PREPARATION OF DEUTERIUM LABELED PHENYLALANINE DERIVATIVES AND THE SOLID-PHASE PEPTIDE SYNTHESIS OF [3-DL-[α-$^{2}H_{1}$]PHENYLALANINE,8-ARGININE]VASOPRESSIN

by

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STATEMENT BY AUTHOR

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No man passes through this life alone; there are always those who share his triumphs and his disappointments. My wife, Rusti, has stood beside me and made sacrifices so that this would would come to fruition. To that extent, this work is as much hers as mine.

TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

LIST OF ILLUSTRATIONS

LIST OF TABLES

ABSTRACT

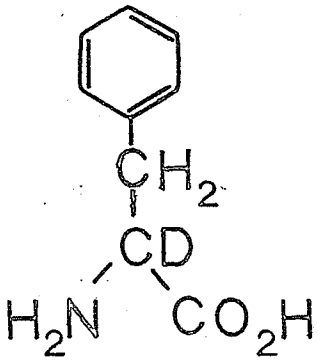
The preparation of three carbon-deuterium labeled residues through the use of solvent exchange via enzyme-related reactions is described. DL-[α-$^{2}H_{1}$]Phenylalanine (I), DL-[β,β-$^{2}H_{2}$]phenylalanine (II), and DL-[α,β,β-$^{2}H_{1}$]phenylalanine (III) are prepared using pyridoxal-metal catalysis with the degree of deuterium exchange being both temperature and pH dependent. Boc-DL-[α-$^{2}H_{1}$]phenylalanine is prepared from (I) and used in the solid-phase peptide synthesis of [3-DL-[α-$^{2}H_{1}$]phenylalanine,8-arginine]vasopressin (V). The diastereomers of the cyclic nonapeptide hormone are separated via partition chromatography and bioassay results of the pure L-isomer using the milk ejecting assay are identical to previously published results. Compound (III) is also resolved using the traditional hog renal acylase procedure to give pure L-[α,β,β-$^{2}H_{3}$]phenylalanine (IV).

INTRODUCTION

For years now, the chemical literature has contained accounts describing various methods by which amino acids can be labeled. Carbon-deuterium labeled amino acids have been used to synthesize polypeptides and proteins in order to study various properties. The syntheses of such labeled polypeptides and proteins have given the chemist a useful handle for studying the chemical, physical, and biological properties of these macromolecules. A carbon-deuterium label can provide an unambiguous method of peak assignment in both proton (Kopple et al. 1972; Hruby 1972, 1974; Brewster and Hruby 1973; Brewster, Hruby, Glasel, and Tonelli 1973) and carbon-13 (Bovey 1972; Brewster, Hruby, Spatola, and Bovey 1973) nuclear magnetic resonance studies. Since the substitution of deuterium for a proton on a carbon atom of an amino acid exhibits little, if any, added steric constraints, it has also been used as a probe for the study of the tertiary structure of proteins (Crespi et al. 1972; Katz and Crespi 1972; Jardetsky and Wade-Jardetsky 1971; Cohen, Feil, and Chaiken 1971; Jardetsky et al. 1971). Various carbon-deuterated analogs of polypeptide hormones, such as oxytocin, have been used to study the binding of the hormones to the neurophysin proteins (Glasel et al. 1973). Such labels have also provided an insight into the

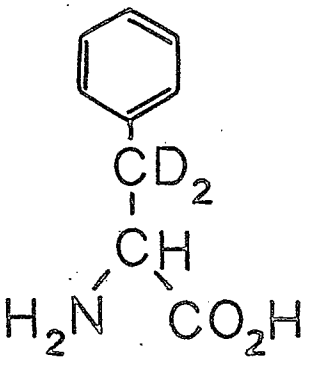
microdynamic properties of these macromolecules (Glasel et al. 1973). The carbon-deuterium label has thus proven itself to be an invaluable handle in the studies of polypeptides and proteins.

Four carbon-deuterium labeled derivatives of phenylalanine have been prepared in this study: DL-[α-$^{2}H_{1}$]-phenylalanine (I); DL-[β,β-$^{2}H_{2}$]phenylalanine (II); DL-[α,β,β-$^{2}H_{3}$]phenylalanine (III); and L-[α,β,β-$^{2}H_{3}$]phenylalanine (IV) (Figure 1). However, the method used was found not to extend to the specific labeling of tyrosine (Cohen et al. 1971). DL-[α-$^{2}H_{1}$]Phenylalanine (I) has been used in the solid-phase peptide synthesis (SPPS; Merrifield 1963, 1969) of [3-DL-[α-$^{2}H_{1}$]phenylalanine,8-arginine]vasopressin (V) (Figure 2) with the diastereomers being separated via partition chromatography (Yamashiro 1964; Yamashiro, Gillessen, and Du Vigneaud 1966; Spatola et al. 1974; Upson 1975; Upson and Hruby 1976).



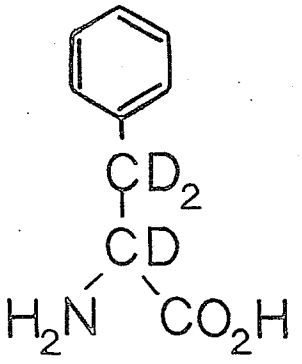
I

DL-[α-$^{2}H_{1}$]phenylalanine



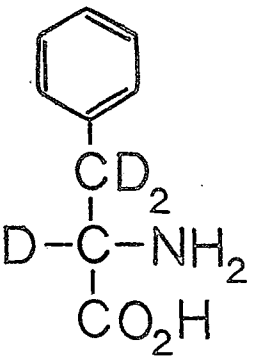
II

DL-[β,β-$^{2}H_{2}$]phenylalanine



III

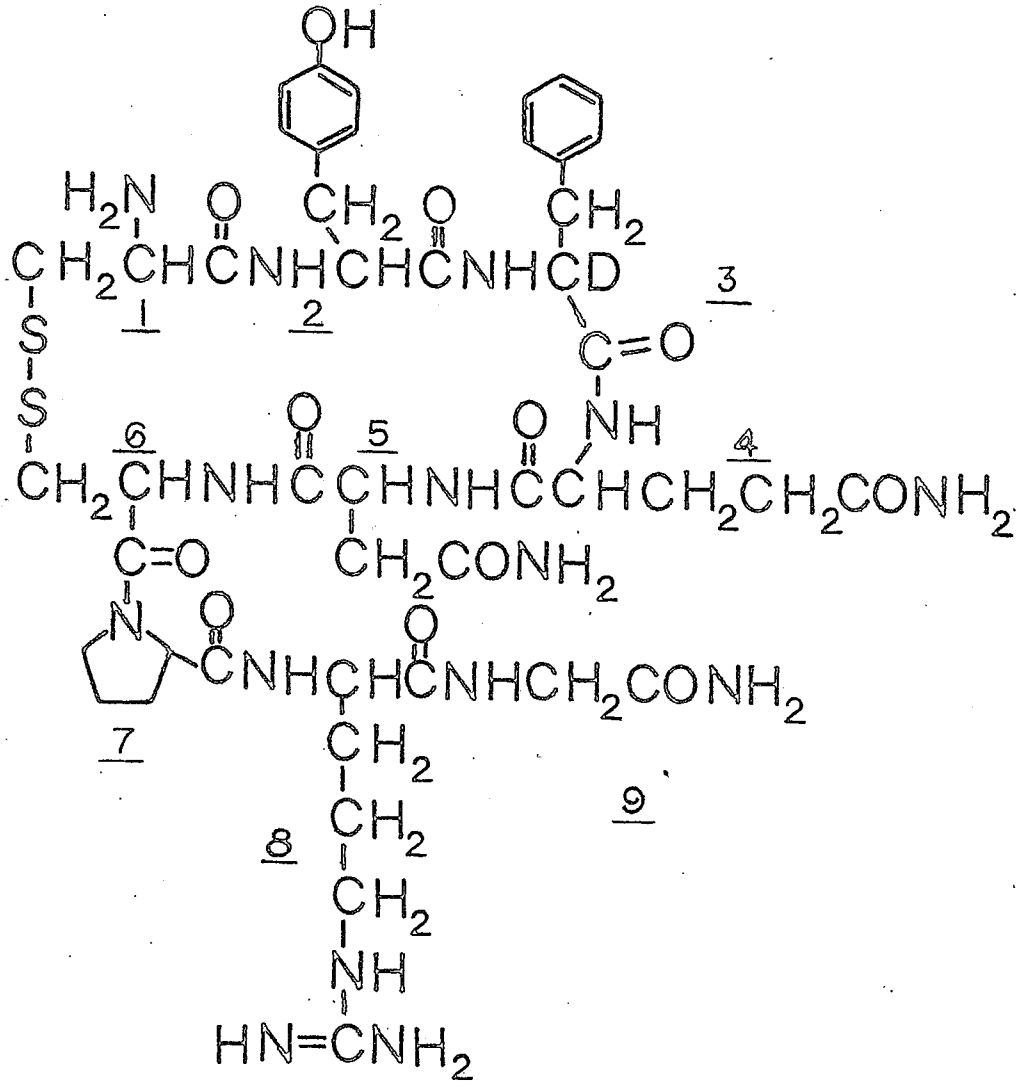
DL-[α,β,β-$^{2}H_{3}$]phenylalanine



IV

L-[α,β,β-$^{2}H_{3}$]phenylalanine

Figure 1. Carbon-deuterium labeled analogs of phenylalanine.



V

Figure 2. [3-DL-Iα-$^{2}H_{1}$]Phenylalanine,8-arginine]-vasopressin.

DISCUSSION

Since amino- and carboxyl-protons of amino acids are labile in aqueous solution (Junk and Svec 1963), any isotopic labeling of amino acids must, in order to be of practical use, occur at a "non-exchangeable" position, i.e., a carbon atom. Poor yields, lack of specificity, lack of generality, an excessive time requirement, or unsuitability for quantitative preparations are but a few of the disadvantages which have hampered many of the previous attempts to deuterium label amino acids at a carbon atom. The literature reports of such amino acid labeling can, in general, be divided into five categories: (1) synthesis by the reduction of appropriate precursors (Hanson et al. 1971, Ife and Haslam 1971, Kirby and Michael 1971, Suzuki et al. 1959); (2) acid- and base-catalyzed exchange procedures (Suzuki, Shimanouchi, and Tsubori 1963); (3) enzymatic transaminations (Cammarata and Cohen 1950, Hird and Rosell 1950, Suzuki et al. 1959); (4) chemical mimicking of the enzymatic process involving the formation of Schiff bases (Metzler and Snell 1952; Metzler, Ikawa, and Snell 1954; Junk and Svec 1964; Johns and Whelan 1966; Abbott and Martell 1969); and (5) incorporation of solvent isotopes during synthesis (Manning 1970, Thanassi 1971, Upson 1975, Upson and Hruby 1976).

