Design and Synthesis of 1-Aminocycloalkane-1-carboxylic Acid-Substituted Deltorphin Analogues: Unique δ and μ Opioid Activity in Modified Peptides

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Deltorphin analogues were substituted by a series of achiral $C^{\alpha,\alpha}$-dialkyl cyclic α-amino acids (1-aminocycloalkane-1-carboxylic acids, Ac_xc, where $_x$ = a hexane, pentane, or propane cycloalkane ring) in position 2, 3, 4, or 2 and 3 in deltorphin C, and in position 2 in [Ac_6c^2,-des-Phe^3]deltorphin C hexapeptide. Receptor assays indicated that even though Ac_6c^2 and Ac_6c^3 exhibited a diminished $K_i\delta$ by ca. 20-fold (2.5−3.3 nM) relative to deltorphin C ($K_i\delta$ = 0.15 nM), selectivity was marginally elevated ($K_i\mu/K_i\delta$ = 1250) or enhanced by about 70%, and both peptides fitted stringent iterative calculations for a two-site binding model (η = 0.625 and 0.766, respectively, $P < 0.0001$). The disubstituted [$Ac_6c^{2,3}$]- or [Ac_6c^2,des-Phe^3]deltorphin analogues yielded peptides with decreased $K_i\delta$, such that the latter peptide was essentially inactive. The presence of Ac_5c or Ac_3c in place of Phe^3 further diminished $K_i\delta$ (15.4 to 19.0 nM), yet δ selectivity only fell about one-half ($K_i\mu/K_i\delta$ = 440 and 535, respectively), and only the former peptide fitted a two-site binding model (η = 0.799). The replacement of Asp^4 by Ac_6c, Ac_5c, or Ac_3c produced essentially nonselective analogues through the acquisition of high μ affinities (2.5, 0.58, and 0.27 nM, respectively) while maintaining high δ affinities ($K_i\delta$ = 0.045−0.054 nM) which were about 3-fold greater than that of deltorphin C. Using pharmacological assays *in vitro* (mouse vas deferens and guinea pig ileum), position 3-substituted analogues all indicated substantial losses in bioactivity, whereas substitution by 1-aminocycloalkanes at the fourth position retained high δ activity. In fact, the bioactivity of [Ac_3c^4]deltorphin C indicated a peptide with relatively weak δ selectivity, which was comparable to the observations with the receptor binding data. In summary, the data confirmed that (i) δ selectivity occurs in the absence of D-chirality at position 2, (ii) the aromaticity of Phe^3 is replaceable by an achiral residue with a hydrophobic ring-saturated side chain, and (iii) the acquisition of dual high-affinity analogues occurs through the elimination of the anionic function at position 4 and replacement by an amino acid with a hydrophobic side chain.

Introduction[1]

Deltorphins are a group of opioid heptapeptides from amphibian skin that interact with high affinity and selectivity for the δ opioid receptor. In contrast to the enkephalin analogues, the deltorphins and μ specific dermorphins contain the common N-terminal tripeptide sequence H-Tyr^1-D-Xaa^2-Phe^3, where D-Xaa^2 is either D-Met^2 in deltorphin A (termed deltorphin,[2] dermorphin gene-associated peptide,[3] or dermenkephalin[4]), D-Ala^2 in dermorphins and deltorphins B and C ([Glu^4]- and [Asp^4]deltorphins II and I, respectively[2]), or D-Leu^2 in a deltorphin-like tridecapeptide which is not an active opioid ligand.[5] An L-isomer at the second residue virtually inactivates these peptides.[2,3] Investigations in which normal or unusual amino acid analogues were substituted for residues in the N-terminal domain (positions 1−4) of deltorphin revealed that δ affinity and discrimination between δ and μ receptors (δ selectivity) depended on the properties of the side chain and the chirality of the amino acid,[8−13] as seen, by way of example, through the extensive alteration to or replacement of Phe^3.[8,9,12,13] On the other hand, analogues in which the acidic function at position 4 was substituted by amino acids with aliphatic side chains produced peptides with minor changes in δ affinities, although δ selectivity was nearly ablated due to the acquisition of greatly enhanced μ affinities.[6,7,14−16] Other studies on sequentially C-terminal abbreviated peptides indicated that the N-terminal sequence tetrapeptides contained the crucial elements required for the expression of opioid activity since receptor selectivity underwent a reversal from δ to μ.[4,7,14,17] *In toto*, the observations with opioid agonists re-enforced the universality that the opioid "message" concept was confined within the N-terminal sequence, which came into prominence through the development of opioidmimetic di-,[18] tri-,[18,19] and tetrapeptide antagonists.[19]

Rationale

The essential amino acid sequence in deltorphin B or C lies within the N-terminal tetrapeptide region H-Tyr^1-D-Ala^2-Phe^3-(Glu/Asp)^4, in which the two essential aromatic side chains are separated by a key D-isomer. However, the introduction of Tic in deltorphin at the second position[12,20] and the formation of abbreviated Tic^2-containing opioidmimetic peptides provided new insights that the residue at the second position could

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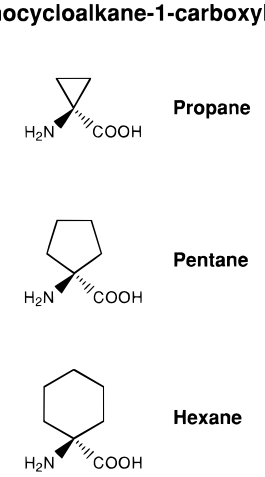


Figure 1. Structures of the 1-aminocycloalkane-1-carboxylic acids showing the alkane rings of propane, pentane, and hexane.

be replaced by a heterocyclic residue to produce opioid peptides that behaved as δ antagonists.[18,19] Further, modification of the physicochemical properties of the phenylalanyl side chain, such as alteration of the electronic configuration of Phe through *para* substitution by halogens and amino and nitro groups[13] or spatial displacement of the aromatic ring and replacement by various amino acids,[8,9] supported the view that aromaticity at position 3 was responsible for receptor binding. Molecular dynamics conformations[13,21,22] and ^{1}H-NMR[23] analyses provided evidence on the existence of β-turns involving H-Tyr-D-Ala-Phe and revealed that the D-chirality of the Ala side chain apparently stabilized the β-turn.

In our attempt to further understand the structural and chemical features of the residues in the 2−4 positions in deltorphin heptapeptides that influence binding to opioid receptors, we systematically substituted the achiral ring-saturated $C^{\alpha,\alpha}$-dialkyl cyclic α-amino acids (1-aminocycloalkane-1-carboxylic acids) for D-Ala^2, Phe^3, or the anion at position 4; the cycloalkane contained either a three-, five-, or six-membered saturated side chain ring (Figure 1). (It is noteworthy that 1-aminocyclopropane-1-carboxylic acid is a natural amino acid found in plants[24] as an intermediate in the conversion of methionine to ethylene[25,26] and interesting in that it acts as a partial agonist on the NMDA receptor and prevents tolerance to μ and δ agonists *in vivo*.[27]) Studies with model peptides have shown that the 1-aminocyclopropane substitution can be accommodated into γ as well as helical turns (3_{10}- or α-helices) in small peptides,[28,29] although all $C^{\alpha,\alpha}$-dialkylated glycyl-containing peptides folded into a type II β-turn (C_{10}-helix) based on crystallographic data.[29] Therefore, since it is well established by ^{1}H-NMR studies[23] and conformational motifs developed by molecular dynamics simulations[21,22] that deltorphins contain a β-turn in their N-terminus, the possibility exists that the cyclic $C^{\alpha,\alpha}$-dialkyl residues might then represent ring-saturated analogues of Phe^3 without major disruptive effects on receptor properties. Our data demonstrate several unique properties imparted to deltorphin analogues by the incorporation of 1-aminocycloalkane-1-carboxylic amino acids in the N-terminal sequence of positions 2−4.

Results and Discussion

Receptor Binding Analyses. The effect of substituting the 1-aminocycloalkane-1-carboxylic acids at position 2 (compound **2**), 3 (compounds **3**, **7**, and **9**), 4 (compounds **4**, **8**, and **10**), or 2 and 3 (compound **5**) in deltorphin C heptapeptides or position 2 in [Ac_6c^2,des-Phe^3]deltorphin C hexapeptide (compound **6**) resulted in distinct differences in receptor binding parameters (Table 1).

