Synthesis and Opiate Activity of Pseudo-Tetrapeptides Containing Chiral Piperazin-2-one and Piperazine Derivatives

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Enantiomeric piperazin-2-one derivatives, *N,N'*-ethylene-bridged alanylphenylalanines (1a or 1b), were synthesized using (*S*)- or (*R*)-alanine and phenylalanine as starting materials, and were inserted into the second and third positions of enantiomeric pseudo-tetrapeptides (P1a- or P1b-OEt). The corresponding piperazine derivatives (1a-or 1b-sRed) obtained by selective BH_3 reduction of the amide carbonyl groups of 1a or 1b were similarly inserted into the same positions of tetrapeptides (P1a- and P1b-sRed). Enantiomeric *N,N'*-ethylene-bridged tyrosyltyrosine derivatives (2a or 2b) obtained from (*S*)- or (*R*)-tyrosine were also inserted into the first and second positions of two pairs of enantiomeric tetrapeptides (P2a- and P2b-OEt or P'2a- and P'2b-OEt). The opiate activities of the eight peptides thus obtained were studied by use of the mouse vas deferens and the guinea pig ileum assays in order to elucidate the structure–activity relationships of these peptides, especially with respect to stereochemistry.

Key words pseudo-tetrapeptide; opiate activity; selective BH_3 reduction; *N,N'*-ethylene-bridged dipeptide; piperazine derivative; structure–activity relationship

Recently, we inserted chiral piperazin-2-one derivatives (*N,N'*-ethylene-bridged dipeptides; eXX')[1,2] and the corresponding piperazine derivatives (eXX'–Red)[2] into the fourth and fifth positions of Leu- and Met-enkephalin analogues[1] [H–Tyr-(*R*)-Ala–Gly–eXX'–OEt; X = (*R*)- or (*S*)-F (phenylalanine) and X' = (*R*)- or (*S*)-L (leucine) or M (methionine)][1] and into the third and fourth positions of shorter dermorphin ones[2] [H–Tyr-(*R*)-Ala–eXX'–OEt or –eXX'–Red; X = (*R*)- or (*S*)-F and X' = F or G (glycine)]. These pseudo-peptides were assayed in order to examine their structure–activity relationships. It was found in the guinea pig ileum (GPI) assays of dermorphin analogues[2] that the replacement of the piperazin-2-one ring with the piperazine ring markedly enhances the opiate activities of these analogues, in addition to the effect of the change from (*S*)- to (*R*)-configuration of Phe residues. Also, DiMaio *et al.*[3] inserted *N,N'*-ethylene-bridged (*R*)- and (*S*)-tyrosylglycine [eY(*R* and *S*)G] into the first and second positions of Leu-enkephalin, and found that only a Leu-enkephalin analogue containing eY(*R*)G displayed morphinomimetic activity (mouse hot plate test). This paper describes the synthesis of four kinds of pseudo-tetrapeptides containing (*S*)-tyrosine or *N,N'*-ethylene-bridged (*S*)-tyrosyl-(*S*)-tyrosine residues [**2a** = eYY(*S,S*)] prepared earlier[4] as their amino-terminal residues [Tyr–eAF(*S,S*)-Gly-(**P1a**), Tyr–eAF(*S,S*)-sRed–Gly-(**P1a**-sRed), H–eYY-(*S*)-Phe–Gly-(**P2a**) and H–eYY-(*R*)-Phe–Gly-(**P'2a**) OEt]. A peptide unit [**1a**-sRed = eAF(*S,S*)-sRed] used here was obtained by selective BH_3 reduction of the amide carbonyl group of *N,N'*-ethylene-bridged (*S*)-alanyl-(*S*)-phenylalanine [**1a** = eAF(*S,S*)]. Previously, Shemyakin *et al.*[5,6] compared the biological activities of cyclic peptides such as Gly[5,10]gramicidin S[5] or acyclic dipeptides[6] such as Ac–Leu–Tyr–$NHCH_3$ with those of the corresponding enantio- and/or retro-isomers, respectively, in order to study the topochemistry of peptides.

Bodanszky *et al.*[7] examined the biological activities of a cyclic pentapeptide disulfide, [5-valine]malformin, and its enantiomer for a similar purpose and concluded that the natural peptide is about 10 times as active as its synthetic enantiomer. In this work, we synthesized four kinds of enantio-tetrapeptides (**P1b**, **P1b**-sRed, **P2b** and **P'2b**) corresponding to **P1a**, **P1a**-sRed, **P2a** and **P'2a**, which are acyclic and not retro-forms. The opiate activities of the eight tetrapeptides thus obtained were examined by means of the mouse vas deferens (MVD) and the GPI assays. The results showed that differences of the configuration of α-amino acid residues and the replacement of the piperazin-2-one ring with the piperazine ring influence the activities of these peptides, though the effects are not marked in comparison with those observed in the dermorphin analogues[2] reported earlier.

