echinoside A gave a desulfated derivative (**4**), $C_{54}H_{88}O_{23}\cdot 2H_2O$, mp 237—239°. On methanolysis, the trideca-O-methyl derivative (**4a**) [four β-anomeric protons: all doublets at δ 4.33 (J=7 Hz), 4.37 (J=8 Hz), 4.66 (J=7 Hz), 4.68 (J=7 Hz)] liberated one part each of Me 3,4-di-O-Me-xylopyranoside, Me 2,3-di-O-Me-quinovopyranoside, Me 2,4,6-tri-O-Me-glucopyranoside, and Me 2,3,4,6-tetra-O-Me-glucopyranoside. Consequently, the chemical structure of echinoside A has been elucidated as **5**.

The structures **3** and **5** proposed for echinoside B and A are further supported by the ^{13}C-NMR data for echinoside B, desulfated echinoside B, and echinoside A which will be reported in detail later together with the antifungal activities of echinoside A (**5**), B (**3**), and their derivatives.

Acknowledgement The authors are grateful to Dr. M. Yamaguchi of Ryukyu University for identification of the sea cucumber *Actinopyga echinites* (JAEGER), and to Foundation for the Promotion of Research on medicinal Resources for a grant.

Faculty of Pharmaceutical Sciences,
Osaka University,
133-1 Yamada-kami, Suita,
Osaka 565 Japan

ISAO KITAGAWA
TATSUYA INAMOTO
MASAKO FUCHIDA
SHINJI OKADA
MOTOMASA KOBAYASHI
TAKAO NISHINO

Institute for Protein Research,
Osaka University,
5311, Yamada-kami, Suita,
Osaka 565, Japan

YOSHIMASA KYOGOKU

Received March 3, 1980

[Chem. Pharm. Bull. 28(5)1653—1655(1980)]

Formation of *m*-Tyrosine and *o*-Tyrosine from L-Phenylalanine by Rat Brain Homogenate

Incubation of L-phenylalanine with rat brain homogenate in the presence of a pteridine co-factor and 2-mercaptoethanol gave rise to three hydroxylated products which were identified with high-performance liquid chromatography as *p*-tyrosine, *m*-tyrosine and *o*-tyrosine.

Keywords——enzymatic hydroxylation of phenylalanine; *m*-tyrosine; *o*-tyrosine; rat brain; fluorescence high-performance liquid chromatography

It is well known that phenylalanine is in large part metabolized by conversion to *p*-tyrosine (tyrosine) in mammal.[1] In addition, Tong *et al.*[2] reported the formation of *m*-tyrosine (*m*-hydroxyphenylalanine) from phenylalanine by beef adrenal medulla preparation *in vitro*. However, the formation of *o*-tyrosine (*o*-hydroxyphenylalanine) by mammalian tissues has not been found. The present communication describes that when phenylalanine is incubated with rat brain homogenate in the presence of a pteridine co-factor, besides *p*- and *m*-tyrosines, *o*-tyrosine is also formed in the reaction mixture.

1) A. Meister (ed.), "Biochemistry of the Amino Acids," Vol. 2, Academic Press, New York, 1965, p. 909.
2) J.H. Tong, A.D' Iorio, and N.L. Benoiton, *Biochem. Biophys. Res. Commun.*, **44**, 229 (1971).

Male Wistar rats (150—200 g) were killed by decapitation, and the brain was removed and homogenized with a waring blender in two part volume of isotonic KCl solution at pH 7.0. The mixture was centrifuged at 2000 ***g*** for 10 min, and the supernatant was used as rat brain homogenate in this study. The initial reaction mixture contained 8 μmol of phenylalanine, 0.6 μmol of 6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$), 20 μmol of 2-mercaptoethanol and 100 μmol of citrate buffer (pH 6.0) in a total volume of 2.0 ml. These were incubated in air in a shaking water bath at 37°, and the reaction was terminated by the addition of 0.5 ml of 0.1 N HCl. After boiling the mixture and centrifuging down the precipitate, 50 μl of the supernatant was subjected to a fluorescence high-performance liquid chromatography (HPLC) according to the method described in our previous paper.[3)]

The chromatographic analysis indicated the formation of *o*-, *m*-, and *p*-tyrosines by the reaction of phenylalanine with rat brain homogenate in the presence of $DMPH_4$. A typical chromatographic pattern is shown in Fig. 1. Under this chromatographic conditions, the retention time of 3,4-dihydroxyphenylalanine, catecholamines, phenylpyruvic acid and 3-methoxytyrosine, which might be formed in the reaction mixture, clealy differed from those of *o*-, *m*-, and *p*-tyrosine.

