Synthesis and Opioid Activity of Conformationally Constrained Dynorphin A Analogues. 2.[1] Conformational Constraint in the "Address" Sequence[†,‡]

Seksiri Arttamangkul,[§] Jane E. Ishmael,[§] Thomas F. Murray,[§] David K. Grandy,[∥] Gary E. DeLander,[§] Brigitte L. Kieffer,[⊥] and Jane V. Aldrich*[,§,#]

College of Pharmacy and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, Oregon 97331, Vollum Institute for Advanced Biomedical Research and Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland, Oregon 97201, and Laboratoire Protéines et Récepteurs Membranaires, Ecole Supérieure de Biotechnologie de Strasbourg, F-67400 Illkirch, France

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Several cyclic lactam analogues of Dyn A-(1−13)NH_2 were prepared in order to reduce the conformational flexibility in different regions of the native linear peptide. Cyclo[D-Asp^i,Dap^{i+3}]Dyn A-(1−13)NH_2 (Dap = α,β-diaminopropionic acid) analogues were designed on the basis of molecular modeling using AMBER, which suggested that this constraint may be compatible with an α-helix. The cyclic portion of these constrained analogues spanned from residues 3 to 9, a region proposed by Schwyzer (*Biochemistry* **1986**, *25*, 4281) to adopt a helical conformation at κ receptor sites. Analogues containing Dab (α,γ-diaminobutyric acid) or Orn in position $i + 3$ were also synthesized to examine the effects of larger ring size. The cyclic peptides exhibited marked differences in binding affinities for κ, μ, and δ receptors and in opioid activity in the guinea pig ileum (GPI). Cyclo[D-Asp^6,Dap^9]Dyn A-(1−13)NH_2 showed both high κ receptor affinity and potent agonist activity in the GPI, while cyclo[D-Asp^3,Dap^6]Dyn A-(1−13)NH_2 exhibited very weak binding affinity at all opioid receptors as well as very weak opioid activity in the GPI. Cyclo[D-Asp^5,Dap^8]Dyn A-(1−13)NH_2 showed moderate binding affinity for κ receptors and was the most κ selective ligand in this study, but this peptide exhibited very weak agonist activity in the GPI assay. Compared to the corresponding linear peptides, all of the cyclic peptides exhibited decreased μ receptor affinity, while κ receptor affinity was retained or improved. Therefore the corresponding linear peptides were generally μ selective while the cyclic constrained peptides demonstrated slight selectivity for κ vs μ receptors or were nonselective. Increasing the ring size by incorporating Dab or Orn in positions 6, 8, or 9 did not significantly affect the binding affinity for the three opioid receptor types nor the opioid activity observed in the GPI. Circular dichroism spectra of the cyclo[D-Asp^i,Dap^{i+3}] derivatives in 80% trifluoroethanol at 25 and 5 °C suggested differences in the stability of a helical structure when the constraint was incorporated near the N-terminus vs in the middle of the peptide.

Introduction

The study of the physiology and pharmacology of the opioids has been complicated by the existence of multiple forms of both opioid receptors and their endogenous ligands.[2] All of the mammalian endogenous opioid peptides are flexible linear molecules which can interact with more than one type of opioid receptor. The ability of these peptides to adopt numerous conformations may account for their significant affinity for multiple opioid receptors. One goal in opioid research is to develop ligands highly selective for each receptor subtype. These ligands may represent both potential therapeutic agents and valuable pharmacological tools for understanding the biological effects produced by different opioid receptor types. Conformational constraint is an approach which can be used to restrict the flexibility of peptide molecules and to provide information on the topographical requirements of receptors.[3] Highly potent and selective ligands may also be found when appropriate conformational constraints are incorporated.

Dynorphin A (Dyn A) is a highly potent opioid peptide which is postulated to be an endogenous ligand for κ opioid receptors.[4] The selectivity of Dyn A for the κ opioid receptors is low, however, and the peptide retains significant affinity for μ and δ opioid receptors. Dyn A contains an N-terminal tetrapeptide sequence (the "message" sequence)[5] identical to other mammalian opioid peptides with different receptor selectivity. A number of studies[5−9] have examined Dyn A fragments and amino acid replacements to determine the relative importance and structure−activity relationships for individual residues of Dyn A. The tetrapeptide sequence at the C-terminus (residues 14−17) of Dyn A is

* Author to whom correspondence should be addressed. Current address: Department of Pharmaceutical Sciences, School of Pharmacy, 20 N. Pine St., University of Maryland, Baltimore, MD 21201.

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‡ Abbreviations used for amino acids follow the rules of the IUPAC−IUB Joint Commission of Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *138*, 9−37. Amino acids are in the L-configuration except when indicated otherwise. Additional abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; CHO, Chinese hamster ovary; ClZ, 2-chlorobenzyloxycarbonyl; Dab, α,γ-diaminobutyric acid; Dap, α,β-diaminopropionic acid; DAMGO, [D-Ala^2,MePhe^4,Gly-ol]enkephalin; DCM, dichloromethane; DIC, *N*,*N*′-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMA, *N*,*N*-dimethylacetamide; DMF, *N*,*N*-dimethylformamide; DPDPE, cyclo[D-Pen^2,D-Pen^5]enkephalin; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; GPB, guinea pig brain; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; NMR, nuclear magnetic resonance; PAL resin, peptide amide linker or a methylbenzhydrylamine resin with a 5-(4-(aminomethyl)-3,5-dimethoxyphenoxy)valeric acid linker; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PyBOP, (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; Tos, 4-toluenesulfonyl; Z, benzyloxycarbonyl.

§ College of Pharmacy, Oregon State University.