Results and Discussion

Recently, we[1] reported a convenient method *via* acid-catalyzed cyclization for obtaining chiral *N,N'*-ethylene-bridged dipeptides (eFL, eFM, *etc.*), which were used as the units of Leu- and Met-enkephalin analogues, from commercial α-amino acids. In this work, we prepared *N,N'*-ethylene-bridged dipeptides (**1a**) from (*S*)-alanine and -phenylalanine according to a method similar to our previous one,[1] as shown in Chart 1.

The cyclic compounds thus obtained were separated chromatographically on silica gel, providing eAF(*S,S*)-OEt predominantly over its retro-form [eFA(*S,S*)-OEt] in a ratio of 8 : 1. On the other hand, eFF(*S,S*)-OEt was obtained in a yield similar to that of eAF(*S,S*)-OEt, but eAA(*S,S*)-OEt was not formed. The result of this selective cyclization [eAF(*S,S*)-OEt *vs.* eFA(*S,S*)-OEt] is contrary to that of the previous cyclization[8] [eF(*S*)G-OEt *vs.* eGF(*S*)-OEt] in which only eF(*S*)G-OEt was obtained, but not its retro-form [eGF(*S*)-OEt]. This result can not be explained by the difference of steric hindrance between α-amino acid residues because eFL (eFM)-OEt and its

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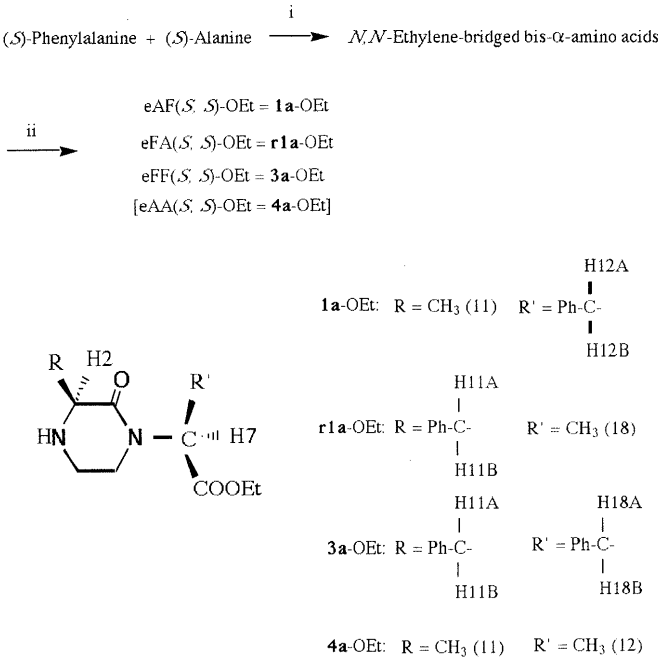
retro-form, eLF (eMF)-OEt, were previously[1] obtained in the proportions of 1:3 for (*S*,*S*)-form and 1:1 for its diastereomer. The structures of species eAF–OEt and its retro-form (eFA–OEt) were established by ^{1}H-NMR measurements including two-dimensional correlated spectroscopy, with reference to the ^{1}H-NMR data of eFF(**3a**)-OEt[1] and eAA(**4a**)-OEt[9] obtained earlier. Table 1 shows the chemical shifts and coupling constants of the α- and β-protons of the Ala and Phe residues of species **1a**-, **r1a**-, **3a**- and **4a**-OEt obtained by ^{1}H-NMR measurements. The chemical shifts of the α-protons of the Ala and Phe residues for **1a**-OEt are 3.51 and 5.08 (ppm), and those for **r1a**-OEt are 5.22 and 3.70 (ppm), respectively, supporting our belief that the Ala residue of **1a**-OEt is situated on the piperazin-2-one ring, and the Phe residue is outside the ring. Recently, we[2] inserted the piperazine derivatives (amino alcohols) obtained by the BH_3 reduction of both amide and ester carbonyl groups of eFG- and eFF-OEt into the third and fourth positions of shorter dermorphin analogues (tetrapeptides), and found marked changes in the structure/opiate activity relationships in these analogues.



