Function of Negative Charge in the "Address Domain" of Deltorphins

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Deltorphins A, B, and C exhibit high δ-affinities (K_i = 0.12–0.31 nM) and very high δ-receptor binding selectivities ($K_i\mu/K_i\delta$ = 1800–4100). A study of the δ-receptor binding properties of 15 deltorphin analogues focused primarily on the influence of the anionic group in the C-terminal tetrapeptide. Amidation of the β-carboxyl groups of [Asp⁷], [Glu⁴], or [Asp⁴] in deltorphins A, B, and C, respectively, yielded peptides with enhanced μ-receptor affinities and minor changes in δ-affinities ($K_i\delta$ = 0.20–0.65 nM), but 5–8-fold diminished δ-selectivities. A free C-terminal carboxyl group markedly reduced δ-affinities and decreased δ-selectivities 6–11-fold; thus, the C-terminal amide group critically facilitates δ-affinity. Modifications in the anionic charged group or hydrophobic residues in the C-terminal tetrapeptide address domain of deltorphin A altered spatial distributions critical for δ-affinity and selectivity; e.g., [Nle⁶]deltorphin A enhanced μ-affinity and lowered δ-selectivity by two-thirds; the progressive, step-wise repositioning of Asp in deltorphin C (from position 4 to 7) was accompanied by linear decreases in δ-affinities and -selectivities, and increased μ-affinities of these peptides; enhancement of the charge density to −2, in [Asp⁶,Asp-OH⁷]deltorphin A, decreased δ-affinity and -selectivity, while [Asp⁴,⁵,His⁷]deltorphin A bound to neither μ- nor δ-sites. These results demonstrate that while an anionic group may occassionally facilitate high δ-receptor affinity, it represents an absolute requirement for the high δ-binding selectivity of these peptides. The locations of the charged groups relative to hydrophobic residues in the address domain of the peptide are also critical determinants for both δ-affinity and -selectivity.

Introduction[1]

Prior efforts to correlate the structures of specific ligands with their abilities to bind to various opioid receptor types were conducted with opiate alkaloids,[2] several non-peptide opiate compounds,[3,4] a large variety of enkephalin-related analogues, and other opioid peptides,[5] as well as novel μ-opioid antagonists structurally related to somatostatin.[6] One key to the development of selective enkephalin analogues involved the introduction of a D enantiomer at position 2 in flexible[5,7-9] or conformationally constrained analogues;[5,10-12] this enabled the peptides to assume unique spatial conformations and charge distributions[11-13] and conferred enhanced stability in pharmacological and receptor assays.[14] The isolation of dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2; **21**) in 1981[15] provided evidence of the existence of a natural opioid peptide which contained a D-Ala² residue. In μ-opioid bioassays with peripheral tissues[15,16] and receptor assays with brain membranes,[17-19] the dermorphin family of peptides exhibits high affinities and selectivities comparable to those of several enkephalin analogues.[5,14]

The recognition of a peptide sequence encoded within a single clone of the dermorphin gene from skin of the frog *Phyllomedusa sauvagei*[20] eventually led to the discovery of a class of heptapeptides with high affinities and exceptional selectivities for δ-receptors;[21-28] sequences for two related peptides[24] were also subsequently found to be encoded in other skin cDNA clones along with other dermorphin-related peptides.[31] The first of these δ-receptor specific peptides to be characterized was named "deltorphin" (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2; **1**),[22] which was also called "dermorphin-gene-associated peptide"[21] and "dermenkephalin";[25-28] two other related peptides, [D-Ala²]deltorphin I (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2; **13**) and [D-Ala²]deltorphin II (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2; **10**),[24] were isolated and characterized. For the sake of clarity, these three peptides are designated in this report as deltorphin A (**1**), deltorphin B (**10**), and deltorphin C (**13**). The D configuration around the α-carbon at residue 2 is assumed to indicate post-translational modification since the peptides are encoded by a normal codon for the L form of that amino acid.[20,31]

Deltorphin A is capable of eliciting receptor-specific pharmacological responses in guinea pig ileum and mouse

