Articles

Synthesis Using a Fmoc-Based Strategy and Biological Activities of Some Reduced Peptide Bond Pseudopeptide Analogues of Dynorphin A[1]

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Eight analogues of Dyn A(1-11)-NH_2 incorporating the enzymatically stable $\Psi(CH_2-NH)$ isosteric peptide bond replacement were synthesized and tested for binding affinity at the central opioid μ, δ, and κ receptors in guinea pig brain (GPB) homogenates and for activity at the peripheral κ (and μ) receptors in the guinea pig ileum (GPI). The peptidic analogues were synthesized by solid phase techniques using a Fmoc/*tert*-butyl strategy, and the $\Psi(CH_2-NH)$ bond, or reduced bond, was introduced via reductive alkylation of the N-terminal amino group of the growing peptide with a Fmoc-N^{α}-protected amino aldehyde. The synthesis of Fmoc-N^{α}-protected amino aldehydes also is described. Several other peptides have been previously synthesized incorporating this modification and showed for instance increased enzymatic stability and antagonist properties. Results obtained in the GPB show that modifications of the peptide bond in the address site (analogues **4-9**) do not affect the binding at the κ receptor and, with a few exceptions, at the μ and δ receptors. On the other hand, analogues **2** and **3**, modified in the message segment of Dyn A(1-11)-NH_2, show a decrease in binding affinity at all three receptors. In the GPI, the results are more varied as the influence of the peptide bond modification seems to be more important than in the GPB. Finally, selected analogues were tested with no indication for antagonist activity at the κ peripheral receptor.

Introduction

Dynorphin A (Dyn A) is a 17 amino acid peptide that interacts preferentially with κ opioid receptors in a variety of tissue preparations and is thus postulated to be an endogenous ligand for these receptors.[2] It was first isolated and identified from the porcine pituitary.[3] Dyn A (sequence shown below) is formed by a message

Dyn A: H-Tyr[1]-Gly-Gly-Phe-Leu[5]-Arg-Arg-Ile-Arg-Pro[10]-Lys-Leu-Lys-Trp-Asp[15]-Asn-Gln[17]-OH

site (residues 1-4, triggering the physiological effects) and an address site (residues 5-17, responsible for the κ site selectivity).

κ receptors have been shown to mediate analgesia.[4] To further understand the pharmacology of κ receptors and their ligands, which involve a lower abuse potential and a milder form of dependence and withdrawal symptoms in comparison to the prototypic μ opiate morphine, it is necessary to develop stable, highly potent, and selective ligands for these receptors and their subtypes,[5,6] as well as antagonists.[7] Also, selective κ ligands could be of therapeutic importance as new treatments for head injury and stroke.[8] Nevertheless, adverse side effects have been implicated with the κ opioid ligands, e.g. dysphoria, psychotomimesis, and diuresis.[9] It also has been reported that Dyn A can induce a hindlimb paralysis and spinal cord injury in the rat that is not opioid-receptor-mediated.[10] Finally, the κ receptor has been recently cloned from the mouse brain[11] and is found to belong, as do the μ and δ receptors,[11,12] to the G-protein coupled receptor family characterized by seven transmembrane helical segments, for which a proposed three-dimensional model exists for some of the members.[13] No model of Dyn A docked into the κ receptor has yet been proposed, and only a few studies regarding the possible interactions between the ligand and its receptor have been reported.[14] Structure-function relationships of dynorphin-related peptides have been reviewed extensively.[15] Especially, sequential removal of amino acids from the C terminus has shown that deletion of residues 14-17 or even 12-17 does not significantly affect Dyn A potency,[16] thus allowing us to use the shorter analogue Dyn A(1-11)-NH_2 as a template.[17,18]

Several pseudo-peptide analogues of tetragastrin, somatostatin, CCK, bombesin, substance P, growth hormone releasing factor, secretin, neurotensin, and peptide T incorporating the enzymatically stable $\Psi(CH_2-NH)$, or reduced bond, peptide bond replacement have been synthesized in the last decade, both by solution and solid-phase peptide synthesis methods.[19-27] Biological results obtained with these analogues were used to probe the importance of the peptide bonds and backbone, as well as the importance of enzymatic stabilization induced by the reduced bond. Interestingly, a number of the analogues mentioned turned out to be antagonists at their receptors.[19,22,23,25]

Similarly, synthesizing and testing Dyn A analogues incorporating this isosteric replacement seemed very attractive to us, as it would (1) lead to more enzymatically stable peptides (a stable analogue of Dyn A(1-8)

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has been reported to induce analgesia and diuresis in the rat[9,28,29]); (2) allow us to assess the relative importance of the amide bonds and explain further the mode of interaction between Dyn A and its receptor, thus leading possibly to the docking of this peptidic ligand into its receptor; and (3) possibly yield a Dyn A peptide analogue, which would in turn help in understanding the action of Dyn A at the κ receptor. Some antagonists exist to date, but they are neither very potent nor highly selective.[7] Since several analogues of secretin, tetragastrin, bombesin, and substance P incorporating such a peptide bond surrogate have been reported to be antagonists, this could prove to be also the case for Dyn A-like peptides.