i: K_2CO_3, NaOH, $BrCH_2CH_2Br$, water, aq. HCl; ii: H_2SO_4, EtOH

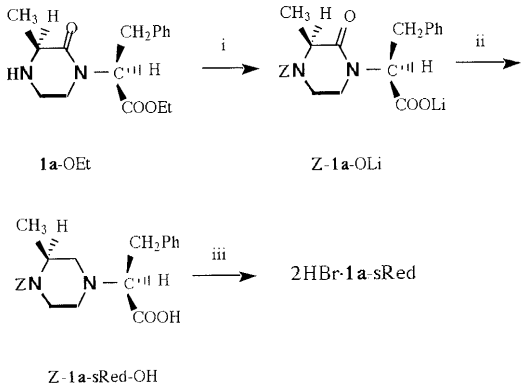
Chart 1

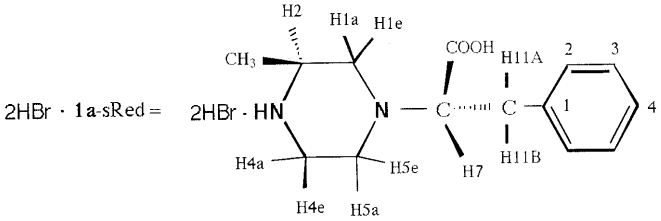
However, these piperazine derivatives can be used only in the carboxyl terminal positions of peptides because they have amino groups, but not carboxyl groups. Therefore, we needed a new method for obtaining piperazine derivatives (amino acids) with both amino and carboxyl groups, which can be inserted in any position in the main chains of peptides. Previously, Roeske *et al.*[10] reported the selective BH_3 reduction of the amide carbonyl groups of *N*-protected dipeptide esters, but the yields of selectively reduced dipeptides were unsatisfactory. Almquist *et al.*[11] also synthesized a reduced type of tripeptide (an inhibitor of angiotensin-converting enzyme). In their work, the amide carbonyl group of the Phe residue of the parent peptide (*N*-benzoyl-Phe–Gly–Pro–OH) was transformed to the methylene group, through several steps, including the BH_3 reduction of both amide and carboxyl groups and the CrO_3 oxidation of the alcoholic hydroxyl group to a carboxyl group. Similarly, Ohfune and Tomita[12] converted an intermediate with both amide and ester groups to another intermediate without the amide group by the use of BH_3 reduction and pyridinium dichromate oxidation in their total synthesis of (−)-domoic acid. We tried the selective BH_3 reduction of the isopropyl and isobutyl esters of eAF (*S*,*S*)-OH (**1a**-OH). However, the yield of a desired piperazine derivative [eAF(*S*,*S*)-sRed (**1a**-sRed)] was unsatisfactory because of the low selectivity, and the separation and purification of **1a**-sRed from its by-products by chromatography were not easy. On the other hand, we found that the lithium salt (Z-**1a**-OLi) of *N*-benzyloxycarbonylated **1a**-OH is readily soluble not only in water, but also in common organic solvents such as tetrahydrofuran (THF).

Therefore, the selective reduction of the amide carbonyl group of Z-**1a**-OLi was carried out with 2 eq. of BH_3 in dry THF for 5 h at room temperature. Silica gel chromatography separated the desired piperazine derivative (Z-**1a**-sRed) in 50% yield from various by-products. In order to obtain more precise analytical, physical and spectroscopic data, this oily material (Z-**1a**-sRed) was transformed, through the removal of the Z-group of Z-**1a**-sRed in 25% hydrobromic acid (HBr)–acetic acid (AcOH), into 2HBr·**1a**-sRed. The structure of this salt (2HBr·**1a**-sRed), after recrystallization from isopro-

Table 1. Chemical Shifts and Coupling Constants of α- and β-Protons of Ala and Phe Residues of **1a**-, **r1a**-, **3a**- and **4a**-OEt in $CDCl_3$ at Room Temperature

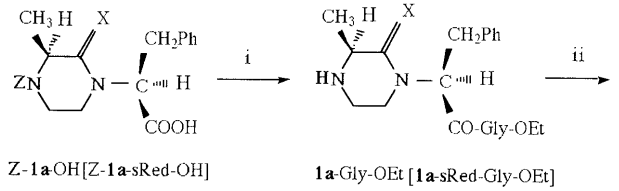
Compound	Chemical shift (δ, ppm)						Coupling constant (*J*/Hz)			
	H2	H11A	H11B	H7	H18A (H12A)	H18B (H12B)	H2-H11A	H2-H11B	H7-H18A (H7-H12A)	H7-H18B (H7-H12B)
1a-OEt				5.08	(3.38)	(3.11)			(5.5)	(11.0)
r1a-OEt	3.70	3.46	2.85				3.7	9.8		
3a-OEt	3.63	3.33	2.60	5.11	3.39	3.12	3.7	9.8	5.5	11.0
	H2	CH_3(11)		H7	CH_3(18) [CH_3(12)]		H2-CH_3(11)		H7-CH_3(11) [H7-CH_3(12)]	
1a-OEt	3.51	1.20					6.7			
r1a-OEt				5.22	1.41				7.3	
4a-OEt	3.59	1.39		5.14	[1.42]		6.7		[7.3]	

