H, 1.41; N, 34.37. UV λ_{max}^{EtOH} nm (ε): 251 (8600), 259 (9100), 294 (5600). IR ν_{max}^{KBr} cm^{-1}: 3062 (arom. C-H), 2237. 2217 (C≡N), 1629 (C=N), 1413, 851, 794. MS *m/e*: 162 (M^+).

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Synthesis of the Nonadecapeptide corresponding to Porcine δ-Endorphin[1]

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The nonadecapeptide corresponding to δ-endorphin, a plasmin hydrolysate of porcine β-lipotropin, was synthesized using protecting groups removable by methanesulfonic acid. Analgesic activity of the synthetic peptide was estimated as active as Leu-enkephalin by the tail pinch method.

Keywords——synthesis of porcine δ-endorphin; synthesis of human β-endorphin; analgesic peptide; methanesulfonic acid deprotection; tail pinch method

In 1976, Gráf *et al.*[3] isolated, as one of the plasmin hydrolysates of porcine lipotropin (LPH), a new analgesic peptide corresponding to positions 61—79 of the parent hormone. This nonadecapeptide fragment, LPH-(61—79), was analogically termed as δ-endorphin, since other fragments, LPH-(61—91), LPH-(61—76) and LPH-(61—77), were proposed to designate as β, α and γ-endorphins respectively by Li and Chung[4] and Guillemin *et al.*[5]

We wish to report the synthesis of the nonadecapeptide corresponding to the entire amino acid sequence of δ-endorphin. This segment of porcine LPH is common to those of other mamalian LPHs so far known.[6] Thus available five peptide fragments employed for our previous synthesis[7] of human β-endorphin[8] served as a building blocks to construct the entire peptide chain in a conventional manner as shown in Fig. 1.

1) Amino acids and peptides are of the L-configuration. Following abbreviations were used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, DCC=dicyclohexylcarbodiimide, HOBT=1-hydroxybenzotriazole, MSA=methanesulfonic acid, DMF=dimethylformamide, DMSO=dimethylsulfoxide, THF=tetrahydrofuran. TFA=trifluoroacetic acid.
2) Location: *a) Minamifunabori-cho, Edogawa-ku, Tokyo, 132 Japan*; *b) Sakyo-ku, Kyoto, 606 Japan.*
3) L. Gráf, J.I. Szekely, A.Z. Ronai, Z.D. Kovacs and S. Bajusz, *Nature* (London), **263**, 240 (1976).
4) C.H. Li and D. Chung, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1145 (1976).
5) N. Ling, R. Burgus and R. Guillemin, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3942 (1976).
6) C.H. Li, *Arch. Biochem. Biophys.*, **183**, 592 (1977); C.H. Li and D. Chung, *Nature* (London), **260**, 622 (1976).
7) M. Kubota, T. Hirayama, O. Nagase and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 2139 (1978).
8) C.H. Li, D. Chung and B.A. Doneen, *Biochem. Biophys. Res. Comm.*, **72**, 1542 (1976); N. Dragon, N.G. Seidah, M. Lis, R. Routhler and M. Chretien, *Can. J. Biochem.*, **55**, 666 (1977).

