Notes

Opioid Receptor Binding Requirements for the δ-Selective Peptide Deltorphin I: Phe^3 Replacement with Ring-Substituted and Heterocyclic Amino Acids

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In order to assess steric, lipophilic, and electronic influences on opioid binding affinity, analogs of the δ receptor selective peptide deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$) were prepared in which the residue 3 phenylalanine was replaced with lipophilic fluoro– and methyl-substituted phenylalanines or with the heterocyclic aromatic amino acids 3-(2-thienyl)alanine, 3-(2-pyridyl)alanine, 3-(3-pyridyl)alanine, histidine, and 3-(4-thiazolyl)alanine. μ binding was variable, with K_is in excess of 10 000 nM for most analogs, and all of the analogs bound poorly to k receptors. Among the phenyl ring-substituted analogs, those containing the smaller and electron-withdrawing halogens were favored over those with larger, electron-releasing methyl groups, although δ opioid binding affinity was reduced in all cases. The *m*-fluorophenylalanine analog demonstrated the best δ binding of the group, with a K_i of 4.79 nM. Within the group of heterocyclic analogs, 3-(2-thienyl)alanine proved to be the best modification, displaying a δ receptor K_i of 1.38 nM, while the polar histidine analog suffered the greatest loss in δ binding (K_i = 317). Compounds containing pyridylalanine and thiazolylalanine were intermediate in binding affinity, with δ K_is ranging from 39.5 to 62.4 nM. The major factor influencing the opioid binding of the similar-sized heterocyclic compounds was relative lipophilicity, which outweighed electronic character.

Introduction

It is well-accepted that opioid peptides interact with at least three distinct types of receptors,[1] and it is likely that these μ, δ, and κ receptors mediate different pharmacological responses. In an effort to enhance opioid potency while eliminating undesirable effects, the search continues for receptor-specific peptide ligands which would allow the elucidation of structural/conformational requirements for binding and the roles of each receptor type. Various synthetic ligands, such as cyclic pentapeptides related to [D-Pen^2,D-Pen^5]enkephalin (DPDPE)[2] and tetrapeptides lacking Gly^3 (particularly, Tyr-c[D-Cys-Phe-D-Pen]-OH or JOM-13[3] and Tyr-c[D-Orn-Phe-Asp/Glu]-NH$_2$[4]), currently are being employed in this investigation.

A family of three naturally-occurring opioid heptapeptide agonists lacking Gly^3 and possessing a D-amino acid at the second residue has been isolated from amphibian skin. These peptides, dermenkephalin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH$_2$), deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH$_2$), and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH$_2$),[5] demonstrate remarkable affinity and selectivity for the δ opioid receptor. Structure–activity studies on natural peptides which display this inherent selectivity may allow the assessment of specific interactions between functional groups of the peptide and the receptor at the molecular level. These peptides also represent an ideal starting point for the development of receptor-specific peptidomimetic drugs.

The N-terminal Tyr-D-aa-Phe message portion of the deltorphins appears to be involved in a type-II β turn[6] while the C-terminal tetrapeptide (the "address" region) plays a conformational role,[7] perhaps similar to that played by conformational restriction in the cyclic tetrapeptide JOM-13.[8] Additional conformational constraint imposed upon deltorphin analogs by lactam cyclization between residues 2 and 4, 4 and 7, or 2 and 7 has been reported to produce less selective analogs resulting from a reduction in δ binding affinity.[9] The topographical relationship of the Tyr and Phe aromatic side chains in opioid peptides is important in receptor discrimination and binding, and it is conceivable that the nature of their relative conformational relationship may vary between enkephalins and deltorphins by virtue of the different distances separating these critical residues; in fact, some disparities in structure–activity relationships for the two classes of peptides[10–15] imply impossible differences in the binding interaction. Substitution of tetrahydroisoquinoline (Tic) at the third residue of deltorphin has been shown to reduce δ binding, while potent and δ-selective analogs have been developed by replacement of phenylalanine with amino acids such as 2-aminoindan-2-carboxylic acid (Aic) and 2-aminotetralin-2-carboxylic acid (Atc).[9] Each of these possesses a side chain constrained by virtue of the bicyclic system, which produces severe limitations on side chain rotational freedom and local backbone flexibility; data therefore confirm the importance of the orientation of the residue three side chain in the binding interaction. In addition, the electronic and lipopilic character of the Phe residue in deltorphin peptides has

