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SYNTHESIS OF HIGH SPECIFIC ACTIVE TRITIATED LEU-ENKEPHALIN IN THE LEUCINE RESIDUE

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SUMMARY

Leu-enkephalin labelled with tritium in the Leu residue has been prepared. Synthesis of the precursor peptide, [4,5-dehydroLeu^5-]Leu-enkephalin, was carried out by solid phase synthesis using Fmoc amino acid derivatives. The peptide was tritiated catalytically yielding ^{3}H-Leu-enkephalin with a specific radioactivity of 4.39 TBq/mmol. The distribution of tritium label was investigated by reversed-phase high performance liquid chromatography with a synchronized accumulating radioisotope detector following acidic and enzymatic hydrolysis, which confirmed that the tritium label was entirely located at Leu residue.

Key Words: Leu-enkephalin, solid phase synthesis, [4,5-dehydroLeu^5-]-Leu-enkephalin, [4,5-^{3}H-Leu^5-]Leu-enkephalin, catalytic tritiation, radio HPLC

INTRODUCTION

The leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu, Leu-enk) is a natural endogenous ligand for opiate receptors. Intracranial injection of Leu-enk exhibits an analgesic activity (1). However, the activity is weak and short lasting probably because of its hydrolysis into inactive metabolites. The in vitro studies using brain homogenates and plasma have confirmed rapid degradation (2). The most rapid enzymatic attack is cleavage of the Tyr-Gly amide bond, but other amide bonds are also cleaved (3). Furthermore, the in vivo studies have shown that the degradation of Leu-enk is extremely rapid after the intraveneous administration in rat (4). In the in vitro and in vivo

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studies, Leu-enk labelled with tritium in the Tyr residue ([3,5-^{3}H-Tyr^1-]Leu-enkephalin, [^{3}H-Tyr^1-]Leu-enk) was used as the substrate coupled with chromatographic procedures.

Several investigators have reported that metabolic fragments of neuropeptides may also have biological activities (5). Recently, Weinberger et al. suggested that [des-Tyr^1-]Leu-enk (Gly-Gly-Phe-Leu), the main metabolite of Leu-enk, produced opposite effect of the parent peptide on shock-induced locomotor activity in mice (6). Leu-enk, at 100 μg/kg i.p., resulted in increased locomotion, while [des-Tyr^1-]Leu-enk, at a dose equimolar to 100 μg/kg of Leu-enk, resulted in significantly decreased locomotion. However, metabolic studies of [des-Tyr^1-]Leu-enk have been limited by the lack of a specific and sensitive assay. Commercially available [^{3}H-Tyr^1-]Leu-enk cannot be used as substrate to address this problem since ^{3}H-Tyr release from [^{3}H-Tyr^1-]Leu-enk makes it impossible to elucidate the following degradation of Gly-Gly-Phe-Leu. The need arose for Leu-enk labelled near the C-terminus rather than the N-terminus.

We have undertaken studies to assess to characterize the metabolic profile of C-terminal fragment of Leu-enk. In the present study, we describe the synthesis of tritiated Leu-enk in the Leu residue.

EXPERIMENTAL

^{1}H-NMR spectra were determined on a Brucker AM-400 400 MHz FT-NMR spectrometer. Optical density was determined on a Shimadzu UV-260 spectrophotometer with 10 mm path-length cell. The liquid scintillation counter was an Aloka LSC-900. Amino acid analysis was performed on a Hitachi L-8500 Amino Acid Analyzer. The two high performance liquid chromatograph systems were used as follows. Analytical and preparative high performance liquid chromatography (HPLC) of the non-labelled compounds were performed on a Waters M600 multisolvent delivery system and a Waters 481 lambda-max variable wavelength UV detector. For the analysis of the labelled compounds, the system consisted of a Shimadzu LC-6A pump, a Shimadzu SPD-6A variable wavelength UV detector, a Shimadzu RF-535 fluorescence detector and an Aloka synchronized accumulating radioisotope detector (SARD) (7,8) having five counting cells

(cell volumes: 0.5 ml) and five pairs of photoelectron multiplier tubes. Analytical HPLC was carried out on a LiChrosorb RP-18 column (5 μm, 4 x 250 mm; E.Merck) in the following solvent systems (all v/v): (A) methanol-H_2O-acetonitrile-trifluoroacetic acid (TFA), 50:30:20:0.05; (B) H_2O-acetonitrile-TFA, 75:25:0.1 and (C) 10 mM acetate buffer (pH 4.0)-acetonitrile-methanol, 50:35:15. The flow rate was 1 ml/min. Preparative HPLC was carried out on μ-Bondasphere C-18 (5μm, 100Å, 19 x 150 mm; Waters) in the solvent system (B). The flow rate was 10 ml/min.

