Structure-Activity Relationships of Cyclic Opioid Peptide Analogues Containing a Phenylalanine Residue in the 3-Position

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Ten analogues of the highly μ-receptor selective cyclic opioid peptide H-Tyr-D-Orn-Phe-Asp-NH_2 (1) were synthesized by the solid-phase method and were characterized in vitro in μ- and δ-receptor representative binding assays and bioassays. These cyclic analogues are structurally related to the linear opioid peptides dermorphin and β-casomorphin (morphiceptin), which also contain a phenylalanine residue in the 3-position of the peptide sequence. The obtained results indicate that analogous structural modifications (configurational inversion at positions 2, 3, and 4 or N^{α}-methylation of Phe^3) in cyclic peptide 1 and in dermorphin-related peptides had qualitatively the same effect on opioid activity, whereas the corresponding modifications in β-casomorphins had the opposite effect. These findings can be interpreted to indicate that the mode of receptor binding of H-Tyr-D-Orn-Phe-Asp-NH_2 is identical with that of dermorphin, but differs from that of β-casomorphins. The side-chain length of the aromatic residue in position 3 of cyclic analogue 1 was shown to be critical for receptor affinity and selectivity, suggesting that μ- and δ-receptors differ from one another in the relative topographical disposition of the binding sites for the Tyr^1 tyramine moiety and the Phe^3 aromatic ring. Cyclic lactam analogue H-Tyr-D-Asp-Phe-A_2bu-NH_2, containing a reduced-size (12-membered) ring structure, showed increased μ-receptor selectivity, whereas the more flexible, cystine-containing analogue H-Tyr-D-Cys-Phe-Cys-NH_2 (11-membered ring) was less selective. The latter results indicate that both ring size and ring flexibility affect receptor affinity and selectivity.

Recently, we developed a potent cyclic opioid peptide analogue, H-Tyr-D-Orn-Phe-Asp-NH_2 (1), which shows high selectivity for μ-opioid receptors.[2] Whereas most naturally occurring opioid peptides contain a phenylalanine residue in the 4-position of the peptide sequence, the Phe residue in cyclic analogue 1 is located in position 3, as it is also the case with the dermorphins (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro(or Hyp)-Ser-NH_2) and with the β-casomorphins (H-Tyr-Pro-Phe-Pro-NH_2 (morphiceptin), H-Tyr-Pro-Phe-Pro-Gly-NH_2 (β-casomorphin-5), etc.). Compound 1 contains a rather rigid 13-membered ring structure, and its μ-receptor preference is due to very weak affinity for the δ-receptor. Pharmacologic comparison with a corresponding open-chain analogue revealed that the high μ-receptor selectivity of this cyclic analogue is a direct consequence of the conformational restriction introduced into the peptide through ring formation.[2]

In the present paper we describe the syntheses and the in vitro opioid activity profiles of several cyclic opioid peptide analogues that are structurally derived from the cyclic parent peptide 1. In particular, it was of interest to establish whether analogous structural modifications in cyclic peptide 1 and in linear opioid peptides with a Phe residue in the 3-position (dermorphins and β-casomorphins) have the same effect on biological activity. Structure-activity data of this type might indicate whether or not the cyclic and linear peptides have the same mode of binding to the receptor. Thus, configurational requirements in positions 2, 3, and 4 of cyclic peptide 1 were investigated by synthesis and pharmacologic characterization of analogues **2**, **3**, and **4** (Table I) and were compared with those of dermorphin and β-casomorphin. Modification of the important Phe^3 residue was achieved through substitution of a nitro substituent in the para position of the aromatic ring (analogue **5**) and through shortening (analogue **6**) and lengthening (analogue **7**) of the side chain. Previously, it had been observed that expansion of the 13-membered ring structure in analogue 1 to the 15-membered ring contained in H-Tyr-D-Lys-Phe-Glu-NH_2 produced a drastic loss in receptor selectivity due to the more relaxed conformational constraint present in the latter peptide.[2] In view of this observation it was of interest to investigate the effect of further conformational restriction of the ring portion in compound 1 on receptor affinity and selectivity. To this end an analogue with an N-methylated Phe^3 residue (compound **8**) and analogues containing reduced-size (12- and 11-membered) ring structures (compounds **10** and **11**) were also synthesized and characterized.

