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Structure-Activity Relationships of 13- and 14-Membered Cyclic Partial Retro-Inverso Pentapeptides Related to Enkephalin

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A series of 13- and 14-membered cyclic enkephalin analogs based on the moderately μ selective prototype compound Tyr-C[D-A_2bu-Gly-Phe-Leu] 8a were synthesized to investigate the structure-activity relationship. The modifications of sequence were mainly focused on two positions 3 and 5, critical for the selective recognition for μ and δ opioid receptors. The substitution of hydrophobic Leu^5 with hydrophilic Asp^5 derivatives led to Tyr-C[D-A_2bu-Gly-Phe-Asp(*N*-Me)] 7 and Tyr-C[D-Glu-Phe-*g*Phe-*r*Asp(*O*-Me)] 5, the peptides with a large affinity losses at both μ and δ receptors. The substitution of Phe^3 with Gly^3 led to Tyr-C[D-Glu-Gly-*g*Phe-*r*Leu] 3 and Tyr-C[D-Glu-Gly-*g*Phe-D-*r*Leu] 4, the peptides with large affinity losses at μ receptors, indicating the critical role of phenyl ring of Phe^3 for μ receptor affinities. One atom reduction of the ring size from 14-membered analogs Tyr-C[D-Glu-Phe-*g*Phe-(L and D)-*r*Leu] 6a, 6b to 13-membered analogs Tyr-C[D-Asp-Phe-*g*Phe-(L and D)-*r*Leu] 1, 2 reduced the affinity at both μ and δ receptors, but increased the potency in the nociceptive assay, indicating the ring constrain is attributed to high nociceptive potency of the analogs. For the influence of D- or L-chirality of Leu^5 on the receptor selectivity, regardless of chirality and ring size, all cyclic diastereomers displayed marked μ selectivity with low potencies at the δ receptor. The retro-inverso analogs display similar or more active at μ receptor, but less active at δ receptor than the parent analogs.

Key Words: Cyclic-enkephalin, Synthesis, Bioactivity

Introduction

Since the first endogenous opioid peptide, enkephalins were isolated from human and animal nerve tissue in 1974,[1] various analogs were synthesized based on the enkephalin sequences Tyr-Gly-Gly-Phe-Leu(Met). The binding studies with the numerous opioids are now well revealed that opioids interact with at least three different types of opioid receptors (μ, δ and k).[1,2] The μ receptor involves in pain control at the supraspinal level [3-5] while the δ receptor implicates in the behavioral effects of opioids.[6,7] The enkephalins interact preferentially with δ receptors but also bind to μ receptors, albeit with somewhat lower affinity.[2] The dual interaction of enkephalins with two distinct binding sites means that the endogenous opioid peptides are able to adopt different conformations at different receptor sites.[8] This is consistent with the high flexibility of these linear pentapeptides.[9,10]

In attempt to improve the receptor selectivity of opioid peptides and reduce the flexibility of the linear enkephalin, several investigators have prepared conformationally constrained opioid peptides through cyclization in a various ways:[11-14] lactam,[25,26] lanthionine,[27,28] disulfide,[29,30] urea,[31] amine,[32] and -CH_2-CH_2-[33,34] bridge formation. For example, cyclic pentapeptide Tyr-C[D-A_2bu-Gly-Phe-Leu] (A_2bu: 2,4-diaminobutyric acid),[15] and cyclic tetrapeptide Tyr-C[Orn-Phe-Gly][35] represent the cyclic lactam bridged enkephalin. The two cyclic peptides were highly μ selective, while the linear precursors were non-selective. The first analog of lanthionine bridged enkephalin is Tyr-C[D-Ala-Gly-Phe-D-Ala], highly active at both μ and δ receptors. The well known analog of disulfide bridged enkephalin is DPDPE Tyr-C[D-Pen-Gly-Phe-D-Pen] (Pen: penicillamine), extraordinary active at δ receptor.[16] Dicarba Tyr-C[D-Cys-Gly-Phe-Cys]-NH_2 has recently been reported as a first analog of -CH_2-CH_2- bridged enkephalin, highly active at both μ and δ receptors.[33]

The enkephalins contain a phenylalanine residue in the 4-position of the peptide sequence, whereas the μ selective opioid peptides, dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), deltorphin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2), β-casomorphine (Tyr-Pro-Phe-Val-Glu-Pro-Ile-OH), and morphiceptin (Tyr-Pro-Phe-Pro-NH_2) have the phenylalanine in the 3-position.

The structure activity relationship studies and the conformational analysis of various cyclic opioid peptides revealed the correlation between structural requirements and receptor selectivity. Followings are summary: (1) In enkephalin family containing Phe,[4] the μ receptor selective analogs require the spatial orientation of the two aromatic rings of Tyr^1 and Phe^4 in an extended away, whereas the δ receptor selective peptides adopt conformations in which the two aromatic residues are folded in close proximity.[17-19] (2) In dermorphine family containing Phe,[3] a tilted stacking arrangement of the two aromatic rings of Tyr^1 and Phe^3 represent a structural requirement for μ receptor affinity.[20,21,36] (3) The backbone flexibility of the cyclic enkephalin is the important determinant for the receptor selectivity. The flexible backbone adopts the preferable conformation to δ receptor, while the rigid backbone is required for activity at the μ receptor.[26-28]

As an example, the conformational analysis of the μ selective cyclic enkephalin analog Tyr-C[D-A_2bu-Gly-Phe-L-Leu] reveals that backbone ring is constrained and allows the Tyr^1 and Phe^4 rings far part in an extended conformation. Reversal of the Leu^5 backbone chirality increases the backbone flexibility, which allows Tyr^1 and Phe^4 rings close proximity in a folded structure and results in nonselective receptor binding.[22,26] Another conformational studies with the two cyclic enkephalin analogs Tyr-C[D-A_2bu-Gly-Phe-Leu][23] and Tyr-C[D-Glu-Gly-

Phe-*g*Leu],[24] where *g* indicates a *gem*-diaminoalkyl residue, have also revealed that the 14-membered ring retains some flexibility and various intramolecular hydrogen bonds are constantly formed, broken, and reformed again.