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(1) Symbols and standard abbreviations for amino acids and peptides are as recommended by the IUPAC–IUB Joint Commission on Biochemical Nomenclature (JCBN) (*Biochem. J.* 1984, *219*, 345. *Europ. J. Biochem.* 1984, *138*, 9). Additional abbreviations are as follows: Boc, *tert*-butoxycarbonyl; BUBU, [D-Ser²(OtBu),Leu⁵]enkephalyl-Thr⁶(OtBu), where OtBu is O-*tert*-butyl ether; BUBUC, [D-Cys²(OtBu),Leu⁵]enkephalyl-Thr⁶(OtBu); Bum, *N*-(π)-*tert*-butoxymethyl; DAGO, [D-Ala²,NMePhe⁴,Gly-ol⁵]enkephalin; DADLE, [D-Ala²,D-Leu⁵]-enkephalin; DIC, diisopropylcarbodiimide, DMF, *N*,*N*'-dimethylformamide; DPDPE, H-Tyr-D-Pen-Gly-Phe-D-Pen-OH, where Pen, penicillamine, is β,β-dimethylcysteine; DSLET, [D-Ser²]enkephalyl-Thr⁶; DSBULET, [D-Ser²(OtBu)]enkephalyl-Thr⁶; Fmoc, *N*-[[(9-fluorenylmethyl)oxy]carbonyl]; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole, HPLC, high-performance liquid chromatography; Nle, norleucine; OtBu, *tert*-butyl ester; OSu, *N*-succinimidoyl ester; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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vas deferens preparations[22-25] and is reported to undergo coprocessing with a dermorphin-like peptide in mammalian tissues, including brain,[29] and may exert an effect on memory.[30]

The dermorphins and deltorphins share common generalized N-terminal tripeptide sequences, H-Tyr^1-D-Xxx^2-Phe^3 (where D-Xxx is either D-Ala^2 or D-Met^2), which comprise the message domains of these peptides.[3,32] The critical structural differences between these two classes of bioactive peptides lie in the C-terminal tetrapeptide region, the address domain,[3,32] adjacent to the N-terminal message domain. The initial definition of the role of a C-terminal tripeptide sequence in determining the selectivity characteristics of the deltorphins was provided by studies employing analogues lacking the charged functionality of deltorphin A[23,26-28] and a deltorphin A–dermorphin hybrid peptide.[26] Those data and that from dermorphin binding studies[17-19] appeared to fit the "membrane-assisted opioid receptor selection" receptor model which proposed (a) that selective interaction results from discriminatory recognition by the address and message specific binding components in the membrane receptor and (b) that δ-site selectivity involved ligand interaction with a positively charged membrane compartment.[33] Selective binding at the μ-site was suggested to be facilitated by an N-terminal β-turn in the peptide,[33] a conformation interestingly shared by dermorphin,[23,34-36] deltorphin,[23,34,35,37] enkephalin,[38,39] and the conformationally constrained DPDPE.[40]

Table I. Binding Parameters of Opioid Peptides[a]

no.	peptide	[^{3}H]DAGO; $K_i\mu$, nM	[^{3}H]DADLE; $K_i\delta$, nM	$K_i\mu/K_i\delta$
1	deltorphin A	335.9 ± 19.2	0.12 ± 0.02	2,799
2	[Asn^7]-A	118.6 ± 14.7	0.22 ± 0.06	539
3	[Asp-OH^7]-A	2,687 ± 120	10.76 ± 0.43	250
4	[Asp-Me^7]-A	3,221 ± 175	155.1 ± 5.99	21
5	[Asp^4]-A	10,571 ± 550	7.71 ± 0.53	1,371
6	[Asp^6]-A	3,408 ± 663	3.08 ± 0.78	1,106
7	[Asp^6,Asp-OH^7]-A	2,094 ± 175	6.01 ± 1.55	348
8	[Asp^{4,5},His^7]-A	1,571 ± 480	1,896 ± 11.6	0.8
9	[Nle^6]-A	206.3 ± 21.3	0.22 ± 0.02	938
10	deltorphin B	1,280 ± 192	0.31 ± 0.05	4,129
11	[Gln^4]-B	152.8 ± 33.2	0.20 ± 0.05	764
12	[Gly-OH^7]-B	5,849 ± 537	7.97 ± 0.54	734
13	deltorphin C	387.3 ± 39.4	0.21 ± 0.04	1,844
14	[Asn^4]-C	175.6 ± 27.7	0.65 ± 0.08	270
15	[Gly-OH^7]-C	1,640 ± 381	6.01 ± 0.87	273
16	[Val^4,Asp^5]-C	275.5 ± 56.8	1.23 ± 0.44	224
17	[Val^4,Asp^6]-C	149.5 ± 28.3	9.42 ± 0.51	16
18	[Gly^4,Asp^7]-C	57.5 ± 6.4	8.79 ± 0.72	7
19	DADLE	8.16 ± 0.59	0.26 ± 0.04	31
20	DAGO	0.66 ± 0.06	180.4 ± 45.9	0.004
21	dermorphin	0.70 ± 0.10	134.0 ± 24.4	0.005

[a] K_i values are means ± SEM with n = 3–11.