The inclusion of Ac_6c at position 2 (peptide **2**) yields a peptide lacking D-chirality and induced analogous decreases in both δ and μ affinities (ca. 20−30-fold) to that of Ac_6c at position 3: That peptide (**3**) lacks one of the two aromatic side chains and increased in δ selectivity. However, in comparison to peptide **1**, the δ selectivity of peptide **2** increased *ca.* 30% and that of peptide **3** rose 70%. The simultaneous inclusion of Ac_6c at positions 2 and 3 (compound **5**), which concomitantly lost both chirality and the aromaticity associated with Phe^3, yielded a peptide with substantially diminished δ affinity and δ selectivity.

The [Ac_6c^2,des-Phe^3]deltorphin hexapeptide **6** enabled direct observation on the effect of 1-aminocyclohexane in the absence of an aromatic side chain at the third residue, being substituted by a branched aliphatic side chain in Val; δ receptor binding activity was essentially eliminated, more so than in hexapeptides containing Phe^3 in which the C-terminal Gly^7 residue was omitted.[4,6,17] These binding data would tend to suggest that even though 1-aminocyclohexane can effectively replace D-Ala^2 (peptide **2**), an aliphatic side chain containing substituent at position 3 fails to allow proper alignment with the ligand-binding domain of the δ receptor with a hexapeptide. Whereas the substitutions of Phe^3 by Ac_5c (peptide **7**) and Ac_3c (peptide **9**) also decreased δ and μ affinities, δ selectivities only diminished by about one-half.

The receptor binding data demonstrate marginal affects on affinity under the following conditions: (i) the loss of chirality of the residue at the second position occurs in the presence of Phe^3 and (ii) the replacement of Phe^3 by a ring-saturated hydrophobic side chain led to only moderate changes in δ selectivity. However, an aromatic residue in the third position of deltorphin C heptapeptides enhanced δ affinity (peptide **1**).[8,9,15]

The elimination of the negatively charged Asp^4 by either Ac_6c (peptide **4**), Ac_5c (peptide **8**), or Ac_3c (peptide **10**) produced deltorphin analogues with remarkable and unanticipated properties, namely, the simultaneous attainment of *both* high δ and μ affinities (Table 1). The augmentation of μ affinities relative to deltorphin C (peptide **1**) was 60-, 250-, and 540-fold for Ac_6c, Ac_5c, and Ac_3c, respectively, such that the latter two analogues were essentially nonselective for either receptor type. Thus, replacement of the acidic function produced peptides with dual high affinities,[21] a phenomenon we termed "opioid infidelity";[16] this was similarly observed with other position 4 deltorphin analogues containing hydrophobic side chain residues with aliphatic,[15,16,21] cyclic imino,[21] indole,[14] or aromatic properties.[14] However, charge neutralization by the substitution of Asp or Glu by Asn or Gln, respectively, which moderately enhanced μ affinity, failed to eliminate δ selectivity even though δ affinity remained high.[6,15,30] The inference provided by these data is that a negatively charged

Table 1. Receptor Binding Parameters of 1-Aminocycloalkane-1-carboxylic Acid-Substituted Deltorphin Analogues

no.	peptide	$K_i\delta$ (nM)	η	$K_i\mu$	η	δ selectivity (μ/δ)
1	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.15 ± 0.03 (8)	−0.951	147 ± 29 (11)	nd[a]	980
2	Tyr-**Ac_6c**-Phe-Asp-Val-Val-Gly-NH_2	2.48 ± 0.58 (5)	−0.625	3110 ± 88.0 (2)	nd[a]	1250
3	Tyr-D-Ala-**Ac_6c**-Asp-Val-Val-Gly-NH_2	3.28 ± 0.76 (7)	−0.766	5465 ± 1259 (4)	nd[a]	1670
4	Tyr-D-Ala-Phe-**Ac_6c**-Val-Val-Gly-NH_2	0.045 ± 0.02 (4)	−1.025	2.45 ± 0.37 (4)	−1.012	54
5	Tyr-**Ac_6c**-**Ac_6c**-Asp-Val-Val-Gly-NH_2	87.8 ± 28.6 (4)	nd[a]	14 824 ± 2787 (3)	nd[a]	170
6	Tyr-**Ac_6c**-Asp-Val-Val-Gly-NH_2	5264 ± 1073 (6)	nd[a]	440 667 ± 49 512 (3)	nd[a]	84
7	Tyr-D-Ala-**Ac_5c**-Asp-Val-Val-Gly-NH_2	15.4 ± 1.9 (4)	−0.799	6822 ± 1041 (4)	nd[a]	440
8	Tyr-D-Ala-Phe-**Ac_5c**-Val-Val-Gly-NH_2	0.054 ± 0.01 (4)	−1.128	0.58 ± 0.08 (5)	−1.004	10
9	Tyr-D-Ala-**Ac_3c**-Asp-Val-Val-Gly-NH_2	19.0 ± 2.7 (4)	−0.956	10 167 ± 2241 (3)	nd[a]	535
10	Tyr-D-Ala-Phe-**Ac_3c**-Val-Val-Gly-NH_2	0.054 ± 0.012 (3)	−0.929	0.27 ± 0.03 (3)	−1.076	5

[a] nd = not determined. The numbers in parentheses indicate the *n* values.

residue causes an electrostatic repulsion between the ligand and the μ receptor which apparently contains an Asp residue at the binding site.[31] A corollary to this proposal is that an anion in deltorphin or the opioid-mimetic peptides[18] plays a minor role in the acquisition of δ affinity,[6,14,30] serving only to discriminate between δ and μ receptors.

In spite of the reduction of δ affinity with deltorphin analogue **2**, the maintenance of δ selectivity succinctly implies that the D-stereoconfiguration at position 2 is not an absolute requirement as previously considered.[2,3,7,20] Moreover, the configuration at the α carbon of this residue may impart new properties on the peptide; *i.e.*, the change in chirality of Tic^2 from D to L configuration reversed the selectivity in dermorphin analogues.[32] The aromaticity of the side chain at position 3 (peptides **3**, **7**, and **9**) is partially replaceable by a saturated alkane ring as long as the heptapeptide contains D-Ala at position 2. In other words, the alkane ring cannot concurrently replace *both* residues (vis-à-vis peptide **5**). This bisubstitution in the N-terminal region could adversely affect the secondary structure, perhaps by the elimination of the existing β-turn.[21,22] Despite the decrease in high δ affinities in peptides **2** and **3**, their receptor binding capabilities were quite comparable to that seen with many enkephalin-derived analogues.

Binding Site Models. The analyses of Hill coefficients (η) and fits to one- or two-site binding model paradigms based on iterative calculations according to the stringent criteria of Attila *et al.*[33] and Bryant *et al.*[21] [$\eta < 0.85$ with narrow log 95% confidence intervals (*ca.* 0.1) and $P > 0.0001$] indicated that, with the exception of peptides **2**, **3**, and **7**, the analogues fitted one-site binding models; the η for peptides **2**, **3**, and **7** ranged from 0.625 to 0.799, in which compound **2** contains a cyclohexane residue in lieu of D-Ala^2 and the latter two analogues contain cyclohexane and cyclopentane substitutions for Phe^3, respectively. These values are in keeping with the η observed for [Pro^4]- and [D-Asp^4]-deltorphin and deltorphin B,[21] in addition to several Dmt-Tic-Ala tripeptides,[18b] which were found previously to be the only analogues that fit a two-site binding model based on these stringent criteria.[21] A low-energy conformer of [Ac_6c^3]deltorphin C (compound **3**)[34] determined using molecular dynamics stimulations indicated that the solution conformation resembles that of peptides which fit two-site binding models[21] and differed from those interacting with a single binding site paradigm. Furthermore, even though peptides **3** and **7** interact with the δ receptor in a complex, heterogeneous manner, they nonetheless associate with μ sites through a simple bimolecular interaction of a one-site binding model as observed with other deltorphin analogues.[21] In this respect, deltorphin analogues with high μ affinities (peptides **8** and **10**) interact with μ receptors in a manner comparable to that of dermorphin.[33]

Table 2. Bioassays of Position 3-Substituted 1-Aminocycloalkane-1-carboxylic Acid Deltorphin Analogues[a]

peptide	IC_{50} (nM) MVD	IC_{50} (nM) GPI
1, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.46 ± 0.24	337 ± 68
3, Tyr-D-Ala-**Ac_6c**-Asp-Val-Val-Gly-NH_2	540 ± 130	>10 μM
4, Tyr-D-Ala-Phe-**Ac_6c**-Val-Val-Gly-NH_2	0.52 ± 0.06	558 ± 10
7, Tyr-D-Ala-**Ac_5c**-Asp-Val-Val-Gly-NH_2	1640 ± 260	>10 μM
8, Tyr-D-Ala-Phe-**Ac_5c**-Val-Val-Gly-NH_2	0.19 ± 0.01	140 ± 3.9
9, Tyr-D-Ala-**Ac_3c**-Asp-Val-Val-Gly-NH_2	458 ± 40	>10 μM
10, Tyr-D-Ala-Phe-**Ac_3c**-Val-Val-Gly-NH_2	0.43 ± 0.05	15 ± 1

[a] The IC_{50} values (nM) represent the mean ± SE of four independently conducted experiments using mouse vas deferens (MVD) and guinea pig ileum (GPI) as described in the Experimental Section. The K_e values for the antagonists naloxone and ICI 174,864 were both within the range of 1−2 nM.