In this paper, in efforts aimed at elucidating the topochemical requirements for the binding of the μ and δ receptors of cyclic enkephalin analogs, we have synthesized a series of 13- and 14-membered cyclic lactam bridged pentapeptides based on the moderately μ selective cyclic analog Tyr-C[D-Glu-Gly-Phe-gLeu]. The modifications of sequence were mainly focused on two positions 3 and 5, critical for the selective recognition for μ and δ opioid receptors. In addition, the retro-inverso modifications are also applied to the backbone between Phe[4]-Leu.[5] This applications to backbone are expected to provide the useful information about the functional importance of the amide bond and protection against cleavage by peptidase.[25,26] And also, the two 13-membered cyclic pentapeptides, which have the common sequence of the 14-membered cyclic analogs, appear to explain the constrained nature of ring size affecting on the receptor selectivity.

Experimental Procedures

The melting points were determined in open glass capillaries using Thomas-Hoover melting point apparatus and were uncorrected. Specific rotations were measured on a Perkin Elmer 141 polarimeter at the sodium D-line with a 10 cm path length water-jacketed cell. Proton nmr (^{1}H-nmr) spectra were recorded on a General Electric GN-500 spectrometer using tetramethylsilane as an internal standard (δ scale). Data are reported as follows; chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad), coupling constants (hertz), and integration. Fast atom bombardment mass spectra (FAB MS) were carried out at University of California, San Diego. Flash column chromatography was performed on Merk silica gel 60 (0.040 - 0.063) using nitrogen pressure. Analytical thin-layer chromatography (TLC) was carried out on precoated (0.25 mm) Merk silica gel F-254 plates. R_f values of TLC and purity were determined in the following solvent systems: A, chloroform/methanol (9/1); B, chloroform/methanol (4/1); C, chloroform/methanol/acetic acid (8/1/1); D, chloroform/methanol/acetic acid (7/2/1); E, butanol/acetic acid/water (4/1/1); and F, butanol/pyridine/acetic acid/water (1/2/1/2). Compounds were visualized by ultraviolet light, ninhydrin, or cholin/tolidine reagents.

Reversed phase HPLC was performed on a LiChrograph system utilizing a Merk column (25 × 0.4 cm) packed with LiChrospher 100 RP-118 (10 μm) and methanol-water/1% trifluoroacetic acid solvent system.

***N*-Benzyloxycarbonyl-L-phenylalanyl-*gem*-L-phenylalanine amide, *Z*-Phe-*g*Phe-NH$_2$, 1.** Phe-NH$_2$, (164 mg, 1 mmol) and *Z*-Phe-OH (265 mg, 1 mmol) were dissolved in DMF (20 mL) and cooled to 0 °C. To this solution were added triethylamine (0.4 mL, 2 mmol), HOBt (200 mg, 1.3 mmol), and EDC (250 mg, 1.3 mmol). After 15 min, ice bath was removed and the mixture was stirred at room temperature for 12 hrs and then concentrated. The residue was dissolved in chloroform (150 mL) and washed with saturated aqueous NaHCO$_3$ (25 mL × 3), 5% citric acid in water (25 mL × 3), saturated aqueous NaCl (25 mL × 3) and dried over magnesium sulfate. The concentration afforded the white solid. Purification by flash chromatography with elution of 5% methanol in dichloromethane gave 277 mg of the title compound as a white crystalline solid. Yield, 95%, R_f (D) 0.71, mp 150 - 152 °C, ^{1}H-NMR (DMSO-d_6) δ 8.1 (d, 1H, NH), 7.5 (d, 1H, NH), 7.4 (s, 2H, NH), 7.2 (m, 15H, 3ph), 5.0 (s, 2, CH$_2$), 4.5 (m, 1H, CH), 4.2 (m, 1H, CH), 2.7-3.1 (m, 4H, CH$_2$).

***N*-Benzyloxycarbonyl-L-phenylalanyl-*gem*-L-phenylalanine, *Z*-Phe-*g*Phe·HCl, 2.** To a solution of iodobenzene bis-trifluoroacetate (IBTFA, 430 mg, 1 mmol) in acetonitrile /water (20 mL, 4/1 v/v), *Z*-Phe-*g*Phe-NH$_2$ **1** (445 mg, 1 mmol) was added at room temperature. Reaction mixture was stirred for 3 hrs. To the reaction mixture was added 1N HCl (1.1 mL) in dioxane and stirred for 10 min. The solvent was evaporated under vacuo and the residue dissolved in 100 mL of ethylacetate. The organic phase was washed with saturated aqueous NaHCO$_3$ (25 mL × 3), 5% citric acid in water (25 mL × 3), saturated aqueous NaCl (25 mL × 3), and dried over magnesium sulfate. Organic phase was concentrated to give a crude product. Purification by flash chromatography with elution of 10% methanol in dichloromethane gave 371 mg of the title compound as a white crystalline solid. Yield, 82%, mp 150 °C (decomposed), R_f (D) 0.55, ^{1}H-NMR (D$_2$O/DMSO-d_6) δ 8.1 (d, 1H, NH), 7.7 (d, 1H, NH), 7.6 (s, 2H, NH), 7.2 (m, 15H, 3ph), 5.0 (s, 2, CH$_2$), 4.5 (m, 1H, CH), 4.2 (m, 1H, CH), 2.7-3.1(m, 4H, CH$_2$).

***N*-Benzyloxycarbonyl-L-phenylalanyl-*gem*-L-phenylalanyl-*t*-butoxycarbonyl-*retro*-L-leucine, *Z*-Phe-*g*Phe-*r*Leu-Boc, 3.** Triethylamine (0.4 mL, 3 mmol), hydroxybenzotriazole monohydrate (HOBt) (153 mg, 1 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (192 mg 1.1 mmol) were added to a chilled solution (−20 °C) of Boc-Leu-OH (249 mg, 1 mmol) and *Z*-Phe-*g*Phe·HCl **2** (453 mg, 1 mmol) in 50 mL of DMF. After the reaction mixture was stirred for 2 hrs at 0 °C and 6 hrs at room temperature, DMF was removed under reduced pressure. The residue was partitioned between 200 mL of ethylacetate and 30 mL of water. The organic phase was washed with saturated aqueous NaHCO$_3$ (25 mL × 3), 5% citric acid in water (25 mL × 3), saturated aqueous NaCl (25 mL × 3), and dried over magnesium sulfate. Organic phase was concentrated to give a crude product. Purification by flash chromatography with elution of 2% methanol in dichloromethane gave 570 mg of the title compound as a white crystalline solid. Yield, 88%, R_f (D) 0.65, mp 225 °C, ^{1}H-NMR (D$_2$O/DMSO-d_6) δ 8.4 (d, 1H, NH), 8.2 (d, 1H, NH), 7.5 (d, 1H, NH), 6.8 (d, 1H, NH), 7.2-7.4 (m, 15H, 5ph), 5.5 (q, 1H, CH), 5.0 (d, 2H, CH$_2$), 4.3 (q, 1H, CH), 3.9 (q, 1H, CH), 2.9 (m, 2H, CH$_2$), 2.7 (q, 2H, CH$_2$), 1.2-1.4 (m, 2H, CH$_2$), 1.4 (s, 9H, Boc), 0.8 (d, 4H, CH$_2$).

