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Activity Profiles of Linear, Cyclic Monomer and Cyclic Dimer of Enkephalin

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The cyclic dimers of enkephalin were isolated as minor components during the solution synthesis of the corresponding cyclic monomers. The ratio of cyclic dimer to monomer was approximately 1:4 from the percent of yields. In the receptor binding assay of two cyclic dimmers, (Tyr_2-C[D-Glu-Phe-gPhe$]_2$ **6**, Tyr_2-C[D-Asp-Phe-gPhe-rLeu$]_2$ **8**), both analogs exhibited the high preference for δ receptor compared to monocyclic counterparts. In the nociceptive activity, both showed about 5 times less potent than the cyclic monomers. The repeated synthesis of 14-membered cyclic analog, Tyr-C[D-Glu-Phe-gPhe-D-rLeu] **14**, which was known as having three distinct *cis-trans* isomers, gave rise to apparently different conformational analog arousing only *trans* isomer. In the receptor binding assay, it showed tremendously high selectivity toward μ receptor (δ/μ = 160).

Key Words : Cyclic-enkephalin, Monomer, Dimer, Bioactivity

Introduction

The discovery of enkephalin has provided the opportunities to understand the topochemical requirement of receptors for opioids.[1,2] However, the linear enkephalin analogs have hampered attempts to elucidate the spatial disposition of the critical pharmacophores due to their conformational flexibility permitting adaptation to the different conformations at different receptor sites.[3] Thus, various ways of restriction methods were designed as limiting the inherent flexibility of the opioid peptides.[4-13] The most drastic restriction was achieved by the cyclization of linear opiate peptides. The incorporation of constraints for the linear peptides through cyclization not only reduces the conformational flexibility, but also leads to more potent and/or highly μ or δ receptor selective analogs.[14-17]

In an attempt to inhibit the enzymatic cleavage and degradation of the native enkephalins, the retro-inverso modification reversing the direction of the peptide bond was developed by Goodman *et al*.[18,19] The partially retro-inverso modified enkephalin analogs are found to be 2-15 times more active than the parent enkephalins and exhibit a much longer duration of action. Recently, we reported similar results of the structure activity relationship for the 13- and 14-membered cyclic enkephalin analogs adopted the retro-inverso modification. The study showed their high *in vivo* potency and receptor selectivity toward either δ or δ receptors.[20,21]

The structure activity relationship studies and the conformational analysis of cyclic enkephalins to date revealed the correlation between structural requirement and receptor selectivity. Followings are summaries: (1) The μ receptor selective analogs require the spatial orientation of the aromatic rings of tyrosine at position 1 and phenylalanine at position 4 in an extended conformation with maximal separation between the two, whereas the δ receptor selective peptides adopt conformations in which the two aromatic residues are folded toward each other. The flexible cyclic backbone allows the side chains of phenylalanine and tyrosine to approach each other and form a folded structure. But, the rigid cyclic backbone formed by a possible intramolecular hydrogen bonds help maintain an extended away conformation.[22-24] (2) The dermorphine like cyclic enkephalin analogs in which glycine was substituted by phenylalanine at position three showed superactivities *in vitro* at one or both of the μ and δ receptors. The conformational studies of the dermorphine like analogs revealed that both the distance between the side chain of tyrosine and leucine and the proximity of the aromatic rings are important for recognition and activity at the δ receptor. A tilted stacking arrangement of the two aromatic rings in position 1 and 3 represent a structural requirement for μ receptor affinity.[19-21,30,36]

As an example, the conformational analysis of the μ selective analogs, Tyr-C[D-A_2bu-Gly-Phe-Leu], reveals that backbone ring structure is constrained and prefer the Tyr[1] and Phe[4] rings far part in an extended conformation. Reversal of the Leu[5] backbone chirality increased the flexibility, which allows Tyr[1] and Phe[4] rings close proximity in a folded structure and results in nonselective receptor binding.[5,25,26] Other conformational studies focused on 14-membered cyclic pentapeptides have also revealed that the 14-membered ring structure still retains some flexibility.[6,27]

To reduce the ring flexibility by minimization of the ring size and elucidate the minimum structure requirements for the binding of the μ and δ receptors of cyclic enkephalin analogs, several investigators[25,28] adopted the cyclic tetramer lacking in leucine residue at position 5. The biological activity of 13-membered cyclic tetrapeptide, Tyr-C[D-Lys-Phe-Phe], that incorporate two phenylalanyl residues at position three and four displays high activity at both the μ and δ opioid receptors and is non-selective. Reducing the

ring size from 13 atoms to 12 atoms as in Tyr-C[D-Orn-Phe-Phe], greatly decreased the activity of the molecule at the δ receptor and results in high μ opioid receptor selectivity.[29] We have also carried out similar study focused on the constrained nature of ring sizes with various 13- and 14-membered cyclic pentapeptides formed by side chain to backbone coupling through amide bond. The comparison study revealed that their affinity for the receptors does not depend significantly on small variation of the flexibility of the main chain ring itself.[20,21]

In this paper, in efforts aimed at elucidating the minimum structure requirements for the binding of the μ and δ receptors of cyclic enkephalin analogs and limiting the conformational flexibility, we have synthesized the 11-membered, Tyr-C[D-Glu-Phe-gPhe], and 13-membered, Tyr-C[D-Asp-Phe-gPhe-rLeu], cyclic lactam bridged peptides based on the moderately μ selective 14-membered cyclic analog, Tyr-C[D-Glu-Phe-gPhe-rLeu].[21] The retro-inverso modification is applied to the backbone between Phe^4-$rLeu^5$. This applications to backbone are expected to alter intramolecular hydrogen bond pattern and provide the useful information about the functional importance of the amide bond.[5,6] Since these analogs resemble the μ selective opioid peptide, dermorphine (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), in which phenylalanine residue place at the 3-position, they are expected to exhibit high preference for μ receptor.[30]

During the synthetic process of the cyclic monomer, the considerable amount of cyclic dimmer was isolated as a second major component. It is conceivable that the conformational restriction imposed on the cyclic dimer obviously differs from that present in the cyclic monomer. In this connection, the opioid activities of cyclic dimmers and monomers were measured and also compared with those of the corresponding linear analogs.

