Synthesis of Met- and Leu-Enkephalin Analogues containing Chiral *N,N'*-Ethylene-bridged Phenylalanyl-Methionine and -Leucine

Hiroshi Takenaka,[a] Hiroyuki Miyake,[a] Yoshitane Kojima,*[,a] Masahide Yasuda,[b] Munekazu Gemba[c] and Tetsushi Yamashita*[a]

[a] *Department of Chemistry, Faculty of Science, Osaka City University, Sumiyoshi-ku, Sugimoto 3-3-138, Osaka 558, Japan*

[b] *Center for Laboratory Animals, Osaka University of Pharmaceutical Sciences, Kawai, Matsubara, Osaka 580, Japan*

[c] *Division of Pharmacology, Osaka University of Pharmaceutical Sciences, Kawai, Matsubara, Osaka 580, Japan*

Conformationally constrained *N,N'*-ethylene-bridged dipeptides (eXX') were conveniently synthesized in two steps using (*R*)- or (*S*)-phenylalanine (X = F) and -methionine (X' = M) [-leucine (X' = L)] as starting materials.

These dipeptides are diastereoisomeric or enantiomeric, and were used as the carboxyl terminal residues of enkephalin analogues [H–tyrosyl–D-alanyl–glycyl–eXX'–OEt]. The opiate activities of these pentapeptides were examined preliminarily by the mouse vas deferens assay, suggesting that these enkephalin analogues possess receptor-binding affinities.

Conformationally restricted and lipophilic *N,N'*-ethylene-bridged dipeptides [eXX'; piperazin-2-one (MKP) derivatives], which resist enzymatic hydrolysis, have been used as basic units of enkephalin analogues,[1,2] but these dipeptides were non-stereoselectively synthesized by alkylation of piperazin-2-one.[3,4] In these works, Moon *et al.*[2] examined the opiate activities of H–tyrosyl(Tyr)–D-alanyl(Ala)–glycyl(Gly)-eXX'–NH_2 by using the mouse tail-flick method. *N,N'*-Ethylene-bridged phenylalanyl-leucine (eFL) used as the units of their pentapeptides were racemic [(*R,R*)/(*S,S*) and (*R,S*)/(*S,R*)].

On the other hand, DiMaio and Belleau[5] reported the synthesis of chiral *N,N'*-ethylene-bridged tyrosyl-glycine, *etc.* However, their method is not necessarily suitable for the preparation of eXX' species constructed from sulfur-containing α-amino acids such as methionine because of the catalytic reduction with $Pd(OH)_2$ used in the synthetic route. Also, the diastereoselective introduction of the side chain outside the MKP ring is difficult.

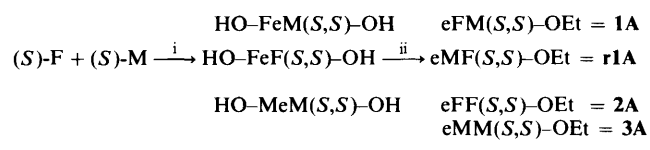
Recently, we reported a simple method[6] for preparing optically active species eXX from [HO_2C–CH(R)–NH–CH_2–]$_2$ (*N,N'*-ethylene-bridged bis-α-amino acid; HO–XeX–OH) through acid-catalysed cyclizations. Applying this procedure, chiral species eFM- and eFL–OEt were synthesized from (*R*)- or (*S*)-F(Phe) and -M(Met)[-L(Leu)]. Eight kinds of enkephalin analogues (H–Tyr–D-Ala–Gly–eXX'–OEt) were obtained using these dipeptides as their carboxyl-terminal residues, and showed receptor-binding affinities.

Moreover, the corresponding retro(r) dipeptides (eX'X) were obtained simultaneously in the preparations of dipeptides eXX', and were used as the units of retro-enkephalin analogues (H–eX'X–Gly-D-Ala–Tyr–OEt). However, these pentapeptides showed no receptor-binding affinities.

Results and Discussion

Scheme 1 shows a synthetic route for preparing species eFM-, eMF-, eFF- and eMM-OEt (eFM = unit of enkephalin analogue, eMF = unit of retro-enkephalin analogue) at the same time, in which (*S*)-Phe and -Met are used as starting materials.