L-Phenylalanyl-*gem*-L-phenylalanyl-*t*-butoxycarbonyl-*retro*-L-leucine, Phe-*g*Phe-*r*Leu-Boc, 4. To a solution of fully protected tripeptide **3** (648 mg, 1 mmol) in 20 mL of methanol/DMF (1/9, v/v) was added a 50 mg of 10% Pd-C. The reaction mixture was stirred under an atmospheric pressure of hydrogen for 5 hrs at room temperature. The reaction of hydrogenolysis was monitored by thin layer chromatography (20% methanol in dichloromethane). The suspension was filtered through cellite and washed with methanol several times (10 mL × 10) and concentrated. The residue dried over P_2O_5 in vacuo to give crude product. Purification by flash chromatography with elution of 10% methanol

in dichloromethane gave 488 mg of the title compound as a white crystalline solid. Yield, 95%, R_f(D) 0.48, mp 141 - 143 °C, ^{1}H-NMR (DMSO-d_6) δ 8.4 (d, 1H, NH), 8.3 (d, 1H, NH), 7.0 (d, 1H, NH), 7.4 (m, 10H, ph), 5.8 (m, 1H, CH), 4.1 (m, 1H, CH), 3.0 (m, 2H, CH_2), 2.7 (m, 2H, CH_2), 1.9 (m, 1H, CH), 1.4-1.6 (m, 4H, CH_2), 1.5 (s, 9H, Boc), 1.0 (m 4H, CH_2).

Benzyloxycarbonyl-*O*-*t*-butyl-D-aspartyl-L-phenylalanyl-*gem*-L-phenylalanyl-*t*-butoxycarbonyl-*retro*-L-leucine, *Z*-D-Asp(*O*-*t*Bu)-Phe-*g*Phe-*r*Leu-Boc, 5. The coupling reaction was carried out in the same manner described for the synthesis of **3** using *Z*-D-Asp(O-*t*Bu)-OH (323 mg, 1 mmol), Phe-*g*Phe-*r*Leu-Boc (514 mg, 1 mmol), EDC (240 mg, 1.2 mmol), HOBt (184 mg, 1.2 mmol). Flash column with elution of 3% methanol in dichloromethane afforded the product as oil. Yield, 753 mg (92%), R_f(D) 0.62, mp 218 - 220 °C, ^{1}H-NMR (DMSO-d_6) δ 8.9 (d, 1H, NH), 8.5 (d, 1H, NH), 8.3 (d, 1H, NH), 8.0 (d, 1H, NH), 7.9 (d, 1H, NH), 7.2-7.4 (m, 15H, ph), 5.5 (q, 1H, CH), 5.0 (q, 2H, CH_2), 4.2 (m, 1H, CH), 3.6 (m, 1H, CH), 3.0 (d, 4H, CH_2), 2.6-2.9 (m, 6H, CH_2), 1.4 (m, 2H, CH), 1.3 (s, 9H, *t*Bu), 1.2 (s, 9H, Boc), 0.8 (m, 2H, CH_2).

Benzyloxycarbonyl-L-tyrosyl-*O*-*t*-butyl-D-aspartyl-L-phenylalanyl-*gem*-L-phenylala-nyl-*t*-butoxycarbonyl-*retro*-L-leucine, *Z*-Tyr-D-Asp(*O*-*t*Bu)-Phe-*g*Phe-*r*Leu-Boc, 6. The deprotection of benzyloxycarbonyl group of tetrapeptide **5** (819 mg, 1 mmol) was carried out in the same manner described for the synthesis of **4**. The following coupling reaction was carried out in the same manner described for the synthesis of 3 using *Z*-Tyr-OH (315 mg, 1 mmol), D-Asp(*O*-*t*Bu)-Phe-*g*Phe-*r*Leu-Boc (685 mg, 1 mmol), EDC (240 mg, 1.2 mmol), HOBt (184 mg, 1.2 mmol). Flash column with elution of 3% methanol in dichloromethane afforded the product as oil. Yield, 736 mg (91%), mp 215 - 216 °C, R_f(D) 0.62, ^{1}H-NMR (DMSO-d_6) δ 9.2 (s, 1H, OH), 8.9 (d, 1H, NH), 8.5 (d, 1H, NH), 8.3 (d, 1H, NH), 7.9 (d, 1H, NH), 7.2-7.4 (m, 15H, ph), 7.0-6.8 (2d, 4H, ph), 5.5 (q, 1H, CH), 5.0 (q, 2H, CH_2), 4.4 (m, 2H, CH), 4.2 (m, 1H, CH), 3.6 (m, 1H, CH), 3.0 (d, 4H, CH_2), 2.6-2.9 (m, 6H, CH_2), 1.4 (m, 2H, CH), 1.3 (s, 9H, *t*Bu), 1.2 (s, 9H, Boc), 0.8 (m, 2H, CH_2).

