Synthesis and Structure-Activity Relationships of Deltorphin Analogues

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In order to study the structure-activity relationships of natural opioid deltorphins (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 and H-Tyr-D-Ala-Phe-Asp[or Glu]-Val-Val-Gly-NH_2), 15 analogues were synthesized by the solution method. Their activities were determined in binding studies based on displacement of μ- and δ-receptor selective radiolabels from rat brain membranes and in two bioassays, using guinea pig ileum and mouse vas deferens. The obtained data indicate that the high δ-selectivity of deltorphins can be due to the constitution/conformation of the C-terminal part and, at least in part, to preselection by charge.

The search for opioid receptor ligands with a high affinity and selectivity toward the different receptor sites (μ, δ, κ, etc.) is believed to be of key importance for elucidation of the functions of opioid receptor types and potential subtypes.

The recent discovery of deltorphin,[1,2] also named dermenkephalin[3] or dermorphin gene-associated peptide,[4] a new natural δ-peptide opioid, may furnish important clues for a better understanding of the differences between δ- and μ-opioid receptors. Deltorphin (DEL) is a heptapeptide of sequence H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, present in the skin of *Phyllomedusa sauvagei*, a South American tree frog.[2] The heptapeptide sequence was first detected[1] in one of the clones that codes the polypeptide precursor of dermorphin (DER, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), a well known μ-opioid peptide,[5,6] characterized by the unusual presence of D-Ala in the second position.[7] The presence of the same processing signals flanking the sequences of dermorphin and of the novel heptapeptide in the precursor polypeptide suggested[1] that it could also be produced in vivo. The synthesis of two analogues, containing either L-Met[2] or D-Met[2], did prove that, similarly to DER, the new natural peptide, present in the frog's skin, contains D-Met[2], but contrary to DER, this new opioid has an activity prevalently δ instead of μ.[2,4,8]

More recently[9] two other deltorphins (H-Tyr-D-Ala-Phe-Asp[or Glu]-Val-Val-Gly-NH_2, henceforth called DELI and DELII), isolated from skin extracts of *Phyllomedusa bicolor*, showed an even higher affinity for δ-sites than DEL.

Indeed, comparison with the synthetic peptides normally used for δ-selectivity tests proved that deltorphins exhibit an affinity and a selectivity for the δ-receptors[2,4,5,9] which are at least equal to those of the prototypical δ-probes DPDPE, DSTBULET, and BUBU.[10-12] The high δ-selectivity of deltorphins may furnish crucial information on the features that impart δ- or μ-specificity to opioid peptides, also in comparison with DER, which has a high μ-selectivity, in spite of the same N-terminal sequence Tyr-D-Xaa-Phe. This statement is particularly stressed by our ^{1}H NMR investigations of deltorphins.[13-15] In fact this study showed that the conformational preferences of DER and deltorphins are, at least in the N-terminal part, similar, suggesting that the different selectivity toward opioid receptors could be due to different charge present in the heptapeptides and/or to the constitution of the C-terminal sequence. The importance of charges in orienting the specificity of opioid peptides has already been pointed out by the theory on the catalytic role of membranes for peptide-receptor interactions.[16] This model predicts that the receptor selectivity of opioid peptides is governed by their net charge and/or amphiphilic moment in addition to their ability to fulfill the structural and conformational requirements of a particular receptor site. Thus, opioid peptides carrying a net positive charge would be accumulated in the vicinity of the μ-receptor and, therefore, would show μ-receptor preference, whereas neutral and negatively charged peptides would preferentially interact with the δ-site.

To test the validity of the catalytic role of membranes, we designed, and synthesized a series of deltorphin analogues containing a different charged side chain in the 4- and/or 7-positions (Table I).

The importance of preselection by the membrane has been strongly emphasized by a recent investigation on DER(1-4) tetrapeptide analogues.[17]

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Table I. Analytical Data of Deltorphin Peptides[a]

no.	compd	$[\alpha]_D^{20}$ [b]	TLC, R_f A	TLC, R_f B	FAB-MS calcd	FAB-MS found
1	H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	−9.5	0.45	0.38	956	956
2	H----------OH	−6.3	0.41	0.30	957	957
3	H----------Asn-NH_2	−8.2	0.57	0.40	955	955
4	H----------Asn-OH	−14.3	0.58	0.39	956	956
5	H----------Abu-NH_2	−17.2	0.51	0.53	941	941
6	H----------Asp--------Asp-OH	−10.5	0.39	0.28	935	935
7	H----------Gly----------NH_2	−5.2	0.52	0.41	876	876
8	H----------Phe----------NH_2	−17.7	0.50	0.43	966	966
9	H----------Tyr----------NH_2	−6.3	0.51	0.42	982	982
10	H----------Lys----------NH_2	−3.7	0.53	0.44	947	947
11	H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	−16.0	0.50	0.41	770	770
12	H----------NH_2	−2.5	0.71	0.43	713	713
13	H----------NH_2	−7.9	0.49	0.44	613	613
14	H----------NH_2	+15.2	0.67	0.52	514	514
15	H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	−15.2	0.52	0.45	784	784
16	H----------OH	−8.1	0.50	0.41	785	785
17	H----------Gln----------NH_2	−4.0	0.54	0.36	783	783
18	H----------Gly--------Asp-NH_2	−16.2	0.51	0.40	870	870

[a] All the peptides showed correct amino acid analysis. [b] *c* 1.0 in methanol, values in degrees. [c] Thin-layer chromatography in solvent system A and B.

