (143.0582 calcd for $C_6H_9NO_3$).

2-Acetamido-3,3-dimethoxypropanoic acid methyl ester (7). A solution of sodium metal (1.85 g, 80.5 mmol) in 150 mL dry methanol was added to **3** (14.3 g, 80.6 mmol) and the resulting solution was refluxed for 13 h. The methanol was removed *in vacuo* and saturated KHCO$_3$ solution was added. This was extracted with CHCl$_3$, the extracts were dried (Na$_2$SO$_4$) and concentrated *in vacuo* to give 15.7 g (96%) of the dimethoxy compound **7** as an oil. Ir (film) 3290, 1750, 1665 cm^{-1}; ^{1}H nmr (80 MHz, CDCl$_3$) δ 2.05 (s, 3H, COC*H*$_3$), 3.45 (s, 6H, C*H*$_3$O-CH), 3.78 (s, 3H, CO$_2$C*H*$_3$), 4.61 (d, *J* = 3.5 Hz, 1H, (MeO)$_2$ C*H*), 4.88 (dd, *J* = 3.5, 8.0 Hz, 1H, C*H*-N), 6.33 (br d, *J* = 8.0 Hz, 1H, N*H*); exact mass: 174.0768 (174.0766 calcd for $C_7H_{12}NO_4$ (M^+-OCH$_3$)). Anal. calcd for $C_8H_{15}NO_5$: C, 46.82; H, 7.37; N, 6.83. Found: C, 46.59; H, 7.51; N, 6.55.

2-Methyl-4-oxazolecarboxylic acid methyl ester (8). To **6**, prepared as in the synthesis of **3** from 1.06 g (7.41 mmol) of **5**, was added a solution, made from sodium metal (0.361 g, 15.7 mmol) and dry MeOH (40 mL). This was refluxed for 22 h, 100 mL of saturated KHCO$_3$ was added, and the mixture was extracted with 3 x 25 mL of CH$_2$Cl$_2$. The organic extracts were dried (Na$_2$SO$_4$) and the solvent was removed to give an oil which was purified by column chromatography (CHCl$_3$) to give **8** (0.140 g, 13%). Ir (CHCl$_3$ cast) 1730, 1590 cm^{-1}; ^{1}H nmr (80 MHz, CDCl$_3$) δ 2.53 (s, 3H, C*H*$_3$), 3.90 (s, 3H, OC*H*$_3$), 8.18 (s, 1H, vinyl-*H*); exact mass: 141.0424 (141.0426 calcd for $C_6H_7NO_3$).

2-Acetamidopropanoic acid 2,2,2-trichloroethyl ester (10). The method of Swern and Jordan[75] was adapted. A solution of *N*-acetyl DL-alanine (20.0 g, 0.153 mol), 2,2,2-trichloroethanol (Aldrich, 29.9 g, 0.200 mol), and *p*-toluenesulphonic acid (500 mg) in benzene (500 mL) was refluxed for 12 h with azeotropic removal of water with a Dean-Stark apparatus. The solution was allowed to cool to room temperature and filtered. The precipitate, after drying, was *N*-acetyl DL-alanine (16.3 g, 82% recovered). The solvent was removed from the filtrate and the residue was taken up in saturated NaCl (200 mL). Saturated $KHCO_3$ (25 mL) was added carefully, the aqueous layer was extracted with $CHCl_3$ (3 x 50 mL), and the organic layer was dried ($MgSO_4$). The solvent was removed *in vacuo* to give a yellow oil which was heated at 51 °C (2.5 torr) to remove unreacted 2,2,2-trichloroethanol. The remaining residue is **10** (5.56 g, 76% based on recovered starting material). Mp 69-72 °C; ir ($CHCl_3$ cast) 3280, 1764, 1654, 1515 cm^{-1}; ^1H nmr (80 MHz, $CDCl_3$) δ 1.50 (d, J = 7.0 Hz, 3H, CH_3-CH), 2.03 (s, 3H, CH_3CO), 4.44 (d, J = 11.8 Hz, 1H, C*H*HCCl$_3$), 4.53 (m, 1H, C*H*), 4.85 (d, J = 11.8 Hz, 1H, C*H*HCCl$_3$), 6.45 (br, 1H, N*H*); exact mass: 260.9724 (260.9726 calcd for $C_7H_{10}Cl_3NO_3$). Anal. calcd for $C_7H_{10}Cl_3NO_3$: C, 32.19; H, 3.86; N, 5.37. Found: C, 31.84; H, 3.81; N, 5.30.

2-Acetamidoacrylic acid 2,2,2-trichloroethyl ester (11). The procedure for the formation of **5** was followed. A solution of **10** (11.5 g, 44.1 mmol) in 17 mL of dry MeOH was stirred at 5 °C. *tert*-Butyl hypochlorite (6.64 mL, 55.7 mmol) was added followed by 0.13 mL of a 1% (w/v) solution made from sodium metal and methanol. This was let stand at 0 °C overnight before the solvent was removed *in vacuo*. The residue was dissolved in 20 mL of CH_2Cl_2, washed with saturated NaCl solution (5 mL), dried ($MgSO_4$), and filtered. The filtrate was diluted to 100 mL with CH_2Cl_2. The flask was equipped with a reflux condensor and DABCO (4.94 g, 44.1 mmol) was added at a rate to bring about gentle reflux. The solution was refluxed another 10 min, then cooled at -20 °C for 2 h. This was filtered and the filtrate was washed with 15 mL each of H_2O, 2 x 1N HCl, saturated $KHCO_3$, and H_2O. The solution was dried ($MgSO_4$) and the solvent was removed to give **11** (9.61 g, 84%) as an oil. Ir ($CHCl_3$ cast) 3300, 1736, 1675, 1518 cm^{-1}; ^1H nmr (80 MHz, $CDCl_3$) δ 2.03 (s, 3H, CH_3CO), 4.83 (s, 2H, CH_2), 6.03 (s, 1H, vinyl *H*), 6.65 (s, 1H, vinyl *H*), 7.63 (s, 1H, N*H*); exact mass: 258.9596 (258.9570 calcd for $C_7H_8Cl_3NO_3$).

2-Acetamido-3-chloroacrylic acid 2,2,2-trichloroethyl ester (12). The method employed for the formation of 3 was used. Chlorine gas was passed through a room temperature solution of **11** (9.08 g, 35.1 mmol) in CCl_4 (100 mL) until a yellow color remained. This was stirred 10 min and the solvent was removed *in vacuo*. The residue was dissolved in CH_2Cl_2 (50 mL) and DABCO (3.93 g, 35.0 mmol) was added with stirring. The solvent was removed, the residue was taken up in H_2O (75 mL), acidified with 1N HCl, and the organic layer was separated. The aqueous layer was extracted with ether (3 x 25 mL), saturated with NaCl, and extracted with ether (3 x 25 mL). The combined organic layers were dried ($MgSO_4$) and the solvent was removed to yield **12** (5.34 g, 52%) as an oil. Ir ($CHCl_3$ cast) 3250, 1728, 1670 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 2.15 (s, 3H, CH_3CO), 4.80 (s, 2H, CH_2), 7.10 (s, 0.59H, vinyl-*H*), 7.38 (s, 1H, N*H*), 7.75 (s, 0.41H, vinyl-*H*); exact mass: 292.9169 (292.9180 calcd for $C_7H_7Cl_4NO_3$).

***Z*-2-Acetamido-3-methoxyacrylic acid phenylmethyl ester (13).** To 23.1 mg (145. μmol) of **1** in 10 mL of CH_2Cl_2 was added a solution of phenyldiazomethane (**14**) in CH_2Cl_2 until a permanent orange color persisted. This was stirred at room temperature for 1 h, phenyldiazomethane being added as neccessary to maintain an orange color. H_2O and 1N acetic acid were added until the color disappeared, and the solvent was removed *in vacuo*. The residue was purified on a prep. tlc plate ($CHCl_3$) to give 29.3 mg (118. μmol, 81%) of **13**.[79] Ir ($CHCl_3$ cast) 3258, 1712, 1661 cm^{-1}; ^1H nmr (80 MHz, $CDCl_3$) δ 2.05 (s, 3H, CH_3CO), 3.88 (s, 3H, CH_3O), 5.18 (s, 2H, CH_2), 6.45 (br s, 1H, N*H*), 7.30 (s, 1H, vinyl *H*), 7.35 (s, 5H, aryl *H*); exact mass: 249.1005 (249.1001 calcd for $C_{13}H_{15}NO_4$).

Phenyldiazomethane (14). According to the method of Overberger and Anselme,[254] benzaldehyde (21.2 g, 0.200 mol) was added with stirring and ice bath cooling to hydrazine hydrate (20.0 g, 0.383 mol). The temperature was maintained at 40 °C with external cooling. The resulting solution was extracted with ether, the ether extracts were dried ($MgSO_4$), and the solvent was removed *in vacuo* to give a yellow oil. This was dissolved in CH_2Cl_2 (200 mL), $MgSO_4$ (20.0 g, 0.166 mol) was added, and the solution was cooled with an ice bath. To this stirred solution was added MnO_2 (80.0 g, 0.920 mol) in portions and stirring was continued for 1 h at 20 °C. This was filtered, the precipitate was washed with CH_2Cl_2, and the bright red filtrate was stored at -20 °C overnight. This was filtered again, stored at -20 °C, and used without further purification.

[2,3-$^2H_2$]-*N*-Acetyl-*O*-methyl-DL-serine methyl ester (15). This was prepared analogously to the unlabelled material from **2** (103.3 mg, 0.596 mmol) which had been exchanged by dissolving in ^2H_2O and lyophilizing. This was added to a mixture of 5% Rh(C) (53.3 mg) predeuterated for 3 h, and Et_3N (40 µL, 0.29 mmol) in a total volume of 50 mL of ether. After isolation and purification, 28.0 mg (27%) of pure **15** was obtained. Mp 66-68 °C; ir ($CHCl_3$ cast) 3285, 1745, 1545 cm^{-1}; ^1H nmr (200 MHz, $CDCl_3$) δ 2.04 (s, 3H, $COCH_3$), 3.32 (s, 3H, OCH_3), 3.77 (s, 4H, CO_2CH_3 and CHD), 4.71 (br d, 0.2H, CH), 6.38 (br s, 1H, NH); exact mass: 177.0972 (177.0968 calcd for $C_7H_{11}{}^2H_2NO_4$). Anal. calcd for $C_7H_{11}{}^2H_2NO_4$: C, 47.45; N, 7.90. Found: C, 47.14; N, 7.61.

Unlabelled *N*-acetyl-*O*-methyl-DL-serine methyl ester. In dry ether (250 mL) was suspended **2** (1.00 g, 5.77 mmol) and 198 mg of 5% Rh/C. Triethylamine (0.40 mL, 2.9 mmol) was added, and this was shaken for 3.5 h at 49 psi of H_2. This was filtered, through celite and the solvent was removed to give a mixture of *N*-acetyl-*O*-methyl-DL-serine methyl ester and *N*-acetyl-DL-alanine methyl ester (**4**) (3:1 by nmr). The desired *N*-acetyl-*O*-methyl-DL-serine methyl ester could be purified by prep. tlc (4% MeOH in $CHCl_3$, run twice, R_f 0.55) to give 0.223 g (22%). Mp 66-67 °C; ir ($CHCl_3$ cast) 3280, 1745, 1550 cm^{-1}; ^1H nmr (80 MHz, $CDCl_3$) δ 2.08 (s, 3H, $COCH_3$), 3.40 (s, 3H, OCH_3), 3.65 (dd, J = 3.8, 9.6 Hz, 1H, C*H*H), 3.83 (s, 3H, CO_2CH_3), 3.90 (dd, J = 3.8, 9.6 Hz, 1H, C*H*H), 4.80 (t, J = 3.8 Hz, 1H, C*H*), 6.53 (br s, 1H, N*H*); exact mass: 175.0839 (175.0844 calcd for $C_7H_{13}NO_4$). Anal. calcd for $C_7H_{13}NO_4$: C, 47.99; H, 7.48; N, 8.00. Found: C, 47.84; H, 7.45; N, 7.81.

