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Studies on Peptides. XCIV.[1,2] Synthesis and Activity of Kyotorphin and Its Analogs

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A dipeptide named kyotorphin, H–Tyr–Arg–OH, and its retro isomer were synthesized using N^G-mesitylene-2-sulfonylarginine. Two corresponding stereoisomers containing D-Arg were also synthesized. Among these, H–Tyr–D-Arg–OH was found to possess an analgesic effect 6 times higher than that of the corresponding L-isomer.

Keywords——analgesic dipeptide, Tyr–Arg; synthesis of kyotorphin; stereoisomer of kyotorphin; retro-isomer of kyotorphin; tail-pinch test of synthetic dipeptides

Recently, Takagi *et al.*[4] isolated a morphine-like dipeptide, H–Tyr–Arg–OH, named kyotorphin from bovine brain. We have synthesized this biologically active dipeptide, its retro-peptide and their D-isomers. Biological evaluations of these synthetic compounds are reported in this paper.

Though various methods are available for the synthesis of these compounds, we selected a new arginine derivative, Arg-(Mts)[5] bearing a protecting group removable by MSA,[6] for the synthesis of kyotorphin and its retro-isomer, as shown in Chart 1.

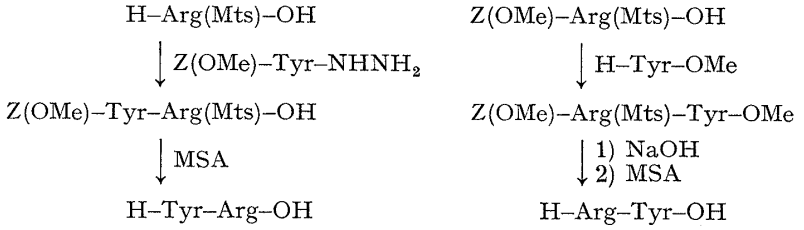

Chart 1. Synthetic Scheme for Kyotorphin and Its Retro-isomer

The azide condensation[7] of Z(OMe)–Tyr–$NHNH_2$[8] with H–Arg–(Mts)–OH derived from the corresponding Z(OMe)-derivative afforded Z(OMe)-Tyr–Arg(Mts)–OH, from which the two protecting groups, Mts and Z(OMe), were removed by MSA treatment. A mixture of scavengers, anisole–thioanisole–*o*-cresol, was employed to suppress a side reaction, *i.e.*, O-mesitylene-2-eulfonylation at the Tyr residue.[5] The protected dipeptide was converted to the corresponding acetate and purified by column chromatography on CM-cellulose. The desired compound was eluted by gradient elution with 0.1 M NH_4HCO_3 buffer at pH 7.9. The

1) Part XCIII: N. Fujii and H. Yajima *J. Chem. Soc. Perkin I*, submitted.
2) Unless otherwise mentioned, Tyr and Arg are of the L-configuration. The following abbreviations are used: Z(OMe)=*p*-methoxybenzyloxycarbonyl, Z=benzyloxycarbonyl, Mts=mesitylene-2-sulfonyl, DCC=dicyclohexylcarbodiimide, TFA=trifluoroacetic acid, MSA=methanesulfonic acid, DMF=dimethylformamide, THF=tetrahydrofuran.
3) Location: *Sakyo-ku, Kyoto, 606, Japan.*
4) H. Takagi, H. Shiomi, H. Ueda, and H. Amano, *Eur. J. Pharmacol.*, **55**, 109 (1979).
5) H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J.C.S. Chem. Commun.*, **1978**, 482; H. Yajima, M. Takeyama, J. Kanaki, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.*, **26**, 3752 (1978).
6) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, **23**, 1164 (1975).
7) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
8) N. Fujii and H. Yajima, *Chem. Pharm. Bull.*, **23**, 2446 (1975).

homogeneity of the synthetic kyotorphin was assessed by TLC, acid hydrolysis and elemental analysis. Identity of the synthetic dipeptide with natural kyotorphin was established by comparison of their *Rf* values and activities.[4]

In order to prepare the retro-dipeptide, H–Arg–Tyr–OH, Z(OMe)–Arg(Mts)–OH was condensed with H–Tyr–OMe by DCC.[9] Attempts to crystallize the resulting dipeptide ester, Z(OMe)–Arg(Mts)–Tyr–OMe, were unsuccessful. Though alkaline saponification of the methyl ester from Arg-peptides is not desirable, careful treatment of this oily product with sodium hydroxide according to Hofmann *et al.*[10] afforded Z(OMe)–Arg(Mts)–Tyr–OH as a powder, which, after characterization by elemental analysis, was subjected to deprotection with MSA as described above. The deprotected peptide was purified by ion-exchange chromatography on CM-cellulose as described above.