In addition to the study of the minimum structure requirement of cyclic enkephalin analogs, we have repeated the synthesis of Tyr-C[D-Glu-Phe-gPhe-D-rLeu] to assure the existence of three distinct *cis-trans* isomers on 14-membered cyclic analog, which was reported by Said *et al.* for the first time.[6,36]

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-L-phenylalaninamide, Z-Phe-Phe-NH_2, 1.** Phenylalanin amide, (164 mg, 1 mmol) and Z-Phe-OH (299 mmol, 1mmol) 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 422 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-gPhe, 2.** To a solution of iodobenzene bistrifluoroacetate (IBTFA, 430 mg, 1 mmol) in acetonitrile/ater (20 mL, 4/1 v/v), Z-Phe-gPhe-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 1 N 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 341 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_2O/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).

Benzyloxycarbonyl-L-phenylalanyl-*gem*-L-phenylalanyl-*t*-butoxycarbonyl-D-glutamyl-α-benzylester, Boc-D-Glu(-gPhe-Phe-Z)-OBzl, 3. The coupling reaction was carried out in the same manner described for the synthesis of **1** using Boc-D-Glu(α-OBzl)-OH (337 mg, 1 mmol), Z-Phe-gPhe (417 mg, 1 mmol), EDC (240 mg, 1.2 mmol), HOBt

(190 mg, 1.2 mmol). Flash column with elution of 2% methanol in dichloromethane afforded the product. Yield, 685 mg (93%), R_f (D) 0.65, ^{1}H-NMR (DMSO-d_6) δ 8.3 (d, 1H, NH), 7.6 (d, 1H, NH), 6.9 (d, 1H, NH), 7.0-7.3 (m, 20H, ph), 5.5 (q, 1H, CH), 5.1 (s, 2H, CH_2), 5.0 (s, 2H, CH_2), 4.9 (d, 2H, CH_2), 4.8 (d, 2H, CH_2), 4.1 (q, 1H, CH), 3.9 (q, 1H, CH), 2.9 (m, 2H, CH_2), 2.6 (m 2H, CH_2), 2.0 (m, 2H, CH_2), 1.6 (m, 2H, CH_2), 1.3 (s, 9H, Boc).

Benzyloxycarbonyl-L-phenylalanyl-*gem*-L-phenylalanyl-*t*-butoxycarbonyl-tyrosyl-D-glutamyl-α-benzylester, Boc-Tyr-D-Glu(-gPhe-Phe-Z)-OBzl, 4. A solution of fully protected tripeptide **3** (736 mg, 1 mmol) in 50% trifluoroacetic acid in dichloromethane (20 mL) was stirred at room temperature for 45 min. Ten 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 773 mg (95%) of the title compound as a white solid. Then, the coupling reaction was carried out in the same manner described for the synthesis **1** using Boc-Tyr-OH (282 mg, 1 mmol), D-Glu(-gPhe-Phe-Z)-OBzl (636 mg, 1 mmol), EDC (240 mg, 1.2 mmol), HOBt (190 mg, 1.2 mmol). Flash column with elution of 2% methanol in dichloromethane afforded the product. Yield, 838 mg (93%), R_f (D) 0.65, ^{1}H-NMR (DMSO-d_6) δ 9.2 (s, 1H, OH), 8.3 (d, 1H, NH), 8.2 (s, 1H, NH), 7.6 (d, 1H, NH), 6.9 (d, 1H, NH), 7.0-7.3 (m, 20H, ph), 6.6-7.0 (2d, 4H, ph), 5.5 (q, 1H, CH), 5.2 (s, 2H, CH_2), 5.1 (s, 2H, CH_2), 4.1 (q, 1H, CH), 3.9 (q, 1H, CH), 2.9 (m, 2H, CH_2), 2.6 (m 2H, CH_2), 2.0 (m, 2H, CH_2), 1.6 (m, 2H, CH_2), 1.3 (s, 9H, Boc).

L-Tyrosyl-cyclic-D-glutamyl-L-phenylalanyl-*gem*-L-phenylalanine Monomer, Tyr-C[D-Glu-Phe-gPhe], 5. To a solution of fully protected tetrapeptide, Boc-Tyr-D-Glu (-gPhe-Phe-Z)-OBzl, **4** (901 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 643 mg (95%) of the title compound as a white crystalline solid. To a solution of the linear pentapeptide, Boc-Tyr-D-Glu-(-gPhe-Phe)-OH (677 mg, 1 mmol, 1 equiv.) in dry degassed DMF (210 mL, 7 × 10^{-3} M) maintained at −20 °C, $NaHCO_3$ (420 mg, 5 mmol, 5 equiv.) and BOP (650 mg, 1.5 mmol, 1.5 equiv.) 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 243 mg (yield of cyclic monomer; 38%) of the monocyclic pentapeptide as an amorphous solid. The deprotection of *t*-butoxycarbonyl group of monocyclic pentapeptide (250 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 199 mg (yield; 94%) of the title compound as an amorphous solid. R_f (D) 0.50, ^{1}H-NMR (DMSO-d_6) δ 9.2 (s, 1H, OH), 8.4 (d, 1H, NH), 8.2 (d, 1H, NH), 7.6 (d, 1H, NH), 6.9 (d, 1H, NH), 7.2-7.4 (m, 10H, ph), 6.8-7.0 (2d, 4H, ph), 5.5 (q, 1H, CH), 4.9 (q, 1H, CH), 4.2 (q, 1H, CH), 3.9 (q, 1H, CH), 2.9 (m, 4H, CH_2), 2.8 (m, 2H, CH_2), 2.0 (m 1H, CH), 1.8 (m, 2H, CH_2), 1.3 (m, 2H, CH_2), FAB-MS; 557 (M^+).