Suzuki and his coworkers (Suzuki et al. 1959) electrolytically reduced α-hydroxyiminopropanoic acid in the presence of 85% D_2SO_4 and 99.9% D_2O, but were only able to achieve a 40% yield of [α-$^2H_1$]alanine. Catalytic hydrogenation of acylaminocinnamic acids has been used (Kirby and Michael 1971) to stereoselectively label the β-methylene groups and obtain [βR-$^3H_1$]- and [βS-$^3H_1$]tyrosine in order to show that hydroxylation in the biosynthesis of haemanthamine occurred with retention of configuration. The base catalyzed attempts of Suzuki and his coworkers to form [α-$^2H_1$]glycine and [α-$^2H_1$]alanine (Suzuki et al. 1963) resulted in poor yields on the order of 10-20%. Various groups (Cammarata and Cohen 1950, Hird and Roswell 1950, Suzuki et al. 1959) have shown that the enzymatic process of transamination while being of general applicability in providing a label at the α-carbon position is far too tedious and laborious to be of much use for large scale preparations. In their work, Cammarata and Cohen established the existence of more than one type of transaminase, and also ascertained the existence and identity of a coenzyme, pyridoxal phosphate. This led others to explore the action of the coenzyme. Use of pyridoxal in non-enzymatic transaminations has led to various carbon-deuterium labeled derivatives of valine (Abbott and Martell 1969, Johns and Whelan 1966) and leucine (Junk and Svec 1964), while salicylaldehyde has been used to label

glycine, alanine, isoleucine, and phenylalanine (Johns and Whelan 1966), all with somewhat varying results and degrees of success. Finally, Thanassi has determined that the alkylation of certain acylaminomalonic esters followed by saponification and decarboxylation passes through an intermediate which will incorporate solvent isotopes at the α-carbon position (Thanassi 1971).

The literature contains several attempts to deuterium label phenylalanine. In studying the action of the enzyme, L-phenylalanine ammonia lyase, which converts L-phenylalanine into trans-cinnamic acid, two groups (Hanson et al. 1971, Ife and Haslam 1971) attempted to ascertain the stereochemistry of the elimination reaction. It was necessary to obtain an L-phenylalanine derivative containing a stereospecific deuterium in the β-position. Hanson and his coworkers devised two syntheses by which they could stereoselectively label the β-position of L-phenylalanine. Both syntheses originate with 1-deuteriobenzaldehyde (Cohen and Song 1965): the first involves formation of an azlactone and the second involves the action of the enzyme liver alcohol dehydrogenase. The work of Ife and Haslam resembles the second synthesis of Hanson's group except in the choice of malonic ester alkylation conditions. The extent of labeling, however, was less (86% vs. 98%). The conclusion of Hanson's group was that the elimination was stereospecific and occurred in an antiperiplanar manner

eliminating the 3-S hydrogen. One other preparation of an α-carbon labeled phenylalanine residue has been presented in literature. The method (Thanassi 1971) uses either tritium or deuterium labels and incorporates the isotope from the solvent during the decarboxylation of aminomalonate precursors.

For this study, the deuterated analogs of phenylalanine were prepared using L-phenylalanine as the starting material and a non-enzymatic transamination to achieve the desired deuterium labeling. Pyridoxal-metal catalysis was used to prepare three deuterated derivatives: DL-[α-$^{2}H_{1}$]-phenylalanine, DL-[β,β-$^{2}H_{2}$]phenylalanine, and DL-[α,β,β-$^{2}H_{3}$]-phenylalanine.

Junk and Svec (1964) have used pyridoxal-potassium alum catalysis in refluxing deuterium oxide to label leucine in the α- and β-positions, and used mass spectral data to show that the leucine contained 97% α-deuterium and was 92% trideuterium labeled. While they did not actually investigate the mechanism, Junk and Svec believed their work was consistent with a pyridoxal-metal-amino acid reaction mechanism previously proposed (Greenstein and Winitz 1961, p. 593) and shown in Figure 3. Both the α-proton and the β-protons (shown in Figure 4) are exchanged for deuterium through the tautomerization of the appropriate Schiff base intermediate.

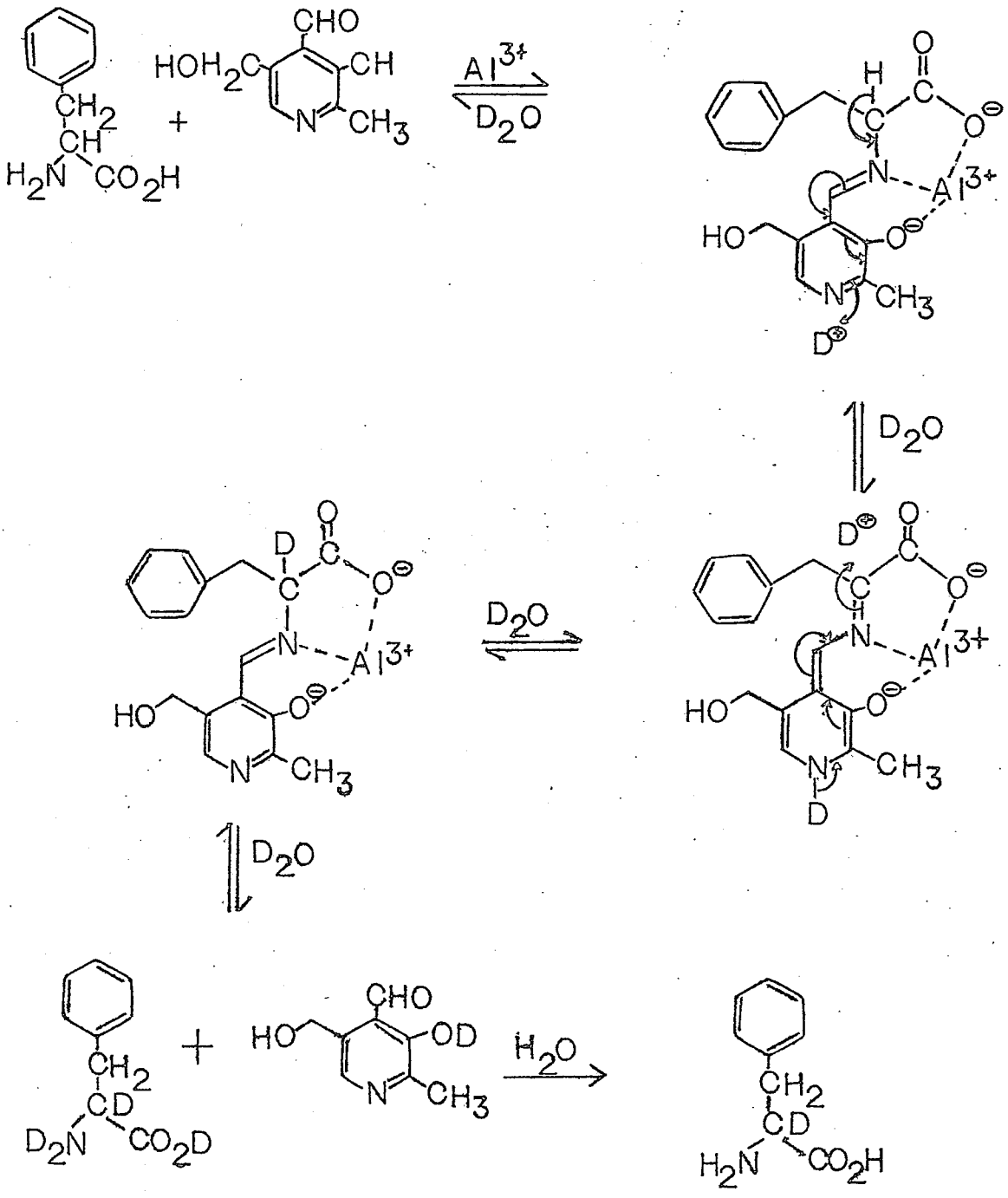


Figure 3. Mechanism for α-exchange involving phenylalanine.

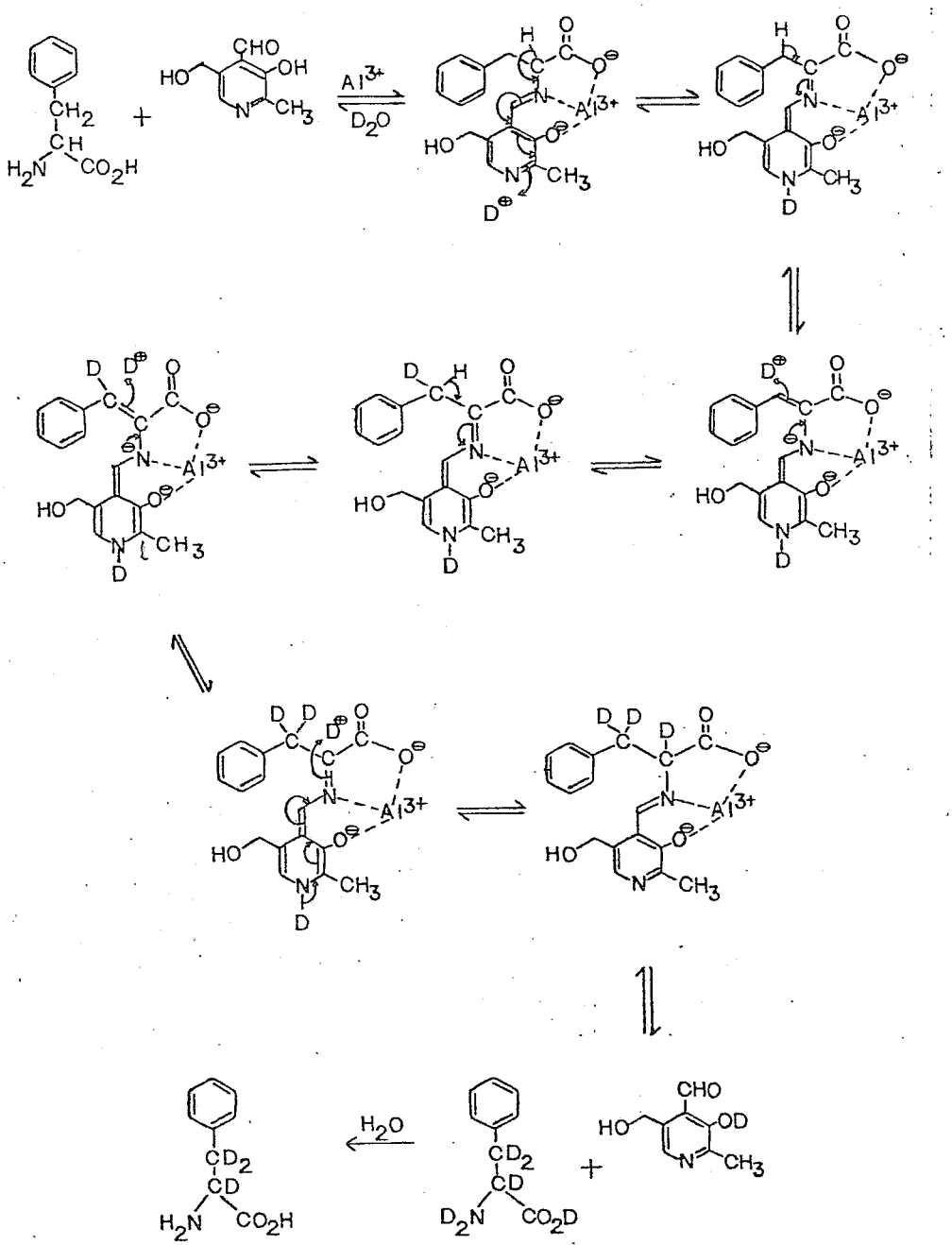


Figure 4. Mechanism for β-exchange involving phenylalanine using Al(III)-pyridoxal catalysis.

Junk and Svec (1964) suggested their method was applicable to the labeling of all amino acids; however, Johns and Whelan (1966) obtained evidence which questions the generality and specificity of these methods. In attempting to carbon-deuterium label valine in the α- and β-positions by the method of Junk and Svec, Johns and Whelan were not able to obtain a dideuterium labeled derivative. They did, however, note a temperature dependence. At temperatures of 120-140°C they were able to label the α-position of valine, but at 100°C no exchange was obtained. Similar observations were noted when salicylaldehyde and Cu(II) were used to catalyze the exchange reaction. Tamiya and Ohshima (1962) obtained DL-[α,β,β-$^{2}H_{3}$]-aspartic acid using this mode of catalysis, but again Johns and Whelan were only able to obtain α-carbon-deuterium labeled amino acids when they attempted to extend the method to glycine, DL-alanine, DL- -aminobutyric acid, DL-valine, DL-isoleucine, DL-phenylalanine, and L-glutamic acid.