∥ Vollum Institute for Advanced Biomedical Research and Department of Cell Biology and Anatomy.

⊥ Laboratoire Protéines et Récepteurs Membranaires.

Center for Gene Research and Biotechnology, Oregon State University.

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not necessary for opioid binding affinity and opioid activity,[10] and therefore, Dyn A-(1−13) has been utilized more frequently as a prototype for further modification. Elimination of one amino acid at a time from the C-terminus suggested that the unusually high potency and the κ receptor selectivity of Dyn A-(1−13) resulted from the C-terminal sequence (residues 6−13, the "address" sequence).[5] Examination of truncated fragments,[5] an alanine scan of residues 1−11,[6] and substitutions of natural and unnatural amino acids at several positions[7−9] have supported the importance of the basic residues Arg[7] and Lys[11] for both affinity and selectivity for κ receptors, and Arg[6] for selectivity.

Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-LysOH

Dyn A-(1−13)

Like most linear peptides, Dyn A-(1−13) is capable of assuming a number of different conformations. In an aqueous environment, CD and 2D-NMR indicate that Dyn A-(1−13) does not have an ordered conformation.[11] Conformationally constrained Dyn A analogues have been prepared in order to reduce this flexibility. The first conformationally constrained analogue of Dyn A-(1−13), prepared by Schiller and his colleagues,[12] was the disulfide-containing analogue cyclized between D-Cys[2] and Cys[5]. Although this cyclic analogue is more potent than the linear parent peptide in the GPI assay, selective tolerance studies suggested that it does not interact well with κ receptors.[13] Other cyclic disulfide-containing analogues of Dyn A have involved modifications in the address segment.[14−16] These cyclic analogues contained disulfide linkages between Cys, D-Cys, Pen (penicillamine), or D-Pen in positions 5 and 9, 10, 11, or 13; 6 and 10; and 8 and 12 or 13. Several of these cyclic Dyn A analogues exhibit high binding affinities for κ and μ receptors in guinea pig brain (GPB) membrane preparations, while their opioid activities in the guinea pig ileum (GPI) assay are very weak.[14−16] This significant difference in IC_{50} values for opioid receptors in GPB vs GPI suggested the possible existence of different subtypes of κ and μ receptors in the brain and peripheral systems. Cyclization via lactam bridges between D- or L-Orn and Asp has also been performed to give cyclo[D-Orn[2],Asp[5]]Dyn A-(1−8)NH_2 and cyclo-[Orn[5],Asp[8]]-, cyclo[Orn[5],Asp[10]]-, and cyclo[Orn[5],Asp[13]]-Dyn A-(1−13)NH_2.[17] These analogues showed high affinity for μ receptors, however, and antagonism of their opioid activity in the GPI by low doses of naloxone suggested that these peptides did not interact significantly with κ receptors.

In his hypothesis of membrane compartmentalization for opioid receptors, Schwyzer proposed that κ receptor sites are buried in the hydrophobic region of the lipid membrane bilayer.[18] Using equilibrium thermodynamic and kinetic estimations, he examined the ability of the N-terminal message sequence of Dyn A-(1−13) to insert into this hydrophobic environment and proposed that Dyn A-(1−13) adopts a helical conformation extending from Tyr[1] through Arg[9].[19] Recent NMR studies of Dyn A confirmed that the sequence from Gly[3] through Arg[9] adopted a helical conformation when the peptide was bound to lipid micelles.[20] We were interested in stabilizing this suggested helical structure of Dyn A through a lactam linkage. From our preliminary study of possible conformational constraints using the molecular modeling program AMBER,[21−23] we found that a four-atom bridge containing an amide bond between residues *i* (D-configuration) and *i* + *3* (L-configuration) appeared to be optimal for spanning the distance between C_i^{α} and C_{i+3}^{α} in an α-helix. We originally incorporated this constraint into the message sequence to give the analogue cyclo[D-Asp[2],Dap[5]]Dyn A-(1−13)NH_2 (Dap = α,β-diaminopropionic acid) containing this bridge between residues 2 and 5;[1] the analogues with five- and six-atom bridges containing Dab (α,γ-diaminobutyric acid) and Orn, respectively, in position 5 of the peptides were also synthesized in order to evaluate the effect on opioid activity and receptor affinity of varying the bridge length. All of the cyclic [2,5] Dyn A analogues were highly potent agonists in the GPI assay and had high affinity for κ receptors. Cyclo[D-Asp[2],Dap[5]]Dyn A-(1−13)NH_2 exhibited a slight preference for κ over μ receptors whereas increasing the ring size to give cyclo-[D-Asp[2],Dab[5]]- and cyclo[D-Asp[2],Orn[5]]Dyn A-(1−13)NH_2 resulted in peptides which were nonselective for κ vs μ receptors and slightly μ selective, respectively.

We further examined the effects of this constraint in different regions of Dyn A-(1−13)NH_2. The amino acid residues involved in the constraint were carefully selected such that they were not critical residues for κ receptor recognition. The cyclic constraints spanned the sequence from residues 3 to 9, the region of Dyn A-(1−13) which has been proposed to be in an α-helix. The cyclic peptides synthesized were cyclo[D-Asp[3],Dap[6]]-, cyclo[D-Asp[5],Dap[8]]-, and cyclo[D-Asp[6],Dap[9]]Dyn A-(1−13)NH_2. Similar to the [2,5] cyclic series, the larger ring sizes with five- and six-atom bridges were also prepared to investigate the effect of bridge length. We evaluated the cyclic peptides and their corresponding linear peptides (Table 1) for affinity for cloned κ, μ, and δ opioid receptors and for opioid activity in the GPI assay. The marked effect of these constraints on opioid receptor affinities and opioid activity is discussed in detail below.