Chemistry. The cyclic lactam analogues (**2–7**, **9**, **10**) were prepared by the solid-phase method on a *p*-methylbenzhydrylamine resin according to a scheme described in detail elsewhere.[3] The C-terminal peptide segment to be cyclized was assembled by using N^{α}-Fmoc amino acids with Boc and *tert*-butyl protection for the side chains of Orn (A_2bu) and Asp, respectively. The side chain protecting groups of the C-terminal tripeptide were removed by treatment with TFA, and side chain to side chain cyclization of the still-resin-bound peptide was then performed in DMF with DCC/HOBt in fivefold excess as coupling agents. The time required for amide bond formation to be complete varied from 1 to 6 days. After the cyclization step, the N-terminal Fmoc protecting group was removed and the peptide chains were completed by coupling Boc-Tyr(Boc)-OH. Following the removal of the Boc groups, the cyclic 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 each case two

(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: A_2bu, α,γ-diaminobutyric acid; Boc, *tert*-butoxycarbonyl; 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; Hfe, homophenylalanine; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Hyp, 4-hydroxyproline; MVD, mouse vas deferens; Phe(NMe), N^{α}-methylphenylalanine; Phe(pNO_2), *p*-nitrophenylalanine; Phg, phenylglycine; TFA, trifluoroacetic acid.

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

no.	compd	[^{3}H]DAGO K_i^μ, nM	[^{3}H]DAGO rel potency[b]	[^{3}H]DSLET K_i^δ, nM	[^{3}H]DSLET rel potency[b]	K_i^δ/K_i^μ
1	H-Tyr-D-Orn-Phe-Asp-NH_2	10.4 ± 3.7	0.907 ± 0.032	2220 ± 65	0.001 14 ± 0.000 03	213
2	H-Tyr-Orn-Phe-Asp-NH_2	4830 ± 610	0.001 95 ± 0.000 25	>60 000	<0.000 042 1	>12.4
3	H-Tyr-D-Orn-D-Phe-Asp-NH_2	3040 ± 470	0.003 10 ± 0.000 48	>380 000	<0.000 006 7	>125
4	H-Tyr-D-Orn-Phe-D-Asp-NH_2	21.7 ± 3.2	0.435 ± 0.064	422 ± 17	0.006 00 ± 0.000 24	19.4
5	H-Tyr-D-Orn-Phe(pNO_2)-Asp-NH_2	273 ± 21	0.0345 ± 0.0027	5060 ± 540	0.000 500 ± 0.000 053	18.5
6	H-Tyr-D-Orn-Phg-Asp-NH_2	445 ± 92	0.0212 ± 0.0044	3570 ± 370	0.000 709 ± 0.000 073	8.02
7	H-Tyr-D-Orn-Hfe-Asp-NH_2	11.9 ± 0.7	0.792 ± 0.047	849 ± 189	0.002 98 ± 0.000 66	71.3
8	H-Tyr-D-Orn-Phe(NMe)-Asp-NH_2	3570 ± 500	0.002 64 ± 0.000 37	28 100 ± 2530	0.000 090 0 ± 0.000 008 1	7.87
9	H-Tyr-D-Asp-Phe-Orn-NH_2	9.55 ± 2.52	0.987 ± 0.261	1320 ± 150	0.001 92 ± 0.000 22	138
10	H-Tyr-D-Asp-Phe-A_2bu-NH_2	24.8 ± 1.1	0.380 ± 0.017	4170 ± 430	0.000 607 ± 0.000 062	168
11	H-Tyr-D-Cys-Phe-Cys-NH_2	11.0 ± 0.3	0.857 ± 0.023	373 ± 63	0.006 78 ± 0.001 15	33.9
12	[Leu^5]enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1	0.268

[a] Mean of three determinations ± SEM. [b] Potency relative to [Leu^5]enkephalin.

major peptide components were obtained and easily separated by reversed-phase chromatography. The structural characterization of the two components obtained in the synthesis of cyclic parent peptide **1** has been reported previously.[4] Both products had been shown to have the correct amino acid composition; however, analysis by FAB mass spectrometry had revealed that, whereas the faster eluting component had the correct molecular weight expected for the cyclic monomer (MH^+ = 539), the molecular weight of the slower eluting component was exactly doubled (MH^+ = 1078). It had thus become clear that the first component corresponded to the desired cyclic monomer, whereas the second component represented the side chain linked antiparallel cyclic dimer, which had been formed through intersite reaction on the resin. In the syntheses of cyclic peptides **2–10**, the faster eluting components were in each case confirmed as the desired cyclic monomers by amino acid analysis and FAB mass spectrometry. In all cases the faster and the slower eluting components had the same amino acid composition. The dimeric nature of the slower eluting component was confirmed by FAB mass spectrometry in some cases (peptides **5**, **7**, **8**, and **10**).