Table II. Binding Assays of Opioid Peptide Analogues

no.	compd	IC_{50}, nM [^{3}H]DAGO(μ)[a]	IC_{50}, nM [^{3}H]DSLET(δ)[a]	IC_{50}, nM [^{3}H]DPDPE(δ)[b]	[^{3}H]DAGO/[^{3}H]DSLET IC_{50} ratio
1	H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	1200 ± 50	9.0 ± 1.0	6.1 ± 0.9	135
2	H----------OH	1500 ± 70	4.5 ± 0.5	2.4 ± 0.5	333
3	H----------Asn-NH_2	380 ± 40	20 ± 1.8	18 ± 2.5	19
4	H----------Asn-OH	660 ± 80	22 ● 3.1	16 ± 2.1	30
5	H----------Abu-NH_2	360 ± 12	18 ± 0.9	7.4 ± 1.5	20
6	H----------Asp--------Asp-OH	>30000	80 ● 10	55 ± 7.3	>375
7	H----------Gly----------NH_2	161 ± 7	14 ± 2.1	8.0 ± 2.3	11.5
8	H----------Phe----------NH_2	450 ± 15	15 ± 2.6	11 ± 3.4	30
9	H----------Tyr----------NH_2	158 ± 8	9.1 ± 1.3	5.9 ± 0.8	17
10	H----------Lys----------NH_2	6000 ± 512	550 ● 48	380 ± 22	11
11	H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	910 ± 95	1.5 ± 0.2	1.1 ± 0.1	607
12	H----------NH_2	700 ± 60	3.9 ± 0.1		179
13	H----------NH_2	640 ± 70	99 ± 11		6.5
14	H----------NH_2	100 ± 6	960 ± 110		0.1
15	H-Try-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	3200 ± 200	3.8 ± 0.4	3.6 ± 0.8	842
16	H----------OH	14500 ± 1300	184 ± 16		78
17	H----------Gln----------NH_2	280 ± 16	3.8 ± 0.5		73
18	H----------Gly--------Asp-NH_2	130 ± 6	29.5 ± 5.2		4.5
dermorphin		3.2 ± 0.2	294 ± 20	320 ± 25	0.01
DPDPE		1300 ± 60	5.8 ± 0.6		224
morphine		3.9 ± 0.7	205 ± 30		0.02

[a] Mean of six determinations ± SEM. [b] Mean of three determinations ± SEM.

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

no.	compd	IC_{50}, nM GPI	IC_{50}, nM MVD	GPI/MVD IC_{50} ratio
1	H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	1900 ± 250	1.30 ± 0.27	1461
2	H----------OH	6000 ± 1580	1.40 ± 0.31	4900
3	H----------Asn-NH_2	245 ± 28	8.00 ± 1.81	30
4	H----------Asn-OH	350 ± 41	9.00 ± 1.65	39
5	H----------Abu-NH_2	300 ± 37	1.50 ± 0.50	200
6	H----------Asp--------Asp-OH	>15000	380 ± 40	>40
7	H----------Gly----------NH_2	100 ± 14	1.65 ± 0.40	62
8	H----------Phe----------NH_2	75 ± 9.5	1.60 ± 0.34	47
9	H----------Tyr----------NH_2	100 ± 16	1.02 ± 0.19	100
10	H----------Lys----------Nh_2	7500 ± 1700	1000 ± 290	7.5
11	H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	420 ± 95	0.14 ± 0.06	3000
12	H----------NH_2	320 ± 61	7.50 ± 1.90	43
13	H----------NH_2	650 ± 118	220 ± 25	3
14	H----------NH_2	160 ± 28	1000 ± 120	0.1
15	H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	5200 ± 1430	0.75 ± 0.10	6933
16	H----------OH	9000 ± 2100	5.55 ± 1.69	1620
17	H----------Gln----------NH_2	280 ± 64	1.51 ± 0.40	185
18	H----------Gly--------Asp-NH_2	130 ± 28	11.1 ± 2.60	12
dermorphin		1.41 ± 0.16	19.3 ± 3.4	0.07
DPDPE		5500 ± 1400	2.65 ± 0.41	2075
morphine		38 ± 8.4		

[a] Mean of five determination ± SEM.

The biological and receptor binding properties of DEL-related peptides are compared with those of deltorphins, DER, and other opioids (Tables II and III).