Bioactivity. The replacement of Phe^3 (peptide **1**) by Ac_6c (peptide **3**), Ac_5c (peptide **7**), and Ac_3c (peptide **9**) on the activity recorded with mouse vas deferens proved to be more detrimental than binding to brain membrane receptor sites. On the other hand, substitution by 1-aminocycloalkanes at position 4 in place of Asp produced peptide analogues (**4**, Ac_6c; **8**, Ac_5c; and **10**, Ac_3c) in which the bioactivity on mouse vas deferens reflected their high δ affinities (Table 1). However, the dual high δ and μ receptor affinities observed with peptides **4** and **8** were not replicated in the guinea pig ileum assay suggesting the possible existence of a different peptide conformation which is poorly recognized by the μ receptors associated with this tissue. In contrast, peptide **10** (Ac_3c^4) demonstrated relatively high affinity in both bioassays (Table 2). Whereas δ affinity maximally decreased about 120-fold in peptide **9** (Table 1), its bioactivity fell *ca.* 1000-fold relative to deltorphin C (Table 2). This further suggests the possibility that peripheral δ receptors differ from those in the central nervous system or that the lack of agonist activity could indicate possible acquisition of antagonist activity, which is observed in analogues modified at position 3 due to alteration of their conformation determined by molecular dynamics simulations.[33]

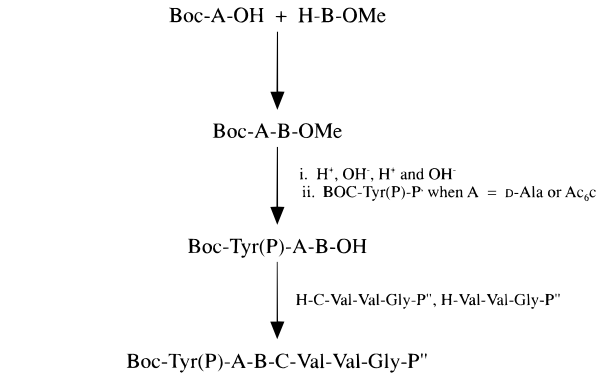
Conclusions

The $C^{\alpha,\alpha}$-dialkylated cyclic amino acid-substituted deltorphin analogues provide sufficient data to conclude the following interactions with the ligand-binding domain of opioid receptors: (i) the retention of δ selectivity occurred in the absence of D-chirality at position 2, and the requirement for a D-isomer is no longer consider

crucial for receptor binding; (ii) the partial replacement of the aromaticity associated with Phe[3] by ring-saturated side chain suggests that hydrophobic interaction *per se* may be an equal or more important element than the aromatic side chain, although peptide conformation may impart an additional effect on the association between the aminocycloalkane-containing ligand and the receptor; (iii) the elimination of the anionic function which yielded heptapeptides with high affinity for both δ and μ receptor sites with the concomitant loss of δ selectivity provides further evidence that the peptide anion causes a repulsion from the μ receptor, confirming the presence of a negatively charged group in the receptor binding pocket;[31] (iv) compounds **2**, **3**, and **7** fit a two-site binding model which is considered to be related to the δ_2 receptor subtype due to modification in the topographical conformation of the peptide[21] in confirmation that $C^{\alpha,\alpha}$-dialkylated α-amino acids induce and maintain helical conformation in model peptides;[28,29] and (v) the simultaneous deletion of second position D-chirality and third position aromaticity in a hexapeptide analogue that lost both δ and μ affinities suggests that the even though the hydrophobic nature of this sequence is maintained, the change in conformation is incompatible with that of the receptor pocket.

A saturated alkane ring at position 2 or 3 enabled the peptide to acquire the specific conformation necessary for receptor recognition through the maintenance of appropriate β-turns in the N-terminal region.[13,21,22] Since data established that 1-aminocyclopropane-1-carboxylic acid, and $C^{\alpha,\alpha}$-dialkyl α-amino acids in general,[29] permitted the formation of helical turns in model peptides,[28] retention of opioid binding parameters in the absence of the essential D-chirality in the second position[2−4] suggests that if a substituted amino acid side chain permits the N-terminal sequence to sustain the necessary β-turn found in deltorphin,[21,22] the analogue will exhibit appropriate binding parameters. The unprecedented presence of high dual affinity in heptapeptide analogues upon elimination of the negative charge at position 4 was similarly observed by substitution with amino acids containing an aliphatic side chain[21] and supported by *in vitro* pharmacological bioassays with peptide **10**. The enhancement of μ affinity confirms earlier observations that hydrophobic factors strongly influence binding to μ receptor sites.[15,17,18] The ability to bind with near equal avidity between two distinct receptor molecules (δ and μ) provides valuable clues on conformational differences that are brought into play during binding of opioid ligands by their respective receptors.[16,21,22,34] Furthermore, the high-affinity interaction with the μ ligand-binding domain by peptides **8** and **10** through a one-site binding model also implies that their conformation must partially resemble that of dermorphin. Whereas spatial orientation must also be ideally suited for interaction with the ligand-binding domain of the receptors, peptide sequence[6,15] and amino acid composition[4,6−9,12,14,15] allows for differentiation. Further, Hill coefficients based on stringent iterative calculations in combination with molecular dynamics simulations[21,34] provide additional clues on the potential conformation of peptides that are recognized by common factors in the disparate δ and μ receptors of rat brain membranes.

Scheme 1. Synthetic Scheme for the Synthesis of 1-Aminocycloalkane-1-carboxylic Acid Analogues of Deltorphin C (Protective groups or substituents (P, P′, P″) on the Tyr residue are designated in the key)



Key

A = D-Ala, Ac_6c, Tyr-Ac_6c, Tyr-D-Ala
B = Phe, Ac_xc (x = 3, 5, 6), Phe-Ac_xc (x = 3, 6)
C = Asp(O*t*Bu), Ac_5c
P = O*t*Bu, H
P′ = OSu, OH
P″ = NH_2, NH-resin

Experimental Section

Materials. Enkephalin-derived peptides, cyclic [D-Pen[2,5]] (DPDPE) and [D-Ala[2],N$^{\alpha}$MePhe[4],Gly-ol[5]] (DAGO), were products of Bachem (Torrance, CA); [^{3}H]DPDPE (60.0 Ci/mmol) was from Amersham (Arlington Heights, IL) and [^{3}H]DAGO (37.1 Ci/mmol) from NEN-DuPont (Billrica, MA). The following compounds were purchased from Sigma Chemical Co. (St. Louis, MO): bacitracin, bestatin, bovine serum albumin (RIA grade), HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], phenylmethanesulfonyl fluoride, soybean trypsin inhibitor, and ultrapure sucrose. Glass microfiber (GF/C) filters were from Whatman International, Ltd. (Maidstone, U.K.). Amino acids and protected amino acids containing the protecting groups Fmoc [N^{α}-[(9-fluorenylmethyl)oxy]carbonyl], Boc (N^{α}-*tert*-butoxycarbonyl), OtBu (*tert*-butyl ester), and OBzl (benzyl ester) were products of Bachem (Switzerland), and the resin Fmoc-PAL-PEG-PS was from Millipore (Waltham, MA). All other chemicals were of the highest purity grade available.

Peptide Synthesis. All deltorphin C ([Asp[4]]deltorphin I) analogues were synthesized by solution methods and, in some cases, by mixed solution−solid phase methods as depicted in Scheme 1.

