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Enkephalin Analogs Containing the Dipeptide Unit Tyr-Arg (Kyotorphin)[1]

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Eight Met- and Leu-enkephalin analogs substituted with L- or D-Arg at position 2 were synthesized using N^G-mesitylene-2-sulfonylarginine. Among them, H–Tyr-D-Arg–Gly–Phe–Met–OH was found to possess an analgesic effect 2.4 times higher than that of morphine on a molar basis, when injected intracisternally. This compound was also found to produce analgesia when administered intravenously.

Keywords——Met-enkephalin; Leu-enkephalin; kyotorphin; Arg(mesitylene-2-sulfonyl); methanesulfonic acid deprotection; anisole-*o*-cresol or thioanisole-*o*-cresol as cation scavengers; analgesic effect

In 1979, Takagi *et al.*[3a,b] isolated from bovine brain a morphine-like dipeptide, Tyr-Arg, named kyotorphin. At the same time, this dipeptide and its stereoisomers were synthesized by Yajima *et al.*[4]

We synthesized eight pentapeptide analogs having this dipeptide unit at the N-terminal portion of Met-enkephalin (H–Tyr–Gly–Gly–Phe–Met–OH) and Leu-enkephalin (H–Tyr–Gly–Gly–Phe–Leu–OH). Since Hughes *et al.*[5] isolated two analgesic peptides, Met- and Leu-enkephalin in 1975, syntheses of these pentapeptides and a number of analogs have been reported.[6] However, the activities of analogs having a Tyr–Arg unit in the enkephalin sequence have not previously been evaluated. It thus seemed of interest to evaluate the pharmacological changes caused by replacement of the N-terminal dipeptide unit of enkephalin by kyotorphin.

The first compound, [Arg^2]–Met–enkephalin (**1**), was synthesized starting with Z(OMe)–Gly–Phe–Met(O)–OH.[7] This, after removing the Z(OMe) group with TFA,[8] was condensed successively with Z(OMe)–Arg(Mts)–OH[9] and Z(OMe)–Tyr–$NHNH_2$[10] by the TCP[11] and azide[12] procedures, respectively, as shown in Fig. 1.

1) The following abbreviations are used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, DCC=dicyclohexylcarbodiimide, HOBt=1-hydroxybenzotriazole, MSA=methanesulfonic acid, DMF=dimethylformamide, DMSO=dimethylsulfoxide, THF=tetrahydrofuran, TFA=trifluoroacetic acid, Mts=mesitylene-2-sulfonyl, ONP=*p*-nitrophenyl ester, OTCP=2,4,5-trichlorophenyl ester, CHA=cyclohexylamine, Tos–OH=*p*-toluenesulfonic acid.
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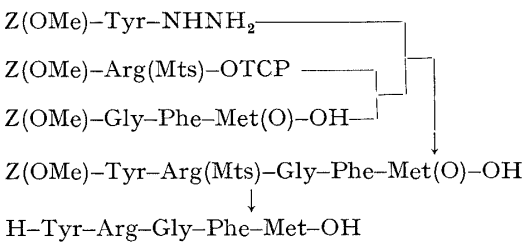

Fig. 1. Synthetic Scheme for[Arg^2]–Met-enkephalin (**1**)

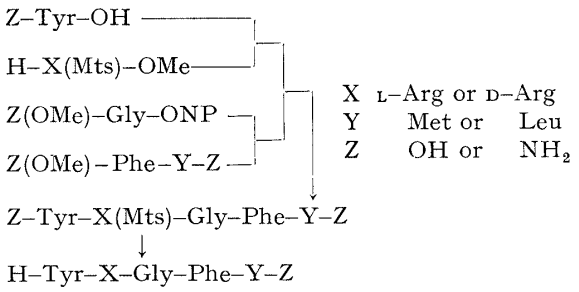

Fig. 2. Synthetic Scheme for[Arg^2]–Met-enkephalin and [Arg^2]–Leu-enkephalin Analogs

Z(OMe) and Mts were removed from the resulting protected pentapeptide by MSA treatment. In order to suppress a possible side reaction at the Tyr residue, *i. e.*, O-mesitylenesulfonylation,[9] a mixture of scavengers, anisole–*o*-cresol, was employed. The Met(O) residue of the deprotected peptide was then reduced with mercaptoethanol[13] and the reduced product was purified by column chromatography on CM-cellulose. Gradient elution with 0.1 M NH_4HCO_3 buffer was employed and after lyophilization, the desired product was obtained as a fluffy white powder. Its homogeneity was ascertained by thin layer chromatography, and amino acid and elemental analyses.