9-Fluorenylmethyloxycarbonyl (Fmoc-) amino acid derivatives, p-benzyloxybenzyl alcohol resin (0.7 meq OH g^{-1} resin, polystyrene with 1% divinylbenzene copolymer) and Fmoc-Leu-p-benzyloxybenzyl ester resin (0.37 mmol Fmoc-Leu g^{-1} resin) were purchased from Kokusan Chemical Works Co (Tokyo, Japan). Leu-enk was purchased from Peptide Institute (Osaka, Japan). [des-Leu^5-]Leu-enk (Tyr-Gly-Gly-Phe) and carboxypeptidase Y (from Bakers Yeast) were purchased from Sigma (St. Louis, MO, USA).

Stability of Fmoc-4,5-dehydroLeu in a mixture of TFA-CH_2Cl_2-dioxane

To 1 ml of a mixture containing TFA, CH_2Cl_2 and dioxane were added 0.5 μmol of Fmoc-4,5-dehydroLeu (9,10) and 0.5 μmol of Fmoc-Leu as an internal standard. After the mixture was incubated for 1 hr at room temperature, an aliquot of the sample was subjected to analytical HPLC (mobile phase: A, detection: UV 280 nm).

Cleavage of Fmoc-Leu-p-benzyloxybenzyl ester resin with a mixture of TFA-CH_2Cl_2-dioxane

Fmoc-Leu-p-benzyloxybenzyl ester resin (0.37 mmol Fmoc-Leu g^{-1} resin, 10 mg) was treated with a mixture of TFA-CH_2Cl_2-dioxane (1 ml) for 1 hr at room temperature, followed by filtration and washing the resin with methanol. The combined filtrates were evaporated under nitrogen stream to dryness. To the residue was added Fmoc-4,5-dehydroLeu (0.1 mmol) in methanol (1 ml) as an internal standard, and subjected to analytical HPLC (mobile phase: A, detection: UV 280 nm).

Preparation of Fmoc-amino acid benzotriazole ester (Fmoc-amino acid-OBT)

To a solution of Fmoc-amino acid (3.5 mmol) and 1-hydroxybenzotriazole (3.5 mmol) in 20 ml of CH_2Cl_2-DMF (5:1) was added N,N'-dicyclohexylcarbodiimide (3.5 mmol) at 0° with stirring. The mixture was stirred for 1 hr at 0° and for 1 hr at room temperature. The precipitated urea was removed by filtration and washed with CH_2Cl_2. The combined filtrates were concentrated under reduced pressure, redissolved in DMF (15 ml) followed by applying to peptide synthesis.

Fmoc-4,5-dehydroLeu-p-benzyloxybenzyl ester resin

p-Benzyloxybenzyl alcohol resin (0.7 meq g^{-1} resin, 1.0 g) was washed with CH_2Cl_2 (3 x 15 ml) and DMF (3 x 15 ml) in a reaction vessel. Fmoc-4,5-dehydroLeu-OBT (3.5 mmol) in DMF (15 ml) and 4-dimethylaminopyridine (0.7 mmol) in DMF (2 ml) were added to the reaction vessel and the mixture was stirred for 2 hr. After filtration, the resin was washed with DMF (3 x 15 ml), CH_2Cl_2 (3 x 15 ml) and DMF (3 x 15 ml). Fmoc-4,5-dehydroLeu was recoupled and then the esterified resin was washed with DMF (6 x 15 ml) and CH_2Cl_2 (6 x 15 ml), followed by drying in vacuo. Fmoc-4,5-dehydroLeu-p-benzyloxybenzyl ester resin (10 mg, approximately) was treated with 50% piperidine in CH_2Cl_2 (1 ml) for 30 min. The mixture was filtered and washed with CH_2Cl_2. The optical density at 301 nm (ε = 7800) of the combined filtrates was measured to show a substitution of 0.64 mmol Fmoc-4,5-dehydroLeu g^{-1} resin.

Peptide synthesis

Solid phase synthesis was carried out manually. The protocol was shown in Table 1. Fmoc deprotection was achieved by treatment with 20% piperidine in DMF. Coupling reactions were carried out with 5 equiv. of Fmoc-amino acid-OBT. All coupling reaction were monitored by Kaiser test (11).

