Synthesis of [D-Ala²]Leu-enkephalin and [D-Ala², D-Leu⁵]Leu-enkephalin with High Specific Tritiated Activity in the Leucine Residue

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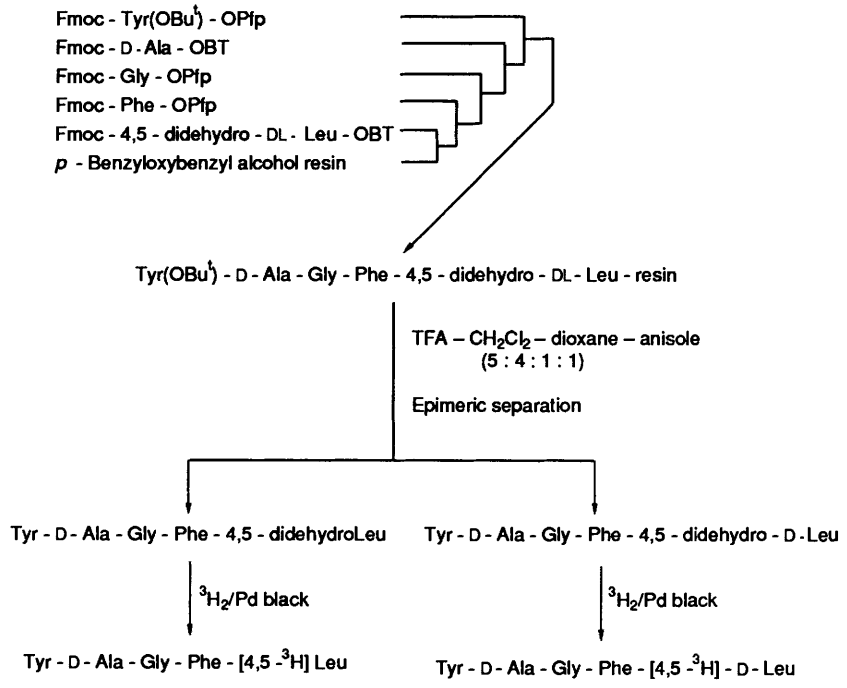
[D-Ala²]Leu-enkephalin [DALE] and [D-Ala², D-Leu⁵]Leu-enkephalin (DADLE) labelled with tritium in the leucine residue have been prepared. Synthesis of the precursor peptides, [D-Ala², 4,5-didehydro-L-Leu⁵]Leu-enkephalin and [D-Ala², 4,5-didehydro-D-Leu⁵]Leu enkephalin, was carried out by solid-phase synthesis using Fmoc amino acid derivatives, followed by diastereoisomeric separation on HPLC. These peptides were tritiated catalytically to yield [³H]DALE with a specific activity of 5.35 TBq mmol^{-1} and [³H]DADLE with that of 5.43 TBq mmol^{-1}, respectively. The distribution of tritium label was investigated by HPLC with a radioisotope detector following acidic hydrolysis, which confirmed that the tritium label in both labelled peptides was exclusively located at the leucine residue.

The leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu, Leu-enk) is a natural endogenous ligand for opiate receptors. Leu-enk is rapidly metabolized *in vivo* and *in vitro*.[1] Many analogues of Leu-enk have been synthesized and tested for morphine-like activity and for stability. Replacements of Gly at position 2 by D-Ala, [D-Ala²]Leu-enk (DALE), together with substitution of Leu at position 5 by D-Leu, [D-Ala², D-Leu⁵]Leu-enk (DADLE) have yielded very stable and more potent pentapeptides.[2] The DALE and DADLE labelled with tritium to high specific radioactivity were required for receptor-binding studies and metabolic studies. Commercially available [³H-Tyr¹]DALE and [³H-Tyr¹]DADLE do not have sufficiently high specific radioactivity. Furthermore, these labelled peptides cannot be used as substrates to elucidate the *C*-terminal fragments. The present paper describes the synthesis of specifically tritiated DALE and DADLE with high radioactivity in the Leu residue.

