A POTENTIALLY GENERAL SYNTHESIS OF HIGH SPECIFIC ACTIVITY SPECIFICALLY LABELLED TRITIATED PEPTIDES: SYNTHESIS OF [D-3-(2-NAPHTHYL)-[2,3-^{3}H]ALANINE^6]LHRH*

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SUMMARY

High specific activity (43.7 Ci/mmol) D-3-(2-naphthyl)-[2,3,-^{3}H]alanine (7) was obtained by reduction of methyl 2-N-acetylamino-3-(2-naphthyl)acrylate (5) with carrier free tritium in the presence of $(Ph_3P)_3RhCl$ followed by enzymatic resolution and hydrolysis. Reductions of 5 and other substrates catalyzed by Pd/C afforded products of low to medium specific activity (0.1 - 17 Ci/mmol).

A synthetic approach which maximizes the specific activity, guarantees specificity of label, and may be applied toward the preparation of any tritiated (or ^{14}C) peptide is suggested.

The synthesis of the decapeptide [D-3-(2-naphthyl)-[2,3-^{3}H]alanine^6]LHRH (12) at 45.7 Ci/mmol, using this methodology, is described.

Key Words: Tritiated peptides, homogenous catalysis, LHRH.

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INTRODUCTION

The decapeptide [D-3-(2-naphthyl)-alanine[6]]LHRH* is an extremely potent LHRH agonist which exhibits varied and important pharmacological properties.[1] A high specific activity tritiated analogue of this peptide, 12, was required for metabolism studies and radioimmunoassay development. A major constraint in the synthesis of 12 was the requirement that the label be associated exclusively with the D-NAL residue. The metabolic fate of this unnatural amino acid inresidue. The metabolic fate of this unnatural amino acid in the critical 6-position[1,2] could then be determined. Early tritium labelling of peptides utilized the Wilzbach method[3] which affords randomly labelled products of very low specific activity.[4,5,6] More recent exchange methods offer much greater complexity but only marginally improved results[7].

A more refined approach was later adopted by some workers in which a peptide containing either an unsaturated[6] or halogenated[8-11] residue was reduced with tritium gas to a desired labelled peptide. This method affords higher specific activities and greater specificity of labelling. However, labelling methods which depend on catalytic reduction (with tritium gas) of an amino acid precursor already contained in the fully elaborated peptide suffer from three major deficiencies.

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*The residue 3-(2-naphthyl)-alanine = NAL.

First of all, total specificity of labelling cannot be guaranteed. Any aromatic amino acids in the system will almost certainly incorporate tritium to some extent by exchange.[9,10]

Secondly, it is impossible to predict, with any degree of certainty, the specific activity of the final product. Many examples exist in which reduction of, ostensibly, similar precursors afford products having vastly different specific activities.[9a,11]

Finally, if high specific activity is desired, labelling methodology has to be optimized using scarce peptide precursors which are available only through laborious syntheses.

We report here our success in circumventing all of those potential problems by concentrating our efforts, first, on the high specific activity synthesis of the desired labelled residue, [2,3-^{3}H]NAL, and then using this material (without dilution with carrier) to prepare 12 by standard methods.*[5,12]

RESULTS AND DISCUSSION

Our initial attempts to prepare high specific activity 6 centered around the reductive dehalogenation of various

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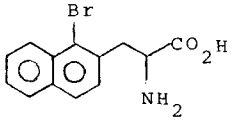
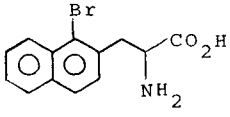
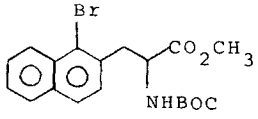
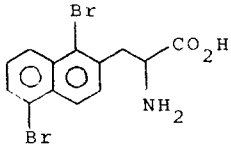
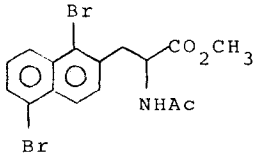
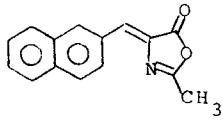
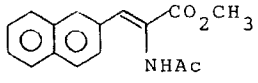
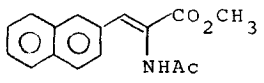
*A similar approach was employed in references 5 and 12. However, these workers did not optimize conditions to get maximum specific activity and labelled amino acid was diluted, affording a low specific activity peptide as the final product.

