Helix-Inducing α-Aminoisobutyric Acid in Opioid Mimetic Deltorphin C Analogues

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The achiral symmetric α-aminoisobutyric acid (Aib) replaced the critical N-terminal residues of the amphibian skin opioid deltorphin C (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) without detriment to the physicochemical requirements for δ opioid receptor recognition. Substitutions by the α,α-dialkyl amino acid in place of D-Ala[2] or Phe[3], or both, exhibited high δ receptor affinity ($K_i\delta$ = 0.12−3.6 nM) and 5−9-fold greater selectivity ($K_i\mu/K_i\delta$ = 5000−8500) than the parent compound. This is the first definitive demonstration that the D-chirality of alanine and the aromaticity of phenylalanine are replaceable by an achiral α,α-dialkylated residue without detrimental effects on ligand binding. Incorporation of the mono-α-alkyl amino acid L- or D-Ala at the third position also produced highly selective δ ligands ($K_i\mu/K_i\delta$ = 2000−3500), albeit with reduced δ affinities ($K_i\delta$ = 6−15 nM). Replacement of the anionic residue Asp[4] by Aib yielded an opioid peptide that fit two-site binding models for the δ receptor (η = 0.763; $P < 0.0001$) and displayed dual high affinity for both δ and μ receptors, emphasizing the repulsive effect by a negative charge at μ receptor sites and the insignificance of Asp for δ affinity. Molecular dynamics conformational analyses suggested that Aib residues caused distinct changes in deltorphin C secondary structure when substituted for D-Ala[2], Asp[4], and simultaneously D-Ala[2] and Phe[3] but not when substituted for Phe[3]. These conformational changes might be critical factors for the proper orientation of reactive constituents of residues in the N-terminal region of deltorphin C. Disparities between binding data and functional bioassays of [Aib[3]] indicated that Phe[3] was required for bioactivity in mouse vas deferens but not for interaction with δ opioid receptors in rat brain membranes.

Introduction[1]

An α,α-dialkylated residue, α-aminoisobutyric acid (α-methylalanine, Aib), typically found in peptaibol antibiotics,[2] is known to stabilize α- and 3_{10}-helices in peptides with 7−20 residues.[2−7] X-ray diffraction studies demonstrated that heptapeptides with Aib positioned adjacent to non-helix-forming valine residues adopted helical conformations.[2−5] Modifications of peptides with Aib substitutions are useful for exploring the impact of secondary conformational changes on the structure−activity relationships between ligands and their receptors based on biological activity[5,8] and receptor binding.[9−11]

The structure−activity relationships of opioid receptors and their ligands are of great interest since the distinct location and conformation of the binding sites of the three (δ, κ, μ) seven-transmembrane G-protein-coupled opioid receptor subtypes are not known and the bioactive conformations of their ligands remain to be elucidated. Furthermore the design of highly selective peptides is critical for determining the distinguishable functions between the opioid receptor subtypes as well as providing potential therapeutics associated with the opioid system.[12] Examples of such applications include modulation of immunity,[13] pain abatement,[14] prevention of morphine tolerance,[15] alcohol dependency,[16] and treatment of autism.[17]

The amphibian skin peptides, deltorphins A (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2), B (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2), and C (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), provide scaffolds for probing opioid structure−activity relationships since they exhibit higher affinity and selectivity for the δ opioid receptor[18] than the endogenous enkephalins (H-Tyr-Gly-Gly-Phe-Leu-OH and H-Tyr-Gly-Gly-Phe-Met-OH).[19] Although there are several studies concerning Aib containing enkephalin derivatives[9−11] with moderate opioid activity, there are none at the time of this study involving the more δ selective deltorphins. Here we present Aib substitutions in deltorphin C to explore its effect on δ opioid receptor binding properties, biological activity, and ligand conformation.

Rationale

The primary amino acid sequence responsible for high opioid receptor selectivity lies within the N-terminal tetrapeptide domain of the naturally occurring deltorphins,[20] which share the common sequence H-Tyr[1]-D-Xaa[2]-Phe[3] with the μ selective dermorphins.[18] Changing the chirality of the first three to four residues reduced receptor binding by orders of magnitude[21−27] and virtually eliminated bioactivity.[23,25] Modification of electronic properties of the Phe[3] benzyl side chain[28]

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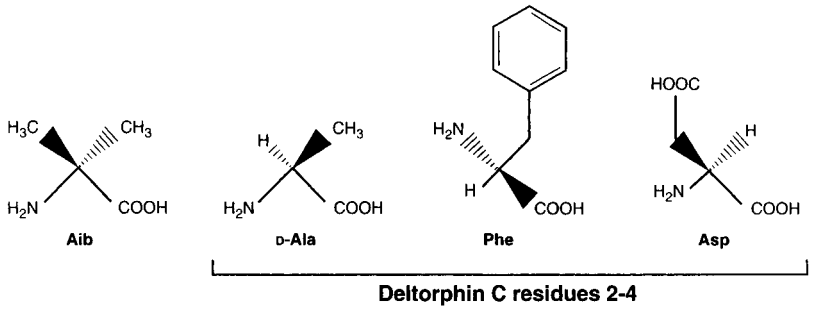

Figure 1. Structures of α-aminoisobutyric acid (Aib) and the amino acids that it replaced in deltorphin C.

or replacement by a variety of unnatural Phe-related amino acids and heterocyclic compounds[29−31] both increased and decreased the δ affinity of deltorphin C. Substitutions by noncharged amino acids for the anionic Glu and Asp residues in the fourth position of deltorphins B and C, respectively, maintained δ affinity and increased μ affinity resulting in nonselective analogues.[27,32,33]

