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Unique sequence in deltorphin C confers structural requirement for δ opioid receptor selectivity

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Summary — A series of deltorphin C (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) analogues were synthesized to assess the consequences of changing anionic and hydrophobic residues on δ receptor selectivity. Analogues with altered C-terminal groups, inverted sequences, or esterified with *tert*-butyl, benzyl, or ethyl groups revealed that high δ selectivity required an unmodified amino acid sequence. Shifts of Asp and hydrophobic residues decreased δ selectivity due to loss in δ affinity (5- to ≈ 700-fold); μ affinity was unchanged or increased 14-fold. Suppression of charge or deamidation diminished δ selectivity through reduced δ and modified μ affinities. Data provide evidence that a negative charge does not *a priori* guarantee high selectivity and specific alignment of anionic and hydrophobic residues might facilitate optimum spatial configuration which complements the δ receptor binding site.

deltorphin C / peptide synthesis / opioid receptors

Introduction

The deltorphins represent a family of amphibian peptides [1–3] which contain a D-amino acid at position 2 and exhibit high selectivity for δ opioid receptors when tested in pharmacological assays on isolated tissues or brain synaptosome preparations [2, 4–10]. In the context of the hypothesis that peptide hormones contain two structural domains [11, 12], the deltorphins also appear to be composed of structural elements that effect binding parameters to μ and δ opioid receptor binding sites: a) a N-terminal tripeptide contains the generalized sequence, H-Tyr-D-Xaa-Phe (where D-Xaa[2] is D-Met[2] in DEL A or D-Ala[2] in deltorphins B and C [2], and dermorphins [13]), which specifies μ binding [8–10, 14–16]; and b) a C-terminal tetrapeptide region which apparently determines binding to the δ receptor site [8, 10, 15, 17, 18]. Conformational analyses by ^{1}H-NMR spectroscopy [14, 19–23] indicate that a β-turn occurs in the backbone structure in the N-terminal tetrapeptide which appears to form H-bonds with residues in the C-terminal tripeptide of deltorphin [22, 23] to maintain a preferred solution conformation.

Structure–activity studies of deltorphin analogues primarily modified in the C-terminal region focused on the involvement of amino acids whose side-chains impart a negative charge [8, 15, 17, 22] or confer hydrophobic properties [24, 25] to influence δ receptor affinity and selectivity. The goal of this study therefore was to assess and delineate the role of specific residues that facilitate binding to δ and μ opioid

Abbreviations: Boc, Nα-*tert*-butoxycarbonyl; BSA, bovine serum albumin; DAGO [D-Ala[2],*N*-Me-Phe[4],Gly-ol]enkephalin; DCC, *N,N'*-dicyclohexylcarbodiimide; DEL, deltorphin; DEL A, deltorphin A (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2), which is also referred to as 'dermenkephlin' [7] and 'dermorphin gene associated peptide' [4]); DEL B, deltorphin B, H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2; DIEA, *N,N'*-diisopropylethylamine; DMF, dimethylformamide; DPDPE, cyclic [D-Pen[2,5]]enkephalin; EtOAc, ethyl acetate; Fmoc, *N*α-9-fluorenylmethyloxycarbonyl; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MA, mixed anhydrides; NMM, *N*-methylmorpholine; OBzl, benzyl ester; O*t*Bu, *tert*-butyl ester; OEt, ethyl ester; ONp, *p*-nitrophenol ester; OSu, *N*-succinimidoyl ester; PITC, phenylisothiocyanate; TFA, trifluroacetic acid; Z, benzyloxycarbonyl.

receptors in order to test the hypothesis of Schwyzer [26] whether a negatively charged ligand specifically interacts at the δ receptor binding site. A variety of amino acid inversion and derivatized analogues of DEL C were prepared for this study. DEL C was chosen since its C-terminal sequence is less polar than that of DEL A [2] and yet exhibits twice the receptor selectivity of DEL A [2, 17]. Our results lead to the proposal that δ receptor affinity and selectivity apparently require a ligand with a specific, defined amino acid sequence that would appear to influence spatial conformation: repositioning of residues between 4 through 7, in addition to derivatization of amino acids 4, 7, or both, appear to be critical for peptide–receptor interaction to the receptor site.

