Synthesis and Biological Evaluation of Naloxone and Naltrexone-Derived Hybrid Opioids

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Abstract: The synthesis and biological evaluation of hybrid opioids consisting of Naloxone or Naltrexone and a partial opioid peptide are described. These compounds were synthesized in a homogeneous solution as well as in solid phase. A hydrazone linkage was employed to connect the alkaloids to the tetrapeptides. In synthesizing the peptides some non-traditional methods, which provided excellent results, were explored. The solid phase synthesis was achieved by anchoring the Fmoc-Phe to the 2-chlorotrityl resin, followed by stepwise addition of two Fmoc-Gly units. Each addition step preceded by standard piperidine removal of the Fmoc from the prior amino acid (AA) residue. The final AA, Tyr, was added as its Boc derivative. The Boc-tetrapeptide was then separated from the resin with a TFE/AcOH/CH_2Cl_2 mixture. In the solution synthesis, each peptide elongation step was accomplished by one-pot removal of the Fmoc from the prior AA residue and addition of the next Fmoc-AA. TBAF-thiol was used to cleanly remove the Fmoc, before adding the next Fmoc-AA in the presence of DIPEA and TBTU. All prepared hybrid ligands exhibited high affinities toward all three opioid receptors; moderate preferences for κ and μ receptors over δ receptor were observed. [^{35}S]GTPγS binding assays indicated that these hybrid opioids are δ and μ antagonists but partial κ agonist.

Keywords: Hybrid opiates, Hybrid opioids, kappa agonist, naloxone derivative, naltrexone derivative, opiate agonist/antagonist, peptide synthesis.

1. INTRODUCTION

Opioid receptors and their mediation in the effect of a variety of ligands on the central and peripheral nervous systems have been studied for over a half a century. Availability of at least three different opioid receptors (μ, κ, and δ) was established about thirty five years ago [1]. Since then many medicinal scientists have sought, and examined the therapeutic usefulness of, agonist and antagonist ligands for these receptors [2-4].

In 1975 the discovery and the structures of enkephalins, as endogenous opioid peptides, were reported [5]. Following that, a number of other endogenous opioid peptides, with differing preferences for the three opioid receptors, were identified and characterized [6]. Most of these endogenous opioids contain the same tetrapeptide fragment (Tyr-Gly-Gly-Phe) at their N-terminal but have different C-terminals with varying lengths. Biological studies indicate that the N-terminal tetrapeptide fragment carries the message, which imparts the opiate properties, and the variable C-terminals confer selectivity for the receptors [7]. Thus the N-terminal "message" segment is thought to be involved in signal transduction at the receptor while the C-terminal "address" provides for recognition of the receptor and binding but is not considered essential to the transduction process. This message-address concept has also been shown to apply to some biologically active synthetic non-peptide opioids [8-10]. Although opioid peptides are considered to be potential candidates for therapeutic purposes, their tendency to undergo enzymatic cleavage and their general inability to cross blood-brain barrier (BBB) limit their use as pharmacological agents. However, shorter peptides present smaller targets for the peptidase and their attachment to a non-peptide molecule is expected to give them added protection. Naloxone and Naltrexone are non-peptide opiates that are recognized for their strong affinity for all three receptors and have no known significant undesirable properties. Thus, in order to produce high affinity ligands, bearing the opioid tetrapeptide "message" with anticipated protection from the peptidases and increased potential for penetration to the central nervous system, we have constructed hybrid opioids consisting of either Naloxone or Naltrexone linked to the N-terminal tetrapeptide (Tyr-Gly-Gly-Phe). These compounds show excellent promise and provide a mechanism for employing short therapeutic peptides to be carried and docked at the receptors by non-peptide ligands. As indicated above, however, Naloxone and Naltrexone are not purely "carriers" and participate in transduction as non-selective antagonists. But their well known properties provide a safe base for examining the effects of the added peptide and their potential synergistic benefits. It should be noted that similar hybrid structures, constituting partial "address" peptides as receptor selective modulators, have been studied [11]. But their rates of metabolism were found to be an obstacle in their utilization [12]. In the present case the "message" peptide is generally less functionalize and is anticipated to have improved *in vivo* stability.

