Conjugates of Enkephalin Analogs: Synthesis and Discrimination of μ and δ Opioid Receptors Based on Membrane Compartment Concept

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Opioid Receptors Based on Membrane Compartment Concept

A series of conjugated cyclic and linear enkephalin analogs, Tyr-q[D-A2bu-Gly-Phe-Asp(NH-X)], where X = methyl, stearyl or PEG350, and Tyr-D-Ala-Gly-Phe-Cys(S-X), where X = methyl, octyl, or farnesyl, were synthesized in solution to investigate the receptor selectivity of opioids based on Schwyzer's membrane compartment concepts.2,6 Cyclizations of the target compounds were achieved in high yields (> 60%) employing BOP, NaHCO3 in DMF despite the steric hindrance of the bulky pendant groups. In the binding assay, the hydrophobic fatty acyl conjugates retained μ-receptor selectivity. The unsaturated farnesyl conjugate exhibited the increased binding affinity than the saturated stearyl conjugate for both μ- and δ-opioid receptors. The PEG conjugates displayed the δ-receptor selectivity. The low molecular weight PEG350 conjugate exhibited the increase selectivity than the high molecular weight PEG5000 conjugate to the δ-receptor. The results of this study support the membrane compartment concepts.

Key Words: Enkephalin, Conjugate, Membrane compartment, Bioactivity

Introduction

Enkephalins, Tyr-Gly-Gly-Phe-Leu(Met), are endogenous opioid peptides, which function as neurotransmitters and neuromodulators. Many analogs of the native enkephalins have been synthesized and their biological activities assayed.1 Previous efforts of comparing structure with biological activity have concluded that the amine and phenolic groups in the tyrosine at the first position, and the aromatic group in the phenylalanine at the third or fourth position are required for opiate receptor recognition.2 The incorporation of a D-residue at the second position led to an increase in activity, most likely because of the accompanying decrease in enzymatic degradation. Cyclization of linear enkephalin through backbone to side chain is another advantage to overcome the labile enzymatic cleavage of linear enkephalin analogs as well as possible explanation of opioid receptor subtype as a result of the reduced conformational flexibility of peptide bond.3,4

According to the first concept of membrane compartments, the receptor selectivity of the opioid peptide is governed by its net charge and/or amphiphilic moment in addition to its ability to fulfill the structural and conformational requirements of particular receptor types (μ, δ, γ). The opioid peptides carrying a lipophilic and negatively charged character accumulate in the vicinity of the μ receptor, and therefore, show μ preference. Conversely, hydrophilic and negatively charged peptides preferentially interact with the δ site, which is exposed to the aqueous compartment, since the μ site is situated in the anionic fixed charge surrounded by the hydrophobic compartment.5,6

A number of laboratories have reported the significance of the lipopeptide conjugate on biological activity. Hashimoto et al.7 have reported that palmitoyl insulin conjugate had a longer duration of hypoglycemic effect than parent insulin. Hruby et al.8 reported that fatty acid conjugates of cyclic lactam bridged α-melanotropin (α-MSH) fragment analog exhibited 10-100 times more potent than α-MSH in a lizard skin assay. Portoghese et al.9 also reported on the important role of the fatty acid derivatives of naltrexamine and naltrex-hydrizine in the persistent binding of ligands to their receptors. The results suggest that the actions of readily reversible agonists can be converted to highly prolonged actions using such modifications.

In a similar manner, PEG (polyethylene glycol) has also been used as a carrier polymer.10-18 A variety of proteins have been conjugated to PEG including asparaginase,10 streptokinase,15 interleukin,16 insulin,17 and RGD peptides.18 Although the resultant PEG-conjugates have been shown to have reduced levels of biological activities in vitro, they have possessed several potentially important biochemical/physical advantages such as non-toxicity, reduced antigenicity, improved solubility (both in aqueous and organic phases), increased resistance to proteolytic degradation and modification of pharmacokinetics. The facilitated penetration of the blood brain barrier may be expected to extend to PEG-conjugated opioids.

In an attempt to systematically determine the molecular requirement for receptor selectivity of opioid analogs based upon membrane compartment concepts as well as persistent binding to the membrane, we have designed two enkephalin-conjugated families of molecules. One possesses the lipophilic saturated and unsaturated hydrocarbon chains (saturated fatty acid = octyl, lauryl, and stearyl; unsaturated fatty acid = farnesyl), which are expected to show structural characters more favorable for μ receptor recognition. The other family possesses partially hydrophilic chains with different chain
lengths (X = PEG, molecular weight; 350 and 5,000), which are expected to show δ-preference. Both pending groups are attached to the side chain of C-terminal amino acid by an amide or thioether linkage. These peptides have general structures such as Tyr-c[D-A2bu-Gly-Phe-Asp(NH-X)] and Tyr-D-Ala-Gly-Phe-Cys(S-X)-NH2, where X is pendant. The existence of a D-residue at the second position is known to enhance stability against the cleavage of the enkephalin peptide bond between the tyrosine and second residue by amino peptidase. Amidation of the C-terminal carboxyl group in the model compound is also known to increase the analgesic effect compared to the parent peptide carrying the free carboxyl group. Such designs are expected to afford peptide opioids not only an increased receptor preference in binding assay, but also offer more favorable structural characteristics for delivery across the receptor membrane and blood brain barrier.

So far, many studies have focused on developing prolonged duration time and on stimulation of the agonist, little reports have noticed the important implications for the design of a conjugate for the more efficacious discrimination of opioids to their receptor sites. Herein, in this regard, we describe a series of cyclic and linear enkephalin conjugates that possess characteristic peptide pharmacophore for favorable receptor recognition as well as different natures of pendants for the receptor discrimination through the membrane compartment concept.

**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 (1H-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 the University of California, San Diego. Flash column chromatography was performed on Merk silica gel 60 (0.040-0.063 mm) using nitrogen pressure. Analytical thin-layer chromatography (TLC) was carried out on precoated (0.25 mm) Merk silica gel F-254 plates. Rf values of TLC and purity were determined in the following solvent systems: A, chloroform/methanol (9/1); B, chloroform/methanol (8/2); 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 (1/2/2/1). 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 x 0.4 cm) packed with LiChrospher 100 RP-118 (10 μm) and methanol-water/1% trifluoroacetic acid solvent system.

