Synthesis and Activity Profiles of Novel Cyclic Opioid Peptide Monomers and Dimers

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A new family of cyclic opioid peptide analogues of the type H-Tyr-D-Xxx-Phe-Yyy-NH_2 was obtained through amide bond formation between side chain amino and carboxyl groups of Orn (or Lys) and Asp (or Glu) residues substituted in positions 2 and 4 of the peptide sequence. Peptides were synthesized entirely by solid-phase techniques, and aside from the cyclic monomers, cyclization on the benzhydrylamine resin also produced side chain linked antiparallel cyclic dimers due to intersite reaction. In binding studies based on displacement of μ- and δ-opioid receptor-selective radiolabels from rat brain membranes the highly rigid cyclic monomer H-Tyr-D-Orn-Phe-Asp-NH_2 (1) (containing a 13-membered ring) was shown to be one of the most selective μ-receptor ligands reported to date, whereas the corresponding cyclic dimer, (H-Tyr-D-Orn-Phe-Asp-NH_2)$_2$ (1a), was nonselective. The difference in receptor selectivity observed between 1 and 1a is a consequence of the different conformational constraints present in the cyclic monomer and dimer. In contrast to 1, the conformationally less restricted cyclic analogue H-Tyr-D-Lys-Phe-Glu-NH_2 (3) (15-membered ring) showed no receptor preference. Qualitatively similar potency relationships were observed in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays. However, in the case of analogues 1 and 3 discrepancies observed between potencies determined in the μ-receptor-representative GPI bioassay and in the μ-receptor-selective binding assay seemed to indicate that the conformational constraint present in these compounds may produce an "efficacy" enhancement. Corresponding analogues containing an Asp (or Glu) residue in the 2-position and an Orn (or Lys) residue in the 4-position showed similar selectivity relationships, but better agreement between bio- and binding assay data. These results indicate that incorporation of various conformational constraints into opioid peptides permits manipulation of both receptor selectivity and efficacy.

Both pharmacologic evaluation of opiates in the chronic spinal dog[2] and structure–activity studies performed with opiates and opioid peptides in vitro using various tissue preparations[3] led to the concept of multiple opioid receptors. There is good evidence for the existence of at least three different types of receptors (μ, δ, κ); however, the physiological roles of the individual receptor types remain to be clarified. Highly selective or even totally specific opioid receptor ligands are needed in studies aimed at correlating specific opioid effects with a distinct receptor class. The μ-agonist sufentanil[4] and the κ-agonist U 50,488[5] are examples of selective nonpeptide opioid receptor ligands. Among the naturally occurring opioid peptides the enkephalins have some preference for δ-receptors and peptides belonging to the dynorphin/neoendorphin family show some selectivity for κ-receptors. Efforts to improve the selectivity of the natural opioid peptides through chemical modification so far have mainly focused on the enkephalins. Classical analogue design based on single or multiple amino acid substitutions resulted in compounds with high μ-receptor selectivity (e.g., DAGO[6]) and in analogues showing a somewhat less pronounced preference for δ-receptors (e.g., DSLET[7]). Attempts to obtain a stable dynorphin analogue with retained or enhanced κ-receptor selectivity have been unsuccessful to date.

The lack of selectivity observed with the natural linear opioid peptides is most likely due to their molecular flexibility permitting adaptation to the different topographies of the various opioid receptor types. The results of numerous studies by various physicochemical techniques have shown that [Leu^5]- and [Met^5]enkephalin can assume a number of different low-energy conformations and that at least in aqueous solution these peptides exist in a conformational equilibrium (for a review see ref 8). Furthermore, it has been clearly established that μ- and δ-opioid receptors differ from one another in their conformational requirements.[9] Therefore, reduction of the conformational flexibility of opioid peptides through introduction of conformational constraints has been considered as a potentially useful approach toward developing more selective receptor ligands. Local conformational constraints can be built into the peptide backbone or into side chains in a number of ways; however, the most drastic restriction of the overall peptide conformation is achieved through synthesis of carefully designed cyclic analogues (cf. ref 10). Biologically active cyclic enkephalin analogues were first obtained through substitution of D-α,ω-diamino acids in position 2 of the peptide sequence and cyclization of the ω-amino group to the C-terminal carboxyl group (e.g., H-Tyr-cyclo[-N$^\epsilon$-D-Lys-Gly-Phe-Leu-]).[11,12] These analogues turned out to be potent agonists with significant selectivity for μ-receptors. Side chain to side chain cyclized enkephalin analogues were obtained through substitution of cysteine residues in position 2 (D configuration) and

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.* 1984, *219*, 345. The following other abbreviations were used: Avl, 2-aminovaleric acid (norvaline); Boc, *tert*-butoxycarbonyl; Bzl, benzyl; DAGO, H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; FAB, fast atom bombardment; Fmoc, (fluoren-9-ylmethoxy)carbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Hyp, 4-hydroxyproline; MVD, mouse vas deferens; TFA, trifluoroacetic acid.