[2,3-$^2H_2$]-*N*-Acetyl-*O*-methyl-DL-serine (16) from hydrolysis of 15. To a solution of **15** (19 mg, 0.11 mmol) in THF (1.5 mL) at 5 °C was added 1.06 mL of 0.101M LiOH solution, and this was stirred at 5 °C for 24 h. After removal of the solvent, the free acid of **16** (15 mg, 84%) was isolated by dissolving in water and passing through an AG50x8 H^+ ion exchange resin. ^1H nmr (80 MHz, $CDCl_3$) δ 2.10 (s, 3H, $COCH_3$), 3.45 (s, 3H, OCH_3), 3.77 (br, 0.07H, C*H*D), 3.93 (s, 0.32H, C*H*D), 4.80 (br, 0.2H, C*H*), 6.60 (br s, 1H, N*H*); exact mass: 163.0810 (163.0812 calcd for $C_6H_9{}^2H_2NO_4$).

[2,3-$^2H_2$]-*N*-Acetyl-*O*-methyl-DL-serine (16) from hydrogenation of 1. The tetrabutylammonium salt of **1** was prepared by dissolving 1.99 g (12.5 mmol) in one equivalent of tetrabutylammonium hydroxide and lyophilizing the resulting solution. This was lyophilized 3 times from D_2O and the oil was taken up in 150 mL of CH_3OD and added to 5% Rh(C) (0.796 g) which had been prehydrogenated with deuterium gas for 1 day in 400 mL of ethyl acetate. The resulting solution was stirred under 1 atm of deuterium for 3 h, filtered through Celite, and the solvent was removed *in vacuo*. The free acid was obtained by stirring with acid washed AG50x8 H^+ ion exchange resin, filtering, and lyophilizing to give a white solid (1.54 g, 76%). This is a mixture of *N*-acetyl-*O*-methylserine (**16**)[255] and *N*-acetylalanine (**17**) in a 1:1 ratio. ^1H nmr (80 MHz, $CDCl_3$) δ

2.10 (s, 3H, $\mathrm{COC}H_3$), 3.37 (s, 3H, $\mathrm{OC}H_3$), 3.85 (s, 0.5H, $\mathrm{C}H\mathrm{D}$), 6.63 (br s, 1H, $\mathrm{N}H$); exact mass: 163.0815 (163.0812 calcd for $C_6H_9{}^2H_2NO_4$).

Unlabelled *N*-Acetyl-*O*-methyl-DL-serine. Unlabelled *N*-acetyl-*O*-methyl-DL-serine methyl ester (58.7 mg, 0.335 mmol) was dissolved in THF (3.0 mL) at 5 °C and 0.101M LiOH (3.40 mL) was added. This was stirred at 5 °C for 25 h and the solvent was removed *in vacuo* to give the lithium salt. This was converted with AG50x8 ion exchange resin to give 52.4 mg (96%) of the free acid.[256] Ir ($CHCl_3$ cast) 3320, 1730, 1640, 1580 cm^{-1}; ^1H nmr (200 MHz, $CDCl_3$) δ 2.03 (s, 3H, $\mathrm{COC}H_3$), 3.36 (s, 3H, $\mathrm{OC}H_3$), 3.66 (dd, J = 3.0, 9.6 Hz, 1H, $\mathrm{C}H\mathrm{H}$), 3.88 (dd, J = 3.0, 9.6 Hz, 1H, $\mathrm{C}H\mathrm{H}$), 4.74 (m, 1H, $\mathrm{C}H$), 6.92 (br s, 1H, $\mathrm{N}H$); exact mass: 161.0690 (161.0688 calcd for $C_6H_{11}NO_4$).

(2S,3R)-[2,3-$^2H_2$]-*O*-Methylserine (18). The procedure of Jaeger *et al.*[65] was followed. In 25 mL of water was dissolved amide **16** (1.20 g, 7.35 mmol) and the pH adjusted to 7.9 with 2N NH_4OH and 1N HCl. A solution of hog kidney acylase I in 2 mL of water was added, and the reaction was shaken at 37 °C for 24 h. This was lyophilized, the residue was dissolved in water, the pH was adjusted to 4 with 1N AcOH, and charcoal was added. After filtration and washing of the charcoal with water, the filtrate was lyophilized to give a solid. This was applied to a 125 mL AG50x8 H^+ column. The *N*-acetyl-*O*-methyl-D-serine and *N*-acetyl-D-alanine were eluted with 375 mL water, and the eluent was lyophilized to give 0.532 g.

The column was then eluted with 4% NH_4OH, and the fractions which showed ninhydrin positive spots were combined and lyophilized to give 0.372 g as a mixture of *O*-methyl-L-serine (**18**)[257] and L-alanine which could not be separated easily. ^1H nmr (80 MHz, D_2O) δ 3.43 (s, 3H, $\mathrm{OC}H_3$), 3.83 (brs, 0.5H, $\mathrm{C}H\mathrm{D}$).

(2S,3R)-[2,3-$^2H_2$]-serine (19). The procedure used by Moore[258] for unlabelled material was followed. To the mixture of methyl ether **18** (0.350 g, 2.89 mmol) and L-alanine was added CH_2Cl_2 (20 mL). This was stirred at 0 °C and 1.0M BBr_3 in CH_2Cl_2 (12 mL, 12 mmol) was added. The solution was stirred at room temperature for 15 h, and another 10 mL of CH_2Cl_2 and 2.0 mL (2.0 mmol) of 1M BBr_3 was added. Stirring was continued for a total of 3 days. Ice was added carefully and the organic layer was extracted with 50 mL of water. The combined aqueous layers were lyophilized to give an off-white solid which is a mixture of the desired serine **19** and L-alanine. The serine was isolated by careful ion-exchange chromatography with AG50x8 H^+ resin. A 2 cm x 36 cm column was used with a gradient elution from $H_2O \rightarrow 0.5M$ HCl (300 mL each), then from 0.5M HCl→1M HCl (300 mL each). The fractions which showed only serine were combined and the water was removed *in vacuo* to give **19**[56] as the HCl salt (131.5 mg, 32%). Ir (KBr) 1755 cm^{-1}; ^1H nmr (400 MHz, 10% NaOD/D_2O) δ 3.58 (s, C*HD*); ^2H nmr (61.4 MHz, 10% NaOH/H_2O, $CDCl_3$ int. standard) δ 3.31 (s, 1D, C*D*), 3.59 (s, 0.6D, C*DD*), 3.72 (s, 1D, C*D*H).

***N*-Benzyloxycarbonyl-(2S,3R)-[2,3-$^2H_2$]-serine (20).** According to the literature method,[82] the labelled serine **19** (120 mg, 1.12 mmol) was dissolved in 5 mL of water, the pH was lowered below 5 with HCl (conc.) and then raised to 7.80 with solid $NaHCO_3$. This was stirred vigorously and benzyl chloroformate (0.24 mL, 1.7 mmol) was added over 0.5 h. After stirring for an additional 1 h, the solution was extracted with ether (3 x 2 mL), cooled in an ice bath, and HCl (conc.) was added until the pH dropped below 2. This was extracted with EtOAc (3 x 6 mL), the organic layers were dried ($MgSO_4$), and the solvent was removed *in vacuo* to give the nitrogen protected serine **20**, 125.8 mg (47%). Ir ($CHCl_3$ cast) 3400, 1715, 1522 cm^{-1}; ^1H nmr (400 MHz, $CDCl_3$) δ 3.86 (s, 0.5H, C*HD*), 5.16 (s, 2H, aryl-C*H*$_2$), 7.44 (m, 5H, aryl-*H*); exact mass: 241.0919 (241.0917 calcd for $C_{11}H_{11}{}^2H_2NO_5$).

Cyclization of *N*-benzyloxycarbonyl-L-serines (36), (20), (25) and (30) to β-lactones (24), (21), and (32). As described in the literature[9] a solution of triphenylphosphine (158. mg, 0.602 mmol) in 2 mL of dry tetrahydrofuran was cooled to -78 °C and dimethylazodicarboxylate (Tokyo Kasei, 87.6 mg, 0.600 mmol) was added dropwise. After 10 min a white precipitate formed to which was added over 10 min *N*-benzyloxycarbonyl-L-serine (**36**) (122. mg, 0.508 mmol) in 2 mL of dry tetrahydrofuran. This was stirred at -78 °C for 25 min, then allowed to warm to room temperature, and stirred for an additional 2.5 h. The solvent was removed and the residue was purified using flash silica chromatography (60/40 Skelly B/EtOAc). This gave 48.7 mg (43%) of the β-lactone (**24**)[9] as a white solid. Ir ($CHCl_3$ cast) 3363, 1845, 1685 cm^{-1}; ^1H nmr (400 MHz, CD_2Cl_2) δ 4.48 (d, *J* = 6.0 Hz, 2H, CH_2-CH), 5.08 (dd, *J* = 6.0, 8.1 Hz, 1H, C*H*), 5.15 (s, 2H, CH_2-C_6H_5), 5.50 (br s, 1H, N*H*), 7.37 (s, 5H, aryl-*H*); exact mass: 221.0685 (221.0688 calcd for $C_{11}H_{11}NO_4$).

Similarly, when the ^2H labelled *N*-benzyloxycarbonyl-L-serine (**20**) was used the labelled β-lactone (**21**) was formed and showed the expected chromatographic and spectral properties. ^1H nmr (400 MHz, CD_2Cl_2) δ 4.44 (s, 0.46H, C*HD*), 5.14 (s, 2H, CH_2), 5.50 (br s, 1H, N*H*), 7.38 (s, 5H, aryl-*H*); exact mass: 223.0810 (223.0813 calcd for $C_{11}H_9{}^2H_2NO_4$).

From *N*-benzyloxycarbonyl-L-serine with ^{18}O in the carboxyl oxygen (**25**) (isotopic content: 71±1% ^{18}O) the β-lactone (**32**) was formed with ^{18}O in the carbonyl and lactone oxygens. This had the expected chromatographic and spectral properties when compared to the unlabelled material (**24**). Isotopic content: 73±1% ^{18}O by chemical ionization (NH_3) mass spectrometry (ci-ms).

From *N*-benzyloxycarbonyl-L-serine with ^{18}O in the hydroxyl oxygen (**30**) (isotopic purity: 76% ^{18}O), β-lactone was formed which showed identical properties to **24** and no indication of ^{18}O in the chemical ionization mass spectrum.

(2*S*,3*S*)-[2,3-$^2H_2$]-*N*-Benzyloxycarbonyl-serine (22). To a cooled (0 °C) solution of 0.5 mL tetrahydrofuran, 0.5 mL of water, and 0.194 mL of 0.587M NaOH

(0.114 mmol, 1.05 eq) was added dropwise a solution of the labelled β-lactone **21** (24.2 mg, 0.108 mmol) in 1 mL of tetrahydrofuran. This was stirred 10 min, the tetrahydrofuran was removed *in vacuo*, and the water was lyophilized. The residue was taken up in 1 mL of 1N HCl and extracted with ethyl acetate. The solvent was removed to give 24.5 mg (95%) of the labelled *N*-benzyloxycarbonyl-serine **22** as a white solid. Ir ($CHCl_3$ cast) 3340, 1715 cm^{-1}; ^1H nmr (400 MHz, D_2O) δ: 3.84 (s, 0.4H, C*H*), 5.15 (s, 2H, CH_2), 7.45 (s, 5H, aryl-*H*); exact mass: 241.0921 (241.0919 calcd for $C_{11}H_{11}{}^2H_2NO_5$).

(2*S*,3*S*)-[2,3-$^2H_2$]-Serine (23). A mixture of **22** (21.5 mg, 89.1 μmol), 5% Pd/C (10.0 mg), and 2 mL of 1N HCl was hydrogenated at 1 atm for 1.5 h. The solution was filtered through celite and lyophilized to give the serine **23** (14.0 mg, > 99%) which was analyzed by nmr. ^1H nmr (400 MHz, 10% NaOD/D_2O) δ 3.74 (s, C*H*D); ^2H nmr (61.4 MHz, 10% NaOH/H_2O, $CDCl_3$ int. standard) δ 3.40 (s, 1D, C*D*), 3.79 (s, 1D, C*D*D), 3.93 (s, 0.6D, C*D*H).