Results and discussion

Chemistry

DEL C **1** and analogues **2–12** were synthesized by solution methods involving tri- and tetrapeptides (3 + 4 coupling techniques) using established procedures [22, 27–29] as detailed in figure 1. The tripeptide intermediates were prepared step-wise starting from the C-terminal residue using OSu or ONp. C-Terminal tetrapeptide intermediates were prepared according to the active OSu method followed by a deblocking step involving catalytic hydrogenation. Z-Val-OSu, Z-Gly-OSu or Z-Asp(O*t*Bu)-OSu were allowed to react with aspartic acid O*t*Bu amide, glycine amide, glycine

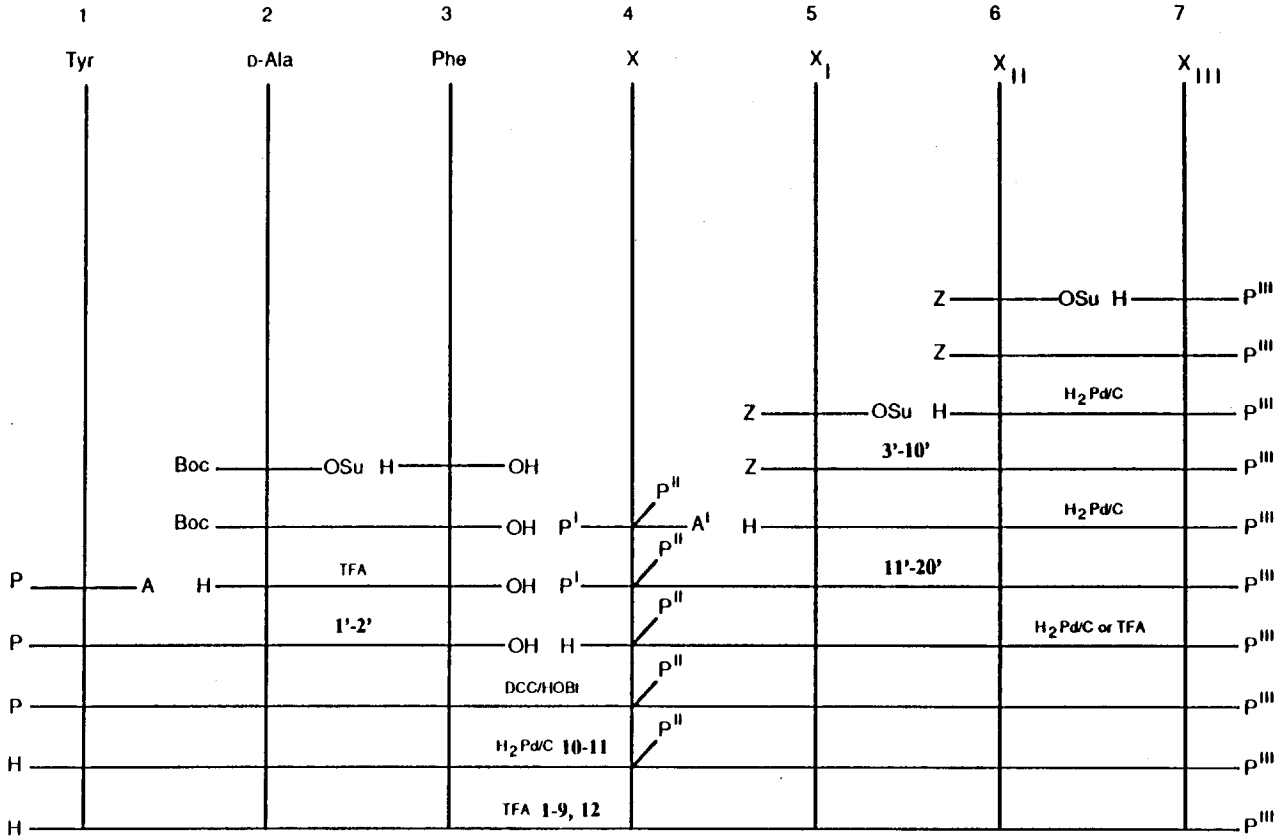

Fig 1. Scheme for the solution synthesis of deltorphin C (peptide^{I}) and analogues **2–12** involving techniques for coupling tri- and tetrapeptides (3 + 4 coupling). P, Protecting group of NH_2-Tyr^1 function, where Z was used in the synthesis of peptides **10** and **11**, and Boc for peptides **1–9**, and **12**. A, Activating group of Tyr^1-COOH function, where ONp was used when P = Z and OSu when P = Boc. P^{I}, Protecting group of NH_2-X (where X = Asp^4, Asn^4, Val^4 or Gly^4), in which Z was for the synthesis of peptides **1–11**, and Boc for compound **12**. P^{II}, Protecting group of Asp^4(COOH) function, where O*t*Bu was used for the synthesis of peptides **1**, **5–8**, **10**, **11** and OBzl for peptide **12**. A^{I}, Activating group of X α-COOH function as follows: MA was used for Asp^4, OSu for Val^4 and Gly^4, and ONp for Asn^4; X_I for Val^5 for peptides **1**, **3**, **4**, **6–12**, Gly^5 and Asp(O*t*Bu)^5 for peptides **2** and **5**; X_{II} for Val^6 peptides **1**, **2**, **4**, **5**, **7–12**, Gly^6 for peptide **6**, and Asp(O*t*Bu)^6 for peptide **3**. P^{III} Protecting group of X_{III}-COOH function, where X_{III} = Gly^7, Val^7, and Asp(O*t*Bu)^7; NH_2 was the protecting group for the synthesis of peptides **1–6**, **10** and **12**; O*t*Bu for peptides **7** and **11**; and OEt for peptides **8** and **9**.

O*t*Bu, glycine OEt, or deprotected peptides H-X_{II}-X_{III}-P^{III}. The last synthesis step was the condensation of the aspartic acid residue by the mixed anhydride method; valine or glycine as OSu activation to give protected tetrapeptides. When X was asparagine, the corresponding tetrapeptide intermediate was synthesized using the active ONp of Z-Asn. The final 3 + 4 condensation was obtained in good yield using DCC in the presence of HOBt [27].

Receptor evaluation