The second compound, [Arg^2]–Met-enkephalin amide (**2**), was synthesized starting with Z(OMe)–Gly–Phe–Met–NH_2 prepared in a stepwise manner (Fig. 2). Recently Kiso *et al.*[14] reported that the MSA–thioanisole deprotecting procedure is useful for the synthesis of Met-containing peptides without masking the functional group. We decided to apply a modification of this procedure to the present synthesis. With a view to the preparation of other analogs having the Tyr–Arg unit, Z–Tyr–Arg(Mts)–$NHNH_2$ was prepared by the DCC condensation[15] of Z–Tyr–OH and H–Arg(Mts)–OMe, followed by the usual hydrazine treatment of the resulting dipeptide ester. Azide condensation of this dipeptide unit with a TFA-treated sample of Z(OMe)–Gly–Phe–Met–NH_2 afforded the protected pentapeptide amide, from which the protecting groups were removed according to Kiso *et al.* without particular difficulty. In addition to thioanisole, *o*-cresol was employed to suppress a possible side reaction mentioned above, and the deprotected peptide was purified by column chromatography on CM-cellulose as described above.

In order to obtain the pentapeptide stereoisomers, [D-Arg^2]–Met-enkephalin (**3**) and its amide (**4**), Z–Tyr–D-Arg(Mts)–$NHNH_2$ was newly synthesized according to the procedure used for the synthesis of the corresponding L-isomer (Fig. 2). This hydrazide was condensed with H–Gly–Phe–Met–OH and H–Gly–Phe–Met–NH_2 by the azide procedure to give the corresponding protected pentapeptides, Z–Tyr–D–Arg(Mts)–Gly–Phe–Met–OH and Z–Tyr–D–Arg(Mts)–Gly–Phe–Met–NH_2. The modified deprotecting procedure was again applied.

For the syntheses of four Leu-enkephalin analogs, [Arg^2]–Leu-enkephalin (**5**), [Arg^2]-Leu-enkephalin amide (**6**), [D–Arg^2]–Leu-enkephalin (**7**), [D–Arg^2]–Leu-enkephalin amide (**8**), the two tripeptides, Z(OMe)–Gly–Phe–Leu–OH and Z(OMe)–Gly–Phe–Leu–NH_2, were first prepared. These tripeptides, after treatment with TFA, were condensed with Z–Tyr–Arg (Mts)–$NHNH_2$ and Z–Tyr–D–Arg(Mts)–$NHNH_2$ by the azide procedure. Deprotection and purification as mentioned above afforded the homogeneous pentapeptide analogs without particular difficulty.

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Table I. The Analgesic Effects of Synthetic Enkephalin Analogs intracisternally administered to Mice

Compound		ED 50 μg/mouse (95% confidence limits)	Naloxone antagonism
[L-Arg²]-Met-enkephalin	(1)	12.5	*n.i.*[a]
[L-Arg²]-Met-enkephalin amide	(2)	61.0 (37.4 — 99.4)	+
[D-Arg²]-Met-enkephalin	(3)	0.169(0.092— 0.309)	+
[D-Arg²]-Met-enkephalin amide	(4)	0.1—1.0[b]	+
[L-Arg²]-Leu-enkephalin	(5)	36.1 (24.4 — 53.4)	*n.i.*
[L-Arg²]-Leu-enkephalin amide	(6)	47.5 (28.1 — 80.3)	+
[D-Arg²]-Leu-enkephalin	(7)	3.4 (1.9 — 6.0)	+
[D-Arg²]-Leu-enkephalin amide	(8)	1.2 (0.74 — 1.82)	+
Met-enkephalin[c]		84.0 (57.0 —123.7)	+
Leu-enkephalin[c]		123 (89.0 —172.4)	+

a) Not investigated.
b) See the text.
c) Data cited from Ueda *et al.*[16]

The analgesic effects of synthetic enkephalin analogs were examined according to the method described by Ueda *et al.*[16] The peptides were dissolved in distilled water (10 μl) and administered with a *J*-shaped needle into the cisterna magna of unanesthetized mice. The analgesic effects were evaluated by the tail-pinch test. Each assay was done with 7—14 mice and the ED 50 values are listed in Table I.

The most potent analgesia was obtained with H–Tyr–D–Arg–Gly–Phe–Met–OH (**3**); its activity was 2.4 times more potent than that of morphine on a molar basis. The corresponding amide analog (**4**) also produced analgesia in 8 out of 19 mice tested at a relatively low dose (0.1 μg/mice), but it showed a similar analgesic potency (9 out of 20 mice) even at a higher dose (1.0 μg/mice). Thus, a definite ED 50 value could not be estimated. Of the analogs tested, only (**3**) produced analgesia in the mouse after intravenous administration (ED 50 value: 60.2 mg/kg).

Experimental

Melting points are uncorrected. Rotations were determined with a Union PM-101 digital polarimeter. The amino acid hydrolysates were analyzed with a Hitachi KLA-5 amino acid analyzer; values are not corrected for amino acid destruction. Solvents were freshly distilled and evaporation procedures were carried out *in vacuo* at a bath temperature of 40—50°. Thin-layer chromatography was performed on silica gel (Kiesel-gel, 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), Rf_3 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1).