Design Rationale

Selection of deltorphin analogues for this study represents an effort to test the hypothesis of Schwyzer[32] that

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flexible peptide hormones contain "sychnologic organization", i.e., the polypeptide sequence is composed of proximal regions which exist as distinct message and address domains. The message domain provides information for signal transduction that leads to a biological response, while the address domain primarily influences binding affinities and accommodates the elements of selectivity without necessarily affecting transduction.[3]

In order to expand our understanding of the characteristics of the C-terminal tetrapeptide address domain of the deltorphins on opioid receptor affinities and selectivities, studies were conducted with several analogues of these peptides in which the spatial distribution of the negatively charged residue was repositioned relative to the hydrophobic residues.

Results and Discussion

The deltorphins exhibited high affinity and exceptionally high selectivities ($K_i\mu/K_i\delta$ = 1800–4100) for δ-receptors (Table I); the order of δ-binding selectivity among deltorphins (B > A > C) was comparable to published data.[21,22,24] The δ-selectivities of deltorphins A, B, and C were from 2–6-fold greater than those of BUBUC[41] and the [D-Cys^2,D-Pen^4]analogue of DPDPE;[42] however, one naltrindole derivative[3] exhibited δ-selectivity similar to that of deltorphin A. In general, δ-selectivities of deltorphins are 1–2 orders of magnitude greater than those of many enkephalin analogues, such as DADLE (Table I), DSLET,[8,21] the sterically constrained[43] DSTBULET,[9,41] and BUBU,[41] or the cyclized, conformationally constrained enkaphalin analogue DPDPE[10,21] and several of its derivatives.[44] Extensive reviews of enkephalin-related peptides appear elsewhere,[5,14] while relevant publications are cited herein.[9,11,13,41-45]

The high δ-receptor affinities of [Asn^7]deltorphin A (**2**) and [Gln^4]deltorphin B (**11**) (K_i = 0.22 and 0.20) were similar to those of the parent peptides (**1** and **10**; Table I), while the δ-affinity of [Asn^4]deltorphin C (**14**) (K_i = 0.65) was only one-third of that found for deltorphin C (**13**). These data indicate that high δ-receptor affinities are essentially unrelated to the presence of a negatively charged amino acid in the address domain; an analogous relationship characterizes the relatively high receptor affinities and δ-selectivities of *tert*-butyl derivatives of enkephalin, e.g., DSTBULET[9,45] and, in particular, BUBUC.[41] The decreased δ-selectivities of the internally amidated peptides (Table I) were consequences of increased μ-receptor affinity, which agrees with a previous observation involving [Asn^7]deltorphin A.[27,28] The δ-selectivities of these amidated deltorphins still equaled or exceeded those of many enkephalin-derived peptides,[5,8-10,21,44] including DADLE (**19**).

Modification of the C-terminal amide group to obtain deltorphin A, B, and C analogues with free carboxylic groups (**3**, **12**, and **15**) resulted in respective declines of δ-receptor affinities by factors of 90-, 26-, and 29-fold, respectively; the change in δ-affinity of deltorphin A-OH was far greater than the 20–50% decreases reported by Mor et al.[27] and Sagan et al.[28] The increases in electronegativities of these analogues correlated with 4–8-fold decreases in μ-receptor affinities and correspondingly diminished δ-selectivities (Table I). Clearly, the C-terminal amide group facilitates high δ-receptor affinities for each deltorphin: a similar correlation was observed for μ-receptor affinity[17,18-21] and bioactivity of dermorphin,[16] as well as for binding of dermorphin-related peptides[19] and the biological activities of μ-receptor specific peptides.[8,9,46] Substitution of the C-terminal amide group of deltorphin A by a methyl ester (**4**) reduced its δ-receptor affinity by nearly 1300-fold while its μ-affinity only diminished about 10-fold; as a consequence, δ-selectivity fell over 2 orders of magnitude (Table I). The changes in affinities of this peptide for μ- and δ-receptor sites represent interesting contrasts with the consequence of substitution of bulky hydrophobic constituents on the C-terminal residue of dermorphin, in which μ- and δ-receptor affinities increased, although the peptides remained μ-selective.[19]

