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Synthesis and Opioid Activity Profiles of Cyclic Dynorphin Analogs

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In an effort to reduce the conformational flexibility of the κ-receptor selective opioid peptide dynorphin A, we synthesized the cyclic analogs [D-Orn^{2}, Asp^{5}]dynorphin$_{A}$-(1-8) (1), [Orn^{5}, Asp^{8}]-dynorphin A-(1-13) (2), [Orn^{5}, Asp^{10}]dynorphin A-(1-13) (3) and [Orn^{5}, Asp^{13}]dynorphin A-(1-13). These side chain-to-side chain cyclized lactam analogs were prepared by the solid-phase method using a protection scheme which permitted cyclization of the peptide still attached to the solid support. In all four cases the desired cyclic analogs were obtained in reasonable yield. Aside from the cyclic monomers, the crude products also contained the side-chain-linked antiparallel cyclic dimer and higher oligomers which had been formed through intersite reaction on the resin. The obtained results demonstrate that cyclic lactam analogs containing relatively large ring structures (up to 31-membered) can be prepared by this method. In the guinea pig ileum assay analog 1 was 45 times more potent than linear dynorphin A-(1-8), whereas analogs 2-4 were 460 - 1,350 times less potent than linear dynorphin A-(1-13). All four analogs showed $\bar{K}_{e}$-values for naloxone as antagonist below 5 nM in the latter assay, indicating that they no longer interact significantly with κ-receptors. In the opioid receptor binding assays cyclic analog 1 displayed extraordinarily high affinity for the μ-receptor and the determined ratios of the binding inhibition constants ($K_{i}^{\delta}/K_{i}^{\mu}$) indicated that all four analogs are μ-receptor selective. It is concluded that the performed cyclizations resulted in overall folded conformations which are incompatible with the conformational requirements of the κ-receptor.

In 1975 Hughes and Kosterlitz described the isolation and characterization of the enkephalins (H-Tyr-Gly-Gly-Phe-Met(or Leu)-OH), the first endogenous opioid receptor ligands to be discovered[2]. Subsequent research revealed that all mammalian opioid peptides known to date are derived from three precursor proteins: proopiomelanocortin, proenkephalin A and proenkephalin B (for a review, see ref.[3]). Furthermore, the results of extensive pharmacologic characterizations of both opioid peptides and opiates indicated the existence of several different classes of opioid receptors (μ, δ, κ, etc.) which differ from one another in their structural requirements (see ref.[3]). Unfortunately, none of the endogenous opioid peptides is very selective for a particular receptor class. Thus, metorphamide, the enkephalins and the dynorphins/neoendorphins are only moderately selective for the μ, δ- and κ-receptor, respectively. Both opioid peptides and classical opiates display a large spectrum of biological activities, including analgesia, respiratory depression, tolerance and physical dependence, euphoria, hypothermia, effects on gut motility, cardiovascular effects, etc. In order to associate a specific receptor class with a distinct biological function, it is of great importance to develop opioid receptor ligands with high selectivity for a particular receptor type. It has been attempted to reach this goal through the synthesis of numerous opioid peptide analogs. In these efforts the classical approach based on amino acid substitutions, additions or deletions represented the most extensively used design principle which led to a few compounds which indeed showed improved receptor selectivity. For

example, H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol (DAGO)[4] and H-Tyr-D-Ser(or Thr)-Gly-Phe-Leu-Thr-OH (DSLET or DTLET)[5] showed considerably improved selectivity for the μ- and the δ-receptor, respectively. The lack of selectivity of the natural opioid peptides and of many of their linear analogs is most likely due to their relatively high structural flexibility which permits conformational adaptation to several different receptor topographies. It has therefore recently been attempted to enhance receptor selectivity through incorporation of conformational constraints into opioid peptides. An effective way of restricting the overall conformation of a peptide is cyclization. Side chain-to-end group or side chain-to-side chain cyclizations of enkephalins via side chains of appropriately substituted residues have resulted in cyclic analogs with greatly improved receptor selectivity. Prototypes of this type of analog are H-Tyr-D-A_2bu-Gly-Phe-Leu[6] (μ-selective), H-Tyr-D-Orn-Phe-Asp-NH_2[7] (μ-selective) and H-Tyr-D-Pen-Gly-Phe-D(or L)-Pen-OH[8] (δ-selective).

