stirred at room temperature for 20 min, the reaction appeared complete as judged by the disappearance of a positive test for primary amino groups (2,4,6-trinitrobenzenesulfonate assay).[11] The DMF was removed in vacuo, and the residue was taken up in H_2O and lyophilized to give 1.97 g of a white powder. The powder was dissolved in 23 mL of H_2O and then 12 mL of 0.1 M barium acetate was added. Addition of 10 mL of EtOH precipitated the barium salt of the blocked product, which was collected by filtration and washed with 4 mL of ice-cold water to give 0.84 g of a damp solid. The damp solid (0.7 g) was dissolved in 7 mL of acetone, and then 7 mL of EtOH was added to reprecipitate the barium salt (0.39 g dry). The salt was dissolved in 8 mL of H_2O, and 0.42 mL of 0.82 M K_2SO_4 was added to precipitate barium sulfate, which was removed by centrifugation. The supernatant contained the potassium salt of the blocked product (11), which was then hydrogenolyzed over 3 h at atmospheric pressure with 100 mg of 10% palladium on carbon as catalyst. Filtration and lyophilization produced 0.17 g of **12** as the trihydrate: ^1H NMR (D_2O with acetone as reference) δ 1.16 and 1.31 (pair of doublets, 3 H, J = 7.3 Hz, CH_3), 1.19 (d, 3 H, J = 7.3 Hz, CH_3), 3.84 and 3.87 (pair of quartets, 1 H, J = .3 Hz, alanyl CH), 4.04 (q, 1 H, J = 7.3 Hz, alanyl CH), 5.75 and 5.80 in a 2:1 ratio (pair of singlets, 1 H, glycyl CH), 6.65 and 6.70 (pair of doublets, 2 H, J = 8.9 Hz, aromatic), 7.48 (d, 2 H, J = 8.8 Hz, aromatic). Anal. ($C_{14}H_{20}N_4O_7SK\cdot 3H_2O$) C, H, N.

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Registry No. **1**, 89625-84-3; **2**, 118597-40-3; **3**, 118597-41-4; L,L-**3**, 118597-42-5; L,D-**3**, 118597-43-6; **4**, 118597-45-8; L,L-**4**, 118597-47-0; L,D-**4**, 118597-49-2; **4** (free acid), 118597-44-7; **5**, 118597-50-5; **5a**, 118597-51-6; **5b**, 118597-52-7; **6**, 118597-53-8; **7**, 118597-54-9; **8**, 118597-55-0; **8** (free acid), 118597-56-1; **9**, 118597-57-2; **9a**, 118597-58-3; **9b**, 118597-59-4; **10**, 89626-22-2; **11**, 118597-60-7; **12**, 118597-61-8; sulfanilic acid, 121-57-3; sulfanilamide, 63-74-1; trimethoprim, 738-70-5; triethylammonium sulfanilate, 51176-58-0; *N*-carbobenzoxy-L-alanine *p*-nitrophenyl ester, 1168-87-2; *N*-carbobenzoxy-L-alanyl-L-alanyl-D,L-2-[(4-sulfophenyl)amino]glycine bis(triethylammonium) salt, 118597-63-0.

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Dermorphin Analogues Carrying an Increased Positive Net Charge in Their "Message" Domain Display Extremely High μ Opioid Receptor Selectivity

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According to the membrane compartment concept the receptor specificity of ligands is based not only on ligand–receptor complementarity but also on specific ligand–membrane interactions. Elaboration of this concept for opioid peptide–receptor interactions had led to the assumption that μ- and δ-receptors are located in anionic and cationic membrane compartments, respectively, and to the prediction that positively charged opioid receptor ligands should display μ-receptor selectivity. To assess the validity of this model, we synthesized a series of dermorphin analogues carrying a net positive charge and tested them in μ- and δ-receptor representative binding assays and bioassays. Some but not all of the prepared compounds showed the receptor-selectivity profile expected on the basis of the membrane compartment concept. In particular, gradual augmentation of the positive charge from 1+ to 3+ in a series of dermorphin-(1–4) tetrapeptide analogues produced an enhancement of μ-receptor affinity and a progressive decrease in δ-receptor affinity, resulting in increasingly higher μ-receptor selectivity. The most selective compound was [D-Arg^2,Lys^4]dermorphin-(1–4)-amide (DALDA), showing a selectivity ratio (K_i^δ/K_i^μ = 11 400) more than 10 times higher than that of DAGO (K_i^δ/K_i^μ = 1050) and, thus, displaying unprecedented μ-receptor specificity. Because of its high positive charge (3+), DALDA may be particularly useful as a very specific agonist for studying peripheral μ-receptor interactions.