Z(OMe)-Arg(Mts)-Gly-Phe-Met(O)-OH——Z(OMe)–Gly–Phe–Met(O)–OH (587 mg, 1.1 mmol) was treated with TFA–anisole (1.7ml—0.6 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF–DMSO (2.5 ml—2 ml) containing Et_3N (0.31 ml, 2.2 mmol). DCC (227 mg, 1.1 mmol) was added to a solution of Z(OMe)–Arg(Mts)–OH (prepared from 682 mg, 1.1 mmol of the CHA salt as usual) and 2,4,5-trichlorophenol (217 mg, 1.1 mmol) in THF (10 ml) and the mixture was stirred for 3 hr, by which time the spot of the starting material on a thin layer chromatogram had disappeared. The filtrate was combined with the above solution containing the tripeptide and the mixture was stirred at room temperature for 24 hr. The solution was concentrated and the residue, after treatment with 0.2 N AcOH, was extracted with *n*-BuOH. The organic phase was washed with H_2O and concentrated. The residue was purified by column chromatography on silica gel (3×20 cm), eluting with $CHCl_3$–MeOH–AcOH (85:10:5). Fractions containing material of Rf_1 0.23 were combined and the solvent was removed by evaporation. Trituration of the residue with ether afforded a powder, which was precipitated from DMF with ether; yield 900 mg (94%), mp 140—145°, $[\alpha]_D^{24}$ −4.0° (*c*=0.4, MeOH), *Anal.* Calcd for $C_{40}H_{53}N_7O_{11}\cdot H_2O$: C, 53.98; H, 6.23; N, 11.02. Found: C, 53.69; H, 5.94; N, 10.67.

Z(OMe)-Tyr-Arg(Mts)-Gly-Phe-Met(O)-OH——Z(OMe)–Arg(Mts)–Gly–Phe–Met(O)–OH (436 mg, 0.5 mmol) was treated with TFA–anisole (0.8 ml—0.3 ml) as usual, then dry ether was added. The resulting

16) H. Ueda, H. Amano, H. Shiomi, and H. Takagi, *Eur. J. Pharmacol.*, **56**, 265 (1979).

powder was washed with ether and dissolved in DMF (2.5 ml) containing Et_3N (0.07 ml, 0.5 mmol). The azide (prepared from 216 mg, 0.6 mmol of Z(OMe)–Tyr–$NHNH_2$) in DMF (2.5 ml) and Et_3N (0.08 ml, 0.6 mmol) were added to the above ice-chilled solution. After stirring at 4° for 48 hr, the solution was concentrated and the residue was treated with 3% AcOH. The resulting powder was washed with 3% AcOH and recrystallized from MeOH and AcOEt; yield 389 mg (75%), mp 121—127°, $[\alpha]_D^{24}$ −13.1° (c=0.7, MeOH), Rf_1 0.15. *Anal.* Calcd for $C_{49}H_{62}N_8O_{13}S$: C, 56.85; H, 6.04; N, 10.83. Found: C, 56.63; H, 6.18; N, 10.72.

H-Tyr-Arg-Gly-Phe-Met-OH, [Arg²]-Met-enkephalin (1)——The above protected pentapeptide (155 mg, 0.15 mmol) was treated with MSA (2.6 ml) in the presence of anisole–*o*-cresol (1: 1, v/v, 1 ml) in an ice-bath for 15 min and at room temperature for 60 min, then dry ether was added. The resulting oily precipitate was washed with ether, dissolved in H_2O (10 ml) and treated with Dowex 1×4 (acetate form, approximately 2 g) for 30 min. The resin was removed by filtration, and the filtrate was lyophilized. The residue was subjected to gel-filtration on Sephadex G-15 (3×110 cm), which was eluted with 0.25 N AcOH. Individual fractions (5 ml each) were collected and the absorption at 275 nm was determined. The desired fractions (tube Nos. 70—78) were combined and the solvent was removed by lyophilization. The resulting powder was dissolved in H_2O (5 ml) and incubated with mercaptoethanol (0.5 ml) at 50° for 24 hr. The solution was applied to a column of CM-cellulose (1.8×10 cm), which was eluted with a gradient up to 0.1 M NH_4HCO_3 (pH 7.9). Individual fractions (5 ml each) were collected. The desired fractions (tube Nos. 68—77) were combined and the solvent was removed by lyophilization. Lyophilization was repeated from 0.2 N AcOH to remove traces of NH_4HCO_3, yielding a fluffy powder; yield 37 mg (37%), $[\alpha]_D^{24}$ −2.9° (c=0.5, 0.2N AcOH), Rf_2 0.51, Rf_3 0.53. Amino acid ratios in 3 N Tos–OH hydrolysate; Arg 0.99, Gly 1.16, Met 0.96, Tyr 0.99, Phe 1.00 (average recovery 98%). *Anal.* Calcd for $C_{31}H_{44}N_8O_7S \cdot CH_3COOH \cdot H_2O$: C, 52.78; H, 6.71; N, 14.92. Found: C, 52.80; H, 6.23; N, 14.40.