In the toluene-*p*-sulfonic acid (TsOH)–EtOH method,[6] the



Scheme 1 *Reagents and conditions:* i, K_2CO_3, NaOH, $BrCH_2CH_2Br$, aq. HCl, 90 °C, 6 h, ~ 30%; ii, TsOH·H_2O, EtOH, reflux, 24 h, ~ 90%.

esterification and cyclization of *N,N'*-ethylene-bridged bis-α-amino acids (HO–FeM–OH, HO–FeF–OH and HO–MeM–OH) proceed simultaneously. The cyclic compounds (**1A**, **r1A**, **2A**[6] and **3A**[6]) thus obtained were separated out chromatographically on silica gel. Their diastereoisomeric and enantiomeric dipeptides [eFM(*R,S*)-**1B**, eMF(*S,R*)-**r1B**, eFM(*S,R*)-**1C**, eMF(*R,S*)-**r1C**, eFM(*R,R*)-**1D** and eMF(*R,R*)-OEt **r1D**] were similarly prepared.

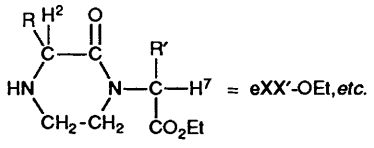
Table 1 shows the physical and analytical data of the hydrochlorides of species **1A**, **r1A**, **1D** and **r1D**, and those of tripeptides Gly-**1B**, Gly-**r1B**, Gly-**1C** and Gly-**r1C**. The hydrochlorides of these tripeptides were obtained by the coupling of Boc-Gly(G)-OH (*tert*-butoxycarbonylglycine) with diastereoisomer **1B** (**r1B**, **1C** and **r1C**) according to the 2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) method in dichloromethane at room temperature, and successively the removal of the *tert*-butoxycarbonyl (Boc) group in 4 mol dm^{-3} HCl–ethyl acetate (EtOAc) containing a small excess of ethyl methyl sulfide as a scavenger. Also, the use of (*R*)- or (*S*)-Leu instead of (*R*)- or (*S*)-Met as starting material afforded eFL(*S,S*)-**1A'** and eLF(*S,S*)-**r1A'**, eFL(*R,S*)-**1B'** and eLF(*S,R*)-**r1B'**, eFL(*S,R*)-**1C'** and eLF(*R,S*)-**r1C'**, and then eFL(*R,R*)-**1D'** and eLF(*R,R*)-OEt **r1D'**, respectively. Table 2 shows the physical and analytical data of their hydrochlorides recrystallized from EtOAc. The structures of species eXX'-OEt and their corresponding retro-dipeptides (eX'X-OEt) were established by ^1H NMR measurements containing two-dimensional correlated spectroscopy.

Table 3 reveals the chemical shifts and coupling constants of α- and β-protons of Phe residues of species **1A**, **r1A**, **1B**, **r1B**, **1A'**, **r1A'**, **1B'**, **r1B'**, **2A** and eFF(*S,S*)-OMe[7] **2A*** obtained by ^1H NMR measurements. The chemical shifts of α-protons of the

Table 1 Physical and analytical data of the hydrochlorides of piperazin-2-one derivatives constructed from (*R*)- or (*S*)-Met and -Phe

Compound	M.p. (°C)	$[\alpha]_D$ in EtOH (10^{-1} deg cm^2 g^{-1})	Formula (molecular weight)	Found (%) (required) C	H	N
1A[a]	162–165[b]	−110	$C_{18}H_{27}ClN_2O_3S$ (386.9)	55.9 (55.87)	7.0 (7.03)	7.3 (7.24)
r1A[a]	134–139[b]	−99	$C_{18}H_{27}ClN_2O_3S$ (386.9)	55.6 (55.87)	7.1 (7.03)	7.2 (7.24)
1D[a]	163–167[b]	+118	$C_{18}H_{27}ClN_2O_3S$ (386.9)	56.15 (55.87)	7.1 (7.03)	7.2 (7.24)
r1D[a]	134–139[b]	+101	$C_{18}H_{27}ClN_2O_3S$ (386.9)	56.0 (55.87)	7.1 (7.03)	7.3 (7.24)
Gly-**1B**[c]	85–90[d]	−100	$C_{20}H_{30}ClN_3O_4S\cdot 4/3H_2O$ (468.0)	51.15 (51.33)	7.0 (7.04)	8.9 (8.98)
Gly-**r1B**[c]	75–82[d]	+127	$C_{20}H_{30}ClN_3O_4S\cdot 5/4H_2O$ (466.5)	51.5 (51.49)	7.1 (7.02)	9.0 (9.01)
Gly-**1C**[d]	85–90[d]	+106	$C_{20}H_{30}ClN_3O_4S\cdot 3/5H_2O$ (454.8)	52.8 (52.82)	7.05 (6.93)	9.2 (9.24)
Gly-**r1C**[c]	79–83[d]	−134	$C_{20}H_{30}ClN_3O_4S\cdot H_2O$ (462.0)	52.1 (51.99)	7.0 (6.98)	9.1 (9.10)

[a] *m/z* 350. [b] Recrystallized from EtOAc. [c] *m/z* 407. [d] Washed thoroughly with dry diethyl ether.