TABLE 1

TRITIUM REDUCTION OF NAL PRECURSORS

Run	Substrate	Catalyst-Solvent[a]	Crude Incorp. mCi[b]	^{3}H-Product(%)[c]	Specific Activity (Ci/mmol)
1		Pd/C-MeOH	42	100	1.3
2		Pd/C-DMF	115	100	3.6
3		Pd/C-EtOAc	1,000	100	11.7
4		Pd/C-DMA	0.2	100	0.01
5		Pd/C-DMA	145	100	12.5
6		Pd/C-EtOAc	330	19	17.0
7		Pd/C-THF	650	88	9.0
8		$(Ph_3P)_3RhCl$-EtOAc/PhH	2,200	80	43.7

a) DMF = N,N-dimethylformamide; DMA = N,N-dimethylacetamide

b) Radioactivity in the crude reaction mixture after removal of labile tritium

c) Refers to corresponding reduced material

bromo-NAL derivatives. The results of these experiments, summarized in Table 1, clearly emphasize the problems often associated with tritium reduction.

In all reductions the bromo-NAL substrates afforded the expected debrominated compounds as the sole product. However, the specific activity of these products was quite low (0.01 - 12.5 Ci/mmol) in light of the high chemical yields. Reductions of mono and dibromo unprotected compounds in both protic and aprotic solvent gave similar results (entries 1,2 and 4). This was surprising in that a reported reduction of 3-iodotyrosine in MeOH-KOH (a clearly unfavorable medium) afforded [3-^{3}H]tyrosine at 14 Ci/mmol.[13]

Protection of both the amino and carboxyl functions resulted in marked improvement in specific activity to 12 Ci/mmol (entry 3). But reduction of a similarly protected dibromo substrate afforded no additional increase (entry 5).

Some additional improvement in specific activity to 17 Ci/mmol was realized upon Pd/C catalyzed reduction of an unsaturated azlactone[13,14a,b] (entry 8). However, we could effect no further increase in specific activity despite the fact that great care was taken to remove moisture and hydrogen from the catalyst surface and to pre-equilibrate the catalyst with excess tritium prior to reduction in all experiments. Even palladium black, generated in situ from palladium oxide and tritium, offered no advantage to commercial Pd/C. These results suggested that hydrogen-tritium exchange was taking

place on the catalyst surface prior to reduction. Although the source of this hydrogen is not clear, we believe that it is not molecular hydrogen.

If this analysis is correct then a reduction catalyzed by a homogenous catalyst, which coordinates only with molecular hydrogen, should be uneffected by extranious proton sources, regardless of their nature. When $(Ph_3P)_3RhCl$ was dissolved in ethyl acetate/benzene (on a vacuum line) the solution retained its original bright red color until tritium was admitted. At that point the color of the solution changed to yellow, indicating the formation of the tritium containing octahedral rhodium complex. And finally, reduction of 5 under these conditions did, indeed, give product having very high specific activity. After purification 6 was obtained at 43.7 Ci/mmol as shown in Scheme 1.*

Enzymatic resolution of 6 with subtilisin[17] was followed by isolation of the desired pure D-N-acetylmethyl ester 6a using only a standard aqueous work-up. Hydrolysis of 6a with 6 N HCl afforded a quantitative yield of pure D-[2,3-^{3}H]NAL (7). The optical purity of 7 was verified by radiochromatographic analysis of the diastereomeric amides 13a and 14a of 1-phenethylamine whose synthesis is shown in Scheme 2.

