Supporting Information

Design, Synthesis, and Evaluation of Opioid Analogues with Nonpeptidic β-Turn Scaffold; Enkephalin and Endomorphin Mimetics

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General Methods. All chemicals were purchased from Aldrich, unless otherwise noted. All amino acid derivatives were purchased from Advanced ChemTech, unless otherwise noted. All synthetic procedure was carried out by using ACT 90 or ACT 396 (Advanced ChemTech), unless otherwise noted. All solvents used in this experiment are spectra grade, and purchased from Aldrich. NMR chemical shifts are expressed in ppm relative to internal solvent peaks. All peaks are assigned as judged by the data from 2D experiments (COSY and/or ROESY).

Experimental Procedure

2-(4-*tert*-Butoxyphenyl)ethanol. A solution of 2-(4-*tert*-Butoxyphenyl)acetic acid methyl ester[1] (1.2 g, 5.4 mmol) in dichloromethane (70 ml) was placed in round-bottomed flask and cooled to –20 °C. To the solution was added DIBAL (1 M solution in toluene, 10.8 ml, 10.8 mmol) at –20 °C. The mixture was warmed to room temperature and stirred for 3 h. The reaction was quenched by addition of methanol at –20 °C, followed by the addition of 10 % $KHSO_4$. The product was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and condensed under reduced pressure. The crude product was purified by column chromatography (silica gel, ethyl acetate : hexanes = 1 : 2) to give the product (755 mg, 72%). The analytical sample was distilled by kugelrohr apparatus (155 °C, 1.5 mmHg): IR (ν max cm^{-1}, neat) 3356, 2976, 1506, 1366, 1235, 1162, 1047, 897; ^1H NMR (500 MHz, $CDCl_3$) δ ppm; 1.33 (s, 9H), 2.82 (t, J = 7 Hz, 2H), 3.82 (t, J = 7 Hz, 2H), 6.93 (m, 2H), 7.10 (m, 2H); ^{13}C NMR(125 MHz, $CDCl_3$) δ ppm, 29.0, 38.7, 63.9, 78.5, 124.5, 129.5, 133.4, 154.0; MS (m/z, APCI) 195 (MH^+); Anal. Calcd for $C_{12}H_{18}O_2$: C, 74.19; H, 9.34. Found: C, 73.90; H, 9.12.

2-(4-Hydroxyl-phenyl)ethoxycarbonyl-β-alanyl-homo-phenylalanine amylamide (7)

2-(4-tert-Butoxyphenyl)ethoxycarbonyl-β-alanine (150 mg) was treated with EDCI (94 mg) and HOBT (75 mg) in DMF at room temperature for 5 min. To the reaction mixture was added a solution of homo-phenylalanine (125 mg) in DMF followed by the addition of DIEA (160 μl) at room temperature. After the reaction mixture was stirred for overnight, ethyl acetate was added to the reaction mixture and the organic layer was washed with 10% $KHSO_4$, sat.$NaHCO_3$, and sat. NaCl, and dried over Na_2SO_4 and condensed to give the oily product. The product was treated with 4N HCl in dioxane at room temperature for 2 hr. After the removal of solvent, the residue was purified by column chromatography (silica gel, ethyl acetate) to give the product (147 mg, 69 % yield). ^1H NMR (500 MHz, $CDCl_3$) δ ppm; 0.89 (br.t, 3H, CH_3-amyl), 1.34 (br.s, 4H, CH_2-amyl), 1.48 (br.s, 2H, CH_2-amyl), 1.93 (m, 1H, βCH_2-hPhe), 2.08 (m, 1H, βCH_2-hPhe), 2.23-2.40 (m, 2H, αCH_2-βAla), 2.63 (br.s, 2H, γCH_2-hPhe), 2.80-2.96 (m, 2H, 4-OH-benzy), 3.10-3.24 (m, 2H, NCH_2-amyl), 3.24-3.40 (m, 2H, βCH_2-βAla), 4.10-4.33 (m, 2H, CH_2O), 4.39 (q, 1H, αCH-hPhe), 5.35 (s, 1H, NH-βAla), 6.30 (s, 1H, NH-amyl), 6.58 (s, 1H, NH-hPhe), 6.65-7.23 (m, 9H, aromatic); MS (m/z, ESI) 484.6 (MH^+).

***N*-Alkylamino-Resin (2)** The bromoacetal resin (**1**) (1.0 g, 0.92 mmol/g) [Advanced ChemTech] and a solution of a primary amine in DMSO (10 ml, 2 M) were placed in 20 ml vial with screw cap. The reaction mixture was shaken at 60 °C using rotating oven [Robbins Scientific] for 10 – 24 h. The resin was collected by filtration, and washed with DMF, then DCM. For the storage, the resin was dried *in vacuo* at room temperature.

Fmoc-α-Amino Acid Coupled Resin (3). A solution of Fmoc-α-amino acid (4 eq.), HOAT [PerSeptive Biosystems] (4 eq.), and DIC (4 eq.) in NMP (Advanced ChemTech) was added to the resin **2**. After the reaction mixture was shaken for 4 h at room temperature, the resin was filtered and washed with DMF, DCM, and then DMF. The loading of the resin was determined at this stage by photometrical calibration of Fmoc chromophore released upon treatment with piperidine. Typical loading of resin is 0.68 – 0.86 mmol.

Fmoc-β-Alanine Coupled Resin (4).To the resin **3** was added 25% piperidine in DMF (10 ml for 1 g of the resin). After the reaction mixture was shaken for 8 min at room temperature, the resin was filtered and washed with DMF, DCM, and then DMF. A solution of Fmoc-β-alanine (4 eq.), HOBT [Advanced ChemTech] (4 eq.), and DIC (4 eq.) in DMF was added to the resin prepared above. After the reaction mixture was shaken for 3 h at room temperature, the resin was filtered and washed with DMF, DCM, and then DMF.