Modification of the hydrophobic properties of deltorphin A was limited to alterations in the penultimate and C-terminal residues, Met^6 and Asp^7. Substitution of Met^6 by Nle (**9**) yielded a peptide which exhibited a relatively high δ-affinity (K_i = 0.22 nM); however, since μ-affinity increased by 40%, δ-selectivity subsequently dropped 3-fold (Table I). The inclusion of Asp at position 4 (**5**), 6 (**6**, **7**), or 4 and 5 (**8**) gave these peptides net negative charges of −1 (**5**, **6**, and **8**) or −2 (**7**), and diminished their δ-affinities by 25–60-fold, while μ-affinities diminished only 15–25-fold. Although their δ-affinities were comparable to those of DSLET,[9] DSTBULET,[9] and DPDPE,[10] peptides **5** and **6** exhibited δ-selectivities greater than those of many other enkephalin-derived peptides.[5,14] Peptide **8** ([Asp^{4,5},His^7]deltorphin A) exhibited very low affinities for either receptor types and was essentially nonselective. These results suggest that Leu^5 in deltorphin A is involved in hydrophobic bonding between the peptide and receptor; however, other interpretations may be that an induced conformational change leads to charge repulsion between the side chains of adjacent Asp residues or to displacement of complementary hydrophobic residues.

Deltorphin C analogues include those in which the Asp residue was repositioned progressively toward the C-terminus (**16–18**). The δ-affinities decreased, approaching 45-fold, as the Asp residue was moved from position 4 to 6 or 7 in these peptides; the μ-affinities of each analogue *increased* to reach nearly 5-fold in [Gly^4,Asp^7]deltorphin C (**18**) (Figure 1). The δ-selectivities of peptides **17** and **18** were more than 2 orders of magnitude less than that of deltorphin C (Table I). These data indicate participation of the amino acid residue at position 4 of deltorphin C in determining receptor selectivity. This interpretation is based upon the following assumptions: (a) the N-terminal tetrapeptide region of [Gly^4,Asp^7]deltorphin C (**18**) is identical with that of dermorphin[15] and should assume an N-terminal β-turn conformation.[23,34-37] In dermorphin, Gly^4 participates in intramolecular hydrogen bonding[38] which leads to a relatively constrained, folded conformation[36] which permits the peptide to bind to the message complement within the receptor.[19] (b) The hydrophobic nature of the Val residues at positions 5 and 6 permits participation in binding to the δ-receptor through nonionic

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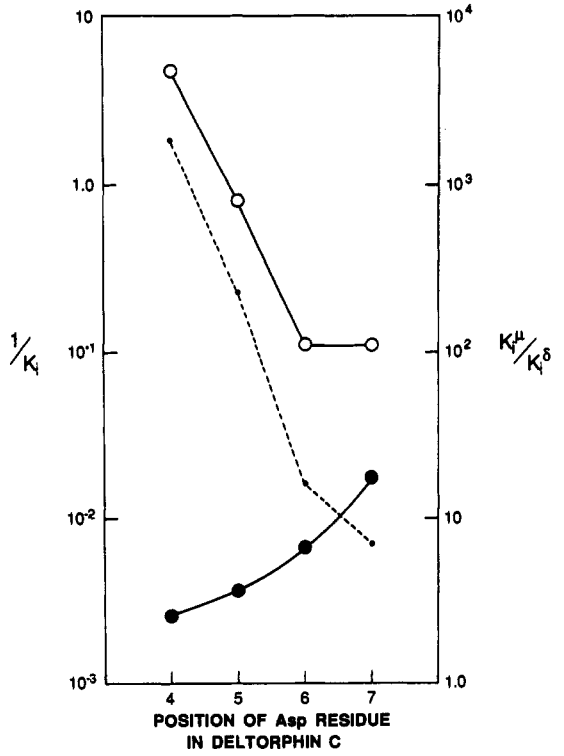

Figure 1. Differential affinities and δ-receptor binding selectivities of deltorphin C analogues. Log values of the reciprocal affinities (K_i) (Table I) are plotted against the position of the Asp residue in deltorphin C. Symbols indicate the following: open circles, δ-affinity; closed circles, μ-affinity; and dashed line, δ-selectivity.

interactions; the high δ-affinity and -selectivity of [Nle^6]deltorphin A (**9**) support this interpretation.

