Synthesis of [2,6-$^3H_2$-Tyr^1]Leucine-enkephalin

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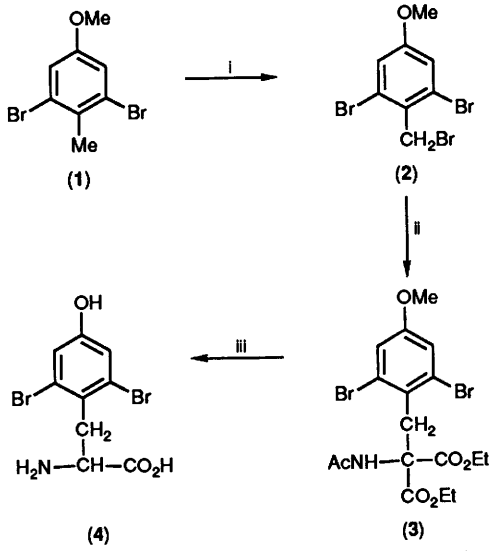
The synthesis of [2,6-$^3H_2$-Tyr^1]leucine-enkephalin by the catalytic tritiation of [2,6-dibromo-Tyr^1]leucine-enkephalin is described. The precursor amino acid, 2,6-dibromo-DL-tyrosine, was synthesized in three steps from 2,6-dibromo-4-methoxytoluene. The protected [2,6-dibromo-Tyr^1]leucine-enkephalin derivative was prepared by solid-phase synthesis, followed by epimeric resolution on HPLC. The peptide was tritiated catalytically to yield [^{3}H-Tyr^1]leucine-enkephalin with a specific radioactivity of 1.37 TBq/mmol. The distribution of tritium was investigated by HPLC with radioisotope detection following enzymatic hydrolysis, and confirmed that the tritium label was entirely located at the tyrosine residue.

The availability of tritium-labelled peptides would be of obvious value for a variety of chemical and biological studies. If the tritiated peptide is to be used for metabolic studies, the label must be placed at an appropriate location in the peptide chain. The specific tritiation of peptides has predominantly been carried out by tritium–halogen exchange of protected peptides containing halogen substituted in histidine,[1,2] phenylalanine,[3] and tyrosine[4] moieties. In the case of tyrosine, usually the ring 3- and 5-position have been tritiated since 3,5-dihalogenated tyrosine peptides are easily available. However, the introduced tritium in these positions may not be always metabolically stable.[5] We turned our attention to the ring 2- and 6-position of the tyrosine residue in the peptide for labelling. These positions would be more stable than the 3- and 5-position. However, there are no reports concerning the syntheses of 2,6-dihalogenated tyrosine and [2,6-$^3H_2$-Tyr^1]peptides.

In the present study, we describe a simple, three-step synthesis of 2,6-dibromo-DL-tyrosine, its incorporation into leucine-enkephalin, and a preparation of [2,6-$^3H_2$-Tyr^1]leucine-enkephalin by tritium–halogen exchange.

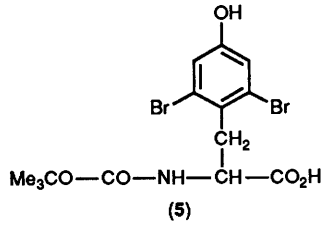


Scheme 1. *Reagents:* i, NBS, *hν*; ii, Na^+ $^-C(CO_2Et)_2NHAc$; iii, c. HBr.

Results and Discussion

The key 2,6-dibromo-DL-tyrosine (**4**) has been prepared in three steps starting with commercially available 2,6-dibromo-4-methoxytoluene (**1**) (Scheme 1). Treatment of compound (**1**) with *N*-bromosuccinimide (NBS) under irradiation effected benzylic bromination to give the benzyl bromide (**2**) in 86% yield. However, the bromination of 2,6-di-iodo-4-methoxytoluene did not occur, presumably from steric hindrance by the iodine atoms. The benzyl bromide (**2**) was treated with sodium diethyl acetamidomalonate in ethanol to give the condensation product (**3**). Treatment of compound (**3**) with concentrated hydrogen bromide caused simultaneous demethylation, hydrolysis, and decarboxylation to give 2,6-dibromo-DL-tyrosine (**4**) in 93% yield.