The opioid peptide dynorphin A-(1-17) (H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH) preferentially interacts with the κ-receptor and shows somewhat reduced, but still considerable, affinity for the μ- and the δ-receptor (for a review see ref.[9]). The pharmacologic characterization of fragments of dynorphin A-(1-17) indicated that dynorphin A-(1-13) retains the high potency and moderate κ-receptor selectivity of the 17-peptide, whereas dynorphin A-(1-8) shows somewhat reduced but still significant preference for κ-receptors. Structure-activity studies with dynorphin A peptides have been relatively scarce to date and have not resulted in analogs with significantly improved κ-receptor selectivity. In the present paper we describe the synthesis and opioid activity profiles of several conformationally restricted dynorphin A analogs containing cyclic structures either in the N-terminal enkephalin segment or in the middle and C-terminal region of the molecule (Fig. 1). For comparative purposes the cyclic enkephalin analog 1a was prepared as well.

These cyclic lactam analogs of dynorphin were obtained through amide bond formation between the side chain groups of appropriately substituted Orn and Asp residues. The substitution sites were chosen based on the observation that amino acid replacements in positions 5, 8, 10 and 13 do not greatly affect the activity profile of linear dynorphin peptides[9-12]. Substitution of D-Ala in dynorphin A-(1-13) had produced a loss of κ-receptor selectivity[13]. However, this may not

(1) H-Tyr-D-Orn-Gly-Phe-Asp-Arg-Arg-Ile-NH_2

(1a) H-Tyr-D-Orn-Gly-Phe-Asp-NH_2

(2) H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Asp-Arg-Pro-Lys-Leu-Lys-NH_2

(3) H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Ile-Arg-Asp-Lys-Leu-Lys-NH_2

(4) H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Asp-NH_2

Fig. 1. Structural formulas of cyclic analogs of dynorphin A-(1-8) (1), enkephalin (1a) and dynorphin A-(1-13) (2, 3 and 4).

necessarily also be the case if a D-amino acid residue with a relatively long side chain contained in a ring structure is present in the 2-position, such as it is the case with cyclic analog 1.

In the present paper we describe the preparation of cyclic analogs 1-4 by the solid-phase method, using the p-methylbenzhydrylamine resin. The C- and N-terminal exocyclic segments can be assembled according to the conventional Boc protection scheme. The peptide segment to be cyclized is built up using the base-labile Fmoc group for α-amino group protection and the Boc and tert-butyl group for the protection of the Orn and Asp side chains engaged in ring formation. Removal of the latter groups by TFA treatment then permits cyclization of the peptide still attached to the resin, using DCC/HOBt as coupling agents. After completion of the cyclization step, the N-terminal Fmoc group is removed in the usual manner by treatment with piperidine and the peptide chain can then be further extended at the N-terminus. The side-chains of the Lys and Arg residues are protected with the 2-chlorobenzyloxycarbonyl and tosyl group, respectively, which are resistant to TFA treatment. After completion of the entire peptide chain the peptide is cleaved from the resin and completely deprotected by treatment with HF in the usual manner. Further details related to this simple scheme for the preparation of cyclic lactam analogs have been reported elsewhere[14]. It should be pointed out that, aside from the desired cyclic peptide monomer, cyclization on the resin also may produce the side-chain-linked antiparallel cyclic dimer due to intersite reaction[14]. So far this method has permitted the synthesis of cyclic lactam analogs containing one or two amino acid residues between the Orn(Lys) and Asp(Glu) residues engaged in ring formation[14,15]. In the present paper we describe for the first time the use of this method for the preparation of cyclic peptides containing larger ring systems (4 and 7 amino acid residues between the ring-forming Orn and Asp residues (analogs 3 and 4)).

For the determination of their in vitro opioid activities the analogs were tested in bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and of the mouse vas deferens (MVD). In the GPI preparation opioid effects are mediated by both μ- and κ-receptors. κ-Receptor interactions in the GPI are characterized by relatively high K_e-values for naloxone as antagonist (> 10 nM)[16], in contrast to the low values (1-2 nM)[17] observed with μ-receptor ligands. The MVD assay is usually taken as being representative for δ-receptor interactions, even though μ- and κ-receptors are also present in this preparation. Opioid receptor binding 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.