Next, D-Arg isomers of Tyr–Arg and Arg–Tyr were prepared. Considering the availability of starting materials, the former compound was prepared through Z(OMe)–Tyr–D–Arg–OH, without using a protecting group for D-Arg, as shown in Chart 2. The azide condensation of Z(OMe)–Tyr–$NHNH_2$ with D-Arg afforded Z(OMe)–Tyr–D-Arg–OH, from which the Z(OMe) group was removed by TFA treatment to give a product showing a single spot on TLC. In order to ensure its homogeneity, the deprotected peptide, after conversion to the corresponding acetate, was purified by column chromatography on CM-cellulose as described above.

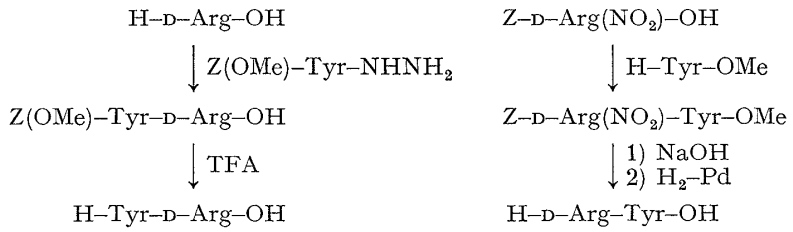

Chart 2. Synthetic Scheme of Two Stereoisomers of Kyotorphin

In order to prepare H–D-Arg–Tyr–OH, protection of the guanidino group of D-Arg is essential during the activation of its carboxyl function. An available derivative, Z–D-Arg–(NO_2)–OH, used for our previous synthesis of a stereoisomer of the active core of α-melanocyte-stimulating hormone,[11] was adopted for the present synthesis. The mixed anhydride procedure[12] was employed to condense Z–D-Arg(NO_2)–OH with H–Tyr–OMe, affording the dipeptide, Z–D-Arg(NO_2)–Tyr–OMe. Referring to the procedure of Hofmann *et al.*,[10] the above dipeptide ester was saponified with sodium hydroxide to give Z–D-Arg(NO_2)–Tyr–OH, which, after characterization by elemental analysis, was subjected to catalytic hydrogenation to remove all protecting groups. The deprotected dipeptide, H–D-Arg–Tyr–OH, was purified by gel-filtration on Sephadex G-10.

Analgesic effects of the synthetic peptides were evaluated by the tail-pinch test.[13] The peptides were dissolved in distilled water (10 μl) and administered with a J-shaped needle into the cisterna magna of unanesthetized mice. Each assay was done with 7—14 mice. Synthetic kyotorphin had the same effect as the natural dipeptide. The three analogs produced analgesic effects similar to that of kyotorphin in a dose-dependent manner, and there were no abnormal behavioral changes such as convulsion or sedation. The ED 50 values are shown in Table I. The most potent analgesia was obtained with H–Tyr–D–Arg–OH.

9) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
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Table I. The Analgesic Effects of Synthetic Kyotorphin and Its Analogs

	ED 50 (intracisternal administration)	
	μg/animal	nmol/animal
L-Tyr-L-Arg	11.7	34.7
L-Tyr-D-Arg	2.1	6.2
L-Arg-L-Tyr	5.4	16.0
D-Arg-L-Tyr	8.5	25.2

Experimental

Thin-layer chromatography was performed on silica gel (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–pyridine–H_2O (4: 1: 1: 2). The azide was prepared according to Honzl and Rudinger.[7]

Z(OMe)-Tyr-Arg(Mts)-OH——The azide (prepared from 1.80 g, 5 mmol of Z(OMe)–Tyr–$NHNH_2$[8]) in DMF (15 ml) and Et_3N (0.7 ml, 5 mmol) were added to an ice-chilled solution of H–Arg(Mts)–OH (1.85 g, 5 mmol) and Et_3N (0.7 ml, 5 mmol) in DMF (10 ml), and the mixture was stirred at 4° for 48 hr. After neutralization with AcOH, the solution was concentrated and the residue was dissolved in *n*-BuOH. The extract was washed with 1 N HCl and H_2O, dried over Na_2SO_4 and concentrated. Trituration of the residue with ether afforded a powder, which was recrystallized from *n*-BuOH and ether; yield 3.09 g (91%), mp 122—127°, $[\alpha]_D^{27}$ −0.5° (*c*=0.6, MeOH), Rf_1 0.36. *Anal.* Calcd for $C_{33}H_{41}N_5O_9S \cdot H_2O$: C, 56.48; H, 6.18; N, 9.98. Found: C, 56.49; H, 6.41; N, 9.97.