L-Tyrosyl-cyclic-D-glutamyl-L-phenylalanyl-*gem*-L-phenylalanine dimer, Tyr$_2$-C[D-Glu-Phe-gPhe]$_2$, 6. Cyclic dimer was isolated simultaneously during the purification process of cyclic monomer **5** from the reaction mixture. Yield of cyclic dimer, 117 mg (9%), R_f (D) 0.52, ^{1}H-NMR (DMSO-d_6) δ 9.3 (s, 1H, OH), 9.0 (d, 1H, NH), 8.5 (d, 1H, NH), 8.2 (d, 1H, NH), 8.0 (d, 1H, NH), 7.2-7.4 (m, 10H, ph), 6.8-7.0 (2d, 4H, ph), 5.5 (q, 1H, CH), 4.9 (q, 1H, CH), 4.4 (q, 1H, CH), 3.8 (q, 1H, CH), 3.3 (m, 2H, CH_2), 3.0 (m, 2H, CH_2), 2.8 (m, 2H, CH_2), 2.2 (m 1H, CH), 1.8 (m, 2H, CH_2), 1.3 (m, 2H, CH_2), FAB-MS; 1114 (M^+).

Benzyloxycarbonyl-L-tyrosyl-cyclic-D-aspartyl-L-phenylalanyl-*gem*-L-phenylalanyl-reto-L-leucine Monomer, Tyr-C[D-Asp-Phe-gPhe-rLeu], 7.[21] The hydrogenation of the benzoxy protecting group of the fully protected linear tetrapeptide, Boc-Tyr-D-Asp(*O*-Bzl)-Phe-gPhe-rLeu-Z with H_2/10% Pd-C was carried out in the same manner described for the synthesis of **5**. Then, cyclization was followed in the same manner described for the synthesis of **5**. From the reaction mixture, the major spot on tlc was isolated by flash chromatography. The following deprotection of Boc group of monocyclic pentapeptide was carried out in the same manner described for the synthesis of **4**. Purification by flash chromatography, gradient elution (2% → 4%, methanol/chloroform) afforded 333 mg of the title compound as an amorphous solid. Yield of cyclic monomer, 36%, R_f (D) 0.52, mp 128-130 °C, ^{1}H-NMR (DMSO-d_6), 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, ph), 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; 656 (M^+).

L-Tyrosyl-cyclic-D-aspartyl-L-phenylalanyl-*gem*-L-phenylalanyl-reto-L-leucine dimer, Tyr$_2$-C[D-Asp-Phe-gPhe-rLeu]$_2$, 8. The deprotection of Boc group of cyclic dimer of pentapeptide (92 mg, 0.045 mmol), which was obtained together with monocyclic pentapeptide **7** in the process of purification (yield of cyclic dimer; 10%), was carried out in the same manner described for the synthesis of **4**. Purification by flash chromatography, gradient elution (5% →

10%, methanol/chloroform) afforded 87 mg of the title compound as an amorphous solid. Yield, 95%, R_f (D) 0.53, ^{1}H-NMR (DMSO-d_6) δ 9.3 (s, 1H, OH), 8.7 (d, 1H, NH), 8.6 (d, 1H, NH), 8.2 (d, 1H, NH), 7.6 (d, 1H, NH), 7.2 (m, 10H, ph), 6.7-7.2 (2d, 4H, ph), 5.4 (q, 1H, CH), 4.4 (m, 1H, CH), 4.2 (m, 2H, 2CH), 3.5 (m, 1H, CH), 3.1 (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; 1312 (M^+).

L-Tyrosyl-D-alanyl-L-phenylalanyl-*gem*-L-phenylalanine, Tyr-D-Ala-Phe-gPhe, 9. The linear tetrapeptide was synthesized by the similar procedure described for the synthesis of **9**. Synthesis of linear tetrapeptide involved 2 + 2 fragment coupling with a succinimide active ester. Rf (D) 0.35, ^{1}H-NMR (DMSO-d_6) δ 9.4 (s, 1H, OH), 8.9 (d, 1H, NH), 8.4 (m, 2H, 2NH), 7.3 (m, 10H, 2ph), 6.6-7.0 (d, 4H, ph), 5.0 (m, 1H, CH), 4.5 (m, 1H, CH), 4.3 (m, 1H, CH), 4.0 (m, 1H, CH), 2.6-3.0 (m, 6H, CH_2), 0.8 (m, 3H, CH_3).

L-Tyrosyl-D-alanyl-L-phenylalanyl-*gem*-L-phenylalanyl-reto-L-leucine, Tyr-D-Ala-Phe-gPhe-rLeu, 10. The overall reaction was followed the synthetic Scheme 1 reported by Hong.[20] Synthesis of linear pentapeptide involved 2 + 3 fragment coupling with a succinimide active ester. R_f (D) 0.37, ^{1}H-NMR (DMSO-d_6) δ 9.7 (s, 1H, OH), 8.9 (d, 1H, NH), 8.7 (d, 1H, NH), 8.4 (m, 2H, 2NH), 8.1 (s, 6H, $2NH_3$), 7.3 (m, 10H, 2ph), 6.6-7.0 (d, 4H, ph), 5.6 (m, 1H, CH), 4.6 (m, 1H, CH), 4.4 (m, 1H, CH), 4.0 (m, 1H, CH), 3.6 (m 1H, CH), 2.6-3.0 (m, 6H, CH_2), 1.2 (m, 1H, CH), 0.9 (m, 6H, CH_3), 0.8 (m, 3H, CH_3).

***N*-Butoxycarbonyl-L-phenylalanyl-*gem*-L-phenylalanyl-benzyloxycarbonyl-retro-D-leucine, Boc-Phe-gPhe-D-rLeu-Z, 11.** Triethylamine (0.4 mL, 3 mmol), hydroxybenzotriazole (HOBt) (200 mg, 1.3 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (250 mg, 1.3 mmol) were added to a chilled solution (–20 °C) of Z-D-Leu-OH (265 mg, 1 mmol) and Boc-Phe-gPhe·HCl (419 mg, 1 mmol) in 50 mL of DMF. The coupling reaction was carried out in the same manner described for the synthesis of **1**. Flash column with elution of 2% methanol in dichloromethane afforded the product. Yield, 554 mg (88%), R_f (D) 0.65, mp 212 °C, ^{1}H-NMR (D_2O/DMSO-d_6) δ 8.3 (m, 2H, 2NH), 7.4 (d, 1H, NH), 6.9 (d, 1H, NH), 7.2-7.4 (m, 15H, 5ph), 5.5 (q, 1H, CH), 5.0 (d, 2H, CH_2), 4.1 (q, 1H, CH), 4.0 (q, 1H, CH), 2.9 (m, 2H, CH_2), 2.6-2.8 (q, 2H, CH_2), 1.6 (m, 2H, CH_2), 1.4 (s, 9H, Boc), 0.8 (d, 4H, CH_2).

