Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXVIII, No. 9

Synthesis of [D-Ala^2,D-Leu^5]enkephalin tritiated in the 2,6-positions of tyrosine

Hiroshi Hasegawa*, Yoshihiko Shinohara and Shigeo Baba

School of Pharmacy, Tokyo University of Pharmacy and Life Science

1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

SUMMARY

[D-Ala^2, D-Leu^5]Enkephalin (DADLE) labelled with tritium in the 2,6-positions of *N*-terminus tyrosine residue has been prepared. Synthesis of the precursor peptide, [2,6-dibromo-Tyr^1]DADLE, was performed by solid phase synthesis using Fmoc strategy. The peptide was tritiated catalytically to yield [2,6-^{3}H-Tyr^1]DADLE with a specific radioactivity of 1.61 TBq/mmol. The distribution of the tritium was investigated by HPLC with radioisotope detection following hydrolysis with aminopeptidase M, and confirmed that the label was entirely located at the tyrosine residue.

Key Words: 2,6-dibromo-tyrosine, [D-Ala^2,D-Leu^5]enkephalin, tritium labelling, tritium-bromine exchange reaction

INTRODUCTION

Leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu, Leu-enk) is a natural endogenous ligand for opiate receptors. Leu-enk is rapidly metabolized *in vitro* (1-6) and *in vivo* (3,7,8). Replacement of Gly at position 2 by D-Ala together with substitution of Leu by D-Leu, [D-Ala^2, D-Leu^5]enkephalin (Tyr-D-Ala-Gly-Phe-D-Leu, DADLE), gained more stability for enkephalin-hydrolyzing enzymes against native enkephalins (6,9-12). Su et al. reported that commercial available [3,5-^{3}H-Tyr^1]DADLE administered intravenously to rat was rapidly metabolized to [^{3}H]Tyr and [^{3}H]water (13). However, it is not obvious whether [^{3}H]water was generated from [3,5-^{3}H-Tyr^1]DADLE by hydrogen-tritium exchange reaction or from [^{3}H]Tyr in the

CCC 0362–4803/96/090825–09

Received 21 February 1996
Revised 14 May 1996

subsequent degradation. If [^{3}H]water was directly generated from [3,5-^{3}H-Tyr^1]DADLE, it would be difficult to estimate the pharmacokinetics of [3,5-^{3}H-Tyr^1]DADLE.

We reported previously the tritium labelling method for 2,6-positions of tyrosine residue of peptide (14). These positions would be chemically and metabolically more stable than the 3,5-positions (15-17). In the present study, we describe the synthesis of tritium labelled DADLE in the 2,6-positions of tyrosine residue.