When the amounts of each hydroxylated phenylalanine in the reaction mixtures during the first 120 min were determined, the amounts of *m*- and *o*-tyrosines accumulated reached a maximum at 10—20 min followed with a steady decline over the next 100 min and in the case of *p*-tyrosine, the maximum level was at 20—30 min (not shown in Figure).

Table I shows the results for 30-min incubations of modifying the standard incubation mixture used. It is seen that the hydroxylation reaction of phenylalanine is accelerated by the addition of ferrous ions, but not of cupric ions. The absence of $DMPH_4$ resulted in reduc-

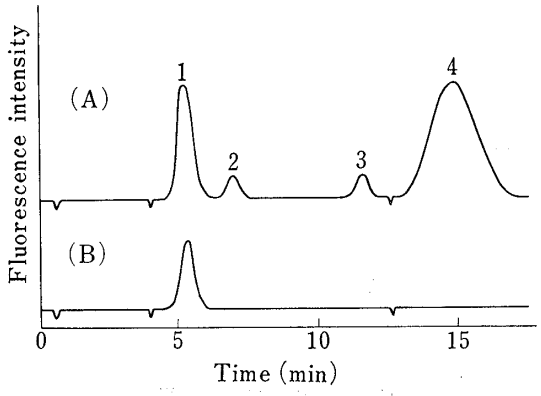

Fig. 1. High Performance Liquid Chromatogram of the Reaction Mixture

(A) Injection sample: After reaction of phenylalanine with rat brain homogenate, pteridine co-factor and 2-mercaptoethanol for 30 min under the conditions described in the text.
(B) Injection sample: Same as (A), except for phenylalanine.
Fifty μl of the sample prepared as described in the text was subjected to HPLC.
HPLC conditions: Hitachi #3011-C resin was packed in 2.1×500 mm i.d. stainless steel column; column temperature, 45°; mobile phase, a mixture of equal volumes of 0.025M sodium acetate and 0.05M acetic acid; flow rate, 0.8 ml/min; detection, excitation at 258 nm and emission at 288 nm for phenylalanine, excitation at 275 nm and emission at 305 nm for *o*-tyrosine, *m*-tyrosine, and *p*-tyrosine.
Peaks: 1=*p*-tyrosine; 2=*m*-tyrosine; 3=*o*-tyrosine; 4=phenylalanine.

Table I. Effect of Various Substances on the Hydroxylation of Phenylalanine by Rat Brain Homogenate[a)]

Modification to digest	Tyrosine formed (μg/2.5 ml)		
	o-	*m*-	*p*-
—	0.93±0.05	0.95±0.06	1.45±0.10
$+Fe^{2+}$[b)]	3.50±0.13	4.33±0.15	3.20±0.20
$+Cu^{2+}$[b)]	0.75±0.05	0.80±0.07	1.00±0.10
$-DMPH_4$	0.30±0.07	0.40±0.08	0.55±0.09
−2-Mercaptoethanol	0.55±0.10	0.68±0.08	0.95±0.10
−2-Mercaptoethanol, $DMPH_4$	0.20±0.05	0.35±0.07	0.50±0.08

a) Incubation mixture, see in the text, for 30 minutes.
b) 4 μmol of $CuSO_4 \cdot 5H_2O$ or $FeSO_4$.
Values are means±S.D. of three experiments.

3) S. Ishimitsu, S. Fujimoto, and A. Ohara, *Chem. Pharm. Bull.*, **28**, 992 (1980).

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.

Kyoto College of Pharmacy
5 Nakauchicho, Misasagi,
Yamashina-ku, Kyoto 607,
Japan

Susumu Ishimitsu
Sadaki Fujimoto
Akira Ohara

Received February 23, 1980

[Chem. Pharm. Bull. 28(5)1655—1658(1980)]

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.

---

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.