Though several other alternatives exist for replacing the CONH peptide bond,[30] the reduced bond has the advantage of being easily incorporated, through reductive alkylation of an unprotected α amino group of the peptide with an α-protected amino aldehyde. This can be readily accomplished by the method developed by Fehrentz and Castro.[31] The fact that this peptide bond replacement can be introduced during the solid phase synthesis of peptides makes it even more attractive.[20,22-25] Nevertheless, one of the drawbacks is that, for all previously synthesized peptides, Boc/benzyl-based strategies were used, making HF (or other harsh acidic media), with all the problems (possible side reactions, danger, and use of special equipment) that go with this method, an almost compulsory choice for the final cleavage/deprotection step.[32] We therefore investigated the possibility of using N^{α}-Fmoc-protected amino acids, with the side chains blocked with *tert*-butyl based protections for (1) the synthesis of N^{α}-Fmoc-protected amino aldehydes and (2) the formation of the CH_2-NH bond, under reductive conditions. The synthesis of several N^{α}-Fmoc-protected amino aldehydes was already published, using a different method (reduction of *S*-benzyl thioesters).[33]

We report here that these two steps can be achieved with the same ease and efficiency as previously published methods for Boc/Benzyl protected amino acids. This Fmoc/*tert*-butyl synthetic strategy was used for the synthesis of eight analogues of Dyn A(1−11)-NH_2. These peptides were tested for their ability to bind at the κ, μ, and δ opioid receptors in guinea pig brain homogenates (GPB) and for agonist, and in some cases antagonist, activity in the guinea pig ileum (GPI).

Results and Discussion

N^{α}-Fmoc-Protected Amino Aldehyde Synthesis. The protected amino aldehydes were prepared in two steps, according to the method developed by Fehrentz and Castro.[31] N^{α}-Fmoc-protected amino acids were reacted in DCM with *N,O*-dimethylhydroxylamine hydrochloride in the presence of BOP reagent and DIEA. The *N,O*-dimethylhydroxamates that were obtained were purified by acid−base washings and transformed without being isolated into the corresponding aldehydes upon action of $LiAlH_4$ in dry THF. The N^{α}-Fmoc-protected amino aldehydes were obtained after hydrolysis of the excess $LiAlH_4$ and acid−base washings. Since N^{α}-Fmoc-protected aldehyde derivatives of arginine with its side chain monoprotected are difficult to obtain in good yield, this residue was replaced by lysine.[34] [Lys^6]-, [Lys^7]-, and [Lys^9]Dyn A(1−11)-NH_2 were synthesized as controls.

Peptide Synthesis. All peptides were synthesized by solid phase methods using a Fmoc/*tert*-butyl strategy on an automated peptide synthesizer.[35] The CH_2-NH isosteres were formed by the reductive alkylation of the preformed protected amino aldehyde with the free amino-terminus of the resin bound peptide, in the presence of an excess $NaCNBH_3$ in 0.5% AcOH in DMF.[20] All components were added at the same time, as advised by Ho et al., to minimize racemization.[36] Completion of the reduction was monitored by the ninhydrin method[37] and was usually completed in less than 3 h. After washing of the resin and N^{α}-Fmoc deprotection, the peptide chain synthesis was completed on the synthesizer. Final deprotection and cleavage was done with a 9/1 TFA/anisole mixture. Purification was achieved by HPLC. Dyn A(1−11)-NH_2 (**1**) was synthesized as a standard. Two peptides that would have belonged in this series, [$^2\Psi^3$,CH_2-NH]Dyn A(1−11)-NH_2 and [$^{10}\Psi^{11}$,CH_2-NH]Dyn A(1−11)-NH_2, could not be obtained. For the first analogue, this was due to dialkylation of Gly^3 by the nonhindered glycine aldehyde, leading to a branched peptide as previously described,[38] and in the second case, we can only postulate that the Pro^{10}-Lys^{11} reduced bond could not be formed either because of a steric problem between the two residues or because of the reduced bond was in a position too close to the polymeric resin support.

Peptides were obtained in sufficient quantities for analysis and biological testing and were found to be single peaks by analytical HPLC (at least 98% pure) at 215 and 280 nm, using two independent gradients, and single spots on TLC in three different solvent systems. The correct mass in each case was observed by fast atom bombardment mass spectroscopy. All analytical results are summarized in Tables 4 and 5 in the Experimental Section.