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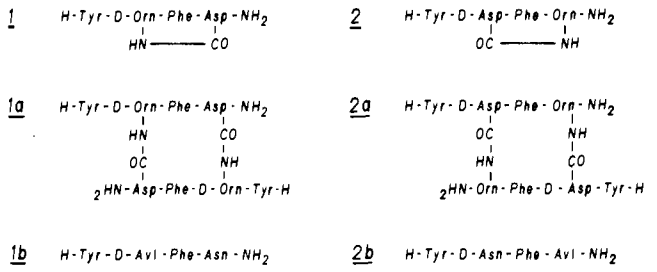

Figure 1. Structural formulas of cyclic monomers 1 and 2, cyclic dimers 1a and 2a, and the corresponding open-chain analogues 1b and 2b.

position 5 (D or L configuration) and subsequent oxidative disulfide bond formation.[13,14] Analogues of the latter type containing a free carboxylic acid function at the C-terminus showed about the same preference for δ-receptors over μ-receptors as the natural enkephalins,[15] whereas the corresponding carboxamides were nonselective.[14] Replacement of the cysteine residues in these analogues by penicillamine residues resulted in compounds with a more rigid 14-membered ring structure that showed the highest selectivity for δ-receptors so far reported.[16] More recently, a new type of enkephalin analogue cyclized through amide bond formation between side chain amino and carboxyl groups of appropriately substituted residues has been described.[17] For example, the analogue H-Tyr-D-Lys-Gly-Phe-Glu-NH_2 was found to be highly potent but nonselective, whereas H-Tyr-D-Glu-Gly-Phe-Lys-NH_2 displayed moderate preference for μ-receptors.

In the present paper we describe the syntheses and activity profiles of cyclic opioid peptides of the latter type which are lacking the glycine residue in position 3 of the enkephalin sequence (Figure 1, compounds 1 and 2). Since these analogues contain a phenylalanine residue in the 3-position, they structurally resemble morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2)[18] and dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro(or Hyp)-Ser-NH_2).[19] Morphiceptin has been reported to be a very selective μ-receptor agonist.[18] From the crude synthetic products of compound 1 and 2 the side chain linked antiparallel cyclic dimers (H-Tyr-D-Orn-Phe-Asp-NH_2)$_2$ (**1a**) and (H-Tyr-D-Asp-Phe-Orn-NH_2)$_2$ (**2a**) were also isolated. These novel dimeric structures are characterized by a 2-fold symmetry axis and are interesting because the conformational restriction imposed on the individual peptide chains obviously differs from that present in the cyclic monomers. In this context it is of interest to note that in one of the crystal forms of [Leu^5]enkephalin ($P2_1$) the peptide molecules are also lined up in an antiparallel fashion and a number of interchain hydrogen bonds are observed.[20] It is quite likely that conformers of the side chain linked antiparallel cyclic dimers are also stabilized by interchain hydrogen bonds. The in vitro opioid activities of **1a** and **2a** were also determined, and in order to assess the effect of the conformational restriction on the activity profiles of the cyclic monomers and dimers, the corresponding linear analogues **1b** and **2b** were synthesized as well and characterized. In comparison to cyclic monomers 1 and 2, the analogues H-Tyr-D-Lys-Phe-Glu-NH_2 (3) and H-Tyr-D-Glu-Phe-Lys-NH_2 (4) contain an additional methylene group in the side chains of the 2- and 4-positions. Since the conformational constraints in these 15-membered cyclic peptides are certain to be different from those present in the more rigid 13-membered cyclic analogues 1 and 2, the in vitro opioid activities of compounds 3 and 4 were also determined for comparison. Finally, in order to clarify the role of the Phe^3 residue in cyclic monomer 1, the analogues H-Tyr-D-Orn-Leu-Asp-NH_2 (5) and H-Tyr-D-Orn-Gly-Glu-NH_2 (6) were synthesized and tested for biological activity.