= eXX′-OEt, *etc.*

1A(B, C and D) R = Ph-C(H^{11A})(H^{11B})- R′ = $MeSCH_2CH_2$

r1A(B, C and D) R = $MeSCH_2CH_2$, R′ = Ph-C(H^{15A})(H^{15B})-

2A (B, C and D) R = Ph-C(H^{11A})(H^{11B})- , R′ = Ph-C(H^{18A})(H^{18B})-

3A(B, C andD) R = R′ = $MeSCH_2CH_2$

1A′ (B′, C′ and D′) R = Ph-C(H^{11A})(H^{11B})- , R′ = Bu^i

r1A′ (B′, C′ and D′) R = Bu^i, R′ = Ph-C(H^{15A})(H^{15B})-

3A′ (B′, C′ and D′) R = R′ = Bu^i

Phe residues are 3.74, 3.67, 3.75 and 3.69 (5.16, 5.04, 5.10 and 4.98) for 2-H (7-H) of species **1A**, **1B**, **1A′** and **1B′** (**r1A**, **r1B**, **r1A′** and **r1B′**), respectively, supporting our belief that the Phe residues of enkephalin units (retro-enkephalin units) are situated on (outside) MKP rings, referring to the data of species **2A** and **2A***.

Met-Enkephalin analogues (EK-**1A**, **-1B**, **-1C** and **-1D**) were obtained by the coupling of *O*-benzyl-*N*-*tert*-butoxycarbonyl-tyrosyl-D-alanyl-glycine [**4** = Boc–Tyr(Bzl)–D-Ala–Gly–OH] with compounds **1A**, **1B**, **1C** or **1D** according to the EEDQ method in tetrahydrofuran (THF), and successively by the deprotection of blocking groups, following the thioanisole–trifluoroacetic acid (TFA) method[8] as shown in Scheme 2.

4 + eFM–OEt $\xrightarrow{i}$

Boc–Tyr(Bzl)–D–Ala–Gly–eFM–OEt $\xrightarrow{ii}$

H–Tyr–D–Ala–Gly–eFM–OEt

Scheme 2 *Reagents and conditions:* i, EEDQ, THF, room temp., 2 days, 90%; ii, TFA, thioanisole, room temp., 3 h, 60%

Similarly, Leu-enkephalin analogues (EK-**1A′**, **-1B′**, **-1C′** and **-1D′**) were prepared *via* the synthetic route in Scheme 2. Table 4 shows the physical and analytical data of compound **4** and the hydrochlorides of Met- and Leu-enkephalin analogues.

Retro-Met-enkephalin analogues (EK-**r1A**, **-r1B**, **-r1C** and **-r1D**) were obtained by the coupling of H–glycyl–D-alanyl–tyrosine ethyl ester (**5** = H–Gly–D-Ala–Tyr–OEt) with Boc–eMF–OH (Boc-**r1A**, **-r1B**, **-r1C** and **-r1D**) according to the dicyclohexylcarbodiimide (DCC)–1-hydroxybenzotriazole (HOBt) method in THF, and successively by the removal of the Boc group with 4 mol dm^{-3} HCl/EtOAc containing MeSEt as indicated in Scheme 3.

5 + Boc–eMF–OH $\xrightarrow{i}$ Boc–eMF–Gly–D-Ala–Tyr–OEt $\xrightarrow{ii}$

HCl·H–eMF–Gly–D-Ala–Tyr–OEt

Scheme 3 *Reagents and conditions:* i, HOBT, DCC, THF, −5 °C to room temp., 2 days, 80%; ii, HCl, EtOAc, MeSEt, room temp., 1 h, 90%

Retro-Leu-enkephalin analogues (EK-**r1A′**, **-r1B′**, **-r1C′** and **-r1D′**) were also prepared using Boc-eLF-OH (Boc-**r1A′**, **-r1B′**, **-r1C′** and **-r1D′**) instead of Boc–eMF–OH. Table 5 indicates the physical and analytical data of Boc-**r1A**, Boc-**r1A′** and the hydrochlorides of compound **5** and retro-Met- and -Leu-enkephalin analogues.