Benzyloxycarbonyl-L-tyrosyl-D-aspartyl-L-phenylalanyl-*gem*-L-phenylalanyl-*retro*-L-leucine hydrochloride salt, *Z*-Tyr-D-Asp-Phe-*g*Phe-*r*Leu·HCl, 7. A solution of fully protected pentapeptide **6** (983 mg, 1 mmol) in 50% trifluoroacetic acid in dichloromethane (20 mL) was stirred at room temperature for 45 min. 10 mL of 4 N HCl in dioxane was added to the reaction mixture and stirred for 5 min. Evaporation gave a yellowish solid. The solid mixture was dissolved in methanol and concentrated several times and dried over P_2O_5 under vacuo for 2 hrs. Purification by flash chromatography with elution of 5% methanol/dichloromethane gave 905 mg of the title compound as a white solid. Yield, 89%, R_f(D) 0.22, mp 205 - 206 °C, ^{1}H-NMR (DMSO-d_6) δ 9.2 (s, 1H, OH), 8.9 (d, 1H, NH), 8.4 (d, 1H, NH), 8.3 (d, 1H, NH), 8.0 (d, 1H, NH), 7.2-7.4 (m, 15H, ph), 7.0-6.8 (2d, 4H, ph), 5.5 (q, 1H, CH), 5.0 (s, 2H, CH_2), 4.4 (m, 2H, CH), 4.2 (m, 1H, CH), 3.6 (m, 1H, CH), 3.0 (d, 4H, CH_2), 2.6-2.9 (m, 6H, CH_2), 1.4 (m, 2H, CH), 0.8 (m, 2H, CH_2).

Benzyloxycarbonyl-L-tyrosyl-cyclo-D-aspartyl-L-phenylalanyl-*gem*-L-phenylalanyl-*retro*-L-leucine, Tyr-C[D-Asp-Phe-*g*Phe-*r*Leu], 8. To a solution of the linear pentapeptide **7** (1018 mg, 1 mmol, 1.0 eq.) in dry degassed DMF (230 mL, 8 mM) maintained at −20 °C, $NaHCO_3$ (420 mg, 5 mmol, 5 eq.) and BOP (650 mg, 1.5 mmol, 1.5 eq.) were added. The reaction mixture was stirred for 3 days at 0 °C. The reaction mixture was then concentrated. The residue was diluted with 100 mL of chloroform, washed with saturated aqueous $NaHCO_3$ (20 mL × 3), 5% citric acid in water (25 mL × 3), saturated aqueous NaCl (25 mL × 3) and dried over magnesium sulfate. Organic phase was concentrated to give yellowish crude solid product. Purification by flash chromatography, gradient elution (1% → 3%, methanol/chloroform) gave 563 mg (yield; 52%) of the monocyclic pentapeptide as an amorphous solid. The deprotection of benzyloxycarbonyl group of monocyclic pentapeptide (366 mg, 0.38 mmol) was carried out in the same manner described for the synthesis of **4**. Purification by flash chromatography, gradient elution (5% → 10%, methanol/chloroform) afforded 296 mg (yield; 94%) of the title compound as an amorphous solid. Overall yield, (54%), R_f(D) 0.52, mp 236 - 237 °C, ^{1}H-NMR (DMSO-d_6) δ 9.3 (s, 1H, OH), 9.0 (d, 1H, NH), 8.7 (d, 1H, NH), 8.3 (m, 1H, NH), 8.1 (d, 1H, NH), 7.9 (2d, 2H, 2NH), 7.3 (m, 10H, 2ph), 6.7-7.2 (2d, 4H, ph), 5.5 (q, 1H, CH), 4.4 (m, 1H, CH), 4.2 (m, 2H, 2CH), 3.6 (m, 1H, CH), 3.2 (m, 4H, CH_2), 2.7-3.0 (m, 6H, CH_2), 2.1 (m, 1H, CH), 1.4 (m, 2H, CH_2), 0.8 (m, 4H, CH_2), FAB-MS; 660 (M^+).

L-Tyrosyl-cyclo-D-aspartyl-L-phenylalanyl-*gem*-L-phenylalanyl-*retro*-D-leucine, Tyr-C[D-Asp-Phe-*g*Phe-D-*r*Leu], 9. The reaction was carried out in the same procedure described for the synthesis of **1** - **8**. Yield, 51%, R_f(D) 0.52, mp 233 - 234 °C, ^{1}H-NMR (DMSO-d_6) δ 9.3 (s, 1H, OH), 8.4 (d, J = 8.0 Hz, 1H, NH), 8.2 (d, J = 8.1 Hz, 1H, NH), 8.1 (d, J = 8.2 Hz, 1H, NH), 7.7 (m, 2H, 2NH), 7.3 (m, 10H, 2ph), 6.7-7.2 (2d, J = 12.0 Hz, 4H, ph), 5.3 (m, 1H, CH), 4.5 (m, 1H, CH), 4.2 (q, J = 3.4 Hz, 1H, CH), 3.9 (q, J = 3.2 Hz, 1H, CH), 3.7 (q, J = 3.1 Hz, 1H, CH), 3.5 (m, 1H, CH), 3.2 (m, 4H, CH_2), 2.8 (m, 2H, CH_2), 2.4-2.6 (m, 6H, CH_2), 1.4-1.7 (m, 4H, CH_2), 0.8 (m, 4H, CH_2), FAB-MS; 660(M^+).

***N*-Benzyloxycarbonyl-glycyl-*gem*-L-phenylalaninamide, *Z*-Gly-*g*Phe-NH_2, 10.** The reaction was carried out in the same procedure described for the synthesis of **1.** Yield, 95%, R_f(D) 0.70, mp 147 - 149 °C, ^{1}H-NMR (DMSO-d_6) δ 8.1 (d, 1H, NH), 7.5 (d, 1H, NH) 7.4 (s, 2H, NH), 7.2 (m, 10H, 3ph), 5.1 (s, 2, CH_2), 4.5 (m, 1H, CH), 3.6 (m, 1H, CH), 2.8-3.0 (m, 4H, CH_2).

***N*-Benzyloxycarbonyl-glycyl-*gem*-L-phenylalanyl-*t*-butoxycarbonyl-*retro*-L-leucine, *Z*-Gly-*g*Phe-*r*Leu-Boc, 11.** The reaction was carried out in the same procedure described for the synthesis of **2-3.** Yield, 87 %, R_f(D) 0.63, mp 220 - 221 °C, ^{1}H-NMR (D_2O/DMSO-d_6) δ 8.2 (m, 2H, 2NH), 7.5 (d, 1H, NH), 6.8 (d, 1H, NH), 7.2-7.4 (m, 10H, 5ph), 5.5 (q, 1H, CH), 5.0 (d, 2H, CH_2), 3.9 (q, 1H, CH), 3.6 (q, 1H, CH), 2.9 (m, 2H, CH_2), 1.2-1.4 (m, 2H, CH_2), 1.4 (s, 9H, Boc), 0.8 (d, 4H, CH_2).