The emergence of the concept of multiple opioid receptors[2,3] has led to numerous efforts aimed at developing highly selective ligands for the various receptor types (μ, δ, κ, etc.). Various strategies were used in the design of such specific ligands. Early studies focussed on modification of the enkephalins (H-Tyr-Gly-Gly-Phe-Met(or Leu)-OH and other opioid peptides, using the classical approach of amino acid substitutions, additions, and deletions. These attempts resulted in agonists showing substantial preference for μ-receptors (e.g. DAGO[4] or PLO17[5]) or considerable δ-receptor selectivity (e.g. DSLET[6] or DSTBULET[7]). In a more recent development, receptor selectivity was achieved through conformational restriction of linear opioid peptides. In particular, peptide cyclizations via side chains led to cyclic opioid peptide analogues displaying quite high μ-receptor selectivity (e.g. H-Tyr-cyclo[-D-A$_2$bu-Gly-Phe-Leu-][8] or H-

(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$_2$bu, α,γ-diaminobutyric acid; Boc, *tert*-butoxycarbonyl; DAGO, H-Tyr-D-Ala-Gly-Phe-(NMe)-Gly-ol; DALDA, H-Tyr-D-Arg-Phe-Lys-NH$_2$; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; DSTBULET, H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr-OH; FAB, fast atom bombardment; Fmoc, (fluoren-9-ylmethoxy)carbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Hyp, 4-hydroxyproline; MVD, mouse vas deferens; Nle, norleucine; Nva, norvaline; Pen, penicillamine; PL017, H-Tyr-Pro-Phe(NMe)-D-Pro-NH$_2$; TFA, trifluoroacetic acid.
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Tyr-D-Orn-Phe-Asp-NH_2[9]), or further improved δ-receptor selectivity (e.g. H-Tyr-D-Pen-Gly-Phe-D-Pen-OH[10]). On the basis of the hypothesis of receptor bridging, various bivalent ligands containing enkephalin-related peptides linked via their C-terminal carboxyl groups through flexible spacers of varying length were prepared and some of these dimers showed pronounced selectivity for either μ- or δ-receptors.[11,12] The "bivalent ligand" approach has recently also been successfully applied to non-peptide opioid ligands by synthesis of bimorphinans which turned out to be potent and very selective κ-antagonists.[13] The μ-agonist sufentanil,[14] the δ-antagonist natrindole,[15] and the κ-agonist U50,488[16] are examples of monovalent non-peptide ligands showing pronounced opioid receptor selectivity.

It is generally recognized that the extent of complementarity between structural moieties of an opioid receptor ligand and topographical features of the various opioid receptor types is of crucial importance for receptor selectivity. More recently, it has been suggested that, in addition to ligand–receptor complementarity, specific interactions of opioid peptides with various membrane compartments might also contribute to their ability to interact selectively with a distinct receptor type.[17] In particular, a model has been proposed according to which the three principal opioid receptor types (μ, δ, κ) are located in different areas of the lipid bilayer membrane of the target cell. The δ-site is supposed to be exposed to the aqueous compartment around the target cell in a cationic environment, whereas the μ-site would be situated in the so-called anionic fixed-charge compartment. The κ-site is assumed to be buried in a hydrophobic region of the membrane adjacent to the anionic fixed-charge compartment. 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 type. 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 analogues would preferentially interact with the δ-receptor. Opioid peptides characterized by an amphiphilic moment of pronounced scalar magnitude (e.g. dynorphins) would be able to bind selectively to the κ-receptor.