Binding in the GPB. No significant difference could be found in the GPB binding assay (or the GPI bioassay) between Dyn A(1−11)-NH_2 and the three controls, [Lys^6]-, [Lys^7]-, and [Lys^9]Dyn A(1−11)-NH_2 (data not shown).

Analogues **4**−**9** exhibit a surprisingly strong binding affinity for the κ central receptor (Table 1), with IC_{50} values ranging from 0.16 to 0.90 nM, to be compared to the 0.58 nM binding affinity of Dyn A(1−11)-NH_2. These results indicate that (1) the presence of an extra positive charge on the secondary amine of the peptide bond surrogate beyond position 4 in the peptide does not affect binding at the κ receptor; (2) the added flexibility due to the reduced bond is not a factor and does not influence binding, which tends to show that the favorable spatial conformation, necessary for the interactions between the κ central receptor and our analogues, can still be obtained; and (3) the backbone, or at least the amide bonds affected by these isosteric replacements, does not seem to be involved in the binding process. Analogues **2** and **3** stand out as exceptions, as a 14- and 30-fold decrease in the binding affinity at the κ receptor (IC_{50} = 8.0 and 17.0 nM, respectively) is observed, when compared to Dyn A(1−11)-NH_2. A similar result to the one obtained for analogue **2** has previously been observed at the δ opioid receptor with a [$^1\Psi^2$,CH=CH]Leu-enkephalin analogue.[39,40] This tends to indicate that the Tyr^1-Gly^2 peptide bond, present in both Dyn A and [Leu]-en-

Table 1. Opioid Receptor Binding Affinities and Selectivities of Various Dyn A Analogues in Guinea Pig Brain Homogenate

analogue	reduced bond between	IC_{50} (nM)[a] κ	μ	δ	selectivity μ/κ	δ/μ
1	none	0.58 ± 0.03	9.9 ± 2.0	26 ± 3	18	44
2	Tyr[1]-Gly[2]	8.0 ± 1.5	96 ± 31	198 ± 6	12	25
3	Gly[3]-Phe[4]	17 ± 3	1,035 ± 222	7,997 ± 1216	61	470
4	Phe[4]-Leu[5]	0.55 ± 0.03	12 ± 4	24 ± 3	22	44
5	Leu[5]-Arg[6]	0.61 ± 0.10	0.64 ± 0.10	32 ± 2	1	52
6	Lys[6]-Arg[7]	0.90 ± 0.24	9.0 ± 1.0	53 ± 19	10	59
7	Lys[7]-Ile[8]	0.16 ± 0.01	9.2 ± 2.5	17 ± 1	58	113
8	Ile[8]-Arg[9]	0.36 ± 0.06	4.0 ± 1.0	13 ± 2	11	37
9	Lys[9]-Pro[10]	0.24 ± 0.07	5.2 ± 1.8	18 ± 4	22	75

[a] The radioligands used were [^{3}H]U-69,593 (κ receptor), [^{3}H]DAMGO (μ receptor) and [^{3}H]c[D-Pen^2,*p*-Cl-Phe^4,D-Pen^5] enkephalin (δ receptor).

kephalin, is important for either the proper orientation of the terminal amino group and the phenol group of tyrosine or necessary *per se* by taking part (by, for instance, via hydrogen bonds) in the interactions with the receptors. The same conclusions can be reached regarding the Gly[3]-Phe[4] peptide bond. In a more general way, since the only analogues to display a significant decrease in binding affinity are the ones modified in the message segment of Dyn A (analogues **2** and **3**), these results underline and strengthen the importance of this portion of the peptide for binding at the central κ receptors, as well as they show on the other hand, that these modifications of the address region of Dyn A do not, at least in this study, influence the peptide–receptor interactions.

As shown in Table 1, the μ and δ vs κ selectivities do not differ very drastically in most cases from what is observed for Dyn A(1–11)-NH_2 (**1**) (μ/κ and δ/κ selectivities of 17 and 44, respectively), indicating that the above conclusions regarding the κ receptor and the reduced bond containing Dyn A-like peptides can be extended to the other two opioid central receptors too, at least with respect to dynorphin peptides. There are nevertheless a few exceptions, like **7**, which is more κ selective, primarily due to a stronger affinity (IC_{50} = 0.16 nM) for this receptor. On the other hand, analogue **5**, with a reduced bond in the 5–6 position, shows a dramatically increased affinity for the μ central receptor to the point of being now equipotent at the μ and κ receptors. No fully satisfactory explanation could be found for this observation. Nevertheless, it has been suggested that opioid selection for the μ receptor can be driven by the net positive charge of the message segment of peptide ligands.[41] Since an extra positive charge is present between Leu[5] and Arg[6], this could account for the higher binding affinity of **5** at the μ receptor. On the other hand, we are perfectly aware of the fact that this does not explain why **2**–**4** (which also have an extra positive charge in their message segment) do not present the same binding pattern. More simply, a reduced bond in position 5–6 might take part in highly favorable interactions with the μ receptor, without playing any role at the κ receptor.

