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Structure and Analgesic Activity Relationship of Cyclo-Tyrosyl-Arginyl and Its Three Stereoisomers[1)]

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Cyclo(–Tyr–Arg–) (I) and its three stereoisomers, in which one (or both) of the constitutive amino acids was replaced by the corresponding D-amino acid, were synthesized. Diketopiperazines were prepared from dipeptide methyl esters by the use of acetic acid as a catalyst. When administered intracerebrally into mice, I exhibited more potent analgesia than its three stereoisomers, and its activity was five times more potent than that of H–Tyr–Arg–OH. The positions of the two side chains of diketopiperazines are discussed on the basis of the proton magnetic resonance spectral data.

Keywords——cyclo(–Tyr–Arg–); cyclo(-D-Tyr–Arg–); cyclo(–Tyr-D-Arg–); cyclo(-D-Tyr-D-Arg–); analgesic activity; intracerebral administration

Kyotorphin, H–Tyr–Arg–OH, isolated from bovine brain by Takagi *et al.*,[2)] exhibited a naloxone-reversible analgesia when administered into the cisterna magna of mice. Yajima *et al.*[3)] synthesized the dipeptide and some analogs including a stereoisomer, H–Tyr-D-Arg–OH, which showed markedly increased activity.

Since a cyclic dipeptide or diketopiperazine is expected to resist peptidases *in vivo*,[4)] it is of interest to evaluate the pharmacological properties of cyclic dipeptide analogs of kyotorphin. In this paper, we describe the synthesis of cyclo(–Tyr–Arg–) and its three stereoisomers, in which one (or both) of the constitutive amino acids is replaced by the corresponding D-amino acid. In addition, the analgesic activities of these compounds after intracerebral administration into mice were determined. The synthetic schemes for the four diketopiperazines are shown in Fig. 1. The benzyl or 2-bromobenzyloxycarbonyl[5)] group was employed for protection of the phenolic hydroxy group in tyrosine, and the guanido group in arginine was protected by protonation. Diketopiperazines were synthesized by cyclization of dipeptide methyl esters according to the procedure described by Suzuki *et al.*[6)]

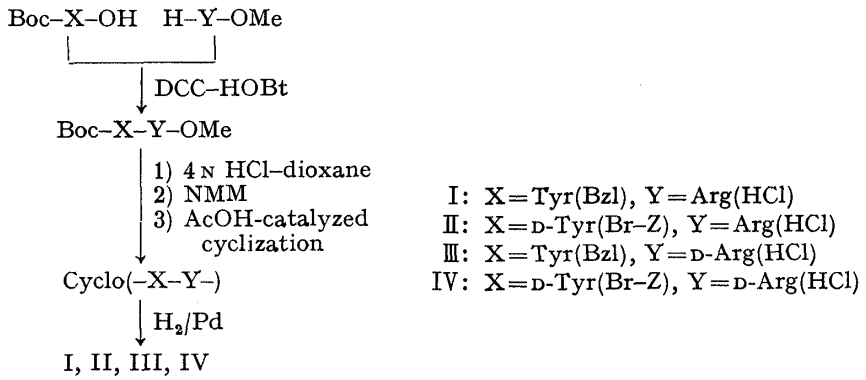

Fig. 1. Synthetic Scheme for Cyclo (–Tyr–Arg–) and Its Stereoisomers

For the preparation of cyclo(–Tyr–Arg–) (I), the benzyl group on the tyrosine residue of cyclo[–Tyr(Bzl)–Arg–]·HCl[6)] was removed by catalytic hydrogenolysis in aqueous methanol. Recrystallization of the resulting product from water gave homogeneous cyclo(–Tyr–Arg–)·

HCl, which was converted to the corresponding acetate by treatment with cation ion-exchange resin (acetate form) to increase the solubility in water.