[^{18}O-Carboxyl]-*N*-benzyloxycarbonyl-serine (25). Sodium metal (10.9 mg, 0.473 mmol) was added cautiously to cold (0 °C) [^{18}O]water (1 mL, 90% isotopic purity) under an argon stream. To this was added 0.5 mL of THF followed by the dropwise addition of a solution of *N*-benzyloxycarbonyl-L-serine-β-lactone (**24**) (99.6 mg, 0.450 mmol) in 3 mL of THF. This was stirred for 0.5 h at 20 °C, the THF was removed *in vacuo*, and the water was lyophilized to give the crude sodium salt of **25**. In preparative experiments the free acid of **25** was obtained by dissolving the sodium salt in water, acidifying with 1N HCl, and extracting with ethyl acetate. The ethyl acetate extracts were dried (Na_2SO_4) and concentrated *in vacuo* to give the pure free acid **25** (89% yield) with expected spectral and chromatographic properties. This had an isotopic ratio ci-ms of 71% ^{18}O (prepared from 90 atom % ^{18}O water). Exact mass: 241.0833 (241.0836 calcd for $C_{11}H_{13}NO_4{}^{18}O$).

Attempted preparation of 25 from exchange of 36 with $H_2{}^{18}O$. To a solution of 1.0 mL of H_2O:$H_2{}^{18}O$:THF (1:1:2) made acidic (pH 2) with HCl (conc.) was added *N*-benzyloxycarbonyl-L-serine (36). This was stirred at room temperature for 6 h and the solvent was lyophilized. The residue was analyzed by ci-ms and showed no peaks corresponding to incorporation of ^{18}O.

[^{18}O-Carboxyl]-*N*-benzyloxycarbonyl-serine methyl ester (26). In studies on the mechanism of β-lactone opening, anhydrous ether (10 mL) and *p*-toluenesulfonic acid monohydrate (85.4 mg, 0.449 mmol) were added to the sodium salt of **25** (0.445 mol). The mixture was treated with an ether solution of diazomethane until a slight yellow color persisted. This was stirred for 0.5 h, filtered, and the solvent was removed *in vacuo* to yield an oil (117.6 mg). This was purified on a silica gel column using Skelly B/ethyl acetate as the eluent to give 56.5 mg (50%) of the methyl ester **26** whose spectral and chromatographic properties were compared to unlabelled material and the corresponding [^{18}O-hydroxy]-compound **31**. The ir and ^1H- nmr were identical; for compound **26**: ^{13}C nmr ($CDCl_3$) δ 52.69 (isotope shift 26 p.p.b. upfield), 56.17, 63.18, 67.25, 128.16, 128.29, 128.60, 136.20, 156.39, 171.17 (isotope shifts 15 and 38 p.p.b. upfield); exact mass: 255.0995 (255.0992 calcd for $C_{12}H_{15}NO_4{}^{18}O$).

Unlabelled *N*-Benzyloxycarbonyl-serine methyl ester. Ethereal diazomethane was added to a solution of **36** (0.263 g, 1.10 mmol) in 10 mL of ether until a yellow color remained. This was stirred for 15 min and the solvent was removed to give 0.279 g (> 99%). Ir ($CHCl_3$ cast) 3350, 1708 cm^{-1}; ^1H nmr (80 MHz, $CDCl_3$) δ 2.96 (br s, 1H, O*H*), 3.73 (s, 3H, C*H*$_3$), 3.90 (br, 2H, C*H*$_2$OH), 4.39 (m, 1H, C*H*), 5.09 (s, 2H, aryl-C*H*$_2$), 5.88 (br, 1H, N*H*), 7.30 (s, 5H, aryl-*H*); exact mass: 253.0953 (253.0950 calcd for $C_{12}H_{15}NO_5$).

Potassium [$^{18}O_2$]acetate (27). This was prepared according to Cane *et al.*[87] To dry acetonitrile (1.14 mL, 20.0 mmol) was added $H_2{}^{18}O$ (1.0 g, 50 mmol) and 19.2 mL (20.0 mmol) of a 1.03M solution of potassium *tert*-butoxide in dry *tert*-butyl alcohol. This was refluxed for 48 h and then cooled. The solvent was removed *in vacuo* to yield **27** (1.97 g, 96%).

***p*-Phenylphenacyl acetate (28).** The procedure used by Risley and Van Etten[83] was followed. A solution of **27** (49.4 mg, 0.484 mmol) in 2 mL of anhydrous ethanol was treated with α-bromo-*p*-phenylacetophenone (121. mg, 0.440 mmol). The mixture was heated to reflux for 3.5 h, water (0.4 mL) was added, and the solution was cooled. The crystaline product was collected and washed with water. This gave **28** (83.9 mg, 74%) as pale yellow crystals. Mass spectrometric (ci) examination gave an isotope ratio of 21.6% $^{18}O_1$ and 76.9% $^{18}O_2$. Mp 105-106 °C (lit.[88] mp 106-107 °C); ir (film) 1724, 1717, 1700, 1600 cm^{-1}; ^1H nmr (400 MHz, $CDCl_3$) δ 2.23 (s, 3H, CH_3), 5.34 (s, 2H, CH_2), 7.35-8.08 (m, 9H, aryl-*H*); exact mass: 258.1031 (258.1029 calcd for $C_{16}H_{14}O^{18}O_2$).

[^{18}O-Acetyl]-*O*-acetyl-*N*-benzyloxycarbonyl-L-serine (29). The method described[9] for opening the β-lactone with acetate was modified. The β-lactone **24** (59.6 mg, 0.270 mmol) was added to a solution of potassium [$^{18}O_2$]acetate (**27**) (142 mg, 1.39 mmol) in dimethylformamide (6 mL) and water (0.5 mL). The mixture was stirred 1.5 h, water (6 mL) was added, and the solution was acidified to pH 2 with 1N HCl. The solution was extracted with chloroform and the extracts were concentrated *in vacuo* to give 77.0 mg (99%) of pure ^{18}O-labelled *O*-acetyl-*N*-benzyloxycarbonyl-L-serine (**29**).[9] Ir ($CHCl_3$ cast) 3140, 1710, 1530 cm-1; ^1H nmr (400 MHz, $CDCl_3$) δ 1.93 (s, 3H, $COCH_3$), 4.40 (br s, 3H, CH and CH_2OAc), 5.05 (s, 2H, CH_2-C_6H_5), 5.95 (br s, 1H, NH), 7.23 (s, 5H, aryl-*H*); exact mass: 285.0987 (285.0984 calcd for $C_{13}H_{15}NO_4{}^{18}O_2$). Isotopic Ratio 21.4% ^{18}O, 77.1% $^{18}O_2$.

Unlabelled *O*-Acetyl-*N*-benzyloxycarbonyl-L-serine.[9] To dry DMF (1.5 mL) was added potassium acetate (25.0 mg, 255 μmol), water (0.2 mL), and β-lactone **24** (10.3 mg, 46.6 μmol). This was stirred 45 min at room temperature, 1.5 mL of water was added, and the solution acidified with 1N HCl. This was extracted with $CHCl_3$ and the solvent was removed from the combined organic layers to give authentic *O*-acetyl-*N*-benzyloxycarbonyl-L-serine (15.8 mg, > 99%). Ir and ^1H nmr were identical with **29**; exact mass: 281.0896 (281.0899 calcd for $C_{13}H_{15}NO_6$).

[Hydroxy-^{18}O]-*N*-benzyloxycarbonyl-L-serine (30). A solution of [^{18}O-acetyl]-*O*-acetyl-*N*-benzyloxycarbonyl-L-serine (**29**) (77 mg, 0.27 mmol), 2 mL THF, 2 mL water, and 0.59M sodium hydroxide (1 mL) was stirred at 4 °C for 2.5 h. This was acidified with 1N HCl and extracted with ethyl acetate. The solvent was removed *in vacuo* to give 53 mg (82%) of pure ^{18}O-labelled *N*-benzyloxycarbonyl-L-serine (**30**). Ir (film) 3320, 1720 cm^{-1}; ^1H nmr (400 MHz, CD_3OD) δ 3.85 (d, J = 4.4 Hz, 2H, CH_2OH), 4.28 (t, J = 4.2 Hz, 1H, CH), 5.09 (s, 2H, CH_2-C_6H_5), 7.31 (s, 5H, aryl-H); exact mass: 241.0830 (241.0836 calcd for $C_{11}H_{13}NO_4{}^{18}O$). Isotopic Purity 76.2% ^{18}O.

[^{18}O-Hydroxyl]-*N*-benzyloxycarbonyl-L-serine methyl ester (31). Ethereal diazomethane was added to a solution of **30** (38.5 mg, 0.160 mmol) in ether until a yellow color remained. This was stirred for 15 min and the solvent was removed to give **31** (40.8 mg, 97%). Exact mass: 255.0995 (255.0992 calcd for $C_{12}H_{15}NO_4{}^{18}O$).

***N*-Benzyloxycarbonyl-*O*-methyl-L-serine methyl esters 33, 34, and 35, from their corresponding *N*-benzyloxycarbonyl-L-serine methyl esters 31, 26, and unlabelled *N*-benzyloxycarbonyl-serine methyl ester.** To an ether solution of the unlabelled *N*-benzyloxycarbonyl-serine methyl ester was added a trace of HBF_4 and excess diazomethane. The solvent was removed and the residue was analyzed directly by mass spectrometry.

For ^{18}O-hydroxyl compound **33** prepared from **29**: exact mass: 269.1127 (269.1149 calcd for $C_{13}H_{17}NO_4{}^{18}O$); 210.0997 (210.1020 calcd for $C_{11}H_{14}NO_2{}^{18}O$ (M^+-COOMe)).

For ^{18}O-carboxyl compound **34**: exact mass: 269.1144 (269.1149 calcd for $C_{13}H_{17}NO_4{}^{18}O$); 208.0980 (208.0980 calcd for $C_{11}H_{14}NO_3$ (M^+-$C^{18}OOMe$)).

For unlabelled compound **35**: exact mass: 267.1104 (267.1107 calcd for $C_{13}H_{17}NO_5$); 208.0972 (208.0980 calcd for $C_{11}H_{14}NO_3$ (M^+-COOMe)).

[^{18}O-Hydroxyl]-*N*-benzyloxycarbonyl-*O*-methyl-L-serine methyl ester (33a) from acidic hydrolysis of 24. A solution of *N*-benzyloxycarbonyl-L-serine-β-lactone (**24**) (72.9 mg, 0.330 mmol) in 1.5 mL THF was added dropwise to a solution of [^{18}O]water (0.70 g, 50% isotopic purity), 0.5 mL THF, and HBF_4 (0.060 mL, 0.33 mmol). This was stirred at room temperature for 11 days, lyophilized, and the residue was taken up in ether. Excess diazomethane was added to the stirred ether solution and the solvent was removed *in vacuo*. The resultant oil **33a** was compared spectrally (ir, ^1H-nmr, ms) to the unlabelled analog **35** and the [carboxy-^{18}O] analog **34**. For compound **33a**: exact mass: 269.1142 (269.1149 calcd for $C_{13}H_{17}NO_4{}^{18}O$); 210.1020 (210.1020 calcd for $C_{11}H_{14}NO_2{}^{18}O$ (M^+-COOMe)). Ci-ms shows peaks at 285 (O), 287 (^{18}O), and 289 ($^{18}O_2$), indicating the presence of ^{18}O in the carboxyl as well as hydroxyl oxygens.