Aib could conceivably replace D-Ala[2] in deltorphin C since the symmetric carbon affords an equal possibility for the L- or D-configuration[2] and Aib adopts (ϕ, ψ) values that overlap those of D-Ala in the Ramachandran map.[3,4] The α,α-dimethyl groups of Aib might further offset the hydrophobicity of the phenyl side chain of Phe[3] since the converse was observed when L-Phe replaced Aib residues in Aib-rich peptides.[5] It is feasible that Aib can substitute for the critical residues in the N-terminal region of deltorphin C without detrimentally affecting the physicochemical requirements for receptor recognition and at the same time induce conformational changes that influence receptor recognition. In other peptides the α,α-dimethyl groups restricted backbone orientation (ϕ, ψ) to the 3_{10}-helical and right and left α-helical regions of the Ramachandran plot,[3,4] and orientation of the peptide bonds (N−C′) preceding and following Aib residues was *trans* (180).[2]

Although the 3-dimensional structure of deltorphin C was investigated using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy[34,35] and computational modeling methods,[27,35,36] its solid state structure remains unresolved. Cumulative data from ^{1}H NMR indicated type I and II′ β-turns in the N-terminal region,[34,35,37,38] while computational modeling,[27,35−37] indicated type I, II′, and III β-turns, demonstrating that deltorphin C is highly flexible in solution, and no firm conclusions exist about its bioactive conformation(s). However, the presence of $C^{\alpha,\alpha}$-dialkyl linear or cyclic α-amino acids[6] and the $C^{\alpha,\alpha}$-dialkyl 1-aminocycloalkane-1-carboxylic acids[39] are known to form 3_{10}- or α-helices in crystalline peptides[2−4] and helices and β-turns in enkephalin analogues.[10,11] The symmetrical dialkylated Aib residue could impart helicity in deltorphin C analogues providing evidence that opioid receptor binding sites interact with helices or at least well-ordered structures which might be an important factor for receptor recognition.[27,30,33,36] This structural information is useful for the refinement of a δ opioid agonist pharmacophore and for further development of highly selective opioid ligands. Thus, this study explores conformational motifs adopted by Aib analogues of deltorphin C (Figure 1) using molecular dynamics simulations without specifying bioactive conformations and assesses the impact of these substitutions on receptor binding and biological activity.

Results

Molecular Dynamics Simulations. The software program Dictionary of Protein Secondary Structure (DSSP)[40] was used to assign secondary structures based on hydrogen-bonding patterns of conformers selected from the molecular dynamics trajectory. The results are shown in Figure 2, and Figure 3 illustrates the backbone superimpositions of deltorphin C and the Aib analogues.

The majority (88%) of deltorphin C conformers (Figure 2a) maintained homogeneous helicity. Similarly, conformers of [Aib[3]] (Figure 2c) were chiefly helical, although the frequency was reduced almost by one-half compared to the parent peptide (Figure 2a); the remaining structures adopted helices with β-turns. [Aib[2,3]] conformers displayed almost equal freqencies between homogeneous helical structures and conformers with helices joined by type III β-turns (Figure 2d). The [Aib[2]] (Figure 2b) and [Aib[4]] (Figure 2e) analogues contrasted most with deltorphin C (Figure 2a), since they lacked homogeneous helical structures. Whereas [Aib[2]] conformers exhibited β-turns and 3_{10}-helix combinations, the majority of the conformers consisted largely of type II′ and III β-turns. The [Aib[4]] conformers (Figure 2e) displayed the most diverse array of secondary structures of any analogue with type I and I′ β-turns and bends joined by 3_{10}-helix and conformers with only type III, III′, and IV β-turns. None of the β-turns adopted by the Aib analogues resembled those of deltorphin C.

Membrane-Bound Receptor Binding. The chemical structure of Aib, compared to those of D-Ala, Phe, and Asp, is shown in Figure 1. Replacement of D-Ala[2] (**2**) or Phe[3] (**3**) by Aib yielded high-δ-affinity analogues with a 6−8-fold greater δ selectivity than deltorphin C (**1**) (Table 1). Although the substitution of Asp[4] (**4** and **5**) by Aib had no demonstrable effect on δ affinity, μ receptor binding increased 340-fold in **4** such that the analogue completely lacked selectivity. The free acid C-terminus of **5** only partially negated the enhanced binding to μ receptors observed in **4**. The replacement of the dipeptide sequence D-Ala[2]-Phe[3] by Aib[2,3] (**6**) reduced δ affinity 20-fold, and that affinity (in the range of 3−4 nM) remains analogous to, if not better than, the affinity of many enkephalin-based opioid peptides.[19] However, the accompanying 100-fold loss in μ binding increased δ selectivity 5-fold relative to deltorphin C (**1**). The Phe[3] deletion analogue **7**, [Aib[2],des-Phe[3]], essentially lost all interaction to both δ and μ opioid receptors. Peptides with Phe[3] replaced by the mono-α-