Table 1. Structure of Cyclic and Linear Dyn A-(1−13)NH_2 Analogues

compd	Dyn A-(1−13)NH_2 analogues
	[3,6] Series
1	cyclo[D-Asp[3],Dap[6]]Dyn A-(1−13)NH_2
2	cyclo[D-Asp[3],Dab[6]]Dyn A-(1−13)NH_2
3	cyclo[D-Asp[3],Orn[6]]Dyn A-(1−13)NH_2
4	[D-Asp[3],Dap[6]]Dyn A-(1−13)NH_2
5	[D-Asp[3]]Dyn A-(1−13)NH_2
	[5,8] Series
6	cyclo[D-Asp[5],Dap[8]]Dyn A-(1−13)NH_2
7	cyclo[D-Asp[5],Dab[8]]Dyn A-(1−13)NH_2
8	cyclo[D-Asp[5],Orn[8]]Dyn A-(1−13)NH_2
9	[D-Asp[5],Dap[8]]Dyn A-(1−13)NH_2
10	[D-Asp[5]]Dyn A-(1−13)NH_2
	[6,9] Series
11	cyclo[D-Asp[6],Dap[9]]Dyn A-(1−13)NH_2
12	cyclo[D-Asp[6],Dab[9]]Dyn A-(1−13)NH_2
13	cyclo[D-Asp[6],Orn[9]]Dyn A-(1−13)NH_2
14	[D-Asp[6],Dap[9]]Dyn A-(1−13)NH_2
15	[D-Asp[6]]Dyn A-(1−13)NH_2
16	Dyn A-(1−13)NH_2

Results and Discussion

Chemistry. All of the cyclic peptides were synthesized on an MBHA resin by procedures similar to those

Table 2. Opioid Receptor Binding Affinities of Dyn A-(1−13)NH_2 Analogues

	Dyn A-(1−13)NH_2 analogues	K_i (nM)[a]			K_i ratio ($\kappa/\mu/\delta$)
		κ	μ	δ	
		[3,6] Series			
1	cyclo[D-Asp^3,Dap^6]	480 (410−580)	910 (470−1800)	2800 (1400−5400)	1/1.9/5.8
2	cyclo[D-Asp^3,Dab^6]	270 (220−330)	210 (150−290)	1000 (610−1600)	1.3/1/4.8
3	cyclo[D-Asp^3,Orn^6]	200 (160−270)	320 (250−410)	320 (140−740)	1/1.6/1.6
4	[D-Asp^3,Dap^6]	300 (240−390)	100 (59−180)	1300 (710−2500)	3/1/13
5	[D-Asp^3]	28 (18−36)	180 (120−280)	430 (320−460)	1/6.4/15
		[5,8] Series			
6	cyclo[D-Asp^5,Dap^8]	8.0 (6.2−10)	75 (61−93)	3300 (2400−4400)	1/9.4/410
7	cyclo[D-Asp^5,Dab^8]	14 (11−18)	40 (32−50)	4100 (3200−5100)	1/2.9/290
8	cyclo[D-Asp^5,Orn^8]	12 (11−14)	62 (50−76)	1900 (1300−2800)	1/5.2/160
9	[D-Asp^5,Dap^8]	20 (14−29)	8.3 (5.9−12)	1600 (1400−2000)	2.4/1/190
10	[D-Asp^5]	58 (40−86)	6.1 (5.2−7.2)	1300 (1100−1600)	9.5/1/210
		[6,9] Series			
11	cyclo[D-Asp^6,Dap^9]	2.6 (2.0−3.4)	4.4 (3.0−6.5)	48 (27−68)	1/1.7/19
12	cyclo[D-Asp^6,Dab^9]	2.5 (1.9−3.3)	4.8 (3.7−6.1)	180 (170−180)	1/1.9/72
13	cyclo[D-Asp^6,Orn^9]	1.5 (1.2−1.9)	3.2 (2.3−4.3)	28 (25−29)	1/2.1/19
14	[D-Asp^6,Dap^9]	12 (8.7−15)	1.5 (1.0−2.5)	8.2 (7.7−8.6)	8/1/5.5
15	[D-Asp^6]	8.0 (6.4−9.9)	1.2 (0.8−1.7)	22 (18−25)	6.7/1/18
16	Dyn A-(1−13)NH_2	0.31 (0.14−0.69)	0.92 (0.57−1.5)	28.4	1/3/92

[a] 95% confidence intervals are shown in parentheses.

described previously.[1] After incorporation of the last amino acid to the peptide chains, the *tert*-butyl protecting groups were removed with TFA and the cyclizations then performed on the resin using either BOP reagent (BOP/DIEA, 3 and 6 equiv, respectively, compounds **1**−**3** and **6**−**8**) or PyBOP reagent (PyBOP/DIEA, 3/6 equiv, compounds **11**−**13**). Cyclization reactions were monitored by ninhydrin and gave negative or slightly positive responses within 2−5 days. The N-terminal Fmoc protecting groups were removed and the peptide amides then liberated from the resin using liquid HF. In all cases, the desired cyclic peptides were the major components (50−60% by reversed phase HPLC). The identities of the cyclic peptides were verified by FAB-MS and amino acid analysis following purification using gel filtration and reversed phase HPLC (see the Supporting Information). The slow cyclization reactions observed in this study were consistent with the result for cyclization of [D-Asp^2,Dap^5]Dyn A-(1−13)NH_2, in which the cyclization using BOP required 3 days to give a nearly complete reaction (Arttamangkul et al., unpublished results). These difficult cyclizations may be due to the tight constraint of the ring portion in which the side chains involved in the cyclization are separated by only two amino acid residues. The different positions and sizes of the ring portion in these cyclic Dyn A analogues did not exhibit any large effect on rate of the cyclization or the purity of the resulting products.