^{1}H NMR data indicated that coupling involving the aromatic ring protons of Tyr^1 and Phe^{3,23,37} and the β- and γ-protons of CH_2 of D-Met^2 in deltorphin A^{23} yielded similar chemical shifts to those involving the methyl protons in D-Ala^2 in dermorphin^{23,35} or in deltorphins B and C.^{37} Therefore, the common sequences, H-Tyr^1-D-Xxx^2-Phe^3, in dermorphin[15] and deltorphins[21-28] are assumed to exhibit similar solution conformations. The findings that deltorphins A^{23} and C^{37} exist in solution as equilibrium mixtures of folded and more extended conformers[23] may indicate that extended linear peptides are capable of adopting a folded conformation upon interaction with an opioid receptor.[46] Similarly, circular dichroism data was correlated with the biological activities of enkephalin analogues to provide support for the arguments that δ-receptors have the capacity to bind both flexible and conformationally constrained peptides.[46]

The consequences of amidation of the anionic functional group at position 4 or 7 of deltorphins A, B, or C (peptides **2**, **11**, and **14**) (Table I) are an enhancement of μ-receptor affinities by 2.5–8-fold with only moderate changes in δ-binding, which led to diminished δ-selectivities of these peptides. These results indicate that the most prominent role of the anionic residue in the deltorphins is to prevent binding to the μ-receptor rather than to enhance δ-affinity. The environment of the binding site of the δ-receptor must be such that at position 4 neither an anionic group (carboxyl) of deltorphins B or C nor a cationic group (imidazole of His) of deltorphin A would prevent binding to the δ-receptor; thus, it seems possible that those charged groups assume a spatial orientation which does not adversely affect association during binding to the complementary surface of the δ-receptor. Our results do not preclude suggestions for peptide-conformation-induced fit,[46] receptor-induced changes following binding the peptide message domain, or binding to nonspecific membrane components.[33] Should the latter concept[33] be invalidated, our results provide support that although μ- and δ-receptors lack identity in their address binding regions, the common message-binding regions of these receptor types are likely to contain considerable structurally homology.[47-49]

Conclusions

Opioid receptor binding of deltorphin, as well as dermorphin, is apparently facilitated by a common sequence in the message domain tripeptide[23,37] which participates in an N-terminal β-turn.[23,35-37] The entire C-terminal tetrapeptide address domain sequences of the deltorphins, rather than just a C-terminal tripeptide,[26-28] are responsible for their high δ-receptor binding affinities and high selectivities. An anionic group in the address domain does not appear essential for high δ-affinity, but is required for the remarkable δ-selectivities of the deltorphins. Repositioning the Asp residue within the address domain of deltorphins A and C modifies binding affinity and selectivity for both opioid receptor types. Residues Leu^5 and Met^6 in deltorphin A and Val^{5,6} in deltorphins B and C provide significant hydrophobic bonding contributions to the conformational alignment of the peptide ligand at the receptor site. The C-terminal amide may facilitate anchoring of the ligand to the receptor through hydrogen bonding or contribute to peptide folding; alternately, amidation may lessen or prevent repulsion by a negatively charged group within the δ-opioid receptor.

The high affinities and remarkable selectivities of the deltorphins for the δ-receptor type make these peptides potentially useful models for studying the interaction of δ-ligands on a variety of biochemical events. Since their δ-opioid receptor affinities generally exceed those of the conformationally restricted cyclic[5,10-12] or flexible enkephalin-derived peptides,[5,7-9,11,12,14,45] alteration of amino acid sequences of the deltorphins can be expected to provide new insights into the nature of the receptor–peptide interactions.

Experimental Section

Overview of Methods for Peptide Synthesis. The syntheses of the deltorphins and their analogues were carried out by solid-phase methods[50-56] developed by Merrifield.[57] Fmoc-Asp(OtBu)-R, Fmoc-Gly-R (R = *p*-(benzyloxy)benzyl alcohol resin[50]),