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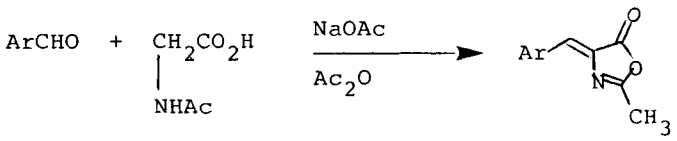
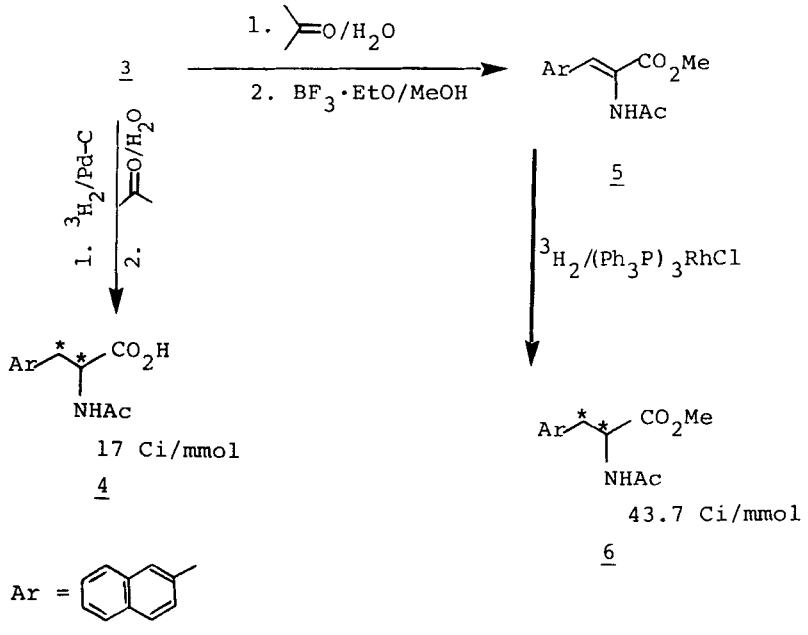
*The use of chiral homogenous catalysts to reduce acrylic and dehydroamino acids has been described by Knowles[15,16]. The use of $(Ph_3P)_3RhCl$ in the synthesis of tritiated amino acids has not, to our knowledge, been previously reported.

SYNTHESIS OF HIGH SPECIFIC ACTIVITY

DL-N-AcetylNAL-^{3}H Methyl Ester 6

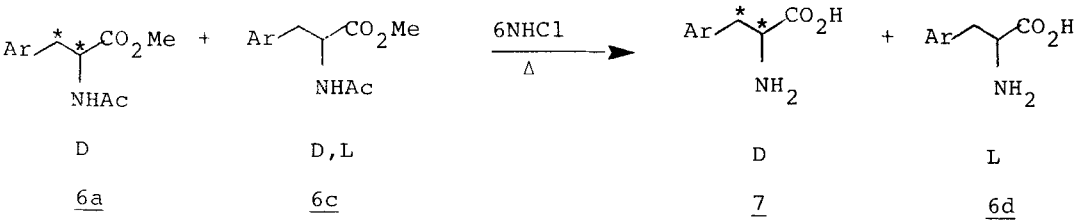


1 2 3

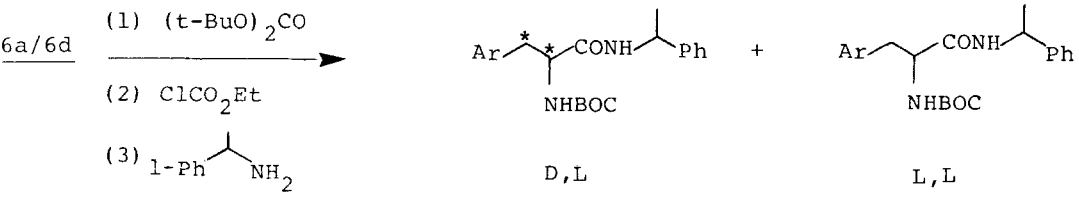


SCHEME 1

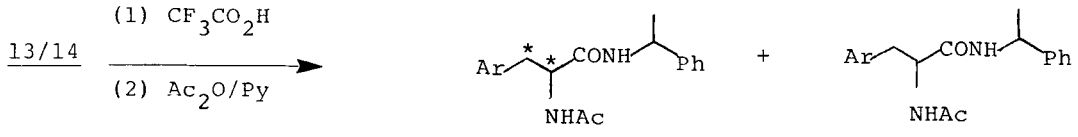
DEMONSTRATION OF OPTICAL PURITY OF D-NAL-^{3}H (6a)



D 6a | D,L 6c | D 7 | L 6d



D,L 13 | L,L 14



D,L 13a | L,L 14a

SCHEME 2

Cold D,L-N-acetylmethyl ester 6c was mixed with a small amount of the labelled D-compound 6a, and the mixture was hydrolyzed with 6N HCl. The amino acids thus obtained were protected as the t-Boc derivatives and condensed with l-phenethylamine to give 13 and 14. The t-Boc group was removed with trifluoroacetic acid and the free amino function was acetylated to pure 13a and 14a.