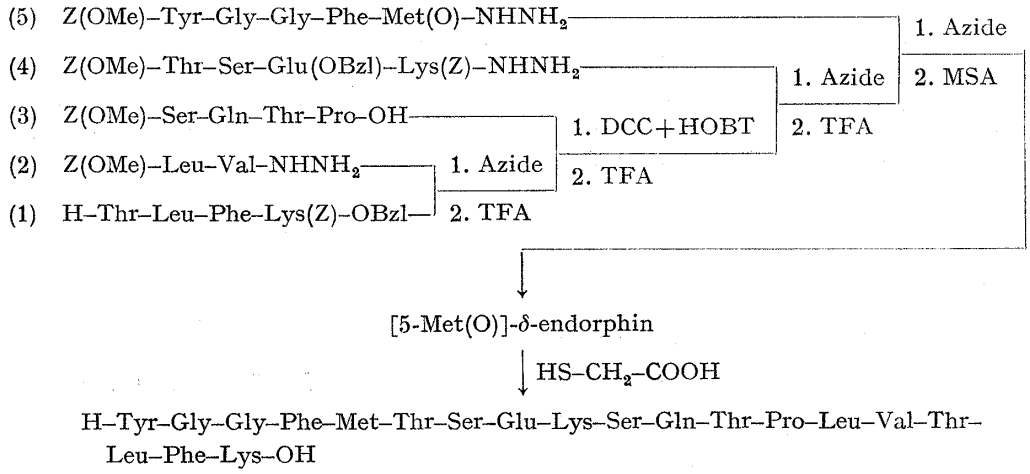

Fig. 1. Synthetic Route to δ-Endorphin

As mentioned previously,[7] amino acid derivatives bearing protecting groups removable by MSA[9] were employed, *i.e.*, Lys(Z) and Glu(OBzl). Methionine in the fragment (5) was reversibly protected as its sulfoxide[10] to prevent the alkylation at its sulfur atom during the final deprotecting step. After removing the α-protecting Z(OMe) group by TFA,[11] the peptide chain was elongated by the Rudinger's azide procedure,[12] except for the fragment (3), Z(OMe)-Ser-Gln-Thr-Pro-OH, for which the DCC plus HOBT procedure[13] was employed. Each protected product was purified by batchwise washing followed by precipitation from DMF with appropriate solvents.

The protected nonadecapeptide was finally treated with MSA and the deprotected peptide was purified in essentially the same manner as described in the human β-endorphin synthesis;[7] *i.e.*, conversion to the corresponding acetate by Dowex 1×4, treatment with dilute ammonia for reversible conversion of the N→O shift, and purification by column chromatography on Sephadex G-25 and subsequently on CM-cellulose. [5-Met(O)]-δ-endorphin] thus obtained was then reduced by thioglycolic acid and the desired product was finally isolated, as a fluffy white powder, after partition chromatography on Sephadex G-25, according to Yamashiro.[14] Its purity was assessed by thin layer chromatography in three different solvent systems and amino acid analyses in 3 N toluenesulfonic acid hydrolysis[15] and aminopeptidase (AP-M)[16] digest.

The relative agonist potency of natural δ-endorphin in logitudinal muscle strip of guinea pig ileum was reported as 0.42 of that of morphin (taken as 1),[3] but the value of the tail pinch method is not available. When our synthetic δ-ednorphin was assayed by the tail pinch method (by Professor H. Takagi), its relative potency was 1/430 of that of morphin (taken as 1). This value was estimated as active as our synthetic Leu-enkephalin.[17] Since synthetic β-endorphin[7] which possesses the additional dodecapeptide chain attached at the C-terminal portion of δ-endorphin exhibited much higher activity than morphin, these results indicated

9) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii and H. Irie, *Chem. Pharm. Bull.* (Tokyo), **23**, 1164 (1975).
10) H. Irie, N. Fujii, H. Ogawa, H. Yajima, M. Fujino and S. Shinagawa, *J.C.S. Chem. Commun.*, **1976**, 922; *idem*, *Chem. Pharm. Bull.* (Tokyo), **25**, 2929 (1977).
11) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).
12) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
13) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
14) D. Yamashiro, *Nature* (London), **201**, 76 (1964).
15) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).
16) G. Pfleiderer and G.P. Celliers, *Biochem. Z.*, **339**, 186 (1963).
17) H. Yajima, H. Ogawa, N. Fujii and S. Funakoshi, *Chem. Pharm. Bull.* (Tokyo), **25**, 740 (1977).

that the C-terminal portion of β-endorphin seems to play an important role for the peptide to exert a high level of the analgesic activity and appears to support the view expressed by Geisow *et al.*[18] that the analgesic activity of lipotropin fragments depends on the C-terminal tetrapeptide.

Experimental

Thin layer chromatography was performed on silicagel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–AcOH–H_2O (4:1:5), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_4 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1)