Benzyloxycarbonyl-*O*-*t*-butyl-D-glutamyl-glycyl-*gem*-L-phenylalanyl-*t*-butoxycarbonyl-*retro*-L-leucine, *Z*-D-Glu(*O*-*t*Bu)-Gly-*g*Phe-*r*Leu-Boc, 12. The reaction was carried out in the same procedure described for the synthesis of **4-5**. Yield, 92%, R_f(D) 0.60, mp 221 - 223 °C, ^{1}H-NMR (DMSO-d_6) δ 8.3 (2d, 1H, 2NH), 8.1 (d, 1H, NH), 7.6 (d, 1H, NH), 6.8 (d, 1H, NH), 7.2-7.4 (m, 10H, ph), 5.5 (q, 1H, CH), 5.0 (q, 2H, CH_2), 3.9 (m, 1H, CH), 3.7 (m, 2H, CH), 2.9 (d, 2H, CH_2), 2.3 (m, 4H, CH_2), 1.7-1.9 (m, 2H, CH), 1.3 (s, 9H, *t*Bu), 1.2 (s, 9H, Boc), 1.2

(m, 4H, CH_2), 0.8 (m, 2H, CH_2).

Benzyloxycarbonyl-L-tyrosyl-*O*-*t*-butyl-D-glutamyl-L-glycyl-*gem*-L-phenylalanyl-*t*-butoxycarbonyl-*retro*-L-leucine, *Z*-Tyr-D-Glu(*O*-*t*Bu)-Gly-*g*Phe-*r*Leu-Boc, 13. The reaction was carried out in the same procedure described for the synthesis of **6**. Yield, 90%, R_f (D) 0.60, mp 212 - 213 °C, ^{1}H-NMR (DMSO-d_6) δ 9.2 (s, 1H, OH), 8.1-8.3 (m, 4H, 4NH), 7.5 (d, 1H, NH), 6.8 (d, 1H, NH), 7.2-7.4 (m, 10H, ph), 7.1-6.6 (2d, 4H, ph), 5.5 (q, 1H, CH), 5.0 (q, 2H, CH_2), 4.3 (m, 2H, CH), 3.9 (m, 1H, CH), 3.7 (m, 1H, CH), 2.9 (d, 4H, CH_2), 2.6 (m, 2H, CH_2), 2.2 (m, 2H, 2H), 1.7-1.9 (m, 2H, CH_2), 1.2 (m, 2H, CH), 1.3 (s, 9H, *t*Bu), 1.2 (s, 9H, Boc), 0.8 (m, 2H, CH_2).

L-Tyrosyl-cyclo-D-glutamyl-glycyl-*gem*-L-phenylalanyl-retro-L-leucine, Tyr-C[D-Glu-Gly-*g*Phe-*r*Leu], 14. The reaction was carried out in the same procedure described for the synthesis of **7** - **8**. Yield, 54%, R_f (D) 0.52, mp 178 - 179 °C, ^{1}H-NMR (DMSO-d_6) δ 9.1(s, 1H, OH), 8.7(d, J = 8.0 Hz, 1H, NH), 8.2 (m, 1H, NH), 8.1 (d, J = 8.1 Hz, 1H, NH), 8.0 (m, 1H, NH), 7.2 (d, J = 7.9 Hz, 1H, NH), 7.1-7.3 (m, 5H, ph), 6.6-7.0 (2d, J = 12.0 Hz, 4H, ph), 5.8 (q, J = 4.2 Hz, 1H, CH), 4.3 (m, 1H, CH), 3.8 (q, J = 3.7 Hz, 1H, CH), 3.7 (d, J = 3.2 Hz, 1H, CH), 3.4 (m, 1H, CH), 3.2 (m, 2H, CH_2), 2.8 (m, 2H, CH_2), 2.2 (m, 2H, CH_2), 1.9 (m, 2H, CH_2), 1.7 (m, 2H,CH_2), 1.2-1.4 (m, 2H, CH_2), 0.8 (m, 4H, CH_2), FAB-MS; 551 (M^+).

L-Tyrosyl-cyclo-D-glutamyl-glycyl-*gem*-L-phenylalanyl-*retro*-D-leucine, Tyr-C[D-Glu-Gly-*g*Phe-D-*r*Leu], 15. The reaction was carried out in the same procedure of **10**. R_f (D) 0.53, mp 177 - 178 °C, ^{1}H-NMR (DMSO-d_6) δ 9.2(s, 1H, OH), 8.8 (d, J = 8.1 Hz, 1H, NH), 8.3 (m, 1H, NH), 8.2 (m, 1H, NH), 8.1 (m, 1H, NH), 7.3 (d, J = 7.9 Hz, 1H, NH), 7.2-7.3 (m, 5H, ph), 6.7-6.9 (2d, J = 12.0 Hz, 4H, ph), 5.9 (q, J = 4.1 Hz, 1H, CH), 4.0 (m, 1H, CH), 3.6 (q, J = 3.9 Hz, 1H, CH), 3.5 (m, 1H, CH), 3.3 (m, 1H, CH), 3.0 (m, 2H, CH_2), 2.9 (m, 2H, CH_2), 2.1 (m, 2H, CH_2), 1.8 (m, 2H, CH_2), 1.6 (m, 2H,CH_2), 1.2-1.4 (m, 2H, CH_2), 0.8 (m, 4H, CH_2), FAB-MS; 551 (M^+).

L-Tyrosyl-cyclo-D-glutamyl-L-phenylalanyl-*gem*-L-phenylalanyl-*retro*-aspartyl-β-methylester, Tyr-C[D-Glu-Phe-*g*Phe-Asp(OMe)], 16. The reaction was carried out in the same procedure described for the synthesis of **1-9**. R_f (D) 0.50, mp 191 - 192 °C, ^{1}H-NMR (DMSO-d_6) δ 9.2 (s, 1H, OH), 9.0 (d, J = 8.2 Hz, 1H, NH), 8.3 (m, 2H, 2NH), 8.1 (d, J = 8.1 Hz, 1H, NH), 7.5 (d, J = 8.3 Hz, 1H, NH), 7.3 (m, 10H, ph), 6.6-7.2 (2d, J = 12.0 Hz, 4H, ph), 5.8 (q, J = 3.1 Hz, 1H, CH), 4.9 (q, J = 2.9 Hz, 1H, CH), 4.7 (m, 1H, CH), 4.3 (m, 1H, CH), 4.2 (m, 1H, CH), 4.1 (q, J = 2.8 Hz, 1H, CH), 3.6 (d, J = 14.6 Hz, 3H, OMe), 3.3 (m, 2H, CH_2), 3.0 (m, 1H, CH), 2.5-2.9 (m, 6H, CH_2), 1.2-1.9 (m, 4H, CH_2), FAB-MS; 639 (M^+).