Synthesis

The heptapeptide analogues listed in Table I were synthesized by the classical solution method by combination of stepwise elongation with fragment condensation by procedures similar to those described and discussed previously for DEL, DELI, and DELII.[15] Whereas hexa- and pentapeptides **12** and **13** were prepared by fragment condensation 3 + 3 and 3 + 2, the shorter tetrapeptide **14** was synthesized by stepwise elongation. Mixed anhydride, active esters, DCC/HOBt, and azide were employed for the coupling method. α-Amino functions were protected by Z, Boc, or Fmoc groups. Side-chain protecting groups were as follows: Asp and Glu, OBu^t; Lys, Boc; Tyr and His were unprotected. The different protecting groups were removed as indicated in the Experimental Section. Purification was achieved by high-performance liquid chromatography (HPLC). Homogeneity of the purified product was accessed by thin-layer chromatography (TLC) and analytical HPLC. Structure identification was achieved by amino acid analysis and fast atom bombardment mass spectroscopy (FAB).

Biological Activity and Binding Property

The peptides listed in Table I were tested in vitro in two isolated organ preparations, guinea pig ileum (GPI)[18] and mouse vas deferens (MVD).[19] In the GPI opioid effects are primarily mediated by μ-receptors, whereas κ-receptors are also present in this tissue. κ-Receptor interactions in the GPI assay are indicated by relatively high K_e values for naloxone as antagonist (20–30 nM),[20] in contrast to the low K_e values (1–2 nM) observed with μ-receptor ligands.[21] The MVD assay is generally taken as being representative for δ-receptor interactions, even though it also contains μ and κ receptors. Both δ and κ-interactions in the MVD are characterized by relatively high K_e values for naloxone as antagonist (~20 nM),[22] whereas low K_e values (<2 nM) are observed with μ-agonist in this preparation.[23] Opioid receptor affinities were determined by displacement of selective radioligands from rat brain membrane preparations. [^{3}H]DAGO served as a μ-receptor-selective radiolabel and the radioligands [^{3}H]DSLET and [^{3}H]DPDPE were used for determining δ-receptor affinities.

Results and Discussion

In the competitive-binding experiments DELI (**11**) and DELII (**15**) display excellent δ-receptor selectivities, as indicated by their very high $IC_{50}\mu/IC_{50}\delta$ ratios, showing an even higher preference for δ-sites than DEL (**1**) and DPDPE (Table II). These data are in agreement with the trend previously observed.[9] The substitution of neutral isosteric residue for aspartic acid in position 7 of **1** (compounds **3** and **5**) produced a 3-fold decrease in μ-receptor affinity and a 2-fold decrease in δ-receptor affinity. Therefore, compounds **3** and **5** show a relatively moderate preference for δ-receptors over μ-receptors. On the contrary, heptapeptide free acids (compounds **2** and **4**) are more selective than the parent amides **1** and **3**, respectively. The analogue C-terminal free acid **6** containing an aspartic residue in position 4 carries a further increased net negative charge of 2–. This compound shows very minimal interaction with μ-receptor but retains an evident affinity and selectivity for the δ-site.

The binding profiles of analogues **1–5** tend to support the membrane-assisted opioid receptor selection model of Schwyzer.[16] The substitution of His in position 4 of **1** by a neutral (glycine) and/or an aromatic residue (phenylalanine and tyrosine) influences the affinity in the binding assay in a similar manner. In fact in comparison to DEL (**1**), compounds **7–9** display comparable δ-receptor affinity and, most interestingly, an increase in μ-receptor affinity. Consequently, **7–9** are about 5–10 times less selective than DEL. Like **7–9**, heptapeptide **10** also contains a substitution in position 4 and, in analogy to **7–9**, it is moderately selective but, in comparison with the latter analogues, displays lower μ- and δ-receptor affinities. Apparently these results can be interpreted to be in disagreement with the predictions made on the basis of the membrane compartment concept. On the other hand, in deltorphins (**1**, **11** and **15**), the presence of positively or negatively charged amino acids in position 4 has little influence on the binding characteristics.

We had already shown[24] that peptides with H-Tyr-D-Xaa-Phe as N-terminal message sequence can be good μ- and/or δ-opioids, with the only difference being that the δ-agonists have larger hydrophobic side chains in the C-terminal residues. Accordingly, the main causes of the selectivity should be sought in the constitution and/or conformation of the C-terminal part. This seems to be supported by the comparison of the binding characteristics that pertain to DELI (**11**) and its shorter homologues **12–14** (Table II). The reduction of the chain length starting from residues 1–7 (compound **11**) to residues 1–6, 1–5, and 1–4 (compounds **12–14**) is accompanied by a progressive decrease in δ-receptor affinity and a stepwise enhancement of μ-receptor affinity. The net result is an inversion from δ- to μ-selectivity. These data illustrate the critical role that the C-terminal residues play in specifying correct addressing of DELI toward δ-receptors. This is in line with the binding properties of compound **18**, which can be considered as a hybrid of dermorphin, DELI or DELII, and DEL, since it has the first four residues of DER, the same hydrophobic part (Val-Val) of DELI/DELII, and the final acidic residue (Asp) of DEL. The hybrid heptapeptide **18** shows a fairly high δ-selectivity, at the expense of both affinities in comparable amounts, confirming the data previously observed.[25] In fact, Sagan et al.[25] have recently shown that it is possible to change the selectivity of dermorphin-like peptides by exchanging the C-terminal parts of typical μ- and δ-peptides; thus the hybrid made up from the N-terminal tetrapeptide of Der plus the C-terminal tripeptide of DEL becomes δ-selective, whereas that made up from the N-terminal tetrapeptide of DEL and the C-terminal tripeptide of DER becomes μ-selective. Finally, through alteration of the C-terminal amide group of DELII to a free acid, i.e. compound **16**, and amidation of Glu in