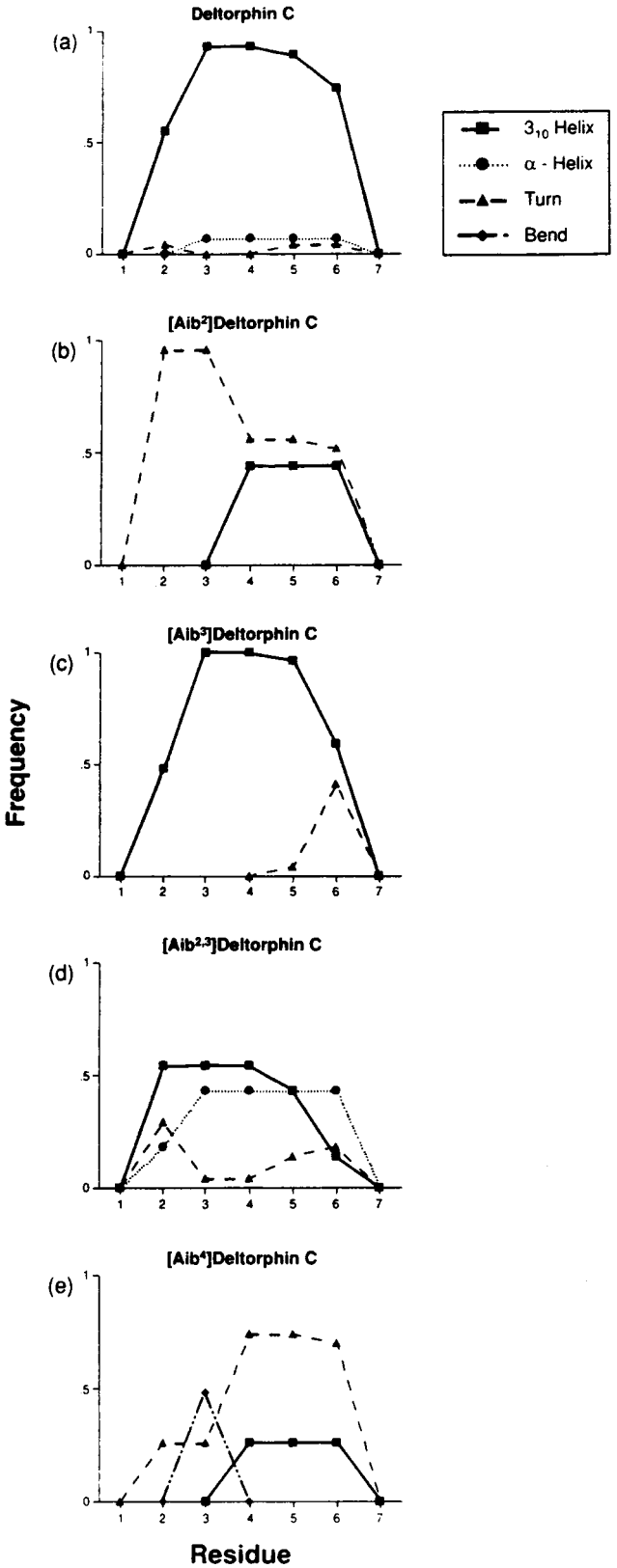

Figure 2. Secondary structural features of deltorphin C analogues analyzed using the program DSSP.[40] Frequency indicates the ratio between the total number of structures analyzed and the number of times that a particular secondary structure was observed.

methyl amino acid L-Ala[3] (**8**) or its D-isomer (**9**) (Table 1) exhibited decreased affinity for both δ and μ receptors but nonetheless exhibited a doubling (**8**) or quadrupling (**9**) of δ selectivity.

The Hill coefficients (η) of **4, 5, 8,** and **9** for the δ receptor displayed heterogeneous binding as illustrated with [Aib[4]]deltorphin C (**4**) (Figure 4). Differences of <0.15 in log of the 95% confidence interval in the η determination with $P < 0.0001$ in which F tests $= 15.1$, 25.1, 25.5, and 14.1 for **4, 5, 8,** and **9**, respectively, indicated fits to a two-site binding model for the δ receptor. Moreover, the fit of **4** to μ receptors corresponded to a one-site binding model ($\eta = 0.999 \pm 0.048$) as previously observed with all of the $C^{\alpha,\alpha}$-dialkyl cyclic amino acids at μ receptors.[32] Analogues **1, 2, 3,** and **6** were described by iterative calculations to fit a simple, bimolecular, one-site binding model (F ranged from 0.0 to 0.5 and $P > 0.6$ are nonsignificant fits for a two-site model).

Functional Biological Activity *in Vitro*. Pharmacological activity *in vitro* of **1, 2,** and **4** on the relaxation of mouse vas deferens (MVD) indicated agonist activities with IC_{50} values and δ selectivity (Table 2) that supported the receptor binding data in rat brain membranes (Table 1). The relationship between the high δ and μ affinity of [Aib[4]]deltorphin (**4**) in rat brain synaptosomes and the high δ and μ activity recorded with MVD and guinea pig ileum (GPI), respectively, indicated a dual mode of action at disparate opioid receptors. However, the low activity in MVD of [Aib[3]] **3** and [Aib[2,3]] **6** was inconsistent with the high δ affinity observed in the synaptosomal preparations. Those data deviated by factors of 70−130 as noted in other bioassay and receptor data.[29,30−32,41−43]

Discussion

Enhanced selectivity and bioactivity exhibited by the Aib analogues suggested that they provided a better fit to the δ opioid receptor binding site than deltorphin C, and that might reflect a preference for more stable, well-ordered ligand binding conformations. Conformers of **2** and **4** contrasted most with deltorphin C since they lacked homogeneous helical structures (Figure 2b,e). The removal of the chiral D-Ala and the bulky Asp residues increased conformational flexibility, while both **2** and **4** exhibited the highest δ affinities (Table 1) and bioactive potencies (Table 2) on MVD. This implied that conformational change through increased flexibility relative to the parent peptide facilitated receptor interaction or perhaps increased its probability of adopting bioactive conformations.

Replacement of the critical D-enantiomer in the second position by Aib substantially enhanced δ selectivity and δ agonist bioactivity. Normally, amino acid replacements at this key position (depending upon the nature of the residue) either caused substantial losses in receptor affinity,[21,22,26,44] were moderately tolerated,[26,32,45] or eliminated bioactivity entirely.[23−25] Interestingly, replacement of D-Ala in deltorphin C with the achiral $C^{\alpha,\alpha}$-dialkyl amino acid 1-aminocyclohexane-1-carboxylic acid[32] decreased δ affinity 16-fold while the Aib residue substitution of a similar scaffold slightly enhanced δ opioid receptor affinity relative to the parent peptide. This phenomenon further underscores the sensitivity of the δ opioid receptor binding site to the physical dimension as well as orientation of the side chain at the second position.