To test the validity of the membrane compartment concept, we designed and synthesized a series of opioid peptide analogues which both carry a net positive charge (2+ or 3+) and fulfill the structural and conformational requirements of the μ-binding site. Such analogues would be expected to display very high μ-receptor selectivity. It is well established that opioid peptides containing a phenylalanine residue in the 3-position of the peptide sequence, such as dermorphins (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro(or Hyp)-Ser-NH_2) or morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2), are generally to some extent μ-receptor selective. We, therefore, synthesized several dermorphin-(1–4) tetrapeptide analogues containing a positively charged side chain in the 4-position (H-Tyr-D-Nva-Phe-Orn-NH_2 (**1**) and H-Tyr-D-Nle-Phe-Orn-NH_2 (**2**)) or in both the 2- and the 4-position (H-Tyr-D-Arg-Phe-A_2bu-NH_2 (**3**), H-Tyr-D-Arg-Phe-Orn-NH_2 (**4**), and H-Tyr-D-Arg-Phe-Lys-NH_2 (**5**)). Recently, a cyclic tetrapeptide analogue, H-Tyr-D-Orn-Phe-Asp-NH_2 (**6a**), containing a phenylalanine residue in the 3-position of the peptide sequence has been reported to be very μ-receptor selective (K_i^δ/K_i^μ = 213).[9] It was, therefore, of interest to establish whether addition of structural elements carrying a positive charge to this cyclic peptide structure would further enhance μ-receptor selectivity. To this end, we synthesized and characterized an analogue of **6a** which was extended at the C-terminus by addition of two positively charged arginine residues (H-Tyr-D-Orn-Phe-Asp-Arg-Arg-NH_2 (**6**)). An analogous C-terminal extension by two arginine residues was performed with the linear tetrapeptide analogue H-Tyr-D-Ala-Phe-Phe-NH_2 (**7a**) by preparing the hexapeptide H-Tyr-D-Ala-Phe-Phe-Arg-Arg-NH_2 (**7**). Analogue **7a** contains a phenylalanine residue in both the 3- and the 4-position and has recently been shown to display high μ-receptor selectivity (K_i^δ/K_i^μ = 409.[18]

Chemistry. Linear peptides were prepared by standard solid-phase techniques on a *p*-methylbenzhydrylamine resin, using Boc- or Fmoc-amino acids. The cyclic hexapeptide analogue H-Tyr-D-Orn-Phe-Asp-Arg-Arg-NH_2 (**6**) was also prepared by the solid-phase method, according to a previously described protection scheme based on side chain to side chain cyclization of the resin-bound peptide.[19] As it had also been the case in the preparation of the cyclic parent peptide H-Tyr-D-Orn-Phe-Asp-NH_2 (**6a**) by the same scheme,[20] cyclodimerization occurred to some extent due to intersite reaction between two peptide chains on the resin. The proportion of peptide chains having formed cyclic monomers and dimers was 34:66, similar to the corresponding proportion previously reported for **6a** (32:68).[20] This result indicates that the C-terminal extension with the exocyclic Arg-Arg- dipeptide segment in the case of analogue **6** does not alter the extent of cyclodimerization occurring in the Orn-Asp side chain to side chain cyclization step.

Bioassays and Binding Assays. 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 radioligand [^{3}H]DSLET was used for determining δ-receptor affinities. In vitro opioid activities of the analogues were determined with bioassays based on inhibition of electrically evoked contractions of the GPI and the MVD.

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

no.	compd	[3H]DAGO K_i^μ, nM	[3H]DAGO rel potency[b]	[3H]DSLET K_i^δ, nM	[3H]DSLET rel potency[b]	K_i^δ/K_i^μ
1	H-Tyr-D-Nva-Phe-Orn-NH_2	1.17 ± 0.28	8.01 ± 1.87	2200 ± 630	0.00115 ± 0.00033	1880
1a	H-Tyr-D-Nva-Phe-Nle-NH_2	3.53 ± 0.46	2.67 ± 0.35	181 ± 8	0.0140 ± 0.0006	51.3
2	H-Tyr-D-Nle-Phe-Orn-NH_2	1.35 ± 0.19	6.97 ± 1.01	2870 ± 30	0.000881 ± 0.000010	2130
3	H-Tyr-D-Arg-Phe-A_2bu-NH_2	5.78 ± 0.53	1.63 ± 0.15	12600 ± 4100	0.000202 ± 0.000065	2180
4	H-Tyr-D-Arg-Phe-Orn-NH_2	1.20 ± 0.26	7.83 ± 1.74	9290 ± 1300	0.000272 ± 0.000039	7740
5	H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA)	1.69 ± 0.25	5.58 ± 0.83	19200 ± 2000	0.000132 ± 0.000014	11400
6	H-Tyr-D-Orn-Phe-Asp-Arg-Arg-NH_2	23.6 ± 1.81	0.399 ± 0.031	1880 ± 70	0.00135 ± 0.00005	79.7
6a	H-Tyr-D-Orn-Phe-Asp-NH_2	10.4 ± 3.7	0.907 ± 0.032	2220 ± 58	0.00114 ± 0.00003	213
7	H-Tyr-D-Ala-Phe-Phe-Arg-Arg-NH_2	2.57 ± 0.87	3.67 ± 1.25	167 ± 39	0.0152 ± 0.0036	65.0
7a	H-Tyr-D-Ala-Phe-Phe-NH_2	1.53 ± 0.59	6.15 ± 2.38	626 ± 162	0.00405 ± 0.00105	409
8	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol (DAGO)	1.22 ± 0.12	7.73 ± 0.76	1280 ± 90	0.00198 ± 0.00014	1050
9	[Leu5]enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1	0.268

[a] Mean of three determinations ± SEM. [b] Potency relative to that of [Leu5]enkephalin.