After the removal of N-terminal Fmoc group, the protected peptide resin was dried in vacuo for 12 hr. Cleavage of the peptide from the resin was performed with 20 ml of TFA-CH_2Cl_2-dioxane-anisole (5:4:1:1) for 1 hr at room temperature. After filtration, the resin was washed with methanol. The combined

Table 1. Schedule of solid phase synthesis using Fmoc protection

	Reagent	Time/repeat
Wash	1 CH_2Cl_2	2 min x 3
	2 DMF	2 min x 3
Deprotection	3 20%Piperidine/DMF	2 min x 1
		+ 30 min x 1
Wash	4 DMF	2 min x 3
	5 CH_2Cl_2	2 min x 3
	6 DMF	2 min x 3
Coupling	7 Fmoc-amino acid-OBT	90 min x 1
Wash	8 DMF	2 min x 3
	9 CH_2Cl_2	2 min x 3
	10 DMF	2 min x 3

Solvent volume, 15 ml per g of resin.

filtrates were evaporated under 30°, redissolved in H_2O and lyophilized.

The crude product was purified by preparative HPLC (detection: UV 210 nm), concentrated and lyophilized to afford the purified [4,5-dehydroLeu^5-]Leu-enk (224mg, 63.2%). This pentapeptide gave the following amino acid analysis after acid hydrolysis by 6N HCl at 110° for 22 hr: Gly, 2.00; Tyr, 0.85; Phe, 1.00.

Catalytic hydrogenation of the pentapeptide (1 mg) in methanol (1 ml) in the presence of palladium black gave a product which was identical with authentic Leu-enk by analytical HPLC (mobile phase: B, detection: UV 210 nm) and amino acid analysis.

Tritiation and purification

[4,5-DehydroLeu^5-]Leu-enk (10 mg, 18 μmol) was dissolved in methanol (10 ml) and reduced using tritium gas (925 GBq) in the presence of 10% palladium on charcoal (1 mg) for 4 hr (Tritium Labelling Service, New England Nuclear Co., MA, USA). After filtration, the labile tritium was removed by successive flash evaporations in methanol. The tritiated product was purified by HPLC using analytical column (mobile phase: B, detection: UV 280 nm), which was chromatographically identical with authentic Leu-enk. The solvent was evaporated to dryness under 30°. The purified ^{3}H-Leu-enk (3.6 μmol, 21%, 4.39 TBq/mmol) was redissolved in methanol (10 ml). Storage of the solution at -20° did not result in any detectable decomposition for more than three months.

Distribution of tritium label

Acid hydrolysis: The ^{3}H-Leu-enk (16.7 KBq) was diluted with non-labelled Leu-enk (10 nmol) and hydrolyzed by 6N HCl (1 ml) at 110°for 22 hr. The solvent was evaporated in vacuo, redissolved with 100 μl of 10 mM borate buffer (pH 9.5) and added 200 μl of 1 mM Fmoc-Cl in acetone. The mixture was stirred at room temperature for 1 min, then washed with n-pentane (1 ml x 3) to remove excess Fmoc-Cl reagent. The aqueous phase was subjected to analytical HPLC (mobile phase: C, detection: fluorescence (excitation; 260nm, emission; 313nm) and SARD).

Digestion with carboxypeptidase Y: The ^{3}H-Leu-enk (16.7 KBq) was diluted with non-labelled Leu-enk (20 nmol) and incubated with 200 μg of carboxypeptidase Y in H_2O (100 μl) at 25°for 4 hr. An aliquot of the incubation mixture was subjected to analytical HPLC (mobile phase: B, detection: UV 210 nm and SARD). Another 10 μl of the incubation mixture was subjected to analytical HPLC after Fmoc derivatization described previously.

RESULTS AND DISCUSSION

The synthetic route of [^{3}H-Leu^5-]Leu-enk was illustrated in Fig. 1. [4,5-DehydroLeu^5-]Leu-enk, the precursor peptide, was prepared by solid phase synthesis using Fmoc-amino acids and p-benzyloxybenzyl alcohol resin. Fmoc-4,5-dehydroLeu (9,10) was prepared from ethyl acetoaminomalonate and 3-chloro-2-methylpropene. Attachment of Fmoc-4,5-dehydroLeu to the resin was carried out with activated ester method using 1-hydroxybenzotriazole in the presence of 4-dimethylaminopyridine. The 4,5-dehydroLeu content anchored to the resin was estimated according to the procedure of Meienhofer et al.(12) and found to be 0.63 mmol per gram of resin. Each coupling of Fmoc-Phe, Fmoc-Gly, Fmoc-Gly and Fmoc-Tyr(OBu^t) was carried out using the corresponding benzotriazole ester until a negative ninhydrin test was obtained. In all cases, no recoupling was necessary.