Analogue **2**, which exhibits a reduced affinity at the κ receptor, does not show a significant variation in its selectivity pattern, strongly suggesting that the Tyr[1]-Gly[2] amide bond is equally important for recognition and binding at all three central opioid receptors. Nevertheless it appears that the replacement of the Gly[3]-Phe[4] amide bond by the $\Psi(CH_2-NH)$ peptide bond isostere affects the binding at the μ and δ receptors more, as shown by the increase in the μ and δ vs κ selectivities (IC_{50} ratios of 61 and 470, respectively) for analogue **3**.

Table 2. Bioassays with the Smooth-Muscle Tissue of the Guinea Pig Ileum

analogue	reduced bond between	IC_{50} (nM) GPI	shift[a]
1	none	1.1 ± 0.3	ns
2	Tyr[1]-Gly[2]	308 ± 78	ns
3	Gly[3]-Phe[4]	20.5 ± 2.8	ns
4	Phe[4]-Leu[5]	16.6 ± 3.6	ns
5	Leu[5]-Arg[6]	21.0 ± 5.9	ns
6	Lys[6]-Arg[7]	70.6 ± 13.1	ns
7	Lys[7]-Ile[8]	2.8 ± 0.5	ns
8	Ile[8]-Arg[9]	3.7 ± 1.6	ns
9	Lys[9]-Pro[10]	2.1 ± 0.4	ns

[a] ns: no significant shift observed with 1000 nM of CTAP used as a μ receptor antagonist.

Table 3. Central (GPB) vs Peripheral (GPI) Nervous Systems Selectivities at the κ Opioid Receptors of Various Dyn A Analogues

analogue	reduced bond between	ratio of IC_{50} GPI/GPB
1	none	1.8
2	Tyr[1]-Gly[2]	38.5
3	Gly[3]-Phe[4]	1.2
4	Phe[4]-Leu[5]	30.2
5	Leu[5]-Arg[6]	30.4
6	Lys[6]-Arg[7]	78.4
7	Lys[7]-Ile[8]	17.3
8	Ile[8]-Arg[9]	10.4
9	Lys[9]-Pro[10]	3.8

The overall important results of this binding study of reduced peptide bond pseudopeptide analogues of Dyn A to the κ, μ, and δ receptors lay in the fact that, except for **2** and **3**, this backbone modification does not lead to strong changes in affinity or selectivity. Often, introducing one reduced bond in a peptide analogue leads to a dramatic loss (2 or even 3 orders of magnitude) in binding affinity at the receptor, as has been the case for bombesin, substance P, and secretin analogues.[22,23,25] The differences in the binding results observed here could indicate that opioid receptors in general, and κ ones in particular, do not use the ligands backbones to create favorable interactions, or at least not as much as some other peptide/receptor pairs. Moreover, it appears that the flexible reduced bond is not able to disrupt the binding conformation of Dyn A(1–11)-NH_2 which has been postulated to be, at the vicinity of membranes at least, α helical.[41–43]

Activities in the GPI and Central vs Peripheral Selectivities (Tables 2 and 3). The results obtained for the bioassay in the guinea pig ileum appear to be more varied and complex to analyze than the ones

outlined above for the central opioid receptors. It is first important to note that all analogues tested, including Dyn A(1−11)-NH_2, interact only with the κ peripheral receptors. Effectively, both μ and κ receptors are present in the GPI. Since no significant shift in activity can be observed upon addition of the potent μ antagonist CTAP,[44] this proves that the effects observed are due to interactions with κ receptors.

Analogues **7**, **8**, and **9** are the only ones to display potencies similar to that of **1** (IC_{50} = 2.8, 3.7, and 2.1, respectively, compared to 1.1 for **1**). Due to slightly better binding affinities in the GPB, the IC_{50} ratios, or selectivity between the central and peripheral receptors, are higher than for Dyn A(1−11)-NH_2 with ratios of 17.3, 10.4 and 3.8, respectively, compared to 1.8 for **1**. All other analogues show much higher decreases in potency, ranging from 16.6 nM for analogue **4** to as weak as 308 nM for analogue **2**. This corresponds to a 15−280-fold drop in potency when compared to **1**. Analogue **2** displays the lowest potency, a result which is similar to the one obtained in the GPB. Surprisingly, analogues **4** to **6**, with high affinities for the central κ receptor, have much lower potencies in the GPI (IC_{50} values ranging from 16.6 to 70.6 nM), outlining the fact that, as observed before for Dyn A-like peptides,[18] the requirements for good interactions between the ligand and the receptor are different at the central and peripheral levels. Illustrating this are the selectivity ratios, as given in Table 3, for analogues **2**, **4**, **5**, and **6**, which range from between approximately 30 and 80.