N-t-Butoxycarbonyl-D-diaminobutyric acid, Boc-D-A2bu-Z-OH, 1. To a solution of iodobenzene bis-trifluoroacetate (IBTFA, 5.1 g, 13 mmol) in dimethylformamide (DMF)/water (1/1), Boc-Gln-OH (2.46 g, 11 mmol) was added at room temperature. After 15 min of stirring, pyridine (1.6 mL, 20 mmol) was added to the reaction mixture, which was stirred for 3 hrs. The solvent was evaporated under vacuo and the residue dissolved in 200 mL of water. Aqueous layer washed extensively with ether (5 × 20 mL) and concentrated in vacuo to give crude product, which was subjected to reverse phase chromatography with elution of 10% methanol in water then, 20% methanol to afforded 2.2 g of pure product. Yields, 84%, m.p. 250 °C (decomposed), Rf (E) 0.67, 1H-NMR (D2O/DMSO-d6) δ 4.5 (m, 1H, αCH), 2.9 (m, 2H, γCH), 1.9-2.0 (m, 2H, βH), 1.4 (s, 9H, Boc).

N-t-Butoxycarbonyl-N-benzyloxy carbonyl-D-diaminobutyric acid, Boc-D-A2bu(Z)-OH, 2. To a solution of dipeptide 3 (423 mg, 1 mmol) in 50% trifluoroacetic acid in dichloromethane (5 mL) was stirred at room temperature for 45 min and concentrated. The amorphous solid was dissolved in methanol and concentrated several times and dried over P2O5 under vacuo for 2 hrs. The resultant deprotected dipeptide was dissolved in 10 mL of DMF and cooled to 0 °C. To this solution were added triethylamine (4 mL, 2 mmol) and Boc-Tyr-ONp (402 mg, 1 mmol). After 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 NaHCO3 (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 white crystalline solid. Yield, 88%, m.p. 121-123 °C; 1H-NMR (D2O/DMSO-d6) δ 8.2 (t, 1H, NH), 7.4 (s, 5H, ph), 7.2 (t, 1H, NH), 7.0 (d, 1H, NH), 5.0 (s, 2H, CH2), 4.0 (m, 1H, αCH), 3.9 (m, 2H, βCH), 3.6 (s, 3H, CH3), 3.1 (m, 2H, γCH), 2.6-2.8 (m, 2H, Boc).
citric acid in water (25 mL x 3), saturated aqueous NaCl (25 mL x 3), and dried over magnesium sulfate. Organic phase was concentrated to give yellow solid purification by flash chromatography with elution of 2% methanol/dichloromethane gave 544 mg of the title compound as a white solid. Yield, 93%, Rf (A) 0.42, m.p. 127-130 °C, 1H-NMR (DMSO-d6) δ 9.1 (s, 1H, OH), 8.9 (s, 1H, NH), 8.2 (m, 2H, NH), 7.4 (s, 5H, CH), 7.2 (t, 1H, NH), 7.1, 6.6 (d, 4H, ph), 7.0 (d, 2H, NH), 5.0 (s, 2H, CH2), 4.3 (m, 1H, αCH), 3.7 (m, 2H, αCH), 2.6-2.8 (m, 2H, βCH), 1.6-1.8 (m, 2H, βCH), 1.3 (s, 9H, Boc).

N-t-Butoxy carbonyl-L-tyrosyl-N-benzylxoy carbonyl-D-diaminobutyryl-glycyl hydrazine, Boc-Tyr-D3Bu(bu)-Z-Gly-NHNH2. 5. To a chilled solution (-10 °C) of tripeptide 4 (2.9 g, 5 mmol) in 10 mL of methanol was added hydrayzine (180 mg, 6 mmol). Temperature was increased gradually to room temperature. Stirring was allowed to continue for 3 hrs at room temperature. The progress of reaction was monitored on using 10% methanol in dichloromethane solvent system. After 3 hrs of reaction time, solvant was evaporated under vacuo with the aid of ethanol several times to give 2.6 g of white solid. Yield, 90%, Rf (A) 0.15, m.p. 172-174 °C, 1H-NMR (DMSO-d6) δ 9.1 (s, 1H, OH), 8.9 (s, 1H, NH), 8.2 (m, 2H, NH), 7.4 (s, 5H, ph), 7.2 (t, 1H, NH), 7.1, 6.6 (d, 4H, ph), 7.0 (d, 2H, NH), 5.0 (s, 2H, CH2), 4.3 (m, 1H, αCH), 3.7 (m, 2H, αCH), 2.6-2.8 (m, 2H, βCH), 1.6-1.8 (m, 2H, βCH), 1.3 (s, 9H, Boc).

N-t-Butoxy carbonyl-N-stearyl-L-aspartyl benzyl ester, Boc-Asp(NH-St)-OBn. 6. To a solution of Boc-Asp-OBn (3.23 g, 10 mmol) and stearylamine (2.98 g, 11 mmol) in 30 mL of DMF were added 4.2 mL of triethylamine (30 mmol), HOBt (10 mmol) and stearylamine (2.98 g, 11 mmol) in 30 mL of DMF. After 3 hrs of reaction time, solvent was evaporated under vacuo with the aid of ethanol several times to give 2.6 g of white solid. The residue was dissolved in 250 mL of DMF were preadjusted to neutral. After the pH of reaction mixture was brought to neutrality, HCl·Phe-Asp(NH-St)-OBn, 6 was added to a solution of tripeptide azide. The pH of the solution was adjusted to neutral. The mixture, after stirred at -20 °C for 12 hrs, was concentrated to give amorphous white solid. The residue was dissolved in chloroform (150 mL) and washed with saturated aqueous NaHCO3 (25 mL x 3), saturated aqueous NaCl (25 mL x 3) and dried over magnesium sulfate. The concentration afforded the white solid. Purification by flash chromatography with elution of 5% methanol in dichloromethane gave 527 mg of the title compound as a white crystalline solid. Yield, 73%, Rf (A) 0.85, m.p. 167-172 °C, 1H-NMR (DMSO-d6) δ 8.4 (d, 1H, NH), 8.0 (t, 1H, NH), 7.4 (s, 5H, ph), 7.2 (s, 5H, ph), 5.1 (s, 2H, CH2), 4.7 (m, 1H, αCH), 4.2 (m, 1H, αCH), 3.0 (m, 2H, γCH), 2.7-2.9 (m, 2H, βCH), 2.6 (m, 2H, βCH), 1.3 (s, 9H, Boc), 1.2 (s, 35H, stearyl).