The alterations in the hydrophobic and anionic properties of DEL C analogues adopted the following strategy: i) sequential repositioning of Asp from residue 4 through 7 (**1–4**); ii) inversion of the Val residues with Gly modified the hydrophobic side-chain spatial localization in the C-terminal sequence (**5** and **6**); iii) changes in C-terminal derivatization (**7** and **8**); iv) esterification of Asp^4 (**10** and **12**); and v) combined modifications of Asp^4 and C-terminus (**9** and **11**). Esterification restricts conformational flexibility [30, 31, 34] and has the potential to modify H-bonding properties [14, 20, 22, 23].

All modifications within the C-terminal tetrapeptide portion of the peptides were detrimental for δ affinities compared to that of DEL C (**1**): the analogues exhibited substantial losses in δ selectivities through diminished δ affinities and variable μ affinities (table I). Movement of Asp from position 4 through position 7 (**2–4**) elicited decreased δ affinities from 5- to 25-fold and progressively improved μ affinities (nearly 14-fold), concomitant with an over 140-fold loss in δ selectivity. Since the N-terminal sequence of peptide **4** is identical to that in the μ selective agonist dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) [13, 32, 33], the C-terminal tripeptide sequence would appear to confer δ selectivity on the deltorphins as suggested earlier [8, 17, 18]. The intercalation of Gly^5 **5** between Asp^4 and the hydrophobic dipeptide, Val^6–Val^7 would alter the spatial relationship between their respective side-chains and decreased δ affinity and selectivity ≈ 700-fold without significantly affecting μ affinity. Importance of a hydrophobic residue at the fifth position was similarly observed in binding studies with analogues of DEL A [25] and DEL B [24] and is supported by ^{1}H-NMR studies [14, 20, 22, 23]: existence of an amino acid with a hydrophobic side-chain at residue 5 appears to be related to the maintenance of a hydrophobic surface above the molecular plane of the N-terminal β-turn [14, 22]. In this regard, the nearly 10-fold difference between δ affinities of peptides **6** and **5** may be due to the presence of the aliphatic side-chains of Val^5. The importance of a hydrophobic residue at position 6 is indicated by the inversion of the C-terminal dipeptide sequence (**6**) which brought about a 100-fold loss in δ selectivity. Interestingly, the corresponding modification in DEL A only resulted in a 7-fold decline (unpublished results), which suggests that the side-chain of residue 6 plays a role in receptor binding in DEL C [24].