Though direct comparison between binding affinity in the GPB and biological activity in the GPI is not possible, the IC_{50} ratios, as given in Table 3, could still be a good preliminary tool to detect antagonist characteristics for some compounds. In addition, large discrepancies between central and peripheral IC_{50} values also could be due to kinetic and efficacy factors. Therefore the antagonist hypothesis has to be further tested (see below). Finally, analogue **3** behaves quite differently from the other peptides tested, as it displays the lowest binding affinity (17 nM for the κ receptor) but only a moderate loss in potency in the GPI (IC_{50} = 20.5 nM), therefore being almost equipotent at the central and peripheral κ receptors. If indeed the two κ receptor types are different, it seems likely that the Tyr^1-Gly^2 and Gly^3-Phe^4 amide bonds interact differently with the central and peripheral receptors, or that at least the magnitude of their effects is variable.

Antagonist Activities in the GPI. Since analogues of bombesin, substance P, and secretin incorporating the $\Psi(CH_2-NH)$ modification have been shown to be antagonists,[22,23,25] some of our analogues were tested in the GPI for possible antagonist activities at the κ and μ receptors. As they display the lowest potencies in the GPI, as well as the highest central vs peripheral selectivity, analogues **2** and **6** were tested in the GPI preparation against Dyn A(1−13)-NH_2, a prototypical κ agonist. No shift in the dose−response curve of Dyn A(1−13)-NH_2 could be observed upon addition of **2** or **6**, therefore indicating these two peptides were not antagonists at the κ receptor.

Compound **5** was surprisingly potent at the μ central receptor (IC_{50} = 0.64 nM), but nevertheless, no shift in potency in the GPI could be observed upon addition of the potent μ antagonist CTAP. A possible explanation was that **5** could actually be an antagonist at the μ receptor. Therefore this peptide was tested in the GPI preparation against the μ agonist PL-017 and did not induced a shift in the dose−response curve of PL-017. This proved the lack of antagonist activity of analogue **5** in the GPI.

These results show that (1), as mentioned before the central and peripheral κ receptors either are different in their requirements for interacting with opioid ligands or other factors, such as efficacy, should be taken into account and (2) the same can be said for the μ receptor: a peptide-like analogue **5** displays a high affinity for the central μ receptor without showing any activity, agonist or antagonist, at the peripheral μ receptor.

Conclusion

This study demonstrates that it is possible (1) to synthesize aldehyde derivatives of common amino acids protected by N^{α}-Fmoc (α-amino-group protections) and *tert*-butyl type groups (side chain protections); and 2) to incorporate those amino aldehydes into a peptide under reductive conditions to create the $\Psi(CH_2-NH)$ peptide bond isostere. No synthetic difficulties were encountered and this adaptation of previous work[31] makes the final deprotection step much easier and safer.

The biological results obtained with the eight Dyn A(1−11)-NH_2 analogues emphasizes the fact that the amide bonds present in the address segment of the peptide (analogues **4**−**9**) do not seem to participate in the binding interaction, at least at the central κ, μ, and δ opioid receptors. On the other hand, analogues **2** and **3**, in which the peptide bond modification occurs in the message segment, show decreased binding affinities by at least 1 order of magnitude at the κ receptor, indicating that the corresponding amide bond in the parent peptide are important either because they interact directly with the receptor (the amide bond is replaced by a positively charged bond) or because they position properly the two aromatic rings of Tyr^1 and Phe^4, as the rigid amide bond is replaced by the more flexible reduced bond.

Incorporating a $\Psi(CH_2-NH)$ peptide bond isostere in Dyn A(1−11)-NH_2 also seems to have a greater effect on the potencies in the periphery, since only three analogues (**7**−**9**) display potencies close to that of **1**, thus indicating a difference between the central and peripheral receptors. Also, selected analogues did not show any antagonist properties at the κ or μ receptors.

All these analogues highlight the importance of some peptide bonds in Dyn A(1−11)-NH_2 for recognition by the opioid receptors and could help us understand the mode of interaction between κ ligands and receptors. Finally, the stability of these analogues is currently being tested, as some of them should be more resistant to enzymatic degradation. This should provide us with new tools to explore the pharmacology of κ receptors and their ligands.

Material and Methods

Amino Aldehyde Synthesis.[31] The synthesis of the aldehyde derivative of N^{α}-Fmoc-Lys(N^{ϵ}-Boc)-OH is given as an example.