The protected [2,6-dibromo-DL-Tyr^1]leucine-enkephalin derivative was prepared by solid-phase synthesis (Scheme 2). Starting with Fmoc-Leu *p*-benzyloxybenzyl ester resin, the peptide chain was elongated manually, according to the sequence steps shown in the Table, *i.e.*, removal of the Fmoc group by 20% piperidine in dimethylformamide (DMF) and condensation of the respective amino acids by the pentafluorophenyl (Pfp) ester procedure.[6] The major exception to these procedures was in the coupling of the last residue 2,6-dibromo-DL-tyrosine. Since the benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) coupling procedure required less excess of protected amino acid,[7] 2,6-dibromo-DL-tyrosine (**4**) was coupled as its *N*-t-butoxycarbonyl (Boc) derivative (**5**) by the BOP procedure.

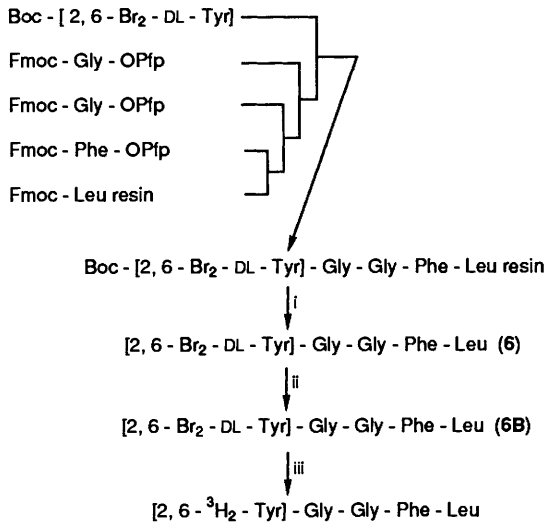


Treatment of the protected pentapeptide resin with 50% trifluoroacetic acid (TFA) in CH_2Cl_2 in the presence of 10% anisole gave an epimeric peptide, [2,6-dibromo-DL-Tyr^1]-leucine-enkephalin (**6**). Analytical HPLC of the racemate (**6**) allowed partial resolution, as shown in Figure 1 [(**6A**) t_R 29.2 min, (**6B**) t_R 30.8 min]. The racemate was purified and resolved into its epimers by preparative HPLC. A check on the purity of these peptides was accomplished by analytical HPLC. Both epimers obtained were found to be >99.9% pure and were not detectably contaminated by the other epimer. To explore the configuration at the tyrosine residue, an aliquot of each epimer

Table. Schedule for solid-phase synthesis.

	Reagent[a]	Time/repeat
Wash	1 CH_2Cl_2	2 min × 3
	2 DMF	2 min × 3
Deprotection	3 20% Piperidine–DMF	2 min × 1
		30 min × 1
Wash	4 DMF	2 min × 3
	5 CH_2Cl_2	2 min × 3
	6 DMF	2 min × 3
Coupling	7 Fmoc-amino acid-OPfp + HOBT[b]	90 min × 1
Wash	8 DMF	2 min × 3
	9 CH_2Cl_2	2 min × 3
	10 DMF	2 min × 3

[a] Solvent volume 15 ml/g resin. [b] In the case of 2,6-dibromo-DL-tyrosine, Boc-2,6-dibromo-DL-tyrosine + BOP reagent + DIPEA.



Scheme 2. *Reagents and conditions:* i, TFA–CH_2Cl_2–anisole (5:4:1); ii, epimeric resolution; iii, $^3H_2$/palladium black.

was catalytically hydrogenated in the presence of palladium black. The retention time-values of these two reduction products in HPLC were almost identical, and were identical with that of authentic leucine-enkephalin. However, the reduction product from epimer (**6B**) was completely hydrolysed with aminopeptidase M (EC 3.4.11.2), whereas the reduction product obtained from epimer (**6A**) was not hydrolysed and remained intact. These results showed that epimers (**6A**) and (**6B**) were identified with [2,6-dibromo-D-Tyr[1]]leucine-enkephalin and its (L)-epimer, respectively.

