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Synthesis and Activity of a newly Isolated Analgesic Pentapeptide, Neo-kyotorphin[1)]

KOUKI KITAGAWA,*,[a] NOBUYUKI KAWAI,[a] YOSHIAKI KISO,[a] TADASHI AKITA,[a] KIYOSHI FUKUI,[b] HIROO AMANO,[c] and HIROSHI TAKAGI[c]

Faculty of Pharmaceutical Sciences, University of Tokushima,[a] Sho-machi, Tokushima 770, Japan, Department of Pharmacology, Kagawa Medical School,[b] Miki-cho, Kida-gun, Kagawa 761-07, Japan, and Faculty of Pharmaceutical Sciences, Kyoto University,[c] Sakyo-ku, Kyoto 606, Japan

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A newly isolated bovine brain pentapeptide, Thr–Ser–Lys–Tyr–Arg (neo-kyotorphin), which contains the Tyr–Arg (kyotorphin) unit at its carboxyl terminal portion, was synthesized by using the TFMSA–TFA–thioanisole deprotecting procedure. The synthetic peptide exhibited dose-dependent analgesic activity in mice at a level approximately equal to that of Leu-enkephalin.

Keywords——peptide synthesis; TFMSA–TFA–thioanisole deprotecting system; kyotorphin; neo-kyotorphin; analgesic activity

In 1979, Takagi *et al.* isolated and identified an analgesic dipeptide, kyotorphin (H–Tyr–Arg–OH), from the bovine brain.[2,3)] During the course of isolation studies of kyotorphin, they found another novel peptide in the methanol-soluble fraction of the bovine brain, and quite recently they isolated and determined its structure as H–Thr–Ser–Lys–Tyr–Arg–OH.[4)]

This peptide contains the kyotorphin sequence at the carboxyl terminal portion and has analgesic activity, like kyotorphin. Thus, this peptide may be a precursor of kyotorphin. Moreover, it is interesting to note that this sequence corresponds to the carboxyl terminal pentapeptide of the α-chain of human (bovine) hemoglobin,[5,6)] while the amino terminal tripeptide, Thr–Ser–Lys–NH_2, has already been isolated from the bovine pineal glands as an antigonadotropic peptide.[7)] However, the relation between this pentapeptide and hemoglobin is not clear.

In this paper, we report the synthesis of this newly isolated pentapeptide, neo-kyotorphin.

The synthetic scheme is outlined in Fig. 1.

First, the protected tripeptide was prepared by the azide condensation of Z(OMe)–Lys(Z)–Tyr–$NHNH_2$ and H–Arg(Tos)–OH. Alternatively, we tried to prepare this tripeptide using the kyotorphin unit, namely by the condensation of Z(OMe)–Lys(Z)–OH and H–Tyr(2,6-Cl_2Bzl)–Arg(Tos)–OH[8)] by HONB active ester method,[9)] but the purification of this tripeptide was very troublesome because of contamination by Z(OMe)–Lys(Z)–OH.

After removal of the Z(OMe) group by TFA, Z(OMe)–Thr–Ser–$NHNH_2$[10)] was condensed by the azide method to afford the protected pentapeptide, Z(OMe)–Thr–Ser–Lys(Z)–Tyr–Arg(Tos)–OH. This peptide was purified by silica gel column chromatography using $CHCl_3$–MeOH (9 : 1) and $CHCl_3$–MeOH–H_2O (8 : 3 : 1) as eluants. Final deprotection of this peptide was performed with the TFMSA–thioanisole in TFA system[11)] in the presence of *o*-cresol to suppress *O*-tosylation at the Tyr residue.[12)] This deprotecting system completely removed the tosyl group of Arg within 2 h at room temperature, as reported previously.[8)] The deprotected peptide was converted to the corresponding acetate and purified by column chromatography on Sephadex G-10 and CM-cellulose. In the former case, 3% acetic acid was