Step 1: Preparation of *N*-Methoxy-*N*-Methyl-N^{α}-Fmoc Hydroxamates: N^{α}-Fmoc-Lys(N^{ϵ}-Boc)-OH (2 mmol, 937 mg), BOP reagent (1.1 equiv, 972 mg), and DIEA (1.15 equiv, 400 μL) were dissolved in 5 mL of DCM. After 5 min of stirring

Table 4. Analytical Properties of Various Dyn A Analogues

analogue	reduced bond between	TLC R_f values[a] I	II	III	K' HPLC values[b] 1	2	FAB-MS[c]
1	none	0.65	0.20	0.80	3.4	5.0	1362 [M$^+$]
2	Tyr^1-Gly^2	0.65	0.15	0.85	3.4	5.0	1348 [M$^+$]
3	Gly^3-Phe^4	0.70	0.20	0.75	3.3	4.9	1348 [M$^+$]
4	Phe^4-Leu^5	0.60	0.25	0.85	3.3	5.2	1348 [M$^+$]
5	Leu^5-Arg^6	0.65	0.20	0.80	3.1	5.1	1348 [M$^+$]
6	Lys^6-Arg^7	0.65	0.25	0.90	3.5	4.8	1320 [M$^+$]
7	Lys^7-Ile^8	0.65	0.25	0.85	3.1	4.2	1348 [M$^+$]
8	Ile^8-Arg^9	0.55	0.20	0.90	3.1	4.6	1348 [M$^+$]
9	Lys^9-Pro^{10}	0.55	0.15	0.85	3.6	4.3	1320 [M$^+$]

[a] Solvent systems: I, 1-butanol/pyridine/acetic acid/water (15/10/3/8); II, 1-butanol/acetic acid/water (4/1/5); III, 2-propanol/concentrated ammonium hydroxide/water (3/10/10). [b] 1 and 2 refer to the HPLC gradients as described below in Materials and Methods. [c] Fast atom bombardment mass spectroscopy.

at room temperature, a solution of *N,O*-dimethylhydroxylamine hydrochloride (1.2 equiv, 200 mg) and DIEA (1.3 equiv, 453 μL) in 5 mL DCM was added. After 1 h, 50 mL of DCM was added and the organic phase was washed twice with 3 N HCl, once with water, and twice with a saturated aqueous solution of $NaHCO_3$, before being dried with brine and $MgSO_4$. The solvent was removed under reduced pressure. The *N,O*-dimethylhydroxamate was obtained as an oily residue that crystallized upon refrigeration. An analytical HPLC analysis showed that the product was at least 95% pure (yield = 92%).

Step 2: Preparation of N^{α}-Fmoc Amino Aldehyde. The *N,O*-dimethylhydroxamate derivative of N^{α}-Fmoc-Lys(N^{ϵ}-Boc)-OH (1.5 mmol, 768 mg) was dissolved in 20 mL of dry THF. Contrary to the N^{α}-Boc derivatives, N^{α}-Fmoc-*N,O*-dimethylhydroxamates were not easily soluble in ether. $LiAlH_4$ (1M solution in ether, 5 mL) was added to the solution for 10 min at room temperature under dry N_2. Then a 10% citric acid aqueous solution was added slowly to destroy the excess hydride. Solvents were removed under reduced pressure, and the oily residue was taken up in DCM. Insoluble materials were filtered off. The organic phase was washed with 3 N HCl, water, and $NaHCO_3$ and dried as described above in step 1. DCM was finally evaporated, yielding the amino aldehyde as a slightly yellow oil. The amino aldehyde was then used without further characterization.

Incorporation of the Ψ(CH_2–NH) Peptide Bond Surrogate via Reductive Alkylation.[36] The N-terminal free peptide was washed three times with 1% AcOH in DMF to remove any residual traces of piperidine used during the Fmoc deprotection step. The amino aldehyde (0.75 mmol, 3 equiv) was dissolved in 10 mL of 1% AcOH in DMF and added to the peptide-resin (0.25 mequiv, theoretical). After a few seconds of mixing, a solution of $NaCNBH_3$ (about 100 mg) in 10 mL of DMF was added. The reductive alkylation generally was complete in 3 h, as shown by a negative ninhydrin test.[37] The resin was then drained, washed with DMF and DCM, and dried *in vacuo*. The peptide synthesis was then continued on the automated synthesizer as described below.

