Conformationally Restricted Deltorphin Analogues^{†,‡}

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Conformationally restricted deltorphin analogues were synthesized either through incorporation of cyclic phenylalanine analogues in position 2 or 3 of the peptide sequence or through various side chain-to-side chain cyclizations. Compounds were tested in μ -, δ -, and κ -receptor selective binding assays and in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays. Replacement of Phe³ in [D-Ala²]deltorphin I with 2-aminoindan-2-carboxylic acid (Aic) or L- or D-2-aminotetralin-2-carboxylic acid (Atc) resulted in agonist compounds which retained the high δ receptor selectivity of the parent peptide. Substitution of a tetrahydroisoquinoline-3-carboxylic acid (Tic) residue in the 2-position of $[D-Ala^2]$ deltorphin I and of $[Phe^4,Nle^6]$ deltorphin produced a partial δ agonist, H-Tyr-Tic-Phe-Asp-Val-Val-Gly-NH₂, and a pure δ antagonist, H-Tyr-Tic-Phe-Phe-Leu-Nle-Asp-NH₂, respectively. The latter antagonist displayed high δ selectivity ($K_i^{\mu}/K_i^{\delta} = 502$) and was a potent antagonist against selective δ agonists in the MVD assay ($K_e \simeq 10$ nM). Various [D-Ala²]deltorphin I analogues cyclized between the side chains of Orn (or Lys) and Asp (or Glu) residues substituted in positions 2 and 4, 4 and 7, and 2 and 7 were essentially nonselective. Comparison with corresponding N-terminal tetrapeptide analogues revealed that the C-terminal tripeptide segment in the deltorphin heptapeptides made a crucial contribution to δ affinity and δ selectivity in the case of the agonist peptides but not in the case of the antagonist.

The development of potent and stable agonists and antagonists with high specificity for each of the three major opioid receptor classes (μ, δ, κ) continues to be an important goal in opioid pharmacology, even though substantial progress has been made in recent years (for recent reviews, see refs 3 and 4). The development of highly selective δ ligands with agonist or antagonist properties represents a particular challenge. Linear analogues of [Leu⁵]enkephalin with selectivity for δ opioid receptors include the hexapeptides H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH (DSLET), H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH (DTLET), and H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)-OH (BUBU).⁵ Among various prepared cyclic opioid peptide

analogues, the pentapeptides H-Tyr-D-Pen-Gly-Phe-Pen-OH (DPLPE), H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE), and H-Tyr-D-Pen-Gly-*p*-ClPhe-D-Pen-OH,^{6,7} and the tetrapeptide H-Tyr-D-Cys-Phe-D-Pen-OH⁸ showed markedly improved δ selectivity. The most potent and most selective δ agonists reported to date are three heptapeptides, H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH₂ (deltorphin), H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂ ([D-Ala²]deltorphin I), and H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂ ([D-Ala²] deltorphin II), which have been isolated from frog skin extracts.^{9,10} The use of various design principles has led to the development of several compounds with δ antagonist properties, including the enkephalin analogue N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864),¹¹ the potent non-

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⁽¹⁾ Symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem.* J. 1984, 219, 345–373. The following other abbreviations were used: Aic, 2-aminoindan-2-carboxylic acid; Atc, 2-aminotetralin-2-carboxylic acid; Boc, tert-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; BUBU, H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)-OH; DAMGO, H-Tyr-D-Ale-Gly-N*MePhe-Gly-ol; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DPDPE,

<sup>H-Tyr-D-Pen-Gly-Phe-D-Pen-OH; DPLPE, H-Tyr-D-Pen-Gly-Phe-Pen-OH; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; DTLET, H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH; DTLET, H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH; Fmoc, (fluoren-9-ylmethoxy)carbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; MVD, mouse vas deferens; Nle, norleucine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP, H-Tyr-Tic-Phe-OH; TIP-NH₂, H-Tyr-Tic-Phe-NH₂; TIPP, H-Tyr-Tic-Phe-OH; TFA, trifluoroacetic acid; U69,593, (5α, 7α,86)-(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-ozaspiro[4.5]dec-8-yl]benzeneacetamide.
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peptide antagonist naltrindole,¹² and the recently discovered, highly δ -selective tetrapeptide H-Tyr-Tic-Phe-Phe-OH (TIPP).13