RESULTS AND DISCUSSION

The assembly of the peptide chains according to the scheme outlined above did not cause any major problems. Cyclization could be brought to completion within four to eight days. After HF cleavage peptides were purified by gel filtration on Sephadex G-25 and by reversed-phase chromatography. In the case of both analog 1 and analog 1a the crude reaction product contained two peptide components

which could easily be separated by reversed-phase chromatography. Both components showed the expected amino acid composition. Analysis by FAB mass spectrometry revealed that in both cases the faster moving component was the desired cyclic monomer, whereas the slower moving product represented the side chain-linked antiparallel cyclic dimer, which was generated as a result of intersite reaction in the cyclization step. In the case of analog 1, 86% of the peptide chains had formed the cyclic monomer and 14% had undergone cyclodimerization. Cyclic monomer formation was also predominant in the case of peptide 1a (76% monomer). The results confirm the rule that monomer formation is favored over cyclodimerization if the side chains to be cyclized are separated by two amino acid residues, whereas with only one residue in between dimer formation is in general predominant. It had previously been established that the extent of cyclodimerization does not depend on the level of resin substitution but rather is governed by conformational factors[15]. From the crude reaction products of analogs 2, 3 and 4 the cyclic monomer was isolated in each case as the fastest moving component in the reversed-phase chromatography purification step and its structure was confirmed by amino acid analysis and FAB mass spectrometry. A slower moving broad peak, presumably containing the dimer and higher oligomers, was obtained in each case and not characterized any further. The dimer/oligomer peak was particularly pronouced in the case of analogs 2 and 3 but relatively minor in the case of peptide 4.

These results indicate that the use of the solid-phase synthesis scheme originally proposed by us[14] permits the preparation of cyclic lactam analogs containing relatively large ring structures (22- and 31-membered in the case of peptides 3 and 4, respectively). Furthermore, this method allows for the relatively easy preparation of side chain-to-side chain cyclized lactam type analogs with linear extensions at the N- and/or the C-terminus.

In the GPI assay cyclic dynorphin analog 1 was found to be 84 times more potent than [Leu^5]enkephalin and 45 times more potent than its parent peptide, dynorphin A-(1-8) (Table 1). Compound 1 is relatively less potent in the MVD assay and, therefore shows moderate preference for μ-receptors over δ-receptors. Both in the GPI and in the MVD assay analog 1 was slightly more potent than the corresponding cyclic enkephalin analog (1a) which lacks the exocyclic C-terminal tripeptide segment of 1. Analogs 1 and 1a showed K_e-values for naloxone as antagonist of 1.49 and 1.98 nM, respectively, which are typical for μ-receptor interactions. On the other hand, the linear dynorphins, dynorphin A-(1-8) and dynorphin A-(1-13), have K_e-values above 10 nM, indicating an interaction with κ-receptors. It is thus clear that cyclic dynorphin 1 no longer shows κ-receptor selectivity but μ-receptor selectivity instead. A loss of κ-receptor characteristics had previously been observed with linear dynorphin analogs containing a D-amino acid residue in the 2-position[13] as well as with the cystine-containing cyclic analog [D-Cys^2, Cys^5]dynorphin A-(1-13)[18]. According to a recently proposed model[19], dynorphin A binds to the κ-receptor with its enkephalin segment ("message sequence") assuming an α-helical conformation. Obviously, the ring structure contained in cyclic dynorphin analog 1 would prevent formation of an α-helical structure of its enkephalin segment. The loss of κ-receptor selectivity observed with 1 is therefore in agreement with this model.