The cyclic $C^{\alpha,\alpha}$-dialkylated residues examined included Ac_3c, Ac_5c, and Ac_6c. Analogues modified at position 3 (peptides **3**, **7**, and **9**) required esterification of the $C^{\alpha,\alpha}$-dialkylated residue followed by soluble carbodiimide condensation with Boc-D-Ala-OH to give a protected dipeptide. The dipeptides were deblocked at the N- and C-termini by treatment with NaOH and TFA and condensed with a protected Tyr residue. Final condensation was achieved between N-protected tripeptides and C-terminal tetrapeptide of deltorphin C by soluble carbodiimide.

Deltorphin C analogues modified at position 4 (peptides **4**, **8**, and **10**) were synthesized adopting different chemical strategies. The Ac_5c analogue was N^{α}-protected with Z and condensed with the common C-terminal tripeptide Val-Val-Gly-NH_2 of deltorphins I and II. Catalytic removal of Z by H_2/Pd, followed by condensation with N-terminal tripeptide Boc-Tyr-D-Ala-Phe-OH, gave the protected heptapeptide analogue. Synthesis of deltorphin peptides containing Ac_3c and Ac_6c at position 4 both required esterification of $C^{\alpha,\alpha}$ residues as reported above and were sequentially condensed with Boc-Phe-OH and Boc-Tyr-D-Ala-OH. Chemical hydrolysis of the ester function and final condensation with the C-terminal tripeptide gave the protected [Ac_3c[4]]deltorphin analogue, while

Table 3. Analytical Properties of 1-Aminocycloalkane-1-carboxylic Acid-Containing Deltorphin Analogues

		chromatographic parameters[a]				
no.	peptide	I	II	$[\alpha]^{20}_D$	K′ [b]	MH^+ [c]
2	Tyr-**Ac_6c**-Phe-Asp-Val-Val-Gly-NH_2	0.33	0.64	−46.4	14.02	823
3	Tyr-D-Ala-**Ac_6c**-Asp-Val-Val-Gly-NH_2	0.34	0.53	−25.2	10.60	747
4	Tyr-D-Ala-Phe-**Ac_6c**-Val-Val-Gly-NH_2	0.39	0.76	+30.0	11.23	779
5	Tyr-**Ac_6c**-**Ac_6c**-Asp-Val-Val-Gly-NH_2	0.27	0.51	−13.7	14.15	801
6	Tyr-**Ac_6c**-Asp-Val-Val-Gly-NH_2	0.28	0.41	−32.3	9.00	676
7	Tyr-D-Ala-**Ac_5c**-Asp-Val-Val-Gly-NH_2	0.31	0.48	−20.9	9.20	733
8	Tyr-D-Ala-Phe-**Ac_5c**-Val-Val-Gly-NH_2	0.60	0.68	+29.4	13.11	765
9	Tyr-D-Ala-**Ac_3c**-Asp-Val-Val-Gly-NH_2	0.21	0.46	−23.5	6.69	705
10	Tyr-D-Ala-Phe-**Ac_3c**-Val-Val-Gly-NH_2	0.67	0.70	+13.2	11.59	737

[a] TLC determination of R_f was conducted with the following solvent systems: I, 1-butanol/acetic acid/H_2O (3:1:1:1, v/v/v); II, ethyl acetate/pyridine acetate/acetic acid/H_2O (12:4:2:2.2, v/v/v/v). [b] *K′* is the capacity factor determined by analytical HPLC. [c] The mass ion (MH^+) was obtained by mass spectrometry.

the intermediate Boc-Tyr-D-Ala-Phe-Ac_6c-OH was used in the solid phase of the acylation of the Val-Val-Gly-resin.

Analogues substituted in position 2 (peptide **2**) required protection as an ester function of Ac_6c as reported and condensation with Boc-Tyr-OH through IBCF. The dipeptide ester function Boc-Tyr-Ac_6c-OMe was normally removed and condensed with Phe-OMe; again the ester function of Boc-Tyr-Ac_6c-Phe-OMe was removed, and the N-protected tripeptide Boc-Tyr-Ac_6c-Phe-OH was condensed with the C-terminal tetrapeptide Asp(OtBu)-Val-Val-Gly-NH_2 of deltorphin C.

The doubly substituted analogue (peptide **5**) was obtained first by condensation via EDC/HOBt with Boc-Ac_6c-OH and H-Ac_6c-OMe to obtain a protected dipeptide. Boc was removed followed by Boc-Tyr(But)-OH condensation to give the protected tripeptide Boc-Tyr(But)-Ac_6c-Ac_6c-OMe, which was hydrolyzed at the C-terminal function followed by one pot-step solid phase acylation of Asp(OtBu)-Val-Val-Gly-resin. Peptide **6** was obtained during the first solid phase synthesis of the doubly substituted analogue; under the conditions outlined below, the coupling between Fmoc-Ac_6c-OH and the H-Ac_6c-resin normally does not proceed in an efficient manner.

Solid Phase Synthesis. Solid phase synthesis was performed in a Milligen 9050 synthesizer using a Rink resin (0.47 mmol/g; 0.1 g in all syntheses) obtained from Bachem (Torrance, CA). The resin was mixed with glass beads (1:15, w/w) purchased from Sigma (St. Louis, MO). Protected amino acids were from either Bachem or NovaBiochem. Peptides were assembled using Fmoc-protected amino acids (4-fold excess) and DIPCDI (4-fold excess) and HOBt (4-fold excess) as coupling agents and coupled for 1 h at each step. The incorporation of Val^5 required double coupling. Final acylation step with Boc-Tyr-

D-Ala-Phe-Ac_6c-OH and Boc-Tyr(But)-Ac_6c-Ac_6c-OH always required 4-fold excess and 14 h during the recycling coupling step. The two analogues were cleaved from the resin by treatment with TFA/H_2O/Et_2SiH (88:5:7, v/v/v) at room temperature for 1 h.

Purification. Crude peptides were purified by preparative reversed-phase HPLC using a Waters Delta Prep 3000 system with a Delta Pak C18 (30 × 3 cm, 300 Å, 15 μm spherical particle size) column. The peptides were eluted with a gradient of 0−100% B in 25 min at a flow rate of 30 mL/min using mobile phase A (10% acetonitrile in 0.1% TFA, v/v) and mobile phase B (60% acetonitrile in 0.1% TFA, v/v).

Analytical HPLC analyses were performed on a Bruker liquid chromatography LC 21-C instrument fitted with a Vydac C18 (218 TP 5415, 5 μm, 4.5 × 175 mm) particle column and equipped with a Bruker LC 313 UV variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (BX-10). Analytic determination and capacity factor (*K′*) of the peptides were determined using HPLC conditions in the above solvent systems programmed at flow rates of 1 mL/min: (a) linear gradients from 50% to 100% solvent B in 25 min and (b) linear gradients from 0% to 100% B in 25 min. All analogues showed <1% impurities when monitored at 220 nm.

Analytical Determinations. Amino acid analyses were carried out using PITC (Pico-Tag) methodology (Millipore) as the amino acid derivatization reagent. Ac_6c and Ac_5c were not quantitatively determined during the amino acid analysis. Lyophilized samples of peptides (50−1000 pmol) were placed in heat-treated borosilicate tubes (50 × 4 mm), sealed, and hydrolyzed using 200 mL of 6 N HCl containing 1% phenol in the Pico-Tag workstation for 1 h at 150 °C. A Pico-Tag column (15 × 3.9 mm) was used to separated the PITC-amino acid derivatives.

TLC was performed in precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: (I) 1-butanol/acetic acid/H_2O (3:1:1, v/v/v), (II) ethyl acetate/pyridine acetate/acetic acid/H_2O (12:4:2:2.2, v/v/v/v), and (III) CH_2Cl_2/methanol/toluene (17:2:1, v/v/v). Ninhydrin (1%) or chlorine iodine spray reagents were employed to detect the peptides.

Melting points were determined on a Reicher-Kofler apparatus and are uncorrected. Optical rotations used a Perkin-Elmer 241 polarimeter with a 10 cm cell using methanol at a peptide concentration of 1%. Chemical characteristics of each peptide were confirmed using ^{1}H-NMR spectroscopy with a 200 MHz Bruker instrument and are recorded in δ units. Molecular weights were determined by a triple-stage quadrapole mass spectrometer (TSQ 700, Finnigan MAT) equipped with a pneumatic electrospray (ionspray) interface, and the data were compiled using a DEC 5000/125 computer (Table 3).

