Formation of Peptide Amides by Peptidylglycine α-Amidating Monooxygenase: A New Assay and Stereochemistry of Hydrogen Loss

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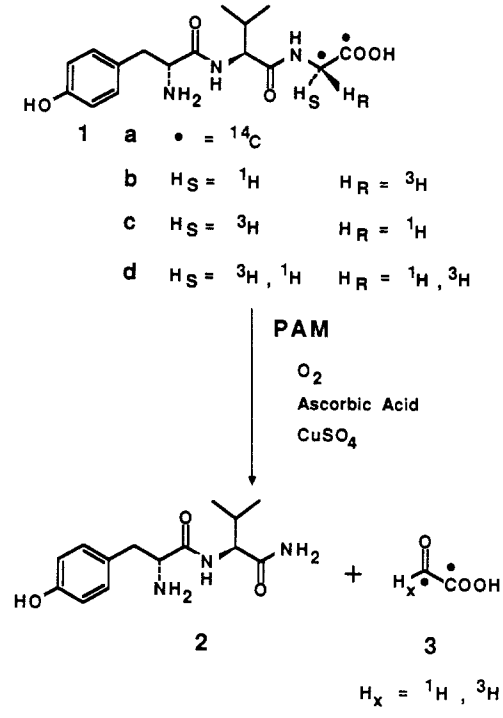
Abstract: A new assay was developed for peptidylglycine α-amidating monooxygenase (PAM) from porcine pituitary, and the stereochemistry of its oxidation of D-tyrosyl-L-valylglycine (**1**) to D-tyrosyl-L-valinamide (**2**) and glyoxylate (**3**) was investigated. This enzyme is believed to be responsible for formation of peptide hormones having a primary amide functionality at the carboxyl terminus. The assay is based on reaction of ^{14}C-labeled glyoxylate formed by PAM with nitrosobenzene to give ^{14}C-labeled *N*-hydroxyformanilide (**4**). (*R*)- and (*S*)-[2-^{3}H]glycines (**5b** and **5c**, respectively) were prepared by one-step 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-oxazin-2-ones (**12** and **13**, respectively) with tritium gas in [^{3}H]water. These were analyzed for stereochemical purity by ^{1}H-decoupled tritium NMR of their (1*S*)-(−)-camphanamide derivatives **14b** and **14c** and by a modified D-amino acid oxidase assay employing nitrosobenzene to capture glyoxylate in a manner analogous to the PAM assay. The stereospecifically labeled [2-^{3}H]glycines were independently transformed to D-tyrosyl-L-valyl[2-^{3}H]glycines; subsequent PAM oxidation demonstrated that the *pro-S* hydrogen of the glycine residue is removed. The possible mechanism of PAM and the significance of these results for determining substrate specificity and for designing inhibitors are discussed.

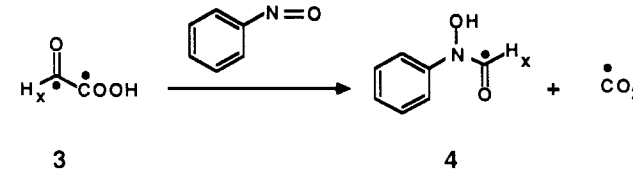
Peptides having a primary amide functionality at the carboxyl terminus are widely distributed in the animal kingdom[1] and elicit a large variety of important physiological effects.[2,3] In mammals, numerous peptide amides occur in the pituitary (e.g., vasopressin, oxytocin), the hypothalamus (e.g., thyrotropin releasing hormone, growth hormone releasing hormone), and the gut (e.g., gastrins). Generally the terminal amide is essential for full biological activity.[4] In the cases studied so far, the peptide amides are generated from precursors bearing a glycine residue at the carboxyl end; these in turn are formed from larger proteins by specific peptidase-catalyzed hydrolyses.[1a,4,5] In 1982 Bradbury et al. first demonstrated that an enzyme in porcine pituitary is capable of cleaving the two terminal carbons of a synthetic tripeptide, D-tyrosyl-L-valylglycine (**1**), to form a dipeptide amide, D-tyrosyl-L-valinamide (**2**) (Scheme I).[1a] Experiments with ^{15}N- and ^{14}C-labeled glycine residues showed that the terminal amide nitrogen of **2** originates from **1** and indicated that the other product was glyoxylate (**3**). It has subsequently been shown that this enzyme, now known as peptidylglycine α-amidating monooxygenase (PAM),[6] requires ascorbate, oxygen, and copper for maximal activity.[7] The enzyme is present in a host of mammalian tissues,[6b,7c,8] has been observed and isolated from frogs (*Xenopus laevis*),[9] and can be produced in cell culture.[10] Multiple forms of PAM with similar activities cooccur[7c,9f] and may be members of a family of closely related enzymes with differing substrate specificities.[7d]

All published assays for the PAM enzyme(s) rely on detection of a specific product amide.[1a,6-11] A major disadvantage of such assays is that they tend to be limited to special substrates and hence are not ideal for rapid comparison of enzyme specificity for a variety of peptides bearing a glycine at the carboxyl terminus. In addition, such assays do not allow examination of events at the carbons and hydrogens of the glycine residue. Since knowledge of the substrate specificity and stereochemistry of enzymatic reactions is very valuable for understanding mechanisms,[12] a PAM assay based on detection of glyoxylate (**3**) appeared highly desirable. The present study describes (1) a new assay for PAM based on the unique reaction of glyoxylate (**3**) with nitrosobenzene;[13] (2) adaptation of a method for synthesis of stereospecifically deuteriated glycines[14] to preparation of the [2-^{3}H] analogues and a new determination of their stereochemical purity; and (3) the stereochemistry of hydrogen removal from the glycine