The high dual affinity for δ and μ receptors by **4** indicated that Aib substitution for Asp accommodated

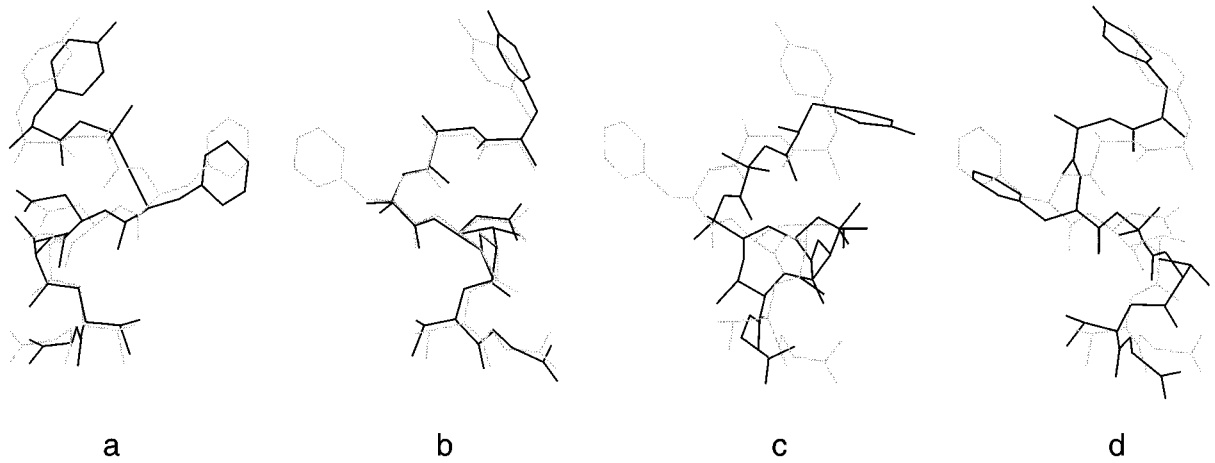

Figure 3. Backbone superimpositions of deltorphin C (gray) with Aib analogues (black): (a) [Aib^2]deltorphin C, (b) [Aib^3]deltorphin C, (c) [Aib^{2,3}]deltorphin C, and (d) [Aib^4]deltorphin C. Root mean square (rms) deviations were 0.9, 0.3, 1.8, and 1.3 Å, respectively. Hydrogen atoms are not shown.

Table 1. Receptor Affinities and Hill Coefficients of Deltorphin C Analogues Containing α-Aminoisobutyric Acid and Alanine Substitutions[a]

		δ receptor properties		μ binding	
no.	peptide	$K_i\delta$ (nM)	η	$K_i\mu$ (nM)	$K_i\mu/K_i\delta$
1	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.15 ± 0.03 (8)	−0.951 ± 0.033	147 ± 29 (11)	980[b]
2	Tyr-**Aib**-Phe-Asp-Val-Val-Gly-NH_2	0.12 ± 0.02 (7)	−0.944 ± 0.044	1015 ± 63 (4)	8460
3	Tyr-D-Ala-**Aib**-Asp-Val-Val-Gly-NH_2	0.80 ± 0.13 (5)	−0.936 ± 0.035	4544 ± 792 (5)	5680
4	Tyr-D-Ala-Phe-**Aib**-Val-Val-Gly-NH_2	0.20 ± 0.07 (9)	−0.763 ± 0.030	0.43 ± 0.11 (4)	2.2
5	Tyr-D-Ala-Phe-**Aib**-Val-Val-**Gly-OH**	0.11 ± 0.002 (4)	−0.624 ± 0.034	4.48 ± 0.79 (3)	41
6	Tyr-**Aib-Aib**-Asp-Val-Val-Gly-NH_2	3.62 ± 0.25 (4)	−0.993 ± 0.058	15200 ± 1300 (3)	4200
7	Tyr-**Aib**-Asp-Val-Val-Gly-NH_2	2628 ± 396 (3)	nd[c]	440700 ± 49500 (3)	168
8	Tyr-D-Ala-**Ala**-Asp-Val-Val-Gly-NH_2	5.72 ± 1.65 (5)	−0.646 ± 0.040	13600 ± 4300 (3)	2380
9	Tyr-D-Ala-D-**Ala**-Asp-Val-Val-Gly-NH_2	15.5 ± 1.76 (5)	−0.748 ± 0.037	54400 ± 12000 (3)	3510

[a] Receptor affinities are listed according to their affinities (K_i) in nM (±standard error), and δ selectivity is the ratio $K_i\mu/K_i\delta$. The number of independent repetitions (n) conducted in duplicate or triplicate for each assay is given in parentheses. The Hill coefficient, η, is a negative value due to the orientation of the slope in the competition binding curves and determined by the iterative calculations.[27,69] [b] Data taken from Breveglieri *et al.*[32] [c] Not determined due to the inability to accurately calculate the η at a low affinity constant. Amino acids denoted in bold represent substitutions in the title peptide **1**.