***N*-Butoxycarbonyl-*O*-benzyl-D-glutamyl-L-phenylalanyl-*gem*-L-phenylalanyl-benzyloxycarbonyl-retro-D-leucine, Boc-D-Glu(O-Bzl)-Phe-gPhe-D-rLeu-Z, 12.** After deprotection of Boc group of Boc-Phe-gPhe-r-D-Leu-Z with TFA, the coupling reaction was carried out in the same manner described for the synthesis of **1** using Boc-D-Glu(O-Bzl)-OH (337 mg, 1 mmol), Phe-gPhe-D-rLeu-Z (530 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 white solid. Yield, 772 mg (91%), R_f (D) 0.62, mp 216-218 °C, ^{1}H-NMR (DMSO-d_6) δ 8.4 (d, 1H, NH), 8.3 (d, 1H, NH), 8.1 (d, 1H, NH), 7.0 (d, 1H, NH), 6.9 (d, 1H, NH), 7.2-7.4 (m, 20H, ph), 5.5 (q, 1H, CH), 5.1 (q, 2H, CH_2), 5.0 (q, 2H, CH_2), 4.6 (m, 1H, CH), 4.0 (m, 2H, 2CH), 3.0 (d, 4H, CH_2), 2.7-3.0 (m, 6H, CH_2), 2.0 (m, 2H, CH_2), 1.7 (m, 2H, CH_2), 1.5 (m, 2H, CH), 1.4 (s, 9H, Boc), 1.2 (m, 2H, CH_2), 0.8 (m, 2H, CH_2).

***N*-Butoxycarbonyl-L-tyrosyl-*O*-benzyl-D-glutamyl-L-phenylalanyl-*gem*-L-phenyl-alanyl-benzyloxycarbonyl-retro-D-leucine, Boc-Tyr(*O-t*-Bu)-D-Glu(O-Bzl)-Phe-gPhe-D-rLeu-Z, 13.** After deprotection of Boc group of Boc-D-Glu(*O*-Bzl)-Phe-gPhe-D-rLeu-Z, the coupling reaction was carried out in the same manner described for the synthesis of **1** using Boc-Tyr(*O-t*Bu)-OH (337 mg, 1 mmol), D-Glu(*O*-Bzl)-Phe-gPhe-rLeu-Z (748 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 white solid. Yield, 970 mg (91%), R_f (D) 0.62, mp 198-201 °C, ^{1}H-NMR (DMSO-d_6) δ 8.5 (d, 1H, NH), 8.4 (m, 2H, 2NH), 8.0 (d, 1H, NH), 7.0 (m, 2H, 2NH), 7.2-7.4 (m, 20H, ph), 6.8-7.1 (m, 4H, ph), 5.5 (q, 1H, CH), 5.1 (q, 2H, CH_2), 5.0 (q, 2H, CH_2), 4.6 (m, 1H, CH), 4.4 (m, 1H, CH), 4.2 (m, 1H, CH), 4.0 (m, 1H, CH), 3.0 (d, 4H, CH_2), 2.7-3.0 (m, 6H, CH_2), 2.0 (m, 2H, CH_2), 1.7 (m, 2H, CH_2), 1.5 (m, 2H, CH), 1.4 (d, 18H, 2*t*Bu), 1.2 (m, 2H, CH_2), 0.8 (m, 2H, CH_2).

L-Tyrosyl-cyclic-D-glutamyl-L-phenylalanyl-*gem*-L-phenylalanyl-reto-D-leucine monomer, Tyr-C[D-Glu-Phe-gPhe-D-rLeu], 14. After deprotection of two benzyl groups of pentapeptide, Boc-Tyr(*O-t*Bu)-D-Glu(*O*-Bzl)-Phe-gPhe-r-D-Leu-Z, by hydrogenolysis, cyclization was carried out in the same manner described for the synthesis of **5** using Boc-Tyr(*O-t*Bu)-D-Glu-Phe-gPhe-r-D-Leu (843 mg, 1 mmol), $NaHCO_3$ (420 mg, 5 mmol, 5 equiv.) and BOP (650 mg, 1.5 mmol, 1.5 equiv.). Flash column with elution of 2% methanol in dichloromethane afforded 404 mg (49%) of the protected product, Boc-Tyr(*O-t*Bu)-C[D-Glu-Phe-gPhe-D-rLeu] as white solid. Deprotection of *t*-Butyl groups afforded the product. R_f (D) 0.36, ^{1}H-NMR (DMSO-d_6) δ 9.3(s, 1H, OH), 8.6 (d, 1H, NH), 8.4 (m, 2H, 2NH), 8.3 (d, 1H, NH), 7.9 (d, 1H, NH), 7.1-7.3 (m, 10H, 2ph), 6.6-7.0 (d, 4H, ph), 5.5 (m, 1H, CH), 4.6 (m, 1H, CH), 4.4 (m, 1H, CH), 4.3 (m, 1H, CH), 4.0 (m, 1H, CH), 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), 1.1 (m, 2H, CH_2), 0.9 (m, 6H, CH_3), FAB-MS; 670 (M^+).