Table 1. Guinea pig ileum (GPI) and mouse vas deferens (MVD) assay of cyclic dynorphin analogs[a]

No.	Compound	GPI IC50 [nM]	GPI K_e (nal.[nM])[b]	MVD IC50 [nM]	MVD/GPI IC50-ratio
1	H-Tyr-D-Orn-Gly-Phe-Asp-Arg-Arg-Ile-NH_2	2.93 ± 0.57	1.49 ± 0.17	8.84 ± 1.85	3.02
1a	H-Tyr-D-Orn-Gly-Phe-Asp-NH_2	4.33 ± 0.35	1.98 ± 0.50	5.45 ± 0.43	1.26
2	H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Asp-Arg-Pro-Lys-Leu-Lys-NH_2	1,970 ± 490	1.84 ± 0.26	2,590 ± 360	1.31
3	H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Ile-Arg-Asp-Lys-Leu-Lys-NH_2	667 ± 63	4.13 ± 0.56	35,100 ± 5,400	52.6
4	H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Asp-NH_2	687 ± 73	1.72 ± 0.15	18,700 ± 3,300	27.2
5	Dynorphin A-(1-8)	131 ± 19	13.4 ± 2.6	13.4 ± 3.4	0.102
6	Dynorphin A-(1-13)	1.46 ± 0.41	29.2 ± 9.2	12.7 ± 5.6	8.70
7	[Leu^5]enkephalin	246 ± 39	1.53 ± 0.43	11.4 ± 1.1	0.0463

[a] Mean of three determinations ± SEM. [b] K_e-values determined with naloxone as antagonist.

Table 2. Binding assays of cyclic dynorphin analogs

No.	Compound	[^{3}H]DAGO displacement K_i^{μ} [nM][a]	[^{3}H]DSLET displacement K_i^{δ} [nM][a]	K_i^{δ}/K_i^{μ}
1	H-Tyr-D-Orn-Gly-Phe-Asp-Arg-Arg-Ile-NH_2	0.0555 ± 0.0036	0.893 ± 0.196	16.1
1a	H-Tyr-D-Orn-Gly-Phe-Asp-NH_2	0.762 ± 0.045	2.71 ± 0.06	3.56
2	H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Asp-Arg-Pro-Lys-Leu-Lys-NH_2	6.84 ± 1.16	253 ± 51	37.0
3	H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Ile-Arg-Asp-Lys-Leu-Lys-NH_2	38.6 ± 1.3	323 ± 34	8.37
4	H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Asp-NH_2	3.39 ± 2.03	163 ± 11	48.1
5	Dynorphin A-(1-8)	1.63 ± 0.25	4.51 ± 0.61	2.77
6	Dynorphin A-(1-13)	3.95 ± 0.36	3.74 ± 0.40	0.947
7	[Leu^5]enkephalin	9.43 ± 2.07	2.53 ± 0.35	0.268

[a] Mean of three determinations ± SEM.

In the μ-receptor representative binding assay ([^{3}H]DAGO displacement) cyclic dynorphin analog 1 showed 170 times higher affinity than [Leu^5]enkephalin, in good agreement with its high potency in the GPI bioassay (Table 2). It should also be noted that 1 has 29 and 71 times higher μ-receptor affinity than dynorphin A-(1-8) and dynorphin A-(1-13), respectively. Since its δ-receptor affinity ([^{3}H]DSLET displacement) is comparable to that of [Leu^5]enkephalin, compound 1 shows a binding inhibition constant ratio (K_i^δ/K_i^μ) of 16 which again indicates its μ-receptor selectivity. The cyclic enkephalin analog 1a displayed about 14 times lower μ-receptor affinity than 1. The extremely high μ-receptor affinity of 1, as compared to 1a, may be due to the two additional positive charges on the arginine residues in positions 6 and 7. The high positive chage (+ 3) of cyclic dynorphin analog 1 may promote its accumulation in the anionic fixed charge membrane compartment which, according to Schwyzer's membrane compartment concept[19], contains primarily μ-binding sites. In addition, the cyclic segment of analog 1 fulfils the conformational requirements at the μ-receptor. Thus, its very high μ-receptor affinity may be due to both its net positive charge and the μ-receptor compatible conformation of its enkephalin segment.

It is of interest to point out that in the case of cyclic dynorphin analog 1 there is good agreement between the μ-receptor affinity observed in the binding assay and the potency determined in the μ-receptor-representative GPI bioassay, in contrast to cyclic enkephalin analog 1a which showed much higher potency on the ileum than was expected on the basis of its μ-receptor affinity. As a tentative explanation of this discrepancy it can be assumed that cyclic peptide 1a has an increased "efficacy" ("intrinsic activity") at the μ-receptor due to more efficient signal transduction. The same phenomenon had been observed with other cyclic enkephalin analogs[15].