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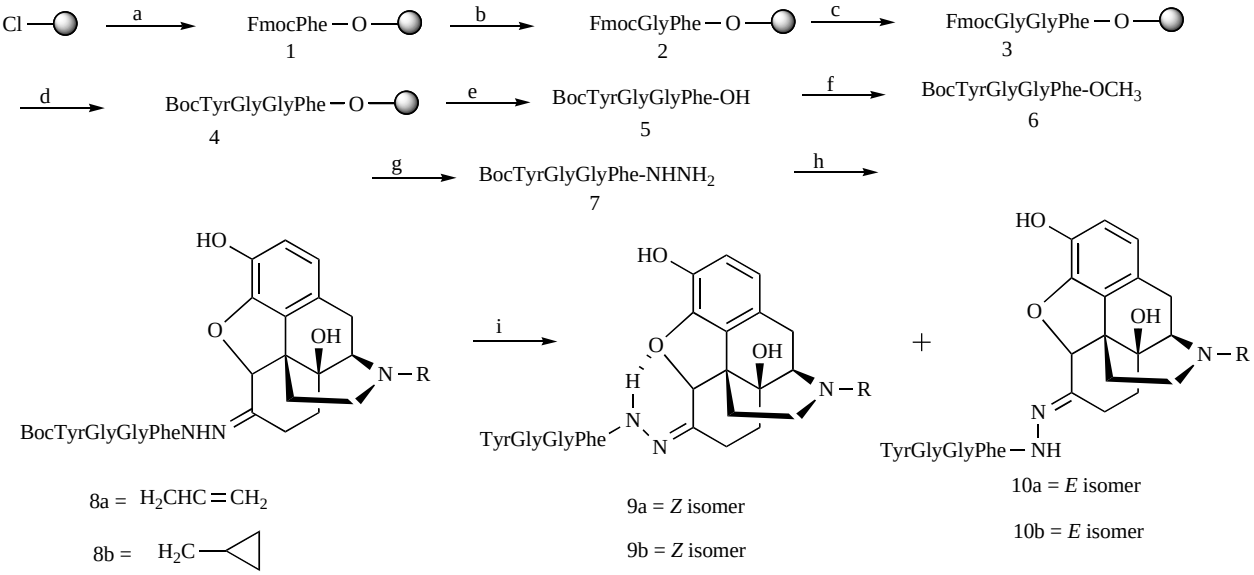


Scheme 1. Reagents and conditions: (a) Fmoc-Phe/DIPEA/CH_2Cl_2/DMF; (b) 20% piperidine/DMF, Fmoc-Gly/DIC/HOBt/DIPEA/CH_2Cl_2; (c) 20% piperidine/DMF, Fmoc-Gly/DIC/HOBt/DIPEA/DMF; (d) 20% piperidine/DMF, Boc-Tyr/DIC/HOBt/DIPEA/DMF; (e) TFE/AcOH/CH_2Cl_2 (1/1/8); (f) Trimethylsilyl diazomethane; (g) H_2NNH_2/EtOH; (h) Naloxone or Naltrexone/AcOH/MeOH; (i) 15% TFA/CH_2Cl_2, Prepararive TLC, CH_2Cl_2/MeOH (10:1).

The total solid phase syntheses of our ligands have already been reported [13]. Here we present their receptor binding and functional evaluation along with alternative syntheses methods, employing partial and total solution syntheses.