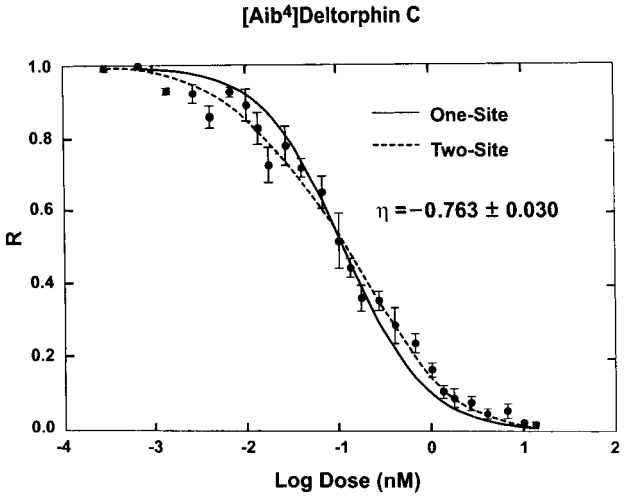

Figure 4. Two-site binding model preference for [Aib^4]-deltorphin C. Differentiation between one- and two-site binding models used Prism and were based on the stringent criteria of Attila *et al.*[69] and Bryant *et al.*[27] in which the Hill coefficient (η) is <0.850, a narrow log of the confidence interval (<0.15) and $P < 0.0001$ in the F test. The points represent the mean ± SEM of five independent experiments conducted in triplicate.

binding conformations compatible to both δ and μ receptor types. Retention of high affinity for δ receptors and increased affinity for μ receptors by elimination of the negatively charged residue (Asp^4; **4** and **5**) supported previous data with deltorphin C analogues modified at the fourth residue[27,32,44,46] and led to the coining of the concept "opioid infidelity", meaning that these peptides exhibited high affinity for two disparate receptors.[33] These data verify that a portion of the binding pocket of δ and μ receptors is quite similar in spite of the fact that the receptors have only approximately 60% sequence identity.[47] Moreover, **4** (Aib^4) exhibited exceptional receptor binding and acted *in vitro* as an agonist in both MVD and GPI bioassays (Table 2), and **4** also mirrored responses observed with other position 4 substitution analogues.[27,32] The conformation of this dual affinity peptide (Figure 3d) lends credence to the notion that its composite solution structure can assist in answering the question of how flexible peptides with comparable N-terminal sequences discriminate between δ and μ receptor binding sites.[27,32,33] Evidence strongly supports the idea that the negatively charged residue Glu^4 or Asp^4 in deltorphin B or C, respectively, is responsible for discrimination between δ and μ receptors.[30,48,49] Furthermore, the di-, tri-, and tetrapeptide opioid mimetics containing Tic in the second position and a C-terminal free acid function succinctly demonstrated that charge accounts for δ selectivity. In addition, neutralization by C-terminal amidation enhanced μ affinity and augmented the shift from δ to μ selective ligands.[50−52]

Phe^3 replacement by Aib did not significantly alter secondary conformations of **3** relative to deltorphin C

Table 2. Bioactivity of Deltorphin C Analogues[a]

no.	peptide	IC_{50} (nM) MVD	IC_{50} (nM) GPI	GPI/MVD
1	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.46 ± 0.24	420 ± 95	913
2	Tyr-**Aib**-Phe-Asp-Val-Val-Gly-NH_2	1.44 ± 0.24	>10 μM	>7000
3	Tyr-D-Ala-**Aib**-Asp-Val-Val-Gly-NH_2	57.3 ± 5.5	>10 μM	>175
4	Tyr-D-Ala-Phe-**Aib**-Val-Val-Gly-NH_2	0.36 ± 0.3	4.5 ± 0.35	13
6	Tyr-**Aib-Aib**-Asp-Val-Val-Gly-NH_2	70 ± 23	>10 μM	>21

[a] Functional bioassays used guinea pig ileum (GPI) and mouse vas deferens (MVD) for μ and δ receptors, respectively, and are listed as the midpoint (50%) in the inhibition curves (IC_{50}) in nM or, when denoted, in μM. Data are the mean of four to six separate determinations ± standard error. Amino acid substitutions in title peptide **1** are in bold.

(Figure 2c) supporting previous observations that Aib was replaceable by Phe in Aib-rich peptides.[5] However, Aib replacement of Phe^3 exhibited a 120-fold loss of MVD potency (Table 2), while it was not detrimental to rat brain δ opioid receptor affinity (Table 1). Enkephalin analogues with modifications at Phe^4 also displayed differentiation between δ opioid receptors in the rat brain and MVD.[53] Apparently the aromaticity of Phe^3 as well as the orientation of the aromatic residue was critical for δ receptor activation in the peripheral tissues, but hydrophobicity was sufficient for interaction with receptors in the rat brain membranes. Phe^3 substitution by other aromatic residues, such as Aic and Atc, resulted in remarkable MVD bioactivities *in vitro* further supporting this observation.[30,41,42] In contrast, hydrophobicity associated with Aib sufficiently replaced the aromatic benzyl ring of Phe^3 for interaction with the rat brain δ opioid receptor, or the geometry of the binding site was more accommodating to the less bulky α,α-dimethyl groups of Aib. In this regard, reduced δ affinities were observed when Phe^3 was replaced by the larger branched chain aliphatic amino acids Val, Leu, and Ile.[31] On the other hand an α-monoalkyl amino acid (L-Ala) replacement of Phe^3 resulted in a 40-fold loss in δ receptor affinity (Table 1), and D-Ala substitution at this position rendered a 100-fold loss (**8** and **9**). The effects of the Ala analogues differed from those of the title peptide **1** and the Aib^3 analogue **3**, although the reduced δ affinities and the two-site binding model (except Ac_5c^3)[32] closely resembled those of the $C^{\alpha,\alpha}$-dialkyl cyclic amino acid-substituted deltorphin analogues (Table 1). It is possible that the side chain of L- or D-Ala^3 either was physically incompatible with the receptor binding pocket or produced conformational changes that affected the interaction of other side chains with the receptor.

Increased flexibility of **6** observed during MD simulations, in which conformers adopted helices as well as various β-turn secondary structures, may account for the diminished δ affinity and dramatic loss in bioactivity. Although δ affinity was decreased 20-fold, this value was still comparable to many enkephalin and deltorphin derivatives.[19,30,31] The dramatic decrease in bioactivity in the peripheral tissue is largely due to the loss of aromatic phenylalanine and possibly a conformational change. As observed in peptide **3** the loss of aromaticity resulted in decreased interaction with the MVD opioid receptors, and modifications of Phe^4 in enkephalin derivatives[53] resulted in decreased MVD bioactivity.