Linear opioid peptides such as the deltorphins are structurally flexible molecules and need to be conformationally restricted in order to obtain insight into their bioactive conformation or to try to establish structural (conformational) relationships with other opioid peptides. Conformational restriction of a peptide can be achieved either locally through incorporation of conformational constraints at a particular amino acid residue or more globally through peptide cyclizations. Various types of cyclizations of opioid peptides resulted in a number of compounds with high selectivity toward either μ or δ receptors (for reviews, see refs 3 and 4). More recently, conformational restriction of phenylalanine residues in opioid peptides has been shown to have drastic effects on receptor selectivity and signal transduction. Thus, substitution of Phe³ in the relatively nonselective cyclic dermorphin analogue H-Tyr-D-Orn-Phe-Glu-NH₂ with 2-aminoindan-2-carboxylic acid (Aic) or 2-aminotetralin-2-carboxylic acid (Atc) produced μ -selective agonists¹⁴ and

opioid tri- and tetrapeptide analogues containing a tetrahydroisoquinoline-3-carboxylic acid (Tic) residue in the 2-position of the peptide sequence turned out to be potent and very selective δ antagonists.¹³

In the present paper we describe novel deltorphin analogues that either resulted from substitution of an Aic, Atc, or Tic residue at the 2- or 3-position, or were obtained through peptide cyclization between the side chains of Orn (or Lys) and Asp (or Glu) residues that had been substituted in various positions of the peptide sequence. We show that some of the Aic^3 and Atc^3 analogues displayed extraordinary δ receptor affinity and δ selectivity and that substitution of a Tic residue in position 2 of deltorphin-related peptides in one case produced a potent, highly selective δ antagonist and in another case resulted in a partial δ agonist. Finally, we report that none of the prepared cyclic deltorphin analogues showed high δ selectivity, primarily as a consequence of their decreased affinity for δ opioid receptors.

Chemistry. The linear deltorphin analogues (1-7, 12, 13) were prepared by the usual solid-phase techniques with N^{α} -Boc-protected amino acids and with dicyclohexylcarbodiimide (DCC)/hydroxybenzotriazole (HOBt) as coupling agents. Aic and Atc were prepared by a modified version of the Strecker synthesis^{15,16} through conversion of 2-indanone or 2-tetralone to the corresponding spirohydantoins and subsequent hydrolysis with 40% alkali,

as described.¹⁴ Atc was obtained in racemic form and incorporated as such into the peptide structure. The resulting diastereoisomeric peptides were isolated separately by semipreparative HPLC. Tic was obtained through condensation of phenylalanine with paraformaldehyde as reported elsewhere.¹⁴

The cyclic deltorphin analogues were prepared by the solid-phase method on a *p*-methylbenzhydrylamine resin according to a scheme described elsewhere.¹⁷ Linear peptide segments were put together as described above, and the peptide segments to be cyclized were assembled by using N^{α} -Fmoc amino acids with Boc and *tert*-butyl protection, respectively, for the side chains of the Orn (Lys) and Asp (Glu) residues engaged in the peptide ring formation. The latter protecting groups were removed by treatment with TFA, and side chain-to-side chain cyclization of the still resin-bound peptide was then performed by reaction with DCC/HOBt or with the BOP [(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate] reagent.¹⁸ The time required for amide bond formation to be complete varied from 12 to 48 h. After the cyclization step, the N-terminal Fmoc protecting group was removed and the peptide chains were completed by adding the N-terminal exocyclic residue(s). In the case of analogue 11, the side chain of the endocyclic Asp⁴ residue had to be protected with the TFA-resistant benzyl group.

Peptides were cleaved from the resin by HF/anisole treatment in the usual manner. Crude products were purified by gel filtration on Sephadex G-25 and by reversed-phase chromatography. In the case of the cyclic analogues cyclodimerization¹⁷ and cyclooligomerization did occur to some extent. Separation of the cyclic monomers from the cyclic dimers and oligomers was easily achieved by reversed-phase chromatography. The desired cyclic monomer was the predominant component (60-90%) in the crude reaction products of analogues 8-11, whereas cyclodimerization was extensive ($\sim 60\%$) in the case of analogue 7.

Receptor Binding Assays and in Vitro Bioassays. Binding affinities for μ and δ opioid receptors were determined by displacing, respectively, [3H]DAMGO and [³H]DSLET from rat brain membrane binding sites, and ĸ opioid receptor affinities were measured by displacement of [³H]U69.593 from guinea pig brain membrane binding sites. For the determination of their in vitro opioid activities, analogues were tested in bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and of the mouse vas deferens (MVD). The GPI assay is usually considered as being representative for μ receptor interactions, even though the ileum does also contain κ receptors. κ receptor interactions in the GPI assay are indicated by relatively high K_e values for naloxone as antagonist (20-30 nM),¹⁹ in contrast to the low K_e values (1-2 nM) observed with μ ligands.²⁰ In the