Perhaps some light has been shed on this apparent disparity by the work of Abbott and Martell (1969). In their nuclear magnetic resonance study of the non-enzymatic transaminations involving valine and α-aminobutyric acid, they observed what they considered a new reaction of pyridoxal catalysis, ß-carbon labeling, and perhaps more importantly, they established the effect of pH during the

exchange experiment. Abbott and Martell observed that β-exchange of protons occurs over the pH range of 4-7, while α-exchange becomes more rapid in comparison to β-exchange as the pH is raised, until at pH values greater than 9.0 it is possible to label an α-carbon atom leaving the β-carbon protons undisturbed. Addition of protons or metal ions, Al(III) or Zn(II), were found to enhance the rate of exchange and Abbott and Martell proposed the mechanisms shown in Figure 4 and Figure 5.

In Figure 5, the rate enhancement provided by deuterium ions can be accounted for by the electropositive nature of the deuterated azomethine nitrogen which attracts the electrons of the β-carbon atom. Acid catalyzed β-exchange is then thought to occur through the imine-enamine tautomerization. The mechanism of metal-catalyzed (Figure 4) β-exchange is analogous to the proton-catalyzed case and involves the formation of a Schiff base-metal chelate. The role of the metal, however, is quite different than the role of the acid. While the metal is a much poorer polarizing agent at a given site than is a deuterium ion (or proton), the metal ion can compensate by its chelating ability. Because it can bind strongly with the carboxylate group and thereby reduce its negative charge density, it enhances the withdrawing effect of the electropositive azomethine nitrogen. The chelated metal ion also forms a five-membered ring with the carboxylate group to further enhance the

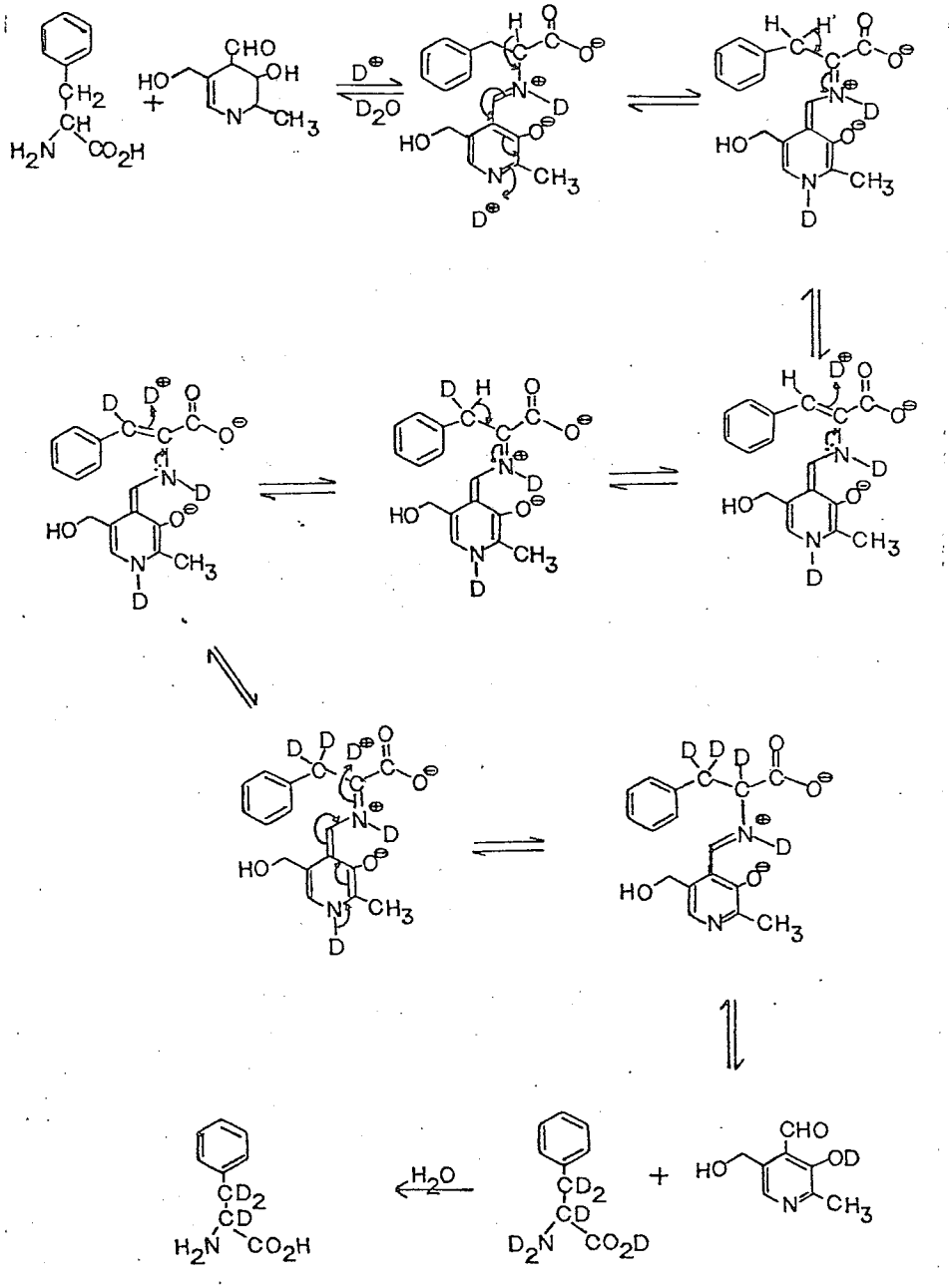


Figure 5. Mechanism for β-exchange involving phenylalanine using pyridoxal-acid catalysis.

stability of the proposed intermediate. With this as a starting point, carbon-deuterium labeling of L-phenylalanine was undertaken using the metal-pyridoxal mode of catalysis.

RESULTS

DL-[α-$^{2}H_{1}$]Phenylalanine was the first carbon-deuterium analog sought. The results of the study are shown in Table 1. The Schiff base-metal-chelate catalysis was studied by varying several reaction parameters in order to obtain a method that would result in a minimum 95% α-carbon-deuterium label, as determined by n.m.r. analysis. It was quickly ascertained that Al(III) was a superior catalyst to Zn(II) in that conditions necessary to achieve the same extent of deuterium labeling were always more vigorous when Zn(II) was used. In several of the experiments, a small amount of precipitate was present in an otherwise clear, bright yellow solution. In the experiments involving a second exchange (TSK-86-1, TSK-98-1, TSK-103-2, and TSK-187-1), a colloidal suspension appeared every time shortly after adjusting the pH to the desired level and vanished when the pH was lowered during isolation and purification procedures. Solid sodium carbonate was used to adjust the pH of the reaction mixtures to avoid the introduction of protons to the mixture.

Except for the experiments TSK-42-1 and TSK-42-2, where the solvent was D_2O/DMSO-d_6 (2:1), the solvent was always 99.8% deuterium oxide.

Table 1. Experiments involving α-carbon-deuterium labeling of phenylalanine.

Expt. No.	Mode of Catalysis[a]	Catalysis Ratio[b]	pH	Time (hr)	Temp	% Exchange[c]
TSK-35-1	Pyridoxal-Zn(II)	10:2:1	9.0	8	RT	0
TSK-36-1	Pyridoxal-Zn(II)	5:1:1	9.5	40	RT	0
TSK-36-2	Pyridoxal-Al(III)	10:2:1	9.4	40	RT	-
TSK-42-1	Pyridoxal-Zn(II)	7:2:1*	9.5	72	RT	-
TSK-42-2	Pyridoxal-Al(III)	7:2:1*	9.6	72	RT	-
TSK-64-2	Pyridoxal-Al(III)	10:2:1	9.5	24	RT	-
TSK-67-1	Pyridoxal-Al(III)	10:2:1	9.5	24	RT	40
TSK-74-1	Pyridoxal-Al(III)	10:2:1	9.5	24	RT	40
TSK-83-1	Pyridoxal-Al(III)	10:2:1	9.4	24	RT	52
			9.4	24	RT	60
TSK-86-1	Pyridoxal-Al(III)	10:2:1	9.3	25.5	RT	85
TSK-94-1	Pyridoxal-Al(III)	10:2:1	9.5	115	RT	86
TSK-95-1	Pyridoxal-Al(III)	10:2:1	9.3	24	100°C	72
TSK-98-1	Pyridoxal-Al(III)	10:2:1	9.1	24	RT	50
			9.7	24	RT	91
TSK-99-2	NaOAc-Al(III)	10:2:1	9.5	24	RT	0
TSK-101-1	Pyridoxal-Al(III)	5:1:1	9.9	24	RT	95
TSK-103-2	Pyridoxal-Al(III)	5:2:2	9.6	24	RT	90
			9.6	24	RT	90
TSK-106-1	Pyridoxal-Al(III)	5:1:1	9.6	24	RT	91
TSK-156-1	Pyridoxal-Al(III)	5:1:1	9.6	24	RT	95
TSK-178-1	Pyridoxal-Al(III)	5:1:1	9.6	24	RT	100
TSK-187-1	Pyridoxal-Al(III)	5:1:1	9.6	69	RT	91
		10:3:3	9.5	36	RT	96
TSK-223-2	Pyridoxal-Al(III)	5:1:1	9.6	24	RT	96
TSK-223-4	Pyridoxal-Al(III)	5:1:1	9.6	24	RT	96
TSK-224-1	Pyridoxal-Al(III)	5:1:1	9.6	24	RT	94

*D_2O/d_6-DMSO (2:1); solvent in all other cases 99.8% D_2O.

Table 1.--Continued

[a]Pyridoxal from pyridoxal hydrochloride; Zn(II) from $ZnCl_2$; Al(III) from $AlK(SO_4)_2 \cdot 12H_2O$.

[b]Phenylalanine/pyridoxal/metal.

[c]Per cent exchange determined by n.m.r. integration.

While Abbott and Martell (1969) observed general base catalysis using acetate ion, no such exchange was noted (TSK-99-2) for L-phenylalanine.

In studying the Al(III) catalyzed exchange reaction, it soon became apparent that pH values in excess of 9.0 led to far greater α-carbon-deuterium labeling than did pH values of 9.0 or slightly lower. The ratio of L-phenylalanine-pyridoxal-Al(III) was at the outset that of Abbott and Martell, that is 10:2:1; however, the best exchange under these conditions resulted in only an 85% α-carbon-deuterium labeled residue which would be insufficient for the purposes of later ^{1}H- and ^{13}C-nuclear magnetic resonance studies. It was, therefore, decided to increase the amount of catalyst present bringing the phenylalanine-pyridoxal-Al(III) ratio to 5:1:1. This very quickly led to the desired level of α-exchange and two experiments resulted in the successful preparation of DL-[α-$^{2}H_{1}$]phenylalanine possessing 96% deuterium in the α-position.