L-Tyrosyl-cyclo-D-diaminobutyryl-glycyl-L-phenylalanyl-*N*-methyl-L-aspartate, Tyr-C[D-A$_2$bu-Gly-Phe-Asp(NH-Me)], 17. The reaction was followed the procedure described by Hong *et. al*.[37] R_f (D) 0.46, mp 160 - 163 °C, ^{1}H-NMR (DMSO-d_6) δ 9.1 (s, 1H, OH), 8.6 (d, J = 7.8 Hz, 1H, NH), 8.3 (t, J = 3.6 Hz, 1H, NH), 8.1 (m, 3H, 3NH), 7.2 (m, J = 9.7 Hz, 5H, ph), 6.6-6.9 (2d, J = 12.0 Hz, 4H, ph), 6.0 (d, J = 8.9 Hz, 1H, NH), 4.5 (m, J = 3.4 Hz, 2H, 2CH), 4.2 (q, J = 3.0 Hz, 1H, CH), 3.6-3.8 (m, J = 7.5 Hz, 2H, CH_2), 2.9-3.1 (m, J = 8.3 Hz, 4H, 2CH_2), 2.9 (s, 3H, CH_3), 2.5 (m, J = 7.4 Hz, 2H, CH_2), 1.6-1.8 (2m, J = 4.8 Hz, 4H, 2CH_2). FAB-MS; 595 (M^+).

L-Tyrosyl-cyclo-D-glutamyl-L-phenylalanyl-*gem*-L-phenylalanyl-*retro*-L-leucinemonomer, Tyr-C[D-Glu-Phe-*g*Phe-*r*Leu], 18. The reaction was carried out in the same procedure described for the synthesis of **1-9**. R_f (D) 0.36, ^{1}H-NMR (DMSO-d_6) δ 9.3 (s, 1H, OH), 8.5 (d, J = 8.0 Hz, 1H, NH), 8.3 (m, 2H, 2NH), 7.9 (d, J = 8.1 Hz, 1H, NH), 7.1-7.3 (m, 10H, 2ph), 6.9 (d, J = 8.2 Hz, 1H, NH), 6.6-7.0 (d, J = 12.0 Hz, 4H, ph), 5.5 (m, J = 3.2 Hz, 1H, CH), 4.5 (m, 1H, CH), 4.3 (m, 1H, CH), 4.1 (m, 2H, 2CH), 3.3 (m, 2H, CH_2), 3.0 (m, 2H, CH_2), 2.7-2.9 (m, 4H, CH_2), 2.5 (m, 2H, CH_2), 1.3-1.6 (m, 6H, CH_2), 0.8 (m, 6H, CH_3), FAB-MS; 672 (M^+).

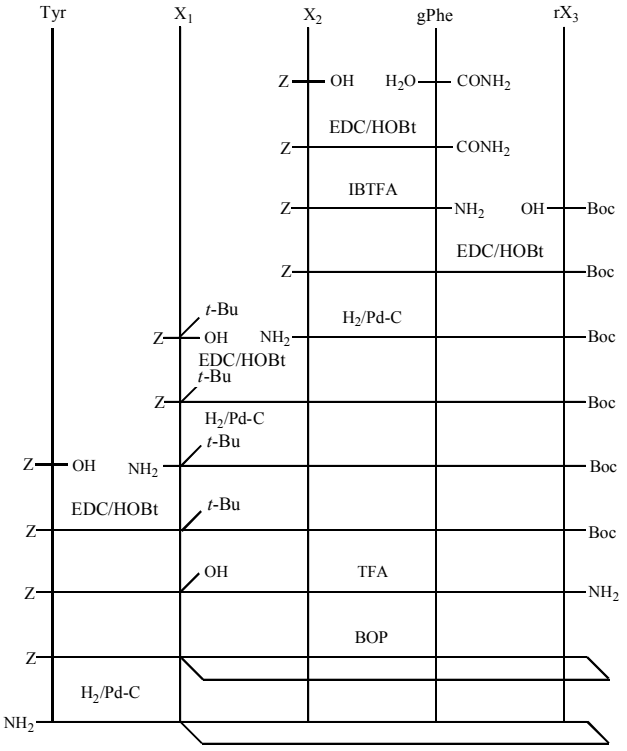
Binding assays. The GPI[38] and MVD[39] bioassays were carried out as reported by P. Schiller.[40,41] A log dose-response curve was determined using [Leu^5]-enkephalin as standard compound. Vas preparation and IC_{50} values for the compounds being tested were normalized according to a published procedure.[42]

Nociceptive assays. Nociceptive response were measured using the 52.5 °C hot plate (HP) and/or the tail flick (TF) test.[43] In the HP model, the latency to lick the hind paw was assayed. Failure to respond within 60 seconds was cause to terminate the experiment and assign that latency as the response measure. In the TF test, the latency to tail withdrawal after being placed over a focused 300 W projection bulb was noted. Cut off time was 6 seconds. For analysis, response latencies were converted to the % of the maximum possible effect (% MPE): % MPE = [(post drug response latency) - (predrug response latency)]/[(cut off time) - (predrug response latency)] × 100.

Result and Discussion

Synthesis. All of the syntheses were carried out in solution. The *tert*-butoxycarbonyl (*t*-Boc) group and benzyloxycarbonyl (Z) were employed to protect the amino group of each amino acid. Water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxybenzotriazole (HOBt) as racemization suppressor were used as coupling reagents in all coupling reactions, apart from steps involving active ester method such as succinimide (-OSu).