Z(OMe)-Leu-Val-Thr-Leu-Phe-Lys(Z)-OBzl——Z(OMe)-Thr-Leu-Phe-Lys(Z)-OBzl (1.81 g) was treated with TFA (2.3 ml) in the presence of anisole (1 ml) in an ice-bath for 60 min and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (4 ml) containing Et_3N (0.3 ml). To this ice-chilled solution was added the azide (prepared from 1.24 g of Z(OMe)-Leu-Val-$NHNH_2$ with 1.76 ml of 3.79 N HCl–DMF, 0.45 ml of isoamylnitrite and 1.4 ml of Et_3N) in DMF (5 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with ether. The resulting powder was washed batchwise with 5% citric acid, 5% $NaHCO_3$ and H_2O and then precipitated from DMF with ether; yield 1.89 g (84%), mp 215—219°, $[\alpha]_D^{24}$ −19.2° (c=0.4, DMF), Rf_1 0.82. Amino acid analysis (6 N HCl hydrolysis): Lys 1.10, Thr 1.04, Val 0.94, Leu 2.11, Phe 1.00 (average recovery 85%). *Anal.* Calcd. $C_{60}H_{81}N_7O_{13}.1/2H_2O$: C, 64.49; H, 7.40; N, 8.78. Found: C, 64.50; H, 7.28; N, 8.82.

Z(OMe)-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys(Z)-OBzl——The above protected hexapeptide ester (1.85 g) was treated with TFA–anisole (2 ml–0.9 ml) as stated above and 3 N HCl–THF (1 ml) was added. Dry ether was then added and the resulting powder was collected by filtration, washed with ether and dissolved in DMF (4 ml) together with Et_3N (0.23 ml), HOBT (0.23 g) and Z(OMe)-Ser-Gln-Thr-Pro-OH (1.0 g). After addition of DCC (0.38 g), the mixture was stirred at room temperature for 48 hr and then condensed. Treatment of the residue with H_2O afforded a powder, which was washed batchwise as mentioned above and precipitated from DMF with ether; yield 1.92 g (76%), mp 213—234°, $[\alpha]_D^{24}$ −31.5° (c=0.3, DMF), Rf_1 0.62. Amino acid analysis (6 N HCl hydrolysis): Lys 1.02, Thr 1.93, Ser 0.87, Glu 0.88, Pro 0.87, Val 1.00, Leu 2.17,-Phe 1.02 (average recovery 95%). *Anal.* Calcd. for $C_{77}H_{108}N_{12}O_{20}$: C, 60.77; H, 7.15; N, 11.05. Found: C, 60.97; H, 7.11; N, 10.66.

Z(OMe)-Thr-Ser-Glu(OBzl)-Lys(Z)-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys(Z)-OBzl——The above protected decapeptide ester (1.90 g) was treated with TFA–anisole (1.5 ml–0.5 ml) as stated above and dry ether was added. The resulting powder was then dissolved in DMSO–DMF (1 ml–3 ml) containing Et_3N (0.35 ml). To this ice-chilled solution was added the azide (prepared from 1.65 g of Z(OMe)-Thr-Ser-Glu-(OBzl)- Lys(Z)-$NHNH_2$ with 1.10 ml of 3.79 N HCl–DMF, 0.28 ml of isoamylnitrite and 0.87 ml of Et_3N) in DMF (5 ml). After stirring at 4° for 48 hr, the solution was condensed and the residue was treated with ether. The resulting powder was washed batchwise as stated above and then precipitated from DMF with MeOH; yield 1.76 g (64%), mp 254—259°, $[\alpha]_D^{24}$ −24.1° (c=0.5, DMF), Rf_1 0.59. Amino acid analysis (6 N HCl hydrolysis): Lys 1.97, Thr 2.84, Ser 1.83, Glu 1.89, Pro 0.97, Val 1.00, Leu 2.01, Phe 2.00 (average recovery 92%). *Anal.* Calcd. for $C_{110}H_{151}N_{17}O_{30}$: C, 60.28; H, 6.95; N, 10.87. Found: C, 60.25; H, 6.81; N, 10.48.