***N*-Benzyloxycarbonyl-*O*-methylserine methyl ester (35).** This material was prepared as described by Bernstein and Ben-Ishai.[259] In 5 mL of water was dissolved *O*-methyl-DL-serine (193.2 mg, 1.62 mmol) and benzyl chloroformate (0.25 mL, 1.8 mmol) was added. This was stirred for 3 h, washed with ~~5 mL of ether, and the~~ aqueous layer was acidified with HCl (conc.). This was extracted with EtOAc, the extracts were dried (Na_2SO_4), and the solvent was removed to give *N*-benzyloxycarbonyl-*O*-methyl-DL-serine (59.4 mg, 15%). This was dissolved in ether and ethereal diazomethane was added until a yellow color persisted. This was stirred an additional 15 min, and the solvent was removed in vacuo to give crude **35**. This was purified by prep. tlc (60:40 EtOAc:hexane). The uv active band from R_f 0.55-0.67 was extracted to give pure **35**[259] (15.0 mg, 3.5% from *O*-methyl-DL-serine). Ir ($CHCl_3$ cast) 3345, 2947, 1723, 1520 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 3.38 (s, 3H, OCH_3), 3.71 (br m, 2H, CH_2OMe), 3.78 (s, 3H, CO_2CH_3), 4.59 (m, 1H, *CH*), 5.15 (s, 2H, aryl-CH_2), 5.60 (br, 1H, *NH*), 7.33 (s, 5H, aryl-*H*); exact mass: 267.1104 (267.1107 calcd for $C_{13}H_{17}NO_5$).

***N*-Benzyloxycarbonyl-L-serine (36).** To 1.12 mL of 0.587M NaOH in 3 mL of water and 3 mL of THF stirred at 0 °C was added **24** (139. mg, 0.626 mmol). This was stirred at 0 °C for 15 min and the THF was removed *in vacuo*. The residue was acidified with 1N HCl and extracted with EtOAc. The EtOAc was dried (Na_2SO_4) and the solvent removed to give **36** (132 mg, 88%). This had spectral and chromatographic properties identical to authentic *N*-benzyloxycarbonyl-L-serine (Sigma). For **36**, $[\alpha]_D^{25}$ +6.7 (c=7, AcOH); for *N*-benzyloxycarbonyl-L-serine from Sigma, $[\alpha]_D^{25}$ +6.7 (c=7, AcOH).

D-Tyrosyl-L-valylglycine (37). The *tert*-butoxycarbonyl group was removed using standard conditions.[260] To *N*-(*tert*-butoxycarbonyl)-D-tyrosylvalylglycine (512. mg, 1.17 mmol) was added 10 mL of CF_3COOH and this was allowed to stand at room temperature for 30 min. The CF_3COOH was removed *in vacuo* and the residue triturated with ether. The off-white solid was collected, dissolved in a minimum amount of 90 °C water, and 2% NH_4OH 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 **37** (295. mg, 75%). Mp 229-230 °C (dec.); ir (KBr) 3280, 1620, 1518 cm^{-1}; ^1H nmr (360 MHz, CD_3COOD) δ 0.88 (2d, J = 6.7 Hz, 6H, $(CH_3)_2$), 3.22 (2d, J = 7.9 Hz, 2H, aryl-CH_2), 4.16 (2d, J = 18 Hz, 2H, NCH_2CO), 4.50 (d, J = 6.7 Hz, 1H, $CHCHMe_2$), 4.55 (t, J = 7.9 Hz, 1H, aryl-CH_2CH), 6.86 (d, J = 8.5 Hz, 2H, aryl-*H*), 7.20 (d, J = 8.5 Hz, 2H, aryl-*H*); ms (POSFAB): 337.98 (337.16 calcd for $C_{16}H_{23}N_3O_5$). Anal. calcd for $C_{16}H_{23}N_3O_5\ H_2O$: C, 54.23; H, 6.83; N, 11.86. Found: C, 54.16; H, 6.77; N, 11.82.

D-Tyrosyl-L-valine amide hydrochloride (38). The procedure[260] used to prepare **37** was followed. To **42** (298 mg, 0.785 mmol) was added 5 mL of CF_3COOH and this was allowed to stand at room temperature for 30 min. The CF_3COOH was removed *in vacuo* and the residue was triturated with ether. The white solid was collected, dissolved in ethanol, and HCl gas was passed through the solution. The solvent was removed, the residue was dissolved in a minimum amount of anhydrous EtOH, and ether was added to precipitate a white solid which was collected and dried to give **38** (173 mg, 70%). Mp >250 °C (dec.); ir (MeOH cast) 1667, 1546 cm^{-1}; ^1H nmr (300 MHz, CD_3COOD) δ 0.79 (2d, J = 7.0 Hz, 6H, ($CH_3)_2$), 2.09 (m, 1H, Me_2CH), 3.18 (m, 2H, aryl-CH_2), 4.30 (d, J = 6.1 Hz, 1H, Me_2CH-CH), 4.67 (t, J = 7.5 Hz, 1H, aryl-CH_2-CH), 6.80 (d, J = 8.5 Hz, 2H, aryl-H), 7.14 (d, J = 8.5 Hz, 2H, aryl-H); ms (POSFAB): 315.07 (315.13 calcd for $C_{14}H_{22}ClN_3O_3$).

***N*-Hydroxyformanilide (39).** The method of Corbett and Corbett[159] was followed. Glyoxylic acid monohydrate (2.30 g, 25.0 mmol) was dissolved in 125 mL of water and the pH was adjusted to 6.0 with 10M NaOH. This was stirred at 40 °C and nitrosobenzene (535 mg, 4.99 mmol) dissolved in a minimum amount of hot 95% EtOH was added. Stirring was continued at 40 °C for 2 h, the mixture was cooled to room temperature, and 12.5 g of NaCl was added. This mixture was extracted with ether, the ether extracts were dried (Na_2SO_4), and the solvent was removed to give a brown residue. This was recrystalized from 1:2:3 acetone:ether:hexane by dissolving in a minimum amount of 1:2 acetone:ether, adding an equal volume of hexane, and cooling to -20 °C. The crystals are collected to give **39** (186. mg, 27%). Mp 68.5-69 °C (lit.[159] mp 69.5-70 °C); ir ($CHCl_3$ cast) 3058, 1708, 1508 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 7.26-7.48 (m, 5H, aryl-H), 8.52 (s, 1H, CH); exact mass: 137.0480 (137.0477 calcd for $C_7H_7NO_2$).

L-Valylglycine methyl ester hydrochloride (40). The literature[261] procedure was adapted. To a slurry of L-valylglycine (1.00 g, 5.74 mmol) in MeOH (30 mL) cooled to

-10 °C was added $SOCl_2$ (1.5 mL, 21 mmol) from a dropping funnel at such a rate as to keep the reaction solution between -7 °C and -11 °C. After the addition was complete the homogeneous solution was stirred at room temperature overnight. The SO_2 and HCl were removed by stirring the solution vigorously under vacuum, and the remaining solvent was removed *in vacuo*. A solid forms after several hours under high vacuum, but this material is extremely hygroscopic and becomes a thick gum after several minutes exposure to air. Yield of **40** is 1.10 g (86%). Ir ($CHCl_3$ cast) 2900, 1750, 1700 cm^{-1}; ^{1}H nmr (80 MHz, CD_3OD) δ 1.15 (d, J = 7.0 Hz, 6H, $(CH_3)_2$), 2.35 (m, 1H, Me_2CH), 3.78 (s, 3H, CO_2CH_3), 3.95 (d, J = 7.0 Hz, 1H, CHN), 4.05 (s, 1H, CHH), 4.13 (s, 1H, CHH); exact mass: 157.0978 (157.0977 calcd for $C_7H_{13}N_2O_2$ (M^+-OCH_3)).

***N*-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valylglycine methyl ester (41) from 40.** The method of Shioiri and Yamada[262] for peptide coupling with diphenylphosphorylazide was used. In a dry flask were placed *N*-(*tert*-butoxycarbonyl)-D-tyrosine (622. mg, 2.21 mmol) and L-valylglycine methyl ester hydrochloride (**40**) (501. mg, 2.23 mmol). This was dried at 50 mtorr over P_2O_5 for 18 h. To this was added dry DMF (7 mL) and the stirred solution was cooled to 0 °C. Diphenylphosphorylazide (0.53 mL, 2.5 mmol) was added followed by a solution of triethylamine (0.65 mL, 4.7 mmol) in DMF (5 mL), and stirring was continued at 0 °C for 6.5 h. The reaction was diluted with 50 mL of benzene and 100 mL of EtOAc. This was washed with 1N HCl (2 x 25 mL), H_2O (25 mL), saturated $NaHCO_3$ (2 x 25 mL), H_2O (25 mL), and saturated NaCl (2 x 25 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed to give an oil. This was purified by silica gel column chromatography using 5% MeOH in $CHCl_3$ as the eluent to give **41** (660. mg, 66%). Mp 87-89 °C; ir ($CHCl_3$ cast) 3306, 1688, 1649, 1516 cm^{-1}; ^{1}H nmr (360 MHz, $CDCl_3$) δ 0.83 (d, J = 6.7 Hz, 6H, $(CH_3)_2$), 1.41 (s, 9H, $(CH_3)_3C$), 2.15 (m, 1H, Me_2CH), 2.99 (m, 2H, aryl-CH_2), 3.72 (s, 3H, CO_2CH_3), 4.04 (br s, 2H, NCH_2CO), 4.40 (m, 2H, NCHCO), 5.48 (d, J = 6.5 Hz, 1H, N*H*), 6.72 (d, *J* = 8.5 Hz, 2H, aryl-*H*), 6.98 (d, *J* = 8.5 Hz, 3H, aryl *H*, N*H*), 7.53 (br s, 1H, N*H*), 7.75 (br s, 1H, O*H*); exact mass: 451.2323 (451.2318 calcd for $C_{22}H_{33}N_3O_7$)

Glycine methyl ester hydrochloride. Dry HCl gas was bubbled through a 0 °C solution of glycine (5.00 g, 66.6 mmol) in dry MeOH (100 mL) for 0.5 h, with the temperature being maintained between 7 and 10 °C by controlling the rate of HCl bubbling. This was allowed to warm to room temperature and stirred for 18 h. After cooling at -20 °C, the precipitate was collected and recrystalized from dry methanol to give pure methyl ester (2.58 g, 31%). Mp 183 °C (lit.[263] mp 175 °C d.); ir (KBr) 1750 br cm^{-1}; ^{1}H nmr (80 MHz, D_2O) δ 3.70 (s, 3H, OC*H*$_3$), 3.79 (s, 2H, C*H*$_2$).

***N*-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valylglycine methyl ester (41) from 43.** The procedure for the preparation of **41** from **40** was followed using **43** (104. mg, 0.273 mmol), glycine methyl ester hydrochloride (38.4 mg, 0.306 mmol), and diphenylphosphorylazide (58. µL, 0.27 mmol) in 1 mL DMF, and adding triethylamine (80. µL, 0.57 mmol, in 1 mL DMF). After washing of the organic layers and removal of the solvent 112. mg (92%) of crude **41** was obtained. This was purified as previously described and gave material with identical properties.

***N*-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valylglycine.** The general procedure[264] for peptide ester hydrolysis was adapted. To a stirred solution of the methyl ester **41** (648. mg, 1.44 mmol) in MeOH (2 mL) in a room temperature water bath was added 3.01 mL of a 1.00 M NaOH solution and the stirring was continued at room temperature. The reaction was followed by tlc (89:10:1 $CHCl_3$:MeOH:HCOOH, R_f **41** 0.56, R_f product 0.27, uv). The reaction was stirred an additional 15 min after the complete disappearance of starting material, then 1 mL of 1N HCl was added and the MeOH was removed *in vacuo*. The residue was further acidified with 2.1 mL of 1N HCl and this was extracted with EtOAc. The combined organic layers were washed with water and saturated NaCl, dried (Na_2SO_4), and the solvent was removed *in vacuo* to give *N*-(*tert*-butoxycarbonyl)-D-tyrosylvalylglycine (543.5 mg, 87%). Ir ($CHCl_3$ cast) 3315, 1722, 1651 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3+CD_3OD$(1 drop)) δ 0.80 (d, *J* = 6.1 Hz, 6H, $(C\mathit{H}_3)_2$), 1.40 (s, 9H, $(C\mathit{H}_3)_3C$), 2.10 (m, 1H, $Me_2C\mathit{H}$), 2.94 (m, 2H, aryl-$C\mathit{H}_2$), 3.88 (d, *J* = 18.3 Hz, 1H, NC*H*HCO), 4.04 (d, *J* = 18.3 Hz, 1H, NC*H*HCO), 4.12 (dd, *J* = 7.3, 15.3 Hz, 1H, aryl-$CH_2C\mathit{H}$), 4.26 (d, *J* = 6.5 Hz, 1H, $C\mathit{H}CHMe_2$), 5.46 (d, *J* = 7.2 Hz, N*H*), 6.70 (d, *J* = 7.9 Hz, 2H, aryl *H*), 6.96 (d, *J* = 7.9 Hz, 2H, aryl-*H*), 7.02 (d, *J* = 7.9 Hz, N*H*), 7.40 (br s, O*H*); exact mass: 320.1377 (320.1372 calcd for $C_{16}H_{20}N_2O_5$ $(M^+-Me_3COCONH_2)$).