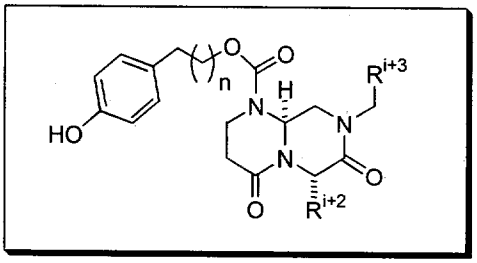
***N*-Alkyloxycarbonyl-β-Alanine Coupled Resin (5)**. To the resin **4** was added 25% piperidine in DMF (10 ml for 1 g of the resin). After the reaction mixture was shaken for 8 min at room temperature, the resin was filtered and washed with DMF, DCM, and then DMF.
p-Nitrophenyl chloroformate (5 eq.) was placed in round-bottomed flask and dissolved in DCM. To the solution was added an alcohol (7.0 eq.) and 2,6-lutidine (6.5 eq.) at room temperature. The reaction mixture was stirred for 4 h at room temperature and added to the resin prepared above. After a solution of DIEA (5 – 6.5 eq.) in DMF was added to the mixture, the suspension was shaken for 3 h at room temperature. The resin was filtered and washed with DMF, DCM, and then MeOH. The resin was dried *in vacuo* at room temperature.

β-Turn mimetic (6). The resin **5** was treated with formic acid (15 ml for 1 g of resin) for 10 h at room temperature. After the resin was removed by filtration, the filtrate was condensed under reduced pressure to give oily residue, which was then purified by column chromatography (silica gel, ethyl acetate-methanol).

References:

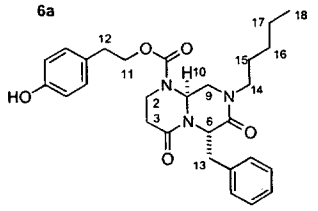
1. Newlander, K. A.; Callahan, J. F.; Moore, M. L.; Tomaszek, T. A.; Huffman, W. F. *J. Med. Chem.* **1993**, *36*, 23321-2331.

Chemical Yields of the Enkephalin and Endomorphin Mimetics



n	R^{i+2}	R^{i+3}	Yield (%)	n	R^{i+2}	R^{i+3}	Yield (%)
1	Bn	Methoxyethyl	29	1	H	nBu	25
1	Bn	nHex	24	1	Me	nBu	22
1	Bn	Ph	30	1	$MeS(CH_2)_2$	nBu	30
1	Bn	2-Pyridylmethyl	36	1	iBu	nBu	21
1	Bn	c.Hex	37	1	iPr	nBu	30
1	Bn	Bn	35	1	sec-Bu	nBu	20
1	Bn	4-Pyridyl	32	1	*p*-OH-Bn	nBu	36
1	Bn	iPr	18	1	Ph	nBu	23
1	Bn	3,4-Dimethoxyl-Bn	37	1	*p*-Cl-Bn	nBu	27
1	Bn	2-Oxo-1-pyrrodinethyl	34	1	*p*-Amino-Bn	nBu	11
1	Bn	iBu	29	1	Bn	nBu	36
1	Bn	nBu	36	1	Phenethyl	nBu	23
1	iBu	iBu	28	2	Bn	nBu	29
1	*p*-OH-Bn	iBu	30	2	Phenethyl	nBu	30
1	H	Bn	29	1	Phenethyl	Bn	29

6a



MS(m/z, ESI) 494.2 (MH^+)

Anal. Calcd for $C_{28}H_{35}N_3O_5$:
C,68.13; H,7.15; N, 8.51.
Found: C, 68.23; H, 6.90; N, 8.64.

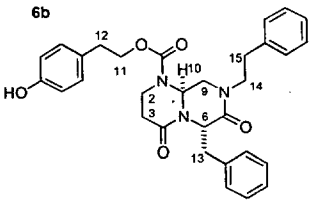
Observed as a mixture of two rotamers in a ratio of 3 : 2 (a : b).

^{1}H NMR (500 MHz, $CDCl_3$, -20°C)

Assignment, δ (ppm)
H (18): 0.88 (m, 3H)
H (16): 1.22 (m, 2H)
H (17): 1.32 (m, 2H)
H (15): 1.48 (m, 2H)
H (3b): 2.21 (m, 0.8H)
H (3a): 2.48 (m, 1.2H)
H (9a, pro-R): 2.67 (m, 0.6H)
H (12a): 2.73 (m, 0.6H)
H (12a): 2.79 (m, 0.6H)
H (12b): 2.90 (m, 0.8H)
H (14): 3.02 (m, 1H)
H (2a, pro-R): 3.04 (m, 0.6H)
H (9b): 3.10 (m, 0.4H)
H (2b) 3.12 (m, 0.4H)
H (13a): 3.23 (dd, J = 5, 14 Hz, 0.6H)
H (9a, pro-S): 3.29 (m, 0.6)
H (13b): 3.30 (m, 0.8H)
H (14b) 3.31 (m, 0.4H)
H (9b): 3.39 (m, 0.4H)
H (13a): 3.41 (m, 0.6H)
H (14a): 3.61 (m, 0.6H)
H (2b): 3.96 (dd, J = 4, 14 Hz, 0.4H)
H (11a): 4.10 (m, 0.6H)
H (2a, pro-S): 4.21 (m, 0.6H)
H (11a and 11b): 4.25 (m, 1H)
H (11b): 4.36 (m, 0.4H)
H (10a): 4.71 (dd, J = 4, 10 Hz, 0.6H)
H (10b): 5.22 (dd, J = 4, 10 Hz, 0.4H)
H (6a): 5.30 (t, J = 5 Hz, 0.6H)
H (6b): 5.38 (t, J = 5 Hz, 0.4H)
H (aromatic): 6.74- 7.34 (m, 9)
H (phenol-OH): 7.61 (br. s, 1H)