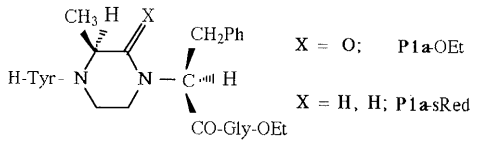




i: a) Z–Cl, CH_2Cl_2, b) LiOH, MeOH–water; ii: BH_3, THF; iii: 25% HBr–AcOH

Chart 2





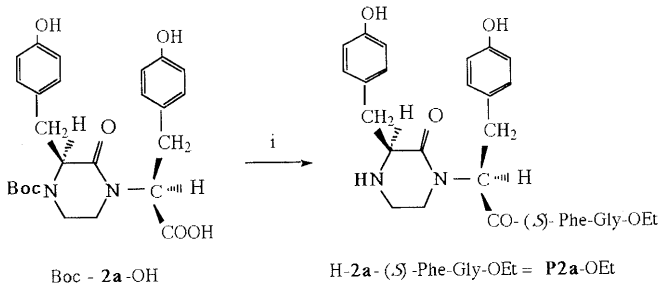
i: a) DCC, HOBT, THF, H–Gly–OEt, b) 25% HBr–AcOH; ii: a) Boc–Tyr–OSu, CH_2Cl_2–pyridine, b) 4 N HCl–DOX, thioanisole

Chart 3

panol–acetonitrile was ascertained by ^{1}H- and ^{13}C-NMR measurements including two-dimensional correlated spectroscopy.

Chart 2 shows the synthetic route to **1a**-sRed from **1a**-OEt. Similarly, its enantiomer [**1b**-sRed = eAF(*R*,*R*)-sRed] was prepared from **1b**-OEt according to the method indicated in Chart 2.

Chart 3 shows the synthetic method for two kinds of tetrapeptides (**P1a**-OEt or **P1a**-sRed-OEt) from *N*-benzyloxycarbonylated(Z) **1a**-OEt or **1a**-sRed. The tripeptides (**1a**-Gly–OEt or **1a**-sRed–Gly–OEt) were obtained by the coupling of Z-**1a**-OH or Z-**1a**-sRed–OH with glycine ethyl ester in THF by the dicyclohexylcarbodiimide (DCC)–1-hydroxybenzotriazole (HOBT) method, followed by the removal of the Z-group in 25% HBr–AcOH. These tripeptides were condensed with *tert*-butoxycarbonylated (*S*)-tyrosine *N*-hydroxysuccinimide ester (Boc–Tyr–OSu) in dichloromethane (CH_2Cl_2)–pyridine, and successively treated with 4 N hydrochloric acid–dioxane



i: a) DCC, HOBT, THF, (*S*)-Phe–Gly–OEt, b) 4 N HCl–DOX, thioanisole

Chart 4

Table 2. Guinea Pig Ileum (GPI) and Mouse vas Deferens (MVD) Assays of Mono- and Di-Hydrochlorides of Pseudo-Tetrapeptide Ethyl Esters (H–Tyr–eAF–Gly–, H–Tyr–eAF–sRed– and H–eYY–Phe–Gly–OEt)

Compound	GPI ($IC_{50}/\mu M$)[a]	MVD ($IC_{50}/\mu M$)[a]	GPI/MVD (IC_{50}-ratio)
H–Tyr–eAF–Gly–OEt			
P1a (*S*,*S*,*S*)	150 (± 4.7)	52 (±1.3)	2.9
P1b (*R*,*R*,*R*)	137 (± 4.1)	11 (±0.12)	12.5
H–Tyr–eAF–sRed–Gly–OEt			
P1a-sRed (*S*,*S*,*S*)	31 (± 0.75)	13 (±0.28)	2.4
P1b-sRed (*R*,*R*,*R*)	34 (± 0.85)	4 (±0.02)	8.5
H–eYY–Phe–Gly–OEt			
P2a (*S*,*S*,*S*)	175 (± 1.3)	28 (±0.67)	6.3
P2b (*R*,*R*,*R*)	988 (±61)	17 (±0.29)	58.1
P′2a (*S*,*S*,*R*)	1198 (±62.3)	165 (±5.5)	7.3
P′2b (*R*,*R*,*S*)	545 (±29.7)	98 (±1.9)	5.6
Leu-enkephalin[13]	0.246 (±0.039)	0.081 (±0.001)	3.0

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

(4 N HCl–DOX) in the presence of thioanisole as a scavenger, yielding **P1a**-OEt or **P1a**-sRed–OEt, respectively. Their enantiomers (**P1b**-OEt or **P1b**-sRed–OEt) were similarly prepared.

Chart 4 shows the preparation of the tetrapeptide **P2a**-OEt (= eYY(*S*,*S*)-(*S*)-Phe–Gly–OEt) containing *N*,*N*′-ethylene-bridged (*S*)-tyrosyl-(*S*)-tyrosine residues (**2a**)[4] in the first and second positions. This tetrapeptide was obtained by the coupling of Boc-**2a**-OH with (*S*)-Phe–Gly–OEt according to the DCC–HOBT method followed by the removal of the Boc group with 4 N HCl–DOX in the presence of thioanisole. Its diastereomer [**P′2a**-OEt = H–eYY(*S*,*S*)-(*R*)-Phe–Gly–OEt] was similarly prepared. Moreover, the corresponding enantio-tetrapeptides [**P2b**- or **P′2b**-OEt] were obtained similarly.