2. RESULTS AND DISCUSSION

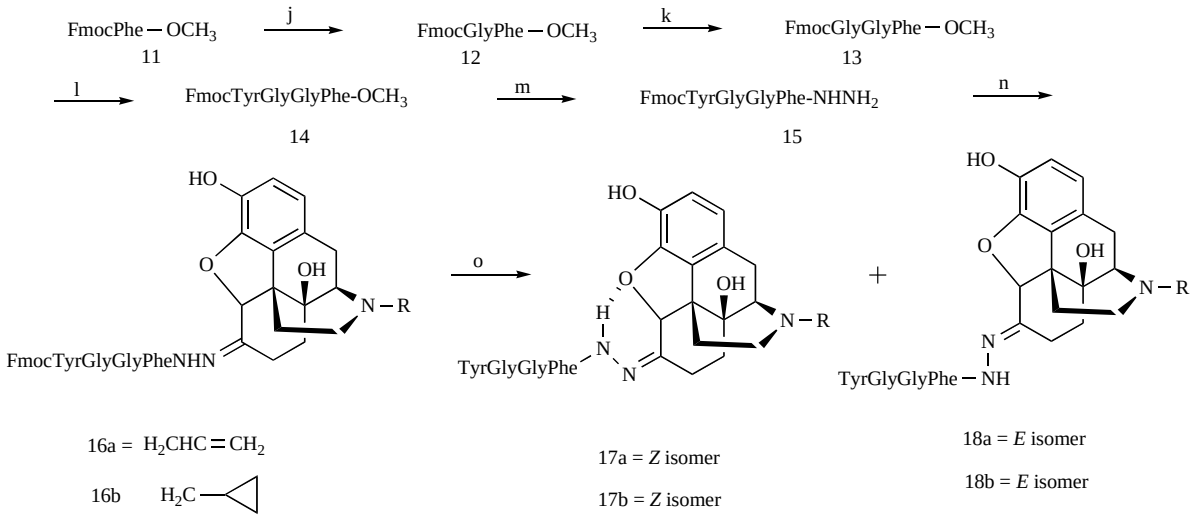
2.1. Chemistry

In a previous publication on this work, we reported the solid phase synthesis of our hybrid opioids, which was accomplished by employing Fmoc strategy, using Wang resin as the polymeric support [13]. This was done by loading the alkaloids onto Wang resin, and the Fmoc-protected amino acids were coupled to the resin-bound moiety via a hydrazone functional group at the alkaloids' 6-position.

Alternatively, here we discuss approaches which began with preparation of the peptide segment followed by its attachment to the alkaloids to create the hybrid structures. The attachment was made by converting the C-terminus of a peptide to a hydrazide, which was then reacted with the 6-ketone functional group of the respective alkaloids, in a homogeneous solution.

The syntheses of the peptide was carried out in two different ways. As shown in Scheme **1**, the first method started with anchoring of the Fmoc-protected C-terminal amino acid onto the 2-chlorotrityl resin with excess DIPEA [14]. The Fmoc-protecting group was removed by the standard piperidine procedure. Subsequent coupling of various Fmoc and Boc-protected amino acids onto the solid support proceeded in the presence of DIC, HOBt, and DIPEA in DMF. Kaiser ninhydrin test was used on the beads of the resin to determine the progress of the coupling. The N-Boc-protected tetrapeptide was cleaved from the resin smoothly by using the mixture of TFE/AcOH/CH_2Cl_2 [15,16]. The C-terminal end of the N-Boc-protected tetrapeptide was converted to the corresponding hydrazide derivative by treatment with Trimethylsilyldiazomethane ($TMSCHN_2$) [15] and anhydrous hydrazine [17,18]. Condensation between the hydrazide and the ketone functional group provided the hybrid structures **8a** and **8b** [19,20] Deprotection of the Boc-protecting group by 15% TFA in CH_2Cl_2 followed by preparative TLC led to the separation of the corresponding *Z* and *E* isomers of the hybrid opiates **9a**, **10a**, and **9b**, **10b**. However, NMR studies showed that the *E* isomers spontaneously change to their corresponding *Z* isomers, on standing in solution.

The Fmoc strategy, which does not use strong acids for deprotection, was the preferred method in solid phase peptide synthesis. However, the use of excess secondary amine for deprotection was not amenable to the solution phase condition. The amine in solution interferes with the next coupling step, and its removal is difficult [21]. Possible subsequent reactions of liberated amino components with dibenzofulvene, the cleavage product of the Fmoc group, is another problem [22]. To overcome these problems in our solution synthesis, we applied the TBAF-thiol system to the cleavage of the Fmoc group [23]. As shown in Scheme **2**, we employed a general one-pot procedure in all the elongations steps, to remove the Fmoc from the preceding amino acid residue and to add the next Fmoc-amino acid. Thus, the synthesis began with addition of excess 1-octanethiol and TBAF to a solution of Fmoc-Phe-OCH3 ester in DMF. Then, bis(1-methyl-1H-tetrazol-5-yl)disulfide was added to oxidize the remaining thiol, in a thiol-disulfide exchange reaction. In addition, the resulting 1-methyl-1H-tetrazol-5-thiol (MTZ-SH) deactivated the TBAF. Addition of Fmoc-Gly with DIPES and TBTU to the mixture resulted in the Fmoc-dipeptide, which was purified by chromatography. Each additional Fmoc-protected amino acid was coupled in a similar way to produce the Fmoc protected tetrapeptide fragment **14**. The C-terminal of the N-Fmoc-protected tetrapeptide was converted to the hydrazide derivative **(15)** and condensed