The GPI assay is usually considered as being representative for μ-receptor interactions, even though the ileum does also contain κ-receptors. κ-Receptor interactions in the GPI assay are indicated by relatively high K_e values for naloxone as antagonist (20–30 nM),[21] in contrast to the low K_e values (1–2 nM) observed with μ-receptor ligands.[3] In the MVD assay opioid effects are primarily mediated by δ-receptors; however, μ- and κ-receptors also exist in this tissue. 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-agonists in this preparation.[23]

Results and Discussion

In the [3H]DAGO binding assay H-Tyr-D-Nva-Phe-Orn-NH_2 (1), carrying a net charge of 2+, showed about 8 times higher μ-receptor affinity than [Leu5]enkephalin, whereas its δ-receptor affinity relative to the latter peptide was nearly 1000 times lower, as determined in the [3H]-DSLET binding assay (Table I). These results indicate that compound 1 displays excellent μ-receptor selectivity, as indicated by the very high ratio of the binding inhibition constants determined at the δ- and the μ-receptor (K_i^δ/K_i^μ = 1880). Reduction of the net positive charge by one unit through substitution of the isosteric norleucine residue for ornithine in position 4 of 1 (compound **1a**) produced a threefold decrease in μ-receptor affinity and a 12-fold increase in δ-receptor affinity. Therefore, compound **1a**, carrying a net charge of 1+, shows only moderate preference for μ-receptors over δ-receptors (K_i^δ/K_i^μ = 51.3). Lengthening the side chain in position 2 of 1 through substitution of norleucine (compound **2**) did not appreciably affect receptor binding affinity and selectivity.

Analogues **3**, **4**, and **5**, containing a D-arginine residue in position 2 and a second basic residue in the 4-position, carry a further increased net positive charge of 3+. These analogues retain similarly high affinity for the μ-receptor as compounds 1 and **2** but, in comparison with the latter analogues, display even lower δ-receptor affinity. The most selective compound turned out to be [D-Arg2,Lys4]-dermorphin-(1–4)-amide (DALDA) (**5**) with a K_i^δ/K_i^μ ratio of 11400. DALDA is 10 times more μ-selective than DAGO (**8**, K_i^δ/K_i^μ = 1050), which hitherto has generally been regarded as one of the most selective μ-agonists available. Dermorphin-(1–4) tetrapeptide analogues containing a D-arginine residue in the 2-position and a neutral residue (glycine or sarcosine) in the 4-position have recently been reported to display high analgesic potency;[24,25] however, the receptor binding selectivity profiles of these compounds had not been determined. Substitution of D-arginine in position 2 of the enkephalin-derived tetrapeptide H-Tyr-Gly-Gly-Phe-OH had resulted in a compound showing only moderate preference for μ-receptors over δ-receptors (selectivity ratio of about 5).[26]

The receptor binding profiles of analogues **1–5** are basically consistent with the membrane compartment concept. Augmenting the positive charge from 1+ (analogue **1a**) to 2+ or 3+ (analogues **1–5**) results in higher μ-receptor affinity, which could be due to increased accumulation of the compounds carrying a high positive charge in the anionic fixed-charge compartment of the membrane, where μ-receptors are supposed to be located.[17] The exception is analogue **3**, which shows slightly lower μ-receptor affinity than **1**, possibly as a consequence of its shorter side chain in the 4-position. Thus, μ-receptor affinity may also depend on the length of the side chain in position 4 of these analogues. More convincingly, the gradual augmentation of the positive charge within this series of compounds leads to a progressive decrease in δ-receptor affinity, presumably due to increasing electrostatic repulsion in the positively charged aqueous compartment which, according to the membrane compartment concept, contains δ-receptors. However, it can, of course, not be ruled out that direct electrostatic peptide–receptor interactions as a consequence of the charge(s) introduced into the peptide may also strengthen or weaken binding at a particular receptor type and, thereby, alter the receptor selectivity profile.

