tion of the amount of tyrosines to be formed. The results suggest the possibility that phenylalanine hydroxylase in rat brain homogenate is able to catalyze the hydroxylation of phenylalanine to *p*-, *m*-, and *o*-tyrosines.

Further studies on the enzymatic hydroxylation of phenylalanine by the other mammalian tissues are in progress, and the results will be reported in the near future.

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Synthesis of Salmon Endorphin

First synthesis of a nonacosapeptide corresponding to the entire amino acid sequence of salmon endorphin, Ac–Tyr–Gly–Gly–Phe–Met–Lys–Pro–Tyr–Thr–Lys–Gln–Ser–His–Lys–Pro–Leu–Ile–Thr–Leu–Leu–Lys–His–Ile–Thr–Leu–Lys–Asn–Glu–Gln–OH, and its des-acetyl derivative was described. Toward the synthesis of this hormone, five fragments, 1—7, 8—15, 16—18, 19—24 and 25—29, were prepared and the fragments were served as the building blocks for the final construction of the entire amino acid sequence of the hormone. The final deprotection of the fully protected peptide was achieved by treatment with trifluoroacetic acid and the purification of the synthetic peptides was effected by a column chromatography on CM-cellulose and then Sephadex LH-20. The synthetic acetylated nonacosapeptide was compared with purified natural salmon endorphin by means of chromatography, electrophoresis and also enzymatic digestion and found to be indistinguishable from natural isolated salmon endorphin. The opiate activity of the synthetic salmon endorphin and des-acetyl salmon endorphin was also described.

Keywords——Salmon endorphin; des-acetyl salmon endorphin; nonacosapeptide; opiate activity; synthesis of peptide; DCC–HONB method

The entire amino acid sequence of endorphin isolated from salmon pituitary glands was recently elucidated by one of the present authors (H.K.) to be[1] Ac–Tyr–Gly–Gly–Phe–Met–Lys–Pro–Tyr–Thr–Lys–Gln–Ser–His–Lys–Pro–Leu–Ile–Thr–Leu–Leu–Lys–His–Ile–Thr–Leu–Lys–Asn–Glu–Gln–OH.[2] This amino acid sequence has only 13 identical residues in the molecule when compared with the sequence of mammalian β-endorphin, and moreover the N-terminus blocked by the acetyl group is interesting to note. In the course of our investigations on the synthesis of peptides related to endorphins, we have synthesized the acetyl and des-acetyl nonacosapeptide corresponding to the entire amino acid sequence of this salmon endorphin (SE). The synthetic acetyl peptide was compared with the natural material and found to be identical and indistinguishable from isolated natural SE, confirming the proposed structure of SE. Moreover, the synthetic des-acetyl SE was found to have approximately 2.5 times greater potency than human β-endorphin (β_h–E) in the rat brain receptor assay.

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1) H. Kawauchi, M. Tubokawa, and K. Muramoto, *Biochem. Biophys. Res. Commun.*, **88**, 1249 (1979).
2) Abbreviations used: Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; OBu^t, *tert*-butyl ester; Ac, acetyl; DCC, dicyclohexylcarbodiimide; HONB, N-hydroxy-5-norbornene-2,3-dicarboximide; DMF, dimethylformamide; SE, salmon endorphin; β_h-E, human β-endorphin.