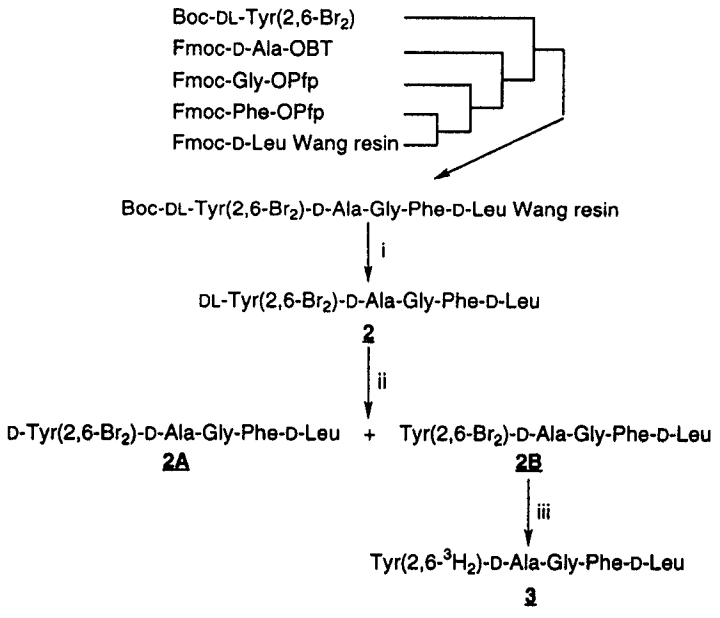
RESULTS AND DISCUSSION

The synthetic route to [2,6-^{3}H-Tyr^1]DADLE **3** is illustrated in Scheme 1. [2,6-dibromo-DL-Tyr^1]DADLE **2** was prepared by solid phase synthesis using Fmoc-strategy (Fmoc = 9-fluorenylmethyloxycarbonyl). Starting with Fmoc-D-Leu *p*-benzyloxybenzyl ester resin (Fmoc-D-Leu Wang resin), the peptide chain elongated manually, according to the sequence steps shown in the Table 1, *i.e.*, removal of Fmoc-group by 20% piperidine in dimethylformamide (DMF) and condensation of the respective Fmoc- amino acids by activated ester procedure using pentafluorophenyl ester (18) and benzotriazole ester. The exception to these procedures was in the

Table 1 Schedule for solid phase synthesis (see Scheme 1)

		Reagent[a]	Time	x	Repeat
Wash	1	CH_2Cl_2	1 min	x	3
	2	DMF	1 min	x	3
Deprotection	3	20% Piperidine/DMF	2 min	x	1
	4	20% Piperidine/DMF	30 min	x	1
Wash	5	DMF	1 min	x	3
	6	CH_2Cl_2	1 min	x	3
	7	DMF	1 min	x	3
Coupling	8	Fmoc-amino acid derivative + coupling reagents	90 min	x	1
Wash	9	DMF	1 min	x	3
	10	CH_2Cl_2	1 min	x	3
	11	DMF	1 min	x	3

[a] Solvent volume 15 ml



Scheme 1. *Reagents and conditions*: i, 90% TFA/H_2O; ii, diastereomeric separation on reversed-phase HPLC [column, μ-Bondasphere C-18; elution, H_2O-MeCN-TFA (67:33:0.1), flow rate, 11 ml min^{-1}; detection, λ 210 nm]; iii, $^3H_2$, Pd black, MeOH

coupling of *N*-terminal residue, 2,6-dibromo-DL-tyrosine **1**, that was coupled as its *N*-*tert*-butyloxycarbonyl (Boc) derivative by the benzotriazolyloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP) procedure (19).

The protected pentapeptide Wang resin was treated with 90% trifluoroacetic acid (TFA) in H_2O to yield an epimeric peptide **2**. Analytical HPLC of the racemate **2** showed excellent separation, as shown in Figure 1 (**2A**: t_R 14.6 min, **2B**: t_R 18.3 min). The racemate was purified and resolved into the epimers by preparative HPLC. To explore the configuration at 2,6-dibromo-tyrosine residue, an aliquot of each epimer was catalytically hydrogenated in the presence of palladium black. Each epimer gave a single reduction product, and the reduction product from **2B** was identical with authentic DADLE by HPLC. These results showed that pentapeptide **2A** and **2B** were identified with [2,6-dibromo-D-Tyr¹]DADLE and its L-epimer, respectively. The assignment of all protons in the pentapeptide **2B** was established by a combination of 2D-NMR experiments (COSY, HOHAHA and NOESY) with the sequence specific assignment procedure introduced by Wüthrich (20)(Table 2).

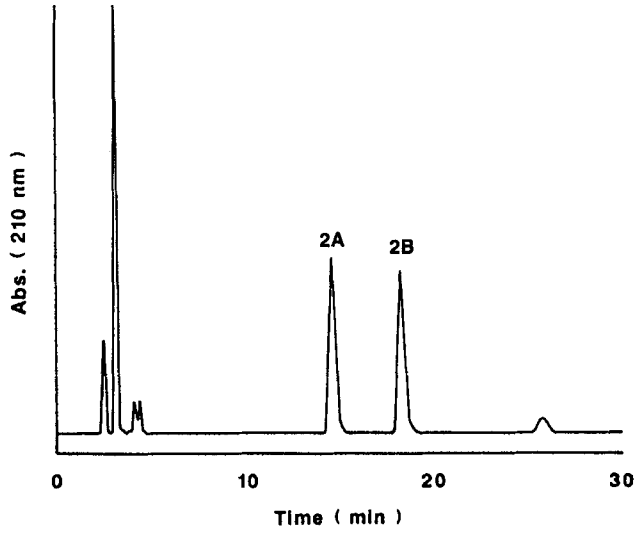


Figure 1 Diastereomeric separation of [2,6-Br_2-DL-Tyr^1]DADLE on reversed phase HPLC. HPLC conditions: column, LiChrospher RP-18 (4 x 250 mm); elution, H_2O-MeCN-TFA (67:33:0.1), flow rate 1 ml min^{-1}; detection, λ 210 nm. **2A**=[2,6-Br_2-D-Tyr^1]DADLE, **2B**=[2,6-Br_2-L-Tyr^1]DADLE