The cyclic hexapeptide analogue **6** was found to be about 3 times less μ-selective than the cyclic parent tetrapeptide analogue **6a**. This is due to the fact that, in comparison with **6a**, the hexapeptide containing two Arg residues at the C-terminus has somewhat lower affinity for the μ-receptor and slightly higher affinity for the δ-receptor. A similar μ-receptor selectivity loss was also observed with the linear hexapeptide analogue **7** relative to the parent tetrapeptide analogue **7a**, again as a consequence of lower μ-receptor affinity and higher δ-receptor affinity. These results can be interpreted to be in disagreement with the predictions made on the basis of the membrane com-

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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 IC50 ratio
		IC50, nM	rel potency[b]	IC50, nM	rel potency[b]	
1	H-Tyr-D-Nva-Phe-Orn-NH_2	104 ± 15	2.36 ± 0.33	271 ± 63	0.0420 ± 0.0098	2.61
1a	H-Tyr-D-Nva-Phe-Nle-NH_2	30.6 ± 2.2	8.04 ± 0.58	153 ± 44	0.0745 ± 0.0212	5.00
2	H-Tyr-D-Nle-Phe-Orn-NH_2	168 ± 33	1.46 ± 0.29	2290 ± 270	0.00498 ± 0.00058	13.6
3	H-Tyr-D-Arg-Phe-A_2bu-NH_2	257 ± 56	0.956 ± 0.208	659 ± 123	0.0173 ± 0.0032	2.56
4	H-Tyr-D-Arg-Phe-Orn-NH_2	346 ± 121	0.712 ± 0.249	1090 ± 279	0.0105 ± 0.0027	3.15
5	H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA)	254 ± 27	0.967 ± 0.102	781 ± 146	0.0146 ± 0.0027	3.07
6	H-Tyr-D-Orn-Phe-Asp-Arg-Arg-NH_2	40.9 ± 12.5	6.01 ± 1.83	745 ± 161	0.0153 ± 0.0033	18.2
6a	H-Tyr-D-Orn-Phe-Asp-NH_2	36.2 ± 3.7	6.80 ± 0.69	3880 ± 840	0.00294 ± 0.00064	107
7	H-Tyr-D-Ala-Phe-Phe-Arg-Arg-NH_2	21.6 ± 1.1	11.4 ± 0.6	1300 ± 400	0.00875 ± 0.00270	60.2
7a	H-Tyr-D-Ala-Phe-Phe-NH_2	3.23 ± 0.14	76.2 ± 3.2	797 ± 240	0.0143 ± 0.0043	247
8	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol (DAGO)	28.3 ± 3.7	8.69 ± 1.14	950 ± 269	0.0120 ± 0.0034	33.6
9	[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.

partment concept. However, it can also be argued that the two arginine residues added at the C-terminus cause some steric interference at the μ-receptor, resulting in lower affinity. Furthermore, the two positively charged Arg residues are at some distance from the N-terminal "message" domain of the peptide and, therefore, repulsion from the proposed cationic environment of the δ-receptor may no longer be effective. In contrast to analogues **6** and **7**, compounds **1–5** contain two or three positive charges in or close to their "message" segments.

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 II). In particular, the obtained IC50(MVD)/IC50(GPI) ratios were in some cases much lower than the corresponding K_i^δ/K_i^μ ratios determined in the binding assays. This was especially the case with analogues **1–5**, due to the fact that these compounds showed much higher potencies in the MVD assay than was expected on the basis of the δ-receptor affinities determined in the [^{3}H]DSLET binding assay and slightly lower potencies in the GPI assays in relation to the measured μ-receptor affinities ([^{3}H]DAGO binding assay). The unexpectedly high potencies of these compounds in the MVD assay are due to the fact that they produce the opioid effect through interaction with μ-receptors which are also present in the vas. That this is indeed the case is clearly indicated by the low K_e values (1.01–1.64 nM) for naloxone as antagonist determined with these compounds in the MVD assay (Table III). Such low K_e values are typical for μ-receptor interactions.[23] Interaction with μ-receptors on the vas is also responsible for the low IC50(MVD)/IC50(GPI) ratio (33.6) observed with DAGO in contrast to its relatively high K_i^δ/K_i^μ ratio (1050) determined in the binding assays (Tables I and II), as suggested previously.[4] To some extent, the discrepancies between binding assay and bioassay data may also be due to a different degree of nonspecific adsorption of these highly charged molecules in the various tissues. In this context, it is of interest to note that analogue **1a**, carrying a net charge of only 1+, shows better agreement between binding assay and bioassay data. Since all examined analogues contain a D-amino acid residue in position 2 and a C-terminal carboxamide function, they can be expected to be equally stable against enzymolysis under the conditions of the binding assays and bioassays[27] and, thus, a different extent of peptide degradation in the various tissues can be ruled out as a factor explaining the differences between bioassay and binding assay data. In contrast to compounds **1–5**, analogues **6**, **6a**, **7**, and **7a** showed