The linear peptides **4**, **9**, and **14** were obtained from the same syntheses as their corresponding cyclic peptides except that the cyclization steps were omitted. Compounds **5**, **10**, and **15** were synthesized on a PAL resin using a standard Fmoc synthetic protocol. In this case, side chains of the amino acids were protected with TFA-labile functional groups. The side-chain protecting groups were removed simultaneously when the peptides were liberated from the resin using Reagent K.[24] Generally, the peptides were obtained in very good yields, and all of them exhibited the expected results by FAB-MS and amino acid analysis (see the Supporting Information). In the case of compound **15**, reversed phase HPLC showed two distinct peptide products which after purification were characterized using FAB-MS. The major component (75%) was the desired peptide **15**, while FAB-MS suggested that the minor component (25%) was the peptide containing one residual Pmc protecting group.

Receptor Affinities and Opioid Activity. The peptides were evaluated for their binding affinity to κ, μ, and δ opioid receptors using equilibrium competition binding assays. The opioid binding affinities of compounds **1**−**4** were determined using cloned receptors transiently expressed on COS-7 (monkey kidney) cells, and the affinities of compounds **5**−**16** were evaluated using cloned receptors stably expressed in CHO (Chinese hamster ovary) cells. The affinities for κ, μ, and δ receptors were determined by competitive inhibition of the radioligands [^{3}H]diprenorphine, [^{3}H]DAMGO, and [^{3}H]-DPDPE, respectively, and are summarized in Table 2.

Cyclic Dyn A-(1−13)NH_2 analogues constrained in different regions exhibited markedly different binding affinities for opioid receptors. The cyclic [6,9] Dyn A analogues (**11**−**13**) exhibited high-affinity binding to κ and μ receptors (K_i = 1.5−4.8 nM). The binding affinities at these two receptor sites decreased when the cyclic contraint spanned residues 5 and 8 (K_i = 8.0−75 nM) and residues 3 and 6 (K_i = 100−910 nM). Increasing ring size in each series generally did not cause large changes in the binding affinities for κ and μ receptors; the affinities varied by less than 2-fold, except for the cyclic [3,6] analogues where the peptides with the longer bridge lengths (**2** and **3**) exhibited 4.3- and 2.8-fold higher affinities, respectively, than **1** for μ receptors. In each series the relative binding affinities of the linear peptides for κ and μ receptors were reversed compared to the corresponding cyclic peptides; the only exception to this was the linear peptide **4**, which like the corresponding cyclic peptide **1** exhibited very low affinity for κ receptors. The cyclic peptides tended to have increased affinity for κ receptors but decreased affinity at μ receptors when compared to the related linear peptides. The δ receptor binding affinities of the [3,6] and [5,8] peptides were very weak (generally >1 μM), whereas the δ receptor affinities for the [6,9] peptides were comparable to or only slightly lower than that of the parent peptide Dyn A-(1−13)NH_2, **16**.

Among the cyclic Dyn A analogues examined in this study, only the cyclic [6,9] analogues, **11**−**13**, exhibited high binding affinity for κ opioid receptors (K_i = 1.5−2.6 nM). These cyclic peptides also had considerable affinity for μ opioid receptors (K_i = 3.2−4.4 nM) and therefore exhibited negligible selectivity for κ vs μ receptors. The related linear peptide [D-Asp^6,Dap^9]Dyn A-(1−13)NH_2, **14**, exhibited a 5-fold decrease in binding affinity for κ receptors, but a 3-fold increase in affinity for μ receptors compared to the cyclic peptide **11**. Thus the linear peptide **14** was moderately μ-selective, whereas the cyclic peptide **11** did not distinguish between κ and μ receptors. Introduction of a residue with the D-configuration and/or a negative charge at position 6 may be responsible for the decreased κ receptor affinity of **14**. Compound **15**, which also contains a D-Asp at position 6, showed a similar binding profile to **14** at κ and μ receptors and bound preferentially to μ over κ receptors. Compared to Dyn A-(1−13)NH_2, **16**, however, compounds **14** and **15** exhibited 40- and 26-fold decreases in κ receptor affinity, respectively, while retaining similar affinities for μ receptors. While it was previously observed that a positive charge on residue 6 was not critical for κ receptor recognition,[7] it is not surprising that a negative charge at this position could adversely affect the binding at κ receptor sites.

The cyclic peptides in the [5,8] series, **6**−**8**, exhibited modest binding affinity for κ receptors (K_i = 8.0−12 nM), but their weaker affinity for μ receptors (K_i = 40−75 nM) resulted in peptides with selectivity for κ over μ receptors comparable to or slightly greater than that of Dyn A-(1−13)NH_2. The cyclic peptide **6** was the most κ selective ligand in this series and exhibited 3-fold higher selectivity for κ over μ receptors than Dyn A-(1−13)NH_2. Compared to the cyclic peptide **6**, the corresponding linear peptide **9** exhibited a 2.5-fold decrease in binding affinity for κ receptors but a 9-fold increase in binding affinity for μ receptors, resulting in a compound with a slight selectivity for μ receptors. The decrease in κ receptor affinity for compound **9** may be the result of both the D-configuration and a negative charge at position 5, since compound **10** also exhibited weak binding affinity for κ receptors. The negative charge on the aspartate residue probably contributed to the adverse effect on κ receptor affinity, since compound **10** had a 190-fold decrease in binding affinity for κ receptors compared to Dyn A-(1−13)NH_2, while inversion of the stereochemistry at position 5 of Dyn A-(1−11)NH_2 to give [D-Leu^5]Dyn A-(1−11)NH_2 (IC_{50} (κ) = 15.3 nM) resulted in only a 26-fold decrease in κ receptor affinity compared to Dyn A-(1−11)NH_2.[16] It is interesting to note that for the linear peptides a D-Asp at position 5 caused a larger decrease in κ receptor affinity than a D-Asp at position 6; K_i's for compounds **10** and **15** were 58 and 8.0 nM, respectively. This charged group in position 5 of compound **10** also decreased the binding affinities at μ and δ receptors 7- and 46-fold, respectively, compared to the parent peptide **16**; negligible effects were observed in the affinities at μ and δ receptors for the negatively charged group in position 6 of **15**. The large decrease in δ receptor affinity for **10** was probably due to the D-configuration at position 5 since [D-Leu^5]Dyn A-(1−11)NH_2 also possesses very weak binding affinity for δ receptors (IC_{50} = 1740 nM).[16] All of the cyclic analogues **6**−**8** containing a D-amino acid in position 5 also exhibited a large decrease (68−150-fold) in δ receptor affinity.