The biological activities of Met- and Leu-enkephalin analogues obtained here were assayed by determining the inhibition of electrically induced contractions in the mouse vas deferens (MVD). As revealed in Table 6, the biological activities (MVD; IC_{50}/nmol dm^{-3} ~4000–10 000) for all enkephalin analogues are similar, and ~400–1000-times less active than Leu-enkephalin[9] (MVD; IC_{50}/nmol dm^{-3} = 11.4 ± 1.1), showing no regular relationships between their receptor-binding affinities.

These results suggest that the configuration (fourth and fifth positions) and the replacement (fifth position, from Met to Leu) of α-amino acid residues are not necessarily important for

Table 2 Physical and analytical data of the hydrochlorides of piperazin-2-one derivatives constructed from (*R*)- or (*S*)-Leu and -Phe

Compound	M.p. (°C)	$[\alpha]_D$ in EtOH (10^{-1} deg cm^2 g^{-1})	Formula (molecular weight)	Found (%) (required) C	H	N
1A'[a]	161–164	−113	$C_{19}H_{29}ClN_2O_3$ (368.9)	61.8 (61.86)	7.95 (7.92)	7.6 (7.59)
rlA'[a]	192–195	−109	$C_{19}H_{29}ClN_2O_3$·1/6H_2O (371.8)	61.3 (61.38)	8.0 (7.95)	7.55 (7.53)
1B'[a]	143–145	+54	$C_{19}H_{29}ClN_2O_3$ (368.9)	61.8 (61.86)	7.9 (7.92)	7.5 (7.59)
rlB'[a]	166–169	+18	$C_{19}H_{29}ClN_2O_3$·1/6H_2O (371.8)	61.3 (61.38)	7.9 (7.95)	7.55 (7.53)
1C'[a]	144–148	−57	$C_{19}H_{29}ClN_2O_3$ (368.9)	61.8 (61.86)	7.9 (7.92)	7.5 (7.59)
rlC'[a]	167–169	−19	$C_{19}H_{29}ClN_2O_3$·1/6H_2O (371.8)	61.4 (61.38)	7.9 (7.95)	7.5 (7.53)
1D'[a]	162–164	+112	$C_{19}H_{29}ClN_2O_3$ (368.9)	61.8 (61.86)	8.0 (7.92)	7.5 (7.59)
rlD'[a]	193–196	+111	$C_{19}H_{29}ClN_2O_3$·1/6H_2O (371.8)	61.2 (61.38)	8.0 (7.95)	7.6 (7.53)

[a] *m/z* 332.

Table 3 Chemical shifts and coupling constants of α- and β-protons of Phe residues of **1A(A')**, **rlA(A')**, **1B(B')**, **rlB(B')**, **2A** and **2A*** in $CDCl_3$ at room temperature

eXX'- and eX'X-OEt	Chemical shift (δ) 2-H	11-H^A	11-H^B	7-H	18-H^A (15-H^A)	18-H^B (15-H^B)	Coupling constant (*J*/Hz) 2-H–11-H^A	2-H–11-H^B	7-H–18-H^A (15-H^A)	7-H–18-H^B (15-H^B)
2A	3.63	3.33	2.60	5.11	3.39	3.12	3.7	9.8	5.5	11.0
2A*	3.62	3.25	2.64	5.09	3.37	3.13	3.7	9.8	5.5	11.0
1A	3.74	3.38	2.95				3.7	9.2		
rlA				5.16	(3.39)[a]	(3.09)[a]			(4.9)[b]	(11.6)[b]
1B	3.67	3.36	2.95				3.7	9.8		
rlB				5.04	(3.37)[a]	(3.10)[a]			(5.5)[b]	(11.0)[b]
1A'	3.75	3.37	2.97				3.7	8.6		
rlA'				5.10	(3.38)[a]	(3.10)[a]			(4.9)[b]	(11.6)[b]
1B'	3.69	3.39	2.97				3.7	9.8		
rlB'				4.98	(3.37)[a]	(3.12)[a]			(5.5)[b]	(11.0)[b]

[a] The chemical shifts of the β-protons of the Phe residues of **rlA**, **rlB**, **rlA'** and **rlB'**. [b] The coupling constants between the α-protons and the β-ones of the Phe residues of **rlA**, **rlB**, **rlA'** and **rlB'**.