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Table I. Receptor Binding A	assays of Deltorphin A	Analogues
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		[³ H]DAMGO		[³ H]DSLET		
no.	compd	K _i [#] , nM ^a	rel potency ^b	Ki ^{\$} , nM ^a	rel potency ^b	$K_{ m i}^{\mu}/K_{ m i}^{\delta}$
1	H-Tyr-D-Ala-Aic-Asp-Val-Val-Gly-NH2	207 ± 32	0.0456 ± 0.0070	4.96 ± 0.97	0.510 ± 0.100	41.7
2	H-Tyr-D-Ala-(D or L)-Atc-Asp-Val-Val- Gly-NH ₂ (I)	671 ± 52	0.0141 ± 0.0011	5.36 ± 0.71	0.472 ± 0.063	125
3	H-Tyr-D-Ala-(D or L)-Atc-Asp-Val-Val- Gly-NH ₂ (II)	1410 ± 190	0.00669 ± 0.00090	6.52 ± 0.44	0.388 ± 0.026	216
4	H-Tyr-D-Ala-Tic-Asp-Val-Val-Gly-NH2	15200 ± 2100	0.000620 ± 0.000086	4960 ± 320	0.000510 ± 0.000033	3.06
5	H-Tyr-Tic-Phe-Asp-Val-Val-Gly-NH ₂	9230 ± 650	0.00102 ± 0.00007	6.49 ± 1.00	0.390 ± 0.060	1420
6	H-Tyr-Tic-Phe-Phe-Leu-Nle-Asp-NH ₂	4150 ± 840	0.00227 ± 0.00046	8.27 🌢 1.52	0.306 ± 0.056	502
7	H-Tyr-D-Orn-Phe-Asp-Val-Val-Gly-NH2	143 ± 11	0.0659 ± 0.0051	290 ± 17	0.00872 ± 0.00051	0.493
7 a	H-Tyr-D-Orn-Phe-Asp-NH2	10.4 ± 3.7	0.907 ± 0.032	2220 ± 58	0.00114 ± 0.00003	0.00468
8	H-Tyr-D-Lys-Phe-Glu-Val-Val-Gly-NH2	17.5 ± 4.5	0.539 🌢 0.139	3.66 ± 0.57	0.691 • 0.108	4.78
9	H-Tyr-D-Ala-Phe-Asp-Val-Val-Orn-NH2	338 ± 79	0.0279 ± 0.0065	562 🖷 30	0.00450 ± 0.00024	0.601
10	H-Tyr-D-Ala-Phe-Glu-Val-Val-Lys-NH2	955 ± 19	0.00987 ± 0.00020	724 单 165	0.00349 ± 0.00080	1.32
11	H-Tyr-D-Lys-Phe-Asp-Val-Val-Glu-NH2	891 ± 26	0.0106 @ 0.0003	248 ± 29	0.0102 ± 0.0012	3.59
12	H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH ₂ (deltorphin)	415 ± 37	0.0227 ± 0.0020	2.13 ± 0.62	1.19 🛳 0.35	195
13	H-Tyr-D-Nle-Phe-His-Leu-Nle-Asp-NH ₂	117 ± 6	0.0806 ± 0.0041	0.665 ± 0.208	3.80 ± 1.19	176
14	H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH ₂ (fD-Ala ²]deltorphin I)	262 🏚 6	0.0360 ± 0.0008	1.44 单 0.28	1.76 ± 0.34	182
1 4a	H-Tyr-D-Ala-D-Phe-Asp-Val-Val-Gly-NH ₂	8650 ± 1420	0.00109 ± 0.00018	67.2 • 6.0	0.0376 ± 0.0034	129
15	[Leu ⁵]enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1	3.73

^a Mean of three determinations \pm SEM. ^b Potency relative to [Leu⁵]enkephalin.

Table II. Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assay of Deltophin Analogues