Z(OMe)-Gly-Phe-Met-NH_2——Z(OMe)–Phe–Met–NH_2 (6.91 g, 15 mmol) was treated with TFA–anisole (17 ml—8 ml) as usual, then *n*-hexane was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* for 3 hr, then dissolved in DMF (30 ml), together with Et_3N (4.3 ml, 31 mmol), HOBt (2.03 g, 15 mmol) and Z(OMe)–Gly–ONP (5.77 g, 16 mmol). After stirring at room temperature for 24 hr, the solution was concentrated and the residue was treated with ether. The resulting powder was washed with 5% citric acid and H_2O, and precipitated from DMF with AcOEt; yield 6.74 g (87%), mp 172—176°, $[\alpha]_D^{24}$ −20.5° (c=0.6, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{25}H_{32}N_4O_6S$: C, 58.12; H, 6.24; N, 10.85. Found: C, 57.92; H, 6.21; N, 10.77.

Z-Tyr-Arg(Mts)-OMe——H–Arg(Mts)–OH (6.77 g, 19 mmol) was methylated with thionylchloride-MeOH in the usual way and the methyl esther HCl salt (8.10 g, 20 mmol) thus obtained was dissolved in DMF (30 ml) together with Et_3N (2.79 ml, 20 mmol). Z–Tyr–OH (6.31 g, 20 mmol) in THF (30 ml) was added to this stirred solution, followed by DCC (4.13 g, 20 mmol). After 18 hr, the solution was filtered, the filtrate was concentrated and the residue was dissolved in AcOEt. The organic phase was washed with 1 N HCl, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then concentrated. Trituration of the residue with *n*-hexane afforded a powder; yield 11.54 g (86%), mp 85—90°, $[\alpha]_D^{24}$ −2.4° (c=0.2, MeOH), Rf_1 0.54. *Anal.* Calcd for $C_{33}H_{41}N_5O_8S$: C, 59.35; H, 6.19; N, 10.49. Found: C, 59.81; H, 6.45; N, 10.09.

Z-Tyr-Arg(Mts)-$NHNH_2$——Z–Tyr–Arg(Mts)–OMe (10.38 g, 15.5 mmol) in DMF (50 ml) was treated with 80% hydrazine hydrate (7.8 ml, 10 equiv.) overnight. Removal of the solvent, followed by trituration of the residue with MeOH afforded a powder, which was precipitated from DMF with MeOH; yield 8.50 g (82%), mp 247—252°, $[\alpha]_D^{24}$ −10.3° (c=0.5, DMF), Rf_1 0.44. *Anal.* Calcd for $C_{32}H_{41}N_7O_7S \cdot 1/2H_2O$: C, 56.79; H, 6.11; N, 14.49. Found: C, 56.65; H, 6.13; N, 14.35.

Z-Tyr-Arg(Mts)-Gly-Phe-Met-NH_2——Z(OMe)–Gly–Phe–Met–NH_2 (1.55 g, 3 mmol) was treated with TFA–anisole (4.6 ml—1.6ml) as usual, then dry ether was added. The resulting powder was washed with ether and dissolved in DMF (8 ml) containing Et_3N (0.42 ml, 3 mmol). The azide (prepared from 2.0 g, 3 mmol of Z–Tyr–Arg(Mts)–$NHNH_2$) in DMF (8 ml) and Et_3N (0.42 ml, 3 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4° for 48 hr. The solvent was removed by evaporation and the residue was treated with 5% citric acid. The resulting powder was washed with 1 N HCl and H_2O, then precipitated from MeOH and ether; yield 2.22 g (75%), mp 138—142°, $[\alpha]_D^{24}$ −19.1° (c=0.2, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{48}H_{61}N_9O_{10}S_2 \cdot 1/2H_2O$: C, 57.81; H, 6.27; N, 12.64; Found: C, 57.78; H, 6.43; N, 12.64.

H-Tyr-Arg-Gly-Phe-Met-NH_2, [Arg²]-Met-enkephalin Amide (2)——The above protected pentapeptide (494 mg, 0.5 mmol) was treated with MSA–TFA (2 ml—1 ml) in the presence of thioanisole (0.5 ml) and *o*-cresol (0.5 ml) in an ice-bath for 45 min. The deprotected peptide was converted to the corresponding acetate, and purified by column chromatography on Sephadex G-15 followed by CM-cellulose as described for the purification of [Arg²]-Met-enkephalin (1); yield 127 mg (38%), $[\alpha]_D^{24}$ −8.3° (c=0.3, 0.2 N AcOH), Rf_2 0.63, Rf_3 0.56. Amino acid ratios in 3 N Tos–OH hydrolysate; Arg 0.95, Gly 1.18, Met 0.95, Tyr 0.99, Phe 1.00 (average recovery 92%). *Anal.* Calcd for $C_{31}H_{45}N_9O_6S \cdot 2CH_3COOH \cdot 1/2H_2O$: C, 52.48; H, 6.80; N, 15.74. Found: C, 52.12; H, 6.47; N, 16.42.