Table 2 summarizes the results of GPI and MVD assays of the mono- and di-hydrochlorides of eight tetrapeptides (**P1a**-, **P1b**-, **P1a**-sRed-, **P1b**-sRed, **P2a**-, **P2b**-, **P′2a**- and **P′2b**-OEt) prepared in this work.

As shown in Table 2, the IC_{50} values of **P1a**-sRed- and **P1b**-sRed–OEt are 13 and 4 μM, and those of **P1a**- and **P1b**-OEt are 52 and 11 μM in the MVD assays, indicating that the activitites of the former two are about 4 and 3 times as high as those of the latter. The GPI assays show that **P1a**-sRed–OEt and **P1b**-sRed–OEt are about 5 and 4 times as active as **P1a**-OEt and **P1b**-OEt. These results suggest that the replacement of the piperazin-2-one ring

with the relatively flexible piperazine ring enhances the activities of these peptides, though their activities are not higher than that observed in the GPI assay of dermorphin analogues.[2] Further, the activities of **P1b**-OEt and **P1b**-sRed–OEt are about 5 and 3 times as high as those of the corresponding enantiomers (**P1a**-OEt and **P1a**-sRed–OEt) from the IC_{50} values (11, 4, 52 and 13 μM for **P1b**-, **P1b**-sRed-, **P1a**- and **P1a**-sRed–OEt) in the MVD assays (Table 2).

However, the differences in their activities are small in the GPI assay. Furthermore, the GPI assay results indicate that **P2a**-OEt is about 6 times as active as its enantiomer (**P2b**-OEt) in terms of the IC_{50} values (175 and 988 μM for **P2a**- and **P2b**-OEt), and about 7 times as active as its diastereomer (**P'2a**-OEt) (IC_{50} 175 and 1198 μM for **P2a**- and **P'2a**-OEt, respectively). These results show that the differences of opiate activities between these peptides and the corresponding enantiomers are not large, as reported previously in the studies of biological activities of enantio-peptides by Shemyakin *et al.*[5,6] and Bodanszky *et al.*[7] The IC_{50}-ratio values in Table 2 show that higher selectivities for δ-receptor than for μ-receptor are exhibited by all the peptides prepared here, and the highest selectivity (IC_{50}-ratio = 58) is found with **P2b**-OEt.

Experimental

Chemistry NMR spectra were measured at room temperature using JEOL GX-400, JEOL LA-300 and VARIAN UNITY-500 spectrometers. The spectra were taken in $CDCl_3$, with tetramethylsilane as an internal standard, except for 2HBr·**1a**-sRed (in D_2O, with 3-(trimethylsilyl)propionic 2, 2, 3, 3-d_4 acid sodium salt as an internal standard). A JASCO DIP-370 was used to measure optical rotations. Infrared spectra were taken on a JASCO IRA-1 spectrometer.

Mass spectra were obtained on a JEOL D-300 spectrometer [MS *m/z* (M^+): in the case of the hydrochlorides (hydrobromides) or their hydrates, MS *m/z* = molecular weight − 1 or 2HCl (HBr) and -nH_2O]. α-Amino acids, their derivatives and reagents for synthesis of peptides were purchased from Peptide Institute Inc., Osaka, and Kokusan Chemical Works Ltd., Tokyo.

Bioassay All tetrapeptides prepared here are effective at inhibiting electrically induced contractions in the bioassays examined. Their IC_{50} values were determined 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 mM) using the vas deferens of ddY mice (8—9 weeks of age) and the ileum of guinea pigs (Hartley strain) (4 weeks of age).

Preparations of Ethyl (2*S*)-2-[(3*S*)-3-Methyl-2-oxopiperazin-1-yl]-3-phenylpropionate and Ethyl (2*S*)-2-[(3*S*)-3-Benzyl-2-oxopiperazin-1-yl]-propionate (1a- and r1a-OEt) 1,2-Dibromoethane (18.8 g, 0.10 mol) and a solution of K_2CO_3 (13.8 g, 0.10 mol) in water (200 ml) were alternately added dropwise to a solution of (*S*)-Phe (16.5 g, 0.10 mol), (*S*)-Ala (8.9 g, 0.10 mol) and NaOH (8.0 g, 0.20 mol) in water (500 ml) with stirring at 90 °C. After 5 h, the reaction mixture was cooled and neutralized with concentrated HCl. The resulting precipitate was filtered off, and dried under reduced pressure.