Table 4 Physical and analytical data of Boc–Tyr(Bzl)–D-Ala–Gly–OH **4** and the hydrochlorides of Met- and Leu-enkephalin analogue ethyl esters

Compound[a]	M.p. (°C)	$[\alpha]_D$ in EtOH (10^{-1} deg cm^2 g^{-1})	Formula (molecular weight)	Found (%) (required) C	H	N
EK-1A[b]	149–153	+88	$C_{32}H_{44}ClN_5O_7S$·4/5H_2O (692.7)	55.6 (55.49)	6.7 (6.64)	9.8 (10.11)
EK-1B[b]	139–143	−47	$C_{32}H_{44}ClN_5O_7S$·3/2H_2O (705.3)	54.6 (54.50)	7.0 (6.72)	9.9 (9.93)
EK-1C[b]	141–145	+122	$C_{32}H_{44}ClN_5O_7S$·3/2H_2O (705.3)	54.7 (54.50)	6.7 (6.72)	9.9 (9.93)
EK-1D[b]	147–152	+4	$C_{32}H_{44}ClN_5O_7S$·H_2O (696.3)	55.0 (55.20)	6.7 (6.66)	10.0 (10.06)
EK-1A'[c]	156–162	+107	$C_{33}H_{46}ClN_5O_7$·3/2H_2O (687.2)	57.7 (57.67)	7.1 (7.19)	10.2 (10.19)
EK-1B'[c]	151–155	−41	$C_{33}H_{46}ClN_5O_7$·7/2H_2O (723.3)	54.8 (54.80)	7.1 (7.38)	9.7 (9.68)
EK-1C'[c]	151–156	+152	$C_{33}H_{46}ClN_5O_7$·5/4H_2O (682.7)	58.0 (58.06)	7.15 (7.16)	10.2 (10.26)
EK-1D'[c]	156–161	−10	$C_{33}H_{46}ClN_5O_7$·3/2H_2O (687.2)	57.6 (57.67)	7.2 (7.19)	10.1 (10.19)
4[d]	100–104	+31	$C_{26}H_{33}N_3O_7$ (499.6)	62.5 (62.23)	6.75 (6.66)	8.2 (8.38)

[a] All samples were washed thoroughly with dry diethyl ether. [b] *m/z* 642. [c] *m/z* 624. [d] *m/z* 500.

enhancing or reducing the receptor-binding affinities of the pentapeptides discussed here. On the other hand, all of the retro-Met- and -Leu-enkephalin analogues showed no biological activity. This fact supports the well known requirement for the presence of the amino and phenolic groups of a Tyr residue at the first position for opioid agonist peptides.[9,10]

Table 5 Physical and analytical data of Boc-**r1A**, Boc-**r1A'** and the hydrochlorides of H-Gly-D-Ala-Tyr-OEt **5** and retro-Met- and -Leu-enkephalin analogue ethyl esters

Compound[a]	M.p. (°C)	$[\alpha]_D$ in EtOH (10^{-1} deg cm^2 g^{-1})	Formula (molecular weight)	Found (%) (required) C	H	N
EK-**r1A**[b]	145–151	−71	$C_{32}H_{44}ClN_5O_7S\cdot 3/4H_2O$ (691.8)	55.5 (55.56)	6.7 (6.63)	10.0 (10.12)
EK-**r1B**[b]	134–139	+65	$C_{32}H_{44}ClN_5O_7S\cdot H_2O$ (696.3)	55.2 (55.20)	6.7 (6.66)	9.8 (10.06)
EK-**r1C**[b]	135–141	−36	$C_{32}H_{44}ClN_5O_7S\cdot H_2O$ (696.3)	55.2 (55.20)	6.7 (6.66)	10.0 (10.06)
EK-**r1D**[b]	128–134	+91	$C_{32}H_{44}ClN_5O_7S\cdot 4/5H_2O$ (692.7)	55.6 (55.49)	6.7 (6.64)	10.0 (10.11)
EK-**r1A'**[c]	148–154	−60	$C_{33}H_{46}ClN_5O_7\cdot H_2O$ (678.2)	58.7 (58.44)	7.2 (7.13)	10.3 (10.33)
EK-**r1B'**[c]	139–145	+72	$C_{33}H_{46}ClN_5O_7\cdot H_2O$ (678.2)	58.7 (58.44)	7.2 (7.13)	10.2 (10.33)
EK-**r1C'**[c]	135–141	−35	$C_{33}H_{46}ClN_5O_7\cdot H_2O$ (678.2)	58.35 (58.44)	7.2 (7.13)	10.3 (10.33)
EK-**r1D'**[c]	134–140	+75	$C_{33}H_{46}ClN_5O_7\cdot 5/3H_2O$ (690.2)	57.2 (57.42)	7.0 (7.20)	10.1 (10.15)
5[d]	159–162	+28	$C_{16}H_{24}ClN_3O_5$ (373.8)	51.4 (51.41)	6.3 (6.47)	11.1 (11.24)
Boc-**r1A**[e]	57–61	−12	$C_{21}H_{30}N_2O_5S\cdot 1/4H_2O$ (427.0)	59.1 (59.06)	7.2 (7.20)	6.7 (6.56)
Boc-**r1A'**[f]	72–79	−20	$C_{22}H_{32}N_2O_5\cdot 1/5H_2O$ (408.1)	64.8 (64.77)	8.0 (8.00)	6.9 (6.86)