Peptide Synthesis and Purification. Peptide syntheses were performed on a 0.25 mmol scale by the solid phase method[32,35,45] utilizing an automated synthesizer (Applied Biosystems Inc. Model 431 A), a Fmoc/*tert*-butyl strategy and a 4-(2′,4′-dimethoxyphenyl-N^{α}-Fmoc-aminomethyl)phenoxy or "Rink" resin (Bachem, Torrance, CA; 0.35 mequiv/g).[46] amino acids (4 equiv) were added sequentially, using diisopropylcarbodiimide with HOBt (4 equiv of each) as coupling reagents in NMP. Side-chain-protected N^{α}-Fmoc amino acids were purchased from Richelieu Biotechnology (Montreal, Canada) and the protections were *O-tert*-butyl for Tyr, Pmc (2,2,5,7,8-pentamethylchroman-6-sulphonyl) for Arg, and N^{ϵ}-Boc for Lys. Deprotection was performed for 2.5 h with 15 mL of a TFA/anisole/water mixture (85/10/5, v/v/v). The resin was filtered off, and the solvents were evaporated under reduced pressure. The oily residue was then taken up in 30% aqueous acetic acid (25 mL) and washed twice with ether (25 mL). After lyophilization, the peptides were purified by HPLC. The yields were not optimized. The structure assignments were corroborated by the results of the amino acid analysis and mass spectroscopy, and the purity of the product was characterized by analytical HPLC and TLC. The analytical data for the purified peptides synthesized are given in Tables 4 and 5.

HPLC was carried out by use of a binary pump (Perkin-Elmer LC 250 model) equipped with an UV/vis detector (Perkin Elmer LC 90 UV model) and integrator (Perkin-Elmer LCI 100 model). For analytical HPLC, the solvent system used was a binary system of water containing 0.1% TFA (pH 2.0) and acetonitrile as the organic modifier, and the solvent programs involved linear gradients as follows: (1) 10 % to 90% acetonitrile over 40 min and (2) 10% to 50% over 40 min. In both cases the flow rate was 1.5 mL/min. The column used had dimensions of 4.5 × 250 mm (Vydac, 10 μm particle size, C-18). HPLC on a semipreparative scale was performed with a reverse-phase column (Vydac 10 × 250 mm, 10 μm particle size, C-18) employing the binary solvent system 1 described above, with a flow rate of 5 mL/min. Mass spectra (fast-atom bombardment, low resolution full scan, glycerol matrix) were performed by the center for Mass Spectroscopy, University of Arizona, Tucson, AZ. Thin-layer chromatography of synthetic peptides was performed on silica plates (0.25 mm, Analtech, Newark, DE) with the solvent systems given in Table 4. Peptides were detected with the ninhydrin reagent.[38] Hydrolyses of the peptides were performed in 4 N methanesulfonic acid (0.2% 3-(2-aminomethyl)indole) at 110 °C for 24 h, and amino acids were analyzed with an automatic analyzer (Bechman Instruments, model 7300). The results are reported in Table 5.

Binding Assays. Membranes were prepared from whole brains taken from adult male guinea pigs (200–400 g) obtained from SASCO. Following decapitation, the brain was removed, dissected and homogenized at 0 °C in 20 volumes of 50 mM Tris-HCl (Sigma, St. Louis, MO) buffer adjusted to pH 7.4 using a Teflon–glass homogenizer. The membrane fraction obtained by centrifugation at 48000*g* for 15 min at 4 °C was resuspended in 20 volumes of fresh Tris-HCl buffer and incubated at 25 °C for 30 min to dissociate any receptor-bound endogenous opioid peptides. The incubated homogenate was centrifuged again as described and the final pellet resuspended in 10 volumes of fresh Tris-HCl buffer. Radioligand binding inhibition assay samples were prepared in an assay buffer consisting of 50 mM Tris-HCl, 1.0 mg/mL bovine serum albumin, 30 μM bestatin, 50 μg/mL bacitracin, 10 μM captopril,

Table 5. Amino Acid Analysis of Various Dyn A Analogues

analogue	reduced bond between	amino acids[a] Tyr	Gly	Phe	Leu	Arg	Ile	Pro	Lys
1	none	1.1 (1)	2.0 (2)	1.1 (1)	1.1 (1)	2.8 (3)	1.1 (1)	0.9 (1)	1.0 (1)
2	Tyr^1-Gly^2		1.0 (1)	0.9 (1)	1.1 (1)	2.9 (3)	0.9 (1)	1.0 (1)	1.0 (1)
3	Gly^3-Phe^4	0.9 (1)	1.1 (1)		1.1 (1)	2.9 (3)	1.1 (1)	1.1 (1)	1.1 (1)
4	Phe^4-Leu^5	1.0 (1)	1.9 (2)			3.0 (3)	0.9 (1)	0.9 (1)	1.0 (1)
5	Leu^5-Arg^6	0.9 (1)	2.1 (2)	1.0 (1)		2.1 (2)	1.1 (1)	0.9 (1)	0.9 (1)
6	Lys^6-Arg^7	1.1 (1)	1.9 (2)	1.1 (1)	1.0 (1)	0.9 (1)	1.1 (1)	1.0 (1)	1.0 (1)
7	Lys^7-Ile^8	1.1 (1)	2.0 (2)	1.0 (1)	1.1 (1)	2.0 (2)		1.0 (1)	0.9 (1)
8	Ile^8-Arg^9	1.0 (1)	2.1 (2)	1.0 (1)	0.9 (1)	1.8 (2)		1.1 (1)	1.0 (1)
9	Lys^9-Pro^{10}	1.0 (1)	2.0 (2)	1.0 (1)	1.1 (1)	1.9 (2)	0.9 (1)		0.9 (1)