Coupling Procedures. Method A. To a solution of the carboxy component (3.50 mmol) in DMF (15 mL) were added the amino acid or amino peptide component (3.26 mmol), TEA (3.26 mmol, if the amino component was in the protonated form), HOBt (3.52 mmol) to reduce racemate formation, and coupling reagent, DCC, or EDC (3.52 mmol), in the above order at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and for 12 h at room temperature. *N′*,*N′*-Dicyclohexylurea was filtered off (if DCC was added) and the solvent evaporated *in vacuo*. The residue was dissolved in ethyl acetate (30 mL) and washed with 10% citric acid, brine, 5% $NaHCO_3$, and brine, successively. The organic phase was dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was crystallized from the appropriate solvents.

Method B. The amino component (amino acid or peptide, 0.62 mmol) and the activated carboxy component (OSu, 0.56 mmol) were dissolved in DMF (6 mL containing 0.62 equiv of TEA if the amino component was protonated), and the mixture was allowed to react at room temperature for 4 h. The solvent was evaporated *in vacuo*, and the residue was worked up as in method A.

Method C. To a solution of the carboxy component (1 mmol) in DMF (2 mL) were added IBCF (1 mmol), the amino acid or the amino peptide component (1 mmol), and TEA (1 or 2 mmol if the amino component was in the protonated form) in the above order at −15 °C. The reaction mixture was stirred for 1 h at −5 °C and for 12 h at room temperature. The solvent was evaporated *in vacuo* and the residue worked up as described in method A.

Protection Procedures. Method D. To a solution of amino acid (20 mmol) in *tert*-butyl alcohol (10 mL) were added 1 N NaOH (20 mL) and a solution of Boc_2O (22 mmol) in *tert*-butyl alcohol (10 mL) in the above order at 0 °C. The reaction mixture was stirred overnight. The turbid solution was diluted

with 20 mL of water and extracted with pentane (30 mL). The aqueous phase was acidified to pH 2−3 with solid citric acid and extracted with three 50 mL portions of ethyl acetate. The organic phase was dried over Na_2SO_4. The solvent was evaporated *in vacuo*, and the residue was crystallized from the appropriate solvents.

Method E. To a solution of the amino acid (5 mmol) in dioxane (5 mL) were added 1 N NaOH (5 mL) and a solution of Z_2O (5.5 mmol) in dioxane (5 mL) in the above order at 0 °C. The reaction mixture was stirred overnight. The turbid solution was worked up as described in method D.

Method F. To a suspension of the amino acid (7 mmol) in methanol (7 mL) was added dichlorosulfoxide (1.7 mL) dropwise at −15 °C. The mixture was stirred at room temperature for 3 h and than refluxed for 4 h. The solvent was evaporated *in vacuo*, and the residue was triturated with diethyl ether. The resulting solid was collected and dried.

Analyses of the Peptide Analogues. 1. Analytical Data of Protected Intermediates Related to [Ac$_6$c^2] Peptides. Boc-Tyr-Ac$_6$c-OH: 79% yield; mp 210−212 °C; $[\alpha]^{20}_D$ −26.6°; R_f 0.90 (II); ^{1}H-NMR ($CDCl_3$) 1.38 (9H, $3CH_3$, s), 1.46 and 1.73−2.02 (10H, $5CH_2$, ms), 2.91 (2H, CH_2, m), 4.25 (1H, CH, m), 6.08 (1H, NH, d, J = 7.29 Hz), 6.70 and 7.03 (2H + 2H, 2d, J = 8.33 Hz), 7.27 (1H, NH, s).

Boc-Tyr-Ac$_6$c-Phe-OH: 89% yield; mp 128−130 °C; $[\alpha]^{20}_D$ +4.2°; R_f 0.88 (II); ^{1}H-NMR ($CDCl_3$ + DMSO) 1.38 (9H, $3CH_3$, s), 1.22−2.08 (10H, $5CH_2$, ms), 2.94 (2H, CH_2, m), 3.10 (2H, CH_2, m), 4.24 (1H, CH, m), 5.70 (1H, NH, d, J = 6.83 Hz), 6.75 and 7.03 (2H + 2H, 2d, J = 8.28 Hz), 6.83 (1H, NH, s), 7.07 (1H, NH, br).

Boc-Tyr-Ac$_6$c-Phe-Asp(OtBu)-Val-Val-Gly-NH$_2$: 71% yield; mp 125−127 °C; $[\alpha]^{20}_D$ −26.7°; R_f 0.33 (III).

2. Analytical Data of Protected Intermediates Related to [Ac$_6$c^2-Ac$_6$c^3] Peptides. Boc-Ac$_6$c-Ac$_6$c-OMe: 56% yield; mp 130−132 °C; R_f 0.76 (III); ^{1}H-NMR ($CDCl_3$) 1.46 (9H, $3CH_3$, s), 1.30−2.11 (20H, $10CH_2$, ms), 3.67 (3H, CH_3, s), 4.67 (1H, NH, s), 7.43 (1H, NH, br s).

Boc-Tyr(OtBu)-Ac$_6$c-Ac$_6$c-OH: 61% yield; mp 145−147 °C; $[\alpha]^{20}_D$ −1.7°; R_f 0.27 (III); ^{1}H-NMR ($CDCl_3$) 1.33 (9H, $3CH_3$, s), 1.39 (9H, $3CH_3$, s), 1.49−2.24 (20H, $10CH_2$, ms), 2.90 and 3.15 (2H, CH^2, AB or ABX, J_{AB} = 14.16 Hz, J_{AX} = 8.45 Hz, J_{BX} = 5.95 Hz), 4.28 (1H, CH, X of ABX, m), 5.09 (1H, NH, br), 6.56 (1H, NH, s), 6.94 and 7.13 (2H + 2H, C_6H_4, 2d, J = 8.42 Hz), 7.59 (1H, NH, s).

Boc-Tyr-D-Ala-Ac$_6$c-Asp(OtBu)-Val-Val-Gly-NH$_2$: 80% yield; mp 123−126 °C; $[\alpha]^{20}_D$ +1.6°; R_f 0.20 (III).

3. Analytical Data of Protected Intermediates Related to [Ac$_x$c^4] Peptides. Boc-Phe-Ac$_3$c-OMe: 85% yield; mp 128−130 °C; $[\alpha]^{20}_D$ −1.84°; R_f 0.76 (III); ^{1}H-NMR ($CDCl_3$) 0.85−1.00 (1H, $^1/_2CH_2$, m), 1.05−1.15 (1H, $^1/_2CH_2$, m), 1.41 (9H, $3CH_3$, s), 1.40−1.73 (2H, CH_2, m), 3.03−3.09 (2H, CH_2, AB of ABX, J_{AB} = 13.5 Hz, J_{BX} = 7.5 Hz), 3.66 (3H, CH_3, s), 4.31 (1H, CH, X of ABX, m), 5.12 (1H, NH, br), 6.39 (1H, NH, s), 7.27 (5H, C_6H_5, s).

Boc-Tyr-D-Ala-OH: 99% yield; mp 190−193 °C; $[\alpha]^{20}_D$ +5.7°; R_f 0.89 (I); ^{1}H-NMR (DMSO) 1.16 (3H, CH_3, d, J = 9.7 Hz), 1.29 (9H, $3CH_3$, s), 2.50−1.85 (2H, CH_2, m), 3.95−4.22 (2H, 2CH, m), 6.64 and 7.01 (2H + 2H, C_6H_4, 2d, J = 8.18 Hz), 6.75 (1H, NH, d, J = 6.75 Hz), 8.14 (1H, NH, d, J = 7.40 Hz), 12.45 (1H, COOH, vbr).

Boc-Tyr-D-Ala-Phe-Ac$_3$c-OMe: 85% yield; mp 110−112 °C; $[\alpha]^{20}_D$ +24.0°; R_f 0.25 (III).

Boc-Tyr-D-Ala-Phe-Ac$_3$c-Val-Val-Gly-NH$_2$: 78% yield; mp 130−132 °C; $[\alpha]^{20}_D$ +5.6°; R_f 0.22 (III).

Z-Ac$_5$c-Val-Val-Gly-NH$_2$: 55% yield; mp 150−153 °C; $[\alpha]^{20}_D$ −6.3°; R_f 0.30 (III).