[a] All hydrochlorides of retro-Met- and -Leu-enkephalin analogue ethyl esters were washed thoroughly with dry diethyl ether. [b] *m/z* 642. [c] *m/z* 624. [d] *m/z* 337. [e] *m/z* 422. [f] *m/z* 404.

Table 6 Mouse vas deferens assay of Met- and Leu-enkephalin analogue ethyl esters

Compound	MVD (IC_{50}/nmol dm^{-3})[a]
HCl·EK-**1A**	8 370 ± 960
HCl·EK-**1B**	10 955 ± 1 000
HCl·EK-**1C**	8 370 ± 960
HCl·EK-**1D**	6 985 ± 230
HCl·EK-**1A'**	6 625 ± 785
HCl·EK-**1B'**	4 475 ± 205
HCl·EK-**1C'**	7 200 ± 1 310
HCl·EK-**1D'**	7 085 ± 325

[a] Results are expressed as means ± S.E. of three experiments.

However, the above conclusions for these enkephalin analogues and their corresponding retro isomers are derived only from our MVD assay, so that other biological tests are required in order to evaluate the receptor-binding affinities of these pentapeptides more fully.

Experimental

All samples were measured at room temperature using a JASCO IRA-1 (IR spectra), a JEOL GX-400 (NMR spectra; solutions in $CDCl_3$, tetramethylsilane as an internal standard) and a JASCO DIP-370 (optical rotations; see Tables 1, 2, 4 and 5). Their mass spectra were obtained on a JEOL D-300 or a Hitachi M-2000 spectrometer [*m/z* (M^+); in the case of the hydrochlorides and/or the hydrates, *m/z* = molecular weight − HCl and/or $-nH_2O$]. Reagents for synthesis of peptides, α-amino acids and their derivatives were purchased from Peptide Institute Inc. Osaka and Kokusan Chemical Works, Ltd. Tokyo.

Bioassays were carried out at 37 °C in Krebs–Ringer solution (NaCl–KCl–$CaCl_2$–KH_2PO_4–$NaHCO_3$–D-glucose = 118:4.75:2.45:1.19:25.0:11.0 mmol dm^{-3}) using the vas deferens of ddY mice (8–9 weeks of age). Compounds **4** and **5** were synthesized by the usual solution method.

Preparation of Ethyl (2S,3'S)-2-(3'*Benzyl*-2'*oxopiperazin*-1'-*yl*)-4-(*methylthio*)-*butanoate* **1A** *and Ethyl* (2S,3'S)-2-[3'-(2-*methylthioethyl*)-2'-*oxopiperazin*-1'-*yl*]-3-*phenylpropanoate* **r1A**. —A mixture of *N,N'*-ethylene-bridged bis-α-amino acids [HO–FeM(*S,S*)–OH, HO–FeF(*S,S*)–OH and HO–MeM-(*S,S*)–OH] (11.0 g ~ 0.03 mol) obtained from (*S*)-Phe (16.5 g, 0.10 mol), (*S*)–Met (14.9 g, 0.10 mol) and 1,2-dibromoethane (18.8 g, 0.10 mol) by a method similar to that of Schöenberg *et al.*[11] was refluxed with $TsOH\cdot H_2O$ (10.5 g, 0.060 mol) in dry ethanol (250 cm^3) for 24 h. The TsOH salt obtained after removal of the solvent was freed by aq. $NaHCO_3$, and the mixture was extracted with CH_2Cl_2. The oily residue (10 g) thus obtained proved to be the mixture of compounds **1A**, **r1A**, ethyl (2*S*,3'*S*)-2-[3'-benzyl-2'-oxopiperazin-1'-yl]-3-phenylpropanoate **2A**[12] and ethyl (2*S*,3'*S*)-4-(methylthio)-2-[3'-(2-methylthioethyl)-2'-oxopiperazin-1'-yl]butanoate **3A**[6] by TLC. NMR and IR spectroscopy, containing negligible amounts of by-products (EtO–XeX'–OEt, *etc.*). The mixture was separated by silica gel chromatography [benzene–EtOAc–MeOH (5:4:1)] into products **1A**, **r1A**, **2A** and **3A** in the proportions (1:3:2:1); compounds **1A** (1.0 g), **r1A** (3.0 g), **2A** (2.0 g) and **3A** (1.0 g) were obtained as oily materials.