In order to prepare cyclo(-D-Tyr–Arg–) (II), Boc-D-Tyr(Br–Z)–OH[5] and H–Arg–OMe·2HCl[7] were coupled by the DCC–HOBt method[8] to give Boc-D-Tyr(Br–Z)–Arg–OMe (V). The Boc group of V was removed by treatment with 4 N HCl–dioxane and the resulting dipeptide methyl ester was cyclized by the method mentioned above, followed by catalytic hydrogenolysis in aqueous methanol to remove the 2-bromobenzyloxycarbonyl group. Since TLC of the resulting product detected a very small amount of contaminants, the product was subjected to column chromatography on carboxymethyl (CM)-cellulose and the desired product was eluted by gradient elution with 0.05 M pyridinium acetate buffer. The product was further purified by partition chromatography on Sephadex G-25[9] to give II.

The other two stereoisomers of I, cyclo(–Tyr-D-Arg–) (III) and cyclo(-D-Tyr-D-Arg–) (IV), were analogously prepared from the corresponding precursors, Boc–Tyr(Bzl)-D-Arg–OMe (VI) and H-D-Tyr(Br–Z)-D-Arg–OMe (VII). The homogeneity of each of the four diketopiperazines prepared was assessed by TLC using two different solvent systems, by amino acid analysis and by elemental analysis.

Analgesic effects of the synthetic peptides were determined by the tail pressure method.[10] The test compounds were dissolved in Ringer's solution and administered intracerebrally to mice. Table I shows the ED_{50} values of the four diketopiperazines together with those of synthetic kyotorphin[11] and its stereoisomer, H–Tyr-D-Arg–OH.[11]

TABLE I. The Analgesic Effects of Cyclo(–Tyr–Arg–) and Its Stereoisomers after Intracerebral Administration to Mice

Compound	ED_{50}[a] (nmol/mouse)	Relative potency
C. (–Tyr–Arg–)	33.0(26.9—40.4)	5.152
C. (–D–Tyr–Arg–)	250.0(172.4—362.5)	0.680
C. (–Tyr–D–Arg–)	435.0(322.2—587.2)	0.391
C. (–D–Tyr–D–Arg–)	85.0(48.6—148.8)	2.000
H–Tyr–Arg–OH[b]	170.0(124.1—232.9)	1
H–Tyr–D–Arg–OH[b]	71.0(59.7—84.5)	2.394

a) 95% confidence limits are given in parentheses.
b) See ref. 11.
C.=cyclo.

Highly potent analgesic activity was observed with cyclo(–Tyr–Arg–) (I), which is twice as potent as H–Tyr-D-Arg–OH and five times more active than kyotorphin in the present assay system. It is noteworthy that a very high dose of naloxone was required to block the analgesic response to I (approximately 20% inhibition at 2 mg/kg intraperitoneally).[12] IV was less active than I, but still showed high potency. II and III showed much lower activity. The analgesic effect of these cyclic dipeptides reached a maximum within 5 min and there were no abnormal behavioral changes such as sedation or convulsion. Administration of Ringer's solution alone had no effect on the biting behavior.

Proton nuclear magnetic resonance (^1H NMR) spectra of I and IV in DMSO-d_6 showed unusually high field shifts of the $C_\beta H$ proton of the arginine (δ: 0.51) and D-arginine (δ: 0.50) residues, respectively. ^1H NMR spectra of II and III showed unusually high field shifts of the $C_\alpha H$ proton of the arginine (δ: 2.7—3.3) and D-arginine (δ: 2.7—3.1) residues, respectively. These results suggest that the aromatic ring of the tyrosine and D-tyrosine residues in the four diketopiperazines faces the diketopiperazine ring.[13] Dreiding model examinations together with the ^1H NMR spectral observations described above indicated that the positions of the two side chains of the constitutive amino acids in I and IV can be quite similar and are different from those in II and III. These observations suggest that the positions of the two side chains may be important for the analgesic activity of these diketopiperazines.