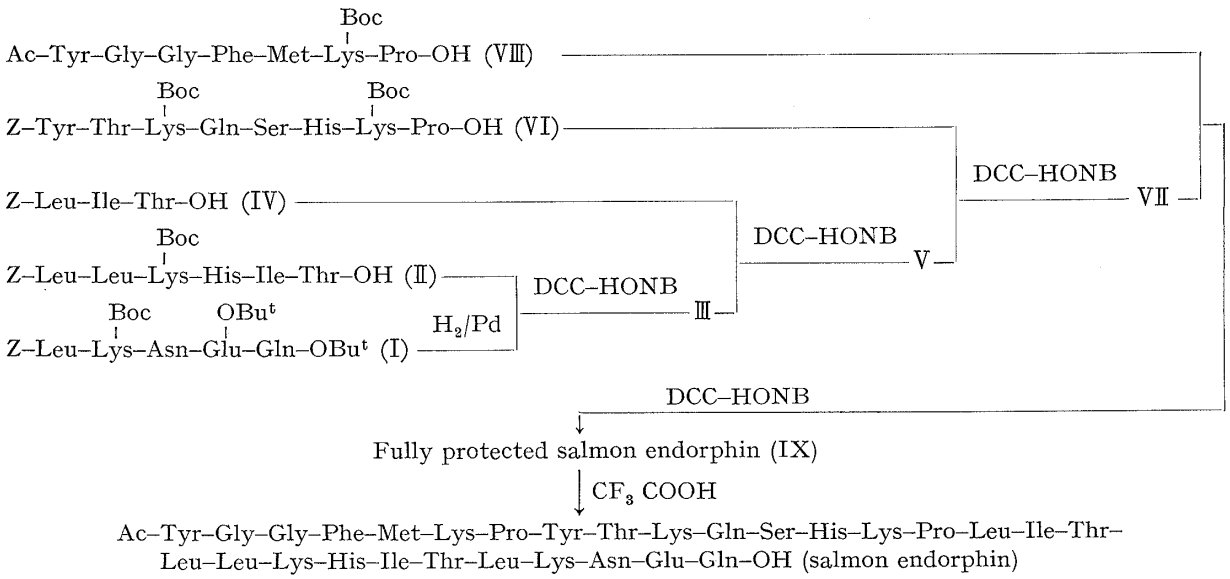

Fig. 1. Synthetic Route to Salmon Endorphin

Our synthesis of SE in solution, as shown in Fig. 1, used amino acid derivatives bearing protecting groups, Boc and OBu^t, which were removable by treatment with trifluoroacetic acid. The α-amino function of most of the intermediates was protected by the Z group which was removable by catalytic hydrogenation. The synthetic strategy of the present synthesis is similar to that of our synthesis of mammalian endorphins,[3] in which for the construction of the total sequence, we chose the synthetic fragments mostly bearing Thr and Ile in the C-terminus to detect the degree of racemization during the coupling reactions by amino acid analysis instantly. In the present synthesis of SE, two peptide fragments (II and IV) having Thr in the C-terminus and two peptide fragments (VI and VIII) having C-terminal Pro were prepared together with the C-terminal fragment I. This choice was based on the observation of little racemizable property of Thr peptides.[3,4] These peptide subunits were prepared by a conventional solution method.

For the synthesis of the C-terminal-protected undecapeptide Z–Leu–Leu–Lys(Boc)–His–Ile–Thr–Leu–Lys(Boc)–Asn–Glu(OBu^t)–Gln–OBu^t (III), the protected pentapeptide I {mp 214—216° (dec.), $[\alpha]_D^{21}$ −22.4° (c=0.98 in DMF), Rf_1 0.53,[5] *Anal.* Calcd for $C_{47}H_{76}N_8O_{14}$: C, 57.75; H, 7.84; N, 11.47. Found: C, 57.69; H, 7.93; N, 11.42.} was hydrogenated over Pd black as a catalyst in DMF and the resulting free base of I was condensed with the partially protected hexapeptide II {mp 227—228°, $[\alpha]_D^{27}$ −39.7° (c=0.4 in MeOH), Rf_2 0.60, *Anal.* Calcd for $C_{47}H_{75}N_9O_{12}\cdot H_2O$: C, 57.83; H, 7.95; N, 12.92. Found: C, 58.08; H, 7.93; N, 13.07.} giving 75.4% yield by the DCC–HONB method[6] in DMF. Undecapeptide III had mp>250°, $[\alpha]_D^{24}$ −19.2° (c=0.5 in DMF), Rf_3 0.65, *Anal.* Calcd for $C_{86}H_{143}N_{17}O_{23}$: C, 57.92; H, 8.08; N, 13.36. Found: C, 57.59; H, 8.31; N, 13.25. Amino acid anal. $Lys_{2.04}$ $His_{0.95}$ $Asp_{1.00}$ $Thr_{0.93}$[7] $Glu_{2.11}$ $Ile_{0.98}$ $Leu_{3.09}$. To prepare the protected tetradecapeptide Z–Leu–Ile–Thr–Leu–Leu–Lys(Boc)–His–Ile–Thr–Leu–Lys(Boc)–Asn–Glu(OBu^t)–Gln–OBu^t (V), III was hydrogenated over Pd black in DMF and the resulting amine was coupled with the partially protected tripep-

3) O. Nishimura, S. Shinagawa, and M. Fujino, *J. Chem. Research* (*S*), **1979**, 352; (M), **1979**, 4365.
4) C. Kitada and M. Fujino, *Chem. Pharm. Bull.*, **26**, 585 (1978).
5) Precoated silica gel, Merck 60 F_{254}. Solvent systems were: Rf_1=$CHCl_3$–MeOH–AcOH (9:1:0.5, v/v), Rf_2=AcOEt–pyridine–AcOH–H_2O (60:20:6:10, v/v), Rf_3=$CHCl_3$–MeOH–H_2O (7:3:0.5, v/v).
6) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.*, **22**, 1857 (1974).
7) D-*Allo*-Thr content: less than 0.88% (amino acid analysis).