N-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valine amide (42). The procedure[262] for the preparation of **41** was followed using *N*-(*tert*-butoxycarbonyl)-D-tyrosine (500. mg, 1.78 mmol), L-valine amide hydrochloride (275. mg, 1.80 mmol), and diphenylphosphorylazide (0.390 mL, 1.81mmol) in 9 mL DMF, and adding triethylamine (0.47 mL, 3.4 mmol, in 5mL DMF). After stirring for 12 h at 0 °C and 12 h at room temperature, 150mL of 2:1 EtOAc:benzene was added. This was washed with the same cycle of 25 mL portions as in the preparation of **41**. The solvent was removed and the residue was purified by column chromatography to give **42** (322. mg, 48%). Mp 103-105 °C; ir ($CHCl_3$ cast) 3310, 1655, 1516 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 0.86 (2d, *J* = 7.2 Hz, 6H, $(C\mathit{H}_3)_2$), 1.44 (s, 9H, $(C\mathit{H}_3)_3C$), 2.10 (m, 1H, $Me_2C\mathit{H}$), 2.84 (dd, *J* = 6.5, 14.4 Hz, 1H, aryl-C*H*H), 3.20 (dd, *J* = 4.3, 14.4 Hz, 1H, aryl-C*H*H), 4.26 (t, *J* = 7.2 Hz, 1H, NC*H*CO), 4.40 (br m, 1H, NC*H*CO), 5.40 (d, *J* = 7.9 Hz, 1H, N*H*), 6.62 (d, *J* = 7.9 Hz, 2H, aryl-*H*), 6.70 (br s, 2H, $N\mathit{H}_2$), 6.90 (d, *J* = 7.9 Hz, 2H, aryl-*H*), 7.48 (br s, 1H, O*H*); exact mass: 379.2116 (379.2107 calcd for $C_{19}H_{29}N_3O_5$).

N-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valine (43). A mixture of **44** (0.750 g, 1.59 mmol) and 5% Pd/C (74.2 mg) in 50 mL EtOAc was hydrogenated under 48 psi of H_2 for 1.5 h. This was filtered through celite and the solvent was removed *in vacuo* to give **43**

(0.467 g, 77%). Mp 97-100 °C; ir ($CHCl_3$ cast) 3320, 1658, 1516 cm^{-1}; ^{1}H nmr (360 MHz, $CDCl_3$) δ 0.89 (m, 6H, $(CH_3)_2$), 1.40 (s, 9H, $(CH_3)_3C$), 2.10 (br, 1H, Me_2CH), 2.88 (br, 1H, aryl-C*H*H), 3.10 (br, 1H, aryl-C*H*H), 4.42 (br, 1H, NC*H*CO), 4.66 (br, 1H, NC*H*CO), 5.46 (br, 1H, N*H*), 6.69 (d, *J* = 8.5 Hz, 2H, aryl-*H*), 6.88 (br, 1H, N*H*), 7.00 (br, 2H, aryl-*H*); exact mass: 380.1949 (380.1947 calcd for $C_{19}H_{28}N_2O_6$).

N-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valine phenylmethyl ester (44). The procedure[262] for the preparation of **41** was followed using *N*-(*tert*-butoxycarbonyl)-D-tyrosine (1.41 g, 5.0 mmol), L-valine phenylmethyl ester hydrochloride (1.34 g, 5.50 mmol), and diphenylphosphorylazide (1.20 mL, 5.57 mmol) in 15 mL DMF, and adding triethylamine (1.50 mL, 11.0 mmol, in 10 mL DMF). After stirring for 5 h at 0 °C, 375 mL of 2:1 EtOAc:benzene was added. This mixture was washed with the same cycle of 25 mL portions as described for the preparation of **41**. The solvent was removed to give an oil which was purified by recrystalization from toluene/Skelly B to give **44** (1.30 g, 55%). Mp 101-102 °C; ir ($CHCl_3$ cast) 3340, 1659, 1516 cm^{-1}; ^{1}H nmr (360 MHz, $CDCl_3$) δ 0.78 (2d, *J* = 6.7 Hz, 6H, $(CH_3)_2$), 1.42 (s, 9H, $(CH_3)_3C$), 2.10 (m, 1H, Me_2CH), 2.99 (br d, *J* = 6.7 Hz, 2H, aryl-CH_2), 4.36 (br s, 1H, NC*H*CO), 4.54 (m, 1H, NC*H*CO), 5.06 (br s, 1H, N*H*), 5.12 (d, *J* = 12.2 Hz, 1H, aryl-C*H*HO), 5.19 (d, *J* = 12.2 Hz, 1H, aryl-C*H*HO), 5.90 (br s, 1H, O*H*), 6.52 (br s, 1H, N*H*), 6.70 (d, *J* = 8.5 Hz, 2H, aryl-*H*), 7.02 (d, *J* = 8.5 Hz, 2H, aryl-*H*), 7.34 (m, 5H, aryl-*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, 66.35; H, 7.29; N, 5.96. Found: C, 66.10; H, 7.17; N, 5.83.

N-Acetyl-[1,2-^{14}C]glycine (47). To the lyophilized residue of [1,2-^{14}C]glycine (1.0×10^7 dpm, 113 mCi/mmol) was added 100 μL of glacial acetic acid. After heating to 100 °C, 1 μL of acetic anhydride was added and this heated for 3 min at 100 °C. This was lyophilized and passed through an Pasteur pipette of AG50x8 H$^+$ ion exchange resin. The column was rinsed and the eluent was lyophlized to give *N*-acetylglycine **47**

(4.4×10^6 dpm, 44%) which co-spotted as a single radioactive spot with authentic material (8:2:2 *n*-BuOH, AcOH, H_2O; R_f 0.44).

***N*-Acetyl-D-tyrosyl-L-valylglycine.** The literature procedure[265] was followed. To a solution of **37** (50.0 mg, 0.148 mmol) in 10 mL of water at 95 °C was added acetic anhydride (3.0 mL, 32. mmol). The solution was heated for a further 40 min, cooled, and lyophilized to give the desired *N*-acetyl tripeptide (28.9 mg, 52%) which is essentially pure by tlc and nmr. Further purification by passing through an AG50x8 H^+ ion exchange column gives analytically pure compound with quantitative recovery. Ir ($CHCl_3$ cast) 3280, 1655, 1518 cm^{-1}; ^1H nmr (360 MHz, D_2O) δ 0.50 (2d, J = 7.3 Hz, 6H, $(CH_3)_2$), 1.82 (s, 3H, $COCH_3$), 1.84 (m, 1H, Me_2CH), 2.74 (dd, J = 9.2, 13.4 Hz, 2H, aryl-C*H*H), 2.84 (dd, J = 6.7, 13.4 Hz, 2H, aryl-C*H*H), 3.77 (m, 1H, Me_2CHCH), 3.79 (m, 1H, aryl-CH_2CH), 4.33 (d, J = 7.3 Hz, 1H, NC*H*HCO), 4.39 (d, J = 7.3 Hz, 1H, NC*H*HCO), 6.64 (d, J = 8.6 Hz, 2H, aryl-*H*), 6.94 (d, J = 8.6 Hz, 2H, aryl-*H*); ms (CI-NH_3): 379.49 (379.17 calcd for $C_{18}H_{25}N_3O_6$). Anal. calcd for $C_{18}H_{25}N_3O_6$: C, 56.97; H, 6.64; N, 11.08. Found: C, 56.69; H, 6.45; N, 11.02.

Synthesis of radiolabelled D-tyrosyl-L-valylglycines 46, 52, 53, and 54. The methods used to prepare unlabelled peptide **37** were adapted for radioactive synthesis. The radioactive glycine was lyophilized from the storage solution (either 0.1 M HCl or 1% ethanol) and the residue was dissolved in dry methanol. Hydrogen chloride gas was passed over the cooled (0 °C) solution for 45 to 60 min, and the resulting solution was stirred at room temperature for 2.5 h to give the methyl ester. (Tlc: 14:3, 96% EtOH:NH_4OH, glycine R_f 0.47; glycine methyl ester R_f 0.71). The solvent was removed *in vacuo*, and to the residue was added 1.5 mL of a solution of *N*-(*tert*-butoxycarbonyl)-D-tyrosyl-L-valine (**43**) in dry DMF (0.93 mM). This was stirred under argon at 0 °C and diphenylphosphorylazide (1.4 µL, 6.7 µmol) was added followed by triethylamine (2.5 µL, 18. µmol). This was stirred at 0 °C for 5 h and 6 mL of 2:1

EtOAc:benzene was added. This was extracted with 2 x 1 mL 1N HCl, 1 mL H_2O, 2 x 1 mL saturated $NaHCO_3$, and 2 x 1 mL saturated NaCl. The organic layer was concentrated *in vacuo* to give the fully protected tripeptide. (Tlc 18:2:0.2, $CHCl_3$:MeOH:HCOOH, R_f 0.42).

To the above residue was added 0.5 mL of MeOH. This was stirred in a room temperature water bath and 100 µL of 0.5 M NaOH was added. After 1.5 h, 60 µL of 1N HCl was added and the methanol was removed *in vacuo*. To the residue was added 1.0 mL of H_2O and this was extracted with 4 x 2.5 mL of EtOAc. The organic layers were concentrated *in vacuo* to give the nitrogen protected tripeptide. (Tlc 18:2:0.2, $CHCl_3$:MeOH:HCOOH, R_f 0.27). CF_3COOH (0.3 mL) was added and, after standing at room temperature for 0.5 h, the CF_3COOH was removed *in vacuo* and water (1.0 mL) was added. This was extracted with ether, lyophilized, and water was added to give a solution of the desired tripeptide. (Tlc 14:6, 95% EtOH:NH_4OH, R_f 0.68; 4:4:14, AcOH:H_2O:*n*-BuOH, R_f 0.47).

Hydrolysis of D-tyrosyl-L-valyl-[2-^{3}H]glycines 53 and 54. The general procedure[186] for the hydrolysis of peptides was adapted for small scale radioactive hydrolysis. Into a capillary melting point tube was placed a solution of the radioactive tripeptide in 50 µL of water. The water was removed by centrifugation under vacuum. To each tube was added 10 µL of constant boiling HCl. The tubes were centrifuged, sealed under vacuum (< 80 mtorr), and heated at 110-120 °C in an oven for 14 h. The tubes were broken near the top, placed top down in a 1.5mL microfuge tube and centrifuged. The other end of the tube was then broken open. The tubes were rinsed with 1 mL of water into the microfuge tube, and the water was removed by centrifugation under high vacuum. The residue was dissolved in 100 µL of water and this was purified by hplc, first on a C_{18} column, then on an NH_2 column to give glycines **50c** and **51c**.

***N*-[1-^{14}C]Acetyl-D-tyrosyl-L-valylglycine(48).** The procedure for the preparation of unlabelled material was adapted. The [1-^{14}C]acetic anhydride (Amersham, 500 μCi, 106 mCi/mmol) was distilled to the bottom of the sealed glass container by cooling the bottom to -78 °C and gently warming the rest of the tube with a heat gun. The tube was left in the cold bath while water (~0.5 mL) was introduced as outlined by the supplier. The water forms a frozen pellet in the bottom of the tube. This was thawed in a room temperature water bath and immediately transfered to a hot (95 °C) solution of 37 (7.51 mg, 19.9 μmol) in 0.5 mL of water. This was then heated for 45 min. The solution was transferred to a round bottom flask with water and lyophilized. Acetic acid (0.1M, 1 mL) was added, and the mixture was lyophilized; and this repeated 2 more times. The residue was taken up in water and passed down a 0.5 mL AG50x8 H$^+$ ion exchange column. The column was rinsed with 3 mL of water and the combined water eluents were lyophilized. A 1.0 mL portion of water was added to the residue. Total activity was 68.1 μCi (55% yield). This was purified by hplc on a C_{18} column (see general experimental); the N-acetyl peptide **48** has a retention time of 15.04 min.