ROE's (-20 °C, $CDCl_3$)
H (2a, pro-R) - H (9a, pro-R)
H (10a) - H (13a)
H (10b) - H (13b)
H (phenol-H) - H (10)
H (phenol-H) - H (10)
H (phenol-H) - H (11)
H (phenol-H) - H (12)
H (phenyl) - H (6)
H (phenyl) - H (10)
H (phenyl) - H (13)

6b



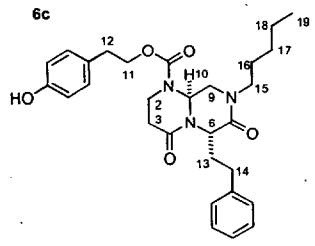
MS(m/z, ESI) 515.7 (MH^+).

Observed as a mixture of two rotamers in a ratio of 3 : 2 (a : b).

^{1}H NMR (500 MHz, $CDCl_3$, -20°C)

Assignment, δ (ppm)
H (3b): 2.12 (m, 0.8H)
H (3a): 2.40 (m, 1.2H)
H (9a): 2.48 (d, J = 11.5 Hz, 0.6H)
H (12a): 2.71 (m, 1.2H)
H (15): 2.80 (m, 2H)
H (2a): 2.82 (m, 0.6H)
H (12b): 2.85 (m, 0.8H)
H (9a): 2.90 (m, 0.6H)
H (2b): 2.92 (m, 0.4H)
H (9b): 2.95 (m, 0.4H)
H (14a): 3.08 (m, 0.6H)
H (9a): 3.10 (m, 0.6H)
H (13): 3.21 - 3.38 (m, 2H)
H (14b): 3.50 (m, 0.4H)
H (14b): 3.61 (m, 0.4H)
H (2b): 3.85 (m, 0.4H)
H (14a): 3.92 (m, 0.6H)
H (2a): 4.12 (m, 0.6H)
H (11a): 4.15 (m, 0.6H)
H (11a): 4.23 (m, 0.6H)
H (11b): 4.23 (m, 0.4H)
H (11b): 4.37 (m, 0.4H)
H (10a): 4.72 (m, 0.6H)
H (10b): 5.28 (m, 0.4H)
H (6a): 5.32 (d, J = 8.5 Hz, 0.6H)
H (6b): 5.39 (d, J = 8.5 Hz, 0.4H)
H (phenol-OH) 6.50 (br. s, 1H)
H (aromatic): 6.71- 7.37 (m, 14H)

6c



MS(m/z, ESI) 508.8 (MH^+)

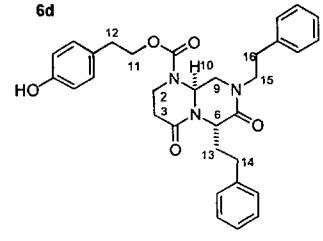
Observed as a mixture of two rotamers in a ratio of 1 : 1 (a : b).

^{1}H NMR (500 MHz, $CDCl_3$, -20°C)

Assignment, δ (ppm)
H (19): 0.89 (m, 3H)
H (17): 1.12 (m, 2H)
H (18): 1.32 (m, 2H)
H (16): 1.48 (m, 2H)
H (13a) 1.92 (m, 0.5H)
H (13b): 2.12 (m, 0.5H)
H (3b): 2.17 (m, 1H)
H (3a): 2.30 (t, J = 12.5 Hz, 1H)
H (13a and 13b): 2.39 (m, 1H)
H (14): 2.58 (m, 1H)
H (14): 2.83 (m, 1H)
H (12): 2.92 (m, 2H)
H (9a): 2.95 (m, 0.5H)
H (15a): 2.98 (m, 0.5H)
H (2a, pro-R): 3.07 (dt, J = 4, 12.5 Hz, 0.5H)
H (2b): 3.19 (m, 0.5H)
H (15b): 3.21 (m, 0.5H)
H (9b) 3.22 (m, 0.5H)
H (9a, pro-R): 3.39 (m, 0.5H)
H (15b): 3.41 (m, 0.5H)
H (9b): 3.42 (m, 0.5H)
H (15a): 3.62 (m, 0.5H)
H (2b): 3.99 (dd, J = 6, 12.5 Hz, 0.5H)
H (2a): 4.13 (dd, J = 6, 12.5 Hz, 0.5H)
H (11): 4.31 (m, 2H)
H (6a): 5.25 (m, 0.5H)
H (6b): 5.28 (m, 0.5H)
H (10a): 5.32 (m, 0.5H)
H (10b): 5.77 (dd, J = 4.5, 11 Hz, 0.5H)
H (phenol-OH): 6.31 (br. s, 1H)
H (aromatic): 6.69 - 7.25 (m, 9H)

ROE's (-20 °C, $CDCl_3$)
H (2a, pro-R) - H (9a, pro-R)
H (10a) - H (13a)
H (10b) - H (13b)
H (phenyl) - H (6)
H (phenyl) - H (14)
H (phenyl) - H (10)

6d



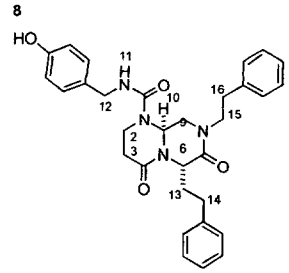
MS(m/z, ESI) 529.8 (MH^+)

Observed as a mixture of two rotamers in a ratio of 1 : 1 (a : b).