Table 2 ^1H NMR chemical shifts of [2,6-Br_2-Tyr^1]DADLE in DMSO-d_6

	NH	$C_\alpha H$	$C_\beta H$	others	
2,6-Br_2-Tyr		3.88	3.13	Ar3,5;	7.05
D-Ala	8.27	4.23	1.00		
Gly	8.03	3.58, 3.68			
Phe	8.01	4.62	2.77, 2.91	Ar;	7.18, 7.21
D-Leu	8.26	4.16	1.43	$C_\gamma H$;	1.38
				$C_\delta H$;	0.76, 0.81

Tritiated DADLE **3** was obtained by reducing the [2,6-dibromo-Tyr^1]DADLE **2B** with tritium gas in the presence of palladium black. After removal of the labile tritium, the preparation was purified by HPLC. The tritiated product **3** was chromatograghically identical with authentic non-labelled DADLE. Radio-HPLC of the tritiated product **3** revealed high radiochemical purity of 99%. The tritiated product **3** had a specific radioactivity of 1.61 TBq/mmol. The distribution of the tritium label among the amino acid residues was investigated. Following incubation

of the tritiated product 3 with aminopeptidase M, which cleaved at Tyr-D-Ala bond of DADLE, the hydrolysate was analyzed by radio-HPLC. Figure 2 showed that the label had been located exclusively at the tyrosine residue, not located at D-Ala-Gly-Phe-D-Leu fragment. No evidence was found for non-specific exchange labelling into other residues.

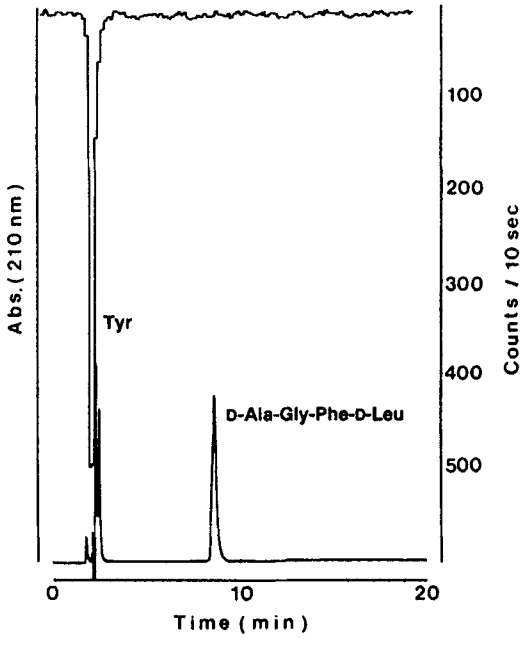


Figure 2 Radio-HPLC analysis of the hydrolysate of [2,6-Br_2-Tyr^1]DADLE with aminopeptidase M. HPLC conditions: column, LiChrospher RP-18 (4 x 250 mm); elution, H_2O-MeCN-TFA (70:30:0.1), flow rate, 1 ml min^{-1}; detection, λ 210 nm (lower) and flow-through radioisotope detector (upper).

EXPERIMENTAL

Amino acid analysis was performed on a Hitachi L-8500 amino acid analyzer. A peptide was hydrolyzed with 6M HCl in a sealed evacuated tube at 110°C for 22 hr. The liquid scintillation counter was an Aloka LSC-900. ^1H NMR spectra were determined on a Bruker AM-400 or AM-500 spectrometer, setting the Me_2SO signal at δ 2.50 ppm. 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-3 pump, a Shimadzu SPD-6A variable wavelength UV 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 x 250 mm; E. Merck) in the following solvent systems (all v/v): (A) H_2O-MeCN-trifluoroacetic acid (TFA), 67:33:0.1; (B) H_2O-MeCN-TFA, 70:30:0.1. The flow rate was 1 ml/min. Preparative HPLC was carried out on a μ-Bondasphere C-18 (5 μm, 100 Å, 19 x 150 mm; Waters) in the solvent system (A). The flow rate was 11 ml/min.