		GPI		MVD		GPI/MVD
no.	compd	IC50, nMª	rel potency ^b	IC50, nm ^a	rel potency ^b	IC50 ratio
1	H-Tyr-D-Ala-Aic-Asp-Val-Val-Gly-NH2	290 ± 52	0.848 ± 0.152	0.0803 ± 0.0090	142 ± 16	3610
2	H-Tyr-D-Ala-(D or L)-Atc-Asp-Val-Val- Gly-NH ₂ (I)	1820 ± 90	0.135 ± 0.007	0.115 • 0.012	99.1 ± 10.3	15800
3	H-Tyr-D-Ala-(D or L)-Atc-Asp-Val-Val- Gly-NH ₂ (II)	1380 ± 160	0.178 ± 0.021	0.163 ± 0.012	69.9 ± 5.1	8470
4	H-Tyr-D-Ala-Tic-Asp-Val-Val-Gly-NH2	14500 ± 2500	0.0170 ± 0.0029	481 ± 45	0.0237 @ 0.0022	30.1
5	H-Tyr-Tic-Phe-Asp-Val-Val-Gly-NH ₂	>65000	<0.00378	$22.8 \pm 2.9^{\circ}$	0.500 ± 0.064	>2850
6	H-Tyr-Tic-Phe-Phe-Leu-Nle-Asp-NH ₂	>10000	<0.0246	>10000 (antagonist)		
7	H-Tyr-D-Orn-Phe-Asp-Val-Val-Gly-NH2	4420 ± 940	0.0557 🛳 0.0118	98.3 ± 9.3	0.116 ± 0.011	45.0
7a	H-Tyr-D-Orn-Phe-Asp-NH2	36.2 ± 3.7	6.80 ± 0.69	3880 ± 840	0.00294 ± 0.00064	0.00933
8	H-Tyr-D-Lys-Phe-Glu-Val-Val-Gly-NH2	102 ± 19	2.41 • 0.45	0.467 ± 0.059	24.4 ± 3.1	218
9	H-Tyr-D-Ala-Phe-Asp-Val-Val-Orn-NH2	251 ± 25	0.980 🛳 0.098	90.5 ± 8.6	0.126 ± 0.012	2.77
10	H-Tyr-D-Ala-Phe-Glu-Val-Val-Lys-NH2	3880 ± 390	0.0634 📾 0.0064	919 ± 82	0.0124 ± 0.0011	4.22
11	H-Tyr-D-Lys-Phe-Asp-Val-Val-Glu-NH ₂	380 ± 60	0.647 ± 0.102	17.3 ± 1.6	0.659 ± 0.061	22.0
1 2	H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH ₂ (deltorphin)	281 ± 29	0.876 ± 0.091	0.695	16.4 ± 2.1	404
13	H-Tyr-D-Nle-Phe-His-Leu-Nle-Asp-NH ₂	241 ± 24	1.02 ± 0.10	0.712 ± 0.067	16.0 ● 1.5	338
14	H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH ₂ ([D-Ala ²]deltorphin I)	854 ± 133	0.288 ± 0.045	0.147 ± 0.019	77.6 ± 10.0	5810
1 4a	H-Tyr-D-Ala-D-Phe-Asp-Val-Val-Gly-NH2	11900 ± 3160	0.0207 0.0055	42.7 ± 6.7	0.267 0.042	279
15	[Leu ⁵]enkephalin	246 🛳 39	1	11.4 ± 1.1	1	21.6

^a Mean of three determinations \pm SEM. ^b Potency relative to [Leu⁵]enkephalin. ^c Partial agonist (IC25; maximal inhibition of contractions was 50%).

MVD assay opioid effects are primarily mediated by δ receptors, even though μ and κ receptors also exist in this tissue.

Results and Discussion

Substitution of the conformationally restricted Aic residue in the 3-position of $[D-Ala^2]$ deltorphin I resulted in a compound (1) which in the opioid receptor binding assays showed about the same μ receptor affinity as the parent peptide 14 but three times lower affinity for δ receptors (Table I). Interestingly, the Aic³ analogue was nearly twice as potent as the native parent peptide in the δ receptor-representative MVD assay and about 3 times as potent in the μ receptor-representative GPI assay (Table II). These results indicate that analogue 1 retains high δ receptor affinity and is only slightly less δ -selective than [D-Ala²]deltorphin I. Both diastereoisomers of H-Tyr-D-Ala-(D or L)-Atc-Asp-Val-Val-Gly-NH₂ (compounds 2 and 3) had about the same δ receptor affinity as the Aic³ analogue (1) but 3–7 times lower affinity for μ receptors. In comparison with [D-Ala²]deltorphin I, analogues 2 and 3 showed about 4 times lower δ receptor affinity in the receptor binding assay but about the same high δ receptor selectivity. In the GPI assay each of the two Atc³-containing diastereoisomeric peptides was about half as potent as parent peptide 14, while showing nearly the same