Boc-Tyr-D-Ala-Phe-Ac$_5$c-Val-Val-Gly-NH$_2$: 42% yield; mp 200−205 °C; $[\alpha]^{20}_D$ +0.39°; R_f 0.35 (III); K' 6.68 (a).

Boc-Phe-Ac$_6$c-OMe: 84% yield; mp 112−113 °C; $[\alpha]^{20}_D$ −17.0°; R_f 0.76 (III); ^{1}H-NMR ($CDCl_3$) 1.43 (9H, $3CH_3$, s), 1.45−2.00 (10H, $5CH_2$, ms), 3.02 and 3.12 (2H, CH_2, AB of ABX, J_{AB} = 14.2 Hz, J_{AX} = 6.50 Hz, J_{BX} = 7.52 Hz), 3.69 (3H, CH_3, s), 4.33 (1H, CH, X of ABX, m), 5.07 (1H, NH, d, J = 6.54 Hz), 7.28 (5H, C_6H_5, s).

Boc-Tyr-D-Ala-Phe-Ac$_6$c-OMe: 46% yield; mp 100−102 °C; $[\alpha]^{20}_D$ +15.8°; R_f 0.57 (III); ^{1}H-NMR ($CDCl_3$) 1.07 (3H, CH_3, d, J = 7.00 Hz), 1.13−1.68 (10H, $5CH_2$, ms), 1.42 (9H, $3CH_3$, s), 2.81−3.22 (4H, $2CH_2$, ms), 3.65 (3H, CH_3, s), 4.17−4.23 (2H, 2CH, m), 4.64 (1H, CH, m), 5.28 (1H, NH, d, J = 7.69 Hz), 6.62 (1H, NH, d, J = 7.36 Hz), 6.72 and 6.98 (2H + 2H, C_6H_4, 2d, J = 8.20 Hz), 7.24 (5H, C_6H_5, s), 7.20−7.25 (1H, NH, br), 7.46 (1H, NH, s), 6.16 (1H, NH, s).

4. Analytical Data of Protected Intermediates Related to [Ac$_x$c^3] Peptides. H-Ac$_3$c-OMe HCl: 91% yield; mp 184−186 °C; R_f 0.48 (III).

Boc-D-Ala-Ac$_3$c-OH: 73% yield; mp 168−170 °C; $[\alpha]^{20}_D$ +20.6°; R_f 0.79 (II); ^{1}H-NMR ($CDCl_3$ + DMSO) 1.39 (9H, $3CH_3$, s), 1.35 (3H, CH_3, d, J = 6.5 Hz), 1.47 (4H, $2CH_2$, 2m), 2.91 (2H, CH_2, m), 4.12 (1H, CH, m), 4.39 (1H, CH, m), 5.76 (1H, NH, br), 6.74 and 7.00 (2H + 2H, 2d, J = 8.28 Hz), 7.29 (1H, NH, br), 7.84 (1H, NH, s).

Boc-D-Ala-Ac$_3$c-Asp(OtBu)-Val-Val-Gly-NH$_2$: 82% yield; mp 138−140 °C; $[\alpha]^{20}_D$ +2.1°; R_f 0.26 (III).

H-Ac$_5$c-OMe·HCl: 99% yield; mp 190−192 °C; R_f 0.45 (III).

Boc-D-Ala-Ac$_5$c-OH: 97% yield; mp 148−150 °C; $[\alpha]^{20}_D$ +24.5°; R_f 0.86 (II); ^{1}H-NMR ($CDCl_3$) 1.35 (3H, CH_3, d, J = 7.0 Hz), 1.44 (9H, $3CH_3$, s), 1.77 and 2.28 (8H, $4CH_2$, m), 4.18 (1H, CH, m), 5.23 (1H, NH, d, J = 7.53 Hz), 7.09 (1H, NH, s).

Boc-Tyr-D-Ala-Ac$_5$c-OH: 94.7% yield; mp 96−98 °C; $[\alpha]^{20}_D$ +35.4°; R_f 0.88 (II); ^{1}H-NMR ($CDCl_3$ + DMSO) 1.38 (9H, $3CH_3$, s), 1.43 (3H, CH_3, d, J = 6.1 Hz), 1.73−2.22 (8H, $4CH_2$, m), 2.88 (2H, CH_2, m), 4.20 (1H, CH, m), 4.42 (1H, CH, m), 5.35 (1H, NH, br), 6.75 and 6.99 (2H + 2H, 2d, J = 8.38 Hz), 7.08 (1H, NH, br), 7.46 (1H, NH, s).

Boc-Tyr-D-Ala-Ac$_5$c-Asp(OtBu)-Val-Val-Gly-NH$_2$: 80% yield; mp 144−146 °C; $[\alpha]^{20}_D$ +3.3°; R_f 0.20 (III).

H-Ac$_6$c-OMe HCl: 94% yield; mp 192−194 °C; R_f 0.49 (III).

Boc-D-Ala-Ac$_6$c-OH: 87% yield; mp 125−126 °C; $[\alpha]^{20}_D$ +38.1°; R_f 0.83 (II); ^{1}H-NMR ($CDCl_3$) 1.36 (3H, CH_3, d, J = 7.0 Hz), 1.45 (9H, $3CH_3$, s), 1.77−2.28 (10H, $5CH_2$, m), 4.21 (1H, CH, m), 5.2 (1H, NH, br), 7.0 (1H, NH, s).

Boc-Tyr-D-Ala-Ac$_6$c-OH: 93% yield; mp 110−113 °C; $[\alpha]^{20}_D$ +40.6°; R_f 0.80 (II); ^{1}H-NMR ($CDCl_3$ + DMSO) 1.38 (9H, $3CH_3$, s), 1.43 (3H, CH_3, d, J = 6.1 Hz), 1.73−2.22 (10H, $5CH_2$, m), 2.88 (2H, CH_2, m), 4.20 (1H, CH, m), 4.42 (1H, CH, m), 5.35 (1H, NH, br), 6.75 and 6.99 (2H + 2H, 2d, J = 8.38 Hz), 7.08 (1H, NH, br), 7.46 (1H, NH, s).

Deprotection Procedures. Method G. Boc and OtBu protecting groups were removed by treating the peptide with aqueous 95% TFA for 30 min. The solvent was evaporated *in vacuo*, and the residue was triturated with diethyl ether. The resulting solid was collected and dried.

Method H (Hydrogenation). Hydrogenation was performed in methanol or acetic acid (10 mL/mmol of peptide) at atmospheric pressure and room temperature in the presence of 5% or 10% palladium on charcoal (catalyst to peptide ratio, 1:9, w/w) generally for 1 h (testing with TLC using solvent I). The catalyst was filtered through paper and evaporated to dryness. The residue was treated as described in method C (*supra vide*).

Method I. The peptide methyl ester (5 mmol) was dissolved in methanol (20 mL) and treated with 1.2 mol equiv of 1 N NaOH for 2 h at room temperature (TLC tests used solvent II). The solution was diluted with water and concentrated *in vacuo* to remove methanol. After cooling at 0 °C, it was acidified with 1 N HCl and the product extracted with ethyl acetate. The organic solution was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The resulting solid peptide was crystallized from the appropriate solvents.

Radioreceptor Assays. Receptor affinities of deltorphin C analogues were assessed using competitive binding assays labeled either with [^{3}H]DPDPE (0.63 nM) for the δ sites or with [^{3}H]DAGO (1.28 nM) for the μ sites according to published methods.[13,15,35] Excess unlabeled peptides (2 μM) saturated the opioid binding sites in order to obtain a base-line value. Duplicate tubes contained preincubated rat brain synaptosomal membranes[3] in equilibrium assays containing 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, glycerol, and protease inhibitors[3,35] for 120 min at room temperature (22−23 °C). Incubation mixtures were trapped in the filters and rapidly washed within 5 s with 3 × 2 mL of ice-cold buffer containing 0.01%

BSA or lactalbumin. In the duplicate assays, labeled peptides were displaced using 6−14 concentrations of each analogue to cover a 1000-fold range in peptide concentration. For determination of the Hill coefficients and statistical analyses of the data, however, assays were conducted in triplicate using 25−35 concentrations of peptide and tested for fits to one- or two-site binding models according to Attila *et al.*[32] and Bryant *et al.*[21] using Prism (v. 1.03, GraphPad, San Diego, CA). Competitive inhibition constants (K_i) were derived from the IC_{50} values based on the equations of Cheng and Prusoff.[36]