Scheme 2. Reagents and conditions: (j) TBAF/1-octanethiol/DMF, bis(1-methyl-1H-tetrazol-5-yl)disulfide, Fmoc-Gly/DIPEA/TBTU/ DMF; (k) TBAF/1-octanethiol/DMF, bis(1-methyl-1H-tetrazol-5-yl)disulfide, Fmoc-Gly/DIPEA/TBTU/DMF; (l) TBAF/1-octanethiol /DMF, bis(1-methyl-1H-tetrazol-5-yl)disulfide, Fmoc-Tyr/DIPEA/TBTU/DMF; (m) H_2NNH_2/EtOH; (n) Naloxone or Naltrexone /AcOH/MeOH; (o) TBAF/1-octanethiol/DMF, bis(1-methyl-1H-tetrazol-5-yl)disulfide, Prepararive TLC, CH_2Cl_2/MeOH (10:1).

with the 6-ketone functional group of the respective alkaloids by the same method described for Scheme **1**. Deprotection of Fmoc group followed by preparative TLC gave the same *Z* and *E* isomers of the hybrid opioids. The methods used to synthesize our peptides present a variation to the traditional methods. These reactions proceeded smoothly and could provide an alternative to the established standard methods.

2.2. Biology

The new ligands were evaluated in receptor binding assays on human opioid receptors transfected into Chinese hamster ovary (CHO) cells by displacement of [^{3}H]Cl-DPDPE (δ), [^{3}H]U69593 (κ), and [^{3}H]DAMGO (μ) [24]. As expected, all ligands have high affinities for all three opioid receptors (Table **1**). K_i values of the ligands indicated moderate preference for κ and μ over δ opioid receptors. However, there does not seem to be a significant difference between the binding of *Z* and *E* (**9a-10a / 17a-18a** and **9b-10b / 17b-18b)** stereoisomer pairs. This is likely due to the spontaneous conversion of *E* to the *Z* isomers, as observed in the NMR studies.

[^{35}S]GTPγS binding assays were applied for functional characterization of the analogues at δ, κ, and μ receptors [25]. All compounds tested in these assays are δ and μ antagonists but partial κ agonists (Tables **2** and **3**). Once again, differences between the stereo isomers (**9a-10a / 17a-18a** and **9b-10b / 17b-18b**) seem to be negligible. The nonspecific binding of these ligands are in line with our expectation, based on the lack of specificity of naloxone and naltrexon. The fact that their functional examination show agonistic properties towards the κ receptor is interesting and may indicate possession of properties similar to that of dynorphine [26]. Furthermore, the antagonist and agonist properties of these ligands may present a useful combination. This is because during the past twenty years or so, κ-agonists have generated some interest for their use in treatment of some drug abuse patients[26-29]; yet, traditional treatments for drug abuse have included the use of Naloxone and Naltrexone as nonspecific antagonists. In addition, some pain studies indicate a potential superiority in analgesic agents with κ-agonist and μ-antagonist properties [30-32]. Therefore, it is possible that our products, which display both properties, could potentially be useful for treatment of drug abuse victims as well as in treatment of pain.

3. EXPERIMENTAL SECTION

3.1. Materials and Abbreviations

The reagents and supplies used in hybrid opioids synthesis were as follows: 2-chlorotrityl resin (1%DVB, 100-200 mesh, 1.1-1.5 mmol/g, AlbatrossChem.Inc. Montreal, Canada); Wang resin (1%DVB, 100-200 mesh, 1.0 mmol/g, Sigma); Fmoc-protected amino acids (SynPep, Dublin, CA); Boc-protected amino acid (Sigma); diisopropylcarbodiimide (DIC); 1-hydroxybenzotriazole (HOBt); N,N-diisopropylethylamine (DIPEA); trifluoroethanol (TFE); trifluoroacetic acid (TFA); acetic acid (AcOH); anhydrous hydrazine (H_2N NH_2); piperidine; tetrabutylammonium fluoride (TBAF); O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluro-nium tetrafluoroborate (TBTU); trimethylsilyldiazomethane ($TMSCHN_2$); dimethyl formamide (DMF); dichloromethane (CH_2Cl_2); methanol (MeOH); ethanol (EtOH), Aldrich. ^{1}H NMR spectra were obtained on a Varian Gemini 2000 300 MHz NMR spectrometer.