Chemistry. Cyclic peptides were synthesized by solid-phase techniques on a benzhydrylamine resin according to a recently published scheme.[21] The C-terminal peptide portion to be cyclized was assembled using N^{α}-Fmoc amino acids with Boc and *tert*-butyl protection for the side chains of Orn and Asp, respectively. While Fmoc protection of the N-terminal amino group was maintained, side chains were deprotected by TFA treatment and cyclization on the resin was subsequently achieved in DMF with DCC/HOBt as coupling agents. Amide bond formation was usually complete after 4–6 days. The use of DCC/HOBt in CH_2Cl_2 resulted in a much slower cyclization that could not be brought to completion. It had previously been shown that this type of side chain to side chain cyclization did not produce significant racemization.[21] Subsequent to the cyclization step, Fmoc protection of the α-amino group was removed and the peptide chains were completed by coupling Boc-Tyr(OBzl)-OH. After final removal of the Boc group, the cyclic peptides were cleaved from the resin and deprotected by HF treatment in the usual manner. Following gel filtration of the crude product on Sephadex G-25, peptide components were separated by reversed-phase chromatography and characterized by amino acid analysis and FAB mass spectrometry. In each case, two major components were identified as the cyclic monomer (**1**, **2**, **5**) and the side chain linked antiparallel cyclic dimer (**1a**, **2a**, **5a**). Since the cyclic dimers showed significantly longer retention times on reversed-phase columns under the conditions used than the cyclic monomers, separation was easily achieved. Obviously, the side chain linked antiparallel cyclic dimers had been formed through an intersite reaction between two adjacent peptide chains on the resin. Other types of intersite reactions had previously been observed (cf. ref 22), indicating the considerable flexibility of the polystyrene–divinylbenzene resin. In the case of analogues 1 and 5 about 30% of the peptide chains formed the cyclic monomer and 70% underwent cyclodimerization. On the other hand, cyclic monomer formation (~60%) was favored in the case of analogue 2. The use of three different benzhydrylamine resins containing 0.4, 0.61, and 1.0 mM/g titratable amine, respectively, in

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Table I. Binding Assays of Opioid Peptide Analogues[a]

no.	compd	[3H]DAGO		[3H]DSLET		
		K_i^μ, nM	potency ratio	K_i^δ, nM	potency ratio	K_i^δ/K_i^μ
1	H-Tyr-D-Orn-Phe-Asp-NH_2	10.4 ± 0.7	0.905 ± 0.062	2220 ± 65	0.00114 ± 0.00003	213
1a	(H-Tyr-D-Orn-Phe-Asp-NH_2)$_2$	25.6 ± 1.9	0.368 ± 0.028	42.2 ± 2.4	0.0600 ± 0.0034	1.65
1b	H-Tyr-D-Avl-Phe-Asn-NH_2	11.7 ± 0.7	0.800 ± 0.048	441 ± 15	0.00574 ± 0.00019	37.7
2	H-Tyr-D-Asp-Phe-Orn-NH_2	9.6 ± 2.5	0.985 ± 0.260	1320 ± 150	0.00192 ± 0.00024	137
2a	(H-Tyr-D-Asp-Phe-Orn-NH_2)$_2$	85.9 ± 15.5	0.110 ± 0.020	129 ± 12	0.0196 ± 0.0018	1.50
2b	H-Tyr-D-Asn-Phe-Avl-NH_2	42.9 ± 15.9	0.220 ± 0.082	2760 ± 90	0.000920 ± 0.000031	64.3
3	H-Tyr-D-Lys-Phe-Glu-NH_2	1.43 ± 0.07	6.58 ± 0.32	4.36 ± 0.46	0.581 ± 0.061	3.05
4	H-Tyr-D-Glu-Phe-Lys-NH_2	0.994 ± 0.329	9.51 ± 3.18	49.3 ± 3.3	0.0513 ± 0.0034	49.6
5	H-Tyr-D-Orn-Leu-Asp-NH_2	6800 ± 1000	0.00138 ± 0.00020	8160	0.000225	1.20
6	H-Tyr-D-Orn-Gly-Glu-NH_2	1760 ± 430	0.00530 ± 0.00127	4990 ± 80	0.000508 ± 0.000008	2.83
7	morphiceptin	22.9 ± 0.6	0.411 ± 0.011	382 ± 87	0.00662 ± 0.00151	16.7
8	[Leu5]enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1	0.268

[a] Mean of three determinations ±SEM.

the preparation of H-Tyr-D-Orn-Phe-Asp-NH_2 did not lead to any significant variation in the monomer/dimer ratio, indicating that the extent of cyclodimerization does not depend on the degree of resin substitution in this range (unpublished results). As previously observed,[21] minor peptide components containing a still benzyl-protected tyrosyl residue were also detected in the crude reaction products. Since the benzyl protecting group is in general easily removed by HF treatment, this observation seems to indicate that deprotection may have been impeded by a strong hydrophobic interaction between the protected tyrosine side chain and the side chain of the D amino acid residue in position 2.

Linear analogues **1b** and **2b** were prepared by the normal solid-phase procedure using Boc amino acids.