H-Tyr-Arg-OH——Z(OMe)–Tyr–Arg(Mts)–OH (1.37 g, 2 mmol) was treated with MSA (3 ml) in the presence of a mixture of anisole–thioanisole–*o*-cresol (1: 1: 1, v/v, 3 ml) at room temperature for 60 min, then dry ether was added. The oily precipitate was dissolved in H_2O (10 ml). The aqueous phase was washed with AcOEt and treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min then filtered. The filtrate was lyophilized. The residue was dissolved in H_2O (3 ml) and the solution was applied to a column of CM-cellulose (2.0×9.0 cm), which was eluted by gradient elution with 0.1 M NH_4HCO_3 (500 ml), pH 7.9, through a mixing flask containing 0.005 M NH_4HCO_3 (500 ml). Fractions of 5.5 ml were collected and the absorption of each at 275 nm was determined. The desired fractions (tube Nos. 41—53) were combined and the solvent was removed by lyophilization. The residue was repeatedly lyophilized to give a fluffy powder; yield 400 mg (53%), $[\alpha]_D^{24}$ +21.4° (*c*=0.6, 0.2 M AcOH), Rf_2 0.33. Amino acid ratios in 6 N HCl hydrolysate: Tyr 0.90, Arg 1.00 (recovery 97%). *Anal.* Calcd for $C_{15}H_{23}N_5O_4 \cdot CH_3COOH \cdot H_2O$: C, 49.15; H, 7.04; N, 16.86. Found: C, 49.09; H, 7.08; N, 16.30.

Z(OMe)-Arg(Mts)-Tyr-OH——DCC (0.89 g, 4.3 mmol) was added to a mixture of Z(OMe)–Arg(Mts)–OH (prepared from 2.32 g, 3.75 mmol of the cyclohexylamine salt) and H–Tyr–OMe (prepared from 0.96 g, 4.1 mmol of the hydrochloride with 0.58 ml, 4.1 mmol of Et_3N) in DMF–AcOEt (8 ml–2 ml). After stirring for 48 hr, the solution was filtered, the filtrate was concentrated and the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated. Attempts to crystallize the protected dipeptide ester were unsuccessful (Rf_1 0.66). The ester was dissolved in MeOH (30 ml) and treated with 1 N NaOH (5.6 ml) at room temperature for 2 hr. After neutralization with AcOH, the solution was concentrated and the residue was dissolved in 1 N NaOH. The aqueous solution was washed with AcOEt and acidified with 1 N HCl. The resulting oily precipitate was extracted with *n*-BuOH. The extract was washed with H_2O, dried over Na_2SO_4 and concentrated. Trituration of the residue with ether afforded a powder; yield 1.43 g (58%), mp 129—134°, $[\alpha]_D^{24}$ +6.2° (*c*=0.7, MeOH), Rf_1 0.32. *Anal.* Calcd for $C_{33}H_{41}N_5O_9S$: C, 57.96; H, 6.04; N, 10.24. Found: C, 57.89; H, 6.25; N, 10.13.

H-Arg-Tyr-OH——Z(OMe)–Arg(Mts)–Tyr–OH (1.37 g, 2 mmol) was treated with MSA (4 ml) in the presence of anisole–thioanisole–*o*-cresol (1: 1: 1, v/v, 3 ml) at room temperature for 60 min, then dry ether was added. The resulting precipitate was purified as described for the purification of H–Tyr–Arg–OH; yield 355 mg (38%), $[\alpha]_D^{24}$ +31.9° (*c*=0.4, H_2O). (lit.[10] +33.3° in H_2O), Rf_2 0.31. *Anal.* Calcd for $C_{15}H_{23}N_5O_4 \cdot CH_3COOH \cdot 1/2H_2O$: C, 50.23; H, 6.94; N, 17.23. Found: C, 50.38; H, 7.04; N, 17.11.