It was necessary to use N-Boc protection when initially forming the amides 13 and 14 to prevent racemization. The N-acetyl amides 13a and 14a were ultimately desired since these compounds were better separated by tlc. Since only D-amino acid in the DL-mixture was labelled then (if 6a was optically pure) only the D,l-diasteromer 13a should contain tritium. Tlc separation of 13a and 14a [(SiO_2), benzene/ethyl acetate

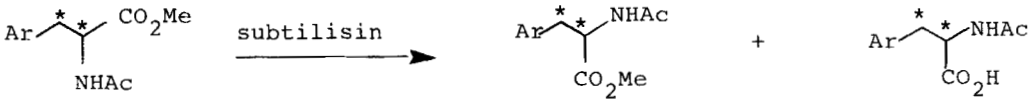
(1:1) developed 3 times] followed by scanning showed that radioactivity was, indeed, associated exclusively with 13.*

Having demonstrated the optical purity of 7 we proceeded with the synthesis of 12 using standard methodology as shown in Scheme 3.

Protection of 7 as the N-Boc derivative 8 and carboxyl activation with ethylchloroformate was followed by coupling with the tetrapeptide 9. The labelled pentapeptide 10 was obtained in 53% yield after column chromatography [(Sephadex

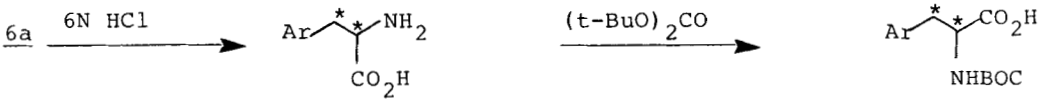
*The relative chromatographic mobilities of 13a and 14a were previously established after synthesis of authentic standards according to Scheme 3 using pure D and L-NAL.

SYNTHESIS OF (D-[2,3-^{3}H]NAL^6)LHRH (12)



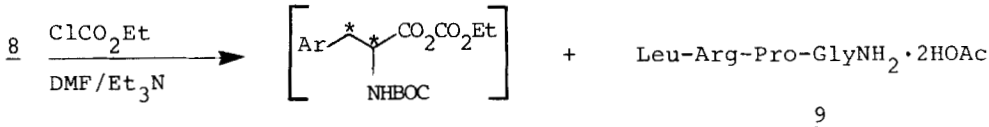
D,l — D — L

6 — 6a — 6b

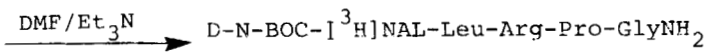


D — D

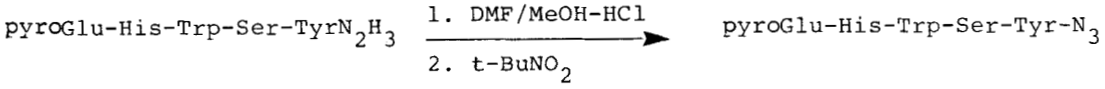
7 — 8



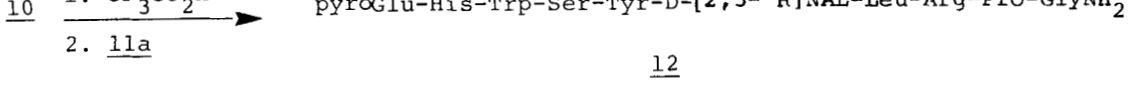
9



10

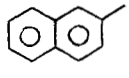


11 — 11a



12

Ar =



SCHEME 3

LH-20), 0-20% MeOH-CH_2Cl_2 gradient]. After removal of the N-Boc group 10 was condensed with the pentapeptide azide 11a.