3.2. Procedures for Route 1

The tetrapeptide fragment was synthesized on a 2-chlorotrityl resin using Fmoc and Boc protected amino acids. Fmoc-L-phenylalanine (1.92g, 5.0 mmol) and DIPEA (1.74 mL, 10.0 mmol) were dissolved in dry CH_2Cl_2 (20 mL). A small amount of DMF was added if the amino acid was not completely dissolved in CH_2Cl_2 (just enough to facilitate dissolution of the amino acid). The resulting solution was

Table 1. Opioid Receptor Binding Affinities of Target Ligands

Compd.	IC50 (nM)			K_i (nM ± SEM)			Hill Slope ± SEM		
	δ	κ	μ	δ	κ	μ	δ	κ	μ
9a	11.88	1.86	5.28	8.18 ± 2.49	1.36 ± 0.48	3.22 ± 0.30	1.15 ± 0.08	1.36 ± 0.35	1.11 ± 0.07
10a	12.26	2.37	4.99	8.44 ± 2.69	1.76 ± 0.34	3.05 ± 0.14	1.09 ± 0.06	1.40 ± 0.15	1.07 ± 0.04
9b	5.14	1.22	1.49	3.54 ± 1.15	0.90 ± 0.24	0.91 ± 0.09	1.16 ± 0.06	1.67 ± 0.05	1.09 ± 0.09
10b	7.15	1.18	1.74	4.93 ± 0.80	0.87 ± 0.16	1.06 ± 0.02	1.42 ± 0.11	1.38 ± 0.20	1.06 ± 0.00

Table 2. Opioid Agonist Properties of Target Ligands in the [35S]GTPγS Assay

Compd.	Agonist					
	EC_{50} (nM) ± SEM			%Stim ± SEM		
	δ	κ	μ	δ	κ	μ
9a	> 10,000	10.34 ± 1.39	> 10,000		52.07 ± 1.42	
10a	> 10,000	7.96 ± 2.70	> 10,000		55.84 ± 2.70	
9b	> 10,000	1.54 ± 0.32	> 10,000		68.65 ± 11.36	
10b	> 10,000	3.32 ± 0.77	> 10,000		50.26 ± 13.36	

Table 3. Opioid Antagonist Properties of Target Ligands in the [^{35}S]GTPγS Assay

Compd.	Antagonist											
	K_e (nM) ± SEM			pA2 ± SEM			Slope ± SEM			n		
	δ	κ	μ	δ	κ	μ	δ	κ	μ	δ	κ	μ
9a	11.33 ± 1.30		2.76 ± 0.05	7.99 ± 0.05		8.59 ± 0.04	-0.92 ± 0.03		-0.95 ± 0.04	5		5
10a	6.23 ± 0.87		2.73 ± 0.24	8.19 ± 0.06		8.65 ± 0.04	-1.07 ± 0.03		-0.87 ± 0.03	5		5
9b	3.56 ± 0.31		0.40 ± 0.03	8.44 ± 0.04		9.43 ± 0.03	-1.02 ± 0.03		-0.97 ± 0.03	5		4
10b	4.49 ± 0.36		0.61 ± 0.08	8.44 ± 0.03		9.23 ± 0.05	-0.87 ± 0.03		-1.00 ± 0.02	6		5

added to 2-chlorotrityl resin (1g, 1.0-1.5 mmol/g). The suspension was shaken for 1h at rt. Subsequently MeOH (10 mL) was added, and shaking was continued for another 20 min. The resin was filtered, washed with CH_2Cl_2 (15 mL), DMF (15 mL), and MeOH (15 mL). The first Fmoc deprotection could be carried out using 5% piperidine in CH_2Cl_2/DMF (1/1) for 10 min. (this gives better resin swelling) followed by 20% piperidine in DMF for 15 min. All subsequent Fmoc deprotections could be done in the usual way with 20% piperidine. Elongation of the peptide chain could be achieved by using Fmoc-Glycine (890 mg, 3.0 mmol) or Boc-L-Tyrosine (843 mg, 3.0 mmol) with DIC (0.47 mL, 3.0 mmol), HOBt (405 mg, 3.0 mmol) and DIPEA (0.87 mL, 5.0 mmol) in DMF. Boc-protected tetrapeptide fragment **5** (355 mg) could be obtained by treating the resin with TFE/AcOH/DCM (1/1/8) at rt for 30 min. ^{1}H NMR (300 MHz, DMSO) δ 8.18-7.95 (4H, m, 4 NH of Tyr, Gly and Phe), 7.21 (5H, m, ArH of Phe), 7.00 (2H, d, J = 8.3 Hz, ArH of Tyr), 6.61 (2H, d, J = 8.3 Hz, ArH of Tyr), 1.25 (9H, s, 3 CH_3 of BOC).