The synthetic routes to [³H-Leu⁵]DALE and [³H-Leu⁵]DADLE are illustrated in Scheme 1. [D-Ala², 4,5-didehydro-DL-Leu⁵]Leu-enk, the precursor peptide, was prepared by solid-phase synthesis using Fmoc-amino acids.* Fmoc-4,5-didehydro-DL-Leu was prepared from *N*-acetyl-4,5-didehydro-DL-leucine[3] by treatment with 2M-LiOH followed by fluoren-9-ylmethyl chloroformate.[4] Attachment of Fmoc-4,5-didehydro-DL-Leu to *p*-benzyloxybenzyl alcohol resin[5] was carried out with the active-ester method using 1-hydroxybenzotriazole in the presence of 4-dimethylaminopyridine (DMAP) according to the procedure of Meienhofer *et al.*[6] The 4,5-didehydro-DL-Leu content anchored to the resin was estimated spectrophotometrically[6] and was found to be 0.59 mmol g^{-1} resin. Each coupling of Fmoc-Phe, Fmoc-Gly, Fmoc-D-Ala and Fmoc-Tyr(OBu^t) was carried out by the

* Fmoc = fluoren-9-ylmethoxycarbonyl.



Scheme 1.

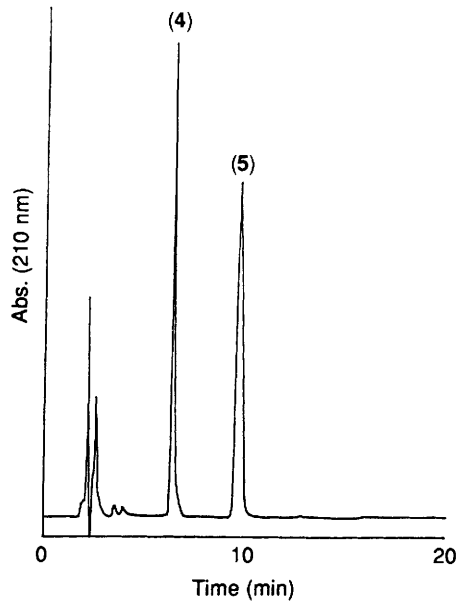


Figure 1. HPLC tracing of [D-Ala^2, 4,5-didehydro-DL-Leu^5]Leu-enkephalin. Column: LiChrospher RP-18 (4 × 250 mm). Elution: water–MeCN–TFA (70:30:0.1), 1 ml min^{-1}. Detection: UV 210 nm. (**4**) = [D-Ala^2, 4,5-didehydro-L-Leu^5]Leu-enkephalin, (**5**) = [D-Ala^2, 4,5-didehydro-D-Leu^5]Leu-enkephalin.

pentafluorophenyl (Pfp) ester procedure [7] or benzotriazole ester procedure until a negative ninhydrin test [8] was obtained. In all cases, no recoupling was necessary.

(**1**) 4,5-Didehydro-DL-Leu
(**2**) Fmoc-4,5-didehydro-DL-Leu
(**3**) [D-Ala^2,4,5-didehydro-DL-Leu^5]Leu-enk
(**4**) [D-Ala^2,4,5-didehydro-L-Leu^5]Leu-enk
(**5**) [D-Ala^2,4,5-didehydro-D-Leu^5]Leu-enk
(**6**) [^{3}H]DALE
(**7**) [^{3}H]DADLE

The protected pentapeptide resin was treated with trifluoroacetic acid (TFA)–CH_2Cl_2–1,4-dioxane–anisole (5:4:1:1) to give an epimeric peptide, [D-Ala^2, 4,5-didehydro-L-Leu^5]Leu-enk (**4**) and [D-Ala^2, 4,5-didehydro-D-Leu^5]Leu-enk (**5**). Analytical HPLC of the racemate showed excellent separation, as shown in Figure 1 [(**4**) t_R 6.6 min; (**5**) t_R 9.9 min]. The racemate was purified and resolved into its epimers by preparative HPLC. The ^{1}H NMR spectra of the two peptides were almost identical, and showed a singlet signal at δ 1.66 [γ-Me protons of 4,5-didehydroLeu residue) and a doublet signal at δ 4.74 (vinylic protons of 4,5-didehydroLeu residue). Amino acid analyses of the peptides gave good results for all amino acids except 4,5-didehydroLeu. To explore the configuration at 4,5-didehydroLeu residue, an aliquot of each epimer was catalytically hydrogenated in the presence of palladium black. Each epimer gave a single reduction product which was identical with authentic DALE and DADLE, respectively, by HPLC. These results showed that compounds (**4**) and (**5**) were identical with [D-Ala^2, 4,5-didehydro-L-Leu^5]Leu-enk and [D-Ala^2, 4,5-didehydro-D-Leu^5]Leu-enk, respectively.