These combined data confirm that the physical dimension, orientation, and hydrophobicity of side chains in the N-terminal region of deltorphin C strongly influence interaction with δ opioid receptors while the aromaticity of the residue located at the third position is necessary for MVD bioactivity. Likewise, peptide conformation is important for positioning the physicochemical properties of the residue side chains in the N-terminal region of deltorphin C (Tables 1 and 2) for optimal interaction with opioid receptors. Other studies on structural mimetics emphasized that amphiphilic structures provide well-defined placement of residues which would enable greater efficiency in receptor binding and that a β-folded structure is more likely present in bioactive peptides.[54] For example, the [Leu^5]enkephalin analogue *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH, a δ opioid antagonist,[9] exhibited a tightly folded double β-bend conformation,[55] while [Aib^3]DPDPE displayed a type I or III′ β-turn at residues 2−5.[10] By analogy the many β-turns observed in **2** and **4** (Figure 2) could represent conformations that are important for opioid ligand−receptor interaction.

Conclusions

Aib substitutions appear to induce conformational changes in deltorphin C analogues and increase peptide hydrophobicity. These properties combined may provide greater compatibility with a binding site tethered in a highly localized lipophilic milieu consisting of residues with aromatic or hydrophobic side chains.[56,57] Although the location and 3-dimensional arrangement of the δ opioid receptor binding site are not known, site-directed mutagenesis of δ receptors indicated that the opioid ligand enters a transmembrane channel to interact with regions of the receptor embedded in the membrane lipid bilayer.[57−59] Ligands adopting α-helical or amphiphilic structures might compliment this binding site. On the other hand, while these data appear to be consistent with the concept of "message" and "address" domains in peptides,[60] in which charge in a membrane moderates opioid peptide recognition,[61] the evidence presented in this paper indicates that ligand conformation plays a definitive role in aligning the peptide within the receptor. For example, in the case of the rat brain receptor assay, while single Aib substitutions for residues at positions 2 and 3 (peptides **2** and **3**) were not detrimental to δ opioid affinity, the simultaneous substitutions of those residues (peptide **6**) resulted in decreased δ opioid affinity indicating that a conformational change influenced δ receptor interaction. Whereas MD conformational analyses do not definitively confirm that Aib induced helical conformations commonly observed in crystalline Aib-containing peptides,[2−8] they do suggest that Aib residues caused distinct changes in deltorphin C secondary structure (Figures 2 and 3) when substituted for D-Ala^2, Asp^4, and simultaneously D-Ala^2 and Phe^3 but not when substituted for Phe^3 alone. These conformational changes are critical factors for the proper

orientation of reactive constituents of residues in the N-terminal region, which includes the -OH of the phenolic side chain of Tyr[1], the free acid of Asp[4], the protonated nitrogen of the N-terminal amine, and the aromaticity of Tyr[1] and Phe[3]. The hydroxyl group of the Tyr side chain and the N-terminal amine are considered to be involved in hydrogen bonding with the receptor,[52,62] while the negative charge of Asp is involved in selectivity between δ and μ receptors[27,32,33,41,45−47] and the aromatic sides chains of Tyr[1] and Phe[3] may influence receptor binding through hydrophobic stacking, $\pi-\pi$ forces, or cation$-\pi$ interactions.[62,63] Thus, peptide conformations in concert with the physicochemical properties of the N-terminal residues are synergistically involved in the proper alignment of opioid ligands in the receptor pocket and act in the differentiation between receptor types.[41,62]

Experimental Section

Materials. Rink resin [4-(2′,4′-dimethoxyphenyl)-Fmoc-(aminomethyl)phenoxy resin, 0.47 mmol/g], Fmoc-Gly-Wang resin (*p*-benzyloxycarbonyl alcohol resin, 0.5 mmol/g), DAGO, and DPDPE were obtained from Bachem California (Torrence, CA); the Fmoc-protected amino acids were either products of Bachem or Novabiochem AG (Germany). The Pico-Tag system was from Millipore (Waltham, MA). [^{3}H]DAGO (60 Ci/mmol) was a product of Amersham (Arlington, IL) and [^{3}H]DPDPE (28.1 Ci/mmol) from NEN-DuPont (Boston, MA). Prism (version 1.03) and InPlot (version 4.03) are programs by GraphPad software (San Diego, CA).

Solid Phase Peptide Synthesis. Peptides were synthesized by solid phase methods using a Milligen 9050 synthesizer using 0.1 g of Rink resin and were mixed with glass beads (1:15, w/w). Peptides were assembled using Fmoc-protected amino acids with 4-fold excess for 1 h with each of the coupling reagents, DIPCI and HOBt. Double coupling was required in the dipeptide sequences Asp-Val, Val-Val, Aib-Val, and Xaa-Aib. Boc-Tyr(*t*-Bu)-Aib-Aib-OH (*infra vide*) was used in 4-fold excess during a 14 h acylation step by solid phase methods. The free acid peptide (compound **5**) and 0.1 g of Fmoc-Gly-Wang resin were mixed with glass beads (1:15, w/w). The procedures for the synthesis of the peptide amides are reported elsewhere.[28,30]

Solution Phase Synthesis of Boc-Tyr(*t*-Bu)-Aib-Aib-OH. The synthesis of compound **6** was prepared by synthesizing the protected tripeptide Boc-Tyr(*t*-Bu)-Aib-Aib-OH by solution methods according to Schmitt and Jung[64] since we initially failed to obtain coupling between Fmoc-Aib and the H-Aib-peptide-resin.