Scheme I



Scheme II



[†] Department of Chemistry.
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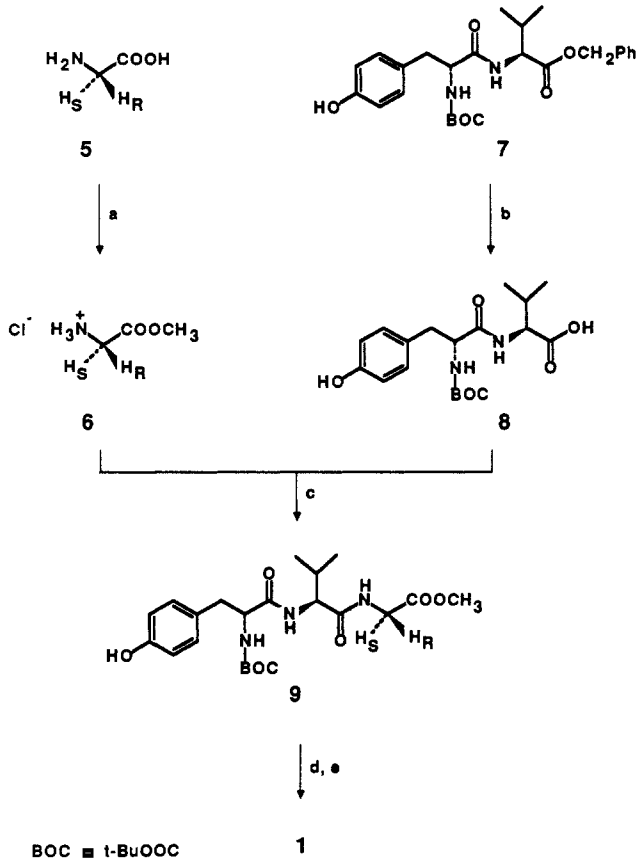
Scheme III[a]



[a] Key: (a) MeOH, HCl; (b) H_2, Pd/C; (c) $(PhO)_2P(O)N_3$, Et_3N; (d) NaOH; (e) CF_3COOH.

residue of **1** by PAM. The resulting information aids in understanding the steric requirements of PAM and may be useful in design of inhibitors or orally active peptide hormone prodrugs.

Results

Development of a Glyoxylate-Based PAM Assay. Although glyoxylic acid is a common metabolite, most of the published procedures for its detection in biochemical systems[15] appeared to lack the necessary sensitivity or specificity (relative to other α-keto acids) or else required extensive sample preparation (e.g., derivatization for HPLC). An alternative assay was therefore developed on the basis of observations of Corbett and Corbett that glyoxylic acid (**3**) reacts with nitrosobenzene under neutral aqueous conditions to form *N*-hydroxyformanilide (**4**) with loss of the carboxyl group as carbon dioxide (Scheme II).[13] This unusual reaction is highly specific for glyoxylic acid (**3**); other α-keto acids (e.g., pyruvate), aldehydes (e.g., formaldehyde), or acids (e.g., formic acid) fail to yield any detectable hydroxamic acid.[13] In addition, our control experiments in D_2O showed that this process does not exchange the formyl hydrogen of **3** or **4** with solvent, thereby indicating that it is suitable for analysis of glyoxylate bearing an isotopically substituted hydrogen. Since *N*-hydroxyformanilide (**4**) is readily soluble in organic solvents (e.g., ethyl ether), it is easily extracted from aqueous media. This assay reaction was optimized for conditions similar (except for temperature) to those required by the PAM enzyme. Simultaneous spectrophotometric monitoring of **4** at 250 nm (ϵ 11 630) and of excess nitrosobenzene at 280 nm (ϵ 9254) showed that the yield after extraction was reproducible (±2%) and above 80% unless ascorbate (required for the PAM reaction) was added. Although the cause is still undetermined, the overall yield of **4** drops to 55 ± 2% when all of the reagents required for maximum activity of the PAM enzyme are added.

To obtain the necessary sensitivity for potentially very small amounts of PAM enzyme and/or glyoxylate production, radioisotopic labeling was used. The tripeptide **1a**, in which both glycine carbons are substituted with ^{14}C, was synthesized by standard solution-phase methods (Scheme III). Reaction of methanol and dry HCl with [1,2-$^{14}C_2$]glycine (**5a**) (113 mCi/mmol, 96% ^{14}C) gave the corresponding methyl ester **6a**, which was coupled to *N*-butoxycarbonyl (BOC)-D-tyrosyl-L-valine (**8**) with use of diphenylphosphoryl azide[16] to yield **9a**. Compound **8** was obtained by analogous coupling of BOC-D-tyrosine with L-valine benzyl ester to give **7**, which was deprotected by hydrogenolysis. Saponification of the methyl ester in **9a** and removal of the BOC group by trifluoroacetic acid afforded free tripeptide **1a**, which could be purified by HPLC if necessary.

The PAM enzyme was isolated by a modified literature procedure[17] employing affinity chromatography with tripeptide **1** bound to Affi-Gel 15 to give protein of an estimated 90–95%

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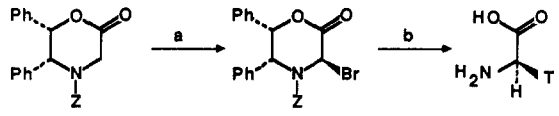
Scheme IV[a]



10 12 5b

Z = $COOCH_2Ph$

[a] Key: (a) *N*-bromosuccinimide; (b) $^3H_2$, $PdCl_2$. Compounds **11** and **13** are enantiomers of **10** and **12**, respectively.

purity. Throughout the isolation, the enzyme was assayed by incubation with the radioactive tripeptide **1a** in the presence of ascorbate, copper sulfate, and potassium iodide at 37 °C. Radioactive glyoxylate (**3a**), which was formed, was trapped by dilution with unlabeled glyoxylic acid and addition of excess nitrosobenzene (4 equiv) to generate ^{14}C-labeled *N*-hydroxyformanilide (**4a**). This was then extracted with ether, dried, and analyzed by scintillation counting. For samples of tripeptide **1a** not purified by HPLC, the addition of aliquots having 80000 dpm to control assays (e.g., enzyme inactivated by boiling) always gave a total activity of less than 200 dpm in the dried *N*-hydroxyformanilide extract. If **1a** was purified by HPLC, such control experiments consistently showed less than 40 dpm in the product extract. Analogous experiments with active enzyme aliquots typically gave 500–3000 dpm in the extract with less than 10% variation in duplicate assays.