***S*-[2-^{3}H]-Glycine (50a) from enzymatic exchange of *RS*-[2-^{3}H]-glycine.** Into a 1.5 mL microfuge tube was placed *RS*-[2-^{3}H]glycine (ICN, 100 μCi, 20 Ci/mmol) and [1,2-^{14}C]glycine (Amersham, 3.75 μCi, 113 mCi/mmol) and this was lyophilized. The ^{3}H/^{14}C ratio of this was determined to be 24. To this was added 200 μL of 0.10M potassium phosphate buffer (pH 7.4), pyridoxal-5'-phosphate (10 μg, 10 μL), and glutamic-pyruvic transaminase (10μL, 10 units, 108 units/mg). This was shaken at 37 °C for 3 days, 40 μL of 50% CCl_3COOH was added, and the protein was removed by centrifugation for 5 min. The supernatant was applied to a 0.5 mL AG50x8 H$^+$ column; the column was washed with 10 mL of water and was eluted with 10 mL of 2N NH_4OH. The eluent was lyophilized to give *S*-[2-^{3}H]-glycine. The total activity was 31μCi ^{3}H, 2.3 μCi ^{14}C, ^{3}H/^{14}C 12.6±0.1. To further increase the stereospecificity this glycine was treated again with glutamic-pyruvic transaminase as described above. Total activity of **50** was 11.6 µCi ^{3}H, 1.2 µCi ^{14}C, ^{3}H/^{14}C 9.4±0.1. This material cospotted with unlabelled material by tlc (70:30 95% EtOH:H_2O, R_f 0.45)

***S*-[2-^{3}H]-Glycine (50b) from reduction of 66.** The procedure was essentially as described for the enantiomeric glycine **51b**. To a 15 mL round bottom flask with side arm, septum inlet, and stirbar was added the (3*S*,5*S*,6*R*)-3-bromo-oxazinone **66** (51.8 mg, 111. µmol) in 3 mL of THF. In a spoon over the flask was placed $PdCl_2$ (19.2 mg, 108. µmol). The flask was attached to the tritiation apparatus and the solution was degassed by stirring under vacuum followed by purging with nitrogen gas. This was repeated and tritiated water (0.55 mL, 50 Ci/mL, 0.91 Ci/mmol) was added through the septum. The flask was frozen in liquid nitrogen and then evacuated for 15 min at 27 mtorr. The flask was allowed to warm to room temperature, nitrogen gas was added, and the procedure was repeated twice more. Carrier free tritium gas was introduced into the reaction flask and the solution was allowed to warm to room temperature. The pressure in the system was maintained at 720-730 torr (atmospheric pressure) by releasing tritium as neccessary. In this way approximately 100 Ci of tritium gas was kept over the reaction; the total volume of the reaction system was 40 mL. The $PdCl_2$ was dropped from the spoon into the solution, and the reaction was stirred for 5 h. A decrease in pressure was observed as tritium gas was consumed. The reaction flask was frozen in liquid nitrogen and the system was evacuated to remove the excess tritium gas. Nitrogen gas was added, the solution was warmed to room temperature, and the THF was removed by stirring the solution under vacuum in a room temperature water bath. Methanol (2 mL) was added and removed by stirring under vacuum. This was repeated 3 more times. The resulting residue was filtered through a borosilicate glass microfiber filter and this was rinsed with water. The filtrates were combined (~5 mL) and applied to a 1 mL AG50x8 H$^+$ ion exchange column. This was rinsed with 3 mL of water and eluted with 10 mL of 3M NH_4OH.

Approximately 20% of this solution was spilled, the remainder was lyophilized overnight. The residue was dissolved in 500 μL of water and filtered through glass wool. The flask was washed with another 100 μL of water to give a total volume of 600 μL.

To determine the total activity of this material, 1 μL was added to 1.0 mL of water and 1 μL of this solution was counted. This gave 1.40×10^5 cpm at 44% efficiency for a total activity of 86 mCi.

To determine the specific activity of the glycine, 10 μL of this solution was injected onto the HPLC and the peak corresponding to glycine was collected. The collected mass was 3.366 g (d=0.85, 3.96 mL). A 1 μL portion of this solution was counted to give 1.32×10^5 cpm or 540. μCi. The mass of the glycine was determined by comparison of the peak area (determined by uv absorbance a 200 nm) to known standards to give 52.2 μg (0.695 μmol); the specific activity was therefore 0.78 Ci/mmol.

The remaining solution was purified by HPLC in three, 150 μL injections. The glycine was collected between 18.30 min and 21.00 min. The combined fractions were lyophilized and dissolved in 2 mL of water. This was 26.6 mCi (31% yield of glycine based on bromide).

Before conversion to the methyl ester, 100 μL of this 2 mL solution was applied to a 0.3 mL AG50x8 H^+ ion exchange column. This was washed with 1 mL of H_2O, eluted with 3 mL of 3M NH_4OH, and the glycine fraction was lyophilized. To the residue was added 1.0 mL of H_2O, 500 μL was removed, and [1,2-^{14}C]glycine (Amersham, 50 μCi, 113 mCi/mmol) was added to the residue. This was analyzed for $^3H/^{14}C$ ratio and found to be 16.702±0.148. Total activity 856 μCi ^3H, 51.2 μCi ^{14}C.

***R*-[2-^{3}H]-Glycine (51a) from enzymatic exchange of glycine.** The procedure of Arigoni and Besmer[169] as described by Aberhart and Russell[172] was used. To 1.0 mL of tritiated water (ICN, 1 Ci/mL, 18 mCi/mmol) was added 50 μL of 2M potassium phosphate buffer (pH 7.4), glycine (10 μL, 10 μmol), pyridoxal-5'-phosphate (100 μg, 10 μL), and glutamic-pyruvic transaminase (100μL, 100 units, 108 units/mg). This was shaken at 37 °C for 3 days. This was lyophilized, water added, and lyophilized again. The residue was taken up in 1.0 mL of water, 0.2 mL of 50% CCl_3COOH was added, and the protein was removed by centrifugation for 5 min. The supernatant was applied to a 0.5 mL AG50x8 H^+ column; the column was washed with 10 mL of water, and was then eluted with 10 mL of 2N NH_4OH. The eluent was lyophilized to give **51**. Total activity was 41μCi. This material cospotted with unlabelled material by tlc (70:30 95% EtOH:H_2O, R_f 0.45)

***R*-[2-^{3}H]-Glycine (51[illegible]) from reduction of 67.** The procedure of Williams *et al* [170] to prepare stereospecifically deuterated glycines was modified. To a 25 mL round bottom flask with side arm, septum inlet, and stirbar was added the (3*R*,5*R*,6*S*)-3-bromo-oxazinone **67** (53.1 mg, 114. μmol) in 3 mL of THF. In a spoon over the flask was placed $PdCl_2$ (19.4 mg, 109. μmol). The flask was attached to the tritiation apparatus, and the solution was degassed by stirring under vacuum followed by purging with nitrogen gas. This was repeated and tritiated water (0.55 mL, 50 Ci/mL, 0.91 Ci/mmol) was added through the septum. The flask was frozen in liquid nitrogen and then evacuated for 15 min at 27 mtorr. The flask was allowed to warm to room temperature, nitrogen gas was added, and the procedure was repeated twice more. Carrier free tritium gas was introduced into the reaction flask, and the solution was allowed to warm to room temperature. The pressure in the system was maintained at 720-730 torr (atmospheric pressure) by releasing tritium as neccessary. In this way approximately 130 Ci of tritium gas was kept over the reaction; the total volume of the reaction system was 50 mL. The $PdCl_2$ was dropped from the spoon into the solution, and the reaction was stirred for 5 h. A decrease in pressure was observed as tritium gas was consumed. The reaction flask was frozen in liquid nitrogen and the system was evacuated to remove the excess tritium gas. Nitrogen gas was added, the solution was warmed to room temperature, and the THF was removed by stirring the solution under vacuum in a room temperature water bath. Methanol (2 mL) was added and removed by stirring under vacuum. This was repeated 3 more times. The resulting residue was filtered through a borosilicate glass microfiber filter, and this was rinsed with water. The filtrates were combined (~5 mL) and applied to a 1 mL AG50x8 H+ ion exchange column. This was rinsed with 3 mL of water, and **51b** was eluted with 10 mL of 3M NH_4OH, and the solution was lyophilized overnight. The residue was dissolved in 1.00 mL of water, filtered through glass wool and the flask washed with another 100. μL of water to give a total volume of 1.10 mL.

To determine the total activity of this material, 1 μL was added to 1.0 mL of water and 1 μL of this solution was counted. This gave 1.30×10^5 cpm at 44% efficiency for a total activity of 147 mCi.

To determine the specific activity of the glycine, 10 μL of this solution was injected onto the HPLC and the peak corresponding to glycine was collected. The collected mass was 2.684 g (d=0.85, 3.15 mL). A 1 μL portion of this solution was counted to give 1.02×10^5 cpm or 332 μCi. The mass of the glycine was determined by comparison of the peak area (determined by uv absorbance at 200 nm) to a standards to give 23.8 μg (0.317 μmol); the specific activity was therefore 1.0 Ci/mmol.

The remaining solution was purified by HPLC in four, 250 μL injections. The glycine was collected between 18.30 min and 21.00 min. The combined fractions were lyophilized and dissolved in 2 mL of water. This gave 33.2 mCi (28% yield of glycine based on bromide).

Before conversion to the methyl ester, 100 μL of this 2 mL solution was applied to a 0.3 mL AG50x8 H^+ ion exchange column. This was washed with 1 mL of H_2O, eluted with 3 mL of 3M NH_4OH, and the glycine fraction was lyophilized. To the residue was added 1.0 mL of H_2O, 500 μL was removed, and [1,2-^{14}C]glycine (Amersham, 50 μCi, 113 mCi/mmol) added to the residue. The $^3H/^{14}C$ ratio of this sample was found to be 18.529±0.056. Total activity was 932μCi ^3H, 50.3 μCi ^{14}C.

(3*S*,5*S*,6*R*)-4-Benzyloxycarbonyl-3-bromo-5,6-diphenyl-2,3,5,6-tetrahydro-1,4-oxazin-2-one (66). This was prepared[10] as for its enantiomer using (5*S*,6*R*)-4-benzyloxycarbonyl-5,6-diphenyl-2,3,5,6-tetrahydro-1,4-oxazin-2-one (**64**)[266] (145. mg, 0.379 mmol), CCl_4 (50 mL), and *N*-bromosuccinimide (80.5 mg, 0.452 mmol) and refluxed for 45 min. Spectral data were identical with those of the 3*R*,5*R*,6*S* enantiomer **67**.

(3*R*,5*R*,6*S*)-4-Benzyloxycarbonyl-3-bromo-5,6-diphenyl-2,3,5,6-tetrahydro-1,4-oxazin-2-one (67). This compound was prepared as described by Williams *et al.*[10] A solution of (5*R*,6*S*)-4-benzyloxycarbonyl-5,6-diphenyl-2,3,5,6-tetrahydro-1,4-oxazin-2-one (**65**)[266] (154. mg, 0.398 mmol) in dry CCl_4 (50 mL) was heated to reflux and after complete dissolution, *N*-bromosuccinimide (85.0 mg, 0.478 mmol) was added. This was refluxed for 35 min, the reaction being followed by tlc (20 $CHCl_3$/0.4 MeOH). After 15 min the tlc indicated complete loss of starting material. The reaction was cooled in an ice bath for 1 h, then filtered under argon through a dry, ice-jacketed glass frit. The solvent was removed to give the bromide **67** (182. mg, 98%) as a white solid. Ir ($CHCl_3$ cast) 1762, 1720 cm^{-1}; ^1H nmr (360 MHz, $Cl_2CDCDCl_2$, 367 °K) δ 5.01-5.21 (m, 3H), 6.53-6.67 (m, 2H), 6.88-7.94 (m, 15H, aryl-*H*); exact mass: 329.1413 (329.1416 calcd for $C_{22}H_{19}NO_2$ (M^+-CHBrCOO)).