Binding Assays. The GPI[11] and MVD[12] bioassays were carried out as reported by P. Shiller.[31,34] 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.[13]

Nociceptive Assays. Nociceptive response were measured using the 52.5 hot plate (HP) and/or the tail flick (TF) test.[13] 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,

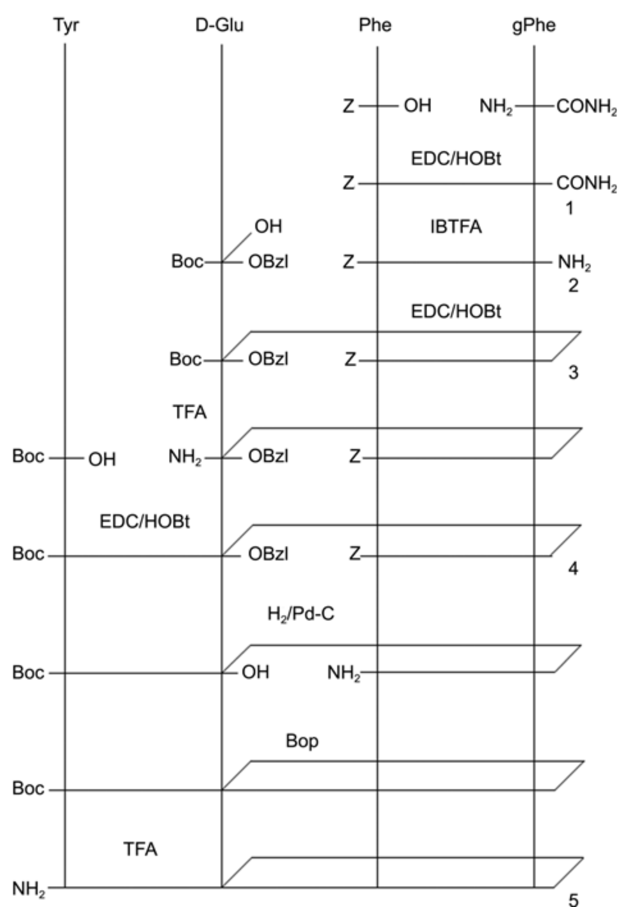
response latencies were converted to the % of the maximum possible effect (% MPE): % MPE = [(post drug response latency) − (predrug response latency)]/[(cut off time) − (predrug response latency)] × 100.

Result and Discussion

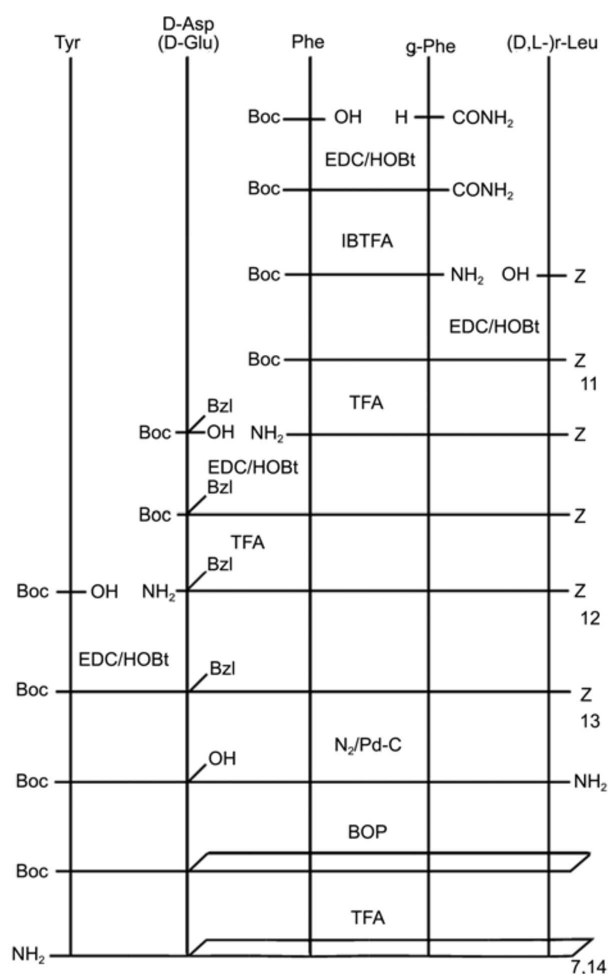
Synthesis. All of the syntheses were carried out in solution. The tert-butoxycarbonyl (*t*-Boc) group and benzyloxycarbonyl (Z) were employed to protect the amino group of each amino acid. Water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxybenzotriazole (HOBt)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

The cyclic enkephalin analog, Tyr-C[D-Glu-Phe-gPhe] **5**, was synthesized according to Scheme 1. The Z-Phe and phenylalanine amide was first coupled into dipeptide amide Z-Phe-Phe-$CONH_2$ **1** using EDC/HOBt. The amide functional group of was rearranged to amine upon treatment with IBTFA. Coupling between dipeptide, Z-Phe-gPhe-NH_2 **2** and Boc-D-Glu-OBzl using EDC/HOBt afforded the tripeptide, Boc-D-Glu(-gPhe-Phe-Z)-OBzl **3**, in which the amid linkage was connected to the β-position of D-Glu. Subsequent stepwise elongation with Boc-Tyr-OH using EDC/HOBt and mild transfer hydrogenation of two benzoxy protecting groups with H_2/10% Pd-C afforded the unprotected linear tetrapeptide, Boc-Tyr-D-Glu(-gPhe-Phe). The backbone to backbone cyclization was achieved with 5 mM of linear tetrapeptide in DMF using BOP. The two cyclic (monomer and dimer) compounds were separated by flash column chromatography in high purities despite of the very close R_f values. The analysis of mass spectrometry confirmed that the fast eluting one was cyclic dimer of tetrapeptide. The ratio of two cyclic tetrapeptides was approximately 1:4 (cyclic dimer:cyclic monomer = 9%:38%) from the percent of yields.

The cyclic enkephalin analog, Tyr-C[D-Glu-Phe-gPhe-D-rLeu] **14**, was synthesized according to Scheme 2. The amide functional group of Boc-Phe-Phe-$CONH_2$ was rearranged to amine upon treatment with IBTFA. The coupling between the dipeptide amine, Boc-Phe-gPhe-NH_2 and Z-D-Leu-OH using EDC/HOBt afforded the protected tripeptide, Boc-Phe-gPhe-D-rLeu-Z **11**. In usual manner, deprotection of Boc group and the subsequent two steps elongations with Boc-D-Glu(O-Bzl)-OH and Boc-Tyr(*O-t*-Bu)-OH using EDC/HOBt afforded the fully protected linear pentapeptide,



Scheme 1. Synthetic scheme of 11-membered cyclic tetrapeptide, Tyr-C[D-Glu-Phe-gPhe] **5** *via* stepwise elongation.