tide IV {mp 146—148°, $[\alpha]_D^{24}$ −36.0° (c=0.2 in MeOH), Rf_1 0.45, *Anal.* Calcd for $C_{24}H_{37}N_3O_7$: C, 60.11; H, 7.78; N, 8.79. Found: C, 59.96; H, 7.59; N, 8.59.} by the DCC–HONB procedure. V was obtained in 78.2% yield, mp >250°, $[\alpha]_D^{24}$ −17.0° (c=0.5 in DMF), Rf_3 0.67, *Anal.* Calcd for $C_{102}H_{172}N_{20}O_{27}\cdot 2H_2O$: C, 57.07; H, 8.26; N, 13.05. Found: C, 57.33; H, 7.97; N, 13.03. Amino acid anal. $Lys_{2.00}$ $His_{0.89}$ $Asp_{0.97}$ $Thr_{1.86}$[8] $Glu_{1.98}$ $Ile_{1.80}$ $Leu_{3.82}$. To prepare the protected docosapeptide Z–Tyr–Thr–Lys(Boc)–Gln–Ser–His–Lys(Boc)–Pro–Leu–Ile–Thr–Leu–Leu–Lys(Boc)–His–Ile–Thr–Leu–Lys(Boc)–Asn–Glu(OBu^t)–Gln–OBu^t (VII), the free amine prepared from V by catalytic hydrogenation in DMF was condensed with the protected octapeptide VI {mp 197—198° (dec.), $[\alpha]_D^{24}$ −21.2° (c=0.8 in DMF), Rf_2 0.12, *Anal.* Calcd for $C_{62}H_{91}N_{13}O_{19}\cdot 2H_2O$: C, 54.82; H, 7.05; N, 13.40. Found: C, 54.72; H, 7.03; N, 13.45.} in DMF. VII was obtained in 99% yield, mp>250°, $[\alpha]_D^{24}$ −10.5° (c=0.5 in DMF), Rf_2 0.41, Rf_3 0.63, *Anal.* Calcd for $C_{156}H_{255}N_{33}O_{43}\cdot 3H_2O$: C, 56.18; H, 7.89; N, 13.86. Found: C, 56.08; H, 7.66; N, 13.78. Amino acid anal. $Lys_{4.09}$ $His_{1.90}$ $Asp_{1.08}$ $Thr_{3.04}$ $Ser_{1.00}$ $Glu_{3.10}$ $Pro_{1.06}$ $Ile_{1.93}$ $Leu_{4.03}$ $Tyr_{0.86}$. To prepare the final protected nonacosapeptide Ac–Tyr–Gly–Gly–Phe–Met–Lys(Boc)–Pro–Tyr–Thr–Lys(Boc)–Gln–Ser–His–Lys(Boc)–Pro–Leu–Ile–Thr–Leu–Leu–Lys-(Boc)–His–Ile–Thr–Leu–Lys(Boc)–Asn–Glu(OBu^t)–Gln–OBu^t (IX) {83.4% yield, mp>250°, $[\alpha]_D^{24}$ −12.8° (c=0.52 in DMF), Rf_2 0.36, Rf_3 0.62, *Anal.* Calcd for $C_{193}H_{311}N_{41}O_{52}S\cdot 6H_2O$: C, 55.48; H, 7.79; N, 13.75; S, 0.77. Found: C, 55.39; H, 7.58; N, 13.55; S, 0.80.}, VII was hydrogenated over Pd black in DMF and the resulting free base was coupled with N-acetyl heptapeptide VIII {mp 146° (dec.), $[\alpha]_D^{24}$ −24.6° (c=0.5 in DMF), Rf_1 0.14, Rf_2 0.44, *Anal.* Calcd for $C_{45}H_{64}N_8O_{12}S$: C, 57.43; H, 6.85; N, 11.91; S, 3.41. Found: C, 57.14; H, 7.10; N, 11.66; S, 3.18.} by the DCC–HONB method in DMF, and the resulting crude IX was purified by column chromatography on Sephadex LH-20 (MeOH). All the protecting groups were removed by 90% aqueous trifluoroacetic acid at 20° for 60 min. The resulting material was immediately converted into the acetate with Amberlite CG-410 (acetate form) and then purified by CM-cellulose column with a gradient elution using pH 6.8 ammonium acetate buffer (0.001→0.3 M) and gel filtration on Sephadex LH-20 (N-acetic acid). A white powder of SE was obtained in 62% yield by lyophilization: $[\alpha]_D^{24}$ −77.7° (c=0.13 in 1 N AcOH),[9] Amino acid anal. $Lys_{5.12}$ $His_{1.91}$ $Asp_{1.01}$ $Thr_{3.10}$ $Ser_{0.96}$ $Glu_{3.19}$ $Pro_{2.02}$ $Gly_{2.17}$ $Met_{0.92}$ $Ile_{1.92}$ $Leu_{4.03}$ $Tyr_{1.97}$ $Phe_{1.00}$ (average recovery, 89%).