Z(OMe)-D-Arg-OH——H-D-Arg-OH HCl (17.07 g, 81 mmol) in H_2O (60 ml) was treated with *p*-methoxybenzyl-S-4,6-dimethylpyrimidin-2-yl-thiocarbonate[17] (29.59 g, 97 mmol) in dioxane (60 ml) in the presence

17) T. Nagasawa, K. Kuroiwa, K. Narita, and Y. Isowa, *Bull. Chem. Soc. Jap.*, **46**, 1269 (1973).

of Et_3N (22.6 ml, 0.16 mol) for 24 hr. After removal of the solvent, the residue was dissolved in H_2O (100 ml). The aqueous phase was washed with AcOEt, the pH of the solution was adjusted to 7 with 1 N HCl, and the solution was concentrated. Storage of the residue in a refrigerator overnight afforded a crystalline mass, which was recrystallized from H_2O; yield 19.82 g (72%), mp 191—194°, $[\alpha]_D^{24}$ +1.6° (c=0.6, AcOH), Rf_1 0.10. *Anal.* Calcd for $C_{15}H_{22}N_4O_5$: C, 53.24; H, 6.55; N, 16.56. Found: C, 53.36; H, 7.00; N, 16.93.

Z(OMe)-D-Arg(Mts)-OH·CHA Salt——Z(OMe)-D-Arg–OH (5.08 g, 15 mmol) was converted to the CHA salt, essentially in the manner used for the preparation of the corresponding L-isomer;[9] yield 5.47 g (59%), mp 150—153°, $[\alpha]_D^{24}$ −6.9° (c=0.2, MeOH), Rf_1 0.41. *Anal.* Calcd for $C_{24}H_{32}N_4O_7S\cdot C_6H_{13}N$: C, 58.14; H, 7.32; N, 11.30. Found: C, 58.67; H, 7.29; N, 11.54.

H-D-Arg(Mts)-OH——Z(OMe)-D-Arg(Mts)–OH (derived from 8.30 g, 13.4 mmol of the CHA salt as usual) provided the desired compound on treatment as described for the preparation of the L-isomer;[9] yield 4.04 g (85%), mp 152—156°, $[\alpha]_D^{24}$ +5.0° (c=0.5, MeOH), Rf_2 0.62. *Anal.* Calcd for $C_{15}H_{24}N_4O_4S\cdot H_2O$: C, 48.11; H, 7.00; N, 14.96. Found: C, 48.31; H, 7.13; N, 14.77.

Z-Tyr-D-Arg(Mts)-OMe——Et_3N (2.37 ml, 17 mmol), Z–Tyr–OH (5.36 g, 17 mmol) in THF (25 ml) and DCC (3.51 g, 17 mmol) were successively added to a solution of H-D-Arg(Mts)–OMe (prepared from 6.89 g, 17 mmol of the hydrochloride as described for the preparation of the L-isomer) in DMF (25 ml), and the mixture was stirred at room temperature for 24 hr. The product was isolated in the manner described for the corresponding L-isomer; yield 9.85 g (87%), mp 84—88°, $[\alpha]_D^{24}$ +3.5° (c=0.9, MeOH), Rf_1 0.54. *Anal.* Calcd for $C_{33}H_{41}N_5O_8S$: C, 59.35; H, 6.19; N, 10.49. Found: C, 59.20; H, 6.50; N, 10.02.

Z-Tyr-D-Arg(Mts)-NHNH$_2$——Z–Tyr-D-Arg(Mts)–OMe (9.08 g, 13.6 mmol) in DMF (50 ml) was treated with 80% hydrazine hydrate (6.8 ml, 10 equiv.) overnight. The product was isolated in the manner used for the corresponding L-isomer; yield 7.40 g (81%), mp 120—123°, $[\alpha]_D^{24}$ −4.8° (c=0.4, DMF), Rf_1 0.44. *Anal.* Calcd for $C_{32}H_{41}N_7O_7S$–$2H_2O$: C, 54.61; H, 6.45; N, 13.93. Found: C, 54.55; H, 6.26; N, 14.11.

Z(OMe)-Gly-Phe-Met-OH——Z(OMe)–Phe–Met–OH (3.64 g, 7.9 mmol) was treated with TFA–anisole (6 ml—3.4 ml) as usual, then dry ether was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF–H_2O (8 ml—10 ml) together with Et_3N (2.2 ml, 15.8 mmol) and Z(OMe)–Gly–ONP (3.13 g, 8.7 mmol). The mixture was stirred at room temperature for 24 hr, then the solvent was removed by evaporation. The residue was dissolved in 5% citric acid and the resulting precipitate was extracted with AcOEt. The extract was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then concentrated. Trituration of the residue with ether afforded a powder which was recrystallized from MeOH and ether; yield 2.23 g (55%), mp 173—176°, $[\alpha]_D^{24}$ −12.6° (c=0.7, DMF), Rf_1 0.42. *Anal.* Calcd for $C_{25}H_{31}N_3O_7S$: C, 58.01; H, 6.04; N, 8.12. Found: C, 58.35; H, 6.08; N, 8.27.