Scheme 2. Synthetic scheme of cyclic pentapeptides, Tyr-C[D-Asp-Phe-gPhe-rLeu] **7** and Tyr-C[D-Glu-Phe-ghe-D-rLue] 14 via stepwise elongation.

Boc-Tyr(*O-t*-Bu)-D-Glu(O-Bzl)-Phe-gPhe-D-rLeu-Z **13**. In this step, Boc-Tyr-(*O-t*Bu)-OH was used to prevent a formation of by-product, in which the phenolic OH of the tyrosine was missing.[21] After the deprotection of two benzoxy groups with H_2/10% Pd-C, the backbone to side chain cyclization by the procedure described for the cyclizations of tetrapeptide **5** in DMF using BOP afforded the cyclic product, Boc-Tyr(*O-t*-Bu)-C[D-Glu-Phe-gPhe-D-rLeu]. Subsequent deprotection of butoxy protecting groups was afforded the final product **14**. In case of **14**, the cyclic dimer of pentapeptide was not detected. Tyr-C[D-Asp-Phe-gPhe-rLeu] **7** was synthesized in the similar manner by stepwise elongation method (Scheme 2).[21] The ratio of two cyclic pentapeptides was approximately 1:4 from the percent of yields (cyclic dimer:cyclic monomer = 10%:36%). Two linear peptides were synthesized by the method described by Hong.[20] Synthesis of two linear compounds, Tyr-D-Ala-Phe-gPhe **9**, Tyr-D-Ala-Phe-gPhe-rLeu **10**, involved 2 + 2 or 2 + 3 fragment coupling with a succinimide active ester.

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.

Biological Activities. The in vitro biological activities of the analogs measured in guinea pig ileum (GPI) and mouse vas deferens (MVD) assays are summarized in Table 1 and 2. The GPI and MVD assays were used for determining the bioactivities at the μ and δ-opioid receptors, respectively.[31] Nociceptive responses (*in vivo* test) were assessed using the 52.5 °C hotplate and/or the tail flick test.[35] 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 pain behavior assays using rats chronically prepared with indwelling intrathecal catheters.

2D ^{1}H-NMR Spectrum of Tyr-C[D-Glu-Phe-gPhe-D-rLeu] 14. The 2D ^{1}H-NMR spectrum for Tyr-C[D-Glu-Phe-gPhe-D-rLeu] obtained in DMSO-d_6 at 30 is shown in Figure 1, which is consistent with the structure of the authentic product. The proton resonances were assigned using two dimensional ROSEY experiments. The NOEs were observed in the ROSEY spectrum measured at mixing times of 300 ms. The vicinal ^{1}H-^{1}H coupling constants for groupings H-N-C$^\alpha$-H and H-C$^\alpha$-C$^\beta$-H were elucidated by analyzing the NH, α- and β-proton signals simultaneously. To elucidate values of other coupling constants, the β-proton are coupled with the r-proton, which is in turn coupled to the δ-protons. The 2D ^{1}H-NMR spectrum allowed for unambiguous assignment of almost all signals. All proton connectivity relationships were exhibited in this spectrum.

Previous ^{1}H-NMR spectrum of Tyr-C[D-Glu-Phe-gPhe-D-

Table 1. Binding affinities and nocicepive activities of linear and cyclic enkephalin analogs

No	Compounds	IC_{50} GPI (μM)	IC_{50} MVD (μM)	MVD/GPI (δ/μ)	ED_{50} (μmol)
5	Tyr-C[D-Glu-Phe-gPhe]	18.4	78.4	42.7	0.9
6	Tyr_2-C[D-Glu-Phe-gPhe]$_2$	147	40.6	0.28	4.3
9	Tyr-D-Ala-Phe-gPhe	22.5	277	12.3	6.1
7	Tyr-C[D-Asp-Phe-gPhe-rLeu][20]	20.5	312	15.1	0.7
8	Tyr_2-C[D-Asp-Phe-gPhe-rLeu]$_2$	8.48	6.83	0.81	3.0
10	Tyr-D-Ala-Phe-gPhe-rLeu	23.4	147	6.7	5.9
	Morphine[21]	58.6	644	11.0	5.6

Table 2. Binding affinites and nociceptive activities of Tyr-C[D-Glu-Phe-gPhe-D-rLeu] **14**

Compounds	IC_{50} GPI (μM)	IC_{50} MVD (μM)	MVD/GPI (δ/μ)	ED_{50} (μmol)
Our product	8.90	1300	161	3.9
Said product[6]	2.75	49.1	17.9	8.4