^{1}H NMR (500 MHz, $CDCl_3$, -20°C)

Assignment, δ (ppm)
H (13b) 1.89 (m, 0.5H)
H (13a): 2.09 (m, 0.5H)
H (3): 2.11-2.22 (m, 2H)
H (13a and 13b): 2.37 (m, 1H)
H (14): 2.59 (m, 1H)
H (9b, pro-R): 2.67 (d, J = 12 Hz, 0.5H)
H (14): 2.81 (m, 1H)
H (9b): 2.82 (m, 0.5H)
H (2a and 2b, pro-R): 2.83 (m, 1H)
H (16): 2.87 (m, 2H)
H (12): 2.89 (m, 2H)
H (9a): 3.09 (m, 1H)
H (15): 3.41 (m, 1H)
H (15): 3.64 (m, 1H)
H (2b): 3.89 (m, 0.5H)
H (2a): 4.00 (m, 0.5H)
H (11): 4.30 (m, 2H)
H (10b): 5.23 (dd, J = 4.5, 12 Hz, 0.5H)
H (6): 5.31 (m, 1H)
H (10a): 5.68 (t, J = 8 Hz, 0.5H)
H (aromatic): 6.65 - 7.28 (m, 14H)

ROE's (-20 °C, $CDCl_3$)
H (2b, pro-R) - H (9b, pro-R)
H (9a) - H (15)
H (10a) - H (13a)
H (10b) - H (13b)
H (phenyl) - H (6)
H (phenyl) - H (12)
H (phenyl) - H (14)
H (phenyl) - H (16)



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^{1}H NMR (400 MHz, $CDCl_3$, 18°C)
Assignment, δ (ppm)

H (13a) 2.07 (m, 1H)
H (3): 2.30-2.35 (m, 2H)
H (13b): 2.38 (m, 1H)
H (14a): 2.64 (m, 1H)
H (14b): 2.82 (m, 1H)
H (16): 2.87 (m, 2H)
H (9): 3.10 (m, 2H)
H (2a): 3.16 (m, 1H)
H (15a): 3.41 (m, 1H)
H (2b): 3.48 (m, 1H)
H (15b): 3.57 (m, 1H)

H (12): 4.33 (dd, J = 1.6, 5.2 Hz, 2H)
H (11): 4.73 (t, J = 5.2 Hz, 1H)
H (6): 5.28 (dd, J = 4.8, 10.4 Hz, 1H)
H (10): 5.70 (dd, J = 6.0, 9.6 Hz, 1H)
H (aromatic): 6.81 - 7.28 (m, 14H)

ROE's (18 °C, $CDCl_3$)

H (10) - H (13a)
H (10) - H (11) (weak)
H (11) - H (2b)
H (phenyl) - H (12)
H (phenyl) - H (14)
H (phenyl) - H (16)

MS(m/z, ESI, positive) 549.3 ($M+Na^+$)
MS(m/z, ESI, negative) 525.1 ($M-H^-$)

Binding Assay[2]

The assay was performed using the following general procedure:

Receptor	Tissue	Reference compound
Opiate (non-selective)	Rat cerebral cortex	Naloxone

The experimental conditions are summarized below:

Receptor	Ligand	Concentration	Nonspecific	Incubation
Opiate (non-selective)	[^{3}H]naloxone	1 nM	Naloxone (1 μM)	40 min/22°C

Following incubation, the membranes were rapidly filtered under vacuum through glass fiber filters (Filtermat A, Wallac). The filters were then washed several times with an ice-cold buffer using a cell harvester (Tomtec).
Bound radioactivity was measured with a scintillation counter (Betaplate, Wallac) using a solid scintillant (MeltiLex B/HS, Wallac).

Experimental protocols

The compounds were first tested at 1 μM and/or 0.1 μM. Compounds **6a-d** were further tested at 9 - 5 concentrations ranging from 1 nM to 0.5 μM in duplicate (**6a**) to obtain competition curves. In each experiment, the reference compound (naloxone) was tested at 8 concentrations in duplicate to obtain a competition curve in order to validate this experiment.

Analysis and expression of results

The specific radioligand binding to the receptors is defined as the difference between total binding and nonspecific binding determined in the presence of an excess of unlabelled ligand. Results are expressed as a percent of control specific binding and as a percent inhibition of control specific binding obtained in the presence of the test compounds. Individual data and mean data are presented in the results section.

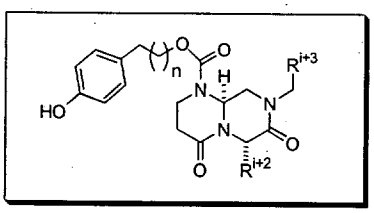
IC_{50} values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (nH) were determined by non-linear regression analysis of the competition curves. These parameters were obtained by Hill equation curve fitting.

The IC_{50} values obtained for the reference compound have passed the required inspections. They are within accepted limits of the historic average obtained ± 0.5 log unit.

Reference:

2. Childers, S. R.; Creese, I.; Snowman, A. M.; Snyder, S. H. Opiate Receptor Binding Affected Differentially by Opiates and Opioid Peptides. *Eur. J. Pharmacol.* **1979**, *55*, 11-18.