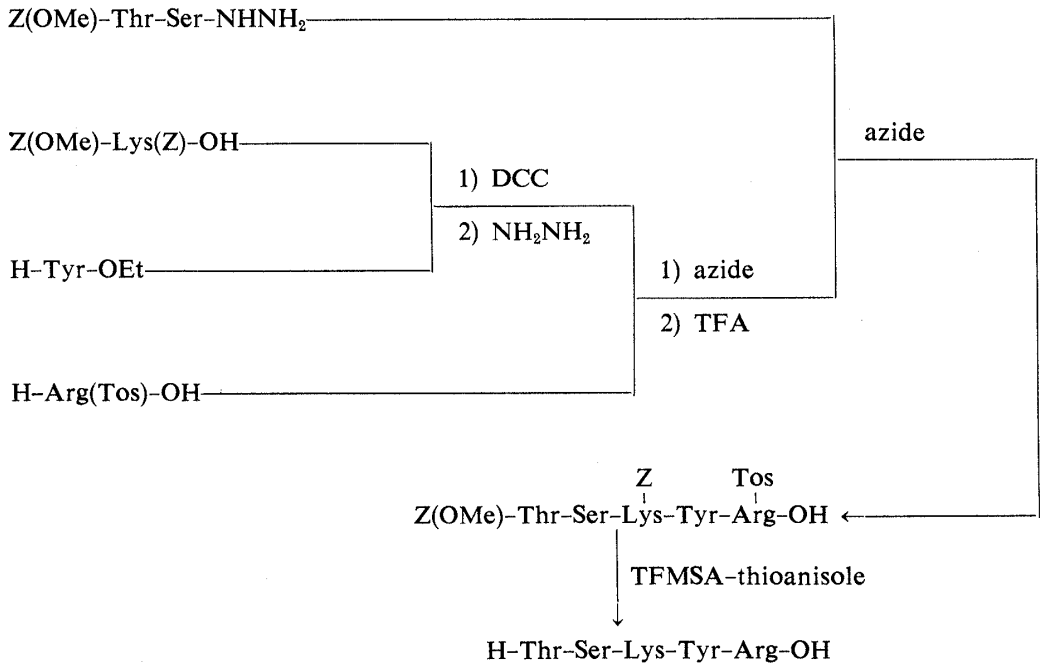

Fig. 1. Synthetic Scheme of Neo-kyotorphin

employed as the eluant and in the latter, gradient elution with 1 M ammonium acetate (pH 6.9) through a mixing flask (400 ml of H_2O) was carried out. Final desalting on Sephadex G-10 and repeated lyophilization gave the homogeneous peptide as a fluffy powder. The homogeneity of the synthetic peptide was assessed by thin layer chromatography (TLC) on a cellulose plate, amino acid analysis of an acid hydrolysate and enzymatic digest, elemental analysis and high performance liquid chromatography (HPLC).

This synthetic peptide was identical to the natural one on high performance thin layer chromatography (HPTLC) ($Rf=0.43$) and high voltage paper electrophoresis ($R_m=0.91$). These results confirm that the structure of neo-kyotorphin is H–Thr–Ser–Lys–Tyr–Arg–OH.

Synthetic neo-kyotorphin exhibited a dose-dependent analgesic effect after intracisternal injection in mice. The ED_{50} value (determined by the tail-pinch method) was 195 nmol/mouse. The analgesic activity of neo-kyotorphin is 5.6 times lower than that of kyotorphin ($ED_{50}=$ 34.7 nmol/mouse), and approximately equal to that of Leu-enkephalin ($ED_{50}=233$ nmol/mouse).

In the ileal contracting assay using isolated guinea-pig ileum, synthetic neo-kyotorphin had no contracting effect even at a dose of 2×10^{-7} M, and it showed no inhibitory effect on the electrically evoked contraction of isolated guinea-pig ileum at a dose of 1.3×10^{-4} M.

In preliminary experiments, it was found that the peptide bond between Lys^3 and Tyr^4 in neo-kyotorphin could be cleaved by a trypsin-like peptidase present in the brain, releasing Tyr–Arg (kyotorphin). Details of the analgesic activity of neo-kyotorphin will be reported elsewhere.

Experimental

The melting points are uncorrected. Rotations were determined with a Union PM-201 polarimeter. Acid hydrolyses were carried out in 6 N HCl in the presence of a small amount of phenol. LAP (Lot. 79C-8110) was purchased from Sigma Chemical Co. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer. Thin layer chromatography was carried out on silica gel (pre-coated Silica gel 60 F_{254}, Merck) or cellulose (pre-coated cellulose F, Merck). Solvent systems used were $Rf_1=CHCl_3$–MeOH–H_2O (8 : 3 : 1), $Rf_2=n$-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24).