1A; ν_{max}(neat)/cm^{-1} 1736 and 1643; *m/z* 350; δ_H 1.27 (3 H, t), 2.11 (3 H, s), 2.00–2.29 (2 H, m), 2.43 (2 H, br s), 2.95 (1 H, dd), 2.98 (1 H, ddd), 3.12 (1 H, ddd), 3.20 (1 H, dt), 3.29 (1 H, dt), 3.38 (1 H, dd), 3.74 (1 H, dd), 4.18 (2 H, q), 5.16 (1 H, dd) and 7.21–7.33 (5 H, br m).

r1A; ν_{max}(neat)/cm^{-1} 1733 and 1640; *m/z* 350; δ_H 1.27 (3 H, t), 1.79 (1 H, ddt), 2.00 (1 H, m), 2.05 (3 H, s), 2.33 (1 H, ddd), 2.41 (1 H, dt), 2.86 (1 H, ddd), 2.98 (1 H, dt), 3.01 (1 H, dt), 3.09 (1 H, dd), 3.30 (1 H, ddd), 3.39 (1 H, dd), 3.58 (1 H, dd), 4.20 (2 H, q), 5.16 (1 H, dd) and 7.21–7.31 (5 H, br m).

2A; ν_{max}(neat)/cm^{-1} 1735 and 1640; *m/z* 366; δ_H 1.27 (3 H, t), 2.60 (1 H, dd), 2.76 (1 H, ddd), 2.93 (1 H, dt), 2.98 (1 H, dt), 3.12 (1 H, dd), 3.31 (1 H, ddd), 3.33 (1 H, dd), 3.39 (1 H, dd), 3.63 (1 H, dd), 4.21 (2 H, q), 5.11 (1 H, dd) and 7.11–7.33 (10 H, br m). Similarly, compounds **1B**, **r1B**, **2B** [eFF(*R,R*)–OEt] and **3B** (=**3A**) were prepared in the proportions (10:10:14:10) by using (*R*)–Phe and (*S*)–Met as starting materials.

Preparation of Ethyl (2S,3'S)-2-[3'-*Benzyl*-2'-*oxopiperazin*-1'-*yl*]-4-*methylpentanoate* **1A'** *and Ethyl* (2S,3'S)-2-[3'-*Isobutyl*-2'-*oxo-piperazin*-1'-*yl*]-3-*phenylpropanoate* **r1A'**.—The piperazin-2-one derivatives constructed from (*S*)-Phe and -Leu were obtained as oily materials in similar overall yields by the use of (*S*)-Phe and -Leu as starting materials at the same scale and under the same conditions as used for the preparation of eXX'–OEt containing Phe and/or Met. The proportions of products **1A'**, **r1A'**, **2A** and ethyl (2*S*,3'*S*) 2-[3'-isobutyl-2'-oxopiperazin-1'-yl]-4-methylpentanoate **3A'** [12,13] separated on silica gel with the chromatographic solvent used for the separation of eFM–OEt, *etc.* were 1:3:3:1.

1A'; ν_{max}(neat)/cm^{-1} 1736 and 1643; *m/z* 332; δ_H 0.94 (3 H, d), 0.96 (3 H, d), 1.27 (3 H, t), 1.45 (1 H, m), 1.68 (2 H, m), 2.97 (1 H, dd), 3.37 (1 H, dd), 2.94–3.22 (4 H, m), 3.75 (1 H, dd), 4.17 (2 H, q), 5.34 (1 H, dd) and 7.20–7.32 (5 H, br m).

r1A'; ν_{max}(neat)/cm^{-1} 1740 and 1640; *m/z* 332; δ_H 0.86 (6 H, d), 1.25 (1 H, m), 1.26 (3 H, t), 1.66 (2 H, m), 3.10 (1 H, dd), 3.38 (1 H, dd), 2.80–3.44 (4 H, m), 3.42 (1 H, dd), 4.19 (2 H, q), 5.10 (1 H, dd) and 7.20–7.30 (5 H, br m).