2,6-Dibromo-DL-tyrosine **1** and its *N-tert*-butyloxycarbonyl (Boc) derivative were synthesized in our laboratory as described previously (14). 9-Fluorenylmethyloxycarbonyl (Fmoc-) amino acid pentafluorophenyl (Pfp) ester derivatives were purchased from Cambridge Research Biochemicals (Cambridge, UK). *p*-Benzyloxybenzyl ester resin (styrene-1% divinylbenzene; Wang resin) was purchased from Kokusan Chemical Works Co (Tokyo, Japan). DADLE was purchased from Peptide Institute (Osaka, Japan). Aminopeptidase M (E.C. 3.4.11.2) was purchased from Sigma (St. Louis, MO, USA).

[2,6-Dibromo-Tyr^1]DADLE **2B**

The solid phase synthesis was carried out manually. Fmoc-D-Leu-Wang resin resin (0.5 g, 0.48 mmol Fmoc-D-Leu/g resin) , which was prepared from Fmoc-D-Leu and Wang resin by the method previously described (21), was loaded into a solid phase synthesis vessel. The pentapeptide was built up according to the sequence steps shown in Table 1. Fmoc deprotection was achieved by treatment with 20% piperidine in DMF. Coupling of Fmoc-Phe and Fmoc-Gly was carried out by the corresponding pentafluorophenyl ester (1.5 mmol) in the presence of 1-hydroxybenzotriazole (0.48 mmol). Fmoc-D-Ala was coupled to the glowing peptide chain by utilization of the corresponding benzotriazole ester. Boc-2,6-dibromo-DL-tyrosine (1 mmol) was coupled to D-Ala-Gly-Phe-D-Leu-Wang resin utilizing BOP reagent (1 mmol) and diisopropylethylamine (1 mmol). Coupling reactions were monitored using the ninhydrin test (22) and no recoupling was necessary. The pentapeptide was cleaved

from the resin by treatment with 15 ml of 90% TFA in H_2O for 1 hr at room temperature. After filtration, the resin was washed with MeOH. The filtrate and washings were combined, evaporated, redissolved in H_2O and lyophilized to give pentapeptide **2** (173 mg). An aliquot of the pentapeptide **2** was analyzed with HPLC [column, LiChrospher RP-18; elution, solvent system (A); detection, λ 210 nm] to show epimeric separation (t_R: **2A**, 14.6 min; **2B** 18.3 min). The epimers **2** were purified by preparative HPLC to give **2A** (67 mg, 38% based on Fmoc-D-Leu Wang resin) and **2B** (61 mg, 35% based on Fmoc-D-Leu Wang resin). Each epimer gave the following amino acid analyses after hydrolysis of 6M HCl at 110°C for 22 hr: **2A**; 2,6-Br_2-D-Tyr, not detectable (Tyr, not detected); Ala 0.98 (1); Gly, 1.00 (1); Phe, 0.97 (1); Leu, 0.96 (1) and **2B**; 2,6-Br_2-Tyr, not detectable (Tyr, not detected); Ala 1.00 (1); Gly, 1.00 (1); Phe, 1.01 (1); Leu, 1.01 (1), respectively.

Each epimer (1 mg) was dissolved in MeOH (1 ml) and reduced with hydrogen in the presence of palladium black (1 mg) for 2 hr. An aliquot of each reduction product was analyzed by HPLC [column, LiChrospher RP-18; elution, solvent system (B); detection, λ 210 nm] to show the different retention time-values (product from **2A**, 8.7 min; product from **2B**, 9.1 min). The retention time-value from the pentapeptide **2B** was identical with authentic DADLE.