Bioactivity. Bioassays were conducted according to Salvadori *et al.*[13] using a 2−3 cm portion of guinea pig ileum (GPI) in a 20 mL organ bath containing 70 μM hexamethonium bromide and 0.125 μM mepyramine maleate aerated with 95% O_2/5% CO_2 at 36 °C for μ receptors as follows: GPI was stimulated transmurally with 0.5 ms square-wave pulses at 0.1 Hz in which the stimulus was 1.5 times that necessary to produce a maximal twitch (∼30 V) and recorded at a magnification ratio of 1:15. For δ receptors, a single mouse vas deferens (MVD) was used suspended in 4 mL of modified Kreb's solution aerated with 95% O_2/5% CO_2 at 33 °C while the twitch induced by field stimulation (0.1 Hz for 1 ms at 40 V) was recorded with a isometric transducer. Dose−response curves were prepared for each analogue in comparison to known compounds for each tissue preparation (dermorphin or morphine for GPI and deltorphin for MVD). The K_e values for naloxone or *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864) were in the range of 1−2 nM as detailed previously.[13]

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References

(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem*. **1972**, *247*, 977). Other abbreviations: Ac_xc, $C^{\alpha,\alpha}$-dialkylated α-amino acids (1-aminocycloalkane-1-carboxylic acid) (hexane, $_x = 6$; pentane, $_x = 5$; propane $_x = 3$); Boc, *tert*-butoxycarbonyl; Boc_2O, Boc-anhydride (di-*tert*-butyl dicarbonate); DCC, dicyclohexylcarbodiimide; DIPCDI, 1,3-diisopropylcarbodiimide; DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide; EDC, ethyldicarbodiimide; Fmoc, (fluoren-9-ylmethoxy)carbonyl; HOBt, 1-hydroxybenzotriazole; IBCF, isobutyl chloroformate; OtBu, *tert*-butyl ester; ^{1}H-NMR, proton nuclear magnetic resonance spectrometry; Rink resin, 4-(2′,4′-dimethoxyphenyl)-Fmoc-aminomethylphenoxy-resin; TEA, triethylamine; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

(2) (a) Erspamer, V. The opioid peptides of the amphibian skin. *Int. J. Dev. Neurosci*. **1992**, *10*, 3−30. (b) Lazarus, L. H.; Attila, M. The toad, ugly and venomous, wears yet a precious jewel in his skin. *Prog. Neurobiol*. **1993**, *41*, 473−507. (c) Lazarus, L. H.; Bryant, S. D.; Attila, M.; Salvadori, S. Frog opioid peptides. A case for environmental mimicry. *Environ. Health Perspect*. **1994**, *102*, 648−654.

(3) Lazarus, L. H.; de Castiglione, R.; Guglietta, A.; Wilson, W. E. Highly selective δ-opioid receptor peptide from preprodermorphin gene sequence. *J. Biol. Chem*. **264**, 3047−3050, 1989.

(4) (a) Mor, A.; Delfour, A.; Sagan, S.; Amiche, M.; Pradelles, P.; Rossier, J.; Nicolas, P. Isolation of dermenkephalin from amphibian skin, a high-affinity δ-selective opioid heptapeptide containing a D-amino acid residue. *FEBS Lett*. **1989**, *255*, 269−274. (b) Sagan, S.; Amiche, M.; Delfour, A.; Camus, A.; Mor, A.; Nicolas, P. Differential contribution of C-terminal regions of dermorphin and dermenkephalin to opioid-sites selection and binding potency. *Biochem. Biophys. Res. Commun*. **1989**, *163*, 726−732.

(5) Barra, D.; Mignogna, G.; Simmaco, M.; Pucci, P.; Severini, C.; Falconieri-Erspamer,G.; Negri, L.; Erspamer, V. [D-Leu^2]-Deltorphin, a 17 amino acid opioid peptide from the skin of the Brazilian hylid frog, *Phyllomedusa burmeisteri*. *Peptides* **1994**, *15*, 199−202.

(6) Lazarus, L. H.; Salvadori, S.; Santagada, V.; Tomatis, R.; Wilson, W. E. Function of negative charge in the "address domain" of deltorphins. *J. Med. Chem*. **1991**, *34*, 1350−1359.

(7) Melchiorri, P.; Negri, L.; Falconieri Erspamer, G.; Severini, C.; Corsi, R.; Soaje, M.; Erspamer, V.; Barra, D. Structure-activity relationships of the δ-opioid-selective agonists, deltorphins. *Eur. J. Pharmacol*. **1991**, *195*, 201−207.

(8) Salvadori, S.; Bryant, S. D.; Bianchi, C.; Balboni, G.; Attila, M.; Lazarus, L. H. Phe^3-substituted analogues of deltorphin C. Spatial conformation and topography of the aromatic ring in peptide recognition by δ opioid receptors. *J. Med. Chem*. **1993**, *36*, 3748−3756.

(9) (a) Heyl, D. L.; Schmitter, S. J.; Bouzit, H.; Johnson, T. W.; Hepp, A. M.; Kurtz, K. R.; Mousigian, C. Substitution of aromatic and nonaromatic amino acids for the Phe^3 residue in the δ-selective opioid peptide deltorphin I: effects on binding affinity and selectivity. *Int. J. Pept. Protein Res*. **1994**, *44*, 420−426. (b) Heyl, D. L.; Dandabathula, M.; Kurtz, K. R.; Mousigian, C. Opioid receptor binding requirements for the δ-selective peptide deltorphin I: Phe^3 replacement with ring-substituted and heterocyclic amino acids. *J. Med. Chem*. **1995**, *38*, 1242−1246.

(10) Dupin, S.; Tafani, J.-A. M.; Mazaguil, H.; Zajac, J.-M. [^{125}I]-[D-Ala^2]Deltorphin-I: a high affinity delta-selective opioid receptor ligand. *Peptides* **1991**, *12*, 825−830.

(11) Marastoni, M.; Salvadori, S.; Balboni, G.; Scaranari, V.; Borea, P. A.; Tomatis, R. Synthesis and opioid activity of tyrosine sulfate containing dermorphin and deltorphin peptides. *Arzneim.-Forsch./Drug Res*. **1995**, *45*, 116−119.

(12) Schiller, P. W.; Weltrowska, G.; Nguyen, T. M.-D.; Wilkes, B. C.; Chung, N. N.; Lemieux, C. Conformationally restricted deltorphin analogues. *J. Med. Chem*. **1992**, *35*, 3956−3561.

(13) Salvadori, S.; Bianchi, C.; Lazarus, L. H.; Scaranari, V.; Attila, M.; Tomatis, R. Para-substituted Phe^3 deltorphin analogues: Enhanced selectivity of halogenated derivatives for δ opioid receptor sites. *J. Med. Chem*. **1992**, *35*, 4651−4657.

(14) Sagan, S.; Charpentier, S.; Delfour, A.; Amiche, M.; Nicolas, P. The aspartic acid in deltorphin I and dermenkephalin promotes targeting to δ-opioid receptor independently of receptor binding. *Biochem. Biophys. Res. Commun*. **1992**, *187*, 1203−1210.

(15) (a) Lazarus, L. H.; Salvadori, S.; Greico, P.; Wilson, W. E.; Tomatis, R. Unique sequence in deltorphin C confers structural requirements for δ opioid receptor selectivity. *Eur. J. Med. Chem*. **1992**, *27*, 791−797. (b) Lazarus, L. H.; Salvadori, S.; Attila, M.; Grieco, P.; Bundy, D. M.; Wilson, W. E.; Tomatis, R. Interaction of deltorphin with opioid receptors: molecular determinants for affinity and selectivity. *Peptides* **1993**, *14*, 21−28.

(16) Lazarus, L. H.; Bryant, S. D.; Salvadori, S.; Attila, M.; Jones, L. S. Opioid infidelity. Novel opioid peptides with dual high affinity for δ and μ receptors. *Trends Neurosci*. **1996**, in press.

(17) Lazarus, L. H.; Salvadori, S.; Tomatis, R.; Wilson, W. E. Opioid receptor selectivity reversal in deltorphin tetrapeptide analogues. *Biochem. Biophys. Res. Commun*. **1991**, *178*, 110−115.