Bioassays and Binding Assays. In vitro opioid activities of the analogues were determined with assays based on inhibition of electrically evoked contractions of the GPI and the MVD. The GPI assay is usually taken as being representative for μ-receptor interactions, even though κ-receptors are also present in this preparation. κ-Receptor interactions on the GPI are indicated by higher K_e values for naloxone as antagonist (20–30 nM)[23] in contrast to the low values (1–2 nM) observed with μ-receptor ligands.[3] In the MVD assay opioid effects are primarily mediated by δ-receptors; however, μ- and κ-receptors also exist in this tissue. K_e values for naloxone as antagonist were determined with the GPI preparation but not in the MVD assay due to the very low potency shown by most analogues on the vas. Relative opioid receptor affinities were determined by displacement of selective radioligands from rat brain membrane preparations. [3H]DAGO served as a highly selective μ-receptor radioligand, and the somewhat less selective radiolabel [3H]DSLET was used for determining relative δ-receptor affinities.

Results and Discussion

In the [3H]DAGO binding assay the cyclic monomer H-Tyr-D-Orn-Phe-Asp-NH_2 (1) is about as potent as [Leu5]enkephalin, whereas in the [3H]DSLET assay it shows nearly 1000 times lower affinity than the natural peptide (Table I). These results demonstrate that cyclic analogue 1 has very high preference for μ-receptors over δ-receptors, as indicated by the high ratio of the binding inhibition constants determined in the two assays (K_i^δ/K_i^μ = 213). The severe conformational restriction in compound 1 obviously does not impair binding to μ-receptors but is highly incompatible with the δ-receptor topography. Compared to cyclic peptide 1, the corresponding open-chain analogue H-Tyr-D-Avl-Phe-Asn-NH_2 (**1b**) shows about the same affinity in the μ-receptor representative binding assay but is about 5 times more potent at the δ-receptor. The fact that the linear peptide **1b** is less μ-receptor selective than cyclic analogue 1 suggests that introduction of the conformational constraint in 1 through ring closure is directly responsible for its very high μ-receptor selectivity and confirms the observation that μ- and δ-opioid receptors differ in their conformational requirements.[9] The corresponding side chain linked antiparallel cyclic dimer (**1a**) is slightly less potent than 1 in the [3H]DAGO binding assay but is 50 times more potent than the cyclic monomer in the [3H]DSLET binding assay and, therefore, is nonselective. Since **1a** is also 10 times more potent than the corresponding linear monomer (**1b**) in the latter assay, it is obvious that the conformational constraint present in the cyclic dimer actually produces an enhancement in the affinity for the δ-receptor, in sharp contrast to the δ-receptor affinity decrease observed with the cyclic monomer. This different behavior of the cyclic monomer and dimer at the δ-receptor is most likely due to the different conformational constraints present in 1 and **1a**. Alternatively, it could also be argued that the different selectivity profile of the cyclic dimer in comparison with the cyclic and linear monomers might be due to the fact that not only one but both individual peptide chains in the dimer could be involved in binding interactions. For example, it is conceivable that one individual chain would bind to the "primary" opioid receptor sites, whereas one or several moieties of the second chain might interact with possible accessory binding sites of either the μ- or δ-receptor and that these additional interactions could be responsible for the change in receptor selectivity.

Transposition of the Orn and Asp residues in cyclic analogue 1 results in a compound (**2**) that in comparison to 1 is about equipotent in the [3H]DAGO binding assay and about twice as potent in the [3H]DSLET binding assay. Therefore, cyclic monomer **2** shows slightly lower preference for μ-receptors over δ-receptors than 1. Interestingly, the linear correlate **2b** has about 5 times lower affinity for μ-receptors and 2 times lower affinity for δ-receptors than **2** and, therefore, is still quite μ-receptor selective. Compared to cyclic monomer **2**, the cyclic dimer (**2a**) shows again lower affinity for the μ-receptor and higher affinity for the δ-receptor and, consequently, is as nonselective as cyclic dimer **1a**.

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Table II. Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assay of Opioid Peptide Analogues[a]

no.	compd	GPI		MVD		MVD/GPI
		IC_{50}, nM	rel potency	IC_{50}, nM	rel potency	IC_{50} ratio
1	H-Tyr-D-Orn-Phe-Asp-NH_2	36.2 ± 3.7	6.79 ± 0.69	3880 ± 840	0.00294 ± 0.00064	107
1a	(H-Tyr-D-Orn-Phe-Asp-NH_2)$_2$	111 ± 14	2.21 ± 0.27	99.1 ± 12.9	0.115 ± 0.015	0.893
1b	H-Tyr-D-Avl-Phe-Asn-NH_2	181 ± 24	1.36 ± 0.18	2210 ± 180	0.00516 ± 0.00041	12.2
2	H-Tyr-D-Asp-Phe-Orn-NH_2	522 ± 102	0.471 ± 0.092	8570 ± 3540	0.00133 ± 0.00055	16.4
2a	(H-Tyr-D-Asp-Phe-Orn-NH_2)$_2$	1160 ± 320	0.212 ± 0.058	579 ± 122	0.0197 ± 0.0042	0.499
2b	H-Tyr-D-Asn-Phe-Avl-NH_2	591 ± 80	0.416 ± 0.056	>22800	<0.000500	>38.6
3	H-Tyr-D-Lys-Phe-Glu-NH_2	2.93 ± 0.30	84.0 ± 8.6	5.21 ± 0.88	2.19 ± 0.37	1.78
4	H-Tyr-D-Glu-Phe-Lys-NH_2	7.99 ± 0.78	30.8 ± 3.0	101 ± 18	0.113 ± 0.020	12.6
5	H-Tyr-D-Orn-Leu-Asp-NH_2	5410 ± 1460	0.0455 ± 0.0123	40400 ± 6200	0.000282 ± 0.000043	7.47
6	H-Tyr-D-Orn-Gly-Glu-NH_2	8750 ± 2520	0.0281 ± 0.0081	>40000	<0.000285	>4.57
7	morphiceptin	552 ± 151	0.446 ± 0.122	3690 ± 740	0.00309 ± 0.00062	6.68
8	[Leu^5]enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a] Mean of three determinations ±SEM.