[2,6-^{3}H-Tyr^1]DADLE **3**

[2,6-Dibromo-Tyr^1]DADLE **2B**(3 mg, 4 μmol) was dissolved in MeOH (0.4 ml) and reduced using tritium gas (2.9 TBq) in the presence of palladium black (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 MeOH. The crude product was purified by HPLC [column, LiChrospher RP-18; elution, solvent system (A); detection, λ 210 nm] to yield the tritiated peptide **3** (1.5 μmol, 37 %, 1.61 TBq/mmol).

Distribution of Tritium Label

The tritiated peptide **3** (2.0 KBq) was diluted with non-labelled DADLE (20 nmol) and incubated with aminopeptidase M (2 μg, 50 munit) in H_2O (120 μl) at 25°C for 4

hr. The reaction mixture was subjected to radio-HPLC [column, LiChrospher RP-18; elution, solvent system (B); detection, λ 210 nm and radioisotope detector].

REFERENCES

1. Sullivan, S., Akil, H. and Barchas, J.D. - Commun. Paychopharmacol, 2: 525 (1978)
2. Roscetti, G., Possenti, R., Bassano, E. and Roda, L.G. - Neurochem. Res., 10: 1393 (1985)
3. Schulteis, G., Weinberger, S.B., and Martinez, J.L. Jr. - Peptides, 10: 913 (1989)
4. Hussain, M.A., Rowe, S.M., Shenvi, A.B. and Aungst, B.J. - Drug Metab. Dispos., 18: 288 (1990)
5. Shibanoki, S., Weinberger, S. B., Ishikawa, K. and Martinez, J. L. Jr. - J. Chromatogr., 532: 249 (1990)
6. Weinberger, S. B. and Martinez, J. L. Jr. - J. Pharmocol. Exp. Ther., 247: 129 (1988)
7. Faraj, J.A., Hussain, A.A., Aramaki, Y., Iseki, K., Kagoshima, M. and Dittert, L.W. - J. Pharm. Sci., 79: 768 (1990)
8. Janak. P. H., Schulteis G. and Martinetz, J.L. Jr. - Peptides, 13: 551 (1992)
9. Pert, C.B., Pert, A., Chang, J.-K. and Fong, B.T.W. - Science, 194: 330 (1976)
10. Miller, R.J., Chang, K.-J. and Cuatrecasas, P. - Biochem. Biophys. Res. Commun., 74: 1311 (1977)
11. Gorin, F.A., Balasubramanian, T.M., Cicero, T.J., Schwietzer, J. and Marshall, G.R. - J. Med. Chem., 23: 1113 (1980)
12. Bunnett, N.W., Walsh, J.H. and Debas, H. T. - Am. J. Physiol., 258: G143 (1990)
13. Su, K.S.E., Campanale, K.M., Mendelsohn, L.G, Kerchner, G.A. and Gries, C.L. - J. Pharm. Sci., 74: 394 (1985)
14. Hasegawa, H., Akagawa, N., Shinohara, Y. and Baba, S. - J. Chem. Soc., Perkin Trans. 1, 2085 (1990)
15. Griffiths, D. V., Feeney, J., Roberts, G. C. K. and Burgen, A. S. V. - Biochim. Biophys. Acta, 446: 479 (1976)

16. Nagatsu, T., Levitt, M. and Udenfriend, S. - Anal. Biochem., 9: 122 (1964)

17 Rosei, M. A., Antonilli, L., Coccia, R. and Foppoli, C. - Biochem. Int., 19: 1183 (1989)

18. Kovacs, J., Kisfaludy, L. and Ceprini, M.Q. - J. Am. Chem. Soc., 89: 183 (1967)

19. Le-Nguyen, D., Seyer, R., Heitz, A. and Castro, B. - J. Chem. Soc., Perkin Trans. 1, 1025 (1985)

20. Wüthrich, K. - NMR of Proteins and Nucleic Acids, Wiley, New York, 1986

21. Hasegawa, N., Shinohara, Y. and Baba, S. - J. Chem. Soc., Perkin Trans. 1, 2641 (1990)

22. Kaiser, E., Colescott, R.L., Bossinger, C.D. and Cook, P.I. - Anal. Biochem., 34: 595 (1970)