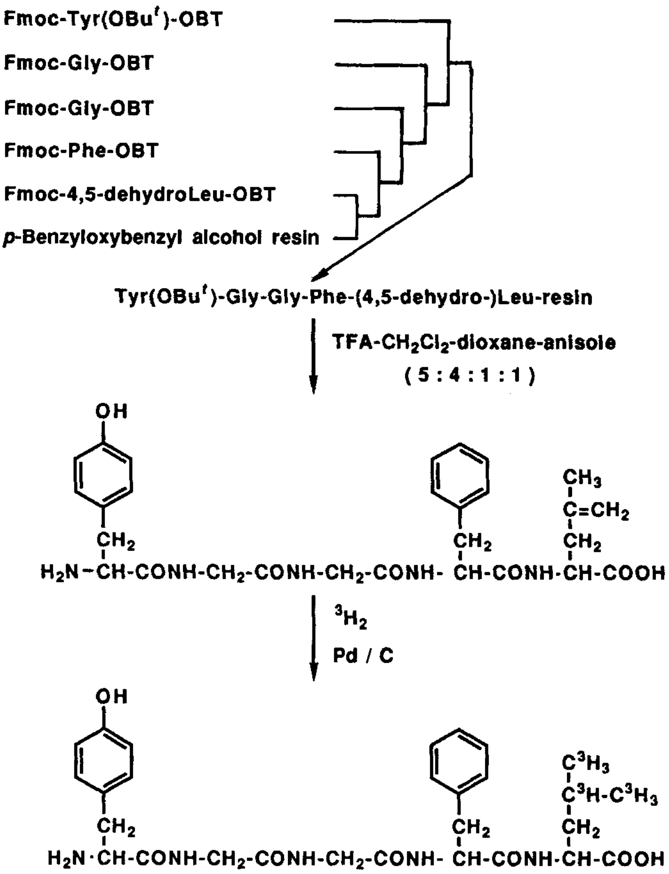

Fig. 1. Synthetic scheme of [^{3}H-Leu^5-]Leu-enkephalin

Since the cleavage of a peptide from p-benzyloxybenzyl alcohol linkage resin is generally carried out by treatment with TFA-scavengers (9:1), the protected pentapeptide resin was treated with TFA-dioxane-anisole (9:1:1). The ^{1}H-NMR spectrum of the major product showed the signals of the vinillic protons at 4,5-dehydroLeu residue to be absent. Two methyl 'singlet' signals were observed at 1.44 and 1.54 ppm, respectively, suggesting that addition of TFA to the double bond had occurred. Therefore, model studies using simple amino acid derivatives were performed to determine the optimum cleavage conditions. The stability of 4,5-dehydroLeu in TFA was first studied. Fmoc-4,5-dehydroLeu was treated with 25 - 100 % TFA in CH$_2$Cl$_2$ for 1 hr, followed by quantification of the remaining Fmoc-4,5-dehydroLeu using HPLC. The results showed that Fmoc-4,5-

dehydroLeu was decomposed in proportion to increase of TFA concentration. In 100, 75 and 50 % TFA, the percentages of Fmoc-4,5-dehydroLeu remained were 13, 33 and 58 %, respectively. However, Fmoc-4,5-dehydroLeu was stable in 37.5 or less TFA. Since dioxane is shown to inhibit the addition of TFA to isobutene (13), the effect of dioxane was also estimated. Fmoc-4,5-dehydroLeu was treated with 50 % TFA in the presence of 10 - 25 % dioxane. In 10, 15 and 25 % dioxane, the percentages of Fmoc-4,5-dehydroLeu remained were 77, 90 and 97 %, respectively. The results showed that addition of dioxane was effective for the stability of 4,5-dehydroLeu. Secondly, the effects of TFA and dioxane on the cleavage of a peptide from the resin were investigated using Fmoc-Leu-p-benzyloxybenzyl ester resin. The results were shown in Table 2. At lower concentration of TFA the cleavage was reduced. On the other hand, increasing the dioxane concentration also decreased the cleavage, presumably by inhibiting the swelling of the resin. This resulted in a decreased availability for peptide-resin-TFA interaction. In view of these observations, the protected pentapeptide resin was treated with TFA-CH_2Cl_2-dioxane-anisole (5:4:1:1) to give a product (224mg, yield 63.2%). ^{1}H-NMR spectrum of the peptide showed a