Experimental

All melting points are uncorrected. TLC was performed on silica gel plates (Kieselgel GF_{254}, Merck) with the following solvent systems: *Rf*(A), *n*-BuOH–AcOH–H_2O (4: 1: 5, upper phase); *Rf*(B), *n*-BuOH–pyridine–AcOH–H_2O (15: 10: 3: 12). The Boc group of V and VI was removed by 4 N HCl–dioxane treatment for TLC. Optical rotation was determined with an Atago Polax. Amino acid analyses were carried out on a Hitachi 835-50 amino acid analyzer. ^1H NMR spectra were recorded on a JEOL JNM FX-60 spectrometer with tetramethylsilane as the internal standard.

Cyclo(-Tyr-Arg-) (I)——Cyclo[-Tyr(Bzl)–Arg-]–HCl[6] (1.37 g) was dissolved in H_2O–MeOH (1: 3, 30 ml) and hydrogenated in the presence of 10% Pd/C (400 mg) for 16 h. The mixture was filtered through cellite, and the filtrate was evaporated to dryness. Recrystallization of the product from H_2O gave I, needles; wt 1.10 g (92%); mp 225—227°C (dec.); $[\alpha]_D^{20}$ +41.0° (c=0.6, 50% AcOH); *Anal.* Calcd for $C_{15}H_{21}N_5O_3 \cdot HCl \cdot 1/2H_2O$: C, 49.35; H, 6.40; N, 19.19. Found: C, 49.31; H, 6.61; N, 18.99.

The product (400 mg) was dissolved in hot 0.5 M AcOH (10 ml) and applied to a column (2×20 cm) of Dowex 1×2 (acetate form), which was eluted with 0.5 M AcOH. Sakaguchi-positive eluates were pooled and lyophilized from H_2O to give the monoacetate monohydrate of I, a colorless fluffy material; wt 410 mg (94% based on the hydrochloride); mp 214—217°C (dec.); $[\alpha]_D^{20}$ +49.2° (c=0.6, H_2O); *Rf*(A) 0.36, *Rf*(B) 0.79; *Anal.* Calcd for $C_{15}H_{21}N_5O_3 \cdot CH_3COOH \cdot H_2O$: C, 51.37; H, 6.85; N, 17.62. Found: C, 50.92; H, 6.76; N, 17.42. Amino acid ratios in acid hydrolysate: Arg 1.00, Tyr 0.98 (recovery 91%). ^1H NMR (DMSO-d_6) δ: 0.51 (1H, m, $C_\beta H$ of Arg), 0.8—1.3 (3H, m, $C_\beta H$ and $C_\gamma H_2$ of Arg), 2.6—3.0 (4H, m, $C_\delta H_2$ of Arg and $C_\beta H_2$ of Tyr), 3.3—3.7 (1H, m, $C_\alpha H$ of Arg).

Boc-D-Tyr(Br-Z)-Arg-OMe (V)——DCC (226 mg) was added to a solution of Boc-D-Tyr(Br–Z)–OH[14] (494 mg), H–Arg–OMe·2HCl[7] (261 mg) and HOBt (135 mg) in DMF (4 ml) and TEA (0.15 ml) at 0°C. The mixture was stirred at 5°C for 20 h, then a few drops of AcOH were added. DC-urea formed was filtered off and the filtrate, after dilution with H_2O (30 ml), was extracted with EtOAc (×2). The extract was washed with 1 N NH_4OH and H_2O, and then dried over $MgSO_4$. Removal of the solvent gave an oil, which was triturated with *n*-hexane to give an amorphous powder; wt 500 mg (75%); mp 95—100°C; $[\alpha]_D^{23}$ −30° (c=1, MeOH); *Rf*(A) 0.37, *Rf*(B) 0.75; *Anal.* Calcd for $C_{29}H_{38}N_5O_8Br$: C, 52.41; H, 5.76; 10.54. Found: C, 52.52; H, 6.26; N, 10.12.