Z-Tyr-D-Arg(Mts)-Gly-Phe-Met-OH——Z(OMe)–Gly–Phe–Met–OH (1.04 g, 2 mmol) was treated with TFA–anisole (2.3 ml—0.9 ml) and the deprotected peptide isolated as described above was dissolved in DMF (8 ml) containing Et_3N (0.28 ml, 2 mmol). The azide (prepared from 1.34 g, 2 mmol of Z–Tyr-D-Arg(Mts)–$NHNH_2$) in DMF (8 ml) and Et_3N (0.28 ml, 2 mmol) were added to the above ice-chilled solution. After stirring at 4° for 48 hr, the solution was concentrated and the residue was treated with 1 N HCl. The resulting powder was washed with H_2O and recrystallized from MeOH and ether; yield 1.70 g (86%), mp 129—132°, $[\alpha]_D^{24}$ −18.3° (c=0.6, DMF), Rf_1 0.42. *Anal.* Calcd for $C_{48}H_{60}N_8O_{11}S_2$: C, 58.28; H, 6.11; N, 11.33. Found: C, 58.31; H, 6.18; N, 11.40.

H-Tyr-D-Arg-Gly-Phe-Met-OH, [D-Arg^2]-Met-enkephalin (3)——The above protected pentapeptide (495 mg, 0.5 mmol) was treated with MSA–TFA (2 ml—1 ml) in the presence of thioanisole–*o*-cresol (0.5 ml—0.5 ml) and the product was purified by gel filtration on Sephadex G-15 followed by column chromatography on CM-cellulose as described for the purification of the corresponding L-isomer; yield 120 mg (37%), $[\alpha]_D^{24}$ +30.0° (c=0.4, 0.25 N AcOH), Rf_2 0.51, Rf_3 0.53. Amino acid ratios in 3 N Tos–OH hydrolysate; Arg 0.90, Gly 1.08; Met 0.95, Tyr 0.88, Phe 1.00 (average recovery 92%). *Anal.* Calcd for $C_{31}H_{44}N_8O_7S\cdot CH_3COOH\cdot H_2O$: C, 52.78; H, 6.71; N, 14.92. Found: C, 52.82; H, 6.66; N, 15.25.

Z-Tyr-D-Arg(Mts)-Gly-Phe-Met-NH$_2$——Z(OMe)–Gly–Phe–Met–NH_2 (1.03 g, 2 mmol) was treated with TFA–anisole (2.5 ml—0.9 ml) as usual, then dry ether was added. The resulting powder was washed with ether and dissolved in DMF (8 ml) containing Et_3N (0.28 ml, 2 mmol). The azide (prepared from 1.34 g, 2 mmol of Z–Tyr–D-Arg(Mts)–$NHNH_2$) in DMF (8 ml) and Et_3N (0.28 ml, 2 mmol) were added to the above ice-chilled solution, and the mixture was stirred at 4° for 48 hr. The solvent was removed by evaporation and the product was isolated as described for the purification of the corresponding L-isomer; yield 1.43 g (72%), $[\alpha]_D^{24}$ −19.2° (c=0.4, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{48}H_{61}N_9O_{10}S_2\cdot 1/2H_2O$: C, 57.81; H, 6.27; N, 12.64. Found: C, 57.74; H, 6.20; N, 12.58.

H-Tyr-D-Arg-Gly-Phe-Met-NH$_2$, [D-Arg^2]-Met-enkephalin Amide (4)——The above protected pentapeptide amide (494 mg, 0.5 mmol) was treated with MSA–TFA (2 ml—1 ml) in the presence of thioanisole–*o*-cresol (0.5 ml—0.5 ml) and the product was isolated as described for the purification of the corresponding L-isomer; yield 184 mg (55%), $[\alpha]_D^{24}$ +38.4° (c=1.0, 0.2 N AcOH), Rf_2 0.63, Rf_3 0.56. Amino acid ratios in 3 N Tos–OH hydrolysate; Arg 0.91, Gly 1.18, Met 0.98, Tyr 0.96, Phe 1.00 (average recovery 95%). *Anal.* Calcd for $C_{31}H_{45}N_9O_6S\cdot 2CH_3COOH\cdot 1/2H_2O$: C, 52.48; H, 6.80; N, 15.74. Found: C, 52.47; H, 6.78; N, 16.26.

Z(OMe)-Gly-Phe-Leu-OH——Z(OMe)–Phe–Leu–OH (5.24 g, 11.8 mmol) was treated with TFA-anisole (18 ml—6.4 ml) as usual, then dry ether was added. The resulting powder was collected by filtration, washed with ether and dissolved in DMF–DMSO (10 ml—2 ml) together with Et_3N (3.3 ml, 23.6 mmol), and Z(OMe)–Gly–ONP (4.25 g, 11.8 mmol). After stirring for 24 hr, the solution was concentrated and the residue was dissolved in 5% $NaHCO_3$. The aqueous phase was washed with AcOEt and acidified with citric acid, and the resulting precipitate was extracted with AcOEt. The extract was washed with H_2O–NaCl, dried over Na_2SO_4 and then concentrated. Trituration of the residue with ether afforded a powder, which was recrystallized from AcOEt and ether; yield 4.04 g (69%), mp 162—165°, $[\alpha]_D^{24}$ −12.7° (c=0.4, MeOH), Rf_1 0.45. *Anal.* Calcd for $C_{26}H_{33}N_3O_7$: C, 62.51; H, 6.66; N, 8.41. Found: C, 62.34; H, 6.64; N, 8.41.