Preliminary purification of this reaction mixture on a CM-Sephadex C-25 column (0.005M NH_4OAc, pH 4.5 to 0.5M NH_4OAc, pH 7 gradient) followed by HPLC purification [Lichrosorb-ODS, 5 micron; 0.03 M NH_4OAc pH 4.5-acetonitrile, 60:40)] afforded pure [D-[2,3-^{3}H]NAL^6]LHRH, 12, having a specific activity of 45.7 Ci/mmol.*

In conclusion, we feel that the approach presented here is the method of choice for the synthesis of high specific activity, specifically labelled peptides for the following reasons:

1. Labelling methodology can be optimized for any amino acid residue of interest.
2. Specificity of labelling in the peptide is guaranteed.
3. A single synthesis of a desired labelled amino acid can be used to prepare a variety of peptides containing that residue.

EXPERIMENTAL

Carrier free tritium gas was purchased from the Oak Ridge National Laboratory. Reagent grade solvents were dried over 3A

*The slight discrepancy between the specific activities of 6a (43.7 Ci/mmol) and 12 (45.7 Ci/mmol) are, we believe, within experimental error.

molecular sieves and used without purification. Melting points are uncorrected. Radiochemical purity was determined by radio-tlc using a Berthold model 2760 Radiochromatography Scanner. Radioassays were obtained using a Packard Tri-Carb Model 574 Scintillation Counter. Proton nmr spectra and mass spectra were recorded on a Varian EM-390 and Varian 112 spectrometers, respectively. Chemical shifts are reported in δ units (ppm downfield from TMS). Specific activities were determined by calculation of the sample concentration from its UV spectrum and directly assaying the UV solution. UV spectra were obtained on a Carey Model 14 spectrophotometer. Reverse phase tlc was performed on Whatman KC-18F plates. HPLC was performed on a 0.6 x 25 cm stainless steel column packed with Merck Lichrosorb-ODS, 5 micron particle size. Subtilisin Carlsberg was obtained from Sigma Chemical Corp. The bromonaphthyl-alanine substrates shown in Table 1 as well as the tetrapeptide 9 and pentapeptide 11 were graciously supplied by Drs. John Nestor and Teresa Ho of Syntex Research, Institute of Bio-Organic Chemistry.

General Conditions for Pd/C Catalyzed Tritiations of ^{3}H-NAL Precursors.

A 10 cc side arm septum flask containing a stirring magnet and catalyst (10-20 mg) was connected to a high vacuum manifold and evacuated overnight. The substrate (0.03 - 0.08 mmol) dissolved in 2 ml of solvent (see Table 1) was injected through the rubber septum. When brominated substrates were reduced the solution contained 50 µl of triethylamine.

The solvent was degassed and carrier free tritium gas was transferred into the reaction vessel by means of a Toepler pump. The reaction was stirred overnight at ambient temperature. Volatile radioactive materials were transferred into a waste flask and the reaction vessel was removed from the vacuum line. The catalyst was removed by filtration through a Teflon Millipore filter. The filtrate was diluted with MeOH and evaporated to dryness three times to ensure removal of exchangeable tritium. The products were purified by HPLC (Lichrosorb 25-40 micron, MeOH/H_2O 80:20) or by silica gel chromatography [ethyl acetate/dichloromethane, (1:4)]. Results of these experiments are summarized in Table 1. All work prior to purification was performed in high flow fume hoods.

2-Methyl-4-(2-naphthal)-5-oxazolone (3)[13a,b]

2-Naphthaldehyde (389 mg, 2.49 mmol), N-acetylglycine (296.7 mg, 2.53 mmol), anhydrous sodium acetate (208 mg, 2.54 mmol) and acetic anhydride (2 ml) were combined and heated at 70-80° for one hour, at 100-110° for 2 hours, then stirring was continued at room temperature overnight. Water was added to the dark yellow mass. Solid materials were filtered, washed with water, and dried. The crude product was extracted from the solid with EtOAc. The title compound was isolated by column chromatography [(SiO_2), hexane]. Crystallization from EtOAc/hexane gave 3 as yellow needles (193 mg, 33%) m 132-133°; nmr ($CDCl_3$), δ8.35 (s, 1H), δ8.13 (α, 1H), δ7.8 (m, 3H), δ7.5 (m, 2H), δ7.25 (s, 1H), δ2.4 (s,3H); mass

spectrum, M^+ 237; μV (EtOH) λmax 278 nm (ε 13,000), 288 nm (ε 15,300), 343 nm (ε 27,300); tlc (SiO_2), hexane-EtOAc (9:1).