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position 4 (compound 17), we have further investigated the influence of charged groups on binding properties of deltorphin peptides. Compared with DELII (15), the corresponding deltorphinoic acid (16) shows lower binding affinity particularly at δ-sites, resulting in an 11-fold decrease in δ-receptor selectivity. This is quite unexpected since the same C-terminal alteration of DEL has been shown to increase δ-receptor recognition (see 2 vs 1). Although this result could be found to disagree with the membrane selection model, it should be pointed out that peptides 2 and 16 carry a different net charge. On the other hand, substitution of the glutamine residue for glutamic acid in position 4 of 15 (peptide 17) did not affect δ-receptor binding affinity but produced a 12-fold increase in μ-receptor affinity: this result suggests that the relatively high μ-receptor affinity of 17 could be due to increased accumulation of the compound carrying a positive charge in the anionic fixed-charge compartment of the membrane, where μ-sites are supposed to be located.[16]

The results of the GPI and MVD bioassays were found to be in qualitative but not always quantitative agreement with the receptor binding data (Table III). Such a trend was already observed for DEL/dermenkephalin[2,8] and by Erspamer et al.[9] for deltorphins, and their findings were similar to those obtained here. The effects of all of the compounds were antagonized by ICI 174, 864, a δ-opioid receptor selective antagonist, and in GPI by naloxone low concentrations ($K_e \approx 2$ nM), indicating that no κ agonist activity is obtained. The discrepancies between binding and bioassays data may be due to a different degree of nonspecific adsorption of peptides in the various tissues or to enhanced intrinsic activity of some compounds at the peripheral δ- and/or μ-receptors. Since all examined compounds contain a D-amino acid residue in position 2 and a C-terminal carboxamide function (except 2, 6, and 16), they can be expected to be equally stable against enzymolysis under the conditions of the bioassays and binding assays,[26] and thus, a different extent of peptide degradation in the various tissues can be ruled out as a factor explaning the differences between bioassays and binding assay data.

Conclusions

The results of our studies concerning this series of deltorphin-related peptides emphasize the crucial contribution of the C-terminal part of these molecules to selectivity. Whereas H-Tyr-D-Ala-Phe-Asp-NH_2 shows a high preference for μ- over δ-receptors, lengthening the peptide chain by stepwise addition of Val, Val, and Gly gives rise to DELI, one of the most potent and selective δ-opioids.

The receptor selectivity profiles of some DEL analogues described in this paper are in agreement with the prediction made on the basis of the membrane compartment concept. On the other hand, some DELII-related peptides and hybrid compounds showed a decrease rather than the expected increase in δ-receptor selectivity relative to their more or less charged parent heptapeptides. Obviously, the structural modifications performed to introduce or delete the charges into the various peptides could also affect the peptide–receptor interaction through steric or direct electrostatic effects[17] or through induced conformational rearrangements of the molecules.

Collectively, results from the present study indicate that various probes are necessary to elucidate the receptor selectivity of deltorphin–dermorphin peptides. However, the main causes of selectivity can be traced to (i) hydrophobicity of the C-terminal part (or address domain), assured no only by the nature of the C-terminal residues but, most of all, by the folded conformations found for all deltorphins,[14,15] which act as a screen even with respect to the charges of Asp^4 and Glu^4 and favor δ-selectivity; (ii) preselection by charge;[16] (iii) the presence of Pro in DER, which prevents a highly folded C-terminal part,[24] thus favoring, indirectly, μ-selectivity; (iv) the presence of negatively charged or polar groups adjacent to the message domain, which is not easily tolerated by the μ-receptor.