The five cyclic enkephalin analogs, Tyr-C[D-X_1-X_2-*g*Phe-*r*X_3] **8-9**, **14-16**, where X_1 = Glu, Asp; X_2 = Phe, Gly; X_3 = (D- or L-)Leu, Asp(OMe), were synthesized by the synthetic Scheme 1. The *Z*-X_2 and phenylalanine amide was first coupled into dipeptide amide using EDC/HOBt. The amide functional group of *Z*-X_2-Phe-$CONH_2$ was rearranged to amine upon treatment with IBTFA. The coupling between the dipeptide amine, *Z*-X_2-*g*Phe-NH_2 and Boc-X_3-OH in usual manner using EDC/HOBt afforded the protected tripeptide, *Z*-X_2-*g*Phe-*r*X_3-Boc. Mild transfer hydrogenation of the *Z*-protecting group over 10% Pd-C gave tripeptide X_2-*g*Phe-*r*X_3-Boc. Subsequent stepwise elongation in usual manner with EDC/HOBt and deprotection of two *t*-butoxy protecting groups with TFA in DMF afforded the unprotected linear pentapeptide. The backbone to side chain cyclizations were achieved with 8 mM of linear pentapeptide in DMF using 1.5 equiv. BOP. The another cyclic D-A$_2$bu[2] analog, Tyr-C[D-A$_2$bu-Gly-Phe-Asp(NH-Me)] **17**, was synthesized by the synthetic procedure described by Hong *et. al*.[37] The linear pentapeptide precursor of the cyclic analog **17** was first prepared by a stepwise elongation from the carboxy terminus to the amino



Scheme 1. Synthetic scheme of 13- and 14-membered cyclic enkephalin analogs, Tyr-C[D-X_1-X_2-*g*Phe-*r*X_3] *via* stepwise elongation. X_1 = D-Glu, D-Asp; X_2 = Phe, Gly; X_3 = (D- or L-)Leu, Asp(*O*-Me), Asp(NH-Me).

terminus as a usual manner using EDC/HOBt. Then, the cyclization was achieved by high dilution method described in Table 1.

Evidences were utilized to confirm the structures of the target molecules. All target molecules were subjected to fast atom bombardment mass spectrometry, which in all cases yielded the appropriate molecular weights. Additionally, all cyclic products were examined in 2D ^{1}H NMR spectroscopy. Linear products were examined in 1D ^{1}H NMR spectroscopy.

As shown in Table 1, we attempted the cyclization in various ways in order to explore the optimum condition. The best result was obtained with BOP/$NaHCO_3$ (1.5equiv./5equiv.). The yield of cyclization was proportional to a function of the reaction time. In case aldrithiol, the product spot was not appeared on tlc with either ninhydrin or uv visualization, because the product have a R_f value identical with that of the aldrithiol. Despite of the ambiguous tlc pattern, attempt to isolate the product by the flash column chromatography was unsuccessful. In case DPPA method, the considerable amount of cyclic side product (> 30%) was detected on the course of the cyclization reaction. According to nmr assignment, the structure of the side product was found to be identical with that of authentic product, except only for the phenolic OH of Tyr[1] eliminated. Interestingly, the similar side reaction was not detected under the same reaction condition with BOP.

Biological activities. The *in vitro* biological activities of the conjugates measured in guinea pig ileum (GPI) and mouse vas deferens (MVD) assays are summarized in Table 2. The GPI and MVD assays were used for determining the bioactivities at the

Table 1. Yields for the synthesis of Boc-Tyr-C[D-Glu-Phe-*g*Phe-*r*Asp (O-Me)] depending on various cyclizaion conditions. (8 mM, 25 °C)

Cyclization agent (1.5 eq.)	Base (5.0 eq.)	Rex.time (day)	Yield (%)
DPPA	NEt_3	3	21
	$NaHCO_3$	5	34
Aldrithiol	NEt_3	3	-
BOP	NEt_3	3 (or 5)	37
	$NaHCO_3$	3	52
	$NAHCO_3$	5	59

DPPA : Diphenylphsphoryl azide, Aldrithiol : 2,2-dipyridyldisulfide, BOP : Benzotriazole-*N*-oxy-tris(dimethylamino)-phesphonium hexafluorophosphate

μ and δ-opioid receptors, respectively.[34] Nociceptive responses (*in vivo* test) were assessed using the 52.5 °C hotplate and/or the tail flick test.[37] Characterization of the receptor mediated the antinociceptive effects of novel opioid peptides and was carried out by examining the effects of spinally administered agents on a selected battery of painbehavior assays using rats chronically prepared with indwelling intrathecal catheters.

Discussion. Pharmacological data for the analogs of the cyclic enkephalin under study are presented in Table 2. The observed μ and δ opioid receptor binding affinities (IC_{50}) and nociceptive potencies (ED_{50}) of the two 13-membered cyclic analogs 1,2 and the four 14-membered cyclic analogs 3,4,5,7 are shown in Table 2, along with the potencies of the reference analogs 6a, 6b, 8a, 8b reported in literatures.[15,26]

All the cyclic analogs are effective at inhibiting the electrically induced contractions in one or both of the bioassays examined. As compared to [Leu^5]-enkephalin, all the cyclic analogs are more potent in the GPI assays. With the exception of the one reference analog 6a, which display higher potency in the δ receptor, the remaining analogs show much lower potencies in the MVD assay than [Leu^5]-enkephalin.

Comparison of the biological activities between the 13-membered cyclic pentapeptides and the corresponding 14-membered cyclic pentapeptides give some interesting insights about the constrained nature of ring size. In the binding studies, all the 13-membered analogs 1,2 display lower potencies at both μ and δ receptors than the 14-membered counterparts 6a, 6b: 10 - 13 times low affinities at the μ receptor (IC_{50}μ(1)/ IC_{50}μ(6a) = 20.5/1.94, IC_{50}μ(2)/ IC_{50}μ(6b) = 40.5/2.75) and 3 - 50 times low affinities at the δ receptor (IC_{50}δ(1)/ IC_{50}δ(6a) = 312/6.5, IC_{50}δ(2)/ IC_{50}δ(6b) = 134/49.1). Contrary to *in vitro* results, in the nociceptive studies, the 13-membered analogs 1,2 show 5 - 13 times higher potencies than 14-membered counterparts 6a, 6b, ED_{50}(1)/ED_{50}(6a) = 0.41/1.9 and ED_{50}(2)/ED_{50}(6b) = 0.63/8.4. Another comparison of the affinities between the 13-membered dermorphin like analogs 1,2 containing Phe^3 and enkephalin like analogs 3,4 containing Phe^4 show almost similar potency profiles at either μ or δ receptors. But, the nociceptive potencies of 13-membered analogs 1,2 are more potent than 14-membered analogs 3,4. Therefore, two comparisons lead us to conclude that one atom decrease in the ring size seems to be responsible for high nociceptive potency of the 13-membered analogs, whereas a decrease in the backbone ring flexibility caused by reducing