[a] Theoretical values in parentheses. Hydrolysis in 4 N methanesulfonic acid (0.2% 3-(2-aminomethyl)indole) at 110 °C for 24 h.

and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 (all from Sigma, St. Louis, MO), except bestatin (Peptides International, Louisville, KY). The radioligands used were [^{3}H]c[D-Pen^2,p-Cl-Phe^4,D-Pen^5]enkephalin[47] (δ receptor) at a concentration of 0.75 nM, [^{3}H]DAMGO (μ receptor) at a concentration of 1.0 nM, and [^{3}H]U-69,593 (κ receptor) at concentration of 1.5 nM (all obtained from New England Nuclear, Boston, MA). Peptide analogues were dissolved in the assay buffer prior to each experiment and added to duplicate assay tubes at 10 different concentrations over a 800-fold concentration range. Control (total) binding was measured in the absence of any inhibitor, while nonspecific binding was measured in the presence of 10 μM naltrexone (Sigma, St. Louis, MO). Nonspecific binding was always less than 10% of the total binding. The final volume of the assay samples was 1.0 mL, of which 10% consisted of the membrane preparation in 0.1 mL Tris-HCl buffer. Incubation were performed at 25 °C for 3 h, after which the samples were filtered through polyethylenimine (0.5% w/v, Sigma, St. Louis, MO) treated GF/B glass fiber filter strips (Brandel, Gaithersburg, MD). The filters were washed three times with 4.0 mL of ice-cold 1 M NaCl solution before being transfered to scintillation vials. The filtrate radioactivity was measured after adding 7–10 mL of cocktail (EcoLiteTM (+), ICN Biomedicals, Inc) to each vial and allowing the samples to equilibrate over 8 h at 4 °C. Binding data were analyzed by a nonlinear least-square regression analysis program named Inplot 4.03 (GraphPadTM, San Diego, CA). Statistical comparisons between one and two site fits were made using the F-ratio test using a p value of 0.05 as the cutoff for significance.[48] Data best fitted by a one-site model were reanalyzed using the logistic equation.[49] Data obtained from at least three independent measurements in duplicate are presented as the arithmetic mean ± SEM.[50] The results are not corrected for the actual peptide content.

In Vitro GPI Bioassay. Electrically induced smooth muscle contraction of strips of guinea pig ileum longitudinal muscle–myenteric plexus was used as a bioassay.[51] Tissues came from male Hartley guinea pigs weighing 250–500 g and were prepared as described previously.[52] The tissues were tied to gold chains with suture silk, suspended in 20 mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.18 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.48 mM glucose), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length previously determined to be 1 g tension and again allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum wire electrodes at 0.1 Hz, 0.4 ms pulses of supramaximal voltage. No peptidase inhibitors were used as no reversal of the initial contraction height inhibition indicative of peptidase activity could be observed with the passage of time. Drugs were added to the baths in 14–60 μL volumes. The agonists remained in contact with the tissue for 3 min before the addition of the next cumulative dose until maximum inhibition was reached. Maximum inhibition of contraction height is reached within 3 min of dosing and longer incubation of the drug would not produce a greater response. Percent inhibition was calculated using the average contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the dose of the agonist. To further define the opioid selectivity of the agonist effect, the μ selective antagonist CTAP was used at a concentration of 1000 nM.[44] To test analogues **2**, **5**, and **6** for κ and μ antagonist activities, a dose causing less than 10% inhibition of contraction height was added to the bath 3 min before begining a cumulative dose–response curve with Dyn A(1–13)-NH_2 (κ receptor) or PL-017 (μ receptor). A shift in the IC_{50} value would reveal antagonist properties.

IC_{50} values represent the mean of two to four tissues. IC_{50} estimates, relative potency estimates, and their associated standard errors were determined by fitting the mean data to the Hill equation by a computerized nonlinear least-squares method.[50] As previously stated, the results are not corrected for the actual peptide content.

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977–983). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations are Dyn A, dynorphin A; Enk, enkephalin; GPB, guinea pig brain; GPI, guinea pig ileum; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; CTAP, c[D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2]; PL-017, Tyr-Pro-N-Methyl-Phe-D-Pro-NH_2; DCM, dichloromethane; U-69,593, *N*-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzo[*b*]furan-4-acetamide; NMP, *N*-methyl-2-pyrrolidinone; DMF, dimethylformamide; DIEA, diisopropylethylamine; BOP, benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate; HOBt, 1*H*-hydroxybenzotriazole.

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