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high potency as 14 in the MVD assay. Consequently, the IC50(GPI)/IC50(MVD) ratios of analogues 2 and 3 were about 2-3 times higher than that of [D-Ala²] deltorphin I, and therefore, these compounds rank among the most selective δ agonists reported to date. The observation that the Aic³ and Atc³ analogues displayed somewhat higher potency in the MVD assay than was expected on the basis of their δ receptor affinities determined in the binding assay can be explained with a relatively better receptor access of the more lipophilic compounds 1-3 as compared to native [D-Ala²]deltorphin I. Alternatively, these discrepancies may also be due to the fact that central and peripheral δ opioid receptors may have somewhat different structural requirements for ligand interactions. Enzymatic degradation can be excluded as a factor affecting the potency relationships of these compounds, since the natural deltorphins have been shown to be quite stable against enzymatic degradation in the assay systems used in the present study 21,22 and since the presence of the conformationally restricted Aic and Atc amino acid residues in the 3-position of the peptide sequence should make these analogues even more resistant to enzymolysis. It was interesting to note that both diastereoisomers of H-Tyr-D-Ala-(D or L)-Atc-Asp-Val-Val-Gly-NH₂ had very similar potencies in all assay systems, in sharp contrast to the observation that the parent peptide [D-Ala2] deltorphin I (14) displayed 13–47 times higher potency in these assays than its D-Phe³-analogue (14a). This loss of stereospecificity as a consequence of conformational restriction at the Phe³ residue may be due to a different process or mode of receptor binding of the D-Atc³ analogue as compared to the D-Phe³ analogue (see ref 14). The deltorphin analogue containing Tic in place of Phe³ (compound 4) showed poor affinity for both μ and δ receptors and had very weak agonist activity in the GPI and MVD bioassays.

The replacement of the Phe residue in [D-Ala²]deltorphin I with an Aic, Atc, or Tic residue produces severe constraints on both side-chain rotational mobility and backbone flexibility at the 3-position residue. In the case of the Aic³ analogue side chain torsional angles are limited to values of $\chi_1 = -80^\circ$, $\chi_2 = -20^\circ$ and $\chi_1 = -160^\circ$, $\chi_2 = +20^\circ$, whereas the backbone torsional angles preferentially assume values of $\phi = -50^{\circ}$, $\psi = -50^{\circ}$ and $\phi =$ +50°, $\psi = +50^{\circ}$. The side-chain conformations possible for the L-Atc and the D-Atc residue are t or g^- and t or g^+ . respectively, and the limitations on the ψ , ϕ angles are the same as in the case of Aic. The ϕ angle of the Tic residue is limited to values around -90° , and the two possible side-chain conformations for this residue are g^+ and g^- . It is interesting to point out that substitution of these same conformationally restricted phenylalanine derivatives for Phe³ in the cyclic opioid peptide analogue H-Tyr-D-

Orn-Phe-Glu-NH₂ produced parallel effects on potency at the μ receptor, insofar as the Aic³, D-Atc³, and L-Atc³ cyclopeptides were potent μ agonists and the Tic³ analogue again showed poor activity.¹⁴ Furthermore, an almost complete loss of stereospecificity had also been observed with the cyclic D- versus L-Atc³ analogues in comparison with the corresponding cyclic D- versus L-Phe³ analogues. This parallel behavior suggests that the μ and δ receptor subsites interacting with the Phe³ side chain of opioid peptides must have very similar structural (conformational) requirements.

Substitution of a Tic residue in the 2-position of deltorphins had most interesting effects on receptor selectivity and on the intrinsic activity ("efficacy") at the δ receptor. A first example is the [D-Ala²]deltorphin I analogue H-Tyr-Tic-Phe-Asp-Val-Val-Gly-NH₂(5), which retained good δ receptor affinity and showed extraordinary δ receptor selectivity ($K_i^{\mu}/K_i^{\delta} = 1420$), being about 8 times more δ -selective than [D-Ala²] deltorphin I (Table I). This compound was virtually inactive in the GPI assay and in the MVD assay was a partial agonist able to inhibit the electrically evoked vas contractions to a maximal extent of only 50% (Table II). This partial agonist effect could be reversed with the δ antagonists naltrindole and TIPP, indicating that it was mediated by δ receptors. Since the tripeptides H-Tyr-Tic-Phe-OH (TIP) and H-Tyr-Tic-Phe- NH_2 (TIP-NH₂) are both pure δ antagonists,¹³ this result indicates that the C-terminal extension with the tetrapeptide segment -Asp-Val-Val-Gly-NH₂ partially restored agonist activity. The potent and highly selective δ antagonist H-Tyr-Tic-Phe-Phe-OH (TIPP)¹³ shows some structural resemblance to deltorphin (12), which contains a Phe and a His residue in the 3- and 4-position, respectively. Therefore, it was of interest to substitute the TIPP tetrapeptide segment for the N-terminal tetrapeptide segment contained in deltorphin. This substitution was carried out with the Nle⁶ analogue of deltorphin, since substitution of Nle for Met in both position 2 and position 6 has no appreciable effect on the activity profile (compound 13) and has the advantage of eliminating the problem of methionine oxidation. The resulting analogue, H-Tyr-Tic-Phe-Phe-Leu-Nle-Asp-NH₂ (6) retained good δ affinity but bound very poorly to μ receptors and, therefore, was very δ -selective (K_i^{μ}/K_i^{δ}) 502). In both the GPI and the MVD assay this compound showed no agonist activity at concentrations up to $10 \,\mu$ M. However, in the MVD assay analogue 6 was a potent δ antagonist against the δ agonists DPDPE and [D-Ala²]deltorphin I with respective K_e values of 10.8 ± 3.1 and 10.4 ± 2.3 nM. No antagonist effect was observed with this compound in the GPI assay at a concentration as high as $10 \,\mu$ M. In comparison with the tetrapeptide antagonist TIPP, analogue 6 had about 6 times lower δ affinity, was about 3 times less δ -selective, and was about 2.5 times less potent as antagonist in the MVD assay. Thus, C-terminal extension of the TIPP tetrapeptide with the C-terminal tripeptide segment of [Nle⁶] deltorphin slightly decreased rather than increased δ receptor affinity and δ selectivity. This finding is in contrast to the observation that the C-terminal tripeptide segment plays a crucial role in determining the high δ affinity and δ selectivity of deltorphins, since the N-terminal tetrapeptide segment of deltorphin has been shown to have weak affinity for δ receptors and to be μ - rather than δ -selective.²³ Analogue 6 is about 15 times less potent as δ antagonist than naltrindole¹² but, on the other hand, has better δ selectivity and no μ antagonist properties.