Table I. Affinities and selectivities of peptide analogues for opioid receptor sites.

No	*Peptide*	$K_i\delta$	$K_i\mu$	$K_i\mu/K_i\delta$
1	Tyr–D–Ala–Phe–Asp–Val–Val–Gly–NH_2	0.32 ± 0.06	461.5 ± 38.7	1442
2	———Val- Asp———	1.58 ± 0.42	154.9 ± 27.4	98
3	———Val—— Asp———	8.05 ± 1.84	83.9 ± 13.8	10
4	———Gly——— Asp ——	3.36 ± 0.16	33.3 ± 3.1	10
5	——— Gly—— Val ——	216.5 ± 16.8	512.7 ± 3.0	2
6	———Gly–Val ——	25.4 ± 5.7	365.6 ± 13.8	14
7	——————— OH	6.78 ± 0.84	1704 ± 269	251
8	———————OEt	2.67 ± 0.87	1116 ± 69	418
9	——— Asn ———OEt	9.00 ± 2.4	146.7 ± 25.3	16
10	——— Asp(O*t*Bu)———	1.85 ± 0.41	320.1 ± 63.1	173
11	——— Asp(O*t*Bu) — Gly(O*t*Bu)	85.9 ± 20.8	1156 ± 174	13
12	——— Asp(OBzl)———	1.16 ± 0.13	23.4 ± 3.0	20

[a]Straight lines indicate sequence homology with deltorphin C. DEL C [17] is also known as [D-Ala^2]deltorphin I [2]. Affinities of peptides for rat brain membrane δ and μ receptors are given by K_i values (nM, mean ± standard error; n = 3–9) determined according to the formulation of Cheng and Prusoff [36]: $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioactive ligand and K_d represents the dissociation constant for either [^{3}H]DAGO or [^{3}H]DPDPE. $K_{i\mu}/K_{i\delta}$ is defined as δ selectivity [33].

Although several of the analogues contain one (**2–6**, **8**) or two (**7**) free carboxyl groups, they exhibit a broad spectrum of δ affinities and selectivities, in contrast to the prevailing hypothesis that δ affinity requires an opioid peptide with an anionic functional group [26]. The decreased δ affinity of peptide **7** may correlate with ^{1}H-NMR data which indicated that a C-terminal carboxylate ion can increase solution conformational stability by formation of an additional H-bond [22, 23]; esterification of the C-terminal carboxyl group in peptide **9** may therefore interfere with formation of such an H-bonded structure. Enhancement of μ affinity accompanies the suppression of the negative charge in deltorphins [6, 9, 15, 17] as seen in peptides **9**, **10** and **12** (table I).

In contrast to the esterification of enkephalin by *tert*-butyl [30, 31], esterification of carboxyl groups at the fourth and/or seventh residues in DEL C (**10** and **11**) diminished both δ binding and selectivity: in particular, the δ affinity of the di-*tert*-butyl ester derivative (**11**) was 270-fold less than that of DEL C. However, esterification of the β-carboxyl of Asp^4 with a *tert*-butyl or benzyl group only decreased δ affinities 4- to 6-fold; on the other hand, the OBzl analogue exhibited ≈ 20-fold greater μ affinity. These modifications in receptor affinities led to a suppression of 8- and 72-fold in the δ selectivities of peptides **10** and **12**, respectively. An increase of μ affinity in the OBzl analogue (**12**) may indicate that an aromatic group in this peptide might interact with the proposed μ receptor site which recognizes enkephalin [34] but which apparently differs from that of dermorphin [33]. Deltorphin conformation and its interaction with receptor sites is apparently influenced by O*t*Bu in a manner that differs from that which occurs in the enkephalins [34]: the *tert*-butylesters sterically constrain enkephalin conformers to yield peptides of relatively high δ selectivity [30, 31] in which the O*t*Bu groups rotate to minimize interference with binding [34].