In comparison to cyclic monomer **1**, the structurally related cyclic analogue **3** is 7 times more potent in the [^{3}H]DAGO binding assay and, most interestingly, 500 times more potent in the [^{3}H]DSLET binding assay. Obviously, expansion of the 13-membered ring structure contained in **1** through incorporation of two additional methylene groups results in a more relaxed conformational constraint in **3** that no longer prevents efficient binding to the δ-receptor. Consequently, **3** is nonselective, as indicated by its low K_i^δ/K_i^μ ratio. Like analogue **3**, cyclic peptide **4** also contains a 15-membered ring structure and, in analogy to **3**, again shows about 10 times higher affinity for μ-receptors than its more constrained cyclic correlate **2**. However, the potency increase observed with **4** in the δ-receptor representative binding assay in comparison to **2** is less dramatic (27-fold) than that shown by **3**, and therefore, analogue **4** is still moderately μ-receptor selective. The difference in receptor selectivity between cyclic analogues **3** and **4** may be due to the different location and direction of the side chain linking amide bond, which may produce distinct patterns of intramolecular hydrogen bonding, resulting in different conformations of the 15-membered ring structures.

Substitution of a leucine or glycine residue for the phenylalanine residue in position 3 of cyclic monomers related to **1** results in compounds (**5** and **6**) showing very low potency in both binding assays. Unlike cyclic analogue **6**, the structurally related linear analogue H-Tyr-D-Met-Gly-NH-CH(CH_3)CH_2CH(CH_3)$_2$ has good affinity for μ-receptors.[24] This discrepancy in potency may indicate that in opioid peptide analogues lacking a second aromatic residue the side chain in position 2 needs to be unrestrained to interact with a specific receptor subsite in order to produce significant binding. Cyclic dimer **5a** also showed very weak affinity (results not shown).

In comparison to H-Tyr-D-Orn-Phe-Asp-NH_2 (**1**), morphiceptin (**7**) is about half as potent in the [^{3}H]DAGO binding assay but 6 times more potent in the [^{3}H]DSLET binding assay. Determination of the K_i^δ/K_i^μ ratios reveals that cyclic analogue **1** is about 13 times more μ-receptor selective than morphiceptin, which is often referred to as a selective μ-receptor agonist.

The results obtained with the analogues in the GPI and MVD bioassays are qualitatively in agreement with the binding data (Table II). However, interesting quantitative discrepancies between bioassay and binding assay data were observed in the case of cyclic analogues **1** and **3**. Compared to its linear correlate **1b**, cyclic monomer **1** is 6 times more potent in the GPI bioassay, whereas in the [^{3}H]DAGO binding assay the two compounds are equipotent. Since both the bioassay and binding assay are representative for μ-receptor interactions, these results could be explained in terms of an enhanced "efficacy" or "intrinsic activity" of the cyclic peptide as compared to the corresponding open-chain analogue. It would thus appear that the conformational constraint introduced in **1** through ring closure would not affect binding at the μ-receptor, but once bound, the cyclic analogue would be more effective in activating the receptor. The discrepancy between the potencies observed in the GPI assay and the [^{3}H]DAGO binding assay is even more pronounced in the case of cyclic analogue **3**, which relative to [Leu^5]enkephalin is 84 times more potent in the bioassay but only 9 times more potent in the binding assay. Much better agreement is observed between potencies determined in the δ-receptor representative bioassay (MVD) and binding assay ([^{3}H]DSLET displacement), insofar as cyclic analogues **1** and **3** show only a slightly enhanced potency in the bioassay as compared to the binding assay. Quite good agreement between bioassay and binding assay data is observed with analogues containing an Asp or Glu residue in the 2-position. It thus appears that only cyclic monomers containing an α,ω-diamino acid in position 2 (compounds **1** and **3**) show an efficacy enhancement at the μ-receptor. Presumably, the long, conformationally constrained aliphatic side chain in position 2 of these analogues interacts with a specific subsite on the receptor, thereby contributing to a conformational change of the receptor protein in a manner that results in highly effective signal transduction. In the case of the linear analogue, interaction of the unrestrained side chain in position 2 with either the same or a different subsite may induce an overall less productive change in receptor conformation. On the basis of their structural features, the cyclic monomers and dimers as well as the corresponding open-chain analogues **1b** and **2b** are stable against enzymatic degradation under the conditions used in the bioassay and binding assay (cf. ref 11 and 25). Therefore, the potency differences observed between the GPI assay and the [^{3}H]DAGO binding assay are not due to a different extent of peptide degradation in the two tissues. Furthermore, all cyclic and linear analogues described in this paper are very similar with regard to the