Effects of the Mimetics on Specific Radioligand ([^{3}H]Naloxone)
Binding to the Relatively Non-Selective Opiate Receptor



n	R^{i+2}	R^{i+3}	Inhibition (%) at 1 μM	n	R^{i+2}	R^{i+3}	Inhibition (%) at 1 μM	Inhibition (%) at 0.1 μM
1	Bn	Methoxyethyl	82	1	H	nBu	51	
1	Bn	nHex	85	1	Me	nBu	42	
1	Bn	Ph	51	1	$MeS(CH_2)_2$	nBu	74	
1	Bn	2-Pyridylmethyl	96	1	iBu	nBu	45	
1	Bn	c.Hex	68	1	iPr	nBu	68	
1	Bn	Bn	96	1	sec-Bu	nBu	27	
1	Bn	4-Pyridyl	89	1	*p*-OH-Bn	nBu	11	
1	Bn	iPr	67	1	Ph	nBu	42	
1	Bn	3,4-Dimethoxyl-Bn	84	1	*p*-Cl-Bn	nBu	74	
1	Bn	2-Oxo-1-pyrrodinethyl	86	1	*p*-Amino-Bn	nBu	94	
1	Bn	iBu	63	1	Bn	nBu	84	
1	Bn	nBu	84	1	Phenethyl	nBu	99	79
1	iBu	iBu	49	2	Bn	nBu		<10
1	*p*-OH-Bn	iBu	<10	2	Phenethyl	nBu		<10
1	H	Bn	<10	1	Phenethyl	Bn		91

Binding assays

The assays were performed using the following general procedures:

Receptors	Origin	Reference compounds	Bibliography
δ *(h)*	CHO cell (h. recombinant)	DPDPE	ref. 3
κ *(h)*	HEK 293 cell (h. recombinant)	U50488	ref. 4
μ *(h)*	CHO cell (h. recombinant)	DAMGO	ref. 5

The experimental conditions are summarized below:

Receptors	Ligand	Conc.	Nonspecific	Incubations
δ *(h)*	[^{3}H]DPDPE	0.5 nM	naltrexone (10 μM)	240 min./22°C
κ *(h)*	[^{3}H]U 69593	0.5 nM	U50488 (10 μM)	60 min./22°C
μ *(h)*	[^{3}H]DAMGO	0.5 nM	naloxone (10 μM)	150 min./22°C

Following incubation, the membranes were rapidly filtered under vacuum through glass fiber filters (GF/B Packard). The filters were then washed several times with an ice-cold buffer using a cell harvester (Packard).
Bound radioactivity was measured with a scintillation counter (Topcount, Packard) using a liquid scintillation cocktail (Microscint 0, Packard).

Experimental protocols

Compounds **6c** and **6d** were tested in each assay at 0.1 μM in duplicate,
In each experiment, the respective reference compound was tested at a minimum of eight concentrations in duplicate to obtain a competition curve in order to validate this experiment.

Analysis and expression of results

The specific radioligand binding to the receptors is defined as the difference between total binding and nonspecific binding determined in the presence of an excess of unlabelled ligand. Results are expressed as a percent of control specific binding and as a percent inhibition of control specific binding obtained in the presence of the test compounds. Individual data and mean data are presented in the results section.

IC_{50} values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (nH) were determined for the reference compounds by non-linear regression analysis of their competition curves. These parameters were obtained by Hill equation curve fitting.

The IC_{50} values obtained for the reference compounds have passed the required inspections. They are within accepted limits of historic averages obtained ± 0.5 log unit.

Results

Effects of the **6c** and **6d** on the Specific Radioligand Binding to the Human Opiate Receptors and IC_{50} Values for the Reference Compounds

Receptors	**6c**	**6d**	Reference compounds		
	0.1 μM	0.1 μM		IC_{50} (nM)	(nH)
δ *(h)*	< 10 %	< 10 %	DPDPE	3.5	(1.1)
κ *(h)*	< 10 %	< 10 %	U 50488	0.69	(0.8)
μ *(h)*	91 ± 0.9 %	101 ± 2.0 %	DAMGO	1.6	(1.1)

The results are expressed as a percent inhibition of specific binding (mean values; n = 2).

References:

3. Malatynska, E.; Wang, Y.; Knapp, R. J.; Santoro, G.; Li, X.; Waite, S.; Roeske, W. R.; Yamamura, H. I. Human δ Opioid Receptor: A Stable Cell Line for Functional Studies of Opioids. *NeuroReport,* **1995**, *6*, 613-616.
4. Simonin, F.; Gaveriaux-Ruff, C.; Befort, K.; Matthes, H.; Lannes, B.; Micheletti, G.; Mattei, M.-G.; Charron, G.; Bloch, B.; Kieffer, B. κ-Opioid Receptor in Humans: cDNA and Genomic Cloning, Chromosomal Assignment, Functional Expression, Pharmacology, and Expression Pattern in the Central Nervous System. *Proc. Natl. Acad. Sci. USA.* **1995**, *92*, 7006-7010.
5. Wang, J.-B; Johnson, P. S.; Persico, A. M.; Hawkins, A. L.;Griffin, C. A.; Uhl, G. R. Human μ-Opiate Receptor. cDNA and Genomic Clones, Pharmacological Characterization and Chromosomal Assignment. *FEBS Lett.* **1994**, *338*, 217-222.

In Vivo Analgesia Assay

Test Article:	
Sample Code:	**6c**
Amount Submitted:	70 mg
Appearance:	White powder
Storage:	Room temperature, protected from light, desiccated
Vehicle:	1,2-Propanediol (Propylene Glycol) and sterile Saline (1:1)
Suppliers:	Sigma Chemical Company (Propylene Glycol) Radix Laboratories (0.9% Saline)
Lot Numbers:	125HO894, Exp 05/2000 (Propylene Glycol) R8J013, Exp. 07/2000 (0.9% Saline)
Appearance:	Clear liquid (Propylene Glycol) Clear liquid (0.9% Saline)
Storage:	Room temperature
Preparation:	Propylene Glycol (PO) and Saline (1: 1) was prepared on 4 Nov 99 as follows: 25 ml PG wag added to 25 ml 0.9% Saline. soluble
Control Article:	
Identity:	Morphine Sulfate
Supplier:	Sigma Chemical Company
Lot Number:	87H0350, Exp 06/2001
Appearance:	White powder
Storage:	Room temperature
Preparation:	Prepared on 4 Nov 99 as follows: 10 mg/kg at 5.0 ml/kg = 2.0 mg/ml 5.0 ml of vehicle (Saline) was added to 10 mg of morphine sulfate, soluble.