Z(OMe)–Lys(Z)–Tyr–OEt——DCC (4.12 g) was added to a solution of Z(OMe)–Lys(Z)–OH (8.90 g) and H–Tyr–OEt (prepared from 4.91 g of the hydrochloride with 2.8 ml of Et_3N) in DMF (80 ml) and the solution was stirred for 48 h at room temperature. The urea derivative was removed by filtration, then the filtrate was concentrated and the residue was dissolved in AcOEt. The solution was washed with 5% Na_2CO_3, 10% citric acid and water successively, dried over Na_2SO_4, and concentrated. The addition of petroleum ether to the oily residue gave a solid product, which was recrystallized from AcOEt–ether. Yield 10.1 g (80.0%), mp 86—88 °C, $[\alpha]_D^{25}$ −6.4° (c=2.5, MeOH), Rf_1 0.70. *Anal.* Calcd for $C_{34}H_{41}N_3O_9$: C, 64.24; H, 6.50; N, 6.61. Found: C, 64.19; H, 6.70; N, 6.53.

Z(OMe)–Lys(Z)–Tyr–NHNH$_2$——Z(OMe)–Lys(Z)–Tyr–OEt (9.53 g) was dissolved in a mixture of MeOH (50 ml) and DMF (50 ml), then treated with hydrazine hydrate (7.5 ml) at room temperature overnight. The reaction mixture was diluted with MeOH and the precipitated product was collected by filtration and washed with MeOH. Yield 8.0 g (84.0%), mp 220—222 °C, $[\alpha]_D^{25}$ −12.0° (c=2.5, DMF), Rf_1 0.68. *Anal.* Calcd for $C_{32}H_{39}N_5O_8$: C, 61.82; H, 6.32; N, 11.27. Found: C, 61.90; H, 6.33; N, 11.02.

Z(OMe)–Lys(Z)–Tyr–Arg(Tos)–OH——The azide (prepared from 3.10 g of Z(OMe)–Lys(Z)–Tyr–NHNH$_2$ with 5.62 ml of 2.7 N HCl–dioxane, 0.67 ml of isoamyl nitrite and 2.1 ml of Et_3N) in DMF (30 ml) was added to a solution of N–Arg (Tos)–OH (3.28 g) in DMF (20 ml) containing Et_3N (1.4 ml). After being stirred for 48 h at 4 °C, the mixture was concentrated and the residue was dissolved in a mixture of AcOEt and 10% citric acid. The organic phase was washed with 10% citric acid, saturated NaCl and water, dried over Na_2SO_4, and concentrated. Addition of ether to the oily residue gave a solid product, which was recrystallized from AcOEt. Yield 4.10 g (89.0%), mp 115—118 °C, $[\alpha]_D^{25}$ −10.0° (c=2.5, MeOH), Rf_1 0.39. *Anal.* Calcd for $C_{45}H_{55}N_7O_{12}S \cdot H_2O$: C, 57.74; H, 6.14; N, 10.48. Found: C, 57.71; H, 6.07; N, 10.05.

Z(OMe)–Thr–Ser–Lys(Z)–Tyr–Arg(Tos)–OH——Z(OMe)–Lys(Z)–Tyr–Arg(Tos)–OH (3.21 g) was treated with TFA (10 ml) in the presence of anisole (2.0 ml) for 45 min at room temperature. Excess TFA was evaporated off *in vacuo* at 35 °C, then ether was added. The precipitated TFA salt was collected by filtration, dried over KOH pellets for 2 h, then dissolved in DMF (20 ml) containing Et_3N (0.5 ml). To this ice-chilled solution was added the azide (prepared from 2.69 g of Z(OMe)–Thr–Ser–NHNH$_2$ with 8.08 ml of 2.7 N HCl–dioxane, 0.93 ml of isoamyl nitrite and 2.94 ml of Et_3N) in DMF (30 ml). After being stirred for 48 h at 4 °C, the mixture was concentrated and the oily residue was dissolved in a mixture of AcOEt and 10% citric acid. The organic phase was washed with 10% citric acid, saturated NaCl and water successively, dried over Na_2So_4, and concentrated. Addition of ether to the residue gave a solid product. For further purification, this product was dissolved in a small amount of $CHCl_3$–MeOH (9 : 1, v/v) and applied to a column of silica (3.3 × 25 cm), which was eluted with the same solvent, followed by $CHCl_3$–MeOH–H_2O (8 : 3 : 1, v/v). The fractions containing the substance of Rf_1 0.22 were combined and the solvent was evaporated off. The residue was triturated with ether and the resulting powder was recrystallized from MeOH–AcOEt. Yield 2.95 g (76.0%), mp 116—119 °C, $[\alpha]_D^{25}$ −12.4° (c=2.5, MeOH), Rf_1 0.22. *Anal.* Calcd for $C_{52}H_{67}N_9O_{16}S \cdot H_2O$: C, 54.68; H, 6.27; N, 11.04. Found: C, 54.90; H, 6.45; N, 10.91. Amino acid analysis of an acid hydrolysate gave the following ratios: Thr 0.93 Ser 0.89 Lys 1.02 Tyr 1.00 Arg 0.89 (average recovery, 91%).