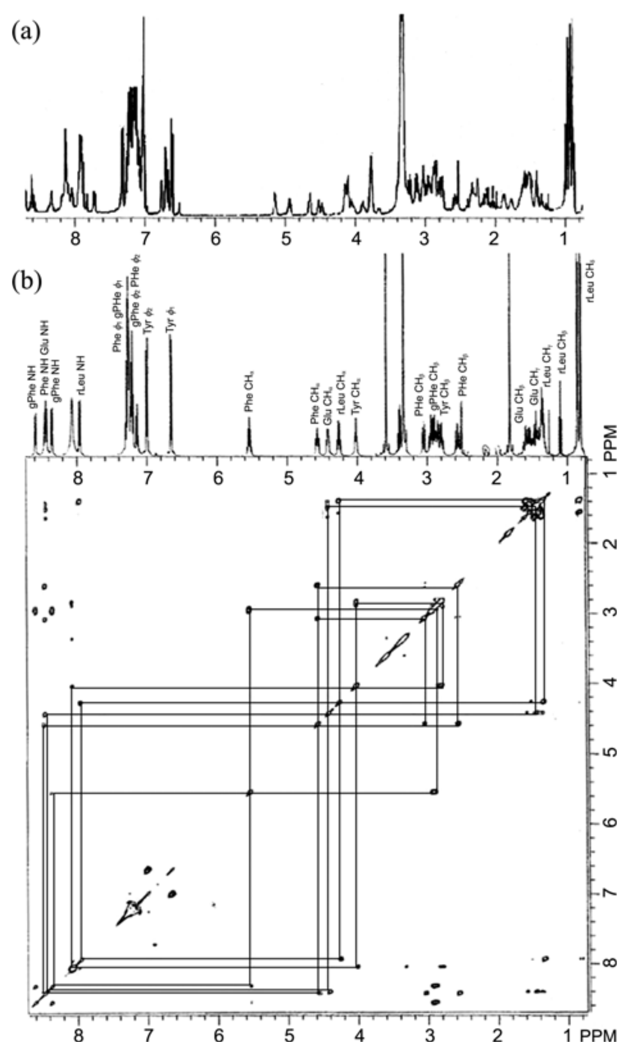


Figure 1. Proton-NMR spectrum of Tyr-C[D-Glu-Phe-gPhe-D-rLeu] **14** measured in DMSO-d_6 [General Electric 500 MHz] FT NMR spectrometer. (a) NMR spectrum reported by Said.[6] (b) Two dimensional NMR spectrum of our product.

rLeu] reported by Said *et al.*[6] indicated that three configurational isomers [28%:51%:21% = *trans:cis:cis*] in DMSO at 30 °C exist simultaneously, even though this analog does not contain proline or any other N-substituted amino acid residues. The configurational isomers are composed of only 28% of *trans* structure with two *cis* containing isomers accounting for 51% and 21%, respectively. The major isomer (51%) has a *cis* amide linkage about the D-Glu-Phe amide bond. The other *cis* isomer (21%) has a *cis* arrangement between Phe-gPhe. Contrary to this data, the result of 2D ^{1}H-NMR spectrum for our synthetic analog (Fig. 1) indicates that there is no existence of *cis* configuration isomer arousing at any amide bond, showing only *trans* structure. It is generally accepted that the peptides containing proline or *N*-substituted amino acids have the characteristic peak pattern of only two different configurations (*cis-trans*) with a different ratio. But, the spectrum of Said *et al.*[6,36] has three different configurational isomers (*cis-trans-cis*) arousing at one compound. Moreover, they have remained the signals arousing between 6.7-6.9 ppm to be ambiguous without any assignment. Considering that those peaks appear only when a certain alkylation on the tyrosine aromatic ring occur, it seems to be due to an existence of small amounts of undesirable side product occurred on tyrosine aromatic ring.

ESI-MS Mass Spectrometry of Tyr-C[D-Glu-Phe-gPhe-D-rLeu] 14. The major peaks of the ESI mass spectrum, those at *m/z* 672 $[M+2H]^+$ and 695 $[M+Na]^+$ (Fig. 2), confirmed a molecular mass of 670 Da, which agrees with the molecular formula $C_{37}H_{46}N_6O_6$. In sequencing by mass spectrometry, two types of fragment ions are produced. Type A is the series resulting from cleavage at each CO-NH amide bond. Type B is the series resulting from fragmentation between CH-CO of each amino acid. The ESI-MS spectrum of the doubly charged Tyr-C[D-Glu-Phe-gPhe-D-rLeu] exhibited additional major fragments at *m/z* 553 and 136. Mass result provided the expected fragments to confirm the sequence of the cyclic pentapeptide. The peak at *m/z* 136 is attributed to the fragmentation between CH-CO of Tyr, with the charge remaining on the CH portion. The mass difference of 119 between two major peaks at *m/z* 553 and 672 is attributed to the mass of the NH-CH-CH_2-C_6H_5 of Phe with the addition of 2H, which support the presence of two phenylalanines.

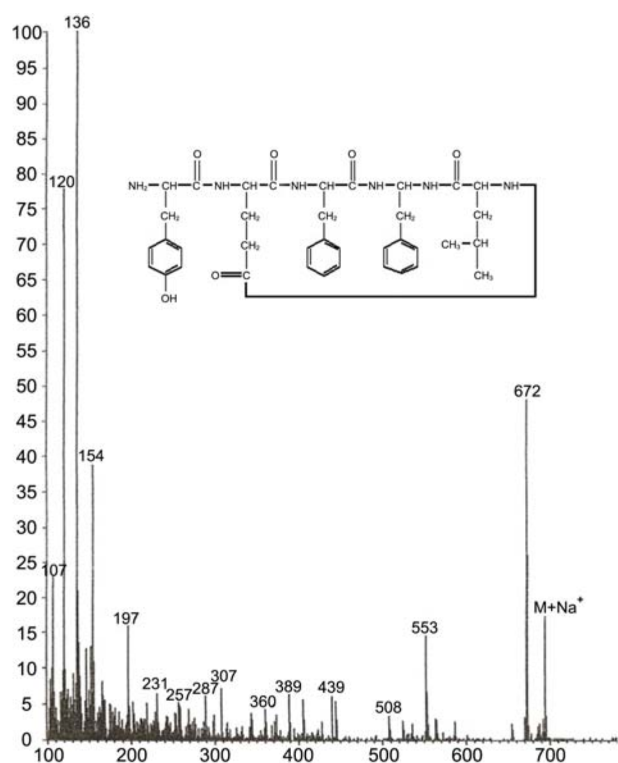


Figure 2. Electroscopy ionizaion mass spectrum of doubly charged Tyr-C[D-Glu-Phe-gPhe-D-rLeu] **14**, $M+2H^+$ ion on exact atomic masses. Peakes at *m/z* 120, 154, 287, and 387 are due to NBA matrix.

Discussion

Pharmacological data for the analogs of the linear and cyclic enkephalins under study are presented in Table 1 and 2. Both tables provide μ (GPI) and δ (MVD) opioid receptor binding potencies (IC_{50}) and nociceptive potencies (ED_{50}) of the analogs.