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Table 1. Opioid Receptor Binding Affinities of Deltorphin I Analogs

peptide[a]	compd no.	binding K_i (nM)[b] DAMGO[c]	DPDPE[d]	U69,593[e]	K_1 (μ)/K_i (δ)
Y-a-F-D-V-V-G-NH_2	deltorphin	677	1.73	>10000	391
Y-a-(*p*FPhe)-D-V-V-G-NH_2	1	10000	703. ± 73	>10000	7
Y-a-(*m*FPhe)-D-V-V-G-NH_2	**2a**	411. ± 21	4.79 ± 0.37	>10000	85.8
Y-a-(*m*FPhe)-D-V-V-G-NH_2	**2b**	3480. ± 1100	73.6 ± 15	>10000	47.3
Y-a-(*p*MePhe)-D-V-V-G-NH_2	**3**	>10000	2650. ± 250	>10000	>3
Y-a-(F_5Phe)-D-V-V-G-NH_2	**4**	>10000	135. ± 15	>10000	>70
Y-a-(Me_5Phe)-D-V-V-G-NH_2	**5a**	>10000	1073. ± 370	>10000	>9
Y-a-(Me_5Phe)-D-V-V-G-NH_2	**5b**	>10000	10000	>10000	>1
Y-a-(Thi)-D-V-V-G-NH_2	**6**	419. ± 69	1.38 ± 0.23	>10000	304
Y-a-(2-Pal)-D-V-V-G-NH_2	**7**	2930. ± 1800	62.4 ± 9.5	>10000	47.0
Y-a-(3-Pal)-D-V-V-G-NH_2	**8**	10000	49.7 ± 4.3	>10000	200
Y-a-(His)-D-V-V-G-NH_2	**9**	>10000	317. ± 52	>10000	>30
Y-a-(Taz)-D-V-V-G-NH_2	**10**	2800. ± 1800	39.5 ± 2.0	>10000	70.9

[a] Y = Tyr = tyrosine; a = D-Ala = D-alanine; D = Asp = aspartic acid; V = Val = valine; G = Gly = glycine. [b] Average values determined from one or two assays performed in triplicate ± standard deviation. [c] DAMGO = [^{3}H][D-Ala^2,NMePhe^4,Gly^5-ol]enkephalin. [d] DPDPE = [^{3}H][D-Pen^2,D-Pen^5]enkephalin. [d] U69,593 = 5a,7a,8b-(−)-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzenacetamide.

Table 2. Physicochemical Data for Deltorphin I Analogs

peptide	compd no.	HPLC I[a]	HPLC II[b]	purity (%)[c]	MS mol wt[d]
Y-a-(*p*FPhe)-D-V-V-G-NH_2	1	2.28	9.62	>99	787.6
Y-a-(*m*FPhe)-D-V-V-G-NH_2	**2a**	3.94	12.1	97	787.6
Y-a-(*m*FPhe)-D-V-V-G-NH_2	**2b**	1.58	8.41	97	787.6
Y-a-(F_5Phe)-D-V-V-G-NH_2	**3**	3.22	10.9	97	859.3
Y-a-(*p*MePhe)-D-V-V-G-NH_2	**4**	4.71	12.0	99	787.4
Y-a-(Me_5Phe)-D-V-V-G-NH_2	**5a**	4.97	12.3	98	839.5
Y-a-(Me_5Phe)-D-V-V-G-NH_2	**5b**	3.82	11.2	98	839.5
Y-a-(Thi)-D-V-V-G-NH_2	**6**	2.53	9.98	95	775.4
Y-a-(2-Pal)-D-V-V-G-NH_2	**7**	1.25	6.98	>99	769.9
Y-a-(3-Pal)-D-V-V-G-NH_2	**8**	1.08	6.83	96	769.9
Y-a-(His)-D-V-V-G-NH_2	**9**	0.78	6.78	99	758.9
Y-a-(Taz)-D-V-V-G-NH_2	**10**	1.53	8.43	95	775.5

[a] HPLC-*k'* on a Vydac 218TP C-18 column (0.46 cm × 25 cm); isocratic conditions at 23% organic component; flow rate of 1 mL/min. Solvent system was 0.1% TFA in water, 0.1% TFA in acetonitrile. Solvent front breakthrough at 3.6 min. [b] HPLC *k'* on a Vydac 218TP C-18 column (0.46 cm × 25 cm); gradient of 0−50% organic component in 50 min; flow rate of 1 mL/min. Solvent system was 0.1% TFA in water, 0.1% TFA in acetonitrile. Solvent front breakthrough at 3.4 min. [c] Purity of final product peptide as assessed by RP-HPLC peak integration at 230 nm. [d] Molecular weight obtained by FAB or electrospray mass spectrometry.