H–Thr–Ser–Lys–Tyr–Arg–OH (Neo-kyotorphin)——The above protected pentapeptide (630 mg; 0.5 mM) was treated with TFA (12 ml) in the presence of thioanisole (3.1 ml; 50 eq.) and *o*-cresol (2.7 ml; 50 eq.) in ice-bath for 30 min, then TFMSA (1.2 ml) was added. The reaction mixture was stirred for 3 h at room temperature, and TFA was evaporated off *in vacuo* at 30 °C. After being washed with *n*-hexane, the oily residue was dried over KOH pellets for 1 h, then dissolved in H_2O (50 ml) and treated with Amberlite IR- 400 (acetate form, approximately 5 g) for 30 min. The resin was removed by filtration and washed with a small amount of 10% AcOH (10 ml). This solution was washed with ether, then the pH was adjusted to 10 with 5% NH_4OH and after 30 min, to 7 with 3% AcOH. The solution was lyophilized and the residue was dissolved in 3% AcOH (3 ml). The solution was applied to a column of Sephadex G-10 (2.6 × 100 cm), which was eluted with 3% AcOH and the absorbancy of each fraction (5 ml) was determined at 275 nm. The first main fractions (tube no. 51—73) were combined and the solvent was removed by lyophilization to give a crude product (155 mg; 41%).

The crude product (95 mg) was dissolved in H_2O (100 ml) and applied to a column of CM-cellulose (2.8 × 28 cm), which was eluted with H_2O (100 ml) and a linear gradient of 1 M $AcONH_4$ (pH 6.9) through a mixing flask (400 ml of H_2O). Individual fractions (10 ml each) were collected and the absorbancy at 275 nm was determined. After a small peak (tube no. 44—47), the main peak (tube no. 48—55) was combined and the solvent was removed by repeated lyophilization. For the final desalting, the lyophilized material was dissolved in 3% AcOH (5 ml) and passed through a column of Sephadex G-10 (2.6 × 100 cm). A white fluffy powder was recovered from a single chromatographic peak fraction after repeated lyophilization (82 mg: 87% recovery). $[\alpha]_D^{25}$ −21.7° (c=1.2, 3% AcOH), Rf_2 0.43 (cellulose plate). *Anal.* Calcd for $C_{28}H_{47}N_9O_9 \cdot 2CH_3COOH \cdot 3H_2O$: C, 46.42; H, 7.43; N, 15.23. Found: C, 46.29; H, 7.18; N, 14.79. Amino acid analysis of an acid hydrolysate gave the following ratios: Thr 0.91 Ser 0.86 Lys 1.00 Tyr 0.98 Arg 0.83 (average recovery; 91%). Amino acid analysis after LAP digestion gave the following ratios: Thr 1.00 Ser 1.00 Lys 1.00 Tyr 0.98 Arg 1.02 (average recovery; 94%). Reverse-phase HPLC was carried out with a Waters compact model, using a μBondapak C_{18} (0.39 × 30 cm) column and a solvent system of 0.1 M KH_2PO_4 containing 2% acetonitrile at a flow rate of 1 ml/min. The synthetic peptide exhibited a single peak at a retention time of 6.70 min under the above conditions.

Bioassay——a) Analgesic Activity: The analgesic effect of the synthetic peptide was examined by using the intracisternal injection technique in mice, according to the method reported previously.[13]

b) Ileal Contracting Activity: Ileal contracting activity was determined by using isolated guinea-pig ileum by the method reported by Segawa *et al.*,[14] and the inhibitory effect on the electrically evoked contraction of the isolated guinea-pig ileum was determined by the method reported by Kosterlitz *et al.*[15]

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References and Notes

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. Abbreviations used are those recommended by the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature: *J. Biol. Chem.*, **247**, 977 (1972). Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, 2,6-Cl_2Bzl = 2,6-dichlorobenzyl, Tos = *p*-toluenesulfonyl, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, HONB = *N*-hydroxy-5-norbornene-2,3-dicarboximide, DCC = *N,N'*-dicyclohexylcarbodiimide, Et_3N = triethylamine, DMF = dimethylformamide.
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