L-Phenylalanine, pyridoxal hydrochloride, and aluminum potassium sulfate dodecahydrate (potassium alum) in the ratio of 5:1:1 were dissolved in a large excess of 99.8% deuterium oxide and the pH adjusted to 9.6. After 24 hrs, the n.m.r. spectrum revealed that the peak corresponding to the α-carbon proton at 4.1-4.3 δ (it should be noted here that the absorption frequency of the α-carbon proton moves down field as the pH is lowered) had essentially

disappeared. Based on the relative peak intensities of the n.m.r. spectrum, the product was found to be DL-[α-$^2H_1$]-phenylalanine possessing 96% deuterium in the α-position and no detectable β-labeling. DCl/D_2O was used as the n.m.r. solvent in order to shift the absorption frequency of the HOD peak downfield so that the α-proton region could be observed. IR analysis indicated that the DL-[α-$^2H_1$]-phenylalanine had been isolated as its hydrochloride salt and this was further confirmed by the formation of a precipitate with a $AgNO_3$ test solution. Comparative tlc experiments using n-butanol-acetic acid-water (4:1:5) as the solvent system gave R_f values of 0.23 for both the DL-[α-$^2H_1$]phenylalanine isolated and an authentic L-phenylalanine sample.

The second successful preparation of DL-[α-$^2H_1$]-phenylalanine involved the utilization of Zn(II) as the metal catalyst. After 24 hrs of refluxing, the product produced an n.m.r. spectrum which indicated 95% deuterium in the α-position and no detectable β-carbon-deuterium labeling.

Obtaining the desired level of exchange of 95% became a problem during some of the preparations involving 2.0 grams or more of phenylalanine. Altering the phenylalanine-pyridoxal-Al(III) ratio seemed to have litte effect on improving the α-carbon-deuterium labeling above the 86% level. A second exchange using a fresh aliquot of 99.8%

deuterium oxide was required (TSK-83-1, TSK-98-1, and TSK-103-2) and even this did not always raise the per cent exchange to the desired 95% level. Since recrystallization of these larger samples was also somewhat more difficult, owing to the greater amount of salt formed in adjusting pH levels of these large preparations, most experiments were done on a 1.0 gram (6.05 mmole) scale.

Synthesis of DL-[α,β,β-$^2H_3$]phenylalanine containing a minimum of 95% deuterium label at both the α- and β-carbons using exchange procedures proved to be a far more formidable task. Reactions run below refluxing temperatures (see Table 2: TSK-38-1, TSK-39-1, TSK-43-1, and TSK-49-1) proved to be totally ineffective at labeling either the α- or β-position of L-phenylalanine, thus substantiating the temperature dependence observed by Johns and Whelan (1966). Temperatures in excess of 100°C (105-110°) were found to be sufficient to produce the desired amount of labeling at both positions in a reaction time of twenty-four hours.

In studies designed to determine the metal to be used for chelate formation, an interesting pH phenomenon was noted in comparing the effectiveness of Al(III) and Zn(II). As mentioned previously, Abbott and Martell (1969) established the pH range 4-7 as necessary to label the β-carbon of valine or α-aminobutyric acid. At pH values outside this range, the β-exchange either did not occur or simply proceeded at an imperceptible rate. The Al(III) and

Table 2. Experiments involving α,β,β-carbon-deuterium labeling of phenylalanine.

Expt. No.	Mode of Catalysis[a]	Catalysis Ratio[b]	pH	Time (hr)	Temp (°C)	% Exchange[c] α	% Exchange[c] β
TSK-38-1	Pyridoxal-Zn(II)	10:2:1	5.9	38	100	<5.0	<5.0
TSK-39-1	Pyridoxal-Zn(II)	10:2:1	6.0	23	82	<5.0	<5.0
TSK-43-1	Pyridoxal-Al(III)	7:2:1	6.1	70	78	<5.0	<5.0
TSK-49-1	Pyridoxal-Zn(II)	8:2:1	5.9	26	81	<5.0	0.0
TSK-57-1	Pyridoxal-Al(III)	200:10:1	3.6	23	100	<5.0	<5.0
TSK-60-1	Pyridoxal-Al(III)	200:10:1	5.8	24	108	<5.0	0.0
TSK-84-1	Pyridoxal-Al(III)	10:2:1	5.8	26	105	80	40
TSK-99-1	PYridoxal-Al(III)	10:2:1	5.8	24	105	80	40
TSK-103-1	Pyridoxal-Al(III)	5:2:2	5.8	24	105	90	0.0
TSK-107-1	Pyridoxal-Al(III)	5:1:1	6.2	24	105	96	27
TSK-107-2	Pyridoxal-Zn(II)	5:1:1	6.3	24	105	83	78
TSK-110-1	Pyridoxal-Zn(II)	5:1:1	5.2	24	105	71	33
TSK-111-1	Pyridoxal-Al(III)	5:1:1	5.5	24	105	85	73
TSK-113-1	Pyridoxal-Al(III)	5:1:1	4.7	24	105	95	88
TSK-114-1	Pyridoxal-Zn(II)	5:1:1	6.0	24	105	83	70
TSK-115-1	Pyridoxal-Al(III)	5:1:1	4.2	24	105	>95	94
TSK-115-2	Pyridoxal-Zn(II)	5:1:1	6.8	24	105	92	76
TSK-117-1	Pyridoxal-Al(III)	5:1:1	4.2	24	105	92	96
TSK-133-1	Pyridoxal-Al(III)	5:1:1	4.1	24	105	>95	>93
TSK-159-1	Pyridoxal-Al(III)	5:1:1	4.0	24	105	~100	95
TSK-177-1	Pyridoxal-Al(III)	5:1:1	4.1	24	105	~100	~100
TSK-205-1	Pyridoxal-Al(III)	5:1:1	4.05	24	105	96	96
TSK-213-1	Pyridoxal-Al(III)	5:1:1	4.05	24	105	96	96
TSK-224-2	Pyridoxal-Al(III)	5:1:1	4.05	24	105	96	98

[a] Pyridoxal from pyridoxal hydrochloride; Zn(II) from $ZnCl_2$; Al(III) from $AlK(SO_4)_2 \cdot 12H_2O$.

[b] Phenylalanine/pyridoxal/metal.

[c] Per cent exchange determined by n.m.r. integration.

Zn(II) catalysis results are summarized in Table 3 and it is immediately obvious that at higher pH values within this range, Zn(II) is the superior catalyst, while Al(III) does a more effective job at the lower pH values. No explanation can be offered for the marked difference in the pH dependence of these two metal ions.

Since Al(III) had proven itself the superior catalyst for the α-exchange, it was decided to use Al(III) as the chelating ion for α,β,β-labeling. Using the same molar ratio as established in the α-exchange experiment (5:1:1), L-phenylalanine, pyridoxal hydrochloride, and aluminum potassium sulfate dodecahydrate were reacted in a large excess of 99.8% deuterium oxide at pH 4.05 and at reflux for 24 hr with the exclusion of moisture. Examination of the n.m.r. spectrum of the bright yellow crude product indicated that α-proton absorption (4.1-4.3 δ) had essentially disappeared as had the β-proton absorption (3.0-3.2 δ). Relative peak areas obtained from integration of the n.m.r. spectrum revealed an α-carbon-deuterium label of 96% and a β,β-carbon-deuterium label of 98%.

Purification of this compound proved to be difficult. Initial attempts at recrystallization using hot deionized water, as in the DL-[α-$^{2}H_{1}$]phenylalanine case, failed, as did recrystallization attempts from a wide variety of solvents. All precipitates obtained by these attempts gave no n.m.r. spectrum and melted above 300°C

Table 3. Zn(II) vs. Al(III) as the metal chelate in α,β,β-carbon-deuterium labeling of phenylalanine.

Expt. No.	Mode of Catalysis[a]	Catalysis Ratio[b]	pH	Time (hr)	Temp (°C)	% Exchange[c] α	% Exchange[c] β
TSK-107-1	Pyridoxal-Al(III)	5:1:1	6.2	24	105	96	27
TSK-111-1	Pyridoxal-Al(III)	5:1:1	5.5	24	105	85	73
TSK-113-1	Pyridoxal-Al(III)	5:1:1	4.7	24	105	95	88
TSK-115-1	Pyridoxal-Al(III)	5:1:1	4.2	24	105	95	94
TSK-117-1	Pyridoxal-Al(III)	5:1:1	4.2	24	105	92	96
TSK-107-2	Pyridoxal-Zn(II)	5:1:1	6.3	24	105	83	78
TSK-110-1	Pyridoxal-Zn(II)	5:1:1	5.2	24	105	71	33
TSK-114-1	Pyridoxal-Zn(II)	5:1:1	6.0	24	105	83	70
TSK-115-2	Pyridoxal-Zn(II)	5:1:1	6.8	24	105	92	76

[a] Pyridoxal from pyridoxal hydrochloride; Zn(II) from $ZnCl_2$; Al(III) from $AlK(SO_4)_2 \cdot 12H_2O$.

[b] Phenylalanine/pyridoxal/metal.

[c] Per cent exchange determined by n.m.r. integration.

indicating they were inorganic salts. It was felt that ion-exchange might prove to be successful in separating the labeled phenylalanine and the salts.

Two previous attempts to purify phenylalanine via ion-exchange chromatography, one involving elution with 1 N hydrochloric acid (Piez, Tooper, and Fosdick 1952) and another involving elution with 2 N ammonia (Carsten 1952) have been reported.

The residue obtained after evaporation of the deuterium oxide solvent in the preparation of DL-[α,β,β-$^2H_3$]phenylalanine was purified by ion exchange chromatography (see Experimental section). The method of Piez et al. (1952) was used and the yield was 40-45%. Since these yields were decidedly low and the methodology very time-consuming, an alternate method of purification was again sought. Returning to recrystallization techniques, a boiling ethanol-water system allowed a recovery of greater than 90% of the DL-[α,β,β-$^2H_3$]phenylalanine.

Having developed a procedure for labeling the α-carbon of phenylalanine as well as for labeling the α- and β-carbons, a combination of the two methods led to a phenylalanine residue containing deuterium exclusively in the β-position. As anticipated, longer reaction times were needed because of the primary isotope effect; otherwise, no significant changes were required in the experimental procedure. DL-[α,β,β-$^2H_3$]Phenylalanine, pyridoxal

hydrochloride, and potassium aluminum dodecahydrate, in a 5:1:1 ratio, were dissolved in a large excess of deionized water and allowed to incubate at a pH of 9.6 for a period of forty-eight hours. The solvent was removed in vacuo and replaced with a fresh aliquot of deionized water. After adjusting the pH to 9.6, the reaction was allowed to incubate for an additional forty-eight hours. Removing the solvent in vacuo and recrystallizing from boiling ethanol-water yielded the desired DL-[β,β-$^{2}H_{2}$]phenylalanine. Examination of the n.m.r. spectrum revealed a deuterium content of 6% in the α-carbon position and 98% in the β-carbon position.

Procedures have thus been developed for the selective labeling of the amino acid phenylalanine on all or some of the alphatic carbons. While the procedures demonstrate selectivity, their generality must be left to further study. The only attempts to extend these methods were performed using L-tyrosine, but difficulties were encountered due to low solubility in water. Under α-exchange conditions, no exchange was observed; however, under refluxing conditions a perdeuterio analog, DL-[α,β,β,3',5'-$^{2}H_{5}$]tyrosine, was apparently isolated. Analysis of the residue by n.m.r. gave a spectrum containing two sharp singlets, one at 7.05 δ and the other at 4.8 δ. The latter peak was probably caused by the absorption of the HOD proton of the solvent. Thin-layer chromatography of this residue and an authentic

sample of L-tyrosine gave identical R_f values of 0.32 for both samples. Unlike the phenylalanine case; base catalysis was successful in the case of tyrosine. Using sodium acetate, identical results were obtained as just outlined for the pyridoxal catalysis of perdeuterio-tyrosine formation. No further studies were undertaken involving tyrosine and thus no claims are made regarding the generality of the procedures cited herein.