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Table II. Analytical Data on Deltorphin Analogues

no.	amino acid analysis							TLC[a] I	TLC[a] II	HPLC:[b] K'	$[\alpha]^{22}_D$,[c] deg
	Tyr	Met	Phe	His	Leu	Nle	Asp				
1	0.97	1.97	0.98	1.02	0.99		1.00	0.45	0.38	6.63	−9.5
2	0.89	2.01	1.00	0.97	0.97		1.01	0.59	0.37	4.76	−8.2
3	1.00	1.99	0.98	0.89	0.94		0.99	0.65	0.14	5.36	−6.3
5	0.94	1.89	0.97		1.0		1.95	0.62	0.50	5.65	−26.3
6	0.89	0.93	1.01	0.94	1.0		1.94	0.38	0.18	4.91	+16.7
7	0.89	1.01	1.00	0.89	1.02		1.99	0.56	0.10	4.47	−11.2
8	0.9	1.95	1.04	0.93			2.01	0.42		4.63	−15.0
9	0.94	0.95	0.98	0.91	0.95	0.90	1.02	0.65	0.43	6.96	−12.3
	Tyr	Ala	Phe	Glu	Val[d]	Gly					
10	0.88	1.00	1.02	1.01	1.77	1.00		0.62	0.45	6.17	−15.2
11	0.94	1.00	1.02	0.98	1.78	0.99		0.66	0.48	5.77	−4.0[e]
12	0.98	1.02	1.00	1.02	1.79	1.03		0.65	0.31	3.43	−3.5[e]
	Tyr	Ala	Phe	Asp	Val[d]	Gly					
13	0.96	1.00	1.01	0.98	1.85	1.03		0.51	0.42	6.34	−16.0
14	1.02	1.00	0.98	0.95	1.79	1.08		0.67	0.52	5.97	−5.1[e]
15	0.95	0.97	0.98	1.01	1.86	1.02		0.54	0.61	3.59	−9.0[e]
16	0.91	0.98	0.97	1.0	1.95	0.97		0.71	0.33	6.43	+0.53[e]
17	0.95	1.02	0.99	1.01	1.76	1.03		0.79	0.38	6.65	−7.2[e]
18	0.89	0.97	0.91	0.89	1.75	1.02		0.51	0.40	6.35	−16.2

[a] TLC solvent systems were as follows: I, 1-butanol/HOAc/H_2O (6:1:5); II, ethyl acetate/pyridine/HOAc/H_2O (60:20:6:11). [b] Capacity factor (K') for the following conditions: a IB01 reversed-phase C_{18} 5-μm column (250 × 45 mm) eluted in a linear gradient from 20% mobile phase A (10% acetonitrile in 0.1% TFA) to 80% mobile phase B (60% acetonitrile in 0.1% TFA) in 30 min at a flow rate of 1.0 mL/min. [c] Optical rotations were either in methanol or DMF as noted. [d] Val was determined after a 72-h hydrolysis. [e] DMF.

and Fmoc-protected amino acids were products of Novabiochem, AG. Resins for peptide amides [CH_3O-Ph(1,4)-CH(NH-Fmoc)-Ph(1,4)-O$(CH_2)_3$-CONH-CH(CH_3)-CONH-CH_2-Ph-polymer[51]] were purchased from Bachem, Hanover, Germany. With the exception of the N-terminal α-Boc-Tyr, α-Fmoc-amino acid derivatives were used in the coupling reactions. Reactive side chains were protected as follows: Asp and Glu with *tert*-butyl ester and His with *tert*-butoxymethyl. Amino acids were double-coupled to the growing peptide chain with preformed symmetrical anhydrides,[52] except Fmoc-Asn-OH, Fmoc-Gln-OH, and Fmoc-His(Bum)-OH, which were coupled by the DIC–HOBt[53] procedure. The N-terminal Boc-Tyr-OH was coupled as a preformed OSu ester.[54] Peptide amides were obtained by removal of Fmoc groups from the resin by the usual protocol, followed by coupling Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, or Fmoc-His(Bum)-OH with DIC-HOBt.[54] Peptide **2**, containing Asn at the C-terminal end was obtained by anchoring Fmoc-Asp-NH_2, with its side chain, to a resin with the DIC-HOBt[53] procedure. All peptides were synthesized with the use of a SAP 4 semiautomatic peptide synthesizer manufactured by SAMPA-Chimie, Paris, France.

Cleavage of Fmoc protective groups, by 55% (v/v) piperidine in DMF, was monitored at each stage by measuring the absorbance of the liberated *N*-(9-fluorenylmethyl)piperidine.[55] Coupling was monitored by the standard ninhydrin test[56] and by amino acid analysis of the acid hydrolysates; coupling was repeated when necessary.

Complete protected peptide resins were treated with 20 mL of TFA/thioanisole/β-mercaptoethanol (10:0.8:0.2) at 20 °C for 3 h; the mixture was filtered and the resin washed with TFA/CH_2Cl_2 (1:1). The combined filtrate and washings were evaporated and the free peptides precipitated with diethyl ether. The crude peptides contained several minor impurities based on HPLC and were purified by gel filtration and HPLC (infra vide). The analytical parameters of the purified peptides are listed in Table II.