In the two cyclic dimers **6**, **8** (Table 1), both analogs exhibited better selectivity ratio to the δ receptor compared to cyclic monomers **5**, **7**. The 26-membered cyclic dimer **8** (δ/μ = 0.81) exhibited 19 times higher selectivity ratio than the 13-membered monocyclic counterpart **7** δ/μ = 15.1). The 22-membered cyclic dimer **6** (δ/μ = 0.28) exhibited 150 times higher selectivity ratio than the 11-membered monocyclic counterpart **5** (δ/μ = 42.7). Considering the general acceptance that all the lactam bridged cyclizations *via* backbone to side-chain of the linear enkephalin analogs increase the μ-selectivity,[6,30,32,33] these are adverse results in binding trend of the cyclic enkephalins. Such adverse changes in binding behavior of the cyclic dimers could be explained by the increased backbone flexibility, which allows the two phenyl rings of Tyr residue at the first position and Phe residue at the fourth position to adopt the folded structure in close proximity. This orientation is required for recognition and activity at the δ receptor.[30,37-39] The better selectivity ratio of cyclic dimers **6**, **8** compared to monocyclic counterparts **5**, **7** was mainly caused by increasing activity toward δ-receptors (IC_{50}δ (6)/IC_{50}δ (5) = 40.6/784, IC_{50}δ (8)/IC_{50}δ (7) = 6.83/312). However, in the GPI assays, the cyclic dimer **6** showed 8 times lower affinity at the μ receptor (IC_{50}μ(6)/ IC_{50}μ(5) = 147/18.4, but the cyclic dimer **8** showed 3 times higher affinity at the μ receptor (IC_{50}μ(8)/IC_{50}μ(7) = 8.48/ 20.5. The receptor binding result of **8** is exception to the general rule that the backbone flexibility of cyclic enkephalin decrease the μ selectivity. The inconsistency of **8** with the general rule needs to require the conformational analysis to find how it fit well in μ receptor. Since the cyclic monomers **5**, **7** are 5 times more potent than the cyclic dimmers **6**, **8** in the nociceptive assay (ED_{50}(5)/ED_{50}(6) = 0.9/4.3 and ED_{50}(7)/ED_{50}(8) = 0.7/3.0), reducing the ring size seems to

be responsible for high nociceptive potency of the cyclic monomers. The observation is consistent with the previous results.[20]

For the comparison of the cyclic monomers **5**, **7** with its linear correlates **9**, **10**, the two cyclic monomers showed IC_{50} values similar to those of the linear analogs in the GPI assays ($IC_{50}\mu(7)/IC_{50}\mu(10) = 20.5/23.4$, $IC_{50}\mu(5)/IC_{50}\mu(9) = 18.4/22.5$), while they showed two times lower IC_{50} values than linear analogs in the MVD assays ($IC_{50}\delta(7)/IC_{50}\delta(10) = 312/147$, $IC_{50}\delta(5)/IC_{50}\delta(9) = 784/277$). The fact that both of the cyclic monomers and linear counterparts show similar affinity in the binding assays ensure the general rule that cyclization increase the μ-selectivity of the enkephalin analogs relative to their linear counterparts caused by reducing activity relative to δ-receptors.[24] The cyclic monomers **5**, **7** showed 9 times higher activities than its linear counterparts **9**, **10** in the *in vivo* studies ($ED_{50}(7)/ED_{50}(10) = 0.7/5.9$, $ED_{50}(5)/ED_{50}(9) = 0.9/6.1$). It is difficult to present a definite explanation for the *in vivo* results observed with linear and monocyclic analogs, but, we can assume that much higher activity of the cyclic monomers in the nociceptive activity might be due to the enhanced efficacy of the cyclic analogs.[32] The high efficacies of cyclic analog might be brought about by formation of a more productive peptide-receptor complex through induction of a conformational change in the receptor molecule.

Two linear analogs **9**, **10** showed the same rank order of potency in both GPI binding assays ($IC_{50}\mu(9)/IC_{50}\mu(10) = 22.5/23.4$) and *in vivo* assays ($ED_{50}(9)/ED_{50}(10) = 6.1/5.9$), with the except of the slight change in MVD assays ($IC_{50}\delta(9)/IC_{50}\delta(10) = 277/147$), indicating that deletion of leucine at position 5 does not affect in orienting the three phenyl rings of linear analogs due to so flexible nature of linear structure, whereas, the spatial orientation of three phenyl rings in the rigid cyclic structure acts as the most important structural determinant for μ and δ receptor differentiation.

It is worthwhile to mention that compounds **6**, **8** (Table 1) represent another two of a few examples of biologically active cyclic enkephalin dimers obtained through lactam bridge formation and are side products obtained accidently among dozens of cyclization attempts of linear enkephalin analogs. The fact that two cyclic dimers described in this paper were found to be opioid activity pattern distinct from those of the cyclic monomers of the corresponding linear analogs suggests that cyclodimerization *via* side chain linkage can be used as valuable tool toward manipulating activity profiles of opioid peptides.

In addition to the study of cyclic dimer, the synthesis of cyclic pentapeptide, Tyr-C[D-Glu-Phe-gPhe-D-rLeu] **14** (Table 2) was repeated to assure the previous report that three different configurational isomers (28%:51%:21% = *trans:cis:cis*) arousing together on the 14-membered cyclic monomer.[6] Our synthetic product was identified as authentic by 2D nmr spectrum (Fig. 1) and mass spectrum (Fig. 2). The nmr spectrum shows only one isomer of *trans*. It seems plausible from the observation that 14-membered cyclic analogs are so fairly flexible that it is hard to allow a *cis-trans* isomerism. The binding potency observed with our product in the GPI assay and MVD assay was found to be extremely high preference for μ receptor over δ receptor ($IC_{50}\mu/IC_{50}\delta = 8.09/1300$), which is caused by complete inactivity at the δ receptor.

Conclusion

The result that the cyclic dimmers **6**, **8** show the better selectivity ratio toward δ receptor compared to the corresponding cyclic monomers **5**, **7** supports strongly the general rule that the more flexible ring structure of cyclic enkephalin allows a positive influence for determining the preferred δ receptor affinity of cyclic enkephalin. In addition, the repeated study of cyclic analog **14** leads to the different result that only *trans* isomerism arise on the 14-membered cyclic enkephalin.

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