The product migrated as a single spot on low or high voltage paper electrophoresis (pH 3.7 or pH 2.0), indistinguishable from the natural material,[10] and thin-layer chromatograms of the synthetic product were identical with and indistinguishable from the natural purified peptide.[11] The tryptic and chymotryptic peptide maps from synthetic and natural SE were also identical.[12]

To prepare the des-acetyl SE, the hydrogenated VII was coupled with Boc-heptapeptide VIII′ {mp 151° (dec.), $[\alpha]_D^{24}$ −28.0° (c=0.52 in DMF), Rf_1 0.25, Rf_2 0.64, *Anal.* Calcd for $C_{48}H_{70}N_8O_{13}S$: C, 57.70; H, 7.06; N, 11.22; S, 3.24. Found: C, 57.68; H, 7.22; N, 10.85; S, 3.14.} by the DCC–HONB method in DMF to give Boc-nonacosapeptide IX′ {84% yield, mp 250°, $[\alpha]_D^{24}$ −13.7° (c=0.50 in DMF), Rf_2 0.46, Rf_3 0.65, *Anal.* Calcd for $C_{196}H_{317}N_{41}O_{53}S\cdot 4H_2O$: C, 56.05; H, 7.80; N, 13.67; S, 0.76. Found: C, 56.19; H, 7.83; N, 13.55; S, 0.78.}. This protected peptide was deblocked and purified by the same manner as described for the preparation

8) D-*Allo*-Thr content: less than 0.33% (amino acid analysis).
9) Purified natural salmon endorphin: $[\alpha]_D^{24}$ −71.7° (c=0.12 in 1N AcOH).
10) The mixture of natural and synthetic salmon endorphin migrated as a single spot in all the systems tested.
11) Rf_4 (cellulose plate, n-BuOH–pyridine–AcOH–H_2O, 30: 20: 6: 24, v/v)=0.60; Rf_5 (cellulose plate, AcOEt-n-BuOH–AcOH–H_2O, 1: 1: 1: 1, v/v)=0.66.
12) To obtain the peptide map, SE was digested with trypsin (Boehringer Mannheim, 100: 0.25, pH 8.0, 37° for 4 hr) or with chymotrypsin (Boehringer Mannheim, 100: 1.6, pH 8.0, 37° for 4 hr). Electrophoresis was carried out with pyridine–acetate buffer (pH 3.7, 400 V, 4 hr) and pyridine-formate buffer (pH 2.0, 2000 V, 1 hr). The color was developed with ninhydrin.

Table I. Receptor Assay of Synthetic Endorphins

Compound	Inhibition of ^{3}H-naloxone stereospecific binding (IC_{50})[a]	
	−NaCl	+100 mM NaCl
	(nM)	(nM)
Morphine	11	710
Met-Enkephalin	19	500
β_h-Endorphin	6.5	580
Salmon endorphin	3800	—
Des-acetyl salmon endorphin	2.4	190

a) The IC_{50} nM values in the presence and absence of sodium ions were measured to determine the sodium shift (+Na/−Na): β_h−E=89; des-acetyl SE=79.

of SE. Des-acetyl SE was obtained in 58% yield: $[\alpha]_D^{24}$ −80.0° (c=0.13 in 1 N AcOH), Rf_4 (cellulose) 0.57, Rf_5 (cellulose) 0.64. Amino acid anal. $Lys_{5.08}$ $His_{1.98}$ $Asp_{1.00}$ $Thr_{3.09}$ $Ser_{0.92}$ $Glu_{3.09}$ $Pro_{2.01}$ $Gly_{2.10}$ $Met_{0.94}$ $Ile_{1.98}$ $Leu_{4.02}$ $Tyr_{1.98}$ $Phe_{1.00}$ (average recovery, 88%).

The opiate activity of the synthetic peptides was performed by the method of Pert and Snyder[13] with modifications by using a membrane fraction from rat brain homogenate and the results are summarized in Table I. In the receptor assay, des-acetyl SE is approximately 2.5 times more potent in comparison with β_h–E, whereas acetyl peptide (SE) appears to be inactive. Recently, Smyth *et al.*[14] have reported the presence of N^α-acetyl endorphins in porcine pituitary as an inactive form of opioid peptides. In salmon pituitary, however, N^α-free endorphin has not yet been found to date. Therefore, it is possible that N^α-acetyl endorphin possesses some other functions. More detailed studies on biological properties of the synthetic peptides are now in progress.

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