Table 2. Cleavage condition of Fmoc-Leu from p-benzyloxybenzyl alcohol resin

%Reagent			%Fmoc-Leu regenarated
TFA	CH_2Cl_2	dioxane	after 1 hr
50	50	0	100.0
50	40	10	100.0
50	35	15	89.2
50	30	20	42.5
50	25	25	15.7
37.5	52.5	10	100.0
37.5	47.5	15	53.1
37.5	42.5	20	23.4
37.5	37.5	25	5.5
25	65	10	87.7
25	60	15	16.3
25	55	20	3.8
25	50	25	2.6

'singlet' signal at 1.74 ppm, δ-CH_3 protons and a 'doublet' signal at 4.84 ppm, vinylic protons of 4,5-dehydroLeu residue. Catalytic hydrogenation of the peptide gave a product which was identical with authentic Leu-enk by HPLC and amino acid analysis. These results indicated that the desired [4,5-dehydroLeu^5-] Leu-enk was obtained in a good yield.

Tritiated Leu-enk was obtained by reducing the [4,5-dehydroLeu^5-]Leu-enk with tritium gas in the presence of palladium on charcoal. After removal of the labile tritium, the preparation was purified by HPLC. The product was rechromatographed giving one single component by UV and radioactivity detection. The product had a radiochemical purity higher than 99%. Since the catalytic hydrogen-tritium exchange reaction might occur in aromatic amino acid residues under the catalytic reduction (14), the distribution of the tritium label among the amino acid residues was investigated. HPLC analysis of the Fmoc amino acid derivatives following acid hydrolysis demonstrated that the label had been located exclusively at the Leu residue, as shown in Fig. 2. No evidence was found for non-specific exchange labelling into the other residues. Enzymatic digestion also confirmed that over 99% of the tritium was located at the Leu residue.

The product had an extremely high specific radioactivity of 4.39 TBq/mmol. It was demonstrated that almost four tritiums were incorporated into the Leu residue in the reduction product. Hardy et al. have reported (15) that catalytic deuterization of N-acetyl-4,5-dehydro-DL-Leu produced N-acetyl-DL-Leu containing from one to seven deuteriums, the trideuterated species being the most abundant. They suggested the occurrence of substantial isotope exchange through a metal η^3 hydride bonded to the propenyl residue during saturation of 4,5-dehydroLeu (16). Thus, we concluded that one to seven tritiums, four tritiums on the average, were incorporated into the δ-methyl groups and γ-methinyl group of Leu residue in the Leu-enk.

The present procedure provides a simple method for the synthesis of selectively tritium labelled peptide with high specific activity. The tritiated Leu-enk should be useful for the metabolic studies of Leu-enk.

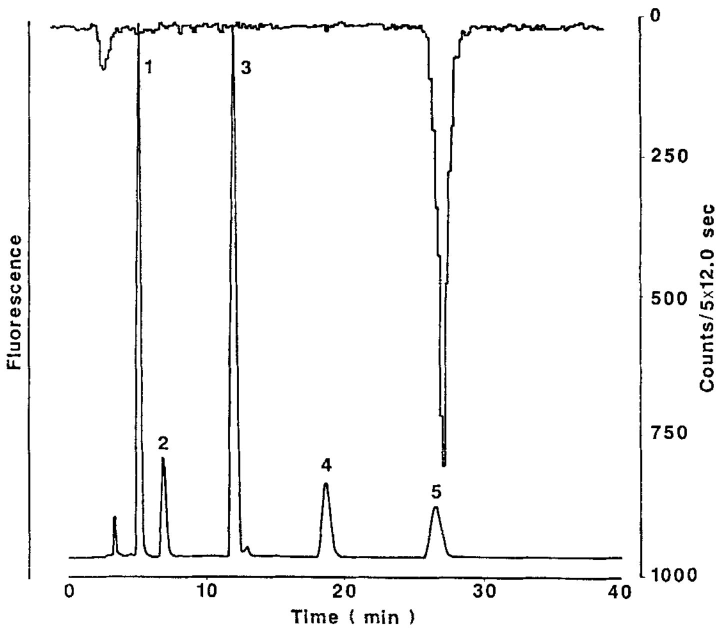

Fig. 2. HPLC tracing of the Fmoc amino acid derivatives following acid hydrolysis of [^{3}H-Leu^5-]Leu-enkephalin. (1) = Fmoc-Gly, (2) = Fmoc-Tyr, (3) = Fmoc-OH, (4) = Fmoc-Phe, (5) = Fmoc-Leu.

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