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Table III. Sensitivities to Naloxone (K_e) of Opioid Peptide Analogues in the Guinea Pig Ileum Assay

no.	compd	K_e,[a] nM
1	H-Tyr-D-Orn-Phe-Asp-NH_2	1.57 ± 0.22
1a	(H-Tyr-D-Orn-Phe-Asp-NH_2)$_2$	1.36 ± 0.32
1b	H-Tyr-D-Avl-Phe-Asn-NH_2	2.08 ± 0.21
2	H-Tyr-D-Asp-Phe-Orn-NH_2	1.28 ± 0.38
2a	(H-Tyr-D-Asp-Phe-Orn-NH_2)$_2$	1.62 ± 0.19
2b	H-Tyr-D-Asn-Phe-Avl-NH_2	2.08 ± 0.14
3	H-Tyr-D-Lys-Phe-Glu-NH_2	1.80 ± 0.18
4	H-Tyr-D-Glu-Phe-Lys-NH_2	0.81 ± 0.04
7	morphiceptin	1.92 ± 0.27
8	[Leu^5]enkephalin	1.53 ± 0.43

[a] Mean of three determinations ±SEM.

chemical moieties they contain and, for this reason, must have about the same partition coefficients. It is thus unlikely that the observed discrepancies between bioassay binding assay data are due to different exposure of the receptors in their lipid environment. It could also be argued that these potency discrepancies are due to the fact that μ-receptors in rat brain membrane preparations differ somewhat in their structural requirements from μ-receptors in the intact GPI. This possibility, however, seems less likely because the rank orders of potencies observed with a large number of linear enkephalin analogues in the GPI assay in general correlate very well with those determined in binding assays based on displacement of μ-receptor-selective radioligands from rat brain membranes. Nevertheless, additional experiments will have to be performed to further corroborate the "efficacy enhancement" hypothesis.

The effects of all analogues on the GPI are completely naloxone reversible, and the apparent dissociation constants (K_e) determined for naloxone as antagonist are all in the range from 0.81 to 2.08 nM (Table III). These low values are typical for μ-receptor interactions[3,23] and rule out the possibility that the enhanced potencies observed with cyclic monomers **1** and **3** in the GPI assay are due to an additional interaction with κ-receptors.

Conclusions

Among the novel side chain to side chain cyclized opioid peptide analogues described in this paper, cyclic monomers **1** and **2** display very high preference for μ-receptors over δ-receptors as a consequence of their poor affinity for δ-sites. In the binding assays cyclic analogue **1** showed a K_i^δ/K_i^μ ratio more than 10 times higher than that determined with the well-known μ-receptor ligand morphiceptin, and it has recently been found to be nearly as μ-receptor selective as the highly selective μ-agonist DAGO.[26] Relaxation of the conformational constraint in **1** and **2** through ring enlargement resulted in nonselective or less μ-selective analogues (**3** and **4**) due to better compatibility with δ-receptors. In comparison with their corresponding linear monomers (**1b** and **2b**) the nonselective cyclic dimers **1a** and **2a** showed 10–20 times higher affinity for δ-receptors, most likely as a consequence of the distinct conformational constraints present in the dimeric structures. Taken together, these results illustrate the great potential of conformational restriction as a tool for manipulating receptor selectivity and confirm the observation that μ- and δ-opioid receptors differ from one another in their conformational requirements.