been determined to be an important element in influencing receptor binding affinity.[16]

In light of this evidence as well as that for the possible existence of subtypes of the δ receptor,[17,18] we undertook an investigation of the δ-selective peptide deltorphin I, primarily by modification of the critical Phe^3 residue to vary steric, electronic, and lipophilic properties. This was accomplished by substitution of the Phe aromatic moiety with ring-substituted analogs and heterocyclic aromatic amino acids.

Results and Discussion

The μ (*versus* [^{3}H]DAMGO]), δ (*versus* [^{3}H]DPDPE), and κ (*versus* [^{3}H]U69,593) receptor binding affinities of all analogs are provided in Table 1, along with the corresponding results for deltorphin I. All K_is obtained for the κ receptor were in excess of 10 000 nM. A measure of δ selectivity is provided as a ratio of μ to δ binding affinities. Physicochemical data are reported in Table 2. Deltorphin I binds with high affinity (K_i = 1.73 nM) to δ opioid receptors and minimally to μ receptors (K_i = 677 nM) in our binding assay, thereby displaying significant selectivity for the δ receptor type. Analogs of this peptide were synthesized with modifications of the Phe^3 residue to explore the role of this residue in deltorphin I for receptor interaction.

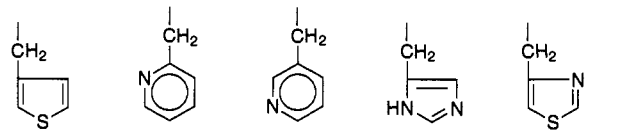
The first set of analogs contained electron-withdrawing (*p*-fluorophenylalanine, *p*FPhe, 1; *m*-fluorophenylalanine, *m*FPhe, **2a** and **2b**; and pentafluorophenylalanine, F_5Phe, **4**) and electron-donating (*p*-methylphenylalanine, *p*MePhe, **3**; pentamethylphenylalanine, Me_5Phe, **5a** and **5b**) substituents on the Phe aromatic ring, altering the character of the aromatic side chain. All of these substituted Phe analogs were lipophilic, as evidenced by relatively late reversed-phase high-performance liquid chromatography (RP-HPLC) elution times. With the exception of **2a**, the methyl-substituted derivatives were slightly more lipophilic than the fluorine-substituted analogs. Several parameters therefore come into play when evaluating binding data for these compounds, including size, lipophilicity, and electronic nature. The methyl-substituted analogs were chosen for study since deltorphin peptides containing Phe^3 substituents which are simultaneously lipophilic and electron releasing have not yet been reported. Although [*p*FPhe^3]deltorphin I has been reported previously along with several *para*-substituted analogs,[19] 1 was synthesized and tested in our binding assay system for comparison and consistency. In the cases of *m*FPhe and Me_5Phe analogs, a diastereomeric pair of peptides was prepared from a racemic mixture of the Boc-D,L-amino acid. The peptides were then separated by RP-HPLC, and the stereocenter was never definitively assigned. However, given the well-accepted preference for an L-amino acid at this position for opioid binding,[13,14] it can be assumed that the first analog listed in each pair (**2a** and **5a**) is that which contains the L-amino acid, based on its higher opioid affinity.

While none of the prepared analogs in this group bound to opioid receptors as well as the lead compound (deltorphin I), those with electron-withdrawing character at the third residue (1, **2**, and **4**) were more favored than those with electron donating substituents (**3** and **5**). The *p*FPhe analog, 1, actually exhibited substantially reduced δ binding (K_i = 703, decreased 406-fold relative to deltorphin I), while that of the *m*FPhe analog, **2a**, was essentially the same as the lead compound (K_i = 4.79). This was an unexpected difference; in fact, it is common for *p*FPhe anlogs to show higher δ affinity than *m*FPhe-containing compounds,[15] apparently due to the different direction of the dipole moment or slightly

higher lipophilicity or both. In this case, relative RP-HPLC elution times for these compounds indicate that **2a** is one of the most lipophilic of those reported here, which may partially account for its favorable binding properties. Substitution at the *para* position of the Phe aromatic ring with a methyl moiety (**3**) resulted in a drastic decline in δ receptor binding (K_i = 2650); the methyl substituent proved much more detrimental to δ receptor interaction than did the fluorine. It is not probable that these results can be attributed to the differing capabilities of the fluoro and methyl substituents for hydrogen bonding since substitution at this position with the electron-donating methoxy group, with similar H-bonding character as a fluoro moiety, also abolished δ binding affinity (K_i > 10 000, unpublished results).