Experimental Section

General Methods. Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter with a 10-cm water-jacketed cell. Molecular weights of the products were determined by FAB-MS on JEOL Model JMS-HX 100 mass spectrometer. HPLC analysis was performed on a Bruker liquid chromatograph LC21-C equipped with a Bruker LC313 UV variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor (Epson computer FX-80X7. A IB01 C-18 column (250 × 4.5 i.d., 5-μm particle size) was used in the HPLC system. All solvents used were UV spectroscopic grade and were filtered and degassed prior to use. Analytical determinations for protected peptides were carried out by a gradient made up of two solvents: A, 10% (v/v) acetonitrile in water; B, 60% (v/v) acetonitrile in water, both containing 0.1% TFA. The gradient program used was as follows: linear gradient from 30% to 100% B in 40 min. Chromatography was performed at a flow rate of 1 mL/min: all analogues showed by analytical HPLC less than 1% impurities while being monitored at 220 and 254 nm. Preparative reverse-phase HPLC was carried out with a Waters Delta Prep 3000 (preparative chromatography system) with a Delta Pak C_{18}-300A° (30 mm × 30 cm, 15 μm, spherical). The gradient program used was as follows: linear fradient from 40% to 70% B in 30 min. Chromatography was performed at a flow rate of 30 mL/min; detector UV, λ = 220 nm. At each step of the synthesis, the lack of significant racemization of a given peptide was checked by ^1H NMR spectroscopy and by HPLC. The amino acid composition was determined with a Carlo Erba (Milano, Italy) 3A29 amino acid analyzer, after acid hydrolysis in constantly boiling HCl containing phenol (1%). TLC was performed on precoated plates of silica gel F254 (from E. Merck, Darmstadt, FRG) with use of the following solvent systems: (A) 1-butanol/AcOH/H_2O (6:1:5), (B) EtOAc/pyridine/AcOH/H_2O (60:20:6:11), (C) CH_2Cl_2/MeOH/benzene (85:10:5), (D) $CHCl_3$/MeOH (1:1), (E) $CHCl_3$/AcOH/benzene (85:10:5), (F) EtOAc/MeOH (1:1), (G) $CHCl_3$/MeOH/30% ammonia (12:8:37). Ninhydrin 1% (Merck), fluorescamine (Hoffman-LaRoche), and/or chlorine reagent were used as spray reagents. Elemental analyses were carried out after the products were dried for 12 h at 50 °C (0.2 Torr). Open-column chromatography was run on silica gel 60 (70–230 mesh, Merck), unless stated otherwise. The following abbreviations are used: Z, benzyloxycarbonyl; Fmoc, (9-fluorenylmethoxy)carbonyl; Boc, *tert*-butyloxycarbonyl; MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; THF, tetrahydrofuran; AcOH, acetic acid; DMF, dimethylformamide; $CHCl_3$, chloroform; PE, petroleum ether; TFA, trifluoroacetic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methylmorpholine; OPfp, pentafluorophenyl ester; OSu, *N*-succinimidyl ester; IBCF, isobutyl chloroformate; HOSu, *N*-hydroxysuccinimide; DCCU, *N,N'*-dicyclohexylurea. Other abbreviations were those recommended by the IUPAC–IUB Commission (*Biochem. J.* 1984, *219*, 345).

Coupling Procedures. Method A. To a stirred solution (0.5–0.8 M) of Boc-protected amino acid or Boc-protected peptide (1 mmol) in DMF was added 1.1 equiv of NMM; the mixture was cooled to −10 °C, treated with IBCF (1.1 equiv), and allowed to react for 2–3 min. A precooled solution of the amino component as the hydrochloride or trifluoroacetate (1.1 mmol) in DMF (0.4–0.6 M) was added to the mixture, followed by NMM (1.1 equiv). The reaction mixture was stirred for 1 h at −10 °C and 2–3 h at 0–10 °C and then diluted with EtOAc (100 mL). The solution or suspension was washed consecutively with brine, 0.5

(26) Munson, P. J.; Rodbard, D. *Anal. Biochem.* 1980, *107*, 220.

N $KHSO_4$, brine, 5% $NaHCO_3$, and brine. The organic phase was dried ($MgSO_4$), filtered, and evaporated to dryness. The residue was crystallized from appropriate solvents or purified by column chromatography.

Method B. The protonated amino component (amino acid or peptide amide) and the activated carboxy component (OSu or ONp) (1 equiv each) were dissolved in DMF (~5 mL/mmol) containing 1 equiv of NMM, and the mixture was allowed to react at room temperature for 4 h. The solution was diluted with EtOAc and worked up as described in method A.

Method C. To a stirred solution (0.5–0.8 M) of Boc- or Fmoc-protected peptide (1 mmol) in DMF were added at −10 °C DCC (1.1 mmol), HOBt (1.1 mmol), and a precooled solution of the protonated amino component (1.1 mmol) in DMF (0.4–0.6 M) containing NMM (1.1 mmol). The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature; DCCU was filtered off, and the solution was diluted with EtOAc (100 mL) and worked up as described in method A.

Method D. The hydrazide component was dissolved in DMF (~4 mL/mmol peptide), cooled to −25 °C, and treated with 2.5 molar equiv of HCl in THF and 1.1 molar equiv of *tert*-butyl nitrite. After 20–30 min at −25 °C, the protonated amino component (1 equiv) dissolved in DMF (4 mL/mmol) was precooled and slowly added together with 3.5 molar equiv of NMM. The temperature was kept at −5 °C for 2 days, while the pH was maintained at 7–8 with NMM. The mixture was diluted with EtOAc and worked up as described in method A.

Hydrazinolysis. Method E. The peptide methyl ester was dissolved in methanol (~10 mL/mmol peptide) and treated with 10 molar equiv of hydrazine hydrate at room temperature for 10–12 h. The solution was evaporated and the product was obtained by repeated crystallization from methanol–diisopropyl ether.