L-Phenylalaninyl-N-stearyl-L-aspartyl benzyl ester hydrochloride salt, HCI·Phe-Asp(NH-St)-OBn. 9. To a solution of Boc-Tyr-D3Bu(bu)-Z-Gly-Phe-Asp(NH-St)-OBn, 9 was added triethylamine (0.6 mL, 3 mmol), Boc-Phe-OH (575 mg, 1 mmol) in 10 mL of DMF. After 3 hrs of reaction time, solvent was evaporated under vacuo with the aid of ethanol several times to give 2.6 g of white solid. Yield, 644 mg (98 %), Rf (A) 0.42, m.p. 95-108 °C, 1H-NMR (CDCl3/DMSO-d6) δ 9.1 (s, 1H, OH), 8.9 (s, 1H, NH), 8.2 (m, 2H, NH), 7.4 (s, 5H, ph), 7.2 (t, 1H, NH), 7.1, 6.6 (d, 4H, ph), 7.0 (d, 2H, NH), 5.0 (s, 2H, CH2), 4.3 (m, 1H, αCH), 3.7 (m, 2H, αCH), 2.6-2.8 (m, 2H, βCH), 1.6-1.8 (m, 2H, βCH), 1.3 (s, 9H, Boc).

N-t-Butoxy carbonyl-L-phenylalaninyl-N-stearyl-L-aspartyl benzyl ester, Boc-Tyr-D3Bu(bu)-Z-Gly-Phe-Asp(NH-St)-OBn. A solution of Boc-Tyr-D3Bu(bu)-Z-Gly-Phe-Asp(NH-St)-OBn, 9, t-Butyl nitrite was added to a stirred solution cooled to -20 °C of Boc-Tyr-D3Bu(bu)-Z-Gly-NHNH2, 5 (586 mg, 1 mmol) in DMF (30 mL) containing 4 N HCl in dioxane (3 mL). The mixture was stirred at -20 °C for 15 min, was cooled at -60 °C and triethylamine was added to adjust pH neutral. HCI·Phe-Asp(NH-St)-OBn, 10 (544 mg, 1 mmol) was added with tripeptide azide. The pH of the solution was preadjusted to neutral. The mixture was stirred at -20 °C for 45 min. 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 NaHCO3 (25 mL x 3), saturated aqueous NaCl (25 mL x 3) and dried over magnesium sulfate. The concentration afforded the white solid. Purification by flash chromatography with elution of 2% methanol/dichloromethane gave 544 mg of the title compound as a white solid. Yield, 93%, Rf (A) 0.42, m.p. 127-130 °C, 1H-NMR (DMSO-d6) δ 9.1 (s, 1H, OH), 8.4 (t, 1H, NH), 8.2 (d, 1H, NH), 7.4 (s, 5H, ph), 7.2 (d, 1H, NH), 6.9 (d, 1H, NH), 6.6-7.0 (d, 4H, ph), 5.9 (s, 2H, -CH2-), 4.4 (m, 1H, αCH), 4.1 (m, 1H, αCH), 3.9 (d, 2H, αCH), 3.6 (s, 3H, CH3), 3.0 (m, 2H, γCH), 2.8-2.6 (m, 2H, βCH), 1.6-1.8 (m, 2H, βCH), 1.3 (s, 9H, Boc), 1.2 (s, 35H, stearyl).
through cellite and washed with methanol several times (10 mL x 10) and concentrated. The residue dried over P2O5 in vacuo to give desired product. Yield, 451 mg (95%), Rf (B) 0.18, Rf(D) 0.43, m.p. 171-175 °C, 1H-NMR (DMSO-d6) δ 9.2(b, 1H, OH), 8.5(d 1H, NH), 8.4(t, 1H, NH), 8.3(t, 1H, NH), 8.1(d, 1H, NH), 7.7(m, 1H, NH), 7.2(s, 5H, ph), 6.6-7.0(d, 4H, ph), 4.3(m, 1H, aCH), 4.3(m, 1H, aCH), 4.1(d, 2H, aCH), 3.9(m, 1H, aCH), 3.5(m, 2H, γCH), 2.4-3.1(m, 6H, 3βCH), 1.7-2.0(m, 2H, βCH), 1.3(s, 9H, Boc), 1.2(s, 35H, stearyl).

N-t-Butoxy carbonyl-L-tyrosyl-cyclo-D-diaminobutyryl-glycyl-L-phenylalanyl-N-stearyl-L-aspartate, Boc-Tyr[d-Asp(NH2)-Bu-Gly-Phe-Asp(NH-St)], 11. To a solution of the deprotected pentapeptide (317 mg, 0.33 mmol, 1 eq.) in dry degassed DMF (70 mL, 7 x 10-3 M) maintained at -20 °C, NaHCO3 (140 mg, 1.65 mmol, 5 eq.) and BOP (175 mg, 0.4 mmol, 1.2 eq.) were added. The reaction mixture was stirred for 3 hrs at 0 °C. The reaction mixture was then concentrated. The residue was diluted with 100 mL of chloroform, washed with saturated NaHCO3 (20 mL x 3) and then dried over magnesium sulfate. Flash column on silica gel with elution of 3% methanol in chloroform afforded the product as oil. Yield, 4.4 g (92%), Rf (A) 0.61, 1H-NMR (DMSO-d6) δ 7.9(m, 4H, ph), 3.8(t, 2H, CH2), 3.7(t, 2H, CH2), 3.5(m, 4H, PEG-CH2), 1.8(m, 2H, βCH), 1.3(s, 9H, Boc), 1.2(s, 35H, stearyl).