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Table III. K_e Values (Naloxone) of Positively Charged Opioid Peptide Analogues in the Guinea Pig Ileum and Mouse Vas Deferens Assay

no.	compd	K_e,[a] nM	
		GPI	MVD
1	H-Tyr-D-Nva-Phe-Orn-NH_2	1.55 ± 0.23	1.47 ± 0.22
1a	H-Tyr-D-Nva-Phe-Nle-NH_2	1.72 ± 0.42	1.27 ± 0.11
2	H-Tyr-D-Nle-Phe-Orn-NH_2	1.01 ± 0.18	1.50 ± 0.41
3	H-Tyr-D-Arg-Phe-A_2bu-NH_2	1.32 ± 0.05	1.01 ± 0.23
4	H-Tyr-D-Arg-Phe-Orn-NH_2	1.24 ± 0.12	1.13 ± 0.15
5	H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA)	1.17 ± 0.08	1.64 ± 0.36
6	H-Tyr-D-Orn-Phe-Asp-Arg-Arg-NH_2	2.03 ± 0.27	2.02 ± 0.52
6a	H-Tyr-D-Orn-Phe-Asp-NH_2	1.57 ± 0.22	2.11 ± 0.44
7	H-Tyr-D-Ala-Phe-Phe-Arg-Arg-NH_2	0.570 ± 0.120	1.27 ± 0.65
7a	H-Tyr-D-Ala-Phe-Phe-NH_2	0.789 ± 0.028	1.31 ± 0.22
8	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol (DAGO)	0.800 ± 0.100	1.64 ± 0.23[b]
9	[Leu^5]enkephalin	1.53 ± 0.43	21.4 ± 3.3[c]

[a] Mean of three determinations ± SEM. [b] Gillan et al.[23] [c] Lord et al.[3]

potency ratios relative to [Leu^5]enkephalin in the GPI assay that were somewhat higher than the corresponding potency ratios observed in the [^{3}H]DAGO binding assay, whereas their potency ratios determined in the MVD assay were not drastically higher and in one case (compound **7**) even lower than the corresponding potency ratios obtained in the [^{3}H]DSLET binding assay. Consequently, the IC50(MVD)/IC50(GPI) ratios of these four analogues turned out to be in relatively good agreement with the corresponding K_i^δ/K_i^μ ratios.

In both the GPI and the MVD assay all analogues showed K_e values for naloxone as antagonist in the range 0.570–2.11 nM (Table III). Such low K_e values are typical for μ-receptor interactions and rule out an additional interaction with κ-receptors, since κ-receptor interactions would be characterized by much higher K_e values.[21]

Conclusions

The receptor selectivity profiles of some of the positively charged dermorphin analogues described in this paper are in agreement with the predictions made on the basis of the membrane compartment concept. In particular, the dermorphin tetrapeptide analogues **1–5**, carrying an effective positive charge in their N-terminal message segment, showed high preference for μ-receptors over δ-receptors. Within the latter series of compounds, increasing the positive charge from 1+ to 2+ to 3+ resulted in progressively higher μ-receptor selectivity. Presumably, the gradual augmentation of the positive charge led to in-

Table IV. Analytical Data of Opioid Peptide Analogues

no.	compd	amino acid anal.	TLC, R_f BAW	TLC, R_f BPAW	HPLC[a] K' value
1	H-Tyr-D-Nva-Phe-Orn-NH_2	Tyr, 0.98; Nva, 0.94; Phe, 1.00; Orn, 1.04	0.27	0.67	1.25
1a	H-Tyr-D-Nva-Phe-Nle-NH_2	Tyr, 1.00; Nva, 0.97; Phe, 1.02; Nle, 0.95	0.80	0.79	3.08
2	H-Tyr-D-Nle-Phe-Orn-NH_2	Tyr, 0.94; Nle, 0.96; Phe, 1.09; Orn, 1.00	0.29	0.68	2.60
3	H-Tyr-D-Arg-Phe-A_2bu-NH_2	Tyr, 1.00; Arg, 0.95; Phe, 1.01; A_2bu, 1.04	0.13	0.58	1.23
4	H-Tyr-D-Arg-Phe-Orn-NH_2	Tyr, 0.97; Arg, 0.96; Phe, 1.00; Orn, 1.05	0.10	0.57	1.19
5	H-Tyr-D-Arg-Phe-Lys-NH_2	Tyr, 0.96; Arg, 1.03; Phe, 0.96; Lys, 1.00	0.13	0.57	1.23
6	H-Tyr-D-Orn-Phe-Asp-Arg-Arg-NH_2	Tyr, 1.00; Orn, 1.07; Phe, 1.01; Asp, 1.05; Arg, 1.92	0.04	0.50	1.54
7	H-Tyr-D-Ala-Phe-Phe-Arg-Arg-NH_2	Tyr, 1.03; Ala, 0.89; Phe, 2.00; Arg, 1.93	0.09	0.61	0.96
7a	H-Tyr-D-Ala-Phe-Phe-NH_2	Tyr, 1.02; Ala, 0.95; Phe, 2.00	0.62	0.79	4.20