Tritiated DALE was obtained by reduction of the [D-Ala^2, 4,5-didehydro-L-Leu^5]Leu-enk (**4**) with tritium gas in the presence of palladium black. After removal of the labile tritium, the preparation was purified by HPLC to give [^{3}H-Leu^5]DALE (41% yield). Radio-HPLC of the product revealed a high radiochemical purity (99%). The distribution of the tritium label among the amino acid residues was investigated using the method essentially identical with those previously developed.[9] HPLC analysis of the Fmoc-amino acid derivatives following acidic hydrolysis demonstrated that the label had been located exclusively at the leucine residue. No evidence was found for non-specific exchange labelling into other residues.

[^{3}H-Leu]DADLE (**7**) was prepared from [D-Ala^2, 4,5-didehydro-D-Leu^5]Leu-enk (**5**) by the same manner as described above. The product (40% yield) had a radiochemical purity higher than 99%. The tritium was exclusively located at the D-leucine residue.

The products showed extremely high radioactivity: 5.35 TBq mmol^{-1} for DALE and 5.43 TBq mmol^{-1} for DADLE. It was demonstrated that almost five tritium atoms were incorporated into each molecule of Leu residue in the products. Such a high specific radioactivity was also reported by Hardy *et al.*[10] in the tritiation of [4,5-didehydroLeu^2]locust adipokinetic hormone. 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.[11] We concluded that from 1–7 tritiums (average 5) were incorporated into the δ-H^3 groups and γ-methinyl group of leucine residue.

The present procedure provides a simple method for the synthesis of selectively tritium-labelled peptide with high specific radioactivity.

Experimental

^{1}H NMR spectra were determined on a Bruker AM-500 (500 MHz) spectrometer, setting the HDO signal at δ 4.80 and Me_2SO signal at δ 2.60. Amino acid analyses were 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-1000. The two HPLC systems were used as follows. Analytical and preparative 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)[12] having five counting cells (cell volume 1.0 ml) and five pairs of photoelectron multiplier tubes. Analytical HPLC was carried out on a LiChrospher RP-18 column (5 μm; 4 × 25 mm; E. Merck) in the following systems (all v/v): (A) water–acetonitrile–TFA, 70:30:0.1; (B) 10 mM-acetate buffer (pH 4.0)–acetonitrile–MeOH, 50:35:15. The flow rate was 1 ml min^{-1}. Preparative HPLC was carried out on a μ-Bondasphere C-18 (5 μm; 100 A; 19 × 150 mm; Waters) in solvent system (A), with a flow rate of 10 ml min^{-1}.

Fluoren-9-ylmethyloxycarbonyl (Fmoc-) amino acid pentafluorophenyl (Pfp) ester derivatives were purchased from Cambridge Research Biochemicals (Cambridge, UK). *p*-Benzyloxybenzyl alcohol resin (0.7 mequiv. OH g^{-1} resin; polystyrene with 1% divinylbenzene copolymer) was purchased from Kokusan Chemical Works Co (Tokyo, Japan). [D-Ala^2, D-Leu^5]Leu-enkephalin (DADLE) was purchased from Peptide Institute (Osaka, Japan). [D-Ala^2]Leu-enkephalin (DALE) was purchased from Sigma (St. Louis, MO, USA).

4,5-*Didehydro*-DL-*leucine* (**1**).—A solution of *N*-acetyl-4,5-didehydro-DL-leucine (4.28 g, 25 mmol) in 2M-LiOH (50 ml) was refluxed for 12 h. After cooling, the precipitate was removed and the filtrate was neutralized with acetic acid. The solution was evaporated under reduced pressure. EtOH (10 ml) was added to the residue and the mixture was left at 0 °C overnight. The

Table. Schedule for solid-phase synthesis.