L-Tyrosyl-cyclo-D-diaminobutyryl-glycyl-L-phenylalanyl-N-stearyl-L-aspartate trifluoroacetic acid salt, TFA-Tyrc-[D-Asp(NH2)-Gly-Phe-Asp(NH-St)]. 12. To a solution of the protected cyclic pentapeptide (150 mg, 0.16 mmol) in 5 mL of trifluoroacetic acid and thioanisole in dichloromethane was stirred at room temperature for 45 min and concentrated. The amorphous solid was dissolved DMF (20 mL) and concentrated several times. Purification by reverse phase column with elution of 30% methanol in water afforded the white solid. It was lyophilized with 10% acetic acid in water. Yield, 0.21 mg (94%), Rf(D) 0.47, m.p. 202-206 °C, 1H-NMR (DMSO-d6) δ 9.1(s, 1H, -OH), 9.0(t, 1H, NH), 7.8(t, 1H, NH), 7.6(d, 1H, NH), 7.3(s, 5H, ph), 7.2(m, 1H, NH), 6.5-7.0(d, 4H, ph), 6.8(d, 1H, NH), 6.7(t, 1H, NH), 4.3(m, 1H, aCH), 4.4(m, 1H, aCH), 4.0(m, 3H, 2aCH), 3.4(m, 1H, aCH), 3.0(m, 2H, γCH2), 2.3-2.9(m, 6H, 3βCH), 1.8(m, 2H, βCH), 1.3(s, 9H, Boc), 1.2(s, 35H, stearyl).

1-Bromoethylen glycol, PEG-Br, 13. Polyethylene glycol (PEG550) (MW 350, 3.5 g, 10 mmol) was dissolved in dichloromethane (20 mL) and cooled to -30 °C under nitrogen. To this stirred solution was added triphenylphosphine (2.62 g, 10 mmol). Bromine (0.6 mL, 11 mmol) was added dropwise to the reaction mixture at room temperature until 2 drops persisted in giving the solution an orange tint. Stirring was continued for 2 hrs at room temperature and then concentrated in vacuo to give oily material. Ph3PO was removed by treating petroleum ether (100 mL) and filtration. Mother liquor was concentrated to give yellowish oil. Purification by flash chromatography with elution of 2% methanol in dichloromethane afforded the product as white oil. Yield, 1.85 g (45%), Rf (A) 0.72, 1H-NMR (DMSO-d6) δ 3.4-3.7(m 47H, PEG-CH2).

Polyethylene glycol phthalimide, PEG-PPI, 14. Potassium-phthalimide (1.9 g, 10 mmol) was added to a solution of PEG-Br (4.13 g, 10 mmol) in DMF (30 mL). Reaction mixture was stirred for 3 hrs in oil bath (90 °C). The precipitated KBr was filtered and washed the precipitate with chloroform (20 mL). Mother liquor was evaporated under vacuo to give yellowish oil, which was dissolved in chloroform (250 mL). Work-up was done with saturated aqueous NaHCO3 (25 mL x 3), saturated aqueous NaCl (25 mL x 3) and dried over magnesium sulfate. Flash column on silica gel with elution of 3% methanol in dichloromethane afforded the product as oil. Yield, 4.4 g (92%), Rf (A) 0.61, 1H-NMR (DMSO-d6) δ 7.9(m, 4H, ph), 3.8(t, 2H, CH2), 3.7(t, 2H, CH2), 3.5(m, 4H, PEG-CH2), 1.8(m, 2H, βCH), 1.3(m, 9H, Boc), 1.2(m, 35H, stearyl).

1-Aminopolyethylene glycol hydrogen chloride, PEG-NH2-HCl, 15. To a boiling solution of PEG-PPI (14 g, 10 mmol) in 95% ethanol (30 mL) was added hydrazine hydrate (99%, 20 mmol) under stirring. The reaction mixture was refluxed for 12 hrs, then cooled to 0 °C. Water (20 mL) was added to the suspension and ethanol was removed by concentration under reduced pressure. Concentrated HCl (5 mL) was added to adjust pH to 1 and refluxed again for 1 hr. The reaction mixture was cooled to 0 °C. The resulted precipitation of N,N-phthaloyl hydrazine was discarded by filtration. The combined solution was evaporated to dryness under vacuo. Purification by flash column with elution of 10% methanol in dichloromethane afforded the oil. Yield, 3.8 g (99%), Rf (B) 0.21, 1H-NMR (DMSO-d6) δ 7.8(b, 3H, NH2-HCl), 3.4-3.6(m, 4H, PEG-CH2), 2.9(t, 2H, CH2).

N-t-Butoxy carbonyl-N-polyethylene glycol-L-aspartyl benzyl ester, Boc-Asp(NH-PEG)-OBn, 16. EDC (250 mg, 1.3 mmol) was added to a solution of Boc-Asp-OBn (323 mg, 1 mmol), PEG-NH2-HCl 15 (3.85 mg, 1 mmol) and HOBt (200 mg, 1.3 mmol) in DMF (15 mL). Then, pH was adjusted to 6-7 with triethylamine at -20 °C. Reaction was allowed to stir for 6 hrs at 0 °C. In the course of reaction pH was kept neutral by adding triethylamine. DMF was evaporated under vacuo to give yellowish oil, which was dissolved in chloroform (100 mL). Organic phase was washed with saturated aqueous NaHCO3 (20 mL x 3), 5% in water (20 mL x 3), saturated aqueous NaCl (20 mL x 3) and then dried over magnesium sulfate. Purification by flash column with elution of 3% methanol in chloroform afforded product as an oil. Yield, 464 mg (71%), Rf (A), 1H-NMR (DMSO-d6) δ 8.0(t, 1H, NH), 7.5(s, 5H, ph), 7.1(d, 1H, NH), 5.1(s, 2H, CH2), 4.5(q, 1H, α CH), 3.5(s, 42H, PEG-CH2), 3.3(s, 3H, CH3), 2.5(m, 2H, β CH), 1.4(s, 9H, Boc).