Test Article Formulation:

Preparation:
All dose levels were prepared on 4 Nov 1999
Stock- 2.0 mg/ml
(1) 10 mg/kg at 5.0 ml/kg = 2.0 mg/ml
10 ml Propylene Glycol was added to 40 mg of test article. The mixture was vortexed and placed in 37°C water bath until it was completely dissolved. 10 ml of 0.9% Saline was added to the volume, vortexed and heated at 37°C in water bath. (Solution A)
(2) 1.0 mg/kg at 5.0 mi/kg = 0.2 mg/ml
1.0 ml of Solution A (2.0 mg) was added to 9 ml of vehicle (Saline). (Solution B)
(3) 0.1 mg/kg at 5.0 mi/kg = 0.02 mg/ml
1.0 ml of Solution B (0.2 mg) was added to 9 ml vehicle (Saline). (Solution C)

All dosing solutions were placed in a 37°C water bath prior to and during dosing. Dosing solutions were prepared in polypropylene centrifuge tubes as requested. All preparations were soluble.

EXPERIMENTAL PROCEDURE

Test System:

Species/strain:	Mouse/Hsd:ICR (CD-1)
Supplier:	Harlan Sprague Dawley
Number of Animals:	60 males
Age at Initiation of Treatment:	34 days
Weight Range at Initiation of Treatment:	22- 28 grams
Justification:	The mouse is a standard species used for the determination of the potential analgesic activity of test articles. The mouse is used since when exposed to an extended time of heat stimulus, a characteristic reaction occurs that can be quantified. The number of animals used in this study is the minimum amount considered to provide valid scientific assessment and is consistent with studies referenced in Methods of Pharmacology, Ciofalo, et al, circa 1959, Chrysalis Internal Document.

Environment and Husbandry:

Temperature:	18-26°C
Relative Humidity:	10-70%
Lighting Cycle:	12 hours light/12 hours dark
Caging:	Mice were housed in groups in compliance with USDA Guidelines.
Diet:	Teklad Certified LM-485 Rodent Diet #7012C, *ad libitum*

Water Provided: *ad libitum*

Contaminants: There are no known contaminants in the diet or water which, at the levels detected, would be expected to have interfered with the conduct or outcome of this study. Certificates of analysis for the diet and drinking water are maintained in the archives at Chrysalis.

Acclimation/Quarantine:

Following arrival at Chrysalis, mice were assessed as to their general health by a member of the veterinary staff or suitable designee. Mice were acclimated/quarantined for 8 days prior to treatment initiation, during which time each mouse was observed at least once daily for any abnormalities or for the development of infectious disease.

Allocation to Treatment Groups:

Mice were identified by color code and assigned to groups as follows:

Group Number	Mouse Number
1	1-10
2	11-20
3	21- 30
4	31 -40
5	41 -50

Experimental Design:

The vehicle, control article and test articles were administered i. v. at 5.0 mi/kg/mouse. Three groups of ten male CD-1 mice were administered 0.1, 1.0 or 10 mg/kg of **6c** intravenously (i.v.) at 5.0 ml/kg. One group of ten male CD-1 mice was administered vehicle (propylene glycol and saline 1 : 1) i.v. at 5.0 ml/kg. Another group of ten male CD-1 mice received the positive control, morphine sulfate at 10 mg/kg, i.v. at 5.0 ml/kg. The mice were sequentially placed on a hot plate analgesia meter (set at 55 °C ± 2°C) and observed for a reaction to the heat stimulus. The mice reacted characteristically to the heat stimulus by licking of the forepaws, rapid fanning of a hind paw or a sudden jump off the hot plate. Any of the three types of reactions were taken as an end point to the heat stimulus. The mice were removed from the hot plate immediately upon displaying the end point. The reaction time was measured quantitatively by the number of seconds that elapsed between the placing of the mouse on the hot plate and the display of a definitive end point. Elapsed time was measured by a stopwatch accurate to at least 1/5 of a second. The reaction times were obtained at 5, 15, 30, 60 and 120 minutes following i.v. administration. The reaction time cut-off point was 30 seconds.

Reaction times (seconds) to the heat stimulus were recorded and percent increase (analgesia) was calculated as follows;

% analgesia = [average response time (treated) / average response time (controls)] -1.0 x 100

Results

Mouse Hot Plate Analgesia Assay (**6c**)

ID	N	Treatment	Predose Mean Reaction Time (sec)	5 min Mean Reaction Time (sec)	Analgesia (%)	15 min Mean Reaction Time (sec)	Analgesia (%)	30 min Mean Reaction Time (sec)	Analgesia (%)	60 min Mean Reaction Time (sec)	Analgesia (%)	120 min Mean Reaction Time (sec)	Analgesia (%)
1	10	Vehicle 5 ml/kg i.v	5.4 ±0.35	5.8 ±0.63	7	5.8 ±0.47	7	5.3 ±0.64	0	4.7 ±0.72	0	NA	NA
2	10	Morphine 10 mg/kg i.v	6.7 ±0.50	25.4* ±1.17	279	22.4* ±2.2	234	17.7* ±1.53	164	14.3* ±1.44	113	8.2* ±0.58	22
3	10	**6c** 0.1 mg/kg i.v	6.3 ±0.66	6.3 ±1.00	0	5.6 ±0.74	0	5.7 ±0.75	0	4.2 ±0.55	0	NA	NA
4	10	**6c** 1.0 mg/kg i.v	6.9 ±0.57	14.1* ±2.23	104	9.4 ±1.26	36	4.5 ±0.61	0	5.9 ±0.77	0	NA	NA
5	10	**6c** 10 mg/kg i.v	5.7 ±0.39	24.9* ±2.37	337	16.5* ±2.84	189	10.0* ±1.57	75	5.0 ±0.45	0	NA	NA