	Reagent*	Time/repeat
Wash	1 CH_2Cl_2	2 min × 3
	2 DMF	2 min × 3
Deprotection	3 20% Piperidine in 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 deriv.	90 min × 1
Wash	8 DMF	2 min × 3
	9 CH_2Cl_2	2 min × 3
	10 DMF	2 min × 3

* Solvent volume 15 ml g^{-1} resin.

precipitate was collected and washed with EtOH to give compound (**1**) (2.14 g, 66%), m.p. 200 °C (decomp.) (Found: C, 55.7; H, 8.6; N, 10.6. $C_6H_{11}NO_2$ requires C, 55.79; H, 8.58; N, 10.85%); δ(500 MHz; D_2O) 1.76 (3 H, s, δ-H_3), 2.48 (1 H, dd, *J* 9.5 and 14.5 Hz, β-C*H*H), 2.67 (1 H, dd, *J* 4.4 and 14.5 Hz, β-CH*H*), 3.82 (1 H, dd, *J* 4.4 and 9.5 Hz, α-CH), and 4.89 and 4.99 (each 1 H, s, together =CH_2).

*Fmoc-4,5-didehydro-*DL*-leucine* (**2**).—To a stirred solution of compound (**1**) (1.94 g, 15 mmol) in a mixture of 10% aq. Na_2CO_3 (45 ml) and dioxane (30 ml) at 0 °C was added a solution of fluoren-9-ylmethyl chloroformate (3.88 g, 15.0 mmol) in dioxane (30 ml) during 30 min. After being stirred for 1 h at 0 °C and for 3 h at room temperature, the solution was poured into water. The resulting solution was washed with Et_2O, acidified with 5% aq. $KHSO_4$ to pH 2–3, and extracted with ethyl acetate. The extracts were washed with water, dried over $MgSO_4$, and evaporated under reduced pressure to give *compound* (**2**) (4.83 g, 91.6%), m.p. 137.5–139 °C (Found: C, 71.5; H, 6.1; N, 4.0. $C_{21}H_{21}NO_4$ requires C, 71.78; H, 6.02; N, 3.99%); *m/z* 351 (M^+).

Fmoc-amino Acid Benzotriazole Ester (*Fmoc-amino Acid-OBT*).—To a stirred solution of Fmoc-amino acid (2.5 mmol) and 1-hydroxybenzotriazole (2.5 mmol) in a mixture of CH_2Cl_2–dimethylformamide (DMF) (5:1) (20 ml) at 0 °C was added *N*,*N*′-dicyclohexylcarbodi-imide (2.5 mmol). The mixture was stirred for 1 h at 0 °C and for 1 h at room temperature. The precipitated urea was removed by filtration and washed with CH_2Cl_2. The combined filtrate and washings were concentrated under reduced pressure, redissolved in DMF (15 ml), and applied to the following peptide synthesis.

*Fmoc-4,5-didehydro-*DL*-leucine* p-*Benzyloxybenzyl Ester Resin.*—*p*-Benzyloxybenzyl alcohol resin (0.7 g) was washed successively with CH_2Cl_2 (15 ml × 3) and DMF (15 ml × 3) in a reaction vessel. A solution of Fmoc-4,5-didehydro-DL-leucine-OBT (2.5 mmol) in DMF (10 ml) and a solution of DMAP (61 mg, 0.5 mmol) in DMF (2 ml) were added to the reaction vessel and the mixture was stirred for 2 h. After filtration, the resin was washed successively with DMF (15 ml × 3), CH_2Cl_2 (15 ml × 3), and DMF (15 ml × 3). Fmoc-4,5-didehydro-DL-leucine was recoupled and then the esterified resin was washed successively with DMF (15 ml × 6) and CH_2Cl_2 (15 ml × 6), and then dried (P_2O_5) *in vacuo*. Fmoc-4,5-didehydro-DL-leucine p-benzyloxybenzyl ester resin (*ca.* 10 mg) 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 7 800 l mol^{-1} cm^{-1}) of the combined filtrates was measured to show a substitution of 0.59 mmol Fmoc-4,5-didehydro-DL-leucine g^{-1} resin.