Tritiated leucine-enkephalin was obtained by reduction of the [2,6-dibromo-Tyr[1]]leucine-enkephalin (**6B**) with tritium gas in the presence of palladium black. After removal of the labile tritium, the preparation was purified by HPLC. The product was chromatographically identical with authentic non-labelled leucine-enkephalin. Radio-HPLC of the product revealed a high radiochemical purity of 99%. The distribution of the tritium label among the amino acid residues was investigated using a method essentially identical with those previously developed.[8] HPLC analysis of the Fmoc amino acid derivatives following enzymatic hydrolysis demonstrated that the label had been located exclusively at the tyrosine residue, as shown in Figure 2. No evidence was found for non-specific exchange labelling into other residues.

The product had a specific radioactivity of 1.37 TBq/mmol. The low incorporation of tritium compared with the theoretical (2.17 TBq/mmol) is probably due to the relatively slow cleavage

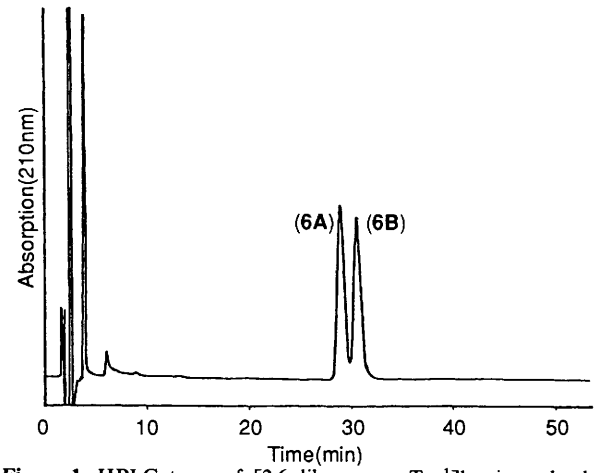


Figure 1. HPLC trace of [2,6-dibromo-DL-Tyr[1]]leucine-enkephalin. Column: LiChrospher RP-18 (4 × 250 mm). Eluant: water–MeCN–TFA (75:25:0.1), 1 ml/min. Detection: λ 210 nm. (**6A**) = [2,6-dibromo-D-Tyr[1]]leucine-enkephalin, (**6B**) = [2,6-dibromo-L-Tyr[1]]leucine-enkephalin.

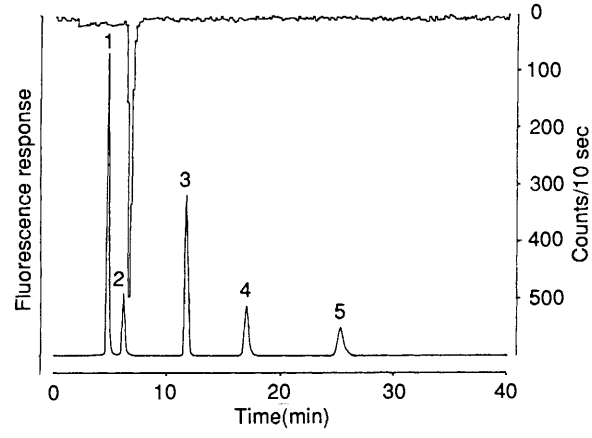


Figure 2. HPLC trace of the Fmoc amino acid derivatives following digestion of [2,6-$^3H_2$-Tyr[1]]leucine-enkephalin with aminopeptidase M. Column: LiChrospher RP-18 (4 × 250 mm). Eluant: 10mM-acetate buffer (pH 4.0)–MeCN–MeOH (50:35:15), 1 ml/min. Detection: fluorescence (excitation 260 nm, emission 313 nm) and radioisotope detector. (1) = Fmoc-Gly, (2) = Fmoc-Tyr, (3) = Fmoc-OH, (4) = Fmoc-Phe, (5) = Fmoc-Leu.

rate of carbon–bromine bonds which allows significant tritium–protium exchange to precede debromination. The effect may be aggravated by released bromide poisoning the catalyst.