Conclusion

Our observations are consistent with the suggestion that the combination of molecular configuration and spatial orientation of the amino acid side-chains in DEL C [18, 26, 34] play a definitive role in determining selectivity: modifications in sequence of DEL C differentially altered δ and μ receptor binding properties and, without exception, decreased δ selectivities. Our results further indicate that the existence of a negatively charged group in the opioid peptide *per se* does not necessarily confer high δ selectivity and that the spatial orientation of the hydrophobic side-chains appear to be important for the receptor selectivity of DEL C, as observed with DEL A [25], in addition to providing a possible reflection of the physical nature of the receptor binding site.

Experimental protocols

Chemical synthesis

General procedures for the chemical synthesis of peptide analogues have been described earlier [27–29]. Amino acid composition was determined as previously published [17]. The properties of the tri- and tetrapeptide intermediates are listed in table II.

Specific solution coupling procedures

Method A
NMM (1.1 equiv) was added to a stirred solution of 0.5–0.8 M DMF containing Boc- or Z-protected amino acid (1 mmol); the mixture was cooled to – 10°C and treated with isobutyl chloroformate (1.1 equiv) and allowed to react for 2–3 min. A precooled solution of amino component (1.1 mmol) in DMF (0.4–0.6 M) was added and the reaction stirred for 1 h at – 10°C and 2–3 h at 0 to – 10°C, then diluted with EtOAc (100 ml). The suspension was washed consecutively with saturated NaCl (brine), 0.5 N $KHSO_4$, brine, 5% $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was crystallized from appropriate solvents or purified by column chromatography [32].

Method B
The following were sequentially added to a solution of the carboxy component (2 mmol) in DMF (10 ml) at 0°C: amino acid component (2 mmol), NMM (2 mmol if the amino component was in the protonated form), HOBt (2 equiv), and DCC (2.1 mmol). The reaction was stirred for 2 h at 0°C and 24 h at room temperature; *N,N'*-dicyclohexylurea was filtered and the solution diluted with EtOAc (100 ml), then worked up as in *Method A*.

Method C
To a cooled (0°C) 1 N NaOH (1 ml) solution of the carboxy component (2 mmol), the Boc-protected amino acid-OSu (4 mmol) in dioxane (6 ml) was added. The mixture was stirred for 2 h at 0°C and 10 h at room temperature, evaporated, and then diluted with 1 N citric acid (50 ml). The product was extracted into EtOAc (3 x 30 ml), back-washed with saturated aqueous NaCl, and then worked up as in *Method A*.

Method D
To a solution of amino component (0.5 mmol) in DMF (5 ml) containing DIEA (1 mmol), the Z-protected amino acid-OSu or ONp (0.6 mmol) and HOBt (0.5 mmol) were added. The reaction was stirred for 30 min at 0°C and overnight at room temperature. After evaporation *in vacuo*, the residue was triturated with 10 ml solvent mixture EtOAc-diethyl ether (40:60, v/v) and washed several times with diethyl ether to yield a white solid.

Deprotection procedures

Method 1
Boc and OtBu protecting groups were removed by treating the peptide with TFA-CH_2Cl_2 (1:1) for 1 h at 0°C. The solvent was removed *in vacuo* and the residue triturated with diethyl ether or petroleum ether; the resulting solid was collected and dried.

Table II. Physicochemical properties of protected peptides.