Cyclo(-D-Tyr-Arg-) (II)——V (465 mg) was treated with 4 N HCl–dioxane (3 ml) for 30 min, then the solvent was evaporated off *in vacuo*. Excess HCl was removed by repeated evaporation with fresh dioxane. The resulting dipeptide methyl ester hydrochloride was cyclized and then hydrogenated as described for the preparation of I. The hydrogenated product was passed through a column (2×15 cm) of Dowex 1×2, which was eluted with H_2O. Sakaguchi-positive eluates were pooled, lyophilized and then applied to a column (2.5×15 cm) of CM-cellulose, which was eluted with a gradient formed from 0.05 M pyridine–AcOH buffer (pH 5.00, 300 ml) through a mixing chamber containing H_2O (300 ml). Fractions of 5.8 ml were collected and tubes No. 55—63 were pooled and lyophilized. A small amount of a minor product having higher *Rf* value (0.83) was detected on TLC. A solution of the resulting product in *n*-BuOH–AcOH–H_2O (4: 1: 5, upper phase, 2 ml) was applied to a column (2.5×50 cm) of Sephadex G-25, which was eluted with the same solvent. Fractions of 6 ml were collected and tubes No. 60—80 were pooled, evaporated to dryness, and lyophilized from H_2O to give a colorless fluffy material; wt 150 mg (67%); mp 166—171°C;[15] $[\alpha]_D^{23}$ −43.8° (c=0.7, H_2O);[15] *Rf*(A) 0.38, *Rf*(B) 0.71; *Anal.* Calcd for $C_{15}H_{21}N_5O_3 \cdot 3/2CH_3COOH \cdot H_2O$: C, 50.57; H, 6.84; N, 16.39. Found: C, 50.76; H, 6.96; N, 15.97. Amino acid ratios in acid hydrolysate: Arg 1.00, D-Tyr (as Tyr) 0.96 (recovery 93%). ^1H NMR (DMSO-d_6) δ: 1.2—2.0 (4H, m, $C_\beta H_2$ and $C_\gamma H_2$ of Arg), 2.7—3.3 (5H, m, $C_\alpha H$ and $C_\delta H_2$ of Arg, and $C_\beta H_2$ of D-Tyr).

Boc-Tyr(Bzl)-D-Arg-OMe (VI)——H-D-Arg–OMe·2HCl was prepared by esterification of H-D-Arg–OH (2.6 g) with $SOCl_2$ in MeOH,[16] reprecipitated from MeOH–abs. ether; wt 3.7 g (95%); mp 193—195°C (dec.); $[\alpha]_D^{19}$ −23.0° (c=1, MeOH); *Rf*(A) 0.14, *Rf*(B) 0.51; *Anal.* Calcd for $C_7H_{16}N_4O_2 \cdot 2HCl \cdot 1/2H_2O$: C, 31.11; H, 7.09; N, 20.73. Found: C, 31.03; H, 7.02; N, 20.56.

The title compound was obtained from Boc–Tyr(Bzl)–OH (371 mg) and H-D-Arg–OMe·2HCl (261 mg) in the same manner as described for the preparation of V; wt 510 mg (94%); mp 98—104°C; $[\alpha]_D^{23}$ +27.0° (c=1, MeOH); *Rf*(A) 0.38, *Rf*(B) 0.75; *Anal.* Calcd for $C_{28}H_{39}N_5O_6 \cdot H_2O$: C, 60.09; H, 7.38; N, 12.52. Found: C, 60.44; H, 7.06; N, 13.08.