Z(OMe)-Tyr-Gly-Gly-Phe-Met(O)-Thr-Ser-Glu(OBzl)-Lys(Z)-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys(Z)-OBzl——The above protected tetradecapeptide ester (1.31 g) was treated with TFA–anisole (1 ml–0.3 ml) and the α-deprotected peptide isolated as stated above was then dissolved in DMSO–DMF (2 ml–4 ml) containing Et_3N (0.17 ml). To this ice-chilled solution was added the azide (prepared from 0.64 g of Z(OMe)-Tyr-Gly-Gly-Phe-Met(O)-$NHNH_2$ with 0.48 ml of 3.79 N HCl–DMF, 0.12 ml of isoamylnitrite and 0.38 ml of Et_3N) in DMF (4 ml). After stirring at 4° for 48 hr, the solution was condensed and the residue was treated with AcOEt. The resulting powder was washed batchwise as mentioned above and precipitated three times from DMF with MeOH; yield 1.24 g (75%), mp 268—274°, $[\alpha]_D^{24}$ −16.2° (c=0.6, DMSO), Rf_1 0.52. Amino acid analysis (6 N HCl hydrolysis with phenol): Lys 2.17, Thr 2.63, Ser 1.76, Glu 1.92, Pro 0.82, Gly 2.14, Val 1.00, Leu 1.98, Tyr 0.98, Phe 2.02 (average recovery 94%). *Anal.* Calcd. for $C_{137}H_{184}N_{22}O_{37}S$: C, 59.55; H, 6.71; N, 11.15. Found: C, 59.54; H, 6.73; N, 10.76.

H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-OH——The above protected nonadecapeptide ester (552 mg) was exposed to MSA (3 ml) in the presence of anisole (1 ml) in an ice-bath for 15 min and then at room temperature for 60 min. Dry ether was added and the resulting oily precipitate was washed with ether and dissolved in H_2O (30 ml). After treatment with Dowex 1×4 (acetate form, approximately 5 g) for 30 min, the solution was filtered and the filtrate was lyophilized. The

18) M.J. Geisow, J.F.W. Deakin, J.O. Dostrovsky and D.G. Smyth, *Nature* (London), **269**, 167 (1977).

residue was dissolved in 2 N NH_4OH (5 ml) and the solution, after standing in an ice-bath for 30 min, was lyophilized. The resulting powder was then dissolved in 0.5 N AcOH (2 ml) and the solution was applied to a column of Sephadex G-25 (3.5 × 145 cm), which was eluted with the same solvent system. Individual fractions (4.2 ml each) were collected and the absorbancy at 275 $m\mu$ was determined. Fractions corresponding to the front main peak (tube No. 126—190) were combined and the solvent was removed by lyophilization. The resulting powder dissoved in H_2O (10 ml) was then applied to a column of CM-cellulose (1.8 × 20 cm), which was eluted with H_2O (240 ml) followed by the gradient elution with 0.5 M NH_4OAc (pH 6.5, 1000 ml) through a mixing flask containing H_2O (500 ml). Individual fractions (10 ml each) were collected and absorbancy at 275 mμ was determined. After elution of a small peak (tube No. 10—17), a main peak (tube No. 33—52) was detected. This desired fractions were combined. The solvent and NH_4OAc were removed by repeated lyophilization to give a white powder (337 mg, 78%). [5-Met(O)]-δ-endorphin thus obtained (100 mg) was dissolved in 5% thioglycolic acid in H_2O (5 ml) and the solution was incubated at 50° for 20 hr. After lyophilization, the residue was dissolved in the upper phase (1 ml) of the solvent consisting of *n*-BuOH–AcOH–H_2O (4:1:5) and the solution was applied to a column of Sephadex G-25 (3 × 60 cm) equillibrated previously with the lower phase of the above solvent system. The column was then eluted with the upper phase and individual fractions (5 ml each) were collected. After determination of the absorbancy at 275 mμ, fractions corresponding to the main peak (tube No. 38—52) were combined and the solvent was evaporated. The residue was dissolved in a small amount of 0.5 N AcOH and lyophilized to give a white fluffy powder; yield 42 mg (reduction and purification steps, 33%), $[\alpha]_D^{24}$ −78.4° (c=0.3, 0.5 N AcOH), Rf_2 0.43, Rf_3 0.58, Rf_4 0.75. Amino acid ratios in 3 N Tos-OH hydrolysate and Ap-M digest (number in parenthesis). Numbers in blacket are theories: Lys 2.13 (2.15) [2], Thr 3.14 [3], Ser 2.00 (2.06) [2], Glu 2.01 (1.06) [1], Pro 1.04 (1.00) [1], Gly 2.00 (2.02) [2], Val 0.94 (1.28) [1], Met 1.01 (0.93) [1], Leu 2.07 (2.07) [2], Tyr 0.97 (0.97) [1], Phe 2.00 (2.00) [2], Thr+Gln (3.75, Calcd. as Thr) [3+1], average recovery 95% (78%).

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