2-N-Acetylamino-3-(2-naphthyl) acrylic acid

The unsaturated azlactone 3 (168 mg, 0.71 mmol) was hydrolyzed by heating at reflux in aqueous acetone for 3.5 hours. Upon cooling to room temperature the title compound crystallized as pale yellow needles. After washing with water and drying under vacuum the pure acid was obtained in 99% yield (178 mg). m 231°; nmr (Me_2SO-d_6): δ9.6 (s, 1H), δ8.3-7.4 (m, 9H), δ2.1 (s, 3H); mass spectrum, M^+255; tlc (SiO_2), CH_2Cl_2/MeOH/HOAc (90:7.5:2.5), toluene/THF/MeOH/HOAc (5:2:1:0.2).

Methyl 2-N-acetylamino-3-(2-naphthyl)acrylate (5)

To a stirred suspension of the acrylic acid prepared above in dry MeOH (2 ml) was added $BF_3 \cdot Et_2O$ (0.2 ml, 1.6 mmol) and the mixture was heated at 80-90° for 6 hours. After cooling to room temperature, water was added and the product was extracted twice with EtOAc. The combined extracts were washed with satd. $NaHCO_3$ and dried over Na_2SO_4. Evaporation of the solvent gave an oil which was crystallized from EtOAc/hexane to afford the title compound (62 mg). mp 145-146°; nmr ($CDCl_3$) δ8.1-7.2 (m, 9H), δ3.9 (s, 3H), δ2.15 (s, 3H); mass spectrum, M^+ 269; tlc [(SiO_2), CH_2Cl_2 EtOAc (4:1), Et_2O EtOAc (99:1)].

D,L-N-Acetyl-3-(2-naphthyl)-[2,3-^{3}H]alanine methyl ester (6)

A 10 cc side-arm septum flask was charged with $(Ph_3P)_3RhCl$ (24 mg) and evacuated on a vacuum line for one hour. Benzene (1 ml) was injected and the red solution was degassed. Carrier free tritium gas (18 Ci, 0.3 mmol) was transferred into the liquid N_2 cooled reaction flask by means of a Toepler pump. When the color of the catalyst solution changed to yellow, a solution of 5 (34 mg, 0.13 mmol) in 3 ml of benzene/ethyl acetate (1:2) was injected and the reaction was stirred for 3 days at ambient temperature. In order to ensure compleat removal of labile tritium the solvent was evaporated to dryness on the vacuum line. The residue was then dissolved in methanol and evaporated to dryness 3 times. Following removal from the vacuum line and aqueous work-up, a total of 2200 mCi of crude product, contaminated with unreduced starting material, was isolated. A portion (830 mCi) of this material was applied to an alumina (Woelm, grade I) column. Elution with chloroform/hexane (2:3) afforded 390 mCi of pure title compound.

Alternatively, a better recovery was achieved by treatment of 280 mCi of crude product with NBS in aqueous acetone (which reacts only with the unsaturated starting material). Column chromatography on silica gel [dichloromethane/ethyl acetate (4:1)] afforded 200 mCi of 6 having a specific activity of 43.7 Ci/mmol.

UV (EtOH) λmax 278 nm (ϵ4825); radio-tlc: (SiO_2), ethyl acetate/dichloromethane (1:4), benzene/ethyl acetate (4:1).

D-N-Acetyl-3-(2-naphthyl)-[2,3-^{3}H]alanine methyl ester (6a)

To the racemic ester 6 (388 mCi, 43.7 Ci/mmol, 9 μmol) dissolved in Me_2SO (0.15 ml), 1.0M KCl (0.06 ml), and water (0.39 ml) was added subtilisin enzyme (20 μg in 0.002 ml 0.1M KCl), and the mixture allowed to stir at room temperature. After 30 minutes 0.2M NaOH (0.025 ml, 5 μmol) was added to maintain the pH at 7.