A mixture of *N,N'*-ethylene-bridged bis-α-amino acids [HO–FeA(*S,S*)–OH, HO–FeF(*S,S*)–OH and HO–AeA (*S,S*)–OH] (11.3 g, *ca.* 0.04 mol) thus obtained was refluxed with concentrated H_2SO_4 (7.9 g, 0.08 mol) in dry ethanol (250 ml) for 24 h, to afford the H_2SO_4 salt after removal of the solvent. It was treated with aqueous $NaHCO_3$, and the mixture was extracted with CH_2Cl_2. The oily residue (7 g) thus obtained proved to consist of **1a**-, **r1a**- and eFF(*S,S*)–OEt[1] by TLC, NMR, MS and IR spectroscopies, and no eAA(*S,S*)–OEt[9] was observed. The mixture was separated by silica gel column chromatography [benzene : EtOAc : MeOH = 5 : 4 : 1] into oily products **1a** (2.4 g)-, **r1a** (0.3 g)- and eFF(*S,S*) (2.0 g)-OEt in the proportions of 8 : 1 : 6. **1a**-OEt: IR (neat) cm^{-1} 1730, 1655. MS *m/z*: 290. ^{1}H-NMR δ: 1.20 (3H, d), 1.26 (3H, t), 2.86 (1H, m), 2.97 (1H, m), 3.01 (1H, m), 3.11 (1H, dd), 3.30 (1H, m), 3.38 (1H, dd), 3.51 (1H, q), 4.20 (2H, q), 5.08 (1H, dd), 7.21—7.31 (5H, br m). **r1a**-OEt: IR (neat) cm^{-1} 1736, 1643. MS *m/z*: 290. ^{1}H-NMR δ: 1.27 (3H, t), 1.41 (3H, d), 2.85 (1H, dd), 2.96 (1H, m), 3.11 (1H, m), 3.18 (1H, m), 3.35 (1H, m), 3.46 (1H, dd), 3.70 (1H, dd), 4.18 (2H, q), 5.22 (1H, q), 7.18—7.33 (5H, br m). The oily materials **1a**-OEt and **r1a**-OEt were transformed into their hydrochlorides, which were recrystallized from benzene. HCl·**1a**-OEt: mp 135—137 °C, $[\alpha]_D$ = −101° (*c* = 1.2, EtOH). *Anal.* Calcd for $C_{16}H_{23}ClN_2O_3$: C, 58.80; H, 7.09; N, 8.57. Found: C, 58.69; H, 7.12; N, 8.55. HCl·**r1a**-OEt: mp 163—167 °C, $[\alpha]_D$ = −87° (*c* = 0.6, EtOH). *Anal.* Calcd for $C_{16}H_{23}ClN_2O_3 \cdot 1/5H_2O$: C, 58.16; H, 7.14; N, 8.48. Found: C, 58.11; H, 7.11; N, 8.38.

Preparation of (2*S*)-2-[(3*S*)-*N*-Benzyloxycarbonyl-3-methylpiperazin-1-yl]-3-phenylpropionic Acid (Z-1a-sRed–OH) An oil, Z-**1a**-OEt (3.4 g, 8 mmol), was obtained in 80% yield from **1a**-OEt (2.9 g, 10 mmol) and Z–Cl (1.7 g, 10 mmol) in CH_2Cl_2 (50 ml) at room temperature for 24 h. This oil was hydrolyzed in a solution of NaOH (0.32 g, 8 mmol) in MeOH–water (1 : 1, 50 ml) for 5 h at room temperature, and the solution was neutralized with $KHSO_4$, and extracted with CH_2Cl_2. The organic solution was washed with water. A powder, Z-**1a**-OH (2.7 g, 7 mmol), obtained in 87% yield was washed thoroughly with dry ether, and transformed, with aqueous LiOH, into its lithium salt (Z-**1a**-OLi). The solution was concentrated and dried under reduced pressure, to afford Z-**1a**-OLi as a powder. Z-**1a**-OH: mp 78—83 °C, $[\alpha]_D$ = −14° (*c* = 1.5, MeOH). MS *m/z*: 396. *Anal.* Calcd for $C_{22}H_{24}N_2O_5$: C, 66.65; H, 6.10; N, 7.07. Found: C, 66.32; H, 6.08; N, 7.01. Z-**1a**-OLi: mp 126—130 °C, $[\alpha]_D$ = +5° (*c* = 2, MeOH). *Anal.* Calcd for $C_{22}H_{23}LiN_2O_5 \cdot 2H_2O$: C, 60.27; H, 6.21; N, 6.39. Found: C, 60.30; H, 6.15; N, 6.41.

Sodium borohydride (0.31 g, 8 mmol) was suspended in dry THF (5 ml) at 0 °C under an argon atmosphere, and methyl iodide (1.14 g, 8 mmol) was added. After 2 h, a THF solution (5 ml) of Z-**1a**-OLi (1.75 g, 4 mmol) was dropped into the BH_3-containing solution with stirring, and the reaction was continued at room temperature for 5 h. Then, MeOH (3 ml) was added slowly to the solution in order to decompose excess BH_3. After removal of the solvent, the residue obtained was taken up in $CHCl_3$ (100 ml), and the insoluble materials were removed by filtration. The solution was concentrated, and the residue containing various by-products was subjected to silica gel column chromatography [$CHCl_3$: MeOH = 60 : 1] to afford Z-**1a**-sRed–OH (0.76 g, 2 mmol) in 50% yield as a syrupy oil. Its structure was examined by IR, MS and NMR spectroscopic measurements. Furthermore, in order to obtain more precise analytical, physical and spectroscopic data, Z-**1a**-sRed–OH (0.38 g, 1.0 mmol) was treated with 25% HBr–AcOH (6 ml) at room temperature for 2 h, and the solution was poured into dry ether (50 ml). The solid that formed was filtered off and recrystallized from CH_3CN–isopropanol, providing 2HBr·**1a**-sRed–OH (0.33 g, 0.8 mmol) in 80% yield.