In all cases 30–50% of the peptide chains formed the cyclic monomer and 50–70% underwent cyclodimerization. As had already been pointed out previously,[4] the level of resin substitution in the range of 0.4–1.0 mM/g of titratable amine had little effect on the monomer/dimer ratio in the case of parent peptide **1**. The extent of cyclodimerization is most likely governed by conformational factors. This assumption is supported by the observation that the number of amino acid residues in between the two residues to be cyclized is of major consequence with regard to the ease with which intramolecular cyclization occurs. Thus, it was generally found that with two residues in between the residues engaged in ring closure cyclic monomer formation was always favored over cyclodimerization (e.g., H-Tyr-D-Orn-Gly-Phe-Asp-NH_2: 86% monomer, 14% dimer) (unpublished results).

The cystine-containing cyclic analogue H-Tyr-D-Cys-Phe-Cys-NH_2 (**11**) was also prepared by the solid-phase method using N^α-Boc amino acids and the *p*-methylbenzyl group for side-chain protection of the Cys residues. After HF cleavage, oxidative disulfide bond formation was carried out at high dilution by reaction with $K_3Fe(CN)_6$. Three major products were isolated and characterized as the cyclic monomer (MH^+ = 533, 25%), a cyclic dimer (antiparallel or parallel, MH^+ = 1065, 43%), and a higher oligomer (32%).

Bioassays and Binding Assays. Opioid receptor affinities were determined by displacement of relatively selective radioligands from rat brain membrane binding sites. [^{3}H]DAGO served as a very selective μ-receptor label, and the somewhat less selective radioligand [^{3}H]-DSLET was used for determining relative δ-receptor affinities. For the determination of their in vitro opioid activities, analogues were tested in bioassays based on inhibition of electrically induced contractions of the guinea pig ileum (GPI) and of the mouse vas deferens (MVD). In the GPI preparation opioid effects are primarily mediated by μ-receptors; however, κ-receptors are also present in this tissue. μ-Receptor interactions in the GPI are characterized by relatively low K_e values for naloxone as antagonist (1–2 nM)[5] in contrast to the considerably higher values (20–30 nM)[6] observed with κ-receptor ligands. The MVD assay is usually taken as being representative for δ-receptor interactions, even though the vas also contains μ- and κ-receptors. K_e values for naloxone as antagonist were determined in the GPI assay but not in the MVD assay because of the very low activity displayed by all analogues on the vas.

Results and Discussion

As previously reported,[2] the cyclic parent peptide **1** displays high μ-receptor selectivity because it retains about the same affinity for μ-receptors as [Leu^5]enkephalin but binds very poorly to δ-receptors. Inversion of the configuration of the ornithine residue in position 2 of the peptide sequence of cyclic analogue **1** resulted in a compound (**2**) with drastically reduced affinity for both the μ- and the δ-receptor (Table I). In linear opioid peptides containing a phenylalanine residue in the 3-position and structurally related to dermorphin, substitution of an L-amino acid residue in position 2 of the peptide sequence had also resulted in a drastic potency loss. Thus, substitution of L-Ala for D-Ala in the active analogue H-Tyr-D-Ala-Phe-NH_2 had produced a compound showing no activity in the