Substitution of the D-Ala² residue in [D-Ala²] deltorphin I with a D-Orn residue and amide bond formation between

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the D-Orn² and Asp⁴ side-chain groups resulted in a cyclic lactam analogue (compound 7), which in comparison with the linear parent peptide had comparable μ receptor affinity but 200 times lower affinity for δ receptors and, thus, was essentially nonselective (Table I). Previously, it had been observed that the corresponding cyclic

tetrapeptide amide H-Tyr-D-Orn-Phe-Asp-NH₂ (7a) was hightly μ -selective $(K_i^{\delta}/K_i^{\mu} = 213)$ (Table I).²⁴ Comparison of 7 and 7a revealed that C-terminal extension of the cyclic tetrapeptide with the -Val-Val-Gly-NH₂ segment resulted in a 14-fold decrease in μ affinity and an 8-fold increase in δ affinity, indicating that, as in the case of the native linear deltorphin, the C-terminal tripeptide ("address" segment) is responsible for shifting the μ/δ selectivity ratio in favor of the δ receptor. Expansion of the 13-membered ring structure in 7 to a 15-membered one (compound 8) produced an 8-fold increase in μ affinity and an 80-fold enhancement of δ affinity. Analogue 8 had quite high δ affinity ($K_i^{\delta} = 3.66 \pm 0.57$ nM) but only slight preference for δ receptors over μ receptors. In the GPI and MVD bioassays, analogues 7 and 8 were both full agonists and showed potency relationships similar to those observed in the receptor binding assays. Two [D-Ala²]deltorphin analogues containing a cyclic tetrapeptide segment at the

C-terminus, H-Tyr-D-Ala-Phe-Asp-Val-Val-Orn-NH₂ (9)

and H-Tyr-D-Ala-Phe-Glu-Val-Val-Lys-NH₂ (10), showed weak affinity for μ and δ receptors, were only slightly δ -selective, and displayed weak potency in the MVD assay. A similar lack of δ affinity and δ selectivity as well as relatively low potency in the MVD assay were also observed

with the cyclic lactam analogue H-Tyr-D-Lys-Phe-Asp-Val-Val-Glu-NH₂ (11).

In summary, none of the cyclic lactam analogues 7-11 retained the high δ affinity and δ selectivity of [D-Ala²]deltorphin I. On the basis of a recently performed NMR study, it has been proposed that the conformation of $[D-Ala^2]$ deltorphin I in DMSO features a type II' β -turn stabilized by a Tyr¹-CO←HN-Asp⁴ hydrogen bond and a type I β -turn stabilized by a Phe³-CO \leftarrow HN-Val⁶ hydrogen bond.²⁵ Inspection of the conformational possibilities of the cyclic peptides described here revealed that a type II' β -turn is not possible with cyclic analogues 7 and 8, whereas analogues 9 and 10 can accommodate the proposed C-terminal type I β -turn. The formation of both turns is possible with analogue 11. However, because the opioid activity profiles of the cyclic analogues described here are different from that of [D-Ala²]deltorphin I, this analysis does not permit an unambiguous assessment of the proposed conformational model in terms of its relevance to the bioactive conformation.

None of the deltorphin analogues displayed significant affinity for κ opioid receptors ($K_i^{\kappa} > 1 \mu M$). The lack of κ receptor interactions was also indicated by the low K_e values (1-2 nM) for naloxone as antagonist that were observed with all compounds in the GPI assay.