Cyclic dynorphin A analogs 2, 3 and 4 contain ring structures in the middle and C-terminal region of the peptide chain. In the GPI assay these three compounds were found to be 3 to 8 times less potent than [Leu^5]enkephalin and 460 - 1,350 times less potent than dynorphin A-(1-13) (Table 1). The low K_e-values for naloxone as antagonist obtained with analogs 2, 3 and 4 in this preparation indicate that they also no longer significantly interact with κ-receptors. In the MVD assay all three analogs were about three orders of magnitude less potent than [Leu^5]enkephalin and the linear dynorphins (5 and 6). In the binding assays cyclic analogs 2, 3 and 4 showed μ-receptor affinities comparable to those of [Leu^5]enkephalin and dynorphin A-(1-13) but weak affinities for the δ-receptor (Table 2). The bioassay and binding assay data are thus in good agreement and it is clear that cyclic analogs 2, 3 and 4 show considerable μ-receptor selectivity, as indicated by their relatively high K_i^δ/K_i^μ-ratios. The lack of κ-receptor binding observed with these three analogs could be due to the fact that the performed cyclizations may result in overall folded conformations which are no longer compatible with the conformational requirements of the κ-receptor. In this context it is of interest to note that fluorescence energy transfer distance measurements performed with dynorphin A-(1-17) in aqueous solution had ruled out a close proximity between the N- and C-terminal segments of the peptide[20].

In conclusion, we have demonstrated that cyclic lactam type analogs containing relatively large

ring structures can be synthesized by solid-phase techniques, using a relatively simple protection scheme[14]. All four cyclic dynorphin A analogs prepared no longer showed the κ-receptor preference typical for native dynorphins, but rather turned out to be μ-receptor selective. Other conformational constraints will have to be built into dynorphin in order to obtain analogs with retained or improved κ-receptor selectivity and, thereby, insight into the bioactive conformation of dynorphin at the κ-receptor.

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 Varian VISTA 5500 liquid chromatograph, utilizing a Waters column (30 x 0.78 cm) packed with C-18 Bondapak reversed-phase (10 μm) material. For amino acid analyses, peptides (0.2 mg) were hydrolyzed in 6N 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 121 C 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, Dept. of Chemistry, University of Montreal).

Fmoc and Boc amino acid derivatives were purchased from IAF Biochem International, Laval, Que., and the following side chain protecting groups were used: tosyl (Arg), 2-chlorobenzyloxycarbonyl (Lys) and Boc (Tyr). The side chains of the Orn and Asp residues to be cyclized were protected with the Boc and tert-butyl group, respectively. All peptides were prepared by the manual solid phase technique, using a p-methylbenzhydrylamine resin (1% cross-linked, 100-200 mesh, 0.38 meq/g titratable amine) also obtained from IAF Biochem International.

Solid-Phase Synthesis and Purification of Cyclic Dynorphin Analogs

The exocyclic C-terminal and N-terminal peptide segments were assembled using Boc amino acids according to a protocol described elsewhere[21]. The cyclic peptide segments were synthesized according to a protection scheme based on the use of Fmoc amino acids[14] by performing the following steps in each cycle: (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[22]); (3) Fmoc deprotection with 50% piperidine in CH_2Cl_2 (1 x 30 min); (4) washing with DMF (3 x 1 min), and EtOH (3 x 1 min). After coupling of the last Fmoc amino acid (Fmoc-D(or L)-Orn(Boc)-OH), Fmoc protection of the N-terminal amino group was retained and the side chains of the Orn and Asp residues to be linked were deprotected by treatment with 50% (v/v) TFA in CH_2Cl_2 (1 x 30 min). Following neutralization with 10% (v/v) DIEA in CH_2Cl_2 (2 x 10 min) and washing with CH_2Cl_2 (3 x 1 min) and DMF (3 x 1 min), cyclization was carried out in DMF at room temperature by addition of DCC (5 equiv.) in the presence of HOBt (5 equiv.). Fresh DCC and HOBt were added every 48 h. Monitoring of the ring closure reaction with the ninhydrin test revealed that cyclization was usually complete after 4-8 days. 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 assembly of the entire peptide chain the resin was washed with CH_2Cl_2 (3 x 1 min) and EtOH (3 x 1 min), and was dried in a dessicator. 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 HF plus 1 ml anisole per gram 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.