N-(t-Butyloxycarbonyl)tyrosylglycylglycylphenylalanine Methyl Ester (6) To a solution of **5** (355 mg, 0.65 mmol) in MeOH (5 mL) was added an excess of trimethylsilyldiazomethane ($TMSCHN_2$). After stirring for 1 h, 1 drop of acetic acid was added and the solvent was evaporated under reduce pressure to yield **6** (353 mg, 97%). ^{1}H NMR (300 MHz, DMSO) δ 8.25-8.15 (4H, m, 4 NH of Tyr, Gly and Phe), 7.33 (5H, m, ArH of Phe), 7.14 (2H, d, J = 8.1 Hz, ArH of Tyr), 6.85 (2H, d, J = 8.1 Hz, ArH of Tyr), 3.41 (3H, s, OCH_3), 1.40 (9H, s, 3 CH_3 of BOC).

N-(t-Butyloxycarbonyl)tyrosylglycylglycylphenylalanine Hydrazide (7) A mixture of **6** (353 mg, 0.635 mmol) and hydrazine hydrate 99% (200 μL, 4 mmol)) in absolute EtOH (5 mL) was stirred at rt overnight. A white precipitate was filtered off, washed with cold EtOH and dried to give the

corresponding hydrazide **7** (293 mg, 83%). ^{1}H NMR (300 MHz, DMSO) δ 9.18 (1H, s, CONHNH$_2$), 8.18-8.01 (4H, m, 4 NH of Tyr, Gly and Phe), 7.21 (5H, m, ArH of Phe), 7.01 (2H, d, *J* = 7.9 Hz, ArH of Tyr), 6.61 (2H, d, *J* = 7.9 Hz, ArH of Tyr), 4.20 (2H, m, CONHNH$_2$), 1.30 (9H, s, 3 CH$_3$ of BOC).

Tyrosylglycylglycylphenylalanine(hydrazino) Naloxone Derivative (9a) and (10a) 145 mg of hydrazide **7** (0.27 mmol) was added to Naloxne (132 mg, 0.4 mmol) in absolute EtOH (5 mL) containing 2 drops of glacial acetic acid. The mixture was refluxed for 2 h and then stirred at rt overnight. A solid product was separated by filtration, washed with EtOH and dried by vacuum to yield 197 mg **8a** (85%). This intermediate was added to 15% TFA solution in CH_2Cl_2 (4 mL) and stirred for 10 min. Aqueous workup followed by preparative TLC purification with CH_2Cl_2/MeOH (10/1) of the crude product led to the separation of the corresponding *Z* and *E* hybrid opiates isomers. **9a** (87 mg, 57%) ^{1}H NMR (300 MHz, DMSO) δ 10.61 (1H, s, hydrazone hydrogen), 8.30-8.00 (4H, m, 4 NH of Tyr, Gly and Phe), 5.90 (1H, m, H-18), 5.59 (2H, m, H-19), 4.98 (1H, s, H-5). **10a** (58 mg, 28%) ^{1}H NMR (300 MHz, DMSO) δ 10.39 (1H, s, hydrazone hydrogen), 8.35-8.01 (4H, m, 4 NH of Tyr, Gly and Phe), 5.82 (1H, m, H-18), 5.20 (2H, m, H-19), 4.79 (1H, s, H-5).

Tyrosylglycylglycylphenylalanine(hydrazino) Naltrexone Derivativ (9b) and (10b) Naltrexone derived intermediate **8b** (186 mg, 79%) was prepared from the condensation of 145 mg hydrazide **7** (0.27 mmol) and Naltrexone (136 mg, 0.4 mmol). *Z* and *E* isomers of **9b and 10b** were prepared by the same procedure as describe above. **9b** (83 mg, 56%) ^{1}H NMR (300 MHz, DMSO) δ 10.87 (1H, s, hydrazone hydrogen), 8.14-7.95 (4H, m, 4 NH of Tyr, Gly and Phe), 5.21 (1H, s, H-5). **10b** (55 mg, 28%) ^{1}H NMR (300 MHz, DMSO) δ 10.28 (1H, s, hydrazone hydrogen), 8.22-7.90 (4H, m, 4 NH of Tyr, Gly and Phe), 4.69 (1H, s, H-5).