Deprotection Procedures. Method F. Boc and OBu^t protecting groups were removed by treating the peptide with aqueous 90% TFA (1:10 w/v) containing anisole (1 mL) for 30–40 min. The solvent was evaporated in vacuo at 0 °C, and the residue was triturated with Et_2O or petroleum ether (PE); the resulting solid peptide was collected and dried.

Method G. Fmoc protecting group was removed by treating the peptide with 10% dimethylamine solution in DMF (1:10 w/v) for 5–10 min. The solution was concentrated in vacuo, and the residue was triturated with Et_2O or PE; the resulting solid peptide was collected and dried.

Method H. Hydrogenations were carried out in AcOH/MeOH (1:3) at atmospheric pressure and room temperature, in the presence of 10% palladized charcoal (catalyst to peptide ratio, 1:9 w/w). The reaction mixture was filtered through a Celite bed and evaporated to dryness. The residue was treated as described above in method G.

Fmoc-Leu-Met-Asp(OBu^t)-OBu^t (19). According to general coupling procedure C, Fmoc-Leu-Met-OH[15] (10 mmol) was reacted with H-Asp(OBu^t)-OMe (11 mmol). Crude **19** was recrystallized from EtOAc/Et_2O (73%): mp 161–163 °C; $[\alpha]^{20}_D$ −35.4° (*c* 1.0, MeOH); TLC R_f (C) 0.69. Anal. ($C_{38}H_{53}N_3O_8S$) C, H, N.

Boc-Tyr-D-Met-Phe-His-Leu-Met-Asp(OBu^t)-OBu^t (20). According to coupling method D, Boc-Tyr-D-Met-Phe-His-NH-NH_2[15] (1.4 g, 2 mmol) was reacted with H-Leu-Met-Asp-(OBu^t)-OBu^t (0.89 g, 2 mmol) (obtained from **19** according to deprotection method G). The mixture was diluted with EtOAc (150 mL), washed, and dried. Crude **20** was purified by column chromatography on silica gel (2 × 50 cm) (eluent CH_2Cl_2/MeOH/benzene 17:1:2). The fractions containing the pure compound were evaporated to dryness and the residue was crystallized from AcOEt/Et_2O (1.89 g, 84%): mp 201–204 °C; $[\alpha]^{20}_D$ +4.1° (*c* 1.0, MeOH); TLC R_f 0.52 (C). Anal. ($C_{57}H_{85}N_9O_{13}S_2$) C, H, N.

Fmoc-Lys(Boc)-Leu-Met-Asp(Obu^t)-NH_2 (21). To a solution of H-Leu-Met-Asp(OBu^t)-NH_2[15] (0.86 g, 2 mmol) in DMF (15 mL) was added Fmoc-Lys(Boc)-OPfp (1.26 g, 2 mmol). The reaction mixture was stirred for 1 h at room temperature and evaporated to dryness. Crude **21** was crystallized from AcOEt/Et_2O (1.4 g, 80%): mp 178–180 °C; $[\alpha]^{20}_D$ −23.0° (*c* 1.0, MeOH); TLC R_f (C) 0.71. Anal. ($C_{45}H_{66}N_6O_8S$) C, H, N.

Boc-D-Met-Phe-OMe (22). According to coupling method B, Boc-D-Met-OSu was reacted with H-Phe-OMe·HCl. Crude **22** was crystallized from Et_2O/PE (83%): mp 92–94 °C; $[\alpha]^{22}_D$ +5.5° (*c* 1.0, MeOH); TLC R_f (C) 0.83. Anal. ($C_{20}H_{30}N_2O_5S$) C, H, N.

Boc-Tyr-D-Met-Phe-OMe (23). According to the deprotection method E, **22** (4.1 g, 10 mmol) was dissolved in TFA (8 mL), and the solution was stirred at room temperature for 30 min. The resulting H-D-Met-Phe-OMe trifluoroacetate in DMF (20 mL) containing NMM (10 mmol) was reacted with Boc-Tyr-OSu (10 mmol) according to coupling method B. Peptide **23** was crystallized from AcOEt/PE (81%): mp 55–57 °C; $[\alpha]^{20}_D$ −6.5° (*c* 1.0, MeOH); TLC R_f (C) 0.65. Anal. ($C_{29}H_{39}N_3O_7S$) C, H, N.

Boc-Tyr-D-Met-Phe-NH-NH_2 (24). Compound **23** (5 mmol) was converted to its hydrazide according to general procedure E. Crude **24** was crystallized from MeOH/diisopropyl ether (85%): mp 126–128 °C; $[\alpha]^{22}_D$ −8.2° (*c* 1.0, MeOH); TLC R_f (C) 0.54. Anal. ($C_{28}H_{39}N_5O_6S$) C, H, N.

Boc-Tyr-D-Met-Phe-Lys(Boc)-Leu-Met-Asp(OBu^t)-NH_2 (25). Compound **21** (3 mmol) was converted to H-Lys(Boc)-Leu-Met-Asp(OBu^t)-NH_2 (deprotection method G) and reacted with the azide Boc-Tyr-D-Met-Phe-N_3 (3 mmol) according to coupling procedure D. Crude **25** was purified by column chromatography on silica gel as **20** and crystallized from AcOEt/Et_2O (81%): mp 212–215 °C; $[\alpha]^{20}_D$ −2.8° (*c* 1.0, MeOH); TLC R_f (C) 0.60. Anal. ($C_{58}H_{91}N_9O_{14}S_2$) C, H, N.