The procedures for the preparation of the deuterium-labeled phenylalanine analogs yielded racemates, and two experimental pathways were undertaken to resolve the enantiomers. The first procedure involved the use of DL-[α-$^2H_1$]phenylalanine in the synthesis of [3-DL-[α-$^2H_1$]-phenylalanine,8-arginine]vasopressin with subsequent separation of diastereomers via partition chromatography (Yamashiro 1964, Yamashiro et al. 1966, Upson 1975). The second method involved preparation of the N-acetyl derivative of the labeled phenylalanine (Greenstein and Winitz 1961, p. 2172) and resolution by the action of hog renal acylase (Greenstein and Winitz 1961, p. 2172).

Enzymatic resolution was accomplished by first converting DL-[α,β,β-$^2H_3$]phenylalanine to N-acetyl-DL-[α,β,β-$^2H_3$]phenylalanine with no loss of deuterium in either position. The N-acylated derivative was then converted to L-[α,β,β-$^2H_3$]phenylalanine by the hog renal acylase procedure.

In order to be used in the solid-phase peptide synthesis of [8-arginine]vasopressin, the labeled phenylalanine residue had to be protected with no appreciable loss of its deuterium label. To this end, DL-[α-$^2H_1$]phenylalanine was N-protected with the t-butyloxycarbonyl group by a slight modification of the method of Schnabel (1967) which allowed the protection of the labeled phenylalanine without prior isolation from the deuterium labeling reaction mixture.

DL-[α-$^2H_1$]Phenylalanine, pyridoxal hydrochloride, and aluminum potassium sulfate dodecahydrate, the residue remaining after the deuterium oxide had been removed in vacuo from the labeling reaction, were dissolved in a 1:1 deionized water/peroxide-free dioxane mixture. Excess hydroxylamine hydrochloride was added to tie up the pyridoxal as a Schiff's base, the pH was adjusted to 10.1, and t-butyloxycarbonyl azide was added. The pH was maintained at 10.1 and the reaction allowed to proceed for a period of 13.5 hours. Workup of the reaction mixture yielded Boc-DL-[α-$^2H_1$]phenylalanine with no detectable loss of the deuterium label. The yield resulting from this method was comparable to that achieved using purified DL-[α-$^2H_1$]phenylalanine.

The solid-phase peptide synthesis (SPPS) of [3-DL-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin was carried out on a Merrifield resin (Merrifield 1963, 1969), a chloromethylated polystyrene resin cross linked with 1%

divinylbenzene containing 0.69 mmole/g of Cl^-. The procedure for this synthesis is summarized in Table 4 and was carried out on an automated Vega Series 95 Synthesizer (Hruby, Barstow, and Linhart 1972). The protected amino acids coupled via dicyclohexylcarbodiimide (DCC) method were used in a three-fold excess; while the protected amino acids coupled via their p-nitrophenyl esters were used in a four-fold excess. Two couplings with a 1.5-fold excess of protected amino acid and DCC were used for each of the amino acid residues except for the deuterium-labeled Boc-DL-[α-$^2H_1$]phenylalanine which was attached through a series of couplings to insure that only a minimum amount would be used. A single coupling step was used to attach the protected amino acids via nitrophenyl ester coupling.

The coupling reactions were monitored for completion using the ninhydrin test method (Kaiser et al. 1970) and were all negative except during the coupling of Boc-DL-[α-$^2H_1$]phenylalanine. Four couplings of this labeled amino acid were performed: two couplings used 0.5 eq. of protected phenylalanine and DCC and two couplings used 0.3 eq. of protected phenylalanine and DCC. After the second coupling about 2% free amine was indicated; 1% was indicated after the third coupling, and the results were negative after the fourth coupling. It is recommended that further attempts at coupling Boc-DL-[α-$^2H_1$]phenylalanine should be done using 1.0 eq. of the protected labeld analog and DCC

Table 4. Procedure utilized in the solid-phase peptide synthesis of [3-DL-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin.

	DCC Coupling			Active Ester Coupling		
Step	Solvent/Reagent	No. of Times	Time (min)	Solvent/Reagent	No. of Times	Time
1	CH_2Cl_2	4	1	CH_2Cl_2	4	1
2	TFA-anisole-CH_2Cl_2	2	6	TFA-anisole-CH_2Cl_2	2	6
3	CH_2Cl_2	3	1	CH_2Cl_2	3	1
4	DIEA-CH_2Cl_2 (7:93)	2	2	DIEA-CH_2Cl_2 (7:93)	2	2
5	CH_2Cl_2	4	1	CH_2Cl_2	4	1
6	Protected residue[a] 1.5 eq in CH_2Cl_2	1	-	Protected residue[c] 4.0 eq in DMF	1	-
7	DCC/CH_2Cl_2[b] 1.5 eq	1	20	DMF	1	x[d]
8	CH_2Cl_2	2	1			
9	Anhydrous EtOH	3	1			
10	CH_2Cl_2	3	1			
11	Protected residue[a] 1.5 eq in CH_2Cl_2	1	-			
12	DCC/CH_2Cl_2[b] 1.5 eq	1	20			
13	CH_2Cl_2	2	1	CH_2Cl_2	2	3
14	Anhydrous EtOH	3	1	Anhydrous EtOH	3	1

[a] N^{α}-Boc amino acids were employed as were Tyr(Bzl), Cys(DMB), and Arg(Tos).

[b] DMF was needed to dissolve N^{α}-Boc-N^{γ}-Tos-arginine.

Table 4.--Continued

[c]Asn-ONp and Gln-ONp.

[d]Reaction time: 18 hrs for Asn-ONp and 20 hrs for Gln-ONp.

and that subsequent couplings use a smaller number of equivalents as is needed to complete the reaction.

The N-terminal Boc-group was removed using the first five steps of the SPPS procedure; at which time H-Cys-(DMB)-Tyr(Bzl)-DL-[α-$^2H_1$]-Phe-Gln-Asn-Cys(DMB)-Pro-Arg(Tos)-Gly-NH_2, the partially protected nonapeptide amide was prepared by ammonolysis (Manning 1970) of the peptide resin ester precursor.

The partially protected peptide amide was deprotected using sodium and liquid ammonia (Sifferd and du Vigneaud 1935) and subsequent oxidative ring formation with potassium ferricyanide (Hope, Murti, and du Vigneaud 1962) produced the desired [3-DL-[α-$^2H_1$]phenylalanine,8-arginine]-vasopressin. After a preliminary desalting with Rexyn 203 (Cl^- cycle), the diastereomers were separated via partition chromatography (Yamashiro 1964, Yamashiro et al. 1966, Upson 1975) of the impure hormone. n-Butanol-ethanol-pyridine-0.1 N acetic acid (4:1:1:7) was used as the partition system and the results are shown in Figure 6. The first peak may be attributed to truncated chains and polymers formed during synthesis; the peak at R_f 0.36 corresponds to the D-isomer, [3-D-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin, and other by-products (Upson 1975), while the peak at R_f 0.17 is that of the L-isomer, [3-L-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin. The fractions corresponding to each isomer were combined and lyophilized

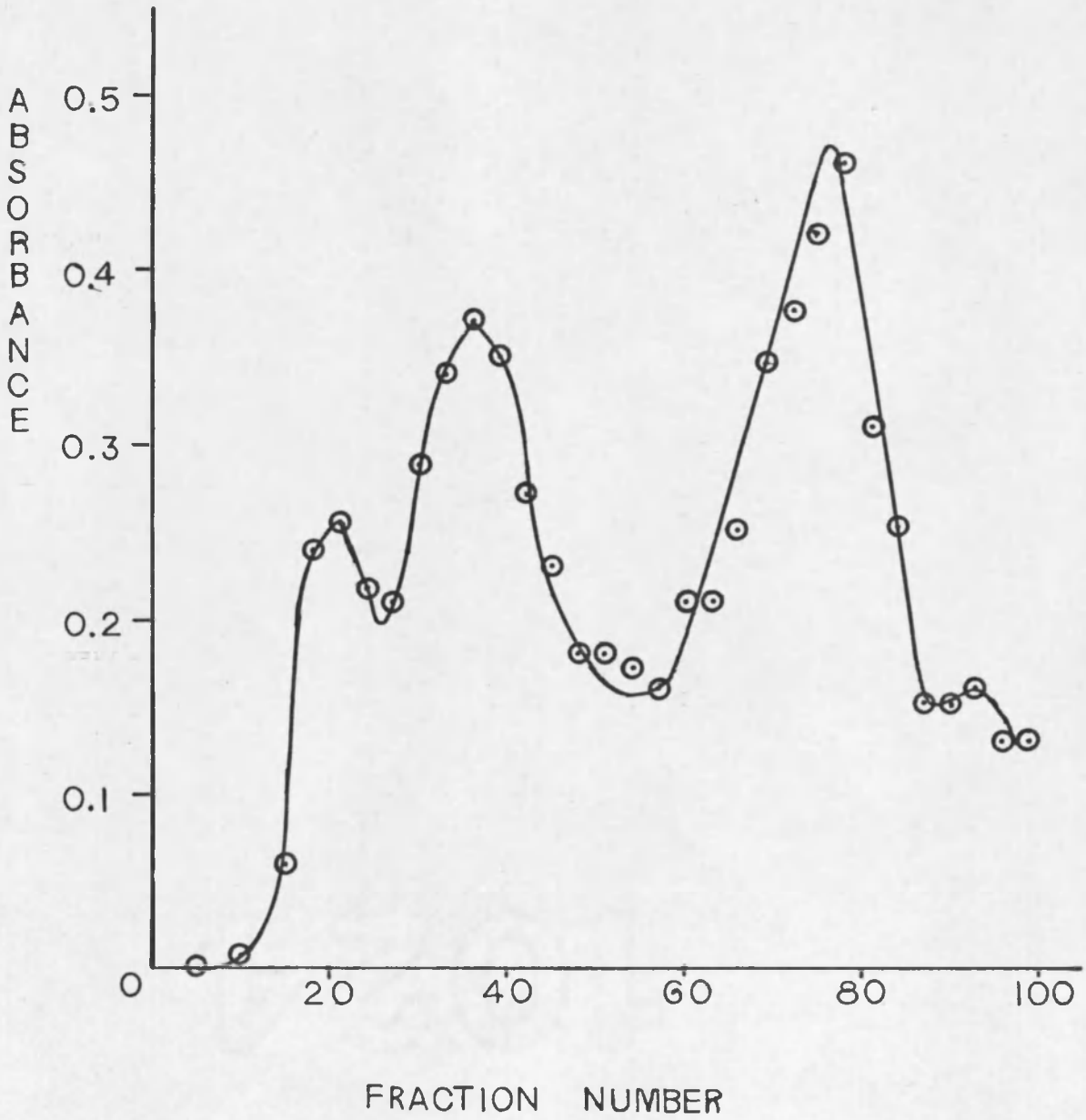


Figure 6. Partition chromatography of [3-DL-[α-$^{2}H_{1}$]phenylalanine,8-arginine]vasopressin.

to dryness and the white powders were further purified by means of gel filtration with 0.2 N acetic acid as the eluant and Sephadex G-25 as the support. The results for the all L-diastereomer are shown in Figure 7.

The final purified deuterium labeled derivatives of [8-arginine]vasopressin were lyophilized to dryness and the white powders were hydrolyzed and amino acid compositions were determined (Spackman, Stein, and Moore 1958) on a Beckman 120C amino acid analyzer. The results are summarized in Table 5.