Z-Tyr-Arg(Mts)-Gly-Phe-Leu-OH——Z(OMe)–Gly–Phe–Leu–OH (1.50 g, 3 mmol) was treated with TFA–anisole (4.6 ml–1.6 ml) as usual, then dry ether was added. The deprotected peptide isolated as described above was dissolved in DMF–H_2O (5 ml–5 ml) containing Et_3N (0.42 ml, 3 mmol). The azide (prepared from 2.0 g, 3 mmol of Z–Tyr–Arg(Mts)–$NHNH_2$) in DMF (8 ml) and Et_3N (0.42 ml, 3 mmol) were added to the above ice-chilled solution. After stirring at 4° for 48 hr, the solution was concentrated and the residue was treated with 1 N HCl and H_2O, then precipitated from MeOH and ether; yield 2.62 g (90%), mp 139—142°, $[\alpha]_D^{24}$ −16.8° (c=0.2, DMF), Rf_1 0.31. *Anal.* Calcd for $C_{49}H_{62}N_8O_{11}S\cdot 1/2H_2O$: C, 60.04; H, 6.48; N, 11.43. Found: C, 59.82; H, 6.49; N, 11.25.

H-Tyr-Arg-Gly-Phe-Leu-OH, [Arg²]-Leu-enkephalin (5)——Z–Tyr–Arg(Mts)–Gly–Phe–Leu–OH (486 mg, 0.5 mmol) was treated with MSA (4 ml) in the presence of thioanisole–*o*-cresol (1: 1, v/v, 1 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was dissolved in H_2O (15 ml) and treated with Dowex 1×4 (acetate form, approximately 3 g) for 30 min. The resin was removed by filtration and the filtrate was lyophilized. The resulting powder was dissolved in a small amount of 0.2 N AcOH and the solution was applied to a column of Sephadex G-15 (3×110 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were collected and the absorption at 275 nm was determined. The fractions corresponding to the main peak (tube Nos. 72—97) were combined and the solvent was removed by lyophilization. The resulting powder was then dissolved in H_2O (50 ml) and the solution was applied to a column of CM-cellulose (1.8×35 cm), which was eluted with H_2O (200 ml) and then with a gradient up to 0.1 M NH_4HCO_3 (pH 7.9) obtained through a mixing chamber containing H_2O (1000 ml). Fractions (10 ml each) were collected and the desired fractions (tube Nos. 65—70), as determined by UV absorption measurement at 275 nm, were combined and the solvent was removed by lyophilization. Lyophilization was repeated from 0.2 N AcOH to remove traces of NH_4HCO_3, yielding a fluffy white powder; yield 138 mg (38%), $[\alpha]_D^{24}$ −5.7° (c=0.3, 0.2 N AcOH), Rf_2 0.60, Rf_3 0.64. Amino acid ratios in 3 N Tos–OH hydrolysate; Arg 0.97, Gly 1.19, Leu 0.99, Tyr 0.99, Phe 1.00 (average recovery 97%). *Anal.* Calcd for $C_{32}H_{46}N_8O_7\cdot CH_3COOH\cdot 1/2H_2O$: C, 56.41; H, 7.10; N, 15.48. Found: C, 56.34; H, 6.84; N, 15.66.

Z-Tyr-Arg(Mts)-Gly-Phe-Leu-NH_2——Z(OMe)–Gly–Phe–Leu–NH_2 (1.48 g, 3 mmol) was treated with TFA–anisole (4.6 ml–1.6 ml) and the deprotected peptide isolated as described above was dissolved in DMF (8 ml) containing Et_3N (0.42 ml, 3 mmol). The azide (prepared from 2.00 g, 3 mmol of Z–Tyr–Arg(Mts)–$NHNH_2$) in DMF (8 ml) and Et_3N (0.42 ml, 3 mmol) were added to the above ice-chilled solution. After stirring at 4° for 48 hr, the solution was concentrated and the residue was treated with H_2O. The resulting powder was washed with 1 N HCl and H_2O, and precipitated from DMF with ether; yield 2.27 g (79%), mp 143—147°, $[\alpha]_D^{24}$ −17.6° (c=0.2, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{49}H_{63}N_9O_{11}S\cdot 1.5H_2O$: C, 59.98; H, 6.78; N, 12.85. Found: C, 59.78; H, 6.54; N, 12.78.