[D-*Ala*^{2}, 4,5-*didehydro*-L-*Leu*^{5}]*Leu-enkephalin* (**4**) *and* [D-*Ala*^{2}, 4,5-*didehydro*-D-*Leu*^{5}]*Leu-enkephalin* (**5**).—The pentapeptide assembly was carried out manually. The protocol is shown in the Table. Fmoc deprotection was achieved by treatment with 20% piperidine in DMF. Coupling reactions were carried out with Fmoc-amino acid pentafluorophenyl ester (5 mol equiv.) in the presence of 1-hydroxybenzotriazole (0.6 mmol), except that Fmoc-D-Ala was coupled to the growing peptide chain by utilization of the corresponding benzotriazole ester. Coupling reactions were monitored using the ninhydrin test[8] and no recoupling was necessary.

After the removal of the *N*-terminal Fmoc group, the protected resin was dried (P_2O_5) *in vacuo* for 12 h. The pentapeptide resin was treated with TFA–CH_2Cl_2–dioxane–anisole (5:4:1:1) (10 ml) for 1 h at room temperature. After filtration, the resin was washed with MeOH. The combined filtrates and washings were evaporated at < 30 °C, redissolved in water, and lyophilized to give compound (**3**) (186 mg, 80%). An aliquot of product (**3**) was analysed by HPLC in solvent system (A) and monitored at UV (210 nm) to show epimeric resolution (Figure 1).

The epimers (**3**) were purified by preparative HPLC to afford the purified [D-Ala^2, 4,5-didehydro-L-Leu^5]Leu-enkephalin (**4**) (63 mg) and [D-Ala^2, 4,5-didehydro-D-Leu^5]Leu-enkephalin (**5**) (57 mg). Each epimer gave the following amino acid analyses after hydrolysis by 6M-HCl at 110 °C for 22 h: Tyr, 0.99 (1); Ala, 1.00 (1); Gly, 1.00 (1); Phe, 1.01 (1); didehydroLeu, not detectable (1); and Tyr, 0.98 (1); Ala, 1.00 (1); Gly, 1.00 (1); Phe, 1.01 (1); didehydroLeu, not detectable (1), respectively.

Each epimer (1 mg) was dissolved in MeOH and reduced with hydrogen in the presence of palladium black (1 mg) for 2 h to give the corresponding reduction products, which were identical with authentic DALE and DADLE, respectively, by analytical HPLC (mobile phase: A, detection: UV 210 nm) and amino acid analyses.

Tritiation and Purification.—[D-Ala^2, 4,5-didehydro-L-Leu^5]Leu-enkephalin (**4**) (5 mg, 9 μmol) was dissolved in MeOH (2 ml) and reduced with tritium gas (2.96 TBq) in the presence of palladium black (10 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 purified by HPLC on an analytical column (mobile phase: A, detection: UV 210 nm), and was chromatographically identical with authentic DALE. The solvent was evaporated off at < 30 °C. The purified [^{3}H]DALE (**6**) (3.6 μmol, 41%; 5.35 TBq $mmol^{-1}$) was stored in MeOH (10 ml) at 0 °C.

[^{3}H]DADLE (**7**) was prepared from [D-Ala^2, 4,5-didehydro-D-Leu^5]Leu-enkephalin (**5**) by the same manner as described above. The purified [^{3}H]DADLE (**7**) (3.5 μmol, 40%; 5.43 TBq $mmol^{-1}$) was stored in MeOH (10 ml) at 0 °C.

Distribution of Tritium Label.—Compound (**6**) (4 kBq) was diluted with non-labelled DALE and hydrolysed by 6M-HCl (1 ml) at 110 °C for 22 h. The solvent was evaporated off under reduced pressure, and the residue was redissolved with 10 mM-borate buffer (pH 9.5) (500 μl) and treated with a solution of 10 mM-Fmoc-Cl in acetone (100 μl). The mixture was stirred at room temperature for 1 min, then washed with pentane (1 ml × 2). The aqueous phase was acidified with 1M-HCl (20 μl) and extracted with ethyl acetate (1 ml × 3). The extracts were evaporated under a N_2 stream, and the residue was redissolved with MeOH (100 μl) and subjected to analytical HPLC with solvent system (B) and monitored by fluorescence detection (excitation; 260 nm, emission; 313 nm) and SARD.

Distribution of tritium label in compound (**7**) was determined analogously.

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