Isotope dilution experiments confirmed that the radioactivity measured in the assay resided in *N*-hydroxyformanilide (**4a**). Unlabeled **4** was added and purified by repeated recrystallization to constant specific activity. The possibility that the tripeptide **1a** was hydrolyzed to radioactive glycine that was then oxidized to labeled glyoxylic acid (**3a**) either with or without enzymatic catalysis was excluded by incubation of [1,2-$^{14}C_2$]glycine (5.8×10^4 dpm, 0.23 nmol) with PAM under the same conditions. The resulting radioactivity (77 dpm) in the extract was not significantly above "background" levels. Since the potential minimum requirement for PAM might be an amide bond with a glycine extension, *N*-acetyl[1,2-$^{14}C_2$]glycine (4.5×10^4 dpm, 0.18 nmol) was exposed to the PAM enzyme under the usual conditions. However, the assay demonstrated that no significant amount of glyoxylate (**3a**) was produced; the radioactivity of the extracts was at background levels (typically 76 dpm).

Substrate and cofactor concentrations were varied to compare the behavior of PAM by the new assay with previous results from other laboratories.[6a,17,18] The Michaelis–Menten constant (K_m) was determined to be 26 μM, and the V_{max} was calculated as 244 pmol/μg per h at an ascorbic acid concentration of 1 mM. A fairly wide range of literature values has been reported: K_m = 7.0 μM, V_{max} = 84 nmol/μg per h with 1.25 mM ascorbate;[7c] K_m = 300 μM, V_{max} = 8100 pmol/μg per h with 1 mM ascorbate;[17] K_m = 42 μM, V_{max} = 39 pmol/μg per h with 0.5 mM ascorbate;[18] K_m = 37 μM, V_{max} = 2.9 pmol/μg per h with unspecified ascorbate concentration.[6a] This variation may be due to differences in the multiple forms of PAM[7c] as well as in enzyme purity and assay methods. At present, the actual cause of these differences is still undetermined. In accord with published results,[6a,7c,17,18] ascorbic acid and copper stimulate enzyme activity up to a certain level (optimum ca. 10 μM Cu, 1 mM ascorbate), but high concentrations inhibit the reaction. In the absence of added copper the PAM enzyme was inactive whereas some activity was observed without added ascorbic acid. The addition of 8 μM diethyldithiocarbamic acid inhibited the reaction[7c,19] by 65%.

Synthesis and Analysis of Stereospecifically Labeled [2-^{3}H]-Glycines. In order to determine the stereochemistry of hydrogen removal from the glycine residue of **1**, samples of glycine **5b** and **5c** bearing stereospecific tritium labels in the *pro-R* and *pro-S* positions, respectively, were synthesized. Since the elegant method of Williams and co-workers for preparation of the deuteriated analogues introduces label in the last step,[14] it was modified for use with tritium gas at atmospheric pressure. The (5*R*,6*S*)-oxazinone derivative **10** and its enantiomer **11** were separately brominated with *N*-bromosuccinimide to give bromo compounds **12** and **13**, respectively (Scheme IV).[20-22] These were reduced[23] with carrier-free (100%) tritium gas with palladium chloride in a mixture of tritiated water (50 Ci/mL, 0.91 Ci/mmol) and tetrahydrofuran to (*R*)-[2-^{3}H]glycine (**5b**) and (*S*)-[2-^{3}H]glycine (**5c**), respectively. The procedure produced **5b** and **5c** in reasonable yield after HPLC purification (28% and 31%) and with good specific activity (1.0 and 0.78 Ci/mmol, respectively).

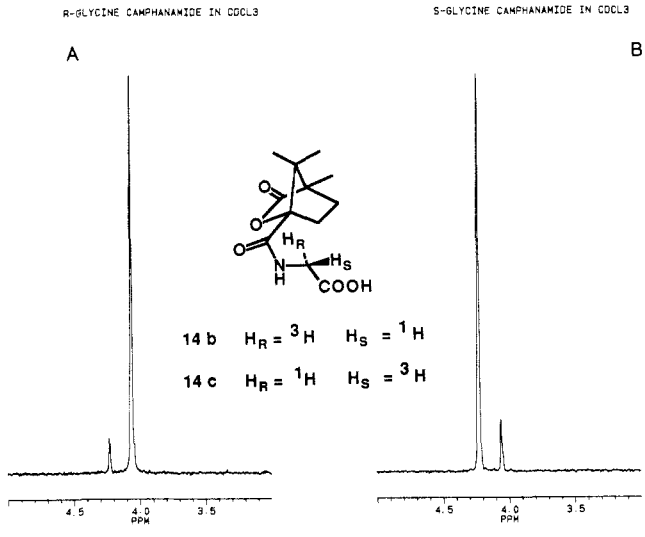


Figure 1. ^{1}H-Decoupled 320.1-MHz ^{3}H NMR spectra of $CDCl_3$ solutions: A, **14b**; B, **14c**. See the Experimental Section.

The stereochemical purity of these glycines was determined by NMR analysis. Portions of labeled glycines **5b** and **5c** were converted[24] to the corresponding (1*S*)-(−)-camphanamides **14b** and **14c**, which were analyzed by 320-MHz ^{1}H-decoupled ^{3}H NMR spectrometry (Figure 1).[25] Since the chemical shifts of the glycine hydrogens in such derivatives are known,[20,24] peak integration indicated that 93% of tritium in **5b** was in the *R* position whereas in **5c** it was 88% in the *S* position. Complete lack of signals due to tritium–tritium coupling as well as mass spectrometric measurements on deuteriated glycine derivatives generated in an analogous manner showed the absence (≤1%) of doubly labeled ([$^3H_2$]glycine camphanamide) species.

A common literature procedure for enzymatic determination of stereochemical purity of [2-^{3}H]glycines employs D-amino acid oxidase to form glyoxylate with stereospecific release of the *pro-S* hydrogen into the aqueous media.[26] The water is then isolated and analyzed for tritium content. Major limitations of this approach are the necessity of quantitative water entrapment and the requirement of complete oxidation of the glycine (a relatively poor substrate) because of the primary isotope effect for the