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GPI assay.[7] Similarly, H-Tyr-D-Arg-Phe-Gly-OH displayed high analgesic activity in the mouse tail pressure test, whereas H-Tyr-L-Arg-Phe-Gly-OH was found to be inactive.[8] β-Casomorphin-4 (morphiceptin) also contains a Phe residue in position 3, but a Pro residue in position 2 of the peptide sequence. In contrast to cyclic analogue 1 and to the dermorphin-related analogues discussed above, morphiceptin requires L configuration at the Pro^2 residue, as indicated by the low activity observed with H-Tyr-D-Pro-Phe-Pro-NH_2 in comparison with morphiceptin.[9] Substitution of D-phenylalanine in position 3 of compound 1 (analogue **3**) also reduced the affinity for both μ- and δ-receptors by more than 2 orders of magnitude. This observation is again in agreement with the drastic potency loss resulting from configurational inversion at the Phe^3 residue in a dermorphin tetrapeptide.[10] Again, the corresponding D-Phe^3 substitution in β-casomorphin has the opposite effect, as indicated by the increased potency of H-Tyr-Pro-D-Phe-Pro-Gly-OH in comparison with H-Tyr-Pro-Phe-Pro-Gly-OH in the GPI and MVD assays.[11] Configurational inversion in the 4-position of cyclic peptide 1 led to a compound (**4**) with only slightly reduced affinity for μ-receptors and with 5 times higher δ-receptor affinity. This relatively minor change in the activity profile is again in agreement with the observation that substitution of D-leucine in position 4 of the dermorphin-related peptide analogue H-Tyr-D-Arg-Phe-Leu-OH did not have a major effect on analgesic potency.[8] On the other hand, the morphiceptin analogue [D-Pro^4]morphiceptin has been reported to be nearly 20 times more potent in the GPI assay than its diastereomer, morphiceptin.[11] Taken together, the receptor binding assay data obtained with analogues **2–4** indicate that cyclic analogue **1** has the same configurational requirements in positions 2, 3, and 4 of the peptide sequence as dermorphin-related peptides, whereas β-casomorphins (morphiceptin) show opposite configurational requirements in all three positions.

Substitution of a nitro group in the para position of the Phe^3 aromatic ring in cyclic peptide 1 (analogue **5**) produced a 30-fold drop in μ-receptor affinity (Table I). Drastically reduced potencies have also been reported for dermorphin and morphiceptin analogues with a *p*-nitrophenylalanine residue substituted in the 3-position.[12] In contrast to these results, introduction of a *p*-nitro substituent in the Phe^4 residue of enkephalin and of enkephalin analogues had resulted in a drastic affinity enhancement, particularly at the μ-receptor.[12,13] These observations had led to the conclusion that the aromatic ring of [Phe^3] opioid peptides might bind to a receptor subsite different from that with which the aromatic ring of Phe^4 in enkephalin analogues interacts. Shortening of the Phe^3 side chain (analogue **6**) produced a 40-fold drop in μ-receptor affinity without much effect on δ-receptor affinity, and, consequently, the μ-receptor selectivity of **6** is considerably lower. These data indicate that the more restricted orientational freedom of the phenylglycine residue may no longer permit an optimal interaction of its aromatic ring with the complementary μ-receptor subsite. On the other hand, lengthening of the side chain in the 3-position of compound **1** resulted in a cyclic analogue (**7**) with unchanged μ-receptor affinity but nearly 3 times higher δ-receptor affinity. Therefore, analogue **7** is about 3 times less μ-receptor selective than parent compound **1**. This result suggests that due to chain lengthening the aromatic ring of homophenylalanine might have somewhat better access to a hydrophobic subsite of the δ-receptor. In agreement with the retained high potency of analogue **7**, a homophenylalanine tripeptide analogue of dermorphin, H-Tyr-D-Ala-Hfe-NH_2 had also been reported to be very potent in the GPI assay.[14]

Further conformational restriction in cyclic analogue **1** was achieved through N-methylation of the Phe^3 residue, resulting in a compound (**8**) that has very low affinity for both the μ- and the δ-receptor. The poor binding properties of analogue **8** may be due either to the additional conformational constraint introduced by incorporation of the methyl group or simply to the bulkiness of the latter group, which may produce steric interference at the receptor. N^{α}-Methylation of the 3-position residue in a dermorphin-related peptide analogue, H-Tyr-D-Ala-Hfe-NH_2, had also produced a considerable potency loss in the GPI assay.[14] Again, the opposite effect was obtained upon methylation of the Phe^3 residue in morphiceptin, as indicated by the observation that H-Tyr-Pro-Phe(NMe)-Pro-NH_2 is about twice as potent as morphiceptin at the μ-receptor.[15,16]