N-t-Butoxy carbonyl-L-phenylalanyl-N-polyethylene glycol-L-aspartyl benzyl ester, Boc-Phe-Asp(NH-PEG)-Obn,
1. Phenylalanyl-N-polyethylene glycol-L-aspartyl benzyloxycarbonyl phenylalanyl-N-polyethylene glycol-L-aspartyl benzyl ester hydrochloride salt, HCl-Phe-Asp(NH-PEG)-OBn, 18. Compound was prepared from 17 (792 mg, 1 mmol) using the standard deprotection procedure with 50% trifluoroacetic acid in dichloromethane followed by the treatment of 4 N HCl in dioxane. Yield, 713 mg (98%), Rf (A) 0.41, 1H-NMR (DMSO-d6) δ 9.0(d, 1H, NH), 8.4(d, 1H, NH), 7.8(d, 1H, NH), 7.4(s, 5H, ph), 7.3(s, 5H, ph), 7.2(d, 1H, NH), 5.1(s, 2H, CH2), 4.7(q, 1H, CH), 4.5(m, J = 4.6, 1H, CH), 4.4(q, 1H, CH), 2.5(d, 3H, CH 3), 2.4(m, 2H, CH 2), 1.6-1.8(2m, 2H, CH2), 1.3(s, 9H, Boc).

2. N-Benzoyloxy carbonyl-L-tyrosyl-N-benzoyloxy carbonyl-D-amino butyryl-glycyl-L-phenylalanyl-N-polyethylene glycol-L-aspartyl benzyl ester, Boc-Tyr-D-A2bu(Z)-Gly-Phc-Asp(NH-PEG)-OBn 19. The compound was prepared in the same manner described for the synthesis of Boc-Tyr-D-A2bu(Z)-Gly-Phc-Asp(NH-St)-OBn. Yield, 2.7 g of white solid. Rf (A), 0.28, 1H-NMR (DMSO-d6) δ 8.4(d, 1H, NH), 8.2(d, 1H, NH), 7.8(d, 1H, NH), 7.4(s, 5H, ph), 7.2(d, 1H, NH), 5.1(s, 2H, CH2), 4.4(q, 1H, CH), 2.5(d, 3H, CH3), 2.4(m, 2H, CH2), 1.3(s, 9H, Boc).

3. N-Benzoyloxy carbonyl-L-tyrosyl-N-benzoyloxy carbonyl glycy l-L-phenylalanyl-N-polyethylene glycol-L-aspartic acid, Boc-Tyr-D-A2bu(Z)-Gly-Phc-Asp(NH-PEG)-OH, 20. Reaction was followed the synthetic method, 10 using compound 9 (885 mg, 0.7 mmol). Yield, 785 mg (89%), Rf (B) 0.052, Rf (D) 0.25, m.p. oily product, 1H-NMR (DMSO-d6) δ 10.1(s, 1H, COOH), 8.6(m, 3H, NH2), 8.4(d, 1H, NH), 8.2(d, 1H, NH), 7.6(d, 1H, NH), 7.2(s, 5H, ph), 7.0-6.8(2d, 4H, ph), 4.4(m, 1H, CH), 4.3(m, 1H, CH), 4.1(m, 1H, CH), 3.9(m, 2H, CH2), 3.5(d, 42H, PEG-CH2), 3.2(d, 3H, CH3), 2.6-3.0(m, 8H, CH), 1.6-1.8(2m, 2H, CH2), 1.3(s, 9H, Boc).

4. N-Benzoyloxy carbonyl-L-tyrosyl-D-amino butyryl-glycyl-L-phenylalanyl-N-polyethylene glycol-L-aspartate trfluoroacetic salt, TF-A Tyr-c[D-A2bu-Gly-Phc-Asp(NH-PEG)]. 21. Cyclization of compound 20 (420 mg, 0.4 mmol) was carried out in the same manner as describe for the synthesis of analog 11. Purification by flash chromatograph gave 210 mg of white solid (61%). Rf (B) 0.43. Deprotection of Boc-protecting group using TFA and thioanisole provided the desired product. Purification procedure was carried out in the similar manner of 9 and 12. Yield, 169 mg (86%), Rf (B) 0.28, 1H-NMR (DMSO-d6) δ 9.1(t, J = 4.2, 1H, NH), 8.9(d, J = 8.0, 1H, NH), 8.1(d, J = 5.3, 1H, NH), 7.9(t, J = 3.4, 1H, NH), 7.2(m, J = 12.1, 5H, ph), 7.1(d, J = 7.1, 1H, NH), 6.6-6.9(2d, J = 12.0, 4H, ph), 6.6(d, J = 8.0, 1H, NH), 4.6(m, J = 4.6, 1H, CH), 4.4(m, J = 3.2, 1H, CH), 4.2(m, J = 3.1, 2H, CH2), 4.1(m, J = 4.9, 1H, CH), 3.9(m, J = 7.6, 1H, CH), 3.5(m, 42H, PEG-CH2), 2.4-3.0(m, J = 7.7, 8H, CH), 1.8-1.9(2m, J = 4.7, 2H, CH), FAB-MS: 9219 (M+), amino acid analysis; Asp 1.05(1), Gly 1(1), Tyr 1.02(1), Phe 1.07(1), Aib 0.98(1).

N-T-Butyloxycarbonyl-N-methyl-L-aspartyl benzylester, Boc-Asp(NH-Me)-OBn, 22. To a chilled solution (-20 °C) of Boc-Asp-OH (3.23 g, 10 mmol), methylamine (4.3 mL, 11 mmol) in DMF (20 mL) was added DCC (2.0 g, 11 mmol), then pH was adjusted to neutral with triethylamine. Temperature was increased gradually to room temperature. Stirring was allowed to continue for 18 hrs. Solvent was removed under reduced pressure to give crude yellowish oil. The residue was dissolved in 250 mL of chloroform, the organic phase were washed with saturated aqueous NaHCO3 (20 mL x 3), 5% in water (25 mL x 3), dried over magnesium sulfate. Organic phase was concentrated to give crude product of yellowish oil. Purification by flash chromatograph gave 2.7 g of white solid. Yield, 81%, Rf (A) 0.67, m.p. 119-120 °C, 1H-NMR (DMSO-d6) δ 7.8(1d, 1H, NH), 7.4(s, 5H, ph), 7.2(d, 1H, NH), 5.4(s, 2H, CH2), 4.4(q, 1H, CH), 2.5(d, 3H, CH3), 2.4(m, 2H, CH2), 1.4(s, 9H, Boc).