The fully halogenated phenyl system in **4** resulted in a 78-fold reduction in δ opioid affinity (K_i = 135), but the adverse effects on binding were 1 order of magnitude greater (K_i = 1070) for the fully-methylated ring system in **5a**. In all cases except for **2a**, μ affinity was reduced as well. The differences in binding affinities between the halogenated and methylated analogs may be attributed to the electron-donating effects of the methyl substituents; previous studies on JOM-13 have found that an electron-poor aromatic system at this residue is preferential to an electron-rich ring.[14,15] It also should be noted, however, that steric bulk was increased to a greater extent for the methyl-substituted phenylalanines than for those containing fluorine, which also may play a role in their decreased affinities for opioid receptors. The small van der Waals radius of the fluorine atom would not likely induce steric or conformational influences. On the other hand, the bulkier modifications, particularly in **5a** and **5b**, impose likely conformational constraint at residue 3 and may interfere with the proposed type-II β turn[6] at the N-terminus of the peptide. The presence of substituents at the 2′ and 6′ positions of the phenyl ring might substantially limit the conformational freedom of the aromatic moiety as well.

We are uncertain as to why the δ binding affinity we obtained consistently for 1 differs so markedly from that reported by others.[19] While the results were obtained in different tissues (guinea pig brain *versus* rat brain), reporting the affinities as K_is rather than IC_{50}s should help to equate the values. Other investigators have reported that [*p*FPhe^3]deltorphin I exhibits high δ receptor affinity, though it does not improve binding over Phe itself and bioassay potency in the mouse vas deferens (MVD) actually declines 25-fold.[19] This is in contrast to effects observed in linear enkphalins, where halogenated Phe^4 analogs display enhanced binding potency.[20] In the halogen-containing series of deltorphin I analogs (*p*FPhe, *p*ClPhe, *p*BrPhe, and *p*IPhe), μ opioid activity roughly correlated with decreasing bulkiness and increasing electronegativity, but δ activity varied.[19] More pronounced unfavorable effects were observed for amino- and nitro-substituted analogs,[19] which are hydrophilic but vary in electronic character, the former substituent being electron-releasing while the latter is electron-withdrawing. It therefore would appear that changes in electronic character, size, and lipophilicity all affect opioid receptor binding to some extent, but it is difficult to determine the degree of influence of each factor since these variables are intertwined. In general, the trend of a preference for smaller electron-withdrawing over larger electron-releasing substituents agrees with that reported for analogous modification in JOM-13 and DPDPE,[13,15,21] though in the cyclic peptides, δ binding actually was enhanced by the presence of the fluoro group and only slightly reduced for the *p*MePhe-containing analogs. In DPDPE analogs, binding to the δ opioid receptor correlated well with increasing electronegativity and decreasing lipophilicity of the halogen substituent at the *para* position of the phenyl ring, again demonstrating the importance of the Phe side chain in the binding interaction.[21] Overall, data suggest that the requirement for a particlar orientation and character of the Phe side chain may actually be more rigid in the linear heptapeptide than in the conformationally constrained analogs.



THi (**6**) 2-Pal (**7**) 3-Pal (**8**) His (**9**) Taz (**10**)

Figure 1. Structures of amino acid side chains for compounds **6**–**10**.