Transposition of the Orn and Asp residues in parent peptide **1** had previously been shown to result in a compound (**9**) with almost identical μ- and δ-receptor affinities[4] (Table I). Like analogue **1**, cyclic peptide **9** contains a rather rigid 13-membered ring structure. Shortening of the side chain in position 4 of **9** by one methylene group through substitution of α,γ-diaminobutyric acid (A_2bu) results in an analogue (**10**) containing an even more rigid, 12-membered ring. As is evident from Table I, this further ring contraction produced a 2.5-fold decrease in μ-receptor affinity and a slightly larger decrease in δ-receptor affinity. Therefore, the μ-receptor selectivity (K_i^{δ}/K_i^{μ}) of compound **10** is higher than that of **9**. The cystine-containing cyclic analogue **11** has an even smaller, 11-membered ring structure. Compound **11** has about the same affinity for the μ-receptor as parent peptide **1** but 6 times higher affinity for the δ-receptor. Thus, despite its smaller ring size, cyclic analogue **11** is less μ-selective than cyclic peptides **1**, **9**, and **10**. This may be due to the fact that the side chain connecting disulfide linkage in **10** is more flexible than the corresponding amide linkages in compounds **1**, **9**, and **10**. This enhanced flexibility may permit somewhat better adaptation to the δ site despite the smaller ring size.

The activities of the cyclic analogues determined with the GPI and MVD bioassay are listed in Table II. In general, the bioassay structure–activity data are in good

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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[b]	IC_{50}, nM	rel potency[b]	IC_{50} ratio
1	H-Tyr-D-Orn-Phe-Asp-NH_2	36.2 ± 3.7	6.80 ± 0.69	3880 ± 840	0.002 94 ± 0.000 64	107
2	H-Tyr-Orn-Phe-Asp-NH_2	16 800 ± 3600	0.0146 ± 0.0031	>100 000	<0.000 114	>5.95
3	H-Tyr-D-Orn-D-Phe-Asp-NH_2	>21 000	<0.0117	>8640	<0.001 32	
4	H-Tyr-D-Orn-Phe-D-Asp-NH_2	632 ± 182	0.389 ± 0.112	4810 ± 550	0.002 37 ± 0.000 27	7.61
5	H-Tyr-D-Orn-Phe(pNO_2)-Asp-NH_2	2340 ± 510	0.105 ± 0.023	>100 000	<0.000 114	>43
6	H-Tyr-D-Orn-Phg-Asp-NH_2	18 400 ± 7100	0.0134 ± 0.0052	1660 ± 30	0.006 87 ± 0.000 12	0.0902
7	H-Tyr-D-Orn-Hfe-Asp-NH_2	111 ± 6	2.22 ± 0.12	1370 ± 520	0.008 32 ± 0.003 16	12.3
8	H-Tyr-D-Orn-Phe(NMe)-Asp-NH_2	6460 ± 1630	0.0381 ± 0.0096	1380 ± 270	0.008 26 ± 0.001 62	0.214
9	H-Tyr-D-Asp-Phe-Orn-NH_2	522 ± 102	0.471 ± 0.092	8570 ± 3540	0.001 33 ± 0.000 55	16.4
10	H-Tyr-D-Asp-Phe-A_2bu-NH_2	279 ± 16	0.882 ± 0.051	2030 ± 690	0.005 62 ± 0.001 91	7.28
11	H-Tyr-D-Cys-Phe-Cys-NH_2	64.7 ± 11.9	3.80 ± 0.70	740 ± 187	0.0154 ± 0.0039	11.4
12	[Leu^5]enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a] Mean of three determinations ± SEM. [b] Potency relative to [Leu^5]enkephalin.

agreement with the receptor binding data. However, some of the cyclic analogues (**1**, **2**, **5**, **7**, **8**, and **11**) showed several-fold higher potency in the GPI assay than was expected on the basis of their μ-receptor affinities determined in the binding assay ([^{3}H]DAGO displacement). These discrepancies may be tentatively interpreted in terms of a higher "efficacy" or "intrinsic activity" of the cyclic analogues at the μ-receptor as compared to linear opioid peptides. However, the validity of this hypothesis needs to be demonstrated by the performance of further experiments. All analogues showed K_e values for naloxone as antagonist between 1 and 2 nM in the GPI assay. These values are typical for μ-receptor interactions and rule out the possibility that the unexpectedly high potencies of the cyclic analogues may be due to an additional interaction with κ-receptors which would result in higher K_e values.