a. Boc-Tyr(*t*-Bu)-Aib-Aib-OMe. Boc-Tyr(*t*-Bu)-OH (3.4 g, 10 mmol) and HOBt (1.7 g, 11 mmol) were dissolved in DMF (50 mL) and activated with water soluble carbodiimide (1.9 g, 11 mmol). After 30 min, the dipeptide ester hydrochloride, H-Aib-Aib-OMe (2.38 g, 10 mmol),[64,65] and triethylamine (1.4 mL, 10 mmol) were added. After 12 h at room temperature, the solvent was removed *in vacuo* and the residue diluted with ethyl acetate (100 mL) and washed sequentially with 10% citric acid, 5% $NaHCO_3$, and a solution of saturated NaCl. After drying over Na_2SO_4, the ethyl acetate was evaporated and the peptide crystallized with ether at −10 °C: yield 3.0 g (58%); mp 135−136 °C; $[\alpha]^{20}_D$ +22.8 (MeOH); R_f (III) 0.75; K' 20.1; ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 6H), 1.40 (s, 9H), 1.42 (s, 9H), 1.49 (s, 3H), 1.51 (s, 3H), 3.02 (d, 2H), 3.70 (s, 3H), 4.15 (q, 1H), 5.0 (m, 1H), 6.15 (bs, 1H), 6.94 (d, 2H, J = 8.44 Hz), 7.05 (d, 2H, J = 8.51 Hz), 7.13 (s, 1H).

b. Boc-Tyr(*t*-Bu)-Aib-Aib-OH. Boc-Tyr(*t*-Bu)-Aib-Aib-OMe (2.5 g, 5 mmol) in methanol (20 mL) was saponified at room temperature with 2 N NaOH (10 mL). After a 24 h reaction, neutralization, and evaporation of the solvent, the solution was acidified and extracted with ethyl acetate, washed with a saturated solution of NaCl, and dried with Na_2SO_4; the tripeptide was crystallized from ether. Analytical values: mp 176−177 °C; $[\alpha]^{20}_D$ +19.1 (MeOH); R_f (I) 0.78, (III) 0.3; K' 19.97. No signals were obtained from the methyl ester at δ 3.70 in the NMR spectra.

Purification. All peptides were cleaved from the resin (solid phase synthesis) by treatment with TFA/H_2O/triethylsilane (88:5:7, v/v/v) at room temperature for 1 h. Crude peptides were purified by reversed-phase chromatography using a Waters Delta Prep 3000 column (30 × 3 cm, 15 μm particle size). The peptides were eluted with a gradient of 0−60% mobile phase B over 25 min at a flow rate of 30 mL/min using mobile phases A (10% acetonitrile in 0.1% TFA) and B (60% acetonitrile in 0.1% TFA). Analytical HPLC analyses were performed on a Bruker liquid chromatography LC-21 instrument using a Waters Pico-Tag C_{18} column (150 × 3.9 mm, 5 μm particle size) 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 (QX-10). The capacity factor (K') of each peptide was determined using HPLC conditions in the above solvent systems with a linear gradient from 0% to 100% mobile phase B in 25 min at a flow rate of 1 mL/min. All analogues showed less than 1% impurities when monitored at 220 nm.

Analytical Determinations. Amino acid analysis was carried out using PITC (Pico-Tag methodology) as the amino acid derivatization reagent. Aib was not quantitatively determined during amino acid analyses. Lyophilized samples of peptides (50−1000 pmol) were placed in heat-treated borosilicate tubes (50 × 4 mm), sealed, and hydrolyzed using 200 μL of 6 N HCl containing 1% phenol in the Pico-Tag workstation for 1 h at 150 °C. The Pico-Tag column was employed to separate the PITC-amino acid derivatives. Thin layer chromatography used precoated plates of silica gel F254 in the following solvent systems: I, 1-butanol/acetic acid/H_2O (3:1:1, v/v/v); II, ethyl acetate/pyridine/acetic acid/H_2O (6:2:0.6:1.1, v/v/v/v); III, chloroform/benzene/methanol (17:1:2). Ninhydrin (1%), fluorescamine, and chlorine reagent were used as detection sprays.

Melting points were determined on a Reicher-Kofler apparatus and are uncorrected. Optical rotations were determined in a Perkin-Elmer 241 polarimeter with a 10 cm cell using methanol at a peptide concentration of 1%. Molecular weights of the compounds were determined by a triple-stage quadrupole mass spectrometer (TSQ 700, Finnigan MAT) equipped with a pneumatic electrospray (ion-spray) interface, and the data were compiled using a DEC 5000/125 computer. Proton NMR resonance spectra were recorded on a Bruker 200 MHz spectrometer using tetramethylsilane as an internal standard.

Molecular Dynamics Simulations. Molecular dynamics (MD) simulations of deltorphin C and [Aib^2]-, [Aib^3]-, [Aib^{2,3}]-, and [Aib^4]deltorphin C analogues were performed on a Silicon Graphics Indigo^2 computer system using AMBER (version 3.0, revision A).[66] The amino acid α-aminoisobutyric acid and the amine functional group (-NH_2) were built using the prep module based on the prep input of glycine and valine already contained in AMBER. Starting structures incorporated the dihedrals ϕ (rotation about N$-$C$^\alpha$), ψ (C′$-$C$^\alpha$), χ_1 (C$^\alpha-$C$^\beta$), and χ_2 (C$^\beta-$C$^\gamma$) derived from ^{1}H NMR data of deltorphin C (unpublished data). Values: ψ_1 = 139°, χ_{11} = 179°, χ_{12} = −106°; ϕ_2 = 55°, ψ_2 = −143°, χ_{21} = 53°; ϕ_3 = −49°, ψ_3 = −41°, χ_{31} = −51°, χ_{32} = −119°; ϕ_4 = −53°, ψ_4 = −24°, χ_{41} = 151°, χ_{42} = 92°; ϕ_5 = −50°, ψ_5 = −38°, χ_{51} = 174°; ϕ_6 = −56°, ψ_6 = −27°, χ_{61} = 179°; ϕ_7 = −63°. The backbone dihedrals (ϕ, ψ) of Aib were assigned the values of the residue it replaced. The total charge on each peptide was neutral.