[a] 0.1% TFA/MeOH: 55/45 (compounds 1, 1a, 2, 7, 7a), 65/35 (compounds 3, 4, 5, 6); flow rate 1.5 mL/min, monitored at λ = 290 nm.

creasing accumulation of the peptides in the μ-receptor containing anionic fixed-charge compartment and progressive repulsion from the positively charged environment of the δ-receptor in the aqueous compartment. On the other hand, the hexapeptide analogues 6 and 7, containing two positively charged Arg residues at the C-terminus, showed a decrease rather than the expected increase in μ-receptor selectivity relative to their less charged parent tetrapeptides. Obviously, the structural modifications performed to introduce positive charges into the various peptides could also affect the peptide–receptor interaction per se, for example through steric or direct electrostatic effects.

The most selective among the tetrapeptide analogues, H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA) (5), shows extremely high preference for μ-receptors over δ-receptors, being more than 10 times as μ-selective as DAGO. In fact, the extraordinary μ-receptor selectivity of DALDA (K_i^{δ}/K_i^{μ} = 11 400) is of the same order of magnitude as that of recently developed somatostatin analogues showing antagonism at the μ-receptor.[28] Thus, both agonists and antagonists with nearly total specificity for the μ-receptor are now available.

Because of its high positive charge, DALDA most likely will not be able to cross the blood–brain barrier to any significant extent. It may thus be the case that, aside from its high μ-receptor preference, this analogue may also show pronounced selectivity for peripheral versus central receptors. Therefore, DALDA may turn out to be a valuable tool for studying peripheral μ-receptor interactions, particularly in relation to peripheral antinociceptive effects and effects on gut motility.

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 × 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. Hydrolysates were analyzed on a Beckman Model 121C amino acid analyzer equipped with a Model 126 Data System integrator. Molecular weights of cyclic peptides 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).

Amino acid derivatives were purchased from IAF Biochem International, Laval, Quebec. Both Boc and Fmoc amino acids were used for the preparation of the various peptides, and side-chain protection was as follows: (a) Boc amino acids, tosyl (Arg), benzyloxycarbonyl (Orn), 2-chlorobenzyloxycarbonyl (Lys), Boc (Tyr); and (b) Fmoc amino acids, Boc (A_2bu, D-Orn), *tert*-butyl (Asp). All peptides were prepared by the manual solid-phase technique, using *p*-methylbenzhydrylamine resin (1% cross-linked, 100–200 mesh, 0.38 mequiv/g of titratable amine) also obtained from IAF Biochem International.

Solid-Phase Synthesis and Purification of Opioid Peptide Analogues. Linear peptides 1, 1a, 2, 4, 5, 7, and 7a were assembled on the resin with Boc amino acids according to a protocol described elsewhere.[29] Fmoc amino acids were used for the assembly of the C-terminal tripeptide segment of peptide 3 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 (completion of the reaction was monitored with the ninhydrin test);[30] (3) Fmoc deprotection with 50% piperidine in CH_2Cl_2 (1 × 30 min); (4) washing with DMF (3 × 1 min), and EtOH (3 × 1 min). After removal of the N-terminal Fmoc group at the tripeptide level the peptide chain was completed by coupling Boc-Tyr(Boc)-OH.

The C-terminal exocyclic dipeptide segment of cyclic peptide 6 was assembled by coupling Boc-Arg(Tos)-OH twice in a row according to the usual protocol.[29] The subsequent tripeptide segment to be cyclized was put together with Fmoc amino acids following the protocol described above. After coupling of Fmoc-D-Orn(Boc)-OH, Fmoc protection of the N-terminal amino group was maintained and the side chains of the Orn and Asp residues were deprotected by treatment with 50% (v/v) TFA in CH_2Cl_2 (1 × 30 min). Following neutralization with 10% (v/v) DIEA in CH_2Cl_2 (2 × 10 min) and washing with CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min), cyclization was 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 essentially complete after 6 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 completed by DCC coupling of Boc–Tyr(Boc)-OH.