In the case of peptide **1**, the side chain linked antiparallel cyclic dimer, (H-Tyr-D-Orn-Phe-Asp-NH_2)$_2$, had previously been shown to be less potent than the cyclic monomer at the μ-receptor and considerably more potent at the δ-receptor.[4] As a result, the cyclic dimer showed no receptor selectivity, in contrast to the high μ-receptor preference observed with the cyclic monomer. The potencies of three selected cyclic dimers of the present series, corresponding to peptides **2**, **10**, and **11**, were also determined in the GPI and MVD assay (data not shown). As in the case of peptide **1**, all three dimers showed reduced potency in the GPI assay and increased potency in the MVD assay when compared with their corresponding cyclic monomers. Consequently, the dimers corresponding to peptides **2**, **10**, and **11** also were relatively nonselective, showing IC_{50}(MVD)/IC_{50}(GPI) ratios of 2.50, 0.829, and 0.222, respectively. As had been previously suggested,[4] the altered activity profile of the dimers in comparison with the cyclic monomers could be either due to the difference in conformational constraints or due to the fact that in the case of the dimers additional interactions with accessory binding sites might alter the activity profile.

Conclusions

The results of the present study indicate that analogous structural modifications in cyclic peptide **1** and in dermorphin-related peptides produce qualitatively the same effect on biological activity, whereas corresponding modifications in β-casomorphins (morphiceptin) in general had the opposite effect. It has previously been proposed that different classes of opiates might have different modes of binding to the receptor.[17] The fact that the configurational requirements of compound **1** and of dermorphin-related peptides in positions 2, 3, and 4 of the peptide sequence are identical is compatible with the assumption that the cyclic analogue and dermorphin interact with the same receptor subsites. The different configurational requirements in the corresponding positions of β-casomorphin in conjunction with the observed opposite effects of Phe^3 N-methylation suggest that the mode of binding of β-casomorphins (morphiceptin) may be different from that of cyclic peptide **1** and of dermorphin.

The length of the side chain of the aromatic residue in position 3 of cyclic analogue **1** was shown to be important for receptor affinity and selectivity. This observation suggests that μ- and δ-receptors differ from one another in the relative spatial disposition of the binding sites for the Tyr^1 tyramine moiety and the Phe^3 aromatic ring.

It had previously been observed that reduction of the ring size in H-Tyr-D-Lys-Phe-Glu-NH_2 (15-membered ring), as achieved by preparation of cyclic analogue **1** (13-membered ring), resulted in lower receptor affinity but increased μ-receptor selectivity.[2] In continuation of this trend, further ring contraction, as obtained by synthesis of cyclic lactam analogue **10** (12-membered ring), produced an additional decrease in receptor affinity and a further increase in μ-receptor selectivity. Cyclic analogue **11** contains an even smaller, 11-membered ring structure and a relatively flexible disulfide bond instead of the side chain linking amide bond present in the cyclic lactam analogues. The lower receptor selectivity of the cystine-containing cyclic analogue relative to cyclic peptides **1**, **9**, and **10** may be due to its more flexible ring structure. This result indicates that both ring size and ring flexibility affect receptor affinity and selectivity.

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 (0.2 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. The cystine-con-

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Table III. 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
2	H-Tyr-Orn-Phe-Asp-NH_2	Tyr, 1.00; Orn, 0.88; Phe, 1.02; Asp, 1.01	0.32	0.68	13.0	539	539
3	H-Tyr-D-Orn-D-Phe-Asp-NH_2	Tyr, 0.88; Orn, 1.00; Phe, 1.02; Asp, 1.00	0.46	0.70	13.5	539	539
4	H-Tyr-D-Orn-Phe-D-Asp-NH_2	Tyr, 1.00; Orn, 1.06; Phe, 1.01; Asp, 0.93	0.41	0.70	13.5	539	539
5	H-Tyr-D-Orn-Phe(pNO_2)-Asp-NH_2	Tyr, 1.00; Orn, 1.10; Phe(pNO_2), 1.13; Asp, 0.94	0.46	0.74	11.0	584	584
	dimer	Tyr, 1.00; Orn, 0.97; Phe(pNO_2), 1.09; Asp, 0.98	0.40	0.71	18.0	1168	1168
6	H-Tyr-D-Orn-Phg-Asp-NH_2	Tyr, 1.00; Orn, 0.96; Phg, 1.02; Asp, 1.06	0.45	0.69	11.0	525	525
7	H-Tyr-D-Orn-Hfe-Asp-NH_2	Tyr, 1.00; Orn + Hfe overlap; Asp, 0.96	0.49	0.73	11.0	553	553
	dimer	Tyr, 1.00; Orn + Hfe overlap; Asp, 0.89	0.36	0.66	16.5	1106	1106
8	H-Tyr-D-Orn-Phe(NMe)-Asp-NH_2	Tyr, 0.98; Orn, 1.01; Phe(NMe), 0.97; Asp, 1.00	0.48	0.73	14.0	553	553
	dimer	Tyr, 0.97; Orn, 1.00; Phe(NMe), 0.97; Asp, 1.12	0.41	0.66	22.5	1106	1106
10	H-Tyr-D-Asp-Phe-A_2bu-NH_2	Tyr, 1.01; A_2bu, 0.95; Phe, 1.10; Asp, 1.00	0.48	0.71	13.5	525	525
	dimer	Tyr, 1.02; A_2bu, 0.95; Phe, 1.00; Asp, 1.00	0.36	0.71	19.0	1050	1050
11	H-Tyr-D-Cys-Phe-Cys-NH_2	Tyr, 0.88; Cys,[a] 1.87; Phe, 1.00	0.68	0.76	12.0	533	533
	dimer	Tyr, 1.00; Cys,[a] 1.88; Phe, 1.08	0.38	0.76	23.5	1065	1065