Table 3. Opioid Activity of Cyclic Dyn A Analogues in the GPI

	Dyn A-(1−13)NH_2 analogues	IC_{50} (nM)[a]
	[3,6] Series	
1	cyclo[D-Asp^3,Dap^6]	1000 (540−2200)
2	cyclo[D-Asp^3,Dab^6]	880 (460−1800)
3	cyclo[D-Asp^3,Orn^6]	2700 (860−9100)
4	[D-Asp^3,Dap^6]	2800 (1600−4900)
5	[D-Asp^3]	440 (270−720)
	[5,8] Series	
6	cyclo[D-Asp^5,Dap^8]	>5000
7	cyclo[D-Asp^5,Dab^8]	1300 (980−1700)
8	cyclo[D-Asp^5,Orn^8]	530 (420−680)
9	[D-Asp^5,Dap^8]	640 (300−1400)
10	[D-Asp^5]	1400 (520−4000)
	[6,9] Series	
11	cyclo[D-Asp^6,Dap^9]	46 (36−60)
12	cyclo[D-Asp^6,Dab^9]	22 (13−35)
13	cyclo[D-Asp^6,Orn^9]	6.8 (4.4−11)
14	[D-Asp^6,Dap^9]	74 (31−180)
15	[D-Asp^6]	29 (20−42)
16	Dyn A-(1−13)NH_2	0.19 (0.13−0.26)

[a] 95% confidence intervals are shown in parentheses.

The cyclic [3,6] Dyn A analogues exhibited large decreases in binding affinities for all three opioid receptor types. The linear peptide **4** containing D-Asp^3 and Dap^6 also exhibited very weak binding affinities at κ, μ, and δ opioid receptors. The binding affinities at κ receptors of the cyclic peptide **1** and its corresponding linear peptide **4** were very similar. The 9-fold decrease in μ receptor affinity of **1** compared to **4** therefore resulted in **1** exhibiting a slight preference for binding at κ over μ receptors, while **4** exhibited a slight preference for μ receptors.

A D-Asp at position 3 in compound **5** decreased binding affinities for κ and μ receptors 100- and 200-fold, respectively, as compared to the parent peptide, **16**. In contrast, the binding affinity for κ receptors of [D-Ala^3]Dyn A-(1−11)NH_2 did not change significantly compared to Dyn A-(1−11)NH_2 while the binding affinity for μ receptors was markedly decreased, resulting in a highly κ selective ligand.[25] Therefore the decrease in κ receptor affinity of compound **5** may be related to the negative charge on residue 3.

A large decrease in κ receptor affinity was unexpectedly observed for compound **4** when Arg^6 of **5** was replaced with Dap^6. Since at physiological pH the side chain of the Dap residue (pK_a = 9.6)[26] could have a positive charge similar to Arg, the difference may be related to the length of the alkyl chain holding this charged group. Schwyzer proposed that the methylene chains of Arg^6, Arg^7, and Arg^9 function as "snorkels" holding the positive charges to the membrane surface.[27] The methylene chain of the side-chain Dap, however, may not be long enough to adopt this snorkel structure.

The opioid activities of the Dyn A analogues were determined in the GPI assay (Table 3). For cyclic [6,9] peptides and their linear analogues the results correlated well with the binding assays; these compounds all had high affinity for both κ and μ receptors and exhibited reasonable agonist potency in the GPI (IC_{50} = 7−46 nM). Interestingly, the ring size in the cyclic [6,9] analogues had a larger affect on opioid activity in the GPI assays (7-fold difference in IC_{50} values) than on binding affinities (<2-fold difference in K_i values);

Table 4. Ratio of Potency in the GPI Assay to Binding Affinity for Opioid Receptors

	Dyn A-(1−13)NH_2	GPI IC_{50}/K_i ratios[a]
	[3,6] Series	
1	cyclo[D-Asp^3,Dap^6]	2
2	cyclo[D-Asp^3,Dab^6]	3
3	cyclo[D-Asp^3,Orn^6]	10
4	[D-Asp^3,Dap^6]	30[b]
5	[D-Asp^3]	16
	[5,8] Series	
6	cyclo[D-Asp^5,Dap^8]	> 600
7	cyclo[D-Asp^5,Dab^8]	90
8	cyclo[D-Asp^5,Orn^8]	40
9	[D-Asp^5,Dap^8]	80[b]
10	[D-Asp^5]	200[b]
	[6,9] Series	
11	cyclo[D-Asp^6,Dap^9]	20
12	cyclo[D-Asp^6,Dab^9]	10
13	cyclo[D-Asp^6,Orn^9]	5
14	[D-Asp^6,Dap^9]	50[b]
15	[D-Asp^6]	20[b]
16	Dyn A-(1−13)NH_2	0.6