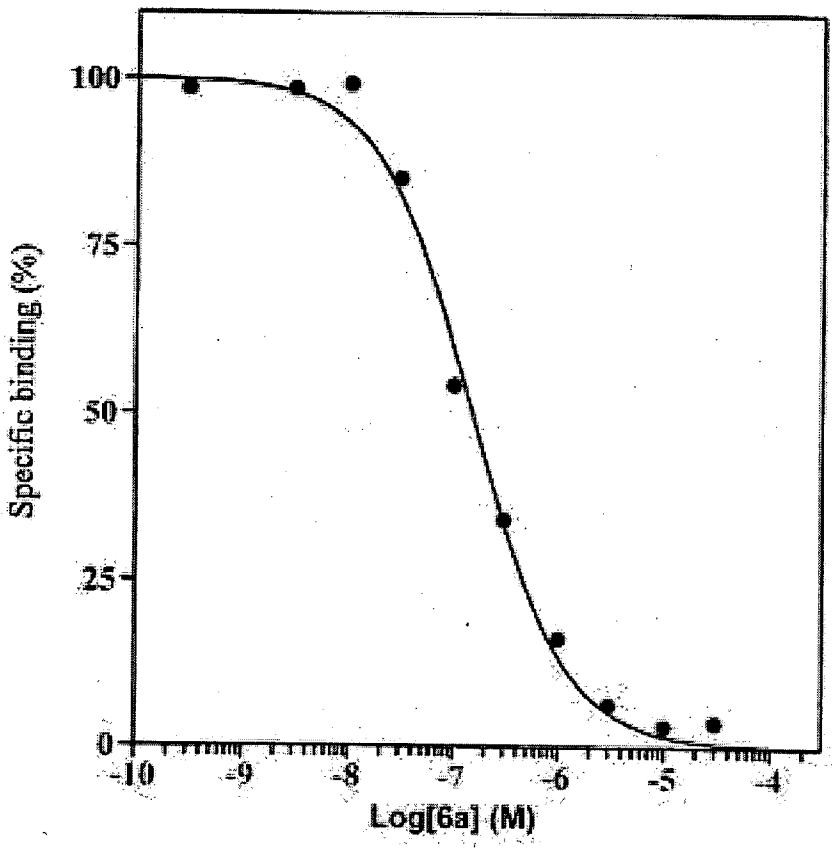
a. Mean ±S.E.M.

- Statistically significant ($p \leq 0.05$) increase in mean reaction time according to paired samples test.