N-T-Butyloxycarbonyl-N-phenylalanyl-N-methyl-L-aspartyl benzylester, Boc-Asp(NH-Me)-OBn, 23. The reaction was carried out in the similar manner as described for the synthesis of analog 9 using Boc-Asp-OH (2.65 g, 10 mmol), Boc-Asp(NH-Me)-OBn (2.35 g, 11 mmol), 25% TFA in dichloromethane (10 mL), EDC (2.0 g, 11 mmol), HOBt (1.6 g, 11 mmol), and triethylamine (pH = 7). Purification by flash column with elution of 2% methanol in dichloromethane afforded the product. Yield, 4.2 g (86%), Rf (A) 0.58, m.p. 117-119 °C, 1H-NMR (DMSO-d6) δ 8.4(d, 1H, NH), 8.0(m, 1H, NH), 7.4(s, 5H, ph), 7.3(s, 5H, ph), 7.0(1d, 1H, NH), 5.1(s, 2H, CH2), 4.7(q, 1H, CH), 2.6-3.0(m, 4H, CH), 2.6(d, 3H, CH3), 1.3(s, 9H, Boc).

N-T-Butyloxycarbonyl-N-phenylalanyl-N-methyl-L-aspartyl benzylester, Boc-Asp(NH-Me)-OBn, 24. The reaction was carried out in the similar manner as described for the synthesis of analog 9 using Boc-Asp(NH-Me)-OBn (2.4 g, 5 mmol), 25% TFA in dichloromethane (10 mL), Boc-Gly-OH (880 mg, 5 mmol), EDC (1.2 g, 6 mmol), HOBt (1.0 g, 6 mmol), and triethylamine (pH = 7). Purification by flash column chromatography with elution of 3% methanol in dichloromethane afforded the product. Yield, 2.4 g (88%), m.p. 116-120 °C. Rf (A) 0.40, 1H-NMR (DMSO-d6) δ 8.4(d, 1H, NH), 7.8(2d, 1H, NH), 7.4(d, 1H, NH), 8.0(m, 1H, NH), 7.4(3H, 5ph), 7.3(3H, 5ph), 7.0(d, 1H, NH), 5.1(s, 2H, CH2), 4.1(6q, 1H, CH), 2.6-3.0(m, 4H, CH), 2.6(d, 3H, CH3), 1.3(s, 9H, Boc).

N-T-Butyloxycarbonyl-N-benzoyloxy carbonyl-D-diaminobut yryl-glycyl-L-phenylalanyl-N-methyl-L-aspartyl benzylester, Boc-D-A2bu(Z)-Gly-Phc-Asp(NH-Me)-OBn, 25. The reaction was carried out in the similar manner described for the synthesis of analog 9 using Boc-Gly-Phc-Asp(NH-Me)-OBn (2.54 g, 5 mmol), 25% TFA in dichloromethane (10 mL), Boc-D-A2bu(Z)-OH (1.6 g, 5 mmol), EDC (1.2 g, 6 mmol), HOBt (1.0 g, 6 mmol), and triethylamine (pH = 7). Purification
by flash column with elution of 3% methanol in dichloromethane afforded the product. Yield, 2.4 g (67%), m.p. 112-118 °C, Rf (A) 0.40, 1H-NMR (DMSO-d6) δ 8.4(d, 1H, NH), 8.1(d, 1H, NH), 8.0(m, 2H, 2NH), 7.4(s, 5H, ph), 7.0(d, 1H, NH), 5.1(s, 2H, CH2), 5.0(s, 2H, CH2), 4.7(q, 1H, CH), 4.5(m, 1H, NH), 4.3-3.8(m, 2H, CH2), 3.9(s, 3H, CH3), 2.6-3.0(m, 8H, CH2), 1.6-1.8(2m, J = 7.4, 4H, CH2), 1.2(s, 9H, Boc).

N-t-Butoxycarbonyl-L-tyrosyl-N-benzylloxycarbonyl-D-diaminobutyrl-L-phenylalanyl-N-methyl-L-aspartyl benzyl ester, Boc-Tyr-D-A2bu-Gly-Phe-Asp(NH-Me)-OBn, 26. The reaction was carried out in the similar manner described for the synthesis of analog 9 using Boc-D-A2bu (Z)-Gly-Phe-Asp(NH-Me)-OBn 25 (1.6 g, 6 mmol), 25% TFA in dichloromethane (10 mL), Boc-Tyr-OH (560 mg, 5 mmol), EDC (1.2 g, 6 mmol), HOBt (1.0 g, 6 mmol) and triethylamine (pH = 7). Purification by flash column with elution of 4% methanol in dichloromethane afforded the product. Yield, 1.6 g (86%), m.p. 159-163 °C, 1H-NMR (DMSO-d6) δ 9.2(s, 1H, OH), 8.7(d, 1H, NH), 8.6(d, 1H, NH), 8.2(m, 2H, 2NH), 7.9(d, 1H, NH), 7.4(s, 5H, ph), 7.2(m, 5H, ph), 6.8(d, 1H, NH), 6.6-7.0(2d, 4H, ph), 5.1(s, 2H, CH2), 5.0(s, 2H, CH2), 4.7(q, 1H, CH), 4.5(m, 1H, CH), 4.3(q, 1H, CH), 4.1(q, 1H, CH), 3.7(m, 2H, CH2), 2.5-3.0(m, 8H, CH3), 2.8(s, 3H, CH3), 1.6-1.8(2m, 2H, CH2), 1.3(s, 9H, Boc).