2HBr·**1a**-sRed–OH: mp 203—208 °C, $[\alpha]_D$ = −23° (*c* = 1.1, MeOH). *Anal.* Calcd for $C_{14}H_{22}Br_2N_2O_2$: C, 40.10; H, 5.41; N, 6.83. Found: C, 40.03; H, 5.38; N, 6.85. MS *m/z*: 248. IR (nujol) cm^{-1}: 1730. ^{1}H-NMR δ: 1.39 (3H, d, *J* = 6.3 Hz, CH_3), 3.23 (1H, dd, *J* = 9.0, 13.9 Hz, H11A), 3.25 (1H, dd, *J* = 12.0, 13.9 Hz, H1a), 3.41 (1H, dd, *J* = 5.9, 13.7 Hz, H11B), 3.42 (1H, ddd, *J* = 2.9, 12.7, 12.9 Hz, H5a), 3.50 (1H, ddd, *J* = 2.7, 12.9, 13.9 Hz, H4a), 3.70—3.75 (1H, m, H1e), 3.71—3.76 (1H, m, H2), 3.78 (1H, ddd, *J* = 2.2, 2.9, 13.9 Hz, H4e), 3.92 (1H, dddd, *J* = 2.2, 2.4, 2.7, 12.7 Hz, H5e), 4.17 (1H, dd, *J* = 5.9, 9.3 Hz, H7), 7.24—7.36 (5H, br m, aromatic). ^{13}C-NMR δ: 17.7 (CH_3), 36.1 (C11), 43.6 (C4), 48.9 (C5), 52.4 (C2), 55.2 (C1), 72.7 (C7), 130.4 (aromatic C4), 131.7 (aromatic C3), 131.9 (aromatic C2), 137.3 (aromatic C1), 173.2 (carboxylic acid C = O).

Preparation of P1a-OEt and P1a-sRed–OEt (1) A solution of DCC (2.1 g, 10.0 mmol) in THF (20 ml) was added to a solution of Z-**1a**-OH (3.9 g, 10.0 mmol), HOBT (1.4 g, 10.0 mmol) and Gly–OEt (1.0 g, 10.0 mmol) in THF (100 ml) at −5 °C, and the reaction was continued at room temperature for 2 d. After removal of the insoluble materials by filtration, the solution was evaporated to dryness and the residue was extracted with CH_2Cl_2. The extract was washed successively with aqueous $KHSO_4$, aqueous $NaHCO_3$ and water, and dried (Na_2SO_4). After removal of the solvent, oily Z-**1a**-Gly–OEt (3.8 g, 8.0 mmol) was obtained in 80% yield. This oil was treated with 25% HBr–AcOH (30 ml) at room temperature for 2 h, and the solution was poured into dry ether (300 ml). The resulting powder was filtered off and washed thoroughly with dry ether to yield the hydrobromide of **1a**-Gly–OEt (2.4 g, 5.6 mmol) as a powder in 70% yield. This powder was used in the next step without further purification.

(2) A solution of HBr·**1a**-Gly–OEt (2.1 g, 5.0 mmol) and commercial Boc-(*S*)-Tyr–OSu (1.8 g, 5.0 mmol) in CH_2Cl_2–pyridine (1 : 1, 50 ml) was stirred at room temperature for 2 d, and evaporated to dryness. The residue obtained was extracted with $CHCl_3$. The extract was washed successively with aqueous $KHSO_4$, aqueous $NaHCO_3$ and water, and dried (Na_2SO_4). After removal of the solvent, Boc–Tyr-**1a**-Gly–OEt (2.7 g, 4.5 mmol), obtained as an oil in 90% yield, was treated with 4 N HCl–DOX (30 ml) at room temperature for 1 h in the presence of thioanisole (0.56 g, 4.5 mmol). The solution was evaporated to dryness, and was purified by silica gel column chromatography [$CHCl_3$: MeOH = 10 : 1] to yield a solid, which was transformed into the hydrochloride. It was dried under reduced pressure to afford HCl·Tyr-**1a**-Gly–OEt (**P1a**-OEt, 2.1 g, 4.0 mmol) was obtained in 88% yield. 2HCl·Tyr-**1a**-sRed–Gly–OEt (2HCl·**P1a**-sRed–OEt) was similarly prepared. In this synthetic route, the extracts of intermediates were washed with aqueous $NaHCO_3$ and water, but not with aqueous $KHSO_4$ because of the basic character of the piperazine units. HCl·**P1a**-OEt: mp 160—167 °C, $[\alpha]_D = -5°$ ($c = 2$, MeOH). MS *m/z*: 510. *Anal.* Calcd for $C_{27}H_{35}ClN_4O_6 \cdot 1.5H_2O$: C, 56.49; H, 6.41; N, 9.76. Found: C, 56.52; H, 6.70; N, 9.63. 2HCl·**P1a**-sRed–OEt: mp 158—161 °C, $[\alpha]_D = -22°$ ($c = 1.2$, MeOH). MS *m/z*: 496. *Anal.* Calcd for $C_{27}H_{38}Cl_2N_4O_5 \cdot 6H_2O$: C, 47.86; H, 7.44; N, 8.27. Found: C, 47.58; H, 7.42; N, 8.54.