H-Tyr-Arg-Gly-Phe-Leu-NH_2, [Arg²]-Leu-enkephalin Amide (6)——The above protected pentapeptide amide (477 mg, 0.5 mmol) was treated with MSA–TFA (3 ml–1 ml) in the presence of thioanisole–*o*-cresol (0.5 ml–0.5 ml) in an ice-bath for 60 min, then dry ether was added. The deprotected peptide was purified as described for the purification of [Arg²]-Leu-enkephalin (5); yield 159 mg (49%), $[\alpha]_D^{24}$ −6.9° (c=0.5, 0.2 N AcOH), Rf_2 0.67, Rf_3 0.58. Amino acid ratios in 3 N Tos–OH hydrolysate; Arg 0.99, Gly 1.19, Leu 0.99, Tyr 1.00, Phe 1.00 (average recovery 92%). *Anal.* Calcd for $C_{32}H_{47}N_9O_6\cdot 2CH_3COOH\cdot H_2O$: C, 54.59; H, 7.25; N, 15.92. Found: C, 54.57; H, 6.64; N, 16.29.

Z-Tyr-D-Arg(Mts)-Gly-Phe-Met-OH——Z(OMe)–Gly–Phe–Leu–OH (1.00 g, 2 mmol) was treated with TFA–anisole (3 ml–1 ml) and the deprotected peptide isolated as described above was dissolved in DMF–H_2O (4 ml–4 ml) containing Et_3N (0.28 ml, 2 mmol). The azide (prepared from 1.34 g, 2 mmol of Z–Tyr-D-Arg-(Mts)–$NHNH_2$) in DMF (8 ml) and Et_3N (0.28 ml, 2 mmol) were added to the above ice-chilled solution. After stirring at 4° for 48 hr, the solution was concentrated and the product was isolated as described for the corresponding L-isomer; yield 1.32 g (68%), mp 133—137°, $[\alpha]_D^{24}$ −11.3° (c=0.5, DMF), Rf_1 0.31. *Anal.* Calcd for $C_{49}H_{62}N_8O_{11}S\cdot 1/2H_2O$: C, 60.04; H, 6.48; N, 11.43. Found: C, 59.83; H, 6.49; N, 11.39.

H-Tyr-D-Arg-Gly-Phe-Leu-OH, [D-Arg²]-Leu-enkephalin (7)——Z–Tyr-D-Arg(Mts)–Gly–Phe–Leu–OH (486 mg, 0.5 mmol) was treated with MSA–TFA (3 ml–1 ml) in the presence of thioanisole–*o*-cresol (0.5 ml–0.5 ml) in an ice-bath for 60 min, and then dry ether was added. The deprotected peptide was purified by gel filtration on Sephadex G-15, followed by column chromatography on CM-cellulose as described for the corresponding L-isomer; yield 118 mg (36%), $[\alpha]_D^{24}$ +42.5° (c=0.3, 0.2 N AcOH), Rf_2 0.60, Rf_3 0.64. Amino acid ratios in 3 N Tos–OH hydrolysate; Arg 1.06, Gly 1.10, Leu 0.99, Tyr 0.99, Phe 1.00 (average recovery (85%). *Anal.* Calcd for $C_{32}H_{46}N_8O_7\cdot CH_3COOH\cdot H_2O$: C, 55.72; H, 7.15; N, 15.29. Found: C, 55.39; H,

6.90; N, 15.89.

Z-Tyr-D-Arg(Mts)-Gly-Phe-Leu-NH_2——Z(OMe)–Gly–Phe–Leu–NH_2 (1.0 g, 2 mmol) was treated with TFA–anisole (3 ml–1 ml) and the deprotected peptide isolated as described above was dissolved in DMF (8 ml) containing Et_3N (0.28 ml, 2 mmol). The azide (prepared from 1.34 g, 2 mmol of Z–Tyr-D-Arg(Mts)–$NHNH_2$) in DMF (8 ml) and Et_3N (0.28 ml, 2 mmol) were added to the above ice-chilled solution. After stirring at 4° for 48 hr, the solution was concentrated and the product was purified essentially in the manner used for the purification of the corresponding L-isomer; yield 1.54 g (81%), mp 139—143°, $[\alpha]_D^{24}$ −17.1° (c=0.2, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{49}H_{63}N_9O_{10}S \cdot 1.5H_2O$: C, 59.98; H, 6.78; N, 12.85. Found: C, 59.82; H, 6.59; N, 12.68.

H-Tyr-D-Arg-Gly-Phe-Leu-NH_2, [D-Arg^2]-Leu-enkephalin Amide (8)——The above protected pentapeptide (477 mg, 0.5 mmol) was deprotected with MSA–TFA (3 ml–1 ml) and the product was purified essentially in the manner used for the purification of the corresponding L-isomer; yield 182 mg (56%), $[\alpha]_D^{24}$ +38.8° (c=0.9, 0.2 N AcOH), Rf_2 0.67, Rf_3 0.58. Amino acid ratios in 3 N Tos–OH hydroalyste; Arg 0.96, Gly 1.19, Leu 0.99, Tyr 0.97, Phe 1.00 (average recovery 83%). *Anal.* Calcd for $C_{32}H_{47}N_9O_6 \cdot 2CH_3COOH \cdot 1.5H_2O$: C, 53.97; H, 7.30; N, 15.74. Found: C, 53.64; H, 7.00; N, 15.40.

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