Cyclo(-Tyr-D-Arg-) (III)——VI (330 mg) was treated with 4 N HCl–dioxane to remove the Boc group as described before. The resulting dipeptide methyl ester was cyclized, hydrogenated and purified by column chromatography on CM-cellulose, then subjected to partition chromatography on Sephadex G-25 as described for the preparation of II to give a colorless fluffy material; wt 183 mg (57%); mp 220—224°C (dec.); $[\alpha]_D^{23}$ +54.3° (c=0.4, H_2O); *Rf*(A) 0.39, *Rf*(B) 0.72; *Anal.* Calcd for $C_{15}H_{21}N_5O_3 \cdot CH_3COOH \cdot 3/2H_2O$: C, 50.23; H, 6.94; N, 17.23. Found: C, 50.62; H, 7.01; N, 17.05. Amino acid ratios in acid hydrolysate: D-Arg (as Arg) 1.00, Tyr 0.96 (recovery 89%). ^1H NMR (DMSO-d_6) δ: 1.2—2.0 (4H, m, $C_\beta H_2$ and $C_\gamma H_2$ of D-Arg), 2.7—3.1 (5H, m, $C_\alpha H$ and $C_\delta H_2$ of D-Arg, and $C_\beta H_2$ of Tyr).

H-D-Tyr(Br-Z)-D-Arg-OMe·2HCl (VII)——Boc-D-Tyr(Br–Z)–OH[14] (494 mg) and H-D-Arg–OMe·2HCl (261 mg) were coupled in the same manner as described for the preparation of V to give Boc-D-Tyr(Br–Z)-D-

Arg–OMe as an oil. Removal of the Boc group of the resulting product by 4 N HCl–dioxane treatment gave VII as a hygroscopic powder; wt 523 mg (82%); mp 116—125°C; $[\alpha]_D^{23}$ +9.8° (c=1, MeOH); *Rf*(A) 0.35, *Rf*(B) 0.74; *Anal.* Calcd for $C_{24}H_{30}N_5O_6Br \cdot 2HCl \cdot 4H_2O$: C, 45.08; H, 6.31; N, 10.95. Found: C, 44.63; H, 6.19; N, 10.57.

Cyclo(-D-Tyr-D-Arg-) (IV)——VII (380 mg) was cyclized, hydrogenated and purified by column chromatography on CM-cellulose in the same manner as described for the preparation of II to give a colorless fluffy material; wt 110 mg (58%); mp 215—217°C (dec.); $[\alpha]_D^{23}$ −43.9° (c=0.57, H_2O); *Rf*(A) 0.37, *Rf*(B) 0.72; *Anal.* Calcd for $C_{15}H_{21}N_5O_3 \cdot CH_3COOH \cdot 3/2H_2O$: C, 50.23; H, 6.94; N, 17.23. Found: C, 50.46; H, 6.61; N, 17.64. Amino acid ratios in acid hydrolysate: D-Arg (as Arg) 1.00, D-Tyr (as Tyr) 0.96 (recovery 90%). ^{1}H NMR (DMSO-d_6) δ: 0.50 (1H, m, $C_\beta H$ of D-Arg), 0.7—1.4 (3H, m, $C_\beta H$ and $C_\gamma H_2$ of D-Arg), 2.6—3.2 (4H, m, $C_\delta H_2$ of D-Arg and $C_\beta H_2$ of D-Tyr), 3.3—3.8 (1H, m, $C_\alpha H$ of D-Arg).

Analgesic Activity Assay——For Analgesic activity assay, ddY strain mice weighing 22—25 g were used. The test compounds were dissolved in Ringer's solution (20 μl) and intracerebrally injected into unanesthetized mice according to the modified method of Brittain and Handley.[17] Only mice which bit the base of the tail or turned round the pressing point in the pressure range of 40—60 mmHg were used. Each assay was done with **10** mice and the analgesic effect was determined to be positive when the tail pressure was increased over 1.5-fold by injection of the test compound. The ED_{50} value of each test compound is shown in Table I.

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References and Notes

1) Unless otherwise stated, Tyr and Arg are of L-configuration. The following abbreviations are used: Boc=*tert*-butoxycarbonyl, Br–Z=2-bromobenzyloxycarbonyl, Bzl=benzyl, Me=methyl, TEA=triethylamine, NMM=N-methylmorpholine, DCC=dicyclohexylcarbodiimide, HOBt=1-hydroxybenzotriazole, DMF=dimethylformamide, TLC=thin-layer chromatogrophy.
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