3.3. Procedures for Route 2: N-(9-Fluorenylmethoxycarbonyl)Glycylphenylalanine Methyl Ester (12)

1-octanethiol (3.8 mL, 22 mmol) followed by TBAF (1.38 g, 4.4 mmol) was added to a solution of **11** (900 mg, 2.2 mmol) in DMF (10 mL) under the nitrogen atmosphere. The mixture was stirred for 10 min. To this was added bis(1-methyl-1H-tetrazol-5-yl) disulfide (3.0 g, 13.2 mmol) and stirring was continued for 3 min. Then, DIPEA (1.4 mL, 8.0 mmol), Fmoc-Gly-OH (980 mg, 3.3 mmol), and TBTU (1.06 g, 3.3 mmol) were added in this order. The mixture was stirred for 10 min. To this 5% $NaHCO_3$ was added and the mixture was extracted with EtOAc and isolated by column chromatography on silica gel using gradient elution with $CHCl_3$ to $CHCl_3$-CH_3OH (20:1) to afford **12** (836 mg, 83%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.39 (1H, m, NH of Gly), 5.40 (1H, m, NH of Phe), 3.76 (3H, s, OCH_3).

N-(9-Fluorenylmethoxycarbonyl)glycylglycylphenylalanine Methyl Ester (13) 711 mg of **13** (79%) was prepared by the same procedure with **12** (800 mg, 1.75 mmol) as the starting material. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.90 (1H, m, NH of Gly), 6.75 (1H, m, NH of Gly), 5.64 (1H, m, NH of Phe), 3.68 (3H, s, OCH_3).

N-(9-Fluorenylmethoxycarbonyl)tyrosylglycylglycylphenylalanine Methyl Ester (14) 640 mg of **14** (75%) was prepared by the same procedure with **13** (650 mg, 1.26 mmol) as the starting material. ^{1}H NMR (300 MHz, DMSO) δ 8.39 (4H, m, 4NH of Tyr, Gly and Phe), 3.67 (3H, s, OCH_3).

N-(9-Fluorenylmethoxycarbonyl)tyrosylglycylglycylphenylalanine Hydrazide (15) 536 mg of **15** (86%) was prepared by the same method of **7** as described above with **14** (625 mg, 0.92 mmol) as the starting material. ^{1}H NMR (300 MHz, DMSO) δ 9.25 (1H, s, CONHNH$_2$), 8.30-8.19 (4H, m, 4NH of Tyr, Gly and Phe), 4.42 (1H, m, CONHNH$_2$).

Tyrosylglycylglycylphenylalanine(hydrazino) Naloxone Derivative (17a) and (18a) 250 mg of hydrazide **15** (0.36 mmol) was added to Naloxne (176 mg, 0.54 mmol) in absolute EtOH (5 mL) containing 2 drops of glacial acetic acid. The mixture was refluxed for 2 h and then stirred at rt overnight. A solid product was separated by filtration, washed with EtOH, and dried by vacuum to yield 309 mg **16a** (87%). 1-octanethiol (520 μL, 3.0 mmol) followed by TBAF (189 mg, 0.6 mmol) was then added to a solution of intermediate **16a** (300 mg, 0.30 mmol) in DMF (5 mL) under the nitrogen atmosphere. The mixture was stirred for 10 min. To this was added bis(1-methyl-1H-tetrazol-5-yl) disulfide (414 mg, 1.8 mmol) and stirring was continued for 3 min. Then 5% $NaHCO_3$ was added and the mixture was extracted with EtOAc. Purification of the crude product by preparative TLC with CH_2Cl_2/MeOH (10/1) yielded the corresponding *Z* and *E* hybrid opiates isomers. **17a** (118 mg, 52%) ^{1}H NMR (300 MHz, DMSO) δ 10.85 (1H, s, hydrazone hydrogen), 8.20-7.95 (4H, m, 4 NH of Tyr, Gly and Phe), 5.81 (1H, m, H-18), 5.19 (2H, m, H-19), 4.87 (1H, s, H-5). **18a** (65 mg, 28%) ^{1}H NMR (300 MHz, DMSO) δ 10.42 (1H, s, hydrazone hydrogen), 8.35-8.09 (4H, m, 4 NH of Tyr, Gly and Phe), 5.88 (1H, m, H-18), 5.30 (2H, m, H-19), 4.85 (1H, s, H-5).