Specific Solid-Phase Synthesis Methods. Fmoc-aa-R [aa = Asp-(OtBu) or Gly] (1 g, 0.79 mmol) or resin for peptide amides (1 g, 0.55 mmol) was washed by two 15-mL portions of DMF; the Fmoc group was cleaved by a single treatment with piperidine in 55% (v/v) DMF for 20 min. The resin was washed with DMF, H_2O/dioxane (1:2), and DMF (2 × 2 min) to which was added 2 equiv of preformed Fmoc-amino acid symmetrical anhydride in CH_2Cl_2/DMF (2:1) for 20 min followed by 1 mL of diisopropylethylamine/g of resin. The couplings were repeated with 2 equiv of freshly preformed anhydride, and after 50–60 min, the resins were washed with CH_2Cl_2/DMF (2 × 2 min). Fmoc-Asn-OH, Fmoc-Gln-OH, and Fmoc-His(Bum)-OH (0.8 mmol) were introduced by coupling with DIC (0.9 mmol) and HOBt (1.5 mmol) in DMF for 2 h. Coupling with DIC/HOBt was also used to insert Fmoc-Asp-NH_2 (0.8 mmol) on the resin through the side chain carboxylic acid group. After cleavage with 90% (v/v) TFA, HPLC analysis of the Fmoc-Asn-NH_2 product indicated nearly quantitative coupling (0.48 mmol/g of resin). The Fmoc group was assessed in each cycle by UV determination at 312 nm; aliquots of Fmoc-peptide resin were removed periodically and cleaved from the resin in 95% TFA (or TFA scavengers, depending on the particular step in the synthesis) and the intermediate peptides assessed for purity by HPLC.

In all peptides, the N-terminal Tyr was double-coupled as preformed Boc-Tyr-ONSu (1 mmol) in DMF for 3 h. In some cases, the residual resin was treated a second time with TFA/β-mercaptoethanol (10:1). The peptides were cleaved from the resin essentially as stated above.

Purification Methods. Peptides were initially purified on Sephadex LH-20 columns (2.5 × 100 cm), using methanol and HOAc/H_2O (2:80) as the mobile phase. Fractions were individually examined by TLC on Merck precoated silica gel plates F_{254} and developed in 1-butanol/HOAc/H_2O (6:1:5) and ethyl acetate/pyridine/HOAc/H_2O (60:20:6:11). Peptides were visualized by ninhydrin followed by Cl_2–starch or with Pauly reagent. The pooled fractions were then purified by preparative reversed-phase HPLC using a Waters Delta Prep 3000 with a Delta Pak C_{18} 300 A 15-μm spherical particle column (30 × 300 mm). Peptides were eluted in a linear gradient from 25% to 50% acetonitrile in 0.1% TFA at a flow rate of 30 mL/min and monitored at 220 nm. Fractions were surveyed by TLC and analytical HPLC; the main components were pooled and repeatedly lyophilized from dilute HOAc. Purified peptides were examined for homogeneity by analytical HPLC using an IB01 C_{18} 5-μm column (4.5 × 250 mm); a 30-min linear gradient was run at 1.0 mL/min from 20% mobile phase A (10% acetonitrile in 0.1% TFA) to 80% mobile phase B (60% acetonitrile in 0.1% TFA). Purification and quantification of peptides were performed with a Bruker LC 21-C HPLC system, equipped with a Bruker LC 313 UV variable-wavelength detector to monitor absorbance at 220 and 254 nm and were recorded on an Epson (QX-10) computer system. All peptides were determined to be at least 99% pure.

The purities of radioligands and peptide esters were estimated with a Spectra Physics Model SP8800 HPLC system and the absorbance at 215 nm was monitored with a variable-wavelength detector (LDC SpectraMonitor III). A Pierce RP-300 10-μm

column (4.8 × 220 mm) was coupled to a precolumn containing the same immobile support; peptides were eluted at 1.0 mL/min using a 1%/min linear gradient from 100% mobile phase A (0.1% TFA) to 56% mobile phase B (90% acetonitrile in 0.1% TFA).