Preparation of P2a- and P′2a-OEt (1) 2-*tert*-Butoxycarbonylthio-4,6-dimethylpyrimidine (2.4 g, 10.0 mmol) was added to a solution of *N*,*N*′-ethylene-bridged (*S*)-tyrosyl-(*S*)-tyrosine ethyl ester[4] (**2a**-OEt, 3.98 g, 10.0 mmol) in CH_2Cl_2 (100 ml) at room temperature. After 2 d, the solution was washed successively with aqueous $KHSO_4$, aqueous $NaHCO_3$ and water, and dried (Na_2SO_4). The oily residue obtained in 95% yield after removal of the solvent was hydrolyzed in a solution of NaOH (0.4 g, 10 mmol) in MeOH–water (1 : 1, 50 ml) for 5 h at room temperature, and then the solution was neutralized with $KHSO_4$. It was extracted with CH_2Cl_2, and washed with water. The powder thus obtained was purified by silica gel column chromatography ($CHCl_3$: MeOH = 10 : 1) to afford Boc-**2a**-OH (4.1 g, 8.6 mmol) in 90% yield. Boc-**2a**-OH: mp 125—128 °C, $[\alpha]_D = -40°$ ($c = 0.8$, EtOH). MS *m/z*: 470. *Anal.* Calcd for $C_{25}H_{30}N_2O_7$: C, 63.82; H, 6.43; N, 5.95. Found: C, 63.75; H, 6.59; N, 5.83.

(2) A solution of DCC (1.1 g, 5.0 mmol) in THF (100 ml) was added to a solution of Boc-**2a**-OH (2.4 g, 5.0 mmol), HOBT (0.7 g, 5.0 mmol) and (*S*)-Phe–Gly–OEt (1.3 g, 5.0 mmol), prepared by a usual solution method, in THF (50 ml) at −5 °C, and the reaction was continued at room temperature for 2 d. After removal of insoluble materials by filtration, the solution was evaporated, and the residue was extracted with CH_2Cl_2. The extract was washed successively with aqueous $KHSO_4$, aqueous $NaHCO_3$ and water. The dried (Na_2SO_4) extract was evaporated to dryness, providing Boc-**2a**-(*S*)-Phe–Gly–OEt (2.8 g, 4.0 mmol) as an oil in 80% yield. This oil was treated with 4 N HCl–DOX (30 ml) at room temperature for 1 h in the presence of thioanisole (0.50 g, 4.0 mmol). After removal of the solvent, the resulting residue was purified by silica gel column chromatography ($CHCl_3$: MeOH = 10 : 1) to yield a solid, which was transformed into the hydrochloride. This was dried under reduced pressure to afford HCl·**2a**-(*S*)-Phe–Gly–OEt (**P2a**-OEt, 2.3 g, 3.6 mmol) in 90% yield.

The diastereomer (**P′2a**-OEt) was similarly prepared using (*R*)-Phe–Gly–OEt instead of (*S*)-Phe–Gly–OEt as a starting material. HCl·**P2a**-OEt: mp 145—150 °C, $[\alpha]_D = -116°$ ($c = 0.5$, MeOH). MS *m/z*: 602. *Anal.* Calcd for $C_{33}H_{39}ClN_4O_7 \cdot 1.3H_2O$: C, 59.76; H, 6.13; N, 8.45. Found: C, 59.76; H, 6.22; N, 8.43. HCl·**P′2a**-OEt: mp 150—155 °C, $[\alpha]_D = -73°$ ($c = 1.0$, MeOH). MS *m/z*: 602. *Anal.* Calcd for $C_{33}H_{39}ClN_4O_7 \cdot 1.6H_2O$: C, 59.33; H, 6.13; N, 8.38. Found: C, 59.47; H, 6.46; N, 8.27.

Enantiomers **1b**, **1b**-sRed, **P1b**, **P1b**-sRed, **2b**, **P2b** and **P′2b** were similarly prepared. Their analytical, physical and spectroscopic data agreed well with those of their counterparts within the experimental errors, except for the opposite sign of their optical rotations.

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