Z-Asp(OBu^t)-Val-Val-NH_2 (26). According to coupling procedure B, Z-Asp(OBu^t)-OSu (5 mmol) was reacted with H-Val-Val-NH_2 acetate[15] (5.5 mmol). Peptide **26** was crystallized from AcOEt (83%): mp 223–225 °C; $[\alpha]^{20}_D$ −15.0° (*c* 1.0, MeOH); TLC R_f (C) 0.76. Anal. ($C_{26}H_{40}N_4O_7$) C, H, N.

Boc-D-Ala-Phe-OMe (27). According to general coupling method A, a mixed anhydride prepared from Boc-D-Ala-OH (5 mmol) was reacted with H-Phe-OMe·HCl (5.5 mmol). The resulting dipeptide **27** was crystallized from Et_2O (84%): mp 78–81 °C; $[\alpha]^{20}_D$ +8.8° (1.3, MeOH); TLC R_f (C) 0.77. Anal. ($C_{18}H_{26}N_2O_5$) C, H, N.

Boc-Tyr-D-Ala-Phe-OMe (28). According to deprotection method E, **28** (4 mmol) was treated with TFA (3 mL). The resulting H-D-Ala-Phe-OMe trifluoroacetate was reacted with Boc-Tyr-OSu (3.8 mmol) according to method B. Crude **28** was crystallized from Et_2O (88%): mp 96–98 °C; $[\alpha]^{22}_D$ −4.5° (*c* 1.1, MeOH); TLC R_f (C) 0.70. Anal. ($C_{27}H_{35}N_3O_7$) C, H, N.

Boc-Tyr-D-Ala-Phe-NH-NH_2 (29). This compound was obtained from **28** (3 mmol) according to procedure F. The hydrazide was crystallized from MeOH (81%): mp 132–134 °C; $[\alpha]^{20}_D$ −9.1° (*c* 1.0, DMF); TLC R_f (C) 0.51. Anal. ($C_{26}H_{35}N_5O_6$) C, H, N.

Boc-Tyr-D-Ala-Phe-Asp(OBu^t)-Val-Val-NH_2 (30). According to general deprotection procedure F, compound **26** (3 mmol) in AcOH/MeOH (1:3, 30 mL) was hydrogenated for 2 h. The resulting H-Asp(OBu^t)-Val-Val-NH_2 acetate was reacted with **29** (3 mmol) according to coupling procedure D. Hexapeptide **30** was purified by column chromatography on silica gel (eluent CH_2Cl_2/MeOH/benzene 15:2:1) and crystallized from AcOEt/Et_2O (71%): mp 208–210 °C; $[\alpha]^{22}_D$ −6.4° (*c* 1.0, DMF); TLC R_f (C) 0.58. Anal. ($C_{44}H_{65}N_7O_{11}$) C, H, N.

Boc-Gln-Val-Val-Gly-NH_2 (31). According to general procedure B, Boc-Gln-ONp (5 mmol) was reacted with H-Val-Val-Gly-NH_2 acetate (5 mmol). The resulting **31** was crystallized from MeOH/Et_2O (71%): mp 233–235 °C; $[\alpha]^{22}_D$ −14.2° (*c* 1.0, DMF); TLC R_f (A) 0.63. Anal. ($C_{22}H_{40}N_6O_7$) C, H, N.

Boc-Tyr-D-Ala-Phe-Gln-Val-Val-Gly-NH_2 (32). Compound **31** (3 mmol) was converted to its trifluoroacetate salt (deprotection procedure F) and reacted with the azide prepared from Boc-Tyr-D-Ala-Phe-NH-NH_2 (**29**; 3 mmol) according to coupling procedure D. Crude heptapeptide **32** was purified as **30** and crystallized from DMF/Et_2O (69%): mp 239–242 °C; $[\alpha]^{20}_D$ −7.6° (*c* 1.0 MeOH); TLC R_f (B) 0.81. Anal. ($C_{43}H_{63}N_9O_{11}$) C, H, N. The other protected heptapeptides were synthesized by similar procedures.

Preparations of Free Peptides 1–18. Each Boc and OBu^t peptide was deprotected according to procedure F. The resulting free compound (1 mmol) was dissolved in excision N acetic acid (4 mL) and passed through a 2 × 30 cm Sephadex G25 column, with solvent system A. The peptide trifluoroacetates were converted into corresponding free bases through anion exchanger resin DE52 Whatman (acetate form) using water as eluent. The

fractions containing the peptide were collected and lyophilized to a constant weight (70–75%). Characterization of free peptides 1–18 is reported in Table I.