Experimental

All m.p.s were determined on a Yamato melting point apparatus and were uncorrected. ^1H NMR spectra were determined on a Varian EM-390 (90 MHz) spectrometer, with tetramethylsilane as internal standard, or on a Bruker AM-400 (400 MHz) spectrometer; the $CHCl_3$ signal was set at δ 7.26, the HDO signal at δ 4.8, and the Me_2SO signal at δ 2.60. Amino acid analysis was performed on a Hitachi L-8500 amino acid analyser. Peptides were hydrolysed with 6M-HCl in a sealed evacuated tube at 110 °C for 22 h. The liquid scintillation counter was an Aloka LSC-900. The two HPLC systems used were as follows. Analytical and preparative HPLC of the non-labelled compounds were performed on a Waters M600 multi-

solvent delivery system and a Waters 481 λ_{max} variable-wavelength UV detector. For analysis of the labelled compounds, the system consisted of a Shimadzu LC-3 pump, a Shimadzu SPD-6A variable-wavelength UV detector, a Shimadzu RF-535 fluorescence detector, and an Aloka RLC-701 radioisotope detector with 1 ml flow cell. Analytical HPLC was carried out on a LiChrospher RP-18 column (5 μm; 4 × 250 mm; E. Merck) in the following solvent systems (all v/v): (A) water–acetonitrile–TFA (75:25:0.1); (B) water–acetonitrile–TFA (70:30:0.1); (C) 10mM-acetate buffer (pH 4.0)–acetonitrile–methanol (50:35:15). The flow rate was 1 ml/min. Preparative HPLC was carried out on a μ-Bondasphere C-18 column (5 μm; 100 Å; 19 × 150 mm; Waters) in solvent system (D); water–acetonitrile–TFA (77:23:0.1). The flow rate was 20 ml/min.

2,6-Dibromo-4-methoxytoluene was purchased from Lancaster Synthesis (Lancaster, UK). Fluoren-9-ylmethoxycarbonyl (Fmoc) amino acid pentafluorophenyl (Pfp) ester derivatives were purchased from Cambridge Research Biochemicals (Cambridge, UK). Fmoc-Leu *p*-benzyloxybenzyl ester resin (0.37 mmol Fmoc-Leu/g resin; styrene–1% divinylbenzene) was purchased from Kokusan Chemical Works Co. (Tokyo, Japan). Leucine-enkephalin was purchased from Peptide Institute (Osaka, Japan). Aminopeptidase M (EC 3.4.11.2) was purchased from Sigma (St. Louis, MO, USA).

2,6-*Dibromo*-4-*methoxybenzyl Bromide* (**2**).—To a solution of 2,6-dibromo-4-methoxytoluene (**1**) (4.85 g, 17.24 mmol) in carbon tetrachloride were added NBS (8.08 g, 45.22 mmol) and benzoyl peroxide (15 mg). The reaction mixture was refluxed under nitrogen and irradiated for 4 h with a 400 W high-pressure mercury lamp with a Pyrex filter sleeve. The succinimide was removed by filtration and washed with carbon tetrachloride. The combined filtrate and washings were washed successively with saturated aq. $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated to give a *yellow product* (**2**) (5.31 g, 86%), m.p. 96.5–97.5 °C (Found: C, 26.9; H, 1.9. $C_8H_7Br_3O$ requires C, 26.78; H, 1.96%); δ(90 MHz; $CDCl_3$) 3.79 (3 H, s, OMe), 4.81 (2 H, s, CH_2Br), and 7.11 (2 H, s, ArH); *m/z* 356, 358, 360, and 362 (1:3:3:1, M^+).