Conclusions

In the present paper we describe the effect on the opioid activity profile of deltorphin-related peptides produced either by local conformational constraints introduced at the 2- and 3-position residues or by more global conformational restriction resulting from various peptide cyclizations. Substitution of an Aic or of an L- or D-Atc residue for Phe³ in [D-Ala²] deltorphin I resulted in agonist compounds that retained the high δ receptor selectivity of the parent peptide. Analogues containing a Tic residue in the 2-position of the peptide sequence showed even higher δ selectivity and either antagonist or partial agonist properties at the δ receptor. In comparison with the tetrapeptide antagonist H-Tyr-Tic-Phe-OH (TIPP), the deltorphin related antagonist H-Tyr-Tic-Phe-Phe-Leu-Nle-Asp-NH₂ (6) showed about 6 times lower δ affinity, 3 times lower δ selectivity, and 2.5 times lower δ antagonist potency in the MVD assay, indicating that the C-terminal tripeptide segment did not contribute to the δ characteristics of the peptide. None of the cyclic deltorphin analogues displayed the high δ selectivity observed with the native, linear deltorphins. It was interesting to note, however, that C-terminal extension of

the μ -selective cyclic tetrapeptide analogue H-Tyr-D-Orn-

Phe-Asp-NH₂ with the C-terminal [D-Ala²]deltorphin I tripeptide segment -Val-Val-Gly-NH₂ reduced μ affinity and increased δ affinity quite drastically. The observation that the C-terminal tripeptide segment plays a differential role in contributing to the δ affinity and δ selectivity of deltorphin-related peptides with agonist properties versus those with antagonist properties suggests a difference in the mode of receptor binding between agonists and antagonists.

Experimental Section

General Methods. Precoated plates (silica gel G, 250 μ m, Analtech, Newark, DE) were used for ascending TLC in the following solvent systems (all v/v): (I) *n*-BuOH/AcOH/H₂O (4/ 1/5, organic phase) and (II) *n*-BuOH/pyridine/AcOH/H₂O (15/ 10/3/12). Reversed-phase HPLC was performed on a Varian VISTA 5500 liquid chromatograph, utilizing a Vydac C₁₈ RP column (25 cm × 10 mm). For amino acid analyses peptides (0.2 mg) were hydrolyzed in 6 N HCl (0.5 mL) containing a small amount of phenol for 24 h at 110 °C in deaerated tubes. Hydrolysates were analyzed on a Beckman Model 121C amino acid analyzer equipped with a Model 126 Data System integrator. Molecular weights of peptides were determined by FAB mass spectrometry on an MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system (Drs. M. Evans and M. Bertrand, Department of Chemistry, University of Montreal).

Solid-Phase Synthesis and Purification of Linear and Cyclic Peptide Analogues. Aic, (D,L)-Atc, and Tic were synthesized as elsewhere described in detail.¹⁴ Other Boc- and Fmoc-amino acids were purchased from BioChem Pharma Inc., Laval, Quebec, Canada, and from Bachem Bioscience, Philadelphia, PA.

Peptide synthesis was performed by the manual solid-phase technique using a p-methylbenzhydrylamine resin (1% crosslinked, 100-200 mesh, 0.2 mmol/g of titratable amine) obtained from United States Biochemical Corp., Cleveland, OH. Linear peptides were assembled using Boc-protected amino acids and dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as coupling agents. Side-chain protection was as follows: benzyl (Asp), tosyl (His), Boc (Tyr). The following steps were performed in each cycle: (1) addition of Boc amino acid in $CH_{2Cl_{2}}$ (2.5 equiv), (2) addition of HOBt (2.5 equiv), (3) addition of DCC (2.5 equiv) and mixing for 2-3 h, (4) washing with $CH_{2}Cl_{2}$ (3 × 1 min), (5) washing with EtOH (1 min), (6) monitoring

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completion of the reaction with the ninhydrin test,²⁶ (7) Boc deprotection with 50% (v/v) TFA in CH₂Cl₂ (30 min), (8) washing with CH₂Cl₂ (5 × 1 min), (9) neutralization with DIEA in CH₂Cl₂ (2 × 5 min), (10) washing with CH₂Cl₂ (5 × 1 min). Double coupling was necessary to complete coupling of the Tyr residue to the Tic residue in the case of analogues 5 and 6.