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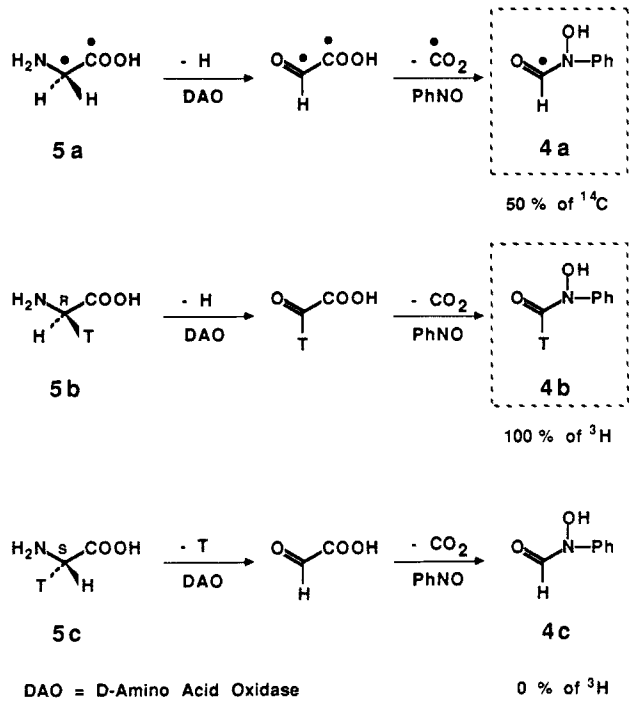

Figure 2. Fate of labeled atoms (● = ^{14}C, T = ^{3}H) during D-amino acid oxidase/nitrosobenzene assay of radioactive glycines.

Table I. Stereochemical Purity of [2-^{3}H]Glycines **5b–g** Determined by D-Amino Acid Oxidase/Nitrosobenzene Assay[a]

glycine	isomer	$^{3}H/^{14}C$ of **5**	$^{3}H/^{14}C$ of **4**	% major isomer[b]
5b	*R*	8.80	15.4	88
5c	*S*	10.6	2.81	86
5d	*RS*	8.14	8.50	(50)
5e	*RS*[c]	6.06	5.07	(50)
5f	*R*	3.26	4.42	68
5g	*S*	4.22	2.83	67

[a] Average results of triplicate assays, $^{3}H/^{14}C$ ratios ±0.10. [b] Calculated directly from change in $^{3}H/^{14}C$ except for **5d** and **5e**. [c] Contains molecules bearing two tritium atoms.

(*S*)-[2-^{3}H]glycine.[26b] These difficulties were circumvented by addition of [1,2-$^{14}C_2$]glycine (**5a**) to the samples of **5b** and **5c** prior to exposure to D-amino acid oxidase and then capture of the resulting labeled glyoxylate by the nitrosobenzene procedure described above. During this process half of the ^{14}C is lost as CO_2, all of the initial tritium in the (*R*)-[2-^{3}H]glycine (which is not subject to a primary isotope effect) is retained, and all of the initial tritium of any (*S*)-[2-^{3}H]glycine oxidized by the enzyme is lost (Figure 2). This modified assay is transparent for the primary tritium isotope effect because the product of the *S* isomer is not radioactive. In this analysis, for (*R*)-[2-^{3}H]glycine the final $^{3}H/^{14}C$ ratio of *N*-hydroxyformanilide will be twice the initial ratio, for (*S*)-[2-^{3}H]glycine the final ratio should be zero, and for a racemic mixture of monotritiated glycines the final ratio should be nearly equal to the starting ratio. These expectations assume that secondary tritium and primary ^{14}C isotope effects are small.

Glycines **5b** and **5c** were separately mixed with [1,2-$^{14}C_2$]glycine (**5a**) and analyzed by this modified amino acid oxidase approach. The results in Table I agree within 5% of those obtained by tritium NMR spectrometry as described above. Interestingly, it is essential that the glycines be singly labeled with tritium for simple analysis. The $^{3}H/^{14}C$ ratio remains nearly constant during assay of monotritiated (*RS*)-[2-$^{3}H_1$]glycine (**5d**) prepared by sodium boro-[^{3}H]hydride reduction of glyoxylate in aqueous ammonia.[27] In contrast, an unexpected 17% decrease in $^{3}H/^{14}C$ ratio was seen during analysis of commercially available[28] "racemic" [2-^{3}H]-glycine (**5e**). Subsequently, information provided by the supplier[28] showed that the latter material was a mixture of singly and doubly tritiated glycines. The primary isotope effect for the *S* tritium is not transparent in this case because it slows the rate of enzymatic oxidation of doubly tritiated glycine molecules also bearing a tritium in the *R* position. This causes a decrease in the $^{3}H/^{14}C$ ratio.

Table II. PAM Oxidation of Labeled Tripeptides **1** and Nitrosobenzene Assay[a]

peptide	^{3}H confign[b]	$^{3}H/^{14}C$ of **1**	$^{3}H/^{14}C$ of **4**
1b	*R*	3.54	4.54
1c	*S*	4.48	3.43
1d	*RS*	7.93	7.56

[a] Average results of triplicate assays, $^{3}H/^{14}C$ ratios ±0.15. [b] Configuration of major isomer; some epimerization occurs during syntheses of **1** (vide infra).

Stereochemistry of the Peptidylglycine α-Amidating Monooxygenase (PAM) Oxidation. The (*R*)-, (*S*)-, and (*RS*)-[2-$^{3}H_1$]glycines (**5b–d**) were individually mixed with [1,2-$^{14}C_2$]glycine (**5a**) and then transformed to the corresponding D-tyrosyl-L-valylglycines **1b–d**, respectively, as shown in Scheme III. These doubly labeled tripeptides[29] were exposed to the purified PAM enzyme from porcine pituitary, the resulting radioactive glyoxylates were captured by the nitrosobenzene assay, and the $^{3}H/^{14}C$ ratios of the *N*-hydroxyformanilides were compared to those of the starting tripeptides. The results clearly show preferential *loss of the pro-S hydrogen* of the glycine residue of **1** and retention of the *pro-R* hydrogen during the PAM reaction (Table II).

Unfortunately the changes in the $^{3}H/^{14}C$ ratio were not as complete as would be expected for the optical purities of the starting glycines **5b** and **5c**. This is due to partial epimerization at the glycine methylene during the synthesis of the tripeptides. Initial evidence for this was noticeable decreases in $^{3}H/^{14}C$ ratio during the final deprotection steps (NaOH and CF_3COOH). The overall decreases in $^{3}H/^{14}C$ ratio were 19% for **1b**, 5% for **1c**, and 3% for **1d**. Since isotope effects and possible preferential deprotonation–reprotonation of chiral intermediates preclude direct determination of stereochemical purity from the decrease in ratios, the peptides **1b** and **1c** were hydrolyzed to regenerate glycines **5f** and **5g**, respectively. These were then stereochemically analyzed by the new D-amino acid oxidase/nitrosobenzene procedure described above. D-Amino acid oxidase removes the *pro-S* hydrogen from glycine. As expected, the relative changes in $^{3}H/^{14}C$ ratio during conversion of glycines **5f** and **5g** to *N*-hydroxyformanilide (Table I) corresponded closely to those observed during PAM oxidation/nitrosobenzene assay of their parent tripeptides **1b** and **1c**. This provides conclusive evidence that the PAM enzyme specifically removes the *pro-S* hydrogen from the glycine residue of **1**.