3A'; ν_{max}(neat)/cm^{-1} 1730 and 1643; *m/z* 298; δ_H 0.86 (6 H, d), 0.93 (3 H, d), 0.97 (3 H, d), 1.25–1.40 (2 H, m), 1.27 (3 H, t), 1.66–1.68 (4 H, m), 2.80–3.22 (4 H, m), 3.42 (1 H, dd), 4.18 (2 H, q) and 5.34 (1 H, dd).

Similarly, compounds **1B'**, **r1B'**, **2B'** and **3B'** (= **3A'**) were obtained in the proportions 2:2:3:1 by using (*R*)-Phe and (*S*)-Leu as starting materials.

Preparation of Boc–eMF–OH and –eLF–OH.—To a solution of compound **r1A** (3.5 g, 0.010 mol) in THF (200 cm^3) was added 2-*tert*-butoxycarbonylthio-4,6-dimethylpyrimidine (Boc-SDP) (2.4 g, 0.010 mol) at room temperature. After 24 h the solvent was removed. The residue obtained was extracted with CH_2Cl_2. The extract was washed successively with aq. $KHSO_4$, aq. $NaHCO_3$ and water, and was then dried over anhydrous Na_2SO_4. The oily residue, obtained in 80% yield after removal of the solvent, was hydrolysed in a solution of NaOH (0.40 g, 0.010 mol) in MeOH–water (1:1; 50 cm^3) at room temperature for 5 h, and was then neutralized with $KHSO_4$. The solution was extracted with $CHCl_3$, and washed with water. Crude Boc-**r1A** obtained as a powder in 90% yield was purified by means of Sephadex LH-20 column chromatography (MeOH).

Similarly, Boc-**r1A'** was prepared in 85% yield by the coupling of compound **r1A'** with Boc–SDP, followed by successive hydrolysis.

Furthermore, their enantiomers and diastereoisomers (Boc-**r1B**, **-r1C**, **-r1D**, **-r1B'**, **-r1C'** and **-r1D'**) were synthesized according to the method described above.

Preparation of Met- and Leu-enkephalin Analogues.—To a solution of compounds **1A** (0.35 g, 1.0 mmol) and **4** (0.50 g, 1.0 mmol) in THF (20 cm^3) was added EEDQ (0.25 g, 1.0 mmol) at room temperature. After 2 days the solution was evaporated to dryness, and the residue was extracted with $CHCl_3$. The extract was washed successively with aq. $KHSO_4$, aq. $NaHCO_3$ and water, and was then dried. After removal of the solvent, *N*,*O*-blocked pentapeptide was obtained as a powder in 90% yield, and this was treated with a solution of thioanisole (1.24 g, 10.0 mmol) in TFA (4 cm^3) at room temperature for 3 h. The reaction mixture was poured into dry diethyl ether (100 cm^3) and the resulting powder was filtered off before being purified by silica gel column chromatography [$CHCl_3$–MeOH (15:1)], to give EK-**1A** in 60% yield, which was transformed into the hydrochloride. EK-**1A'** prepared similarly was also purified as the hydrochloride.

Moreover, the other enkephalin analogues (EK-**1B**, **-1C**, **-1D**, **-1B'**, **-1C'** and **-1D'**) were obtained in a similar manner.

Preparation of Retro-Met- and -Leu-enkephalin Analogues.—To a stirred solution of Boc-**r1A** (0.42 g, 1.0 mmol), HOBT (0.14 g, 1.0 mmol) and compound **5** (0.33 g, 1.0 mmol) in THF (10 cm^3) was added a solution of DCC (0.20 g, 1.0 mmol) in THF (5 cm^3) at −5 °C, and the reaction was allowed to continue at room temperature for 2 days. After removal of the solvent, the reaction mixture was extracted with CH_2Cl_2 and the extract was washed successively with aq. $KHSO_4$, aq. $NaHCO_3$ and water. The dried (Na_2SO_4) extract was evaporated to dryness, yielding *N*-blocked pentapeptide as an oily material in 90% yield. This pentapeptide was treated with 4 mol dm^{-3} HCl–EtOAc (5 cm^3) containing MeSEt (0.090 g, 1.2 mmol) at room temperature for 1 h, and the mixture was poured into dry diethyl ether (50 cm^3). The resulting powder was filtered off, and washed thoroughly with dry diethyl ether, to yield the hydrochloride of EK-**r1A** as a powder in 90% yield.

Compound EK-**r1A'** was similarly obtained as the hydrochloride in 85% yield. Moreover, the other retro-enkephalin analogues (EK-**r1B**, **-r1C**, **-r1D**, **-r1B'**, **-r1C'** and **-r1D'**) were obtained as their hydrochlorides in a similar manner.

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