Z(OMe)-Tyr-D-Arg-OH——The azide (prepared from 1.88 g, 5.2 mmol of Z(OMe)–Tyr–$NHNH_2$) in DMF (10 ml) and Et_3N (0.73 ml, 5.2 mmol) were added to an ice-chilled solution of H-D-Arg–OH (prepared from 1.41 g, 5.7 mmol of the dihydrochloride,[11] with 1.6 ml, 11.4 mmol of Et_3N) in DMF–H_2O (2 ml—5 ml). After stirring at 4° for 48 hr, the solution was neutralized with AcOH and concentrated. The residue was extracted with *n*-BuOH. The organic phase was washed with H_2O, dried over $MgSO_4$ and concentrated. Treatment of the residue with ether afforded a powder; yield 1.72 g (62%), mp 152—157°, $[\alpha]_D^{24}$ −11.4° (*c*=0.4, MeOH), Rf_1 0.23. *Anal.* Calcd for $C_{24}H_{31}N_5O_7 \cdot HCl$: C, 53.58; H, 6.00; N, 13.02. Found: C, 54.02; H, 6.30; N, 12.90.

H-Tyr-D-Arg-OH——Z(OMe)–Tyr–D–Arg–OH (0.64 g, 1.2 mmol) was treated with TFA–anisole (2 ml–0.5 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was dissolved in H_2O (10 ml) and the solution was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min. The resin was removed by filtration, and the filtrate was lyophilized. The resulting powder (0.47 g) was purified by column chromatography on CM-cellulose as performed for the purification of the corresponding L-isomer; yield 338 mg (83%), $[\alpha]_D^{24}$ +57.5° (c=0.6, 0.2 M AcOH), Rf_2 0.33. Amino acid ratios in 6 N HCl hydrolysate: Tyr 1.00, Arg 0.97 (recovery 96%). *Anal.* Calcd for $C_{15}H_{23}N_5O_4 \cdot CH_3COOH \cdot 1/2H_2O$: C, 50.23; H, 6.94; N, 17.23. Found: C, 50.04; H, 7.25; N, 16.76.

Z-D-Arg(NO_2)-Tyr-OMe——A mixed anhydride (prepared from 1.06 g, 3 mmol of Z–D–Arg(NO_2)–OH[11]) in THF (10 ml) was added to an ice-chilled solution of H–Tyr–OMe (prepared from 0.77 g, 3.3 mmol of the hydrochloride with 0.46 ml, 3.3 mmol of Et_3N). After stirring in an ice-bath for 5 hr, the solution was concentrated and the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel (2.0×40 cm) with benzene–AcOEt (2: 5 v/v) as an eluent. Fractions containing material of Rf_1 0.65 were combined and the solvent was evaporated off. Trituration of the residue with ether afforded a powder; yield 1.04 g (65%), mp 107—110°, $[\alpha]_D^{24}$ +1.9° (c=0.4, MeOH), Rf_1 0.65. *Anal.* Calcd for $C_{24}H_{30}N_6O_8$: C, 54.33; H, 5.70; N, 15.84. Found: C, 53.93; H, 5.80; N, 15.69.

Z-D-Arg(NO_2)-Tyr-OH——Z-D-Arg(NO_2)–Tyr–OMe (531 mg, 1 mmol) was treated with 0.5 N NaOH (6 ml) at room temperature for 60 min. The aqueous phase was washed with AcOEt and acidified with 5 N HCl. The resulting oily precipitate solidified on ice-cooling, and was recrystallized from 50% aqueous acetone; 310 mg (60%), mp 125—132°, $[\alpha]_D^{24}$ +13.6° (c=0.6, MeOH), Rf_1 0.17. *Anal.* Calcd for $C_{23}H_{28}N_6O_8 \cdot 1/2H_2O$: C, 52.56; H, 5.56; N, 15.99. Found: C, 52.98; H, 5.36; N, 15.66.

H-D-Arg-Tyr-OH——Z-D-Arg(NO_2)–Tyr–OH (230 mg, 0.45 mmol) in MeOH–AcOH (5 ml–0.5 ml) was hydrogenated over a Pd catalyst for 18 hr. The catalyst was removed by filtration, the filtrate was concentrated and the residue was purified by gel-filtration on Sephadex G-10 (1.8×140 cm), eluting with 0.5 N AcOH. Fractions of 4.5 ml were collected and the absorption of each at 275 nm was determined. The desired fractions (tube Nos. 29—34) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 146 mg (80%), $[\alpha]_D^{24}$ −12.7° (c=0.3, 0.25 N AcOH), Rf_2 0.31. Amino acid ratios in 6 N HCl hydrolysate: Arg 1.00, Tyr 0.98 (recovery 92%). *Anal.* Calcd for $C_{15}H_{23}N_5O_4 \cdot CH_3COOH \cdot 1/2H_2O$: C, 50.23; H, 6.94; N, 17.23. Found: C, 50.31; H, 6.94; N, 17.72.