Table 2. Binding affinities(IC_{50}) and nociceptive activities(ED_{50}) of 13- and 14-membered cyclic enkephalin analogs. Potencies of analogs 6b and 8a, 8b are the values reported in literatures 26, 15, respectively

No	Compounds	Ring Size	IC_{50} (nM)		MVD/GPI (δ/μ)	ED_{50} (μmol)
			GPI(μ)	MVD(δ)		
1	Tyr-C[D-Asp-Phe-*g*Phe-*r*Leu]	13	20.5	312	15.1	0.71
2	Tyr-C[D-Asp-Phe-*g*Phe-D-*r*Leu]	13	40.5	134	3.31	0.93
3	Tyr-C[D-Glu-Gly-*g*Phe-*r*Leu]	14	20.5	717	35	1.0
4	Tyr-C[D-Glu-Gly-*g*Phe-D-*r*Leu]	14	19.4	313	16.1	2.1
5	Tyr-C[D-Glu-Phe-*g*Phe-*r*Asp(*O*-Me)]	14	114	4830	42.4	1.3
6a	Tyr-C[D-Glu-Phe-*g*Phe-*r*Leu]	14	1.94	6.5	3.28	1.9
7	Tyr-C[D-A2bu-Gly-Phe-Asp(*N*-Me)]	14	120	1020	8.5	0.42
8a	Tyr-C[D-A2bu-Gly-Phe-Leu][15]	14	14.1	81.4	5.77	-
9	[Leu]-Enkephalin	-	246	11.4	0.046	-
6b	Tyr-C[D-Glu-Phe-Phe-*g*Phe-D-*r*Leu][26]	14	2.75	49.1	17.9	8.4
8b	Tyr-C[D-A2bu-Gly-Phe-D-Leu][15]	14	66.1	27.1	0.49	-

the ring size from 14 to 13 atoms may not enhance the *in vitro* potency of cyclic analogs at both μ and δ receptors.

In comparison with differences of the receptor potency of 14-membered analogs between the enkephalin like analogs 3,4 and dermorphine like analogs 6a, 6b, all the enkephalin like analogs show lower potencies in both opioid receptors than dermorphine counterparts, indicating the important role of Phe^3 associated with the receptor affinity: 10 times less potent in μ receptor ($IC_{50}\mu(3)/IC_{50}\mu(6a) = 20.5/1.94$) and 110 times less potent in δ receptor ($IC_{50}\delta(3)/IC_{50}\delta(6a) = 717/6.5$) for analog 3;7 times less potent in μ receptor $IC_{50}\mu(4)/ IC_{50}\mu(6b) = 19.4/2.75$ and 6 times less potent in δ receptor ($IC_{50}\delta(4)/IC_{50}\delta(6b) = 313/49.1$) for analog 6. Similar results are also reported by Schiller *et al*. with dermorphine like cyclic tetrapeptides derived from Tyr-[D-Orn-Phe-Asp]-NH_2,[45] which is highly μ receptor selective. Taken together, the results ensure strongly the critical role of Phe^3 residue in binding for the μ receptor in the same way of the μ binding action of dermorphine and favorable interaction with a hydrophobic site at the μ receptor.

Modifications of Leu at the fifth position were carried out to characterize the structure optimizing μ specificity. Replacement of Leu^5 in the analogs 6a,8a by hydrophilic residues, Asp(*O*-Me) and Asp(NH-Me), produce the analogs 5,7. All two show the drastic affinity losses at both μ and δ receptors; in the former, 55 times less potent in μ receptor ($IC_{50}\mu(5)/IC_{50}\mu(6a) = 114/1.94$) and 740 times less potent in δ receptor ($IC_{50}\delta(5)/IC_{50}\delta(6a) = 4830/6.5$) respectively and in the latter, 9 times less potent in μ receptor ($IC_{50}\mu(7)/IC_{50}\mu(8a) = 120/14.1$) and 13 times less potent in δ receptor ($IC_{50}\delta(7)/IC_{50}\delta(8a) = 1020/81.4$) respectively. These results corroborate the negative influence of hydrophilic residue at fifth position on the receptor binding and the important role of hydrophobic nature of Leu.[5] The similar effect was described in the case of the linear enkephalin derivatives.[44]

The chirality of Leu^5 at the fifth position is one of the important structural requirement for opioid receptor differentiation as described in the introduction.[46,47,24] Thus, we examined the influence of Leu^5 chirality on the receptor selectivity with the 13- and 14-membered cyclic diasteromers 1-4. Results reveal that regardless of Leu^5 chirality, all four analogs are highly active at μ receptor with similar potencies. However the ratios, $IC_{50}\delta(1)/IC_{50}\delta(2) = 312/134$ and $(IC_{50}\delta(3)/IC_{50}\delta(4) = 717/313$, are approximately 3, indicating that cyclic D-Leu^5 analogs 2,4 are more δ preference over cyclic L-Leu^5 analogs. As mentioned, this effect may be explained by the backbone flexibility. All four analogs 1-4 adopt the rigid conformation, which allow the two phenyl rings in extend away and results in the marked μ preference.[17-19] But, the cyclic D-Leu^5 backbones 2,4 are relatively somewhat flexible compared to L-Leu^5 backbone because of altered intramolecular hydrogen bond pattern, which undergo subtle variation in conferring the proper orientation to two phenyl rings suitable for the δ receptor recognition and result in the increased δ preference.[46,47]

Application of two reverse amide bond modification between Phe^4-Leu^5 and Leu^5-D-Glu^2(side chain) to cyclic enkephalin analogs 8a, 8b produce analogs 3,4, which possess the altered biological profiles. The retro-inverso analogs 3,4 display similar or 3 times higher potencies in μ receptor, whereas approximately 10 times lower potencies in δ receptor than the parent analogs 8a,8b. The results indicates that the retro-inverso modification lead to the altered intramolecular hydrogen bond pattern and ultimately affect the receptor binding mode of action.

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