Diethyl 2-*Acetamido*-2-(2′,6′-*dibromo*-4′-*methoxybenzyl*)-*malonate* (**3**).—Diethyl acetamidomalonate (6.20 g, 28.5 mmol) was added to a stirred solution of sodium ethoxide [sodium (0.66 g, 28.6 mmol) in absolute EtOH (50 ml)]. A solution of compound (**2**) (10.27 g, 28.53 mmol) in dry benzene (30 ml) was added dropwise during 15 min to the mixture at 0 °C. After being stirred for 12 h at room temperature, the reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water, extracted with $CHCl_3$, and the extract was dried over Na_2SO_4. The $CHCl_3$ was removed to give a yellow residue. After silica gel column chromatography of the residue with $CHCl_3$–ethyl acetate (5:1) as eluting solvent, the purified *product* (**3**) (9.65 g, 68%) was obtained as white crystals following evaporation of the solvent under reduced pressure; m.p. 158–158.5 °C (Found: C, 41.1; H, 4.2; N, 2.8. $C_{17}H_{21}Br_2NO_6$ requires C, 41.24; H, 4.27; N, 2.83%); δ(400 MHz; $CDCl_3$) 1.26 (6 H, t, *J* 7.1 Hz, 2 × CH_2*Me*), 1.98 (3 H, s, COMe), 3.77 (3 H, s, OMe), 4.01 (2 H, s, CH_2), 4.25 (4 H, q, *J* 7.1 Hz, 2 × C*H*$_2$Me), and 7.08 (2 H, s, ArH); *m/z* 495 (M^+).

2,6-*Dibromo*-DL-*tyrosine* (**4**).—A solution of compound (**3**) (2.13 g, 4.3 mmol) in 47% HBr (20 ml) was refluxed for 4 h. After cooling, the solution was neutralized with 30% NH_4OH and was left at 0 °C overnight. The precipitate was collected and washed with water to give the *title compound* (**4**) (1.36 g, 93%), m.p. 253–257 °C (decomp.) (Found: C, 30.5; H, 3.2; N, 3.9. $C_9H_9Br_2NO_3 \cdot H_2O$ requires C, 30.28; H, 3.10; N, 3.92%); δ(400 MHz; 0.1M-NaOD) 3.02 (1 H, dd, *J* 9.4 and 14 Hz, β-CH_2), 3.18 (1 H, dd, *J* 5.9 and 14 Hz, β-CH_2), 3.58 (1 H, dd, *J* 5.9 and 9.4, α-CH), and 6.83 (2 H, s, ArH).

N-*t*-*Butoxycarbonyl*-2,6-*dibromo*-DL-*tyrosine* (**5**).—To a solution of the free amino acid (**4**) (4.07 g, 12.0 mmol) and triethylamine (4 ml, 28.7 mmol) in water (50 ml) was added dropwise a solution of 2-t-butoxycarbonyl-2-phenylacetonitrile (3.67 g, 14.5 mmol) in dioxane (50 ml). After being stirred for 1 h at 0 °C and for 4 h at room temperature, the solution was concentrated to *ca.* 10 ml under reduced pressure. Water and ethyl acetate were added. The aqueous phase was separated, washed with ethyl acetate, acidified with 10% citric acid to pH 2–3, and extracted with ethyl acetate. The ethyl acetate extracts were washed with water, dried over $MgSO_4$, and evaporated under reduced pressure to give *compound* (**5**) (2.98 g, 57%), m.p. 203–205 °C (Found: C, 38.4; H, 4.1; N, 3.2. $C_{14}H_{17}Br_2NO_5$ requires C, 38.29; H, 3.90; N, 3.19%); δ[400 MHz; $(CD_3)_2SO$] 1.31 (9 H, s, Me_3C), 3.09 (1 H, dd, *J* 8.7 and 14.0 Hz, β-CH_2), 4.16 (1 H, m, α-CH), 6.91 (1 H, d, *J* 8.2 Hz, NH), and 7.00 (2 H, s, ArH); *m/z* 439 (M^+).