N-t-Butoxycarbonyl-L-tyrosyl-cyclo-D-diaminobutyrl-glycyl-L-phenylalanyl-N-methyl-L-aspartyl, Boc-Tyr-c[D-A2bu-Gly-Phe-Asp(NH-Me)-OH, 27. After the hydrolysis of analog 26 by the same method of analog 10, cyclization was done by the same method described for the synthesis of analog 11 using Boc-Tyr-D-A2bu-Gly-PheAsp(NH-Me)-OH (330 mg, 0.46 mmol), BOP (220 mg, 0.50 mmol), NaHCO3 (200 mg, 2.3 mmol) in DMF (70 mL). Purification by flash column with elution of 8% methanol in chloroform afforded the product. Yield, 2.4 g (67%), m.p. 159-163 °C, Rf (A) 0.40, 1H-NMR (DMSO-d6) δ 9.1(s, 1H, OH), 8.6(d, 1H, NH), 8.3(m, 3H, 3NH), 7.2(m, 5H, ph), 6.6-6.7(2d, 4H, ph), 6.8(d, 2H, NH), 5.9(m, 1H, NH), 4.4(m, 1H, CH), 4.3(q, 1H, CH), 4.1(q, 1H, CH), 3.6-3.8(m, 2H, CH2), 2.5-3.0(m, 8H, CH3), 2.8(s, 3H, CH3), 1.6-1.8(2m, 2H, CH2), 1.3(s, 9H, Boc).

L-Tyrosyl-cyclo-diaminobutyrl-glycyl-L-phenylalanyl-N-methyl-L-aspartyl trifluoroacetic acid, TFA-Tyr-c[D-A2bu-Gly-Phe-Asp(NH-Me)], 28. Deprotection of Boc protecting group was carried out in the similar manner described for the synthesis of analog 12 using analog 27 (200 mg, 0.29 mmol). Yield, 180 mg (94%), Rf (E) 0.46, m.p. 134-137 °C, 1H-NMR (DMSO-d6) δ 9.1(s, 1H, OH), 8.6(d, 1H, NH), 8.3(m, 3H, 3NH), 7.2(m, 5H, ph), 6.6-6.7(2d, 4H, ph), 6.0(d, 2H, NH), 5.9(m, 1H, NH), 4.9(m, 1H, CH), 4.3(q, 1H, CH), 4.1(q, 1H, CH), 3.6-3.8(m, 2H, CH2), 2.5-3.0(m, 8H, CH3), 2.8(s, 3H, CH3), 1.6-1.8(2m, 2H, CH2), 1.3(s, 9H, Boc).

Synthesis. All of the syntheses were carried out in solution. The tert-butoxycarbonyl (Boc) group was employed to protect the amino group of each amino acid. Water soluble N-ethyl-L-3-(dimethylaminopropl) carbodiimide hydrochloride (EDC) and N-hydroxybenzotriazole (HOBt) as racemization suppressor were used as coupling reagents in all coupling reactions, apart from steps involving azide coupling for the fragment condensation.

Monomethoxy poly(ethylene glycol), MW 350 (PEG350)
was converted to Asp-conjugates which were attached to the Asp side chain by amide linkage. Bromination of the hydroxyl function of PEG was achieved by triphenylphosphin and Br2. Treatment of potassium phthalimide followed by hydrazinolysis gave PEG-NH2. The corresponding PEG 350 amid-linked Asp-conjugate was prepared by the reaction of PEG-NH2 with Boc-Asp-OBn using DCC/HOBt. 1-Amino stearyl was purchased from Aldrich, which was coupled to the Boc-Asp-OBn by the same procedure to give Boc-Asp(NH-stearyl)-OBn. Boc-Asp(NH-CH3)-OBn was synthesized in the same manner as Boc-Asp(NH-stearyl)-OBn with CH3-NH2 using DCC/HOBt as coupling agents. Tyr-c[D-Asp-Gly-Phe-Asp(NH-CH3)] where X = PEG 350 and stearyl, were synthesized by the synthetic scheme 1. The synthesis of fully protected pentapeptide involved 3 + 2 fragment coupling with an azide active ester. Tyr-c[D-Asp-Gly-Phe-Asp(NH-X)], where X = CH3, was synthesized by the synthetic scheme 2.

The synthesis of fully protected pentapeptide involved a stepwise elongation from C-terminal aspartic acid residue. The cyclizations were achieved with 5 mM of linear pentapeptide in DMF using benzotriazole-N-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) to give 60% Yield despite the steric hindrance of the pending groups.

For the synthesis of Cys(S-X)-OCH3, where X = methyl, octyl, and farnesyl, we followed the procedure employed by Hong et al.38 Tyr-D-Ala-Gly-Phe-Cys(S-X)-NH2, where X = methyl, octyl and farnesyl, were obtained from coupling between Boc-Tyr-D-Ala-Gly-Phe-OH and Cys(S-X)-OCH3 using EDC/HOBt followed by amidation and deprotection of the Boc-Protecting group using trifluoroacetic acid (Scheme 3).