After assembly of the desired peptide chains, the resins were washed with CH_2Cl_2 (3 × 1 min) and EtOH (3 × 1 min) and were dried in a dessicator. Peptides were cleaved from the resins 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 per gram of resin). After evaporation of the HF, the resins were extracted three times with diethyl ether, and subsequently, three times with 7% acetic acid. The crude peptides were then obtained in solid form through lyophilization of the acetic acid extracts.

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,[31] using a linear gradient of 0–80% MeOH in 1% TFA. If necessary, further purification to homogeneity was performed by semipreparative reversed-phase HPLC (20–50% MeOH (linear gradient in 0.1% TFA)). In the

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case of cyclic lactam analogue 6, HPLC analysis of the crude product revealed the presence of two major components. Separation was easily achieved and analysis by FAB mass spectrometry indicated that the faster and the slower eluting components had MH^+ values of 852 and 1703, respectively. Obviously, the slower eluting peak corresponded to the side chain linked, antiparallel dimer that had been formed through interchain cyclization. Cyclodimerization was favored over cyclic monomer formation (66% dimer, 34% monomer). Final products were obtained as lyophilisates. Homogeneity was established by TLC and HPLC. All peptides were at least 95% pure, as judged from the HPLC elution profiles. Analytical parameters are presented in Table IV.

The synthesis of analogue **6a** has been described elsewhere.[20] DAGO was purchased from IAF Biochem International.

Binding Assays and Bioassays. Receptor binding studies with rat brain membrane preparations were performed as reported in detail elsewhere.[32] [^{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. IC50 values were determined from log dose–displacement curves and K_i values were calculated from the obtained IC50 values by means of the equation of Cheng and Prusoff,[33] using values of 1.3 and 2.6 nM for the dissociation constants of [^{3}H]DAGO and [^{3}H]DSLET, respectively.[4,34]

The GPI[35] and MVD[36] bioassays were carried out as reported in detail elsewhere.[32,37] A log dose–response curve was determined with [Leu^5]enkephalin as standard for each ileum and vas preparation, and IC50 values of the compounds being tested were normalized according to a published procedure.[38] 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.[39]

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Registry No. 1, 118476-81-6; **1a**, 118476-88-3; **2**, 118476-82-7; **3**, 118476-83-8; **4**, 118476-84-9; **5**, 118476-85-0; **6**, 118476-86-1; **6a**, 96382-72-8; **7**, 118494-42-1; **7a**, 118476-87-2; BOC-Tyr(BOC)-OH, 20866-48-2; BOC-Arg(Tos)-OH, 13836-37-8; Fmoc-D-Orn-(BOC)-OH, 118476-89-4.

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Preparation and Antitumor Activity of Additional Mitomycin A Analogues

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On the basis of qualitative structure–activity relationships developed in the preceding article,[2] a series of 32 new mitomycin A analogues were prepared and tested in antitumor screens. Seven of them gave greater prolongation of life (ILS) than mitomycin C in the mouse P388 leukemia assay. They included examples with 7-O substituents such as cyclic ethers and nitrogen heterocycles. A Hansch analysis was attempted with log P and MR as the independent variables, but no statistically significant correlation could be made. Seven compounds, chosen mainly for their good potency (MED), were tested in the subcutaneous B16 melanoma assay in mice and four of them showed greater ILS than mitomycin C.

The synthesis and antitumor activity of mitomycin C analogues have been extensively investigated, resulting in data on about 500 new compounds. In contrast, relatively few mitomycin A analogues (7-methoxymitosanes) have been prepared, despite the high potency (in terms of minimal effective dose, MED) of mitomycin A against P388 leukemia and subcutaneous B16 melanoma in mice.[1] We recently addressed the question of mitomycin A analogues by preparing and testing a group of 26 compounds in which the 7-methoxy group was replaced by a wide variety of substituted alkoxy groups.[2] Many of these analogues were superior to mitomycin C against the two mouse tumors noted above. Although statistically significant QSAR could not be established for this set of analogues, we suggested that, on the basis of qualitative guidelines, future analogues might emphasize 7-O substituents including hydrophilic straight chains, cyclic ethers, and tertiary amines. In the present research, these guidelines are explored further. As described below, we have prepared and screened a series of 32 new analogues, most of which contain the type of 7-O substituents named above. A few new types of analogues, containing cyclopropyl, aryl, or silyl groups, also have been studied.

Chemistry

Two different methods were used for the preparation of mitomycin A analogues. One involved an alkoxide exchange in which mitomycin A was treated with alkali in

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