(18) (a) Temussi, P. A.; Salvadori, S.; Amodeo, P.; Bianchi, C.; Guerrini, R.; Tomatis, R.; Lazarus, L. H., Picone, D.; Tancredi, T. Selective opioid dipeptides. *Biochem. Biophys. Res. Commun*. **1994**, *198*, 933−939. (b) Salvadori, S.; Attila, M.; Balboni, G.; Bianchi, C.; Bryant, S. D.; Crescenzi, O.; Guerrini, R.; Picone, D.; Tancredi, T.; Temussi, P. A.; Lazarus, L. H. δ Opioidmimetic antagonists: prototypes for designing a new generation of ultraselective opioid peptides. *J. Mol. Med*. **1995**, *1*, 678−689.

(19) (a) Schiller, P. W.; Nguyen, T. M.-D.; Weltrowska, G.; Wilkes, B. C.; Marsden, B. J.; Lemieux, C.; Chung, N. N. Differential stereochemical requirements of μ vs. δ opioid receptors for ligand binding and signal transduction: development of a class of potent and highly δ-selective peptide antagonists. *Proc. Natl. Acad. Sci. U.S.A*. **1992**, *89*, 11871−11875. (b) Schiller, P. W.; Weltroska, G.; Nguyen, T. M.-D.; Wilkes, B. C.; Chung, N. N.; Lemieux, C. TIPP [ψ]: a highly potent and stable pseudopeptide δ opioid receptor antagonist with extraordinary δ selectivity. *J. Med. Chem*. **1993**, *36*, 3182−3187.

(20) (a) Schmidt, R.; Chung, N. N.; Lemieux, C.; Schiller, P. W. Tic^2-Substitution in dermorphin, deltorphin I and dynorphin A analogues: effect on opioid receptor binding and opioid activity in vitro. *Regul. Pept*. **1994**, *54*, 259−260. (b) Schmidt, R.; Kálmán, A.; Chung, N. N.; Lemieux, C.; Horváth, C.; Schiller, P. W. Structure-activity relationships of dermorphin analouges containing *N*-substituted amino acids in the 2-position of the peptide sequence. *Int. J. Pept. Protein Res*. **1995**, *46*, 47−55.

(21) Bryant, S. D.; Attila, M.; Salvadori, S.; Guerrini, R.; Lazarus, L. H. Molecular dynamics conformations of deltorphin analogues advocate δ opioid binding site models. *Pept. Res*. **1994**, *7*, 175−184.

(22) Bryant, S. D.; Salvadori, S.; Attila, M.; Lazarus, L. H. Topographical conformation of the deltorphins predicates δ receptor affinity. *J. Am. Chem. Soc*. **1993**, *115*, 8503−8504.

(23) (a) Temussi, P. A.; Picone, D.; Tancredi, T.; Tomatis, R.; Salvadori, S.; Marastoni, M.; Balboni, G. Conformational properties of deltorphin: New features of the δ-opioid receptors. *FEBS Lett*. **1989**, *247*, 283−288. (b) Balboni, G.; Marastoni, M.; Picone, D.; Salvadori, S.; Tancredi, T.; Temussi, P. A.; Tomatis, R. New features of the δ opioid receptor: Conformational properties of deltorphin I analogues. *Biochem. Biophys. Res. Commun*. **1990**, *169*, 617−622. (c) Tancredi, T.; Temussi, P. A.; Picone, D.; Amodeo, P.; Tomatis, R.; Salvadori, S.; Marastoni, M.; Santagada, V.; Balboni, G. New insights on μ/δ selectivity of opioid peptides: Conformational analysis of deltorphin analogues.

Biopolymers **1991**, *31*, 751−760. (d) Amodeo, P.; Motta, A.; Tancredi, T.; Salvadori, S.; Tomatis, R.; Picone, D.; Saviano, G.; Temussi, P. A. Solution structure of deltorphin I at 265 K: A quantitative NMR study. *Pept. Res.* **1992**, *5*, 48−55.

(24) Burroughs, L. F. 1-Aminocyclopropane-1-carboxylic acid: a new amino-acid in perry pears and cider apples. *Nature* **1957**, *179*, 360−361.

(25) (a) Adams, D. O.; Yang, S. F. Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 170−174. (b) White, M. F.; Vasquez, J.; Yang, S. F.; Kirsch, J. F. Expression of apple 1-aminocyclopropane-1-carboxylate synthase in *Escherichia coli*: kinetic characterization of wild-type and active-site mutant forms. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12428−12432.

(26) (a) Stammer, C. H. Cyclopropane amino acids (2,3- and 3,4-methanoamino acids). *Tetrahedron* **1990**, *46*, 2231−2254. (b) Burgess, K.; Ho, K.-K. Asymmetric syntheses of all four stereoisomers of 2,3-methanomethionine. *J. Org. Chem*. **1992**, *57*, 5931−5936. (c) Burgess, K.; Ho, K.-K.; Ke, C.-Y. Synthesis of a valuable cyclopropyl chiron for preparation of 2,3-methanoamino acids. *J. Org. Chem*. **1993**, *58*, 3767−3768.

(27) Kolesnikov, Y. A.; Maccechini, M.-L.; Pasternak, G. W. 1-Aminocyclopropane carboxylic acid (ACPC) prevents mu and delta opioid tolerance. *Life Sci*. **1994**, *55*, 1393−1398.

(28) Balaji, V. N.; Ramnarayan, K.; Chan, M. F.; Rao, S. N. Conformational studies on model peptides with 1-aminocyclopropane-1-carboxylic acid residues. *Pept. Res.* **1994**, *7*, 60−71.

(29) (a) Benedetti, E.; di Blasio, B.; Pavone, V.; Pedone, C.; Santini, A.; Crisma, M.; Valle, G.; Toniolo, C. Structural versatility of peptides from $C^{\alpha,\alpha}$-dialkylated glycines: linear Ac_3c homo-oligopeptides. *Biopolymers* **1989**, *28*, 175−184. (b) di Blasio, B.; Pavone, V.; Lonbardi, A.; Pedone, C.; Benedetti, E. Noncoded residues as building blocks in the design of specific secondary structures: symmetically disubstituted glycines and β-alanine. *Biopolymers* **1993**, *33*, 1037−1049.

(30) Misicka, A.; Lipkowski, A. W.; Fang, L.; Knapp, R. J.; Davis, P.; Kramer, T.; Burks, T. F.; Yamamura, H. I.; Carr, D. B.; Hruby, V. J. Topographical requirements for delta opioid ligands: presence of a carboxyl group in position 4 is not critical for deltorphin high delta receptor affinity and analgesic activity. *Biochem. Biophys. Res. Commun*. **1991**, *180*, 1290−1297.

(31) Surrat, C. K.; Johnson, P. S.; Moriwaki, A.; Sedleck, B. K.; Blaschak, C. J.; Wang, J. B.; Uhl, G. R. μ Opiate receptor. Charged transmembrane domain amino acids are critical for agonist recognition and intrinsic activity. *J. Biol. Chem*. **1994**, *269*, 20548−20553.

(32) Tancredi, T.; Salvadori, S.; Amodeo, P.; Picone, D.; Lazarus, L. H.; Bryant, S. D.; Guerrini, R.; Marzola, G.; Temussi, P. A. Conversion of enkephalin and dermorphin into δ-selective opioid antagonists by single-residue substitution. *Eur. J. Biochem*. **1994**, *224*, 241−247.

(33) Attila, M.; Salvadori, S.; Balboni, G.; Bryant, S. D.; Lazarus, L. H. Synthesis and receptor binding analysis of dermorphin hepta-, hexa- and pentapeptide analogues. Evidence for one- and two-site binding models for the μ opioid receptor. *Int. J. Pept. Protein Res.* **1993**, *42*, 550−559.

(34) Bryant, S. D.; Salvadori, S.; Guerrini, R.; Attila, M.; Lazarus, L. H. Structural distinctions among aminocycloalkanecarboxylic acid substituted deltorphins determined using molecular dynamics simulations. In *Peptides: Chemistry, Structure and Biology*; Kaumaya, P. T. P., Hodges, R. S., Eds.; Escom: Leiden, 1995, in press.

(35) Lazarus, L. H.; Salvadori, S.; Balboni, G.; Tomatis, R.; Wilson, W. E. Stereospecificity of amino acid side chains in deltorphin defines binding to opioid receptors. *J. Med. Chem*. **1992**, *35*, 1222−1227.

(36) Cheng, Y.-C.; Prusoff, W. H. Relationships between the inhibition constant (K_i) and the concentration of inhibition which cause 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol*. **1973**, *22*, 3099−3108.

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