[a] Determined as cysteic acid.

taining cyclic analogue **11** was oxidized with H_2O_2/formic acid (9:1) prior to hydrolysis. 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 MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system (Drs. M. Evans and M. Bertrand, Department of Chemistry, University of Montreal).

Fmoc and Boc amino acid derivatives were purchased from IAF Biochem International, Laval, Quebec, Canada. The Fmoc derivatives of D-phenylalanine, *p*-nitrophenylalanine, phenylglycine, homophenylalanine, and N^{α}-methylphenylalanine were prepared by reaction with 9-fluoroenylmethyl chloroformate,[18] and their structures were confirmed by NMR spectroscopy, using a Varian EM-390 spectrometer. All peptides were prepared by the manual solid-phase technique using a *p*-methylbenzhydrylamine resin (1% cross-linked, 100–200 mesh, 0.4 mM/g of titratable amine) obtained from United States Biochemical Corp., Cleveland, OH.

Solid-Phase Synthesis and Purification of Cyclic Peptide Analogues. The cyclic lactam analogues **2–8** and **10** were synthesized according to a protection scheme described in detail elsewhere.[3] Subsequent to 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), the C-terminal tripeptide 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 4–24 h (completeness of the reaction was monitored with the ninhydrin test[19]); (3) Fmoc deprotection with 50% piperidine in CH_2Cl_2 (30 min); (4) washing with DMF (3 × 1 min) and EtOH (3 × 1 min). After coupling of the third Fmoc amino acid, Fmoc protection of the N-terminal amino group was retained and the side chains of the Orn (or A_2bu) and Asp 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 CH_2Cl_2 (2 × 10 min) and washing with CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min), cyclization was carried out in DMF at room temperature by addition of DCC (5 equiv) in the presence of HOBt (5 equiv). Monitoring of the ring-closure reaction with the ninhydrin test revealed that cyclization was usually complete after 1–6 days. In cases where the cyclization reaction took longer than 2 days, fresh DCC and HOBt were added every 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, Boc-Tyr(Boc)-OH (2.5 equiv) in CH_2Cl_2 and DCC (2.5 equiv) were added and the resin suspension was mixed for 24 h. After subsequent 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 × 1 min) and was dried in a desiccator. Peptides were cleaved from the resin and deprotected 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 diethyl ether and, subsequently, three times with 7% acetic acid. The crude peptide was then obtained in solid form through lyophilization of the acetic acid extract.

The linear precursor tetrapeptide of the cystine-containing cyclic analogue **11** was assembled on the same resin by using Boc amino acids according to a protocol described elsewhere.[20] The Boc and *p*-methylbenzyl groups were used for the side-chain protection of tyrosine and cysteine, respectively. The peptide was cleaved from the resin and deprotected by treatment with HF as described above. Following three washings with diethyl ether, the crude linear peptide was extracted from the resin with 7% acetic acid, and the resulting solution was diluted with H_2O to a concentration of approximately 0.3 mM. After adjustment of the pH to 8.0, oxidative disulfide bond formation was achieved by reaction with $K_3Fe(CN)_6$ (2.5-fold excess) for 40 min. Following adjustment of the pH to 5.0 with 50% acetic acid, anion-exchange resin (Amberlite 400, OH^- form) was added and the suspension was stirred for 20 min. After filtration, the absence of free sulfhydryl groups was established with the nitroprusside test. Subsequent volume reduction by evaporation and lyophilization yielded the crude product.