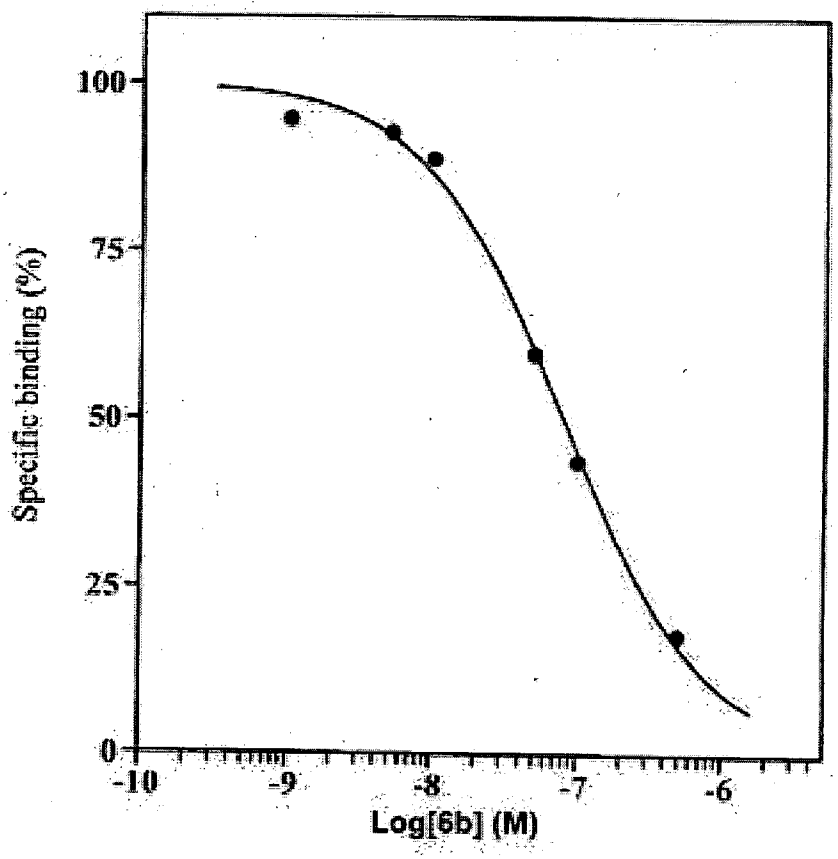
Abbreviations

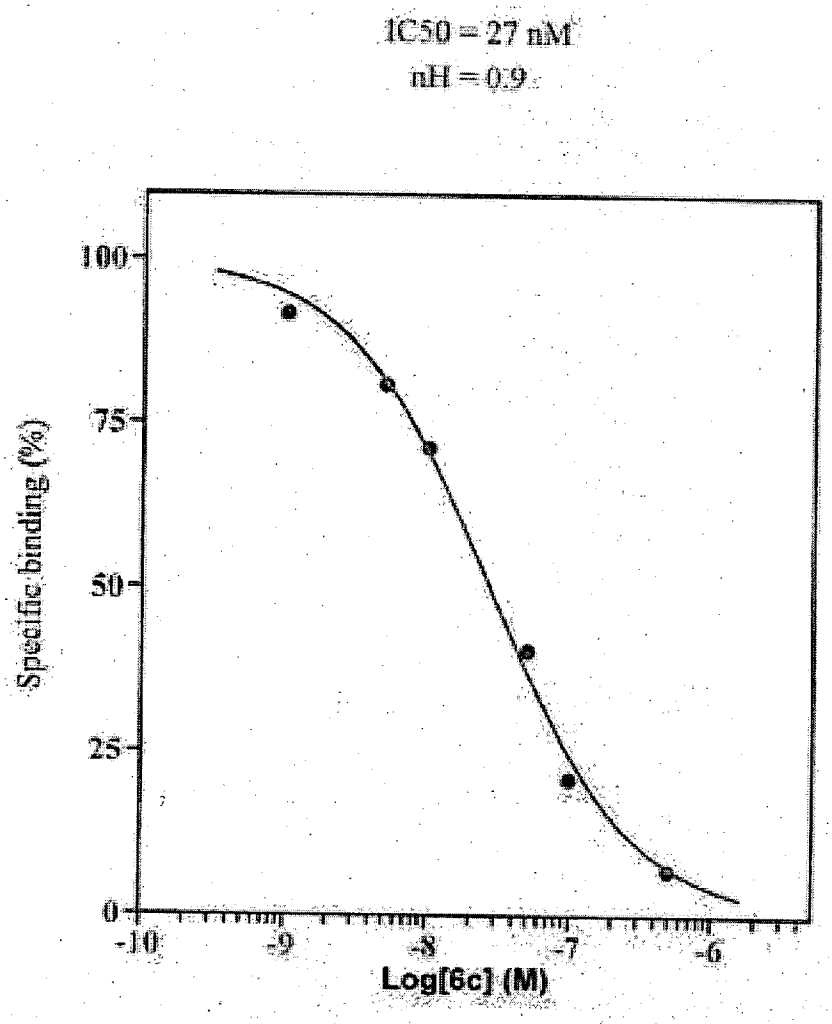
2D-NMR	-	Two-dimensional nuclear magnetic resonance
HOAt	-	7-Aza-1-hydroxybenzotriazole
DIC	-	1,3-Diisopropylcarbodiimide
NMP	-	*N*-Methyl-2-pyridone
DMF	-	*N,N*-Dimethylformamide
DIEA	-	*N,N*-Diisopropylethylamine
DMSO	-	Dimethyl sulfoxide
HOBt	-	1-Hydroxybenzotriazole
TLC	-	Thin-layer chromatography
DPDPE	-	D-Pen 2, D-Pen 5-enkephalin
DAMGO	-	Tyr-D-Ala-Gly-MePhe-Gly(ol)-enkephalin
RMSD	-	Root mean square deviation

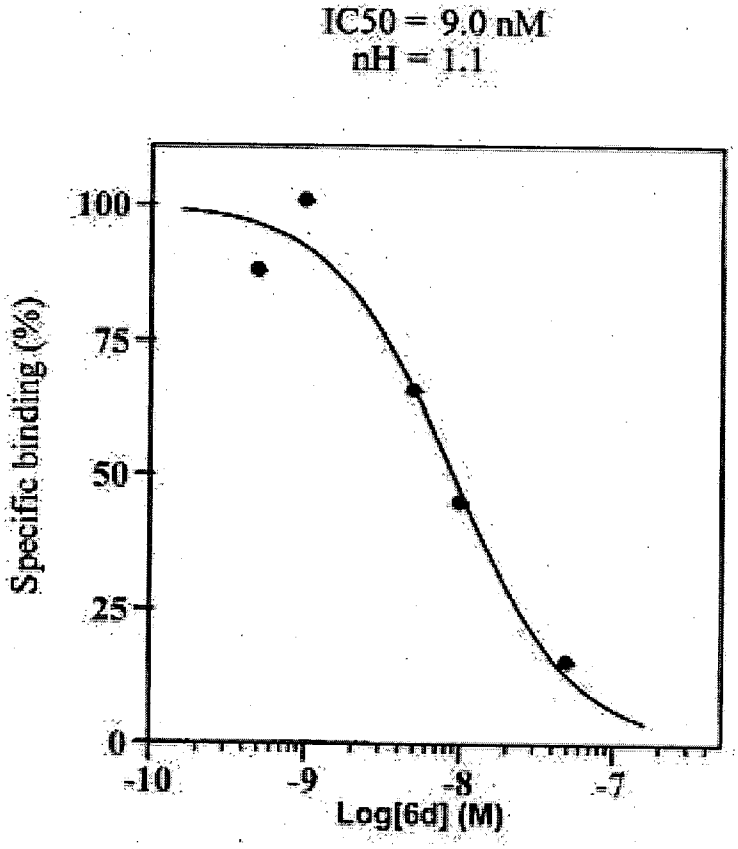
IC50 = 149 nM
nH = 1.0



IC50 = 80 nM
nH = 0.9




IC50 = 27 nM
nH = 0.9
100
75
50
25
0
Specific binding (%)
-10
-9
-8
-7
-6
Log[6c] (M)


IC50 = 9.0 nM
nH = 1.1
100
75
50
25
0
Specific binding (%)
-10
-9
-8
-7
Log[6d] (M)