The next group of analogs (**6**–**10**, Figure 1) included those with aromatic heterocyclic replacements for Phe^3. In general, these analogs were less lipophilic than **1**–**5**, and the amino acid replacements should have minimal effects on the conformation of the peptide. The 3-(2-thienyl)alanine substitution (Thi, **6**) proved to be the best in the series (δ K_i = 1.38, μ K_i = 419), indicating a favorable effect on the δ binding interaction, in spite of possessing an electron-rich aromatic ring. Both δ and μ binding were effectively the same as that of deltorphin I. Since the 3-(3-benzothienyl)alanine (Bth) modification previously reported[20] also led to tight binding, the presence of a sulfur atom appears to be favorable. Thi is similarly accommodated in JOM-13, though δ binding is very slightly reduced.[14] The high affinity of **6** appears somewhat contradictory to observations for compounds with aromatic ring substituents, where data tend to suggest a harmful effect brought about by substitution with an electron rich aromatic system at residue 3[14,15] (as this five-membered heterocyclic system would be expected to be). As described above, electron-withdrawing substituents are more favored than electron-releasing substituents in analogs of this particular sequence containing substituted phenyl moieties at residue 3. However, relative RP-HPLC elution times (Table 2) indicate that this analog is the least polar of the heterocyclic analog group (with a lipophilic nature similar to that of the Bth analog), which may account for its binding properties.

The 3-(2-pyridyl)alanine (2-Pal, **7**) and 3-(3-pyridyl)-alanine (3-Pal, **8**) substitutions resulted in analogs with 30–35-fold reductions in δ binding (K_i = 62.4 and 49.7, respectively). μ binding also was affected adversely. Pal is hydrophilic but uncharged under assay conditions, which may contribute to the deleterious effect on binding. The heterocyclic six-membered ring would be expected to be electron-deficient relative to the five-membered ring analogs, and data generally support the preference for a relatively π-deficient system at this residue. However, in JOM-13, substitution with 3-Pal

at this position severely compromises δ binding;[14] thus, it appears that the hydrophilic nature of this substitution (or lack of lipophilicity) outweighs electronic characteristics in influencing opioid binding affinity.

The histidine-containing peptide (His, **9**) displayed substantially reduced opioid binding relative to the parent compound. The same effect has been observed in deltorphin I by other investigators[16] and in JOM-13[14] and most likely can be attributed to the partial positive charge on the imidazole side chain at the pH of the binding assay (physiological pH); the hydrophilic character of this analog is supported by its early relative RP-HPLC elution time as well. Again, the requirement for lipophilicity at this residue for opioid receptor interaction is well-supported.

Last, 3-(4-thiazolyl)alanine (Taz, **10**), with both nitrogen and sulfur heteroatoms, was substituted for Phe. The resulting compound exhibited reduced but reasonable δ affinity ($K_i = 39.5$) and slightly reduced μ binding ($K_i = 2800$). This binding result is comparable to those of the other analogs with uncharged nitrogen-containing side chains at residue 3 (**7** and **8**). This system, however, would be more electron-rich than **7** and **8** (an adverse influence). As indicated by RP-HPLC, the Taz analog is intermediate in polarity between those peptides containing Pal (**7** and **8**) and Thi (**6**), again implying a strong influence of the lipophilic character of this residue on opioid receptor binding. Within this series of analogs containing a heterocycle, δ affinity correlates well with relative lipophilicity. This is further supported by results obtained for [Trp^3]deltorphin,[16,22] which binds slightly better than these other analogs which contain a nitrogen atom in the residue 3 side chain aromatic ring; the bicyclic system with an additional phenyl ring improves the lipophilicity of this analog relative to **7**–**10**.

In summary, most modifications proved detrimental to δ (and μ) receptor affinity relative to deltorphin I, with the notable exceptions of *m*FPhe (**2a**) and Thi (**6**). As expected, all analogs bound extremely weakly to κ opioid receptors. Many modifications previously reported, whether steric, lipophilic, or electronic, are reasonably accommodated, with only extreme reductions in δ affinity resulting from substitution of Phe with less bulky, nonaromatic amino acids,[22] very bulky amino acids,[23] methyl-substituted analogs, and polar/charged analogs. Our data here suggest that size and electronic character are important influences on the opioid binding of analogs containing substituted phenyl moieties; however, relative lipophilicity within a group of similar analogs of approximately constant size appears to outweigh electronic character as a factor in determining effects on δ receptor binding affinity. In most cases, δ opioid receptor selectivity was reduced, but the Thi-containing analog (**6**) retained excellent selectivity as well as affinity. Most consequences arising from modifying deltorphin I were generally consistent with those observed upon identical modification in the cyclic, δ-selective opioid tetrapeptide, Tyr-c[D-Cys-Phe-D-Pen] (JOM-13), where comparison was possible. Data suggest that the side chain of residue 3 in these peptides interacts with a hydrophobic pocket of defined size in the δ receptor and that electronic character is reasonably important in the binding interaction. It is uncertain as to whether this hydrophobic pocket is the same cleft with which the Phe^4 residue of enkephalins aligns.