_RS_-[2-^{2}H]-Glycine(72). The procedure of White[184] was used. A solution of glyoxylic acid monohydrate (198. mg, 2.15 mmol) in 0.5 mL of water was added to 100 mL of NH_4OH (conc.). This was stirred at 55 °C and $NaBD_4$ (Aldrich, 98% atom D, 98.1 mg, 2.34 mmol) dissolved in 5 mL of NH_4OH (conc.) was added. The reaction temperature was maintained at ~60 °C for 2 h, the mixture was cooled, and the solvent was removed *in vacuo* to give a solid residue. The remaining $NaBD_4$ was destroyed by acidifying with 0.1N HCl, and the resulting solution was applied to an AG50x8 H^+ ion exchange column. The column was washed with water, and then the glycine was eluted with 3M NH_4OH. The solution was concentrated *in vacuo* to give a solid which was recrystalized from water/ethanol. Yield of **72** was 40.6 mg (25%). Chromatographic and spectral properties were identical to authentic unlabelled glycine except ^1H nmr (360 MHz, D_2O) δ 3.60 (t, J = 2.5 Hz, CH*D*), 3.62 (s, CH_2).

Analysis of stereochemical purity of [2-^{3}H]-Glycines 49, 50, 51 with D-Amino Acid Oxidase. The method of Wellner[171] was adapted to be suitable for trapping the glyoxylate. A cocktail was prepared by diluting 45 μL (0.99 mg, 53955 units) of catalase solution and 50 μL (62.5 μg, 0.796 μmol) of a flavin adenine dinucleotide solution to 5.0 mL with buffer (pH 8.3, 0.10 M sodium pyrophosphate). To 200 μL of this was added D-amino acid oxidase (1.0-1.5 units in 10-20 μL), and the solution of radioactive glycine to be analyzed (in 5-15 μL). Control tubes either lacked enzyme or were heated for 5 min at 95 °C. The samples were shaken gently for 12-18 h at 37 °C. To each sample was then added 100 μL of glyoxylic acid (3.74 mM in buffer, pH 6.8, 50 mM sodium phosphate, 0.2 M NaCl) and 5 μL of nitrosobenzene (0.30 M in 95% ethanol). This was shaken at 60 °C for 1 h and then extracted with 3x0.5 mL of ether. The ether extracts passed down a Pasteur pipette filled with Na_2SO_4 into a scintillation vial to be analyzed for radioactivity.

General procedure for the preparation of 1*S*-(-)-camphanamide derivatives 70, 71, 73, 74, 75, and 76. These derivatives were prepared according to the procedure of Armarego *et al.*[173] as described by Williams and co-workers.[170] To 1*S*-(-)-camphanic acid chloride (Aldrich, 32.1 mg, 148. μmol, 2 eq.) in 1.5 mL of toluene at 0 °C was added glycine (5.47 mg, 72.9 μmol, 1 eq.) in 0.1M NaOH (3.6 mL, 0.36 mmol, 5 eq.). This was stirred at 0 °C for 0.5 h and then at room temperature for 4 h. This was washed with 3 x 5 mL of $CHCl_3$, acidified with 0.5 mL of 1N HCl, and extracted with 3 x 5 mL of CH_2Cl_2. The solvent was removed *in vacuo* to give the camphanamide which was analyzed by nmr.

Glycolic acid *p*-bromophenacyl ester (78). The general procedure[161] for preparing phenacyl esters was employed. Triethylamine (1.40 mL, 10.0 mmol) was added to a stirred solution of glycolic acid (0.760 g, 10.0 mmol) and 2,4′-dibromoacetophenone (2.77 g, 10.0 mmol) in 20 mL of EtOAc at room temperature. This was stirred overnight, water (30 mL) and EtOAc (40 mL) were added, and the organic layer was washed with 20 mL water, 20 mL of 0.5M $KHCO_3$, and 20 mL of saturated NaCl. More EtOAc was added as necessary to maintain two phases. The organic layer was dried (Na_2SO_4) and the solvent was removed. The residue was purified by recrystalization (95% EtOH) to give pure **78** (2.57 g, 94%). Mp 138–139.5 °C (lit.[191] mp 142–142.5 °C, lit.[196] mp 138 °C); ir ($CHCl_3$ cast) 1745, 1693 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 2.45 (t, *J* = 5.6 Hz, 1H, O*H*), 4.39 (d, *J* = 5.6 Hz, 2H, C*H*$_2$OH), 5.42 (s, 2H, COOC*H*$_2$CO), 7.66 (m, 2H, aryl-*H*), 7.78 (m, 2H, aryl *H*); exact mass: 271.9688 (271.9685 calcd for $C_{10}H_9BrO_4$). Anal calcd for $C_{10}H_9BrO_4$: C, 43.98; H, 3.32; Br, 29.26. Found: C, 43.80; H, 3.28; N, 29.30.

***O*-(*tert*-Butyldimethylsilyl)-*N*-(*tert*-butoxycarbonyl)-D-tyrosyl-L-valine phenylmethyl ester (79).** The method used by Kendall *et al.*[189] was followed. To *tert*-butyldimethylsilylchloride (0.485 g, 3.22 mmol), **44** (1.26 g, 2.67 mmol), and imidazole (0.364 g, 5.34 mmol) was added 15 mL of dry DMF. This was stirred at room temperature for 5.5 h. A 5% solution of $NaHCO_3$ (125 mL) was added and the aqueous layer was extracted with 3 x 50 mL of 2:1 EtOAc:benzene. The organic layers were washed with 2 x 50 mL of water, 50 mL of saturated NaCl, dried (Na_2SO_4), and the solvent was removed to give an oil. This was purified by flash chromatography with 7:2 Skelly B:EtOAc as the eluent. This gave pure **79** (1.40 g, 90%) as a thick gum. Ir ($CHCl_3$ cast) 3300, 1657, 1511 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 0.18 (s, 3H, C*H*$_3$Si), 0.78 (2d, *J* = 6.6 Hz, 6H, (C*H*$_3$)$_2$), 0.96 (s, 9H, (C*H*$_3$)$_3$CSi), 1.40 (s, 9H, (C*H*$_3$)$_3$CO), 2.10 (m, 1H, Me$_2$C*H*), 2.98 (m, 2H, aryl-C*H*$_2$), 4.34 (m, 1H, NC*H*CO), 4.54 (m, 1H, NC*H*CO), 4.98 (br, 1H, N*H*), 5.10 (d, *J* = 12.0 Hz, 1H, aryl-C*H*HO), 5.18 (d, *J* = 12.0

Hz, 1H, aryl-C*H*HO), 6.46 (br , 1H, N*H*), 6.74 (d, *J* = 6.6 Hz, 2H, aryl-*H*), 7.04 (d, *J* 6.6 Hz, 2H, aryl *H*), 7.32 (m, 5H, aryl *H*); exact mass: 467.2507 (467.2492 calcd for $C_{27}H_{37}NO_4Si$ ($M^+ - Me_3COCONH_2$)); ms (POSFAB) 584.31 (584.33 calcd for $C_{32}H_{48}N_2O_6Si$). Anal. calcd for $C_{32}H_{48}N_2O_6Si$: C, 65.72; H, 8.28; N, 4.79. Found C, 65.65, H, 8.29; N, 4.65.

***O*-(*tert*-Butyldimethylsilyl)-*N*-(*tert*-butoxycarbonyl)-D-tyrosyl-L-valine (80).** A solution of **79** (1.08 g, 1.85 mmol) and 5% Pd/C (120. mg) in 50 mL of EtOAc was hydrogenated for 1 h at 50 psi. This was filtered through a glass frit, H_2S was bubbled through the filtrate for 5 min, the H_2S was removed by stirring the solution under vacuum, and the solution was filtered again. The solvent was removed to give analytically pure acid **80** (0.883 g, 97%). Ir ($CHCl_3$ cast) 3300, 2960, 1722, 1657, 1511 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 0.18 (s, 3H, CH_3Si), 0.84 (br m, 6H, $(CH_3)_2$), 0.96 (s, 9H, $(CH_3)_3CSi$), 1.38 (s, 9H, $(CH_3)_3CO$), 2.10 (br, 1H, Me_2CH), 2.94 (br, 2H, aryl-CH_2), 4.48 (br, 1H, NC*H*CO), 4.68 (br, 1H, NC*H*CO), 5.32 (br, 1H, N*H*), 6.76 (d, *J* = 8.0 Hz, 2H, aryl-*H*), 7.06 (d, *J* = 8.0 Hz, 2H, aryl-*H*); exact mass: 494.2815 (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.

***O*-(*O*-(*tert*-Butyldimethylsilyl)-*N*-(*tert*-butoxycarbonyl)-D-tyrosyl-L-valyl)-glycolic acid *p*-bromophenacyl ester (81).** To a -10 °C solution of **80** (0.455 g, 0.920 mmol) in 10 mL of dry CH_2Cl_2 was added dicyclohexylcarbodiimide (0.196 g, 0.951 mmol), **78** (0.251 g, 0.920 mmol), and 4-dimethylaminopyridine (12.8 mg, 0.105 mmol). The cooling bath was removed and the reaction was stirred at room temperature for 24 h. This was filtered and the precipitate was washed with 2 x 4 mL of CH_2Cl_2. The solvent was removed from the filtrate to give an oil which was purified by flash chromatography using 25:13 Skelly B:EtOAc to give pure **81** (0.453 g, 65%). Ir ($CHCl_3$ cast) 2932, 1706, 1660, 1511 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 0.18 (s, 3H, C*H*$_3$Si), 0.86 (2d, *J* = 7.2 Hz, 6H, (C*H*$_3$)$_2$), 0.96 (s, 9H, (C*H*$_3$)$_3$CSi), 1.40 (s, 9H, (C*H*$_3$)$_3$CO), 2.20 (m, 1H, Me$_2$C*H*), 2.98 (m, 2H, aryl C*H*$_2$), 4.30 (m, 1H, NC*H*CO), 4.56 (m, 1H, NC*H*CO), 4.72 (d, *J* = 14.4 Hz, 1H, OC*H*HCOO), 4.90 (m, 1H, OC*H*HCOO), 4.94 (br, 1H, N*H*), 5.42 (m, 2H, COOC*H*$_2$CO), 6.36 (br, 1H, N*H*), 6.72 (d, *J* = 7.2 Hz, 2H, aryl *H*), 7.02 (m, 2H, aryl *H*), 7.60 (m, 2H, aryl-*H*), 7.72 (m, 2H, aryl-*H*); ms (POSFAB): 748.50 (748.24 calcd for $C_{35}H_{49}BrN_2O_9Si$). Anal. calcd for $C_{35}H_{49}BrN_2O_9Si$ C, 56.07; H, 6.59; N 3.74. Found. C, 56.10; H, 6.55; N, 3.49.

Glycolic acid phenacyl ester (82). As in the preparation of **78**, a solution of α-bromoacetophenone (1.96 g, 9.86 mmol), glycolic acid (0.758 g, 9.96 mmol), and 20 mL EtOAc was stirred at room temperature and triethylamine (1.37 mL, 9.86 mmol) was added. Stirring was continued overnight. Water (20 mL) and EtOAc (30 mL) were added and the organic layer was washed with 20 mL each of water, 0.5M $KHCO_3$, and 2 x saturated NaCl. The organic phase was dried (Na_2SO_4) to give, after removal of the solvent, 1.44 g (75%) of **82**. This could be further purified by recrystalization (*iso*-propyl alcohol). ^{1}H nmr (360 MHz, $CDCl_3$) 2.34 (t, *J* = 6.5 Hz, 1H, O*H*), 4.38 (d, *J* = 6.5 Hz, 2H, C*H*$_2$OH), 5.46 (s, 2H, COOC*H*$_2$CO), 7.48 (m, 2H, aryl-*H*), 7.62 (m, 1H, aryl-*H*), 7.90 (m, 2H, aryl-*H*).