The discrepancies in potency observed between the GPI assay and the [^{3}H]DAGO binding assay in the case of cyclic monomers **1** and **3** can be interpreted to indicate that the conformational constraints present in these compounds may produce an efficacy enhancement. Similar observations had previously been made with enkephalin analogues cyclized between the ω-amino group of a D-α, ω-diamino acid residue, substituted in position 2, and the C-terminal carboxyl group (e.g., H-Tyr-cyclo[-N$^\epsilon$-D-Lys-Gly-Phe-Leu-]).[12] It is conceivable that introduction of conformational constraints into peptides could either enhance or decrease efficacy, thereby producing either superagonists or antagonists. An example of antagonism due to enhanced conformational restriction is provided by the antagonist [Pen^1]oxytocin.[27,28]

Among the cyclic opioid peptides reported to date, cyclic analogue **1** is the most selective μ-agonist. Because of its high selectivity and rigidity, studies of its conformation by various physicochemical techniques will be particularly relevant with regard to the bioactive conformation of opioid peptides at the μ-receptor. Eventually, conformational studies with the highly μ-selective analogue **1** and the highly δ-selective cyclic analogue (D-Pen^2,D-(or L)-Pen^5]enkephalin[16] can be expected to reveal the specific conformational requirements of μ- and δ-opioid receptors.

Experimental Section

General Methods. Precoated plates (silica gel G, 250 μm; Analtech, Newark DE) were used for ascending TLC in the following solvent systems (all v/v): (1) *n*-BuOH/AcOH/H_2O (BAW) (4:1:5, organic phase) and (2) *n*-BuOH/pyridine/AcOH/H_2O (BPAW) (15:10:3:12). Reversed-phase HPLC was performed on a Waters liquid chromatograph (Model 6000 solvent delivery system, Model 660 solvent programmer) equipped with a Model 450 variable-wavelength detector, utilizing a Waters column (30 × 0.78 cm) packed with C-18 Bondapak reversed-phase (10 μm) material. For amino acid analyses, peptides (0.3 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 system AA computing integrator. Molecular weights of the obtained products were determined by FAB mass spectrometry on a Kratos/AEI MS-30 mass spectrometer, interfaced to a DS-55 data system.

Fmoc and Boc amino acid derivatives were purchased from IAF Biochemicals, Laval, Quebec, Canada. Boc-Avl and Boc-D-Avl were prepared with use of di-*tert*-butyl dicarbonate.[29] All peptides were prepared by the manual solid-phase technique using a benzhydrylamine resin (2% cross-linked, 80–180 mesh, 0.4 mM/g of titratable amine) obtained from Chemical Dynamics Corp., South Plainfield, NJ.

Solid-Phase Peptide Synthesis and Purification of Opioid Peptides. Cyclic analogues were synthesized according to a protection scheme described in detail elsewhere.[21] After neutralization of the resin with 10% (v/v) DIEA in CH_2Cl_2 (2 × 10 min) and washing with CH_2Cl_2 (3 × 1 min) and EtOH (3 × 1 min) the C-terminal peptide segment to be cyclized was assembled according to the following protocol: (1) addition of Fmoc amino acid in CH_2Cl_2 (2.5 equiv); (2) addition of DCC (2.5 equiv) and mixing for 5–24 h (completeness of the reaction was verified with the ninhydrin test[30]); (3) Fmoc deprotection with 50% (v/v) piperidine in CH_2Cl_2 (1 × 30 min); (4) washing with DMF (3 ×

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Table IV. Analytical Data of Opioid Peptide Analogues

no.	compd	amino acid anal.	TLC: (R_f) BAW	TLC: (R_f) BPAW	HPLC elut time, min	FAB-MS (MH^+) calcd	FAB-MS (MH^+) found
1	H-Tyr-D-Orn-Phe-Asp-NH_2	Tyr, 1.00; Orn, 1.00; Phe, 1.00; Asp, 0.86	0.56	0.66	15.3	539	539
1a	(H-Tyr-D-Orn-Phe-Asp-NH_2)$_2$	Tyr, 1.00; Orn, 1.02; Phe, 1.02; Asp, 0.89	0.37	0.69	23.1	1078	1078
1b	H-Tyr-D-Avl-Phe-Asn-NH_2	Tyr, 0.88; Avl, 0.94; Phe, 1.03; Asp, 1.00	0.66	0.68	29.8		
2	H-Tyr-D-Asp-Phe-Orn-NH_2	Tyr, 1.01; Asp, 0.88; Phe, 1.00; Orn, 1.01	0.48	0.71	18.4	539	539
2a	(H-Tyr-D-Asp-Phe-Orn-NH_2)$_2$	Tyr, 1.00; Asp, 0.91; Phe, 1.04; Orn, 0.99	0.46	0.72	27.4	1078	1078
2b	H-Tyr-D-Asn-Phe-Avl-NH_2	Tyr, 1.01; Asp, 0.97; Phe, 1.00; Avl, 1.09	0.54	0.72	24.1		
5	H-Tyr-D-Orn-Leu-Asp-NH_2	Tyr, 0.94; Orn, 0.85; Leu, 1.02; Asp, 1.00	0.44	0.66	17.3	505	505
5a	(H-Tyr-D-Orn-Leu-Asp NH_2)$_2$	Tyr, 0.95; Orn, 1.02; Leu, 1.01; Asp, 1.00	0.38	0.74	26.6	1110	1110