[a] The K_i for binding to κ receptors was used except where otherwise noted. [b] K_i values for binding to μ receptors were used in the calculations since the compound had higher affinity for μ than κ receptors.

increasing the ring size increased the agonist potency in the GPI. The pA_2 values (95% confidence interval) for naloxone antagonism of componds **11**, **12**, and **13** were 7.77 (7.45−8.08), 7.62 (7.45−7.79), and 7.87 (7.76−7.98), respectively. These values are comparable to the pA_2 values of 7.65 (7.47−7.83) for Dyn A-(1−13)NH_2, suggesting that compounds **11**−**13** produced their opioid activity in the GPI predominantly by interacting with κ receptors. For the linear peptides **14** and **15**, the pA_2 values for antagonism by naloxone were 7.88 (7.74−8.02) and 8.07 (7.92−8.22), respectively. These pA_2 values are intermediate between those of Dyn A-(1−13)NH_2 and morphine (pA_2 = 8.2 (7.9−8.5))[28] and therefore suggest that both κ and μ receptors were responsible for the opioid activity of these compounds in the GPI. The cyclic [3,6] Dyn A analogues and their related linear peptides were all very weak agonists in the GPI assay (IC_{50} = 0.9−2.7 μM; Table 3), results which also correlated well with those observed in the binding assays.

The cyclic [5,8] Dyn A-(1−13)NH_2 analogues were very weak agonists (IC_{50} = 0.5−5 μM) in the GPI, a result which did not correlate well with their reasonable binding affinity for κ receptors (K_i = 8−12 nM; Table 2). Although compound **6** exhibited little agonist activity in the GPI, this peptide at a concentration of 1 μM did not show any antagonism against Dyn A-(1−13)-NH_2 or morphine. Compound **6** was the most κ selective ligand in this series and also exhibited the most significant difference between binding and opioid activity, with a ratio of >600 for the IC_{50} in the GPI vs K_i at κ receptors (see Table 4). The μ-selective peptide **10** also showed a large ratio (200) for the IC_{50} in the GPI vs K_i at μ receptors. Increasing the ring size in the cyclic [5,8] analogues increased potency in the GPI, which resulted in a decrease in the ratio between the opioid activity in the GPI and the binding affinity for κ receptors for compounds **7** and **8**. Schiller's μ-selective analogue cyclo[Orn^5,Asp^8]Dyn A-(1−13)NH_2 also exhibited this phenomenon, with a ratio of 290 for the IC_{50} in the GPI and K_i at μ receptors.[18] Hruby and his co-workers reported similar significant differences for binding affinity in guinea pig brain and opioid activity in the GPI for some of their cyclic disulfide analogues involving a constraint in the address region, particularly when the residue in position 5 was involved in the constraint.[14−16] After the completion of this research, Hruby and coworkers reported on a series of Dyn A-(1−11)NH_2 lactam derivatives cyclized between residues *i* and *i* + 4.[29] Two of the cyclic peptides, cyclo[D-Asp^3,Lys^7]- and cyclo[Lys^5,D-Asp^9]Dyn A-(1−11)NH_2, exhibited modest affinity for κ opioid receptors (IC_{50} = 4.9−6.6 nM), and cyclo[D-Asp^3,Lys^7]Dyn A-(1−11)NH_2 was selective for κ receptors in the binding assays, but both of these peptides exhibited low potency in the GPI (IC_{50} = 600 and 2500 nM, respectively). These results were postulated to be due to the existence of different receptor subtypes for κ or μ opioid receptors in central and peripheral nervous systems. Alternatively the difference could be a function of partial agonist activity.

Conformational Analysis. Circular dichroism (CD) was used to examine possible conformations of the cyclic peptides. As was observed for the cyclic [2,5] Dyn A analogues,[1] in phosphate buffer (pH 7.4) at room temperature the CD spectra of the cyclic peptides were characteristic of random structures (data not shown). The CD spectra were then determined in 80% trifluoroethanol (TFE, Figure 1) in order to examine whether the secondary structure could be induced in these constrained Dyn A analogues. At 25 °C there was a marked difference between the spectra for the peptides in which the constraint was incorporated into the N-terminus (the [2,5][1] and [3,6] peptides) vs the middle of the sequence (the [5,8] and [6,9] peptides). Like cyclo-[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2, the [3,6] cyclic peptide exhibited evidence of some helical structure at room temperature. When the constraint was incorporated into the middle of the sequence to give either the [5,8] or [6,9] cyclic peptides, however, the spectra at room temperature were more similar to those seen in phosphate buffer, typical of a random structure. CD spectra were also obtained at 5 °C to determine whether evidence of helical structure could be observed for the [5,8] or [6,9] cyclic peptides at a lower temperature (Figure 1b). The effect on the CD spectra of lowering the temperature depended upon the position of the constraint in the peptide. While lowering the temperature appeared to enhance the helicity of the [2,5] cyclic peptide,[1] the effect on the spectra above 200 nm for the [3,6] or [5,8] peptides was small. For the [6,9] cyclic peptide, lowering the temperature decreased the $\Delta\epsilon$ at 222 nm, but the maximum observed at 190 nm was still negative. Thus it appears that neither the [5,8] nor [6,9] cyclic peptides contained significant helical content, even at 5 °C; these peptides contain a D-amino acid in the middle of the sequence, however, and how this affects the CD spectra is not clear. These results suggest that while the cyclo[D-Asp^i,Dap^{i+3}] constraint may be compatible with a helical structure, the position of the constraint can affect the ability of the peptide to adopt a helical conformation. In these Dyn A analogues a helical structure appears to be stable when the constraint is incorporated near the N-terminus, but not when the lactam is present in the middle of the peptide. We are conducting further conformational analysis by NMR in order to examine how incorporation of this