Discussion

Although PAM enzymes capable of converting the synthetic tripeptide **1** to its truncated amide **2** (Scheme I) have been obtained from many tissues[6-11] and may occur in all animals, relatively little is known about substrate preference or structural variations of enzyme from different sources. In bovine pituitary, from which multiple forms of the enzyme have been purified (e.g., PAM A MW 54000, PAM B MW 38000), it is presently unclear whether these proteins have different basic sequences or they are produced by modification of a single gene product.[7c] However, it has recently been shown that in frog skin (*X. laevis*) there are two PAM enzymes that are products of different genes and have primary sequences that are 92% identical in major portions.[9e,f] The new assay using nitrosobenzene to detect glyoxylate should aid rapid comparison of the activities of various PAM enzymes toward a large variety of substrates bearing a carboxy-terminal

(27) White, R. H. *J. Labelled Compd. Radiopharm.* **1983**, *20*, 787–790.

(28) Supplied by ICN Radiochemicals.

(29) The $^{3}H/^{14}C$ ratios of the tripeptides were adjusted by addition of ^{14}C-labeled tripeptide **1a**. The peptides were purified by HPLC immediately before use.

glycine. This may permit assignment of particular biological functions (e.g., synthesis of certain peptide hormones) to different forms of PAM.

The assay can also be used to conveniently examine other glyoxylate-producing systems, as has been done in the analysis of stereospecifically labeled glycines **5b** and **5c** by the modified D-amino acid oxidase assay. However, attempts to extend it to glycolate oxidase[30] encountered severe difficulties because cysteine normally present in the reaction mixture completely prevents formation of *N*-hydroxyformanilide (**4**).[31] This may be due to competing reaction of glyoxylate with cysteine.[32] Although ascorbate does interfere to some extent in the PAM assay, the procedure is still very sensitive and reproducible at the usual concentrations of this cofactor provided that they are kept constant.

A useful feature of the assay is that it is transparent to the primary tritium (or deuterium) isotope effect provided that the precursor to the formyl carbon of glyoxylate (e.g., C_2 of glycine) bears a single isotopic hydrogen. Although breaking a bond to a ^{14}C atom during the nitrosobenzene reaction is presumably slowed by a primary isotope effect,[33] thereby favoring production of tritiated molecules of **4** over ^{14}C-labeled ones, the results show that the influence of this phenomenon in combination with possible secondary tritium isotope effects is relatively small ($\leq 5\%$). This is true even though most of the bond breaking to radioactive carbon occurs between *two* ^{14}C atoms due to the high level of enrichment of **5a** (96% ^{14}C). This feature eliminates a major problem associated with previous determinations of stereochemical purity of 2-deuterio- or 2-tritioglycines using D-amino acid oxidase.[26] The primary hydrogen isotope effect, which previously required complete oxidation of the glycines for accurate results, disappears in the new assay with nitrosobenzene, which gives accurate results with only partial conversion.

Earlier chemical syntheses of stereospecifically deuteriated or tritiated glycines employed multiple steps and were complicated by introduction of label early in the sequence.[24,34] Previously reported enzymatic syntheses utilized single-step exchange of the glycine hydrogen with solvent by serine hydroxymethyltransferase or by alanine aminotransferase.[26a,35] However, in our hands the latter sometimes proved problematic due to excessive nonenzymatic or incomplete exchange, or because of difficulty in product purification. The current adaptation of the procedure of Williams and co-workers[14] to tritiation at 1 atm of pressure is convenient and affords glycines **5b** and **5c** with good stereochemical purity in a single step. The results also show that tritium NMR spectrometry of their chiral derivatives (e.g., *N*-camphanamides **14b** and **14c**) is an effective alternative to enzymatic methods for analysis of the stereochemistry of tritium labeling. Although the subsequent syntheses of the peptides **1b** and **1c** suffer from some epimerization at C-2 of the glycyl residue during deprotection, this could probably be avoided through use of protecting groups that can be removed by hydrogenolysis (e.g., benzyl, benzyloxycarbonyl).

Our results show that PAM from pig pituitaries oxidizes D-tyrosyl-L-valylglycine (**1**) with *loss of the pro-S hydrogen* of the glycyl residue. The *pro-R* hydrogen is retained in glyoxylate and after the nitrosobenzene reaction appears as the formyl hydrogen of *N*-hydroxyformanilide (**4**). The mechanism initially suggested[1a] for PAM begins with dehydrogenation of the glycine-bearing peptide **A** to an *N*-acylimine **C** (Figure 3). This would spontaneously add water to form **B**, which would cleave to the peptide amide and glyoxylate. A more recent and generally accepted proposal involves hydroxylation of carbon to give **B** directly[18,36] in analogy to dopamine β-hydroxylase, which also requires copper, oxygen, and ascorbate.[37] Although perhaps less likely, another possibility may be N-hydroxylation to generate **D** followed by transformation to **C** and **B**. A chemical precedent for this sequence exists in the oxidation of *N*-aroylglycines with lead tetraacetate.[38] The recent report that PAM can transform the glyoxylic acid phenylhydrazone to oxalic acid monohydrazide[36a] disfavors a dehydrogenation mechanism (i.e., **A** → **C**) and supports C-hydroxylation as a first step. Since enzymatic C-hydroxylations generally proceed with retention of configuration,[39] the removal of the *pro-S* hydrogen by this process would require that aminal **B** possess *S* configuration. If correct, this suggests that peptide analogues that bear a hydroxyl in that position but are incapable of further cleavage may be good inhibitors of PAM.[40] Alternatively, the requirement of enzyme-catalyzed stereospecific removal of the *pro-S* hydrogen for an elimination mechanism (**D** → **C**) also offers hints for inhibitor design.