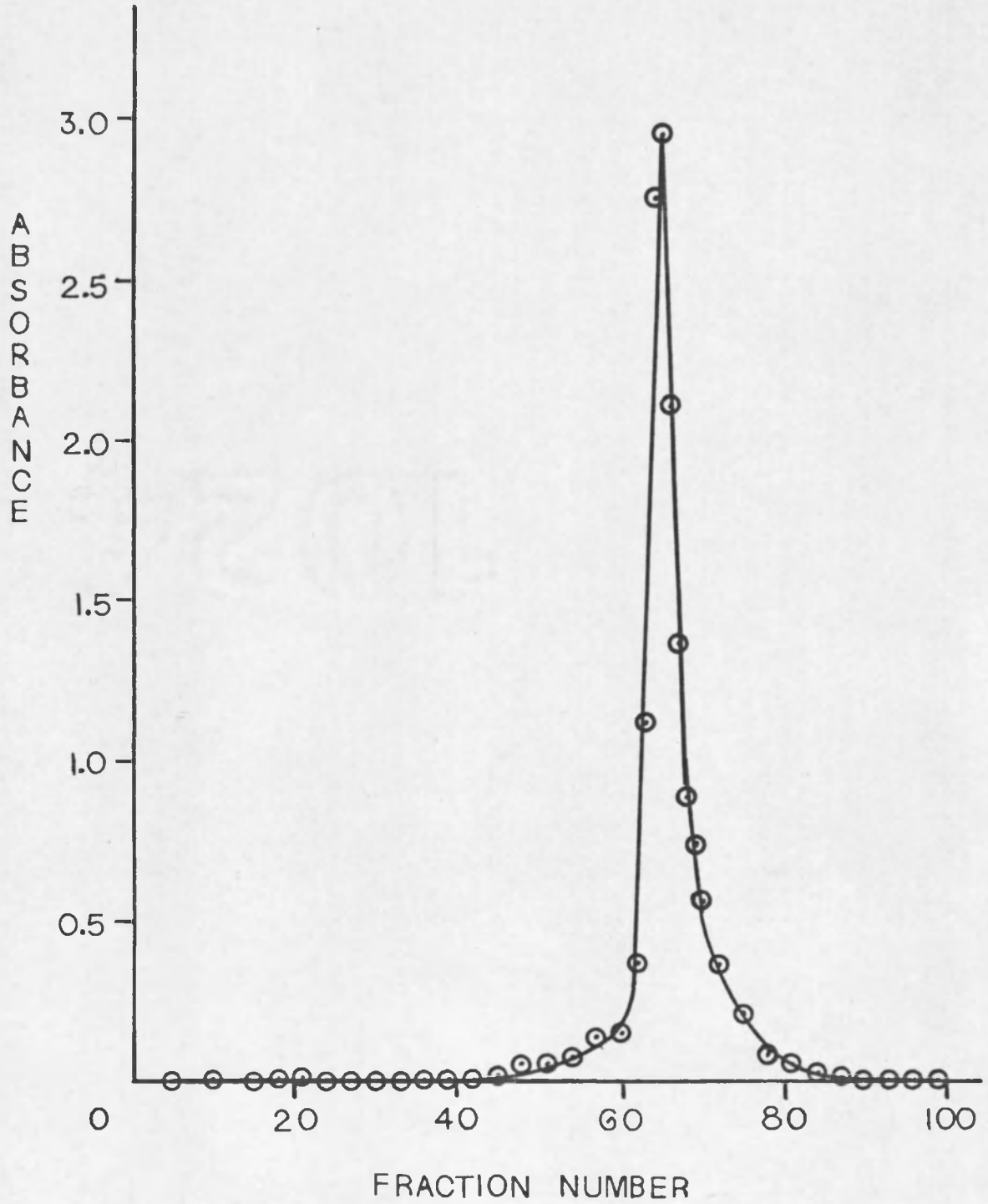


Figure 7. Gel filtration of [3-L-[α-$^{2}H_{1}$]phenylalanine,8-arginine]vasopressin.

Table 5. Amino acid analysis of gel filtration lyophilizates.

Amino acid	[3-D-[α-$^{2}H_{1}$]phenylalanine, 8-arginine]vasopressin plus impurities	[3-L-[α-$^{2}H_{1}$]phenylalanine, 8-arginine]vasopressin
Glycine	1.00	1.00
Arginine	0.90	0.92
Proline	0.81	0.92
Half-cystine	1.38	1.97
Aspartic acid	1.45	1.02
Glutamic acid	1.62	1.07
[α-$^{2}H_{1}$]phenylalanine	1.47	1.05
Tyrosine	0.59	1.00

EXPERIMENTAL

Materials

The n.m.r. spectra were obtained on a Varian Associates T-60 spectrophotometer and except where noted DCl/D_2O was used as the solvent and tetramethylsilane (TMS) as the standard. The solid-phase peptide synthesis of [3-DL-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin was carried out on an automated Vega Series 95 Synthesizer (Hruby et al. 1972). Infrared spectra were recorded using a Perkin-Elmer 237B spectrophotometer by the KBr pellet method. Melting points were carried out on a Thomas-Hoover melting point apparatus and are uncorrected. Thin-layer chromatography (tlc) was performed on Analtech Inc. silica gel G plates using two solvent systems: (A) n-butanol-acetic acid-water (4:1:5) and (B) n-butanol-acetic acid-pyridine-water (15:3:10:12). The amino acid analyses were performed on a Beckman 120C amino acid analyzer after hydrolysis with 6 N HCl (Spackman, Stein, and Moore 1958). Partition chromatography and gel filtration were carried out on Sephadex G-25, 100-200 mesh, block polmerizate. The partition chromatography was followed using the Folin-Lowry reagent (Lowry et al. 1951) on a Bausch and Lomb Spectronic 20. The gel filtration chromatography was followed on a Gilford Spectrophotometer set at 280 nm. A

Copenhagen Radiometer Autoburet was used to monitor and maintain pH during the protection of phenylalanine residues. Optical rotations were performed on an O. C. Rudolph Model 80 polarimeter.

All amino acids except glycine are of the L-configuration unless otherwise noted. Standard abbreviations for amino acids, peptides, and protecting groups are used as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (1972). Additional abbreviations used include: DCC, dicyclohexylcarbodiimide; AVP, [8-arginine]vasopressin; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; DMB, 3,4-dimethylbenzyl; DMF, dimethylformamide; HOAc, acetic acid; and EtOAc, ethyl acetate.

Preparation of DL-[α-$^2H_1$]Phenylalanine Using Pyridoxal-Al(III) Catalysis

L-Phenylalanine (1.00 g, 6.05 mmole), pyridoxal hydrochloride (0.24 g, 1.21 mmole), and aluminum potassium sulfate dodecahydrate (0.57 g, 1.21 mmole) were dissolved in 99.8% deuterium oxide (30 ml). Using solid sodium carbonate, the pH was adjusted to 9.6 and the tightly sealed reaction mixture was incubated for twenty-four hours at room temperature. Using thionyl chloride, the pH was lowered to 1.0; the solvent was removed *in vacuo* and the resulting residue was recrystallized from boiling deionized water. The product, DL-[α-$^2H_1$]phenylalanine, was isolated

as its hydrochloride salt as determined by IR analysis and a positive test with a 1% silver nitrate solution. Purity was established via comparative tlc with authentic L-phenylalanine; single spots, R_f 0.4 and 0.6, where obtained in systems (A) and (B), respectively. Relative peak areas obtained by integration of the n.m.r. spectrum revealed deuterium labeling in the α-position in excess of 95% with no detectable labeling of the β-position. Yield: 0.92 g (91%); mp 225-227°C (lit. mp 271-273°C for the protio analogue, Marvel 1955); n.m.r. (DCl/D_2O) δ: 7.2 (s, 5H), 4.1-4.3 (α-CH, < 5%H), and 3.0-3.2 (β-CH_2, s, 2H); and IR (KBr pellet): 2985 (b), 1735 (s), 1600 (w), 1485 (s), and 1220 (m) cm^{-1}.

Preparation of DL-[α-$^2H_1$]Phenylalanine Using Pyridoxal-Zn(II) Catalysis

L-Phenylalanine (1.65 mg, 1.00 mmole), pyridoxal hydrochloride (40.6 mg, 0.2 mmole), and zinc chloride (26.2 mg, 0.2 mmole) were dissolved in 99.8% deuterium oxide (7.0 ml). After adjusting the pH to 9.6 using solid sodium carbonate, the reaction mixture was refluxed for twenty-four hours. Upon cooling to room temperature, the pH was lowered to 1.5 using thionyl chloride and the solvent removed *in vacuo*. Analysis of the n.m.r. spectrum indicated greater than 95% deuterium labeling in the α-position and no detectable labeling in the β-position. N.m.r. (DCl/D_2O) δ:

7.2 (s, 5H), 4.1-4.3 (α-CH, < 5%H), and 3.0-3.2 (β-CH_2, s, 2H).

Preparation of DL-[α,β,β-$^2H_3$]Phenylalanine Using Pyridoxal-Al(III) Catalysis

L-Phenylalanine (5.00 g, 30.25 mmole), pyridoxal hydrochloride (1.22 g, 6.05 mmole) were dissolved in 99.8% deuterium oxide (150 ml). Using solide sodium carbonate, the pH was adjusted to 4.05 and the reaction mixture refluxed in a system which had been purged using dry nitrogen and equipped with a calcium chloride drying tube to exclude moisture. After twenty-four hours of refluxing at 105°C, nitrogen was slowly passed over the reaction mixture and the solvent allowed to evaporate leaving a yellow residue. The residue was dissolved in a minimal amount of deionized water; the pH was lowered to 1.0 using hydrochloric acid and the solvent removed in vacuo. Ethanol (95%) was added to the resultant residue and the mixture brought to a boil at which time deionized water was added until no more residue appeared to dissolve. The mixture was filtered and anhydrous ethanol (50 ml) was used to wash the residue. The filtrates were combined and reduced in vacuo and the resultant residue was crystallized from hot ethanol-water. Relative peak intensities of the n.m.r spectrum indicated 96% α-deuterium labeling and 98% β-deuterium labeling. Comparative tlc in both systems (A) and (B) gave single spots, R_f values of 0.4 and 0.6, respectively,

identical to authentic L-phenylalanine. Yield: 4.68 g (92%); mp 245-248°C; n.m.r. (DCl/D_2O) δ: 7.3 (s, 5H), 4.1-4.3 (α-CH, < 4%H), and 3.0-3.2 (β-CH_2, < 2%H).

Preparation of DL-[α,β,β-$^2H_2$]Phenylalanine Using Pyridoxal-Al(III) Catalysis

DL-[α,β,β-$^2H_3$]Phenylalanine (1.0 g, 6.05 mmole), pyridoxal hydrochloride (0.24 g, 1.21 mmole), and aluminum potassium sulfate dodecahydrate (0.57 g, 1.21 mmole) were dissolved in deionized water (100 ml). The pH was raised to 9.6 using a 4.0 N NaOH solution; the flask was sealed to the atmosphere and allowed to incubate at room temperature. After forty-eight hours the solvent was removed *in vacuo*, replaced with fresh deionized water (100 ml), and the pH adjusted to 9.6 using 4.0 N NaOH solution. After an additional forty-eight hours of incubation, the pH was lowered to 1.0 using concentrated hydrochloric acid. The solvent was removed *in vacuo* and the resultant residue was recrystallized from boiling ethanol-water. Relative peak areas obtained from the n.m.r. spectrum revealed 6% deuterium remaining in the α-position and 97% deuterium labeling in the β-position. Comparative tlc in both systems (A) and (B) gave single spots, R_f values of 0.4 and 0.6, respectively, identical to authentic L-phenylalanine. Yield: 0.90 g (90%); mp 250-253°C; n.m.r. (DCl/D_2O) δ: 7.4 (s, 5H), 4.1-4.3 (α-CH, 94%H), and 3.0-3.2 (β-CH_2 < 3%H).