Radiochromatography indicated that 50% of the ester had been converted to the acid. The pH was adjusted to 8.5 by addition of $NaHCO_3$ (6 mg) and the volume brought to 3 ml with water. The unreacted D-ester was extracted with ethyl acetate (4 x 2 ml) and the extracts washed with saturated $NaHCO_3$ (1 ml), giving radiochemically pure 6a (180 mCi, 46%). Tlc: (SiO_2) toluene/ethyl acetate (6:4), (RPC-18) acetonitrile/ water/acetic acid (50:50:2).

Determination of the Optical Purity of 6a: D-[^{3}H] and DL-N-Boc-3-(2-naphthyl)-alanine

Unlabelled DL-N-acetyl-3-(2-naphthyl)-alanine methyl ester (6c) was combined with 6a and the mixture hydrolyzed to the free amino acid by heating at reflux in 6N HCl for 2 hours. The reaction mixture was taken to dryness and the residue dissolved in 3 ml of water/acetone (1:2). One ml of this solution (0.3 mCi) was removed and the solvent evaporated. To the residue was added H_2O (0.5 ml), dioxane (0.5 ml) and 20%

aq. NaOH to pH 8-9. Di-t-butyldicarbonate (9 µl) was added and the solution was stirred at room temperature for one hour during which time the pH was periodically adjusted to 8-9 with MgO.

Acidification to pH 2 with $NaHSO_4$ was followed by extraction with EtOAc. The extracts were washed with 5% $NaHSO_4$, water, brine, then dried over Na_2SO_4 to afford a quantitative yield (0.3 mCi) of a mixture of D-N-Boc-3-(2-naphthyl)-[2,3-^{3}H]alanine and the corresponding unlabelled DL-compound. This mixture was radiochemically pure by tlc [(RPC-18), methanol/water/acetic acid (60:40:1); (SiO_2), chloroform/methanol/acetic acid (14:2:0.1)].

(D,L)-[^{3}H] and (L,L)-N-Boc-3-(2-naphthyl)alanine phenethylamide 13 and 14

To an ice cooled solution of the above N-Boc-amino acids (0.3 mCi, 22 µmole) in dichloromethane (0.3 ml) containing triethylamine (8.4 µl, 60 µmole) was added ethylchloroformate (3 µl, 34 µmole). After stirring for 5 minutes, L-(-)-methylbenzylamine (8 µl, 54 µmole) was added. The stirred reaction was allowed to come to room temperature overnight. Dichloromethane (10 ml) was added and the solution was washed with 5 ml each dilute HCl, H_2O, satd. $NaHCO_3$, then dried over Na_2SO_4. Evaporation of the solvent afforded 13 and 14 as a colorless oil (0.25 mCi, 87%). The product was radiochemically pure by tlc [(SiO_2), toluene/ethyl acetate (9:1)].

D,L-[^{3}H] and L,L-N-Acetyl-3-(2-naphthyl)-alanine phenethylamides 13a and 14a

To the above oil was added trifluoroacetic acid (1 ml) and the solution was stirred for 10 minutes. The reaction mixture was concentrated, the residue dissolved in ethyl acetate (5 ml) and evaporated to dryness three times. The resulting oil was dissolved in pyridine (1 ml) and treated with acetic anhydride (0.3 ml). After 30 minutes the reaction was concentrated, the residue dissolved in ethyl acetate (5 ml), concentrated twice more, and finally dissolved in dichloromethane (5 ml). After washing with dilute HCl, water, brine, and drying over Na_2SO_4, the solvent was evaporated leaving 13a and 14a as an off-white solid.

Tlc separation of this mixture was achieved on silica plates developed three times with either ether/ethyl acetate (100:1) or benzene/ethyl acetate (1:1).

Examination of the developed plates on a radioactivity scanner revealed that radioactivity was associated exclusively (>98%) with the D,L diastereomer 13a.

D,L and L,L-amide standards were prepared using pure D-NAL and L-NAL according to the procedures described above. The relative R_f values of 13a and 14a were determined using these standards. The NMR and mass spectra of the standards were consistent with their structures.

D-3-(2-Naphthyl)-[2,3-^{3}H]alanine (7)

To 6 (180 mCi, 4.2 μmol) was added 6 N hydrochloric acid (2 ml) and the solution heated at reflux for 2.5 hours. Following cooling to room temperature the solution was concentrated, the residue dissolved in (1:1) methanol/water (8 ml) and reconcentrated twice more, to give 7 (178 mCi, 99%). Tlc: (SiO_2), ethyl acetate/acetic acid/water (12:1:1), dichloromethane/methanol/acetic acid (9:3:0.5), pyridine/ethanol/water (2:1:1).