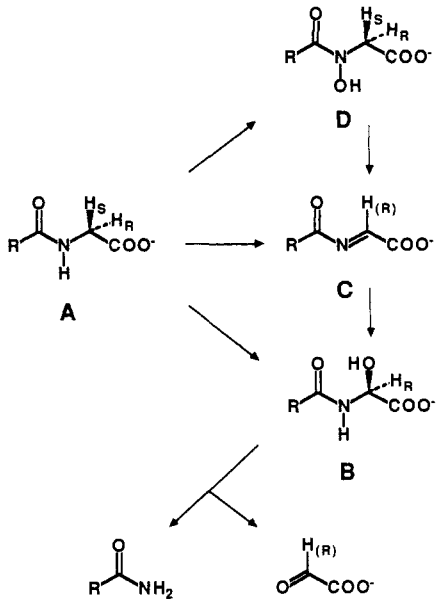

Figure 3. Possible mechanisms of PAM oxidation.

Regardless of the mechanism, the stereochemistry of PAM oxidation clearly shows why this enzyme will not allow replacement of the terminal glycine with L-amino acids[7a,e] but will accept some (though not all) D-amino acids.[7e,41] Since terminal D-amino acid residues are likely to afford some protection against peptidase cleavage, peptide hormones bearing such extensions possess potential to be orally active prodrugs. Investigations of this possibility as well as studies on the mechanism, inhibition, and substrate specificity of PAM enzymes are in progress.

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Experimental Section

Most general procedures and instrumentation have been previously described.[42] Unlabeled D-tyrosyl-L-valylglycine (**1**) and D-tyrosyl-L-valinamide (**2**) were purchased from Bachem (Torrance, CA). Unlabeled *N*-hydroxyformanilide (**4**) was prepared by literature procedures.[13] All other reagents and commercially available enzymes were obtained from Sigma Chemical Co. (St. Louis) unless otherwise indicated. Radiochemicals were purchased from ICN Biomedicals (Cosa Mesa, CA). Radioactivity was determined by standard liquid scintillation procedures in plastic 10-mL scintillation vials (Terochem, Edmonton, AB) with Amersham ACS liquid scintillation cocktail. The instruments used were Beckman LS100C, Beckman 1801, or Packard Tricarb 1500 (National Tritium Labeling Facility) counters. The automatic quench control on the Beckman 1801 was employed to directly determine decompositions per minute by comparison with quench curves prepared from Beckman ^3H and ^{14}C quenched standards. This automatically calculates $^3H/^{14}C$ ratios, but the results were confirmed by analyzing random samples with the addition of standardized [^{14}C]toluene and [^3H]toluene solutions (ICN Radiochemicals). Radioactive TLC plates were analyzed with a Berthold LB2760 TLC scanner. Tritium NMR spectra were obtained[25] on $CDCl_3$ solutions of about 6 mCi of ^3H (10700 scans) at 320.1 MHz with broad-band ^1H decoupling according to the method of Bloxsidge et al.[43] High-pressure liquid chromatography (HPLC) employed the following instruments: Hewlett-Packard 1082B with a variable-wavelength detector; Waters 660 with an 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 loaded in a Z-module compression unit. A two-solvent system was employed with a reversed-phase Radial-Pak C_{18} column (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 (detection at 254 nm). The D-TyrValGly (**1**) has a retention time of 16.8 ± 0.2 min (six runs). For initial purification of glycine after peptide hydrolysis, 0.1% phosphoric acid was used instead of CF_3CO_2H (detection at 200 nm). A binary solvent system was used with a NH_2 Radial-Pak column (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, and 25 min 30% D. Glycine had retention times from 17 to 20 min. All separations used a flow rate of 2 mL/min. Purification of the tritiated glycines[23] employed detection of radioactive compounds with a Berthold LB5026 radiochemical detector connected to a Trace Northern TM7200 multichannel analyzer. HPLC-grade acetonitrile (190-nm cutoff) (Terochem) and buffers (prepared fresh daily) were vacuum filtered before use.

Isolation and Assay of Peptidylglycine α-Amidating Monooxygenase (PAM). The PAM isolation was done at 4 °C and was adapted from the procedure of Kizer et al.[17] Frozen porcine pituitaries (10 g) obtained from Pel-Freeze Biologicals (Rogers, AR) and stored at −60 °C were chopped with a razor blade and suspended in 20 mL of 4 °C buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.2). This was placed in an ice–water bath and homogenized with an IKA Werk Ultra-Turrax homogenizer for four 40-s intervals with 20-s wait between each homogenization. This thick solution was frozen in a −78 °C bath and thawed in a 4 °C water bath twice. The homogenate was centrifuged in a Sorvall RC-5B centrifuge for 30 min at 4 °C and 25000*g*. The clear red supernatant (typically 120 mg of protein) was applied to a Sephadex G-100 column (35 g, 2.5 × 100 cm) at a rate of 1 mL/min and eluted with buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8). Fractions (4 mL) were collected and assayed (see below). The red band that elutes was found to be a good marker for the PAM enzyme. Active fractions were combined (total volume of 100–120 mL; 60 mg of protein; PAM activity 0.19 nmol of D-Tyr-L-ValGly/mg of protein per h) prior to affinity chromatography. The required affinity adsorbent was prepared by shaking Bio-Rad Affi-Gel 15 (5 mL of resin) and D-tyrosyl-L-valylglycine (**1**) (50.7 mg) in 3 mL of buffer (2-*N*-morpholinoethanesulfonic acid, 0.1 M, pH 6.5) at 25 °C for 4 h and then at 4 °C overnight. This material was washed with 25 mL of 6 M urea and then with three cycles of alternating pH 4.0 buffer (0.1 M sodium acetate, 1 M NaCl, 25 mL/cycle) and pH 8.0 buffer (0.1 M sodium borate, 1 M NaCl, 25 mL/cycle). The column was then equilibrated with buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8). The combined active fractions from the Sephadex column were applied to the affinity column at 1 mL/min. The column was washed with 40 mL of buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) and then eluted at 1 mL/min with a pH 4.0 buffer (3 M urea, 1 M glycylglycine, 0.1 M *N*-acetylglycine). Fractions (4 mL) were collected starting exactly 1 min after the elution buffer entered the column. These fractions were immediately transferred to Spectrapor dialysis tubing (Fisher Scientific, 25-mm diameter, 12000–14000 cutoff) and dialyzed against 400 mL of buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8). The buffer was changed after 1, 3, and 7 h total time. The final dialysis was overnight. Protein content (typically 22–53 μg total) was determined by the procedure of Bradford[44] and Bio-Rad protein assay dye. Solutions of bovine serum albumin were used to prepare standard curves (five different concentrations for each curve) for normal (0.2–1.5 mg of protein/mL) and microscale (2.5–25 μg of protein/mL). Absorbance was measured at 590 nm. Enzyme activity was assayed with nitrosobenzene as described below. Most of the activity was in fractions 2 and 3.