In the case of the analogues containing cyclic peptide structures the exocyclic C-terminal and N-terminal peptide segments were assembled using Boc amino acids according to the protocol described above. The cyclic peptide segments were synthesized according to a protection scheme based on the use of Fmoc amino acids¹⁷ by performing the following steps in each cycle: (1)addition of Fmoc amino acid (2.5 equiv) in CH₂Cl₂/DMF (85/15, v/v), (2) addition of HOBt (2.5 equiv), (3) addition of DCC (2.5 equiv) and mixing for 2–3 h, (4) washing with CH_2Cl_2 (3 × 1 min), (5) washing with EtOH (1 min), (6) monitoring completion of the reaction with the ninhydrin test, (7) Fmoc deprotection with 50% (v/v) piperidine in CH_2Cl_2 (30 min), (8) washing with DMF $(3 \times 1 \text{ min})$, (9) washing with EtOH $(3 \times 1 \text{ min})$, (10) washing with CH_2Cl_2 (3 × 1 min). After coupling of the last Fmoc amino acid. Fmoc protection of the N-terminal amino group was retained and the side chains of the Orn (Lys) and Asp (Glu) residues to be linked were deprotected by treatment with 50% (v/v) TFA in CH_2Cl_2 (30 min). Following neutralization with 10% (v/v) DIEA in CH₂Cl₂ (2 × 10 min) and washing with CH₂Cl₂ (3 × 1 min) and DMF (3×1 min), cyclization was carried out in DMF containing 1.5% (v/v) DIEA at room temperature by addition of HOBt/DCC (2.5 equiv) for 12-48 h. Completion of the ringclosure reaction was monitored with the ninhydrin test. If necessary, the reaction was continued and brought to completion by addition of the BOP coupling reagent (2 equiv) and DIEA (3 equiv), again using DMF as solvent. Cyclization was usually complete after 12-48 h. After performance of the cyclization step, the N-terminal Fmoc group was removed as usual and washing of the resin was carried out as described above. Subsequently, the peptide chain was extended at the N-terminus as required. After final deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min), the resin was washed with CH_2Cl_2 (3 × 1 min) and EtOH $(3 \times 1 \text{ min})$ and was dried in a desiccator. Peptides were cleaved from the resin by treatment with HF for 90 min at 0 °C and for 15 min at room temperature (20 mL of HF plus 1 mL of anisole/g of resin). After evaporation of the HF, the resin was extracted three times with Et₂O and, subsequently, three times with 7% AcOH. The crude peptide was then obtained in solid form through lyophilization of the acetic acid extract.

Peptides were purified by gel filtration on a Sephadex-G-25 column in 0.5 N AcOH followed by reversed-phase chromatography on an octadecasilyl silica column,²⁷ with a linear gradient of 0-80% MeOH in 1% TFA. If necessary, further purification to homogeneity was performed by semipreparative HPLC [20-80% MeOH (linear gradient) in 0.1% TFA]. Semipreparative HPLC under the same conditions was also used to separate the two diastereoisomers of the Atc³ analogue. Final products were obtained as lyophilisates. Homogeneity of the peptides was established by TLC and by HPLC under conditions identical with those described above. All peptides were at least 98% pure as judged from the HPLC elution profiles. Analytical data are presented in Table III (Supplementary Material).

Receptor Binding Assays and Bioassays. Opioid receptor binding studies were performed as described in detail elsewhere.²⁸ Binding affinities for μ and δ receptors were determined by displacing, respectively, [³H]DAMGO (New England Nuclear) and [³H]DSLET (Amersham) from rat brain membrane preparations, and κ opioid receptor affinities were measured by displacement of [³H]U69,593 (New England Nuclear) from guinea pig brain membranes. Incubations were performed for 2 h at 0 °C with [³H]DAMGO, [³H]DSLET, and [³H]U69,593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC50 values were determined from log dose-displacement curves, and K_i values were calculated from the obtained IC50 values by means of the equation of Cheng and Prusoff,²⁹ using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [³H]DAMGO, [³H]-DSLET, and [³H]U69,593, respectively.

The GPI³⁰ and MVD³¹ bioassays were carried out as reported in detail elsewhere.^{28,32} A log dose-response curve was determined with [Leu⁵]enkephalin as standard for each ileum and vas preparation and IC50 values of the compounds being tested were normalized according to a published procedure.³³ K_o values for naloxone or for the antagonist H-Tyr-Tic-Phe-Phe-Leu-Nle-Asp-NH₂ (6) were determined from the ratio of IC50 values obtained in the presence and absence of a fixed antagonist concentration.³⁴

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Supplementary Material Available: Table III, listing analytical data of deltorphin analogues (1 page). Ordering information is given on any current masthead page.

Registry No. 1, 143293-95-2; 2, 143293-96-3; 3, 143344-20-1; 4, 143293-97-4; 5, 143293-98-5; 6, 143293-99-6; 7, 143294-00-2; 7a, 96382-72-8; 8, 143294-01-3; 9, 143294-02-4; 10, 143294-03-5; 11, 143294-04-6; 12, 119975-64-3; 13, 143294-05-7; 14, 122752-15-2; 14a, 143294-06-8; 15, 58822-25-6.

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