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[23], using a linear gradient of 0-80% MeOH in 1% TFA. If necessary, further purification to homogeneity was performed by semipreparative reversed-phase HPLC (20-60% MeOH (linear gradient) in 0.1% TFA). Aside from the desired cyclic monomers, the crude reaction products also contained components which corresponded to the side-chain-linked antiparallel cyclic dimer and to higher oligomers which had formed through intersite reaction on the resin. Since the HPLC elution times of the cyclic monomers on the reversed-phase column were considerably shorter than those of the corresponding cyclic dimers and higher oligomers, separation was easily achieved. In the case of cyclic dynorphin A-(1-8) analog 1 and cyclic enkephalin analog 1a, the cyclic dimers were isolated and identified by amino acid analysis and FAB mass spectrometry (MH^+ = 2,043 and 1,192, respectively). Cyclic monomer formation was favored over cyclodimerization in the case of both analog 1 and analog 1a (86% and 76% cyclic monomer, respectively). The cyclic monomer was a minor component in the crude reaction products of 2 and 3 and the major component in the reaction product of 4. 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 95% pure, as judged from the HPLC elution profiles.

H-Tyr-D-Orn-Gly-Phe-Asp-Arg-Arg-Ile-NH_2 (1). TLC Rf 0.16 (BAW), 0.57 (BPAW). Amino acid analysis: Asp 0.97 (1), Gly 0.95 (1), Ile 1.00 (1), Tyr 0.97 (1), Phe 1.04 (1), Orn 1.18 (1), Arg 2.03 (2). FAB mass spectrum: MH^+ calc., 1,022; found 1,022.

H-Tyr-D-Orn-Gly-Phe-Asp-NH_2 (1a). TLC Rf 0.37 (BAW), 0.68 (BPAW). Amino acid analysis: Asp 0.92 (1), Gly 1.00 (1), Tyr 0.98 (1), Phe 0.98 (1), Orn 1.08 (1). FAB mass psectrum: MH^+ calc., 596; found 596.

H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Asp-Arg-Pro-Lys-Leu-Lys-NH_2 (2). TLC Rf 0.00 (BAW), 0.43 (BPAW). Amino acid analysis: Asp 1.00 (1), Pro 1.05 (1), Gly 1.77 (2), Leu 1.14 (1), Tyr 0.95 (1), Phe 0.83 (1), Orn + Lys 3.21 (3), Arg 3.00 (3). FAB mass spectrum: MH^+ calc., 1,589; found 1,589.

H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Ile-Arg-Asp-Lys-Leu-Lys-NH_2 (3). TLC Rf 0.02 (BAW), 0.48 (BPAW). Amino acid analysis: Asp 0.97 (1), Gly 2.00 (2), Ile 0.93 (1), Leu 1.11 (1), Tyr 0.95 (1), Phe 1.03 (1), Orn + Lys 3.20 (3), Arg 3.08 (3). FAB mass spectrum: MH^+ calc., 1,605; found, 1,605.

H-Tyr-Gly-Gly-Phe-Orn-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Asp-NH_2 (4). TLC Rf 0.05 (BAW), 0.51 (BPAW). Amino acid analysis: Asp 1.09 (1), Pro 0.89 (1), Gly 1.90 (2), Ile 0.98 (1), Leu 1.10 (1), Tyr 0.88 (1), Phe 0.99 (1), Orn + Lys 2.09 (2), Arg 3.00 (3). FAB mass spectrum: MH^+ calc., 1,574; found 1,574.

Dynorphin A-(1-13) was synthesized as previously reported[24] and dynorphin A-(1-8) was purchased from IAF Biochem International.

Bioassays and Binding Assays. The GPI[25] and MVD[26] bioassays were carried out as reported in detail elsewhere[27,28]. A log dose-response curve was determined with [Leu^5]enkephalin as standard for each ileum or vas preparation, and IC50 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 IC50 values obtained in the presence and absence of a fixed naloxone concentration[30].

Receptor binding studies with rat brain membrane preparations were performed as reported in detail elsewhere[27]. [^{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 of Cheng and Prusoff[31], using values of 1.3 and 2.6 nM for the dissociation constants of [^{3}H]DAGO and [^{3}H]DSLET, respectively[4,32].

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