PAM Assay with Nitrosobenzene. Solutions of $CuSO_4$ (1.0 mM), KI (5.0 M), and ascorbic acid (0.40 M) were prepared in fully degassed water. A fresh cocktail was prepared for each set of assays by adding to buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 6.8) 50 μL of the copper and KI solutions, 25 μL of the ascorbate solution, and catalase (1 mg) 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 were added 100 μL of the cocktail and D-Tyr-L-Val[1,2-^{14}C]Gly (**1a**) (~80000 dpm in 5 μL). These conditions give final concentrations of 1 mM ascorbic acid, 25 mM KI, 5 μM $CuSO_4$, 0.1 mg/mL catalase, and 1.3 μM **1a**. This solution 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 (0.30 M in 95% EtOH) were added. The solution was mixed at 60 °C for 1 h, and the resulting *N*-hydroxyformanilide was then extracted three times with ether by adding ~0.5 mL of ether, mixing thoroughly, and removing the ether layer with a pipet. The ether extracts were passed through a Pasteur pipet containing ~2.5 g of anhydrous sodium sulfate directly into a scintillation vial. After the third extraction, the drying tube was rinsed with 0.5 mL of ether into the scintillation vial.

(*R*)-[2-^{3}H]Glycine (5b).[23] The procedure of Williams et al.[14] for preparation of deuteriated glycines was modified. To a 25-mL flask with side arm, septum inlet, and stir bar was added (3*R*,5*R*,6*S*)-3-bromooxazinone **12** (53.1 mg, 114 μmol)[20-22] 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 20 °C, nitrogen gas was added, and the procedure was repeated twice. Carrier-free tritium gas was introduced, and the solution was allowed to warm to 20 °C. The pressure was maintained at 720–730 Torr by releasing tritium as necessary. 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 mixture was stirred for 5 h. The 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 under vacuum. Methanol (2 mL) was added and removed by stirring under vacuum. This was repeated three times. The resulting residue was filtered through a borosilicate glass microfiber filter, and this was rinsed with water. The combined filtrates (~5 mL) were applied to a 1-mL AG50×8 H^+ ion-exchange column. This was rinsed with 3 mL of water; **5b** was then eluted with 10 mL of 3 M NH_4OH and lyophilized overnight. The residue was dissolved in water and filtered to give a volume of 1.10 mL with a total activity of 147 mCi. Portions of this solution were purified by HPLC to give a total of 33.2 mCi of **5b** (28% yield based on **12**) with a specific activity of 1.0 Ci/mmol (mass determined by UV absorbance at 200 nm).

(*S*)-[2-^{3}H]Glycine (5c).[23] The procedure used to prepare **5b** was employed to convert the (3*S*,5*S*,6*R*)-3-bromooxazinone **13** (51.8 mg, 111 μmol)[20-22] to the enantiomeric glycine **5c**. After HPLC purification, a total of 26.6 mCi of **5c** with a specific activity of 0.78 Ci/mmol (31% yield) was obtained.

(*RS*)-[2-^{3}H]Glycine (5d). The procedure of White[27] was adapted. Glyoxylic acid (6.20 mg, 84 μmol) was dissolved in concentrated ammonia (2 mL) and the resultant mixture warmed to 55 °C. Sodium boro[^{3}H]hydride (25 mCi, ICN Radiochemicals) was added; the mixture was stirred 2 h and lyophilized in a closed system. The lyophilization was repeated after addition of water (5 mL), and the residue was dissolved in 0.1 M HCl (2 mL). The solution was applied to a column of Bio-Rad AG50 (H^+) cation-exchange resin (5 mL), which was washed with water

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(5 mL) and eluted with 3 M ammonia. Lyophilization of the eluent gave chromatographically pure (*RS*)-[2-$^3H_1$]glycine (**5d**) (1.52 mCi) in 6% yield. This material was repurified immediately before use by HPLC on a NH_2 Radial Pak column as described in the general procedure (specific activity 45 μCi/μmol).

***N*-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valine Benzyl Ester (7).** The method of Shioiri and Yamada[16b] for peptide coupling with diphenylphosphoryl azide was used. In a dry flask were placed *N*-BOC-D-tyrosine (1.41 g, 5.0 mmol) and L-valine benzyl ester hydrochloride (1.34 g, 5.50 mmol). This was dried at 50 mTorr over P_2O_5 for 18 h. To this was added dry DMF (15 mL), and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (1.20 mL, 5.57 mmol) was added followed by a solution of triethylamine (1.50 mL, 11.0 mmol) in DMF (10 mL), and stirring was continued at 0 °C for 5 h. The mixture was diluted with 125 mL of benzene and 250 mL of EtOAc. This was washed with 1 N HCl (2 × 25 mL), H_2O (25 mL), saturated $NaHCO_3$ (2 × 25 mL), H_2O (25 mL), and saturated NaCl (2 × 25 mL). The organic layer was dried (Na_2SO_4), and the solvent was removed to give an oil that was purified by recrystallization from toluene/Skelly B to give **7**: 1.30 g (55%); mp 101–102 °C; IR ($CHCl_3$ cast) 3340, 1659, 1516 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 0.78 (d, J = 6.7 Hz, 6 H, $(CH_3)_2$), 1.42 (s, 9 H, $(CH_3)_3C$), 2.10 (m, 1 H, Me_2CH), 2.99 (br d, J = 6.7 Hz, 2 H, aryl CH_2), 4.36 (br s, 1 H, NCHCO), 4.54 (m, 1 H, NCHCO), 5.06 (br s, 1 H, NH), 5.12 (d, J = 12.2 Hz, 1 H, aryl CHHO), 5.19 (d, J = 12.2 Hz, 1 H, aryl CHHO), 5.90 (br s, 1 H, OH), 6.52 (br s, 1 H, NH), 6.70 (d, J = 8.5 Hz, 2 H, aryl H), 7.02 (d, J = 8.5 Hz, 2 H, aryl H), 7.34 (m, 5 H, 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*-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valine (8).** A mixture of **7** (0.750 g, 1.59 mmol) and 5% Pd/C (74.2 mg) in 50 mL of 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 **8**: 0.467 g (77%); mp 97–100 °C; IR ($CHCl_3$ cast) 3320, 1658, 1516 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 0.89 (m, 6 H, $(CH_3)_2$), 1.40 (s, 9 H, $(CH_3)_3C$), 2.10 (br, 1 H, Me_2CH), 2.88 (br, 1 H, aryl CHH), 3.10 (br, 1 H, aryl CHH), 4.42 (br, 1 H, NCHCO), 4.66 (br, 1 H, NCHCO), 5.46 (br, 1 H, NH), 6.69 (d, J = 8.5 Hz, 2 H, aryl H), 6.88 (br, 1 H, NH), 7.00 (br, 2 H, aryl H); exact mass, 380.1949 (380.1947 calcd for $C_{19}H_{28}N_2O_6$).