3,4-Methylenedioxybenzyl alcohol. To a 0 °C solution of piperonal (4.00 g, 26.6 mmol) in 95% EtOH (40 mL) was added 1.00 g (26.4 mmol) of $NaBH_4$. This was allowed to warm to room temperature, of ice water (40 mL) and 20 mL of saturated NH_4Cl solution were added carefully. Extraction with CH_2Cl_2 and removal of the solvent gave an oily residue which was recrystalized from petroleum ether to give **98** (1.85 g, 46%). Mp 51-52 °C (lit.[267] mp 51-52 °C); ir ($CHCl_3$ cast) 3250, 1500, 1490, 1448 cm^{-1}; ^{1}H nmr (80 MHz, $CDCl_3$) δ 1.61 (t, *J* = 4.5 Hz, 1H, O*H*), 4.58 (d, *J* = 4.5 Hz, 2H, C*H*$_2$OH), 5.93 (s, 2H, OC*H*$_2$O), 6.83 (m, 3H, aryl-*H*); exact mass: 152.0469 (152.0473 calcd for $C_8H_8O_3$).

Glycolic acid 3,4-methylenedioxybenzyl ester (83). 3,4-Methylenedioxybenzyl chloride was prepared according to Jaegfeldt and coworkers.[268] In a dry flask was suspended PCl_5 (797 mg, 3.83 mmol) and $CaCO_3$ (330. mg, 3.29 mmol) in 25 mL ether. The solution was cooled (0 °C) and 3,4-methylenedioxybenzyl alcohol (500. mg, 3.29 mmol) was added. Stirring was continued for 10 min at 0 °C. This was decanted into a separatory funnel, washed with 3 x 7 mL of 0 °C saturated Na_2CO_3, dried ($MgSO_4$) at 0 °C for 1 h, and filtered. The solvent was removed to give the chloro compound as a clear oil (394. mg, 70%). This was coupled with glycolic acid as decribed[197] for nitrogen protected amino acids. To the chloride was added dry DMF (5 mL), 173. mg (2.28 mmol) of glycolic acid, and 0.32 mL (2.3 mmol) of dry triethylamine. After stirring overnight at room temperature the DMF was removed under high vacuum (50 mTorr) and the residue was taken up in 10 mL of water and 25 mL of EtOAc. The organic layer was washed with 5 mL of 1N HCl, 2 x 5 mL of water, and 2 x 5 mL of saturated NaCl. The solvent was removed to give a yellow oil **83** (227 mg, 33% from the alcohol). ^1H nmr (400 MHz, $CDCl_3$) δ 2.80 (br, 1H, O*H*), 4.23 (s, 2H, CH_2OH), 5.06 (s, 2H, aryl-CH_2), 5.91 (s, 2H, OCH_2O), 6.76 (m, 3H, aryl-*H*); exact mass: 210.0520 (210.0528 calcd for $C_{10}H_{10}O_5$).

Preparation of gel-filtration column. Sephadex G-100 (35 g, 40-120 µ particle size) was slurried in 700 mL of degassed buffer (50 mM sodium phosphate, 0.2M NaCl, pH 6.8) 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, 300 mL of 0.05% NaN_3 in buffer was passed through the column.

Preparation of affinity column . As described[162] previously, Bio-Rad affigel-15 was allowed to settle at -20 °C in a graduated cylinder to measure the volume of resin used. Approximately 5 mL of settled resin was transfered to a Buchner funnel and immediately washed with 15 mL of isopropyl alcohol and 15 mL of 4 °C water. The dry resin was added to a solution of D-tyrosyl-L-valylglycine (37) (50.7 mg) in 3 mL of buffer (2-(*N*-morpholino)ethanesulfonic acid, 0.1M, pH 6.5) in a test tube, and this was shaken at room temperature for 4 h and then overnight at 4 °C. After transfer to a 10 mL disposable syringe, the resulting column was washed with 25 mL of 6M urea, then with 3 cycles of alternating pH 4.0 buffer (0.1M sodium acetate, 1M NaCl, 25 mL/cycle) and pH 8.0 buffer (0.1M sodium borate, 1M NaCl, 25 mL/cycle). The column was then equilibrated with buffer (50 mM sodium phosphate, 0.2M NaCl, pH 6.8).

Isolation of peptidyglycine α-amidating monoxygenase (PAM). The isolation of PAM followed the procedure described by Kizer *et al.*[133] The entire procedure is done at 4 °C. Frozen pig pituitaries (Pel-Freez, 10 g) were chopped with a razor blade and suspended in 20 mL of 4 °C buffer (50 mM sodium phosphate, 0.2M NaCl, pH 6.2). This was placed in an ice-water bath and homogenized with a IKA homogenizer for four, 40 s intervals with 20 s wait 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, 0.2M NaCl, pH 6.8). Fractions (4 mL) were collected. The red band which elutes, presumably hemoglobin, was found to be a good marker for the PAM enzyme. Every 5 th 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 100-120 mL and the resulting solution was applied to the affinity column at 1 mL/min. After application the column was washed with 40 mL of buffer (50 mM sodium phosphate, 0.2M NaCl, pH 6.8) and then eluted at 1 mL/min with a pH 4.0 buffer (3M urea, 1M glycylglycine, 0.1M *N*-acetylglycine). Fractions (4 mL) were collected starting exactly 1 min after the elution buffer entered the column. These fractions were immediately transferred to dialysis tubing and dialysed against 400 mL of buffer (50 mM sodium phosphate, 0.2M NaCl, pH 6.8), the buffer being changed after 1 h, 3 h, and 7 h total time. The final dialysis was overnight. Most of the enzyme activity was in fractions 2 and 3.

PAM assay with nitrosobenzene. The assays for PAM activity were as follows. Solutions of $CuSO_4$ (1.0mM), KI (5.0M), and ascorbic acid (0.40M) were prepared in fully degassed water. These solution were stored tightly stoppered at 4 °C for up to 1 week. A cocktail was prepared fresh for each set of assays by adding to buffer (50 mM sodium phosphate, 0.2M NaCl, pH 6.8) 50 μL of the copper and KI solutions, 25 μL of the ascorbate solution, and catalase (1mg), and diluting to 5.0 mL with more buffer. To 100 μL of the enzyme solution to be assayed in a 1.5 mL microfuge tube was added 100 μL of the cocktail and D-TyrVal-[1,2-^{14}C]Gly (**46**) (~80000 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-TyrVal-[1,2-^{14}C]Gly (**46**). This was shaken at 37 °C for 2 h and 100 μL of a glyoxylic acid solution (3.7 mmol in pH 6.8 buffer) and 5 μL of nitrosobenzene (0.30M in 95% EtOH) were added. After shaking at 60 °C for 1 h, the solution was extracted three times with ether by adding ~0.5 mL of ether, vortexing thoroughly, and removing the ether layer with a pipette. The ether extracts were passed down a Pasteur pipette containing ~2.5 g of Na_2SO_4 directly into a scintillation vial. After the third extraction, the drying tube was rinsed with 0.5 mL of ether into the scintillation vial.

Isotope Dilution of Product of PAM Assay. In order to confirm that the radioactivity measured from the PAM assay was from NHF as proposed an isotope dilution experiment was performed. The assay was done using twice the normal volumes (200 μL cocktail, 200 μL enzyme solution) and 7.2x10^5 dpm of D-TyrVal-[1,2-^{14}C]Gly (**46**, 2.9 nmol). After drying, the ether was added directly to 45.2 mg of NHF. The solvent was removed *in vacuo*, 2.0 mL of ether was added and 100 μL was counted (179 counts per minute, cpm) to give a specific activity of 79.2 cpm/mg. This was repeatedly recrystalized and portions were weighed and counted for activity. Successive specific activities of 76.9 cpm/mg (166 cpm, 2.16 mg), 81.1 cpm/mg (163 cpm, 2.01 mg), 88.5 cpm/mg (177 cpm, 2.00 mg), and 88.1 cpm/mg (177 cpm, 2.01 mg) were obtained showing that all of the radioactivity co-purifies with NHF.

PAM Assay with *N*-[1-^{14}C]acetyl-D-TyrValGly 48. The assay was a modification of the literature procedure.[135] Into a 1.5 mL microfuge tube with screw cap was placed 100 μL of enzyme solution, 100 μL of cocktail (prepared as described for the assay with PhNO), and 5 μL of **48** (1.0x10^5 dpm, 872 pmol). This was incubated at 37 °C for 2 h and extracted with 3x0.5 mL of ethyl acetate layer. The organic extracts were passed down a Pastuer pipette filled with Na_2SO_4 and analyzed for radioactivity.

Determination of protein content. The method of Bradford[162] was used to determine protein concentration with Bio-Rad protein assay dye reagent. A standard solution of bovine serum albumin was used for preparing 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 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 dilute reagent (2.00 mL).

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

Typical standard curves are shown below.

Macro Assay		Micro Assay	
[ ] (mg/mL)	absorbance	[ ] (μg/mL)	absorbance
1.48	1.022	26.6	0.495
0.96	0.738	20.7	0.416
0.67	0.585	14.8	0.339
0.44	0.414	8.88	0.214
0.19	0.160	2.96	0.083

Assay of Glycolate Oxidase. A standard curve for the hydrazone was prepared by adding 100 μL of 0.1 M phenylhydrazine hydrochloride (10 μmol) and varying amounts of 5 mM glyoxlic acid to 2.0 mL of buffer (0.1 M potassium phosphate, pH 8.3).[153] The uv absorbance was measured against a solution in which the glyoxylic acid was omitted. After 8 min stable readings were obtained. The following absorbances were recorded; 109 μM (1.34), 54 μM (0.81), 27 μM (0.51), 11 μM (0.34).

The phenylhydrazine assay was as described by Baker and Tolbert.[153] Into a test tube was placed 2.1 mL of buffer (0.10M potassium phosphate, pH 8.3), 25 μL (5 μmol) of 0.20M glycolic acid, 10 μL of catalase (22 mg/mL, 54500 units/mg), and 1.5 μL of glycolate oxidase (Sigma, 2.9 mg/mL, 30 units/mg). In control experiments glycolate oxidase was not added. This was vortexed, allowed to stand 20 min at room temperature, and treated with 4M HCl (50 μL) to stop the reaction. For the phenyhydrazine assay 200 μL of buffer (2M potassium phosphate, pH 6.5) and 25 μL (2.5μmol) of 0.10M phenylhydrazine hydrochloride were added and the absorbance measured at 324 nm against the control experiment.

For the nitrosobenzene assay, the above procedure was followed except calcium 2-^{14}C-glycolate (ICN, 5 μL, 35 mCi/mmol, ~70000 dpm) was added along with the unlabelled glycolate. After 20 min at room temperature, 200 μL of 12.7 mM glyoxylic acid (2.5 μmol, in 2M potassium phosphate buffer, pH 6.2) and 30 μL (10.5 μmol) of 0.35M nitorosbenzene in 95% EtOH was added and this was shaken at 60 °C for 1 h. The solution was extracted three times with ether by adding ~0.5 mL of ether, vortexing thoroughly, and removing the ether layer with a pipette. The ether extracts were passed down a Pasteur pipette containing ~2.5 g of Na_2SO_4 directly into a scintillation vial. After the third extraction, the drying tube was rinsed with 0.5 mL of ether into the scintillation vial.

^{2}H Decoupled ^{1}H-^{13}C chemical shift correlation nuclear magnetic resonance spectra. Heteronuclear proton-carbon shift correlations with broad band deuterium decoupling employed previously described procedures[225] on a Bruker WH400 spectrometer (400 MHz ^{1}H, 100.6 MHz ^{13}C) equipped with a ^{19}F lock on C_6F_6. Samples were dissolved in C_6D_6 (ca. 0.2 M for ^{13}C, ^{2}H labeled compounds) in 5 mm tubes at 25 °C. Complete (non-selective)[225] correlations were obtained using the Bruker 'XHCORR.AU' program, with a relaxation delay (D1) of 2.0 s, sweep widths; f_2 (^{13}C) 1136 Hz, and f_1 (^{1}H) 600 Hz, 256 experiments of size 1 K. The spectra were processed with zero-filling in both dimensions, using Gaussian windows, as power spectra (MC2 = P), with LB (f_2) = 1.5, LB (f_1) = -5.0, GB (f_2) = 0.0, GB = 0.3 (f_1).

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