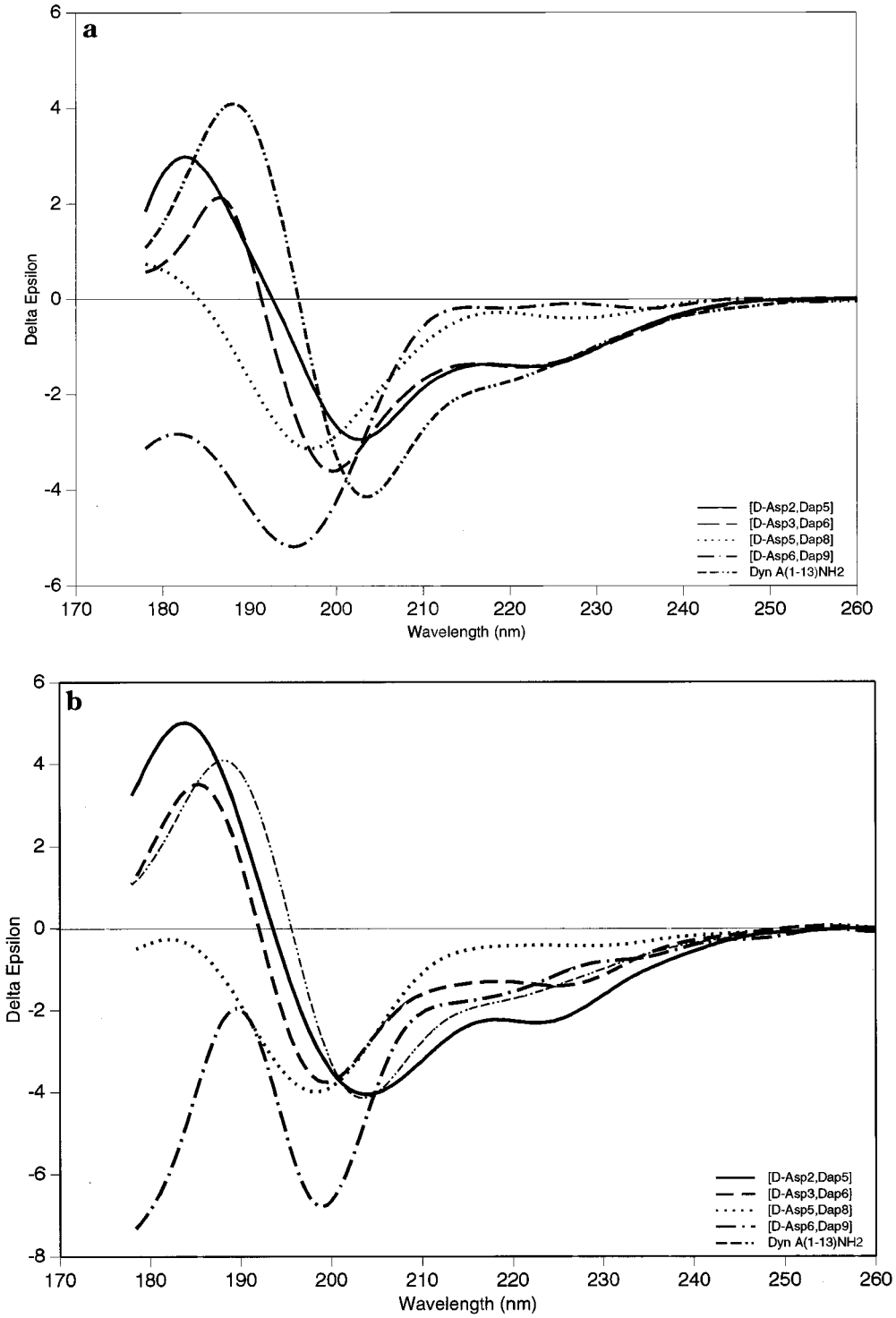

Figure 1. Circular dichroism spectra of cyclo[D-Asp^{i},Dap^{i+3}]Dyn A-(1−13)NH_2 analogues in 80% TFE in 10 mM potassium phosphate buffer (pH 7.4) at (a) 25 °C and (b) 5 °C.

[i,i+3] constraint in different regions of Dyn A affects the resulting peptides' conformation.

Conclusions

Cyclic constraints incorporated in different regions of Dyn A-(1−13)NH_2 resulted in differences in binding profiles for κ and μ receptors and in opioid activity in the GPI assay. The cyclic [D-Asp^{i},Dap^{i+3}] constraint needs to be incorporated into appropriate positions in order to retain high binding affinity for κ receptors and high potency in the GPI. Cyclo[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2 gave the best results, with a K_i for κ receptors and an IC_{50} in the GPI of less than 1 nM,[1] whereas cyclo[D-Asp^3,Dap^6]Dyn A-(1−13)NH_2 exhibited the lowest binding affinity and potency in the GPI. Constraints in the address region between positions 5 and 8, or 6 and 9 also provided useful information for further analogue design, since these peptides still bound to κ receptors with modest to high affinity.

Cyclo[D-Asp^5,Dap^8]Dyn A-(1−13)NH_2 was an interesting compound because the binding affinity for cloned receptors did not correlate well with opioid activity in the GPI. In contrast, cyclo[D-Asp^6,Dap^9]Dyn A-(1−13)-NH_2 exhibited both high κ receptor affinity and modest

opioid activity in the GPI, although it was not as potent in either assay as cyclo[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2.[1] Increasing ring size in the [5,8] and [6,9] series increased potency in the GPI, while the binding affinities for κ and μ receptors did not change substantially. Incorporation of D-Asp alone was not well tolerated at any of the positions, and all of the linear peptides containing D-Asp had lower binding affinity for κ receptors and weaker opioid activity in the GPI compared to the parent peptide, **16**.