***N*-(*tert*-Butoxycarbonyl)-D-tyrosyl-L-valylglycine Methyl Ester (9).** The procedure used for the preparation of **7** was adapted to couple glycine methyl ester hydrochloride (**6**) (38.4 mg, 0.306 mmol) to *N*-BOC-D-tyrosyl-L-valine (**8**) (104 mg, 0.273 mmol) with diphenylphosphoryl azide[16b] (58 μL, 0.27 mmol) in DMF (2 mL) containing triethylamine (80 μL, 0.57 mmol). After 6 h at 0 °C the reaction mixture was diluted with 50 mL of benzene and 100 mL of EtOAc. This was washed with 1 N HCl (2 × 25 mL), H_2O (25 mL), saturated $NaHCO_3$ (2 × 25 mL), H_2O (25 mL), and saturated NaCl (2 × 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 **9**: 112 mg (92%); mp 87–89 °C; IR ($CHCl_3$ cast) 3306, 1688, 1649, 1516 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 0.83 (d, J = 6.7 Hz, 6 H, $(CH_3)_2$), 1.41 (s, 9 H, $(CH_3)_3C$), 2.15 (m, 1 H, Me_2CH), 2.99 (m, 2 H, aryl CH_2), 3.72 (s, 3 H, CO_2CH_3), 4.04 (br s, 2 H, NCH_2CO), 4.40 (m, 2 H, NCHCO), 5.48 (d, J = 6.5 Hz, 1 H, NH), 6.72 (d, J = 8.5 Hz, 2 H, aryl H), 6.98 (d, J = 8.5 Hz, 3 H, aryl H, NH), 7.53 (br s, 1 H, NH), 7.75 (br s, 1 H, OH); exact mass, 451.2323 (451.2318 calcd for $C_{22}H_{33}N_3O_7$).

Synthesis of Labeled D-Tyrosyl-L-valylglycines 1a–d. The radioactive glycine (**5a–d**) 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 45–60 min, and the resulting solution was stirred at room temperature for 2.5 h to give pure 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*-BOC-D-tyrosyl-L-valine (**8**) in dry DMF (0.93 mM). This was stirred under argon at 0 °C, and diphenylphosphoryl azide (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 1 N HCl (2 × 1 mL), H_2O (1 mL), saturated $NaHCO_3$ (2 × 1 mL), and saturated NaCl (2 × 1 mL). The organic layer was concentrated in vacuo to give the 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 1 N HCl was added and methanol was removed in vacuo. To the residue was added 1.0 mL of H_2O, and this was extracted with EtOAc (4 × 2.5 mL). The organic layers were concentrated in vacuo to give the *N*-BOC tripeptide (TLC: (18/2/0.2 $CHCl_3$–MeOH–HCOOH) R_f 0.27). Trifluoroacetic acid (0.3 mL) was added, the mixture was allowed to stand for 0.5 h at 20 °C, the solvent was removed in vacuo, and water (1.0 mL) was added. This was extracted with ether and 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). Tripeptides prepared in this way were chromatographically identical with unlabeled **1** obtained commercially (Bachem) or prepared analogously from unlabeled **9**. They were purified by HPLC as described in the general procedure immediately before use in enzymatic reactions.

Hydrolysis of D-Tyrosyl-L-valyl[2-^{3}H]glycines 1b and 1c to [2-^{3}H]-Glycines 5f and 5g. The general procedure[45] for the cleavage of peptides was adapted for small-scale radioactive hydrolysis. Into a capillary melting point tube sealed at one end was placed a solution of the radioactive tripeptide **1b** or **1c** 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 cooled tubes were broken near the top, placed top down in a 1.5-mL microfuge tube, and centrifuged. The other end of the capillary tube was then opened. 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 and then on an NH_2 column (see the general procedure) to give pure glycines **5f** or **5g**.

D-Amino Acid Oxidase Analysis of Stereochemical Purity of [2-^{3}H]-Glycines 5b–g. The method of Wellner[26a] was adapted to be suitable for trapping the glyoxylate. A cocktail was prepared by diluting 45 μL (0.99 mg, 54 000 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). 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 were 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 ether (3 × 0.5 mL). The ether extracts were passed down a Pasteur pipet filled with Na_2SO_4 into a scintillation vial and were analyzed for radioactivity.

***N*-((1*S*)-(−)-Camphanoyl)glycines 14b and 14c.** These derivatives were prepared according to the procedure of Armarego et al.[24] as described by Williams and co-workers.[14] To (1*S*)-(−)-camphanic acid chloride (Aldrich; 32.1 mg, 148 μmol, 2 equiv) in 1.5 mL of toluene at 0 °C was added glycine **1b** or **1c** (5.47 mg, 72.9 μmol, 1 equiv) in 0.1 M NaOH (3.6 mL, 0.36 mmol, 5 equiv). This was stirred at 0 °C for 0.5 h and then at room temperature for 4 h. The solution was washed with $CHCl_3$ (3 × 5 mL), acidified with 0.5 mL of 1 N HCl, and extracted with CH_2Cl_2 (3 × 5 mL). The solvent was removed in vacuo to give the chromatographically pure camphanamide, which was analyzed by tritium NMR.

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