Tyr-D-Ala-Phe-Gly-NH-Stearyl and Tyr-D-Ala-Gly-Phe-

**Table 1.** Binding affinities and nociceptive activities of conjugates of linear and cyclic enkephalin analogs.

<table>
<thead>
<tr>
<th>No</th>
<th>compounds</th>
<th>IC50 (GPI(µ))</th>
<th>IC50/IC50 (δ/µ)</th>
<th>ED50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Try-c[D-Asp-Gly-Phe-Asp(NH-CH3)]</td>
<td>120</td>
<td>1020</td>
<td>8.5</td>
</tr>
<tr>
<td>2</td>
<td>Try-c[D-Asp-Gly-Phe-Asp(NH-stearyl)]</td>
<td>2410</td>
<td>4670</td>
<td>1.9</td>
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<tr>
<td>3</td>
<td>Try-c[D-Asp-Gly-Phe-Asp(NH-PEG350)]</td>
<td>1090</td>
<td>300</td>
<td>0.27</td>
</tr>
<tr>
<td>4</td>
<td>Try-D-Ala-Gly-Phe-Cys(S-CH3)-NH2</td>
<td>17.3</td>
<td>33.7</td>
<td>1.95</td>
</tr>
<tr>
<td>5</td>
<td>Try-D-Ala-Gly-Phe-Cys(S-octyl)-NH2</td>
<td>41.9</td>
<td>42.5</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>Try-D-Ala-Gly-Phe-Cys(S-farnesyl)-NH2</td>
<td>63.1</td>
<td>41.5</td>
<td>0.66</td>
</tr>
<tr>
<td>7</td>
<td>Try-D-Ala-Gly-Phe-NH2</td>
<td>45.2</td>
<td>510</td>
<td>11.3</td>
</tr>
<tr>
<td>8</td>
<td>Try-D-Ala-Gly-Phe-NH-stearyl</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>Try-D-Ala-Gly-Phe-NH2</td>
<td>89</td>
<td>126</td>
<td>1.4</td>
</tr>
<tr>
<td>10</td>
<td>Try-D-Ala-Gly-Phe-NH-lauryl</td>
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<td>2158</td>
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<td>11</td>
<td>Try-D-Ala-Gly-Phe-Leu-NH2</td>
<td>7.63</td>
<td>8.26</td>
<td>1.1</td>
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<tr>
<td>12</td>
<td>Try-D-Ala-Gly-Phe-Leu-NH-PEG500</td>
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<tr>
<td>13</td>
<td>Morphine</td>
<td>58.6</td>
<td>644</td>
<td>11.0</td>
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</table>

*Insoluble even in 20% cyclodextrin media. Data were not shown in references. PEG: polyethylene glycol.
Leu-NH-PEG5000 were synthesized by a stepwise elongation from the carboxy terminus to the amin terminus as a usual manner using EDC/HOBt.

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

**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 1. The GPI and MVD assays were used for determining the bioactivities at the µ and δ-opioid receptors, respectively. 34 Nociceptive responses (*in vitro* 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 spatially administered agents on a selected battery of pain behavior assays using rats chronically prepared with indwelling intrathecal catheters.

**Discussion**

In view of evidence regarding the involvement of lipids in the operation of opioid receptors, 39-41 it is conceivable that the lipids is closely related to the opioid receptor system. It is also proposed that the membrane may act as a reservoir for the long-chain fatty acid moiety of the fatty acyl opioid product. In this regard, the trapped opioid may have access to the receptor as a result of lateral diffusion in the membrane bilayer.

Pharmacological data for the conjugate analogs of the linear and cyclic enkephalin under study are presented in Table 1. Evaluation of the data reveals several interesting contrasts between the hydrophobic fatty acyl pendants and PEG series. Table 1 provides µ and δ opioid receptor binding affinities (IC$_{50}$) and nociceptive potencies (ED$_{50}$) of both conjugates series. Also presented in Table 1 are previously reported binding and *in vivo* results for some analogs 7, 9, 10, 11.

The saturated fatty acyl conjugates series 2, 5, 8, 10, all analogs are weakly active in both the GPI and MVD tests compared to the unconjugated reference analogs 1, 4, 7, 9, but, displayed greater preference for µ-receptor based on the comparison to the unconjugated reference analogs 1, 4, 7, 9, but, weakened analogs are weakly active in both the GPI and MVD tests 10, 11.

In this regard, the trapped opioid may have access to the receptor as a result of lateral diffusion in the membrane bilayer.

In the PEG conjugates series 3, 12, both analogs exhibited better selectivity at the δ-receptor. The cyclic PEG$_{5300}$ conjugate 3 exhibited 3.5 times as much as selective at the δ-receptor (IC$_{50}$(δ)(3)/IC$_{50}$(µ)(3) = 300/1090). The longer PEG$_{5000}$ conjugate 12 exhibited 10 times as much as selective at the δ-receptor (IC$_{50}$(δ)(12)/IC$_{50}$(µ)(12) = 121/1220). The PEG$_{5300}$ pendant attachment to the µ receptor selective peptide moiety 1 (IC$_{50}$(δ)(1)/IC$_{50}$(µ)(1) = 8.5) led to a dramatic change in receptor selectivity (IC$_{50}$(δ)(3)/IC$_{50}$(µ)(3) = 0.27). Such an adverse change in binding behavior can be explained by a major role of PEG pendant, which is believed to confer δ receptor propensity on peptide moiety.

For the comparison of the δ-receptor affinity ratio with the unconjugated analogs between two PEG conjugates 3, 12, the high molecular weight PEG$_{5000}$ 12 exhibited the decreased δ-receptor affinity compared to low molecular PEG$_{30}$ 3. The PEG$_{5000}$ conjugate 3 exhibited 3.5 times increased δ-receptor affinity than the unconjugated reference analog 1 (IC$_{50}$(δ)(1)/IC$_{50}$(δ)(3) = 1020/300). Whereas, the PEG$_{5000}$ conjugate 12 exhibited approximately 15 times decreased δ-receptor affinity than the unconjugated reference analog 11 (IC$_{50}$(δ)(11)/IC$_{50}$(δ)(12) = 8.26/121). The binding studies proposed that the presence of high molecular weight PEG pendant negatively contribute to binding of opioid moiety for the δ-receptor, as the similar manner observed with fatty acyl conjugates. In summary, the conjugation of opioid with partially hydrophilic PEG enhance δ-receptor selectivity whether it is short or long, but, the long chain of PEG pendant (PEG$_{5000}$) decrease the δ-receptor selectivity.

With regard to the *in vivo* data (ED$_{50}$), the saturated fatty acyl conjugates was found to have so poor solubility even in 20% cyclodextrin media that nociceptive activity test was not possible. Whereas, the PEG conjugates 3, 12 showed the strong *in vivo* activity as well as the improved solubility in aqueous media.
Consequently, although the conjugation of fatty acyl or PEG pendants to the enkephalin moieties led to the decrease in the binding affinities to both $\mu$ and $\delta$ receptors, the study support the Schwyzer’s hypothesis of the preferred receptor binding affinity of opioid ligand assisted by receptor membrane compartment.5,6

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References