Experimental Section

Peptide Synthesis. Most protected amino acids and coupling agents were purchased from Bachem California (*p*FPhe, *m*FPhe, F_5Phe, Thi) and Bachem Bioscience (Me_5Phe); protected 2-Pal, 3-Pal, and Taz were purchased from Synthetec, and *p*MePhe was purchased from Chemalog. Solvents and deprotecting agents were obtainded from Fisher Scientific and Aldrich Chemical Co. Radioligands were purchased from New England Nuclear, Multiple Peptide Systems, and Amersham, and frozen guinea pig brains were obtained from Rockland, Inc. The peptides were prepared on a Milligen or St. John's Associates manual shaker using standard solid phase techniques for *N*-α-*tert*-butyloxycarbonyl- (Boc) protected amino acids on *p*-methylbenzhydrylamine (MBHA) resin (1.1 mmol/g). The side chains of Tyr and Asp were protected as the 2,6-dichlorocarbobenzyloxy and benzyl derivatives, respectively. The His imidazole nitrogen was protected with a benzyloxymethyl (BOM) group. The deprotection solution was 30% trifluoroacetic acid (TFA) in dichloromethane (DCM). Dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt) were used as coupling agents. The protocol for peptide synthesis in each cycle was as follows: (1) addition of Boc amino acid in DCM (3 equiv), (2) addition of HOBt (2.4 equiv), (3) addition of DCC (2.4 equiv), (4) mixing/shaking for 4 h, (5) washing with DCM (3 × 2 min), (6) checking for completion of reaction with ninhydrin test,[24] (7) Boc deprotection with 30% TFA in DCM (30 min), (8) washing with DCM (3 × 2 min), (9) neutralization with diisopropylethylamine (DIEA) in DCM (10 min), (10) washing with DCM (3 × 2 min). Simultaneous deprotection and cleavage from the resin were accomplished by treatment with 90% anhydrous HF and 10% anisole scavenger (10 mL of HF and 1 mL of anisole per gram of resin) at 0 °C for 1 h. After evaporation of the HF, the peptide resin was washed with diethyl ether and the peptide was extracted with 70% acteonitrile/30% water (with 0.1% TFA), concentrated under reduced pressure, diluted with water, and lyophilized. Crude peptides were purified to homogeneity by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a Vydac C18 column (2.2 × 25.0 cm, 10 mL/min) with a linear gradient of water (0.1% TFA) to 50% acetonitrile (0.1% TFA), followed by lyophilization. For compounds **2a** and **2b** and **5a** and **5b**, a diasteromeric pair of peptides was prepared using a racemic mixture of the Boc-D,L-*m*FPhe or Boc-D,L-Me_5Phe, respectively, at the appropriate coupling. The peptides were then separated by RP-HPLC under the conditions described above.

Peptide Analysis. Peptide purity was assessed by analytical RP-HPLC. Peaks were monitored at 230 and 280 nm. All compounds were at least 95% pure as analyzed by peak integration. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on a Bruker spectrometer at 250 MHz. Samples (ca. 1 mg) were dissolved in DMSO. Diagnostic resonances and peak patterns confirmed the presence of all indicated residues. Fast atom bombardment-mass spectroscopy (FAB-MS) or electrospray MS confirmed the appropriate molecular weights.

Opioid Receptor Binding Assays. Receptor binding assays measured displacement by the test compounds of radiolabelled receptor-selective ligands from guinea pig brain homogenates, using 1.2 nM [^{3}H]DAMGO for the μ receptor, 2.5 nM [^{3}H]DPDPE for the δ receptor and 1.0 nM [^{3}H]U69,-593 for the κ receptor. This protocol has been described previously.[13] IC_{50} values were obtained by linear regression from plots relating inhibition of specific binding to the log of 12 different ligand concentrations, using the RADLIG computer software program (Biosoft Software).[25] For binding to κ receptors, which was expected to be weak, the protocol was altered to include only five ligand concentrations and was performed in duplicate. K_i values were calculated using values for K_D of each ligand. Saturation binding experiments determined the K_D range of each ligand as follows: [^{3}H]DAMGO = 1.31–1.35 nM; [^{3}H]DPDPE = 1.60–1.72 nM; [^{3}H]U69,593 =

1.13−1.25 nM. K_i values reported represent the mean of one or two determinations, each performed in triplicate.

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