1 min), CH_2Cl_2 (3 × 1 min), and EtOH (3 × 1 min). After the last Fmoc amino acid was coupled, Fmoc protection of the N-terminal amino group was retained and the side chains of the Orn and Asp residues to be cyclized were deprotected by treatment with 50% (v/v) TFA in CH_2Cl_2 (1 × 30 min). Following neutralization with 10% (v/v) DIEA in CH_2Cl_2 (2 × 10 min) and washing with CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min), cyclization was performed in DMF at room temperature by addition of DCC (5 equiv) in the presence of HOBt (5 equiv). Progress of ring closure was monitored by the ninhydrin test, and the cyclization reaction was usually complete after 4–6 days. Fresh DCC and HOBt were added every 48 h. After completion 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, Boc-Tyr(OBzl)-OH (2.5 equiv) in CH_2Cl_2 and DCC (2.5 equiv) were added, and the resin suspension was mixed for 5–12 h. After subsequent deprotection with 50% (v/v) TFA in CH_2Cl_2 (1 × 30 min) and washing with CH_2Cl_2 (3 × 1 min) and EtOH (3 × 1 min), the resin was dried in a desiccator. Linear peptides **1b** and **2b** were prepared on the same benzhydrylamine resin with Boc amino acids according to a protocol described elsewhere.[31] HF treatment was used for cleaving of the peptide from the resin and for concomitant deprotection of the tyrosyl residue. The reaction was carried out for 90 min at 0 °C and for 15 min at room temperature with 20 mL of HF and in the presence of 1 mL of anisole/g of resin. After evaporation of the HF, the resin was extracted three times with diethyl ether and, subsequently, three times with 7% acetic acid. Lyophilization of the acetic acid extract provided the crude product in solid form.

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,[32] using a linear gradient of 0–80% MeOH in 1% TFA. If necessary, further purification to homogeneity was performed by semipreparative reversed-phase HPLC (20–50% MeOH (linear gradient) in 0.1% TFA). In the case of cyclic peptides 1, **2**, and **5** a second major component corresponding to the side chain linked antiparallel cyclic dimer (compounds **1a**, **2a**, and **5a**) was also isolated from the crude reaction products. The elution times of the cyclic dimers on the reversed-phase column were longer than those of the corresponding cyclic monomers (cf. Table IV), and therefore, separation was easily achieved. The proportions of peptide chains having formed cyclic monomers and dimers during the cyclization step were 32:68 in the case of analogue 1, 58:42 in the case of analogue **2**, and 24:76 in the case of analogue **3**, as determined by peak integration of HPLC chromatograms obtained with the crude products. In each case a minor component was identified as the cyclic monomer containing a still protected tyrosyl residue. Due to their longer elution times on the reversed-phase column these contaminants could easily be separated from the free peptides. Final products were obtained as lyophilisates. Homogeneity of the peptides was established by TLC and HPLC under conditions identical with those described above. All peptides were at least 95% pure, as judged from the HPLC elution profiles. On the basis of the amount of C-terminal amino acid coupled to the resin, overall reaction yields after purification to homogeneity were as follows: **1** + **1a**, 24%; **1b**, 28%; **2** + **2a**, 32%; **2b**, 17%; **5** + **5a**, 19%. Analytical data are presented in Table IV.

The syntheses of peptides **3**, **4**, and **6** have been reported elsewhere.[21] Morphiceptin was purchased from IAF Biochemicals, Laval, Quebec, Canada.

Bioassays. The bioassays based on inhibition of electrically evoked contractions of the GPI[33] and of the MVD[34] were carried out as reported in detail elsewhere.[12,35] A log dose–response curve was determined with [Leu^5]enkephalin as standard for each ileum or vas preparation, and IC_{50} values of the opioid peptide analogues being tested were normalized according to a published procedure.[36] K_e values for naloxone as antagonist were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed naloxone concentration (5 nM).[37]

Binding studies with rat brain membrane preparations were carried out as described in detail elsewhere.[35] [^{3}H]DAGO and [^{3}H]DSLET at respective concentrations of 0.71 and 0.98 nM were used as radioligands, and incubations were performed at 0 °C for 2 h. K_i values were calculated on the basis of Cheng and Prusoff's equation[38] using values of 1.3 and 2.6 nM for the dissociation constants of [^{3}H]DAGO and [^{3}H]DSLET, respectively.[6,39]

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Registry No. 1, 96382-72-8; **1a**, 98049-10-6; **1b**, 96382-73-9; **2**, 98048-58-9; **2a**, 98049-11-7; **2b**, 97974-51-1; **3**, 95610-94-9; **4**, 95610-98-3; **5**, 97974-52-2; **5a**, 98048-58-9; **6**, 95610-96-1.

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