Preparation of Boc-DL-[α-$^2H_1$]Phenylalanine

DL-[α-$^2H_1$]Phenylalanine (1.89 g, 9.4 mmole) was dissolved in deionized water (10 ml) and peroxide-free dioxane (10 ml). The pH was raised to 10.1 (Schnabel 1967) with 4.0 N NaOH and tert-butylazidoformate (2.05 g, 10.3 mmole) was added. The pH was monitored and maintained at 10.1 using a Copenhagen Radiometer Autoburet with 4.0 N NaOH. After 13.5 hours, deionized water (50 ml) was added to the reaction mixture and this aqueous solution subsequently extracted with ethyl ether (30 ml, 3x). Solid citric was used to lower the pH to 3.0 and the product extracted from the aqueous solution with ethyl acetate (45 ml, 4x). The combined organic extracts were back extracted with saturated sodium chloride solution and then dried over anhydrous magnesium sulfate.

The magnesium sulfate was filtered off and washed with anhydrous ethyl aceteate (2o ml, 2x). The filtrate and washings were combined and the solvent removed *in vacuo*, leaving a clear colorless oil. Recrystallization from boiling hexane produced white crystals which were dried *in vacuo* over potassium hydroxide. Comparative tlc in both systems (A) and (B) gave single spots, R_f values of 0.85 and 0.72, respectively, identical to authentic Boc-L-phenylalanine. Relative peak areas of the n.m.r. spectrum revealed no detectable protons in the α-position; the reaction, therefore, proceeded with no detectable loss

of the deuterium label. Yield: 1.43 g (57%); mp 77-79°C (Schnabel [1967] 84-86°C); n.m.r. ($CDCl_3$) δ: 7.2 (ØH, s, 5H), 5.0 (NH, b), 3.1 (β-CH_2, s, 2H), and 1.4 ($C(CH_3)_3$, s, 9H); IR (KBr pellet): 3365 (m), 1740 (s), 1630 (s), 1240 (m), 1140 (m), 770 (m), 750 (m), and 700 (m) cm^{-1}.

Preparation of Boc-L-Phenylalanine from an Exchange Reaction Mixture

L-Phenylalanine (500 mg, 3.0 mmole), pyridoxal hydrochloride (1.22 mg, 0.6 mmole), and aluminum potassium sulfate dodecahydrate (287 mg, 0.6 mmole) were dissolved in deionized water (20 ml) and dioxane (20 ml) which was peroxide-free. Hydroxylamine hydrochloride (41 mg, 1.2 mmole) was added and the pH raised to 10.1 (Schnabel 1967) using 4.0 N NaOH; tert-butylazidoformate (485 mg, 3.44 mmole) was added and the pH monitored and maintained at 10.1 with 4.0 N NaOH. After 13.5 hours, the reaction mixture was extracted with ethyl ether (3x) and the pH of the aqueous phase subsequently lowered to 3.0 using solid citric acid. After increasing the volume of the aqueous phase with deionized water (120 ml), the product was extracted from the aqueous phase with ethyl acetate (45 ml, 4x). The organic phase was back-extracted with a saturated sodium chloride solution (100 ml, 3x) and then dried over anhydrous magnesium sulfate.

The magnesium sulfate was removed by filtration and washed with anhydrous ethyl acetate (20 ml, 2x). The

filtrate and washings were combined and the solvent removed *in vacuo*, leaving a yellowish oil which was subsequently recrystallized from boiling hexane. Yield: 651 mg (80%); mp 77-80°C.

Preparation of N-Boc-S-3,4-Dimethylbenzylcysteine from its Dicyclohexylamine Salt

For convenience, the protected amino acid is stored in its salt form. In order to be utilized in the SPPS technique, the protected amino acid must be freed from its salt. N-Boc-S-3,4-Dimethylbenzylcysteinedicyclohexylamine salt (6.25 g, 12 mmole) was partitioned between ethyl acetate (100 ml) and aqueous citric acid (20%, 100 ml). The system was shaken vigorously for a period of twenty minutes before allowing the layers to equilibrate for several hours. The organic layer was separated and the aqueous layer further extracted with ethyl acetate (50 ml, 2x). The combined organic fractions were washed with saturated sodium chloride (100 ml, 3x). After drying over anhydrous magnesium sulfate for a period of four to five hours, the drying agent was removed via suction filtration, and the solvent removed *in vacuo*. The colorless oil was allowed to dry *in vacuo* over potassium hydroxide.

Synthesis of the Protected Nonapeptide Resin; Cys(DMB)-Try(Bzl)-DL-[α-$^2H_1$]Phe-Gln-Asn-Cys(DMB)-Pro-Arg(Tos)-Gly-Resin

Boc-Glycine resin (CS-VI-30), which was found to contain 0.36 mmole of the amino acid per gram of resin by a modified (Ehler 1972) aldimine test (Esko, Karlsson, and Poroth 1968), was prepared from chloromethylated (0.69 mmole/mg Cl^-) polystyrene crosslinked with 1% divinylbenzene. For the synthesis 2.77 g (1.0 mmole) of the Boc-glycine resin was used. Deprotecting, neutralizing, and coupling with each new protected amino acid were performed as shown in Table 4. All steps were carried out with 34 ml aliquots. The modified procedure for the coupling of asparagine and glutamine nitrophenyl esters is also shown in Table 4.

The coupling of the deuterium labeled residue, DL-[α-$^2H_1$]phenylalanine, was modified in order to insure complete coupling using only a minimal mount of labeled residue. Deprotection and neturalization were accomplished in steps 1-5; however, steps 6 and 7 were altered by using 0.5 equivalents of the protected labeled amino acid and DCC. After the coupling reaction was completed and the resin had been washed (steps 8-10), the modified coupling procedure was repeated again using 0.5 equivalents of both the labeled protected amino acid and DCC. A ninhydrin test (Kaiser et al. 1970) indicated the presence of about 2% free amine. A third and fourth coupling were performed using 0.3

mmole protected amino acid and DCC; the ninhydrin test indicated 1% free amine after the third coupling and was negative after the fourth coupling.

After coupling the final residue, the terminal Boc-group was removed using steps 1-5. The resin was filtered, dried, and found to have had a weight gain of 1.25 g or 86% of the theoretical value.

Synthesis of [3-DL-[α-$^{2}H_{1}$]Phenylalanine,8-arginine]vasopressin and Resolution of Diastereomers

The protected nonapeptide, Cys(DMB)-Tyr(Bzl)-DL-[α-$^{2}H_{1}$]Phe-Gln-Asn-Cys(DNB)-Pro-Arg(Tos)-Gly-NH_2, was cleaved from the resin by ammonolysis. Liquid ammonia was distilled over soidum and used to prepare 150 ml of saturated ammonia/anhydrous methanol solution in which the protected nonapeptide resin was suspended at 0°C. The mixture was stirred for a period of 162 hours (only 72 hours are necessary) in a tightly sealed vessel. The solvent was removed in vacuo and the residue dissolved in nitrogen-treated dimethylformamide (DMF, 50 ml) and stirred for a period of two hours. The resin was removed by filtration, washed with DMF (15 ml, 2x), and then placed in fresh DMF (50 ml) and incubated at 70°C for an additional two hours. The resin was removed by filtration and washed with additional DMF (20 ml, 3x). The combined washings and filtrates were reduced in vacuo and the resultant oil

recrystallized from DMF-deionized water yielding two crops of white crystals. Yield: 373 mg (25%); a further 0.32 g of oil was isolated from the mother liquor.

Deprotection of the partially protected nonapeptide amide was carried out on a 0.2 mmole scale (314 mg) using a solution of freshly distilled ammonia and sodium metal Sifferd and du Vigneaud 1935) until the blue color was found to persist for ninety seconds. The ammonia was removed by evaporation under a nitrogen atmosphere and by lyophilization. The nonapeptide amide lyophilizate was dissolved in 0.1% acetic acid (550 ml) under a nitrogen atmosphere which was maintained until the oxidation was completed (Walti and Hope 1973). The pH was adjusted to 8.5 using 3.0 N NH_4OH and the oxidative cyclization was accomplished with 0.010 N potassium ferricyanide solution (50 ml, Hope et al. 1962). After thirty minutes, the pH of the solution was lowered to 5.0 using glacial acetic acid and 4.0 ml (settled volume) of Rexyn 203 (Cl^- cycle) were added. The solution was stirred for an additional twenty minutes, at which time the nitrogen atmosphere was removed and the ion-exchange resin was removed by filtration. A 10% aqueous acetic acid solution was used to wash the resein (33 ml, 3x); the washings and filtrate were combined and reduced *in vacuo* to an approximate volume of 100 ml at which point the solution was lyophilized to yielding a white powder.

A Sephadex G-25 (100-200 mesh, block polymerizate) column (2.85 x 64 cm) was equilibrated with the lower and upper phases of a n-butanol-ethanol-pyridine-0.1 N acetic acid (4:1:1:7) solvent system, according to the method of Yamashiro (Yamashiro 1964, Yamashiro et al. 1966). The white powder was dissolved in 4 ml of the upper layer and one ml of the lower layer of the solvent system and applied to the column. Ninety-nine 5.8 ml fractions were collected and analyzed by the Folin-Lowry method (Lowry et al. 1951) with the results being shown in Figure 6. Three peaks were obtained: one corresponding to polymers, truncated peptides, and other by-products; one corresponding to contaminated product; and one to pure product. By analogy to previous work in this lab (Spatola et al. 1974, Linn 1974, Upson 1975, Upson and Hruby 1976) the peaks with R_f values of 0.36 and 0.17 should be assigned to [3-D-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin and various by-products and [3-L-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin respectively. Each peak was isolated and lyophilized yielding 66 mg and 81 mg, respectively. Each product was further purified by separate gel filtration on a Sephadex G-25 (200-270 mesh, block polymerizate) column using 0.2 N acetic acid as the eluate. Final yields of the purified products were 64 mg of the L-diastereomer and because of a malfunction of the fraction collector 24 mg of an impure D-isomer. Bioassay of the pure L-isomer gave the following

results using the milk ejecting assay (Hruby and Hadley 1975): 106 ± 20 U/mg (lit: 116 ± 18 U/mg for all the protio derivative, Hruby and Hadley 1975), identical to that previously published.

Thin layer chromatography (tlc) in solvent system (A), upper only; and (B) gave single spots identical to an authentic sample of [8-arginine]vasopressin (Linn 1974). After hydrolysis of each isomer for thirty-six hours at 110°C, an amino acid analysis was performed with the results summarized in Table 5. The D-isomer was repartitioned as previously described with the peak of R_f 0.36 lyophilized to yield 9.4 mg of [3-D-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin.

Preparation of N-Acetyl-DL-[α,β,β-$^2H_3$]-phenylalanine

DL-[α,β,β-$^2H_3$]Phenylalanine (2.52 g, 15.0 mmole) was dissolved in freshly prepared anhydrous acetic acid (150 ml). The solution was brought to a boil and allowed to reflux for a period of five minutes. The slightly turbid solution was allowed to cool for two minutes and 2.5 ml of redistilled anhydrous acetic anhydride (1.8-fold excess) were added. The reaction mixture was again brought to a boil and after refluxing for a period of three minutes, the solution was allowed to cool to room temperature.