[2,6-*Dibromo*-*Tyr*^{1}]*leucine-enkephalin* (**6B**).—The solid-phase synthesis was carried out manually. Fmoc-Leu *p*-benzyloxybenzyl ester resin (1 g; 0.37 mmol Fmoc-Leu/g resin; styrene–1% divinylbenzene) was loaded into a solid-phase synthesis vessel. The pentapeptide was built up according to the sequence steps shown in the Table, using each Fmoc-amino acid Pfp ester (1.5 mmol) in the presence of 1-hydroxybenzotriazole (HOBT) (0.37 mmol), except that Boc-2,6-dibromo-DL-tyrosine (1 mmol) was coupled to the growing peptide chain by means of BOP reagent (1 mmol) and di-isopropylethylamine (DIPEA) (1 mmol). Coupling reactions were monitored by the ninhydrin test[9] and no recoupling was necessary. The pentapeptide was cleaved from the resin by treatment with TFA–CH_2Cl_2–anisole (5:4:1) (15 ml) for 1 h at room temperature. After filtration, the resin was washed with MeOH. The filtrate and washings were combined, evaporated, redissolved in water, and lyophilysed to give peptide (**6**) (240 mg, 91% based on Fmoc-Leu resin). An aliquot of peptide (**6**) was analysed with HPLC in solvent system (A) and monitored at λ 210 nm to show epimeric resolution (Figure 1). The epimers of peptide (**6**) were purified by preparative HPLC to give the L-epimer (**6B**) (54 mg) with no trace (<0.1%) of the D-epimer (**6A**). Amino acid analysis gave the following molar preparations: 2,6-Br_2-Tyr, not detectable (Tyr, not detected); Gly, 2.00; Phe, 1.15; Leu, 1.03.

Identification of the Configuration at Tyrosine Residue of Epimer (**6A**) *and* (**6B**).—Each epimer (**6A**) and (**6B**) (100 μg) in methanol (100 μl) was reduced with hydrogen in the presence of palladium black (1 mg) for 1.5 h. An aliquot of each reduction product was analysed by analytical HPLC in solvent system (A) and monitored at λ 210 nm. The retention time-values of these reduction products in HPLC were almost identical, and were identical with that of authentic leucine-enkephalin.

Each of the remaining mixtures was evaporated. The resulting material was redissolved in water, treated with aminopeptidase M (25 m-unit) at room temperature for 12 h, and subjected to analytical HPLC in solvent system (A) and monitored at λ 210 nm. The reduction product from epimer (**6B**) was completely hydrolysed with the enzyme (thus the L-Tyr compound), whereas the reduction product from epimer (**6A**) was not hydrolysed, and remained intact (thus the D-Tyr compound).

[2,6-$^3H_2$-*Tyr*^{1}]*Leucine-enkephalin*.—[2,6-Dibromo-Tyr^1]-leucine-enkephalin (**6B**) (5 mg, 7.0 μmol) was dissolved in MeOH (1 ml) and reduced with tritium gas (2.59 TBq) in the

presence of palladium black (5 mg) for 5 h (Tritium Labelling Service, New England Nuclear Co., MA, USA). After filtration, the labile tritium was removed by successive flash evaporations in MeOH. The tritiated product was applied to a column of LiChrospher RP-18 with solvent system (B). The eluate was monitored at λ 210 nm. The main fraction (t_R 12.2–12.8 min) was collected and evaporated to dryness at <30 °C to yield [^{3}H]leucine-enkephalin (1.54 μmol, 22%; 1.37 TBq/mmol).

Distribution of Tritium Label.—The [^{3}H]leucine-enkephalin (2 300 Bq) was diluted with non-labelled leucine-enkephalin (20 nmol) and incubated with aminopeptidase M (2 μg, 50 m-unit) in water (120 μl) at 25 °C for 4 h. To the reaction mixture (50 μl) were added 10mM-borate buffer (pH 9.5) (500 μl) and a solution of 1mM-Fmoc-Cl in acetone (100 μl). The mixture was stirred at room temperature for 1 min, then washed with pentane (1 ml × 2) to remove excess of Fmoc-Cl reagent. The aqueous phase was acidified with 1M-HCl (20 μl) and extracted with ethyl acetate (1 ml × 3). The extracts were evaporated under a stream of N_2, redissolved with MeOH (100 μl), and subjected to analytical HPLC with solvent system (C) and monitored at fluorescence (excitation 260 nm, emission 313 nm) and with a radioisotope detector. HPLC analysis of Fmoc amino acid derivatives demonstrated that the label had been located exclusively at Fmoc-Tyr, as shown in Figure 2.

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