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,[21] with 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 the cyclic lactam analogues, a second major component corresponding to the side chain linked antiparallel cyclic dimer was identified in the crude peptide products. Since the HPLC elution times of the cyclic dimers on the reversed-phase column were longer than those of the corresponding cyclic monomers, separation was easily achieved. The elution profile of the crude product of H-Tyr-D-Cys-Phe-Cys-NH_2 (**11**) showed three major peaks. The first and second ones corresponded to the cyclic monomer and a cyclic dimer, respectively, and the third one represented a higher oligomer. Final products were obtained as lyophilisates. Homogeneity of the peptides was established by TLC and by HPLC under conditions identical with those de-

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scribed above. All peptides were at least 95% pure, as judged from the HPLC elution profiles. Analytical data are presented in Table III. The syntheses of peptides 1 and 9 have been reported elsewhere.[4]

Binding Assays and Bioassays. Receptor binding studies with rat brain membrane preparations were performed as reported in detail elsewhere.[22] [^{3}H]DAGO and [^{3}H]DSLET at respective concentrations of 0.72 and 0.78 nM were used as radioligands, and incubations were performed at 0 °C for 2 h. The calculation of the binding inhibition constants (K_i) was based on the equation by Cheng and Prusoff,[23] with values of 1.3 and 2.6 nM for the dissociation constants of [^{3}H]DAGO and [^{3}H]DSLET, respectively.[24,25]

The GPI[26] and MVD[27] bioassays were carried out as reported in detail elsewhere.[22,28] 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 compounds being tested were normalized according to a published procedure.[29] 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).[30]

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2-Phenylpyrroles as Conformationally Restricted Benzamide Analogues. A New Class of Potential Antipsychotics. 1

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2-Phenylpyrroles were synthesized as conformationally restricted analogues of the substituted benzamide sultopride and the butyrophenones haloperidol and fluanisone. Dopamine antagonistic activity is maintained if the 2-phenylpyrrole side chain is linked to the pharmacophoric *N*-ethylpyrrolidine moiety of sultopride or to the 4-substituted piperazine moiety of fluanisone but is lost if the 2-phenylpyrrole is combined with the 4-substituted piperidine moiety of haloperidol. The 2-phenylpyrrole analogue **1** of sultopride is in vitro 0.25 and in vivo 3 times as potent as the parent compound. Its binding to the dopamine D-2 receptors is, in analogy to the substituted benzamides, strongly sodium-dependent. The 2-(4-fluorophenyl)pyrrole analogue **5** of fluanisone is superior in vitro as well as in vivo to the corresponding benzamide **7** and the butyrophenone fluanisone. The increase in activity is not only due to a higher affinity for the D-2 receptors but also to an enhanced oral absorption (ratio po/ip = 4.5 vs 40 for the benzamide and 60 for fluanisone). Compound **5** is further characterized by a high selectivity for the D-2 receptors, in contrast to the benzamide and butyrophenone analogues (ratio D-2/α_1 = 60, 2.0, and 0.3, respectively). The binding to the D-2 receptors has little dependence on sodium. The 2-phenylpyrrole **5** shares with the benzamide **7** a low potential to induce catalepsy, which is in contrast to haloperidol. So, 5-(4-fluorophenyl)-2-[[4-(2-methoxyphenyl)-1-piperazinyl]methyl]pyrrole (**5**) is the prototype of a new class of sodium-independent dopamine D-2 antagonists, which may be particularly useful as potential antipsychotics with a low propensity to induce acute extrapyramidal side effects.

The substituted benzamides have attracted considerable interest as potential antipsychotics with a lower propensity to induce extrapyramidal side effects (EPS) than the classical neuroleptics like haloperidol (I). The "atypical" neuroleptic profile is characterized by a large separation between the doses inhibiting apomorphine-induced behavior patterns (index for antipsychotic activity) and the doses inducing catalepsy (index for acute extrapyramidal side effects).[1] For sulpiride (IIa), the prototype of the substituted benzamides, the atypical neuroleptic profile could be confirmed in humans.[2] Sulpiride, however, is a rather weak antipsychotic drug[3] due to its low bioavailability [3a] and poor penetration into the brain.[3b]

Since the discovery of sulpiride, more lipophilic (sultopride, IIb)[3d] and highly potent [eticlopride (IIc)[4a] and ra-

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