No	*Structure*	*Yield (%)*	*Melting point (°C)*	$[\alpha]_D 20^a$	*TLC*[b]
1'	Z-Tyr-D-Ala-Phe-OH	68	135–137	+ 15.8	0.90
2'	Boc-Tyr-D-Ala-Phe-OH	75	111–113	+ 28.1	0.86
3'	Z-Val-Val-Gly-NH_2	82	184–187	– 9.0	0.22
4'	Z-Val-Val-Gly-O*t*Bu	78	179–182	– 54.7	0.60
5'	Z-Gly-Val-Val-NH_2	80	239–241	– 12.7	0.44
6'	Z-Val-Gly-Val-NH_2	75	250–252	– 147.1	0.33
7'	Z-Val-Val-Gly-OEt	78	190–194	– 31.8	0.77
8'	Z-Asp(O*t*Bu)-Val-Gly-NH_2	81	175–177	– 18.3	0.63
9'	Z-Val-Asp(O*t*Bu)-Gly-NH_2	77	191–193	– 61.4	0.57
10'	Z-Val-Val-Asp(O*t*Bu)-NH_2	65	210–211	– 13.7	0.51
11'	Z-Asp(O*t*Bu)-Val-Val-Gly-NH_2	75	215–217	– 14.3	0.32
12'	Boc-Asp(OBzl)-Val-Val-Gly-NH_2	78	210–213	– 1.6	0.54
13'	Z-Asp(O*t*Bu)-Val-Val-Gly-O*t*Bu	70	180–184	– 11.1	0.63
14'	Z-Asp(O*t*Bu)-Gly-Val-Val-NH_2	74	223–225	– 2.3	0.48
15'	Z-Asp(O*t*Bu)-Val-Gly-Val-NH_2	76	226–228	– 12.1	0.49
16'	Z-Asp(O*t*Bu)-Val-Val-Gly-OEt	78	188–190	– 13.4	0.59
17'	Z-Asn-Val-Val-Gly-OEt	64	245–247	+ 15.3	0.18
18'	Z-Val-Asp(O*t*Bu)-Val-Gly-NH_2	78	183–185	– 22.4	0.48
19'	Z-Val-Val-Asp(O*t*Bu)-Gly-NH_2	79	215–217	– 42.6	0.54
20'	Z-Gly-Val-Val-Asp(O*t*Bu)-NH_2	67	187–191	– 31.4	0.49

[a]Optical rotations were conducted in DMF, except those for intermediates **1'**, **3'**, **4'**, **7'** and **8'** which were carried out in methanol. [b]Solvent system for intermediates **1'** and **2'** was *n*-butanol/acetic acid/water (3:1:1, v/v/v) and that for the remainder of the compounds was methylene chloride/methyl benzene/methanol (17:1:2, v/v/v).

Method 2
Hydrogenations were carried out in HOAc-isopropanol (3:2, v/v) at atmospheric pressure and room temperature in the presence of 5% palladized charcoal (using a catalyst to peptide ratio of 1:9, w/w). The reaction mixture was filtered through a Celite bed and evaporated to dryness. The residue was treated as in *Method D*.

Isolation procedures

All protected peptides (0.7–1 g material) were initially purified on a silica gel column (2 x 70 cm) using a linear gradient from 10% to 50% MeOH in $CHCl_3$. Deprotected peptides [27] were further purified by a combination of Sephadex gel filtration, partition chromatography, and preparative HPLC [17]. Preparative HPLC of 100 mg quantities of peptide was performed on a Delta Pak reverse-phase C_{18} 300 Å 15 µm spherical particle column (10 x 300 mm) using a linear gradient from 20% to 50% acetonitrile containing 0.1% TFA over 40 min at a flow rate of 30 ml/min.

Peptide purification

Analytical HPLC was performed on a Spherisorb 5-ODS2 C_{18} column (4.5 x 250 mm); mobile phases A (10% acetonitrile containing 0.1% TFA) and B (60% acetonitrile containing 0.1% TFA) were employed in the following elution schemes for purification of peptides a flow rates of 1 ml/min: i) linear gradient from 20% to 80% B in 30 min; ii) linear gradient from 50% to 100% B in 15 min; and iii) linear gradient from 50% to 100% B in 15 min, then isocratically for 10 min. Eluants were monitored at 220 and 254 nm. Data were compiled using an Epson (QX-10) computer system. Thin layer chromatography was carried out on Merck precoated 0.25 mm analytical silica gel plates 60 F_{254} using two solvent systems: *n*-butanol/acetic acid/water (3:1:1, v/v/v) and ethyl acetate/acetic acid/pyridine/water (6:0.6:2.2:1.2, v/v/v/v). Purities were estimated at > 99%.

Determination of physical and chemical characteristics

Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were conducted in DMF or methanol as detailed in tables II and III using a 10-cm pathlength cell in a Perkin-Elmer 241 polarimeter. The chemical characteristics of each peptide were routinely monitored by ^{1}H-NMR spectrometry using 200 MHz [20] or 500 MHz [22] Bruker instruments, which were also used to confirm the absence of racemization [35]. The physicochemical properties of the peptides are listed in table III.

Receptor binding assay methods

Preparation of brain membranes
Whole rat brain (minus cerebellum) preparations of synaptosomes (P_2) were obtained by homogenization in a 0.32 M sucrose solution as described using differential centrifugation and an incubation step to remove endogenous opioid peptides [4]. The membranes (25 mg/ml) were stored in 50 mM Hepes (pH 7.5), containing 50 µg/ml soybean trypsin inhibitor and 20% glycerol at – 70°C to – 80°C.