Other Methods. UV spectra were measured on a JASCO Uvidec-510 recoding spectrophotometer. Peptide hydrolysis was performed in constant boiling HCl (110 °C for 22 h) containing 1% phenol; amino acid analyses were performed in a Carlo Erba 3A-29 amino acid analyzer. Optical rotation measurements were conducted in methanol or DMF (Table II) with a 10-cm pathlength in a Perkin-Elmer 241 polarimeter. ^{1}H NMR spectra (taken by Dr. T. Tancredi, Naples, Italy, using a Bruker AM-400 spectrometer equipped with an Aspect 3000 computer) were obtained for each analogue and were consistent with the anticipated sequence and structure of the peptides.[23,37]

The radioligands [^{3}H]DAGO (60.0 Ci/mmol) and [^{3}H]DADLE (44.1 Ci/mmol) were purchased from the Amersham Corp., Arlington Heights, IL; [^{3}H]DADLE (30.0 Ci/mmol) was also obtained from NEN/Du Pont, Bellrica, MA. DAGO and DADLE were products of Bachem, Torrance, CA. Dermorphin was synthesized by solution methods described in detail elsewhere.[58] Esterification of [Asp-OH^7]deltorphin A took place in 50% ethanol with diazomethane in diethyl ether (carried out by Dr. L. A. Levy). Protonated molecular ion determinations were carried out with a VG-ZAB-4F spectrometer, VG Analytical, Altrincham, UK (conducted by Dr. K. Tomer), as published elsewhere,[59] on several peptides: e.g., the observed $(M + H)^+$ for peptides **1**, **5**, **6**, **7**, **8**, and **9**, rounded off to the nearest whole number, were 955, 933, 939, 940, 957, and 937, respectively.

Binding Assays. Receptor binding assays were conducted on a P_2 membrane preparation of rat brain,[60] incubated to reduce the level of endogenous opioids,[18,19,21] based on the studies of Chang et al.[61] The synaptosomal membranes in the P_2 fraction were suspended by homogenization in buffer (50 mM HEPES, pH 7.5) containing 50 μg/mL soybean trypsin inhibitor, 100 mM NaCl, and 10^{-4} M GDP (3 volumes/g wet weight) and kept at 23 °C for 60 min. Following centrifugation at 40000*g* for 20 min at 4 °C, the pellets were washed three times in ice-cold 50 mM HEPES, pH 7.5, containing 50 μg/mL soybean trypsin inhibitor, and the final pellet was resuspended (40 mg/mL) in the same buffer solution containing 20% glycerol (v/v).[18,19,21] The concentrations of [^{3}H]DAGO and [^{3}H]DADLE radioligands in the assay were 0.68 and 0.87 nM, respectively.[19] Incubations with 1.6 mg of synaptosomal protein from three to five separate preparations were carried out for 60 min at 21–23 °C in assay media containing 50 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 8% glycerol, 1 mg BSA, 1 μM bestatin, 4 μg bacitracin, and 32 μg soybean trypsin inhibitor.[18,19,21] Under these conditions there is very little degradation of the peptides; these D-amino acid containing peptides are generally resistant to proteolysis.[24] The δ-binding assays, in addition, contained 2.6 μM [NMe-Phe^3,D-Pro^4]morphiceptin to suppress potential interference with endogenous μ-sites without effecting δ-binding when using [^{3}H]DADLE as the radioligand.[14,61,62] The membranes were trapped on wetted glass-fiber filters (GF/C) and washed with an ice-cold solution of 50 mM Tris-HCl, pH 7.5, and BSA (1 mg/mL) (5 × 1 mL). The IC_{50} values were estimated from semilogrithymic plots of the peptide concentration (nM) versus the percent of receptor-specific bound ligand.

The binding inhibition constants (K_i) were calculated according to the equation of Cheng and Prusoff:[63] $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of the radioactive ligand, and K_d represents the dissociation constant for [^{3}H]DAGO or [^{3}H]DADLE (0.99 ± 0.02 nM and 1.37 ± 0.44 nM, respectively) calculated according to published methods;[64] the K_i values are similar to published data.[14,65]

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Registry No. **1**, 119975-64-3; **2**, 124411-85-4; **3**, 131131-14-1; **4**, 132103-64-1; **5**, 132103-65-2; **6**, 132103-66-3; **7**, 132103-67-4; **8**, 132103-68-5; **9**, 132103-69-6; **10**, 122752-16-3; **11**, 131131-19-6; **12**, 131152-65-3; **13**, 122752-15-2; **14**, 132125-57-6; **15**, 132103-70-9; **16**, 132125-58-7; **17**, 132125-59-8; **18**, 131152-66-4.

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