The reaction mixture was stripped *in vacuo* at 40°C and the faint yellow oil was triturated with deionized water (50 ml) and then removed at 40°C *in vacuo*. Two further triturations yielded white crystals which were further triturated with deionized water (50 ml, 2x). The resultant crystals were recrystallized from hot deionized water and shown by n.m.r. analysis to be N-acetyl-DL-[α,β,β-$^2H_3$]-phenylalanine with no detectable loss of deuterium in either the α- or β-positions. Yield: 2.04 g (65%); mp 143.5-144.5°C (lit.: 146°C for all the protio derivatives; Greenstein and Winitz 1961, p. 2173); n.m.r. (D_2O) δ: 7.2 (ØH, s, 5H), and 1.9 (CH_3, s, 3H).

Preparation of L-[α,β,β-$^2H_3$]Phenylalanine by Enzymatic Action on N-Acetyl-DL-[α,β,β-$^2H_3$]Phenylalanine

N-Acetyl-DL-[α,β,β-$^2H_3$]Phenylalanine (2.00 g, 9.65 mmole) was dissolved in deionized water (80 ml). Using a dilute solution of LiOH, the pH was adjusted to 7.2 at which time hog renal acylase (40 mg, 1210 units/mg at 25°C activity) was added and the reaction mixture incubated at 38°C for a period of forty-four hours. More hog renal acylase (24 mg) was added, the pH checked and found to be 7.1, and the reaction mixture was allowed to incubate for an additional forty-seven hours at 38°C.

The pH was lowered to 5.0 using redistilled glacial acetic acid and the reaction mixture filtered. Deionized

water (25 ml, 2x) was used to ensure complete transfer and to wash the enzyme and the combined filtrate and washings were concentrated *in vacuo* to a volume of approximately 25 ml and refrigerated. Crystals so obtained were collected and washed with cold deionized water. Comparative tlc in both systems (A) and (B) gave single spots, R_f values of 0.4 and 0.6, respectively, identical to that of authentic L-phenylalanine. Yield: 0.59 g (72%); mp 245-246°C; $[\alpha]_D^{24}$ -32.9°. An authentic sample of L-phenylalanine gave an $[\alpha]_D^{24}$ -34.1° (lit. $[\alpha]_D^{24}$ -34.5°, Greenstein and Winitz 1961); n.m.r. (DCl/D_2O) δ: 7.3 (ØH, s).

Preparation of Hydroxide Ion-Exchange Resin

Dowex 1X-8, 20-50 mesh, chloride form, was used to prepare 300 ml settled volume of hydroxide resin. The resin capacity was 1.4 meq/settled ml, providing a column capacity of 420 milliequivalents. The resin was first treated with deionized water and the slurry decanted. This procedure was repeated three times to remove the fines and then the column was packed using deionized water. Hydrochloric acid (1.0 N, 100 ml) was eluted through the column followed by the elution of 1.0 N sodium hydroxide. This procedure was repeated three times to remove the soluble impurities. The column was eluted with 2.0 N sodium hydroxide until the eluate gave a negative test with a 1%

silver nitrate solution. Deionized water was then eluted through the column until the eluate was neutral.

Preparation of a Bicarbonate Ion-Exchange Resin

Dowex 1X-8, 20-50 mesh, chloride form resin (340 ml settled volume), was washed with deionized water and the slurry decanted. The resin was washed three more times in this manner to remove the fines. Four alternate washings of the resin, first with 1.0 N hydrochloric acid and then with 1.0 N sodium hydroxide, were performed to remove any soluble impurities. The resin was then slowly stirred with 400 ml aliquots of saturated sodium bicarbonate solution for a period of five hours. The slurry was allowed to gravity filter and the resin was transferred back to a flask and a fresh aliquot of saturated sodium bicarbonate solution was added. This process was continued until no chloride ions could be detected in the wash using a 1% silver nitrate test solution. The resin was then washed with deionized water until the washings were neutral.

Ion-Exchange Chromatography of DL-[α,β,β-$^2H_3$]Phenylalanine

The residue obtained after evaporating away the deuterium oxide under nitrogen in the preparation of DL-[α,β,β-$^2H_3$]phenylalanine theoretically contained 2.03 g of the deuterated analog (12.1 mmole), 0.50 g of pyridoxal hydrochloride (2.4 mmole), 1.14 g of aluminum potassium

sulfate dodecahydrate (2.4 mmole), and the salt that formed in adjusting the pH. The residue was dissolved in deionized water (150 ml) and the pH adjusted to 8.0 using 2.0 N sodium hydroxide. A brownish residue which would not dissolve was filtered off and the filtrate was transferred to the hydroxide column and eluted at a rate of 0.2 ml/min. Deionized water was used to elute the cations and neutral species present; 1500 ml of 1.0 N hydrochloric acid were then used to elute the DL-[α,β,β-$^2H_3$]phenylalanine and other anions present. This eluate was reduced *in vacuo* and the resultant residue was found to weigh 2.51 g. The bicarbonate residue was placed in a large glass fritted funnel and the 2.51 g of residue dissolved in 50 ml of 1.0 N hydrochloric acid and allowed to gravity filter through the resin. After washing the resin with 100 ml of deionized water, the combined filtrate and washings were removed *in vacuo* and the resultant residue was found to weigh 0.95 g. Further washings of the resin produced no further product. The crude product was recrystallized from hot deionized water. Yield: 0.82 g (40%).

LIST OF REFERENCES

Abbott, Edwin H., and Arthur E. Martell. J. Amer. Chem. Soc., 91, 6931 (1969).

Bovey, F. A. In Chemistry and Biology of Peptides, J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Michigan, 1972, p. 3.

Brewster, A. I. Richard, and Victor J. Hruby. Proc. Natl. Acad. Sci. U.S.A., 70, 3806 (1973).

Brewster, A. I. Richard, Victor J. Hruby, Jay A. Glasel, and A. E. Tonelli. Biochemistry, 12, 5294 (1973).

Brewster, A. I. Richard, Victor J. Hruby, Arno F. Spatola, and F. A. Bovey. Biochemistry, 12, 1643 (1973).

Cammarata, P. S., and Phillip P. Cohen. J. Biol. Chem., 187, 439 (1950).

Carsten, Mary E. J. Amer. Chem. Soc., 74, 5954 (1952).

Cohen, Jack S., Marvin Feil, and Irwin M. Chaiken. Biochim. Biophys. Acta, 236, 468 (1971).

Cohen, Theodore, and Il Hwan Song. J. Amer. Chem. Soc., 87, 3780 (1965).

Crespi, H. L., U. Smith, L. Gajda, T. Tisue, and R. M. Ammeraal. Biochim. Biophys. Acta, 256, 611 (1972).

Ehler, Kenneth W. Ph.D. dissertation, University of Arizona, 1972.

Esko, K., S. Karlsson, and J. Poroth. Acta. Chem. Scand., 22, 3342 (1968).

Glasel, Jay A., Victor J. Hruby, Jeffery F. McKelvy, and Arno F. Spatola. J. Mol. Biol., 79, 555 (1973).

Greenstein, Jesse P., and Milton Winitz. Chemistry of the Amino Acids. John Wiley and Sons, New York, 1961. 1, pp. 593-610, 3, pp. 2172-2174.

Hanson, K. R., R. H. Whightman, J. Staunton, and A. R. Battersby. J. Chem. Soc. D, 4, 185 (1971).

Hird, F. J. R., and E. V. Roswell. Nature, 166, 517 (1950).

Hope, Derek B., V. V. S. Murti, and Vincent du Vigneaud. J. Biol. Chem., 237, 1563 (1962).

Hruby, Victor J. In Structure Activity Relationships of Protein and Peptide Hormones, M. Margoulies and F. C. Greenwood, Ed., Excerpta Medica, Amsterdam, 1972, p. 444.

Hruby, Victor J. In Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, B. Weinstein, Ed., Marcel Dekker, New York, 1974, 3, pp. 1-188.

Hruby, Victor J., Leon Barstow, and Theo Linhart. Anal. Chem., 44, 343 (1972).

Hruby, Victor J., and Mac E. Hadley. In Peptides: Chemistry, Structure and Biology, R. Walter and J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Michigan, 1975, pp. 729-736.

Ife, R., and E. Haslam. J. Chem. Soc., C, 16, 2818 (1971).

IUPAC-IUB Commission on Biochemical Nomenclature. J. Biol. Chem., 247, 977 (1972).

Jardetzky, Oleg, H. Thielmann, Y. Arata, J. L. Markley, and M. N. Williams. Cold Spring Harbor Symp. Quant. Biol., 36, 257 (1971).

Jardetzky, Oleg, and Norma G. Wade-Jardetzky. Ann. Rev. Biochem., 40, 605 (1971).

Johns, R. B., and D. J. Whelan. Aust. J. Chem., 19, 2143 (1966).

Junk, Gregor, and Harry Svec. J. Amer. Chem. Soc., 85, 839 (1963).

Junk, Gregor, and Harry Svec. J. Org. Chem., 29, 944 (1964).

Kaiser, E., R. L. Colescott, C. D. Bossinger, and P. I. Cook. Anal. Biochem., 34, 505 (1970).

Katz, J. J., and H. L. Crespi. Pure Appl. Chem., 32, 221 (1972).

Kirby, G. W., and J. Michael. J. Chem. Soc. D, 4, 187 (1971).

Kopple, Kenneth D., Anita Go, Ralph H. Logan, Jr., and Jaroslav Savrda. J. Amer. Chem. Soc., 94, 973 (1972).

Linn, David. Ph.D. dissertation, University of Arizona, 1974.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. J. Biol. Chem., 193, 265 (1951).

Manning, James M. J. Amer. Chem. Soc., 92, 7449 (1970).

Marvel, Carl S. Organic Synthesis, Collect. Vol. 3, E. C. Horning, Ed., Wiley, New York, 1955, pp. 705-708.

Merrifield, R. B. J. Amer. Chem. Soc., 85, 2149 (1963).

Marrifield, R. B. Adv. Enzymol., 32, 221 (1969).

Metzler, David E., Miyoshi Ikawa, and Esmond Snell. J. Amer. Chem. Soc., 76, 648 (1954).

Metzler, David E., and Esmond Snell. J. Amer. Chem. Soc., 74, 979 (1952).

Piez, K. A., E. B. Tooper, and L. S. Fosdick. J. Biol. Chem., 194, 669 (1952).

Schnabel, Eugen. Justus Lieb. Ann. Chem., 702, 188 (1967).

Sifferd, Robert, and Vincent du Vigneaud. J. Biol. Chem., 108, 753 (1935).

Spackman, Darrel H., William H. Stein, and Stanford Moore. Anal. Chem., 30, 1190 (1958).

Spatola, Arno F., Dennis Cornelius, Victor J. Hruby, and A. T. Blomquist. J. Org. Chem., 39, 2207 (1974).

Suzuki, Shigeko, Taito Ohshima, Nobuo Tamiya, Kunio Fukushima, Takehiko Shimanouchi, and Sanichiro Mizushima. Spectrochim Acta, 15, 969 (1959).

Suzuki, Shigeko, Takehiko Shimanouchi, and Masamichi Tsubori. Spectrochim Acta, 19, 1195 (1963).

Tamiya, Nobuo, and Taito Ohshima. J. Biochem., Tokyo, 51, 78 (1962).

Thanassi, John W. J. Org. Chem., 36, 3019 (1971).

Upson, Donald A. Ph.D. dissertation, University of Arizona, 1975.

Upson, Donald A., and Victor J. Hruby. J. Org. Chem., 41, 1353 (1976).

Walti, M., and D. B. Hope. Experientia, 29, 389 (1973).

Yamashiro, Donald. Nature, 201, 76 (1964).

Yamashiro, Donald, Dieter Gillessen, and Vincent du Vigneaud. J. Amer. Chem. Soc., 88, 1310 (1966).