Table III. Analytical properties of deltorphin C and related analogues.

No	Peptide	TLC (R_f) I[b]	TLC (R_f) II[b]	HPLC (K')[c]	$[\alpha]_D 20$[a]	Melting point (°C)	Amino acid analysis Y	A	F	D	V	G
1	Deltorphin C	0.53	0.44	5.43	− 16	168–170	0.98	1.02	0.98	0.94	1.81	0.99
2	[Val^4,Asp^5]	0.71	0.67	4.65	+ 5.3	172–174	0.89	1.01	0.98	0.95	1.89	1.0
3	[Val^4,Asp^6]	0.79	0.73	4.42	− 7.2	160–162	0.91	0.99	1.02	0.96	1.87	1.0
4	[Gly^4,Asp^7]	0.68	0.75	4.53	− 16.2	158–160	0.95	0.97	0.96	0.93	1.9	1.0
5	[Gly^5,Val^7]	0.74	0.44	5.56	− 8.5	176–178	0.94	0.95	1.04	0.94	1.69	0.97
6	[Gly^6,Val^7]	0.69	0.46	5.49	+ 10	160–162	0.99	0.89	1.02	1.01	1.89	1.0
7	[Gly(OH)^7]	0.54	0.59	5.67	− 9	168–170	0.92	1.01	1.01	0.97	1.88	1.01
8	[Gly(OEt)^7]	0.84	0.39	7.99	− 25	208–210	0.97	0.98	1.03	0.95	1.84	0.99
9	[Asn^4,Gly(OEt)^7]	0.87	0.32	7.34	− 43	242–244	0.92	0.89	1.03	0.90	1.81	0.98
10	[Asp(O*t*Bu)^4]	0.78	0.75	8.87	+ 2.9	210–212	0.95	0.97	0.97	0.95	1.79	1.0
11	[Asp(O*t*Bu)^4, Gly(O*t*Bu)^7]	0.88	0.65	8.96	− 4.4	195–197	1.03	0.95	1.04	0.91	1.85	0.95
12	[Asp(OBzl)^4]	0.77	0.57	5.41	− 1.4	179–181	0.94	1.02	1.03	0.81	1.55	1.0

[a]Optical rotations were collected in DMF, except peptide **1** which was taken in methanol. [b]Solvent systems: I, *n*-butanol/acetic acid/water (3:1:1); II, ethyl acetate/acetic acid/pyridine/pyridine (6:0.6:2.2:1.2). [c]Capacity factor (K') determined with HPLC elution scheme (i) as described in *Experimental protocols*.

Radioreceptor assay

The binding assays for μ and δ receptors were conducted as detailed previously [4, 15–17, 25, 32, 33] and in the presence of 100 μM PMSF under conditions considered optimal for binding. Peptide stock solutions (100 μg peptide/ml) were prepared in 15% ethanol containing 10 mM acetic acid and stored at 4°C; working solutions were diluted in acidic alcohol containing 1 mg BSA/ml. Concentrations of [^{3}H]DAGO and [^{3}H]DPDPE in the μ and δ binding assays were 1.28 ± 0.03 and 0.63 ± 0.05 nM, respectively. The 1.6 mg synaptosome protein used in duplicate assays was rapidly entrapped on a presoaked GF/C filter (50 mM Hepes, pH 7.5, containing 1 mg/ml BSA) and washed within 5 s using three 2-ml aliquots of the ice-cold buffered BSA solution. The filters were dried (≈ 75°C) and the radioactivity determined using 2 ml CytoScint. Peptides were tested at 4–7 concentrations using at least 3–5 synaptosomal membrane preparations in 3–9 separate binding experiments to provide statistical reliability. Specific binding represents the ratio of bound to free labelled ligand; non-specifically bound radioactivity was determined in the presence of excess (2 μM) unlabelled DAGO or DPDPE for μ or δ binding sites, respectively. Graphically determined IC_{50} values, which represented nM peptide required to displace 50% of the radiolabelled ligand, were used to calculate the competitive inhibition constants (K_i) for μ and δ receptors according to Cheng and Prusoff [36].

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