Tyrosylglycylglycylphenylalanine(hydrazino) Naltrexone Derivative (17b) and (18b) Naltrexone derived intermediate **16b** (291 mg, 81%) was prepared from the condensation of 250 mg of hydrazide **15** (0.36 mmol) and Naltrexone (184 mg, 0.54 mmol). *Z* and *E* isomers of **17b and 18b** were prepared by the same procedure. **17b** (115 mg, 51%) ^{1}H NMR (300 MHz, DMSO) δ 10.75 (1H, s, hydrazone hydrogen), 8.01-7.85 (4H, m, 4 NH of Tyr, Gly and Phe), 5.08 (1H, s, H-5). **18b** (63 mg, 27%) ^{1}H NMR (300 MHz, DMSO) δ 10.27 (1H, s, hydrazone hydrogen), 8.21-7.87 (4H, m, 4 NH of Tyr, Gly and Phe), 4.68 (1H, s, H-5).

3.4. Binding Assays

Receptor binding studies were conducted on human opioid transfected into Chinese hamster ovary (CHO) Cells. The μ cell line was maintained in Ham's F-12 supplemented with 10% fetal bovine serum (FBS) and 400 μg/mL Geneticin (G418). The δ and the κ cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS, 400 μg/mL G418, and 0.1% penicillin/streptomycin. All cell lines were grown to confluence and then harvested for membrane preparation. The membrane for functional assay was prepared in buffer A (20 mM HEPES, 10 mM $MgCl_2$, and 100 mM NaCl at pH 7.4); the membrane

for binding assays was prepared in 50 mM Tris buffer (pH 7.7). Cells were scraped from the plates and centrifuged at 500 × *g* for 10 minutes. The cell pellet was homogenized in buffer with a polytron, centrifuged at 20, 000 × *g* for 20 min., washed and recentrifuged, and finally resuspended at 3 mg protein/mL in buffer to determine protein content. The homogenate was then stored at -70℃ in 1 mL aliquots. Binding assays were conducted using [^{3}H] DAMGO, [^{3}H] DPDPE, and [^{3}H] U69, 593 at the μ, δ, and κ receptors, respectively. The assay was performed in triplicate in a 96-well plate. Nonspectific binding was determined with 1.0 μM of the unlabeled counterpart of each radioligand. Cell membranes were incubated with the appreciate radioligand and test compound at 25℃ for 60 min. The incubation was terminated by rapid filtration through glass fiber filter paper on a Tomtec cell harvester. The filters were dried overnight and bagged with 10 mL scitillation cocktail before counting for 2 min on a Wallac Betaplate 1205 liquid scintillation counter. Full characterization of compounds includes analysis of the data for IC_{50} values and Hill coefficients by using the program PRISM. K_i values were calculated using the Cheng Prusoff transformation: $K_i = IC_{50}/ (1 + L/K_d)$ where "L" is the radioligand concentration and "K_d" is the binding affinity of the radioligand.

[^{35}S]GTPγS Binding Assays

Membrane prepared as described above was incubated with [^{35}S]GTPγS (50 pM), GDP (usually 10 μM), and the test compound, in a total volume of 1mL, for 60 min at 25℃ (Traynor and Nahorski, 1995). Samples were filtered over glass fiber filters and counted as described for the binding assays. A dose response curve with a prototypical full agonist (DAMGO, DPDPE, and U69, 593 for μ, δ, and κ receptors, respectively) was conducted in each experiment to identify full and partial agonist compounds. High affinity compounds (K_i value is 200 nM or less) that demonstrate no agonist activity were tested as antagonists. A Schild analysis (Schild, 1949) was conducted, using a full agonist dose response curve in the presence of at least three concentrations of the experiments. If the Schild slope was significantly different from -1.00, the antagonist activity was non-competitive; in such cases, the $_pA_2$ value was not reported. Equilibrium dissociation constants (K_e values) were calculated as: K_e = a/(DR-1) where "a" is the nanomolar concentration of the antagonist and "DR" is the shift of the agonist concentration-response curve to the right in the presence of a given concentration of antagonist.

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