D-N-Boc-[2,3-^{3}H]NAL-Leu-Arg-Pro-Gly NH_2 (10)

The free D-amino acid 7 (178 mCi; 4.2 μmole) was converted to the N-Boc derivative [168 mCi; 4.2 μmol; radio-tlc: (SiO_2), dichloromethane/methanol/acetic acid (12:2:0.1), (RPC-18), acetonitrile/water acetic acid (50:50:2)] as described above and dissolved in DMF (0.2 ml) containing Et_3N (0.85 μl; 6 μmol). After cooling to -5°, ethylchloroformate (0.6 μl, 6.3 μmol) in DMF (0.1 ml) was added and the reaction was stirred for 30 minutes. An identical portion of ethylchloroformate in DMF was added followed by a solution of Leu-Arg-Pro-GlyNH_2 diacetate (12.6 mg, 15.8 μmole) in DMF (0.5 ml). The reaction was stirred at 0° for 3 days then at room temperature for 8 hours. Methanol was added and the solution was concentrated to an oil which solidified upon addition of 7 ml of ethyl acetate/ether (4:3). The supernatant was discarded and the residue dissolved in 0.5 ml of methanol/dichloromethane (1:1). Application to a

Sephadex LH20 column (2 x 60 cm in dichloromethane)) followed by gradient elution using 0-20% methanol in dichloromethane afforded the labelled pentapeptide 10 in 53% yield (90 mCi) and 95% radiochemical purity [radio-tlc (silica) acetonitrile/water/acetic acid (8:1:1), n-butanol/acetic acid/water (4:1:1)].

pyroGlu-His-Trp-Ser-Tyr-D-[2,3-^{3}H]NAL-Leu-Arg-Pro-GlyNH$_2$ (12)

The labelled N-Boc-pentapeptide 10 (90 mCi, 2 µmol) was stirred with trifluoroacetic acid (1 ml) for 2 hours. The reaction was evaporated to dryness 2 times with 5 ml portions of methanol. The deprotected pentapeptide residue was triturated with ether, isolated by centrifugation and dissolved in DMF (0.3 l) containing N-methylmorpholine (1 µl, 9.1 µmol).

In a separate flask, the pentapeptide hydrazide, pyroGlu-His-Trp-Ser-TyrN$_2$H$_3$, (11), (7.9 mg, 11 µmole), was dissolved in DMF (0.3 ml), cooled to -25° and treated with 5.2M methanolic HCl (13 µl, 0.068 µmol). Reaction with t-butylnitrite (1.74 mg, 16 µmol) for 20 minutes served to generate the azide 11a. The pH was adjusted to 7-8 with N-methylmorpholine and the deprotected labelled pentapeptide (described above) was added. The reaction mixture was stirred for 3 days at -10° then evaporated to dryness three times with ethanol-water (1:1). The residue was dissolved in 0.005M NH_4OAc pH 4.5 (1 ml) and applied to a CM-Sephadex C-25 column

(2.5 x 60 cm) which was pre-swelled with 0.5 M NH_4OAc then washed with 0.005M NH_4OAc. Gradient elution with 0.005 M pH 4.5 to 0.5 M pH 7 NH_4OAc afforded the decapeptide 12 still contaminated with several impurities. Final purification was effected by HPLC (Lichrosorb-ODS, 5 micron, 0.6 x 25 cm column) using 0.03M NH_4OAc pH 4.5/acetonitrile (60:40) as the eluting solvent. The desired 12 (18.5 mCi) was obtained at a specific activity of 45.8 Ci/mmol. The product was homogenous by radio-tlc [(Avicel), n-butanol/formic acid/water (20:1:1); (DEAE cellulose), 0.5M NH_4OAc pH 4.5] and by analytical HPLC [Lichrosorb-ODS, 5 micron, 0.03M NH_4OAc pH 4.5/acetonitrile (60:40)]. In addition the UV spectrum of 12 was superimposable with standard. UV (ethanol) λmax 276 nm (ε 11,200).

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