Binding Assays. The homogenates of male rat brains (Wistar, 150–200 g) were prepared as described by Gillan et al.[23] Briefly, animals were sacrificed, brains were removed, and after excision of the cerebellum, the brains tissue was homogenized in a 50 mM tris buffer (pH 7.4) and centrifuged at 48000 rpm for 10 min; the pellet was then resuspended in tris buffer, incubated at 37 °C for 45 min, and centrifuged again. For the binding assays, 1.8 mg of brain tissue was used and the volume made up to 2.0 mL with solutions of the inhibitory cold ligands and the tritiated ligand. [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO; 0.8 nM, 45 Ci/mmol, Amersham) was used as μ-ligand and [^{3}H][D-Ser^2,Leu^5,Thr^7]enkephalin (DSLET; 1.5 nM) or [^{3}H][D-Pen^2,D-Pen^5]enkephalin (DPDPE; 1.0 nM, New England Nuclear) as δ-ligands. The mixture was incubated for 40 min at 25 °C (in the dark for DSLET), filtered through Whatman GF/B glass-fiber filters, using a Brandel cell harvester, and washed three times with ice-cold buffer. From the total binding, specific binding was obtained by deducing the nonspecific binding which was not inhibited by 10^{-3}M bremazocine. In order to determine IC_{50} values (i.e. values for 50% inhibition of specific [^{3}H]DAGO, [^{3}H]DSLET, and [^{3}H]DPDPE binding) of the peptide under examination, the compounds were added, in triplicate, to the binding assays in at least six different concentrations. The IC_{50} values were calculated by means of the IBM program LIGAND.[26]

GPI and MVD Bioassays. A segment of intact ileum, 2–3 cm in length, was removed and mounted in a 20 mL organ bath using the Kosterlitz and Watt method.[18] The tissue was bathed in Krebs' solution containing 70 μmol/L hexamethonium bromide and 0.125 μmol/L mepyramine maleate. The solution was aerated with 95% O_2/5% CO_2 and maintained at 36 °C. The ileum was stimulated transmurally with square-wave electrical pulses of 0.5-ms duration at a frequency of 0.1 Hz. Unless otherwise stated, stimulus strength was 1.5 times that which produced maximal twitch (usually 30 V). Twitchlike contractions were recorded isotonically (magnification 1:15). The IC_{50} is the concentration of compound necessary to inhibit the amplitude of the electrically induced twitch by 50%. Single vas deferens from mature mice (Albino, 30–35 g) were dissected and suspended in 4 mL of modified Krebs' solution[19] aerated with 95% O_2/5%CO_2 and maintained at 33 °C. The twitch induced by field stimulation (0.1 Hz, 1 ms, 40 V) was recorded on an ink-pen recorder via an isometric transducer. Dose–response curves were constructed by addition of appropriate amounts of peptides (in 10–100 μL of Krebs' solution) to the baths. Three to four washings were done with intervals of 10 min between each dose.

A log dose–response curve was determined with DPDPE or morphine as standard for each vas and ileum preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[27] K_e values for naloxone or *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864) as antgonists were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed antagonist concentration.[18]

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Registry No. 1, 131131-13-0; 2, 131131-14-1; 3, 124411-85-4; 4, 131131-15-2; 5, 131152-62-0; 6, 131131-16-3; 7, 131152-63-1; 8, 131131-17-4; 9, 131152-64-2; 10, 131131-18-5; 11, 122752-15-2; 12, 128837-52-5; 13, 128837-53-6; 14, 128837-54-7; 15, 122752-16-3; 16, 131152-65-3; 17, 131131-19-6; 18, 131152-66-4; 19, 131152-67-5; 20, 131152-68-6; 21, 131152-69-7; 22, 99909-57-6; 23, 100572-13-2; 24, 131152-70-0; 25, 131152-71-1; 26, 131131-20-9; 27, 95083-33-3; 28, 124044-55-9; 29, 131131-21-0; 30, 131131-22-1; 31, 131131-23-2; 32, 131131-24-3; Fmoc-Leu-Met-OH, 86961-00-4; H-Asp(OBu-*t*)-OMe, 39895-10-8; H-Leu-Met-Asp(OBu-*t*)-OBu-*t*, 131152-72-2; BOC-Tyr-D-Met-Phe-His-NHNH$_2$, 131131-25-4; H-Leu-Met-Asp(OBu-*t*)-NH$_2$, 131152-73-3; Fmoc-Lys(BOC)hOPfp, 86060-98-2; BOC-D-Met-OSu, 26060-98-0; H-Phe-OMe·HCl, 7524-50-7; H-D-Met-Phe-OMe·TFA, 100572-36-9; BOC-Tyr-OSu, 20866-56-2; H-Lys(BOC)-Leu-Met-Asp(OBu-*t*)-NH$_2$, 131131-26-5; Z-Asp(OBu-*t*)-OSu, 3338-32-7; H-Val-Val-NH$_2$, 128211-98-3; BOC-D-Ala-OH, 7764-95-6; H-D-Ala-Phe-OMe·TFA, 131131-27-6; H-Asp(OBu-*t*)-Val-Val-NH$_2$·HOAc, 131152-75-5; BOC-Gln-ONp, 15387-45-8; H-Val-Val-Gly-NH$_2$·HOAc, 131131-29-8.

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