Energy minimization lasted 500 hundred cycles with a distance dependent dielectric ($\epsilon = r$) and 9 Å nonbonded cutoffs. Restart coordinates were output every 25 cycles, and the nonbonded pair list was updated every 50 steps. Steepest descent and conjugate gradient algorithms were used for the energy minimizations with a step length of 0.001 fs, a convergence criterion of 1×10^{-7} kcal/mol, and a gradient convergence of 0.10 kcal/mol·Å. Peptides were solvated in three shells of TIP3P Monte Carlo water molecules under periodic boundary conditions, and the solvent was energy minimized (before and after MD) for 500 cycles with constant

dielectric ($\epsilon = 1$) followed by 20 ps of MD under constant pressure (1 atm) and temperature (300 K). The time step was 0.001 ps with 1000 steps/run, and a 0.4 ps time constant for heat bath coupling with a 0.6 ps pressure relaxation time and 9 Å nonbonded cutoffs were incorporated. The nonbonded pair list was updated every 20 ps, and information was output every 100 steps. The solvent and peptide systems were energy minimized for 500 cycles with the same parameters as with the solvent energy minimization followed by 100 ps of MD on the peptide and solvent with the same parameters as the solvent dynamics except that the coordinates were written to file every 200 steps. Conformers generated every 4 ps during the 100 ps MD simulation were assigned secondary structures using the program Dictionary of Protein Secondary Structure (DSSP).[40] The DSSP algorithm is principally based on hydrogen-bonding patterns and defines 3_{10}-helical structures as having hydrogen bonds between $O_i \rightarrow H\text{-}N_{i+3}$, α-helical as $O_i \rightarrow H\text{-}N_{i+4}$, turns as $O_i \rightarrow H\text{-}N_{i+3}$, $O_i \rightarrow H\text{-}N_{i+4}$, or $O_i \rightarrow H\text{-}N_{i+5}$, and bends as $O_i \rightarrow H\text{-}N_{i+2}$. Distances, $\alpha C_i - \alpha C_{i+3}$, and dihedral angles, ϕ and ψ, of the $i+1$ and $i+2$ bend residues were measured for structures characterized by DSSP as turns or bends and compared to values reported from X-ray crystallography of 29 proteins[67] for classification of turn types.

Receptor Binding Assays. Receptor affinities of deltorphin C analogues were assessed using competitive binding assays labeled with either [^{3}H]DPDPE (6.3 nM) for the δ sites or [^{3}H]DAGO (1.28 nM) for the μ sites according to published methods.[26−28,30] Excess unlabeled peptides (2 μM) saturated the opioid binding sites in order to obtain a baseline value. Duplicate tubes contained preincubated rat brain synaptosomal membranes in equilibrium assays containing 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, glycerol, and protease inhibitors[21] for 120 min at room temperature (22−23 °C). Incubation mixtures were trapped in the glass fiber filters and rapidly washed within 5 s with 3 × 2 mL of ice-cold buffer containing 0.01% BSA. In the duplicate assays, labeled peptides were displaced using 6−14 concentrations of each analogue to cover a 1000-fold range in peptide dose. Competitive inhibition constants (K_i) were derived from the IC_{50} values based on the equations of Cheng and Prusoff.[68]

Statistical Analysis of Binding Site Models. Determination of the Hill coefficients (η) and statistical analyses of the binding data utilized receptor assays conducted in triplicate using 25−35 peptide dosages covering differences in concentrations of 300−500-fold either side of the K_i. Competition curves were tested for fits to one- or two-site binding models as detailed by Attila *et al.*[69] and Bryant *et al.*[27] using Prism (version 1.02). In the assignment of fits to either a one- or two-site binding model, stringent iterative calculations were only considered valid for the two-site model when the Hill coefficients were <0.85 with a narrow log of the 95% confidence interval (<0.15) and an F test in which $P < 0.0001$.

Bioassays. Functional pharmacological assays were conducted according to Salvadori *et al.*[28] using a 2−3 cm portion of 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. 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 MVD was suspended in 4 mL of modified Kreb's solution aerated with 95% O_2/5% CO_2 at 33 °C with the twitch induced by field stimulation (0.1 Hz for 1 ms at 40 V) recorded with an 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 C 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.[28]

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Supporting Information Available: Analytical properties of the α-aminoisobutyric acid-containing analogues of deltorphin C including the capacity factor (K') and the mass spectrometry molecular weights (MH^+), the AMBER prep files for the Aib and -NH_2 residues, and the secondary structure assignments for the MD conformers (5 pages). Ordering information is given on any current masthead page.

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(1) Symbols and abbreviations are in accord with the recommendation of the IUPAC-IUB Commission of Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). Other abbreviations: AMBER, assisted model building with energy refinement; Aib, α-aminoisobutyric acid; Boc, *tert*-butyloxycarbonyl; DAGO, H-Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-CH_2OH; *t*-Bu, *tert*-butyl; deltorphin A (deltorphin or dermenkephalin), H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2; deltorphin C, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2; deltorphin B, [Glu^4]deltorphin C; dermorphin, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2; DIPCI, 1,3-diisopropylcarbodiimide; DPDPE, [D-Pen^{2,5}]enkephalin, H-Tyr-[D-Pen-Gly-Phe-D-Pen]-OH; DSSP, dictionary of protein secondary structure; O*t*-Bu, *tert*-butyl ester; Fmoc, 9-fluorenylmethyloxycarbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; MD, molecular dynamics; Pen, β,β-dimethylcysteine; Tic, 1,2,3,4-tetrahydro-3-isoquinoline-3-carboxylic acid.

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