All of the cyclic analogues containing a four-atom bridge exhibited some selectivity for κ over μ receptors, although K_i ratios were generally small. Compared to their corresponding linear peptides, which were μ-selective, the constraint of the lactam between D-Asp^i and Dap^{i+3} adversely affected μ receptor affinity while either improving or not affecting binding affinity at κ receptors. For the peptides containing a larger ring size with a five- or six-atom bridge, their selectivity for κ and μ receptors varied depending upon the positions involved.

The cyclic [D-Asp^i,Dap^{i+3}]Dyn A analogues synthesized in our laboratory provide information about the possible conformation Dyn A adopts at κ receptors. The small ring size in cyclo[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2, a peptide which possesses high κ receptor affinity and potent agonist activity in the GPI, suggests that Dyn A-(1−13)NH_2 tends to form a folded structure in the message sequence.[1] A folded structure may occur in the address sequence as well, since cyclo[D-Asp^6,Dap^9]Dyn A-(1−13)NH_2 exhibited high binding affinity at κ receptors and strong opioid activity in the GPI.

The cyclic [6,9] peptides described here along with the cyclic [2,5] analogues we reported previously[1] represent lead compounds which can be further modified in order to characterize the structure− and conformation−activity relationships for Dyn A interacting with κ opioid receptors, studies which are underway in our laboratory. The cyclic [5,8] peptides will also be examined further in order to gain insight into possible reasons for the differences between their binding affinity and opioid activity in the GPI. Further characterization of conformationally constrained Dyn A analogues will help identify the spatial relationships between key pharmacophoric groups in Dyn A and the structural requirements for interaction with κ vs μ or δ opioid receptors.

Experimental Section

Materials. The reagents and instrumentation used were those described previously[1] with the following modifications: the analytical HPLC column used was a C_4 Vydac analytical column (214TP54, 300 Å, 5 μm, 4.6 mm × 25 cm) equipped with a C_4 Vydac guard cartridge or a C_{18} Vydac analytical column (218TP54) with a C_{18} Vydac guard cartridge, and preparative reversed phase HPLC was performed on a Rainin gradient HPLC system using either a Dupont Zorbax Protein Plus preparative column (C_3, 300 Å, 10 μm, 21 mm × 25 cm) with a Dynamax (C_4, 12 μm, 21 mm × 5 cm) guard cartridge or a C_{18} Vydac column (218TP1015, 300 Å, 10−15 μm, 21 mm × 25 cm) with a C_{18} guard cartridge.

General Method for Solid Phase Synthesis. Fmoc-amino acids were assembled on an MBHA resin (0.27 mmol/g resin substitution, 1% cross-linked poly(vinylstyrene)) as described elsewhere,[1] except that backbone couplings were performed with DIC (Aldrich) and HOBt (4 equiv of each). After the last amino acid was attached, the *tert*-butyl side chain protecting groups were removed with 50% (v/v) TFA plus 2% anisole in DCM (5 min followed by 30 min). The resin was washed extensively with DCM and MeOH and then neutralized with 10% DIEA in DCM prior to cyclization.

Cyclizations and Purification of Compounds 1−3 and 6−8. The cyclizations were performed on the peptide resins using BOP/DIEA (3/6 equiv) in DMF. Fresh coupling reagent (BOP/DIEA) was replaced daily until the reaction gave a negative or only a slightly blue response, which occurred after 2−3 days. Then the N-terminal Fmoc protecting group was removed by 30% piperidine in DMA/toluene (1/1). The peptide was cleaved, and all protecting groups were removed simultaneously by treatment with liquid HF plus 10% anisole at 0 °C for 1 h. The crude peptides were desalted on a Sephadex G-10 column using 0.5 M AcOH as the eluent and then further purified by preparative reversed phase HPLC using a gradient of 10−40% B in 45 min at a flow rate of 20 mL/min on a Protein Plus column (C_3) (solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in AcCN). Pure fractions were then pooled and lyophilized, and the homogeneity of the peptides (>98%) was checked by analytical HPLC using a gradient of 0−75% B in 50 min at a flow rate of 1.5 mL/min on a Vydac C_4 column (solvents A and B were the same as above).

Cyclizations and Purification of Compounds 11−13. The cyclizations were performed using fresh PyBOP/DIEA (3/6 equiv) in DMF which was replaced every 24 h. The reaction was terminated after 3−5 days when ninhydrin gave a negative or only a faint positive response. The peptides were deprotected and liberated from the solid support using the same procedure as described above. Crude peptides were desalted on a Sephadex G-10 column using 0.5 M AcOH and then purified on a Vydac C_{18} column using a gradient of 20−50% B in 30 min at a flow rate of 20 mL/min (solvents A and B were the same as above). Additional purification of compounds **11** and **13** was done using isocratic preparative HPLC at 26% B and 20 mL/min to give pure peptides. The purity of peptides (>98%) was determined on an analytical Vydac C_{18} column using the same gradient as described for compounds **1**−**3** and **6**−**8**.

Solid Phase Syntheses of Linear Peptides 4, 9, and 14. Protected peptides **4**, **9**, and **14** were obtained from the same syntheses as compounds **1**, **6**, and **11**, respectively. The N-terminal Fmoc protecting group was removed with 30% piperidine in DMA/toluene (1/1) after the last amino acid derivative was attached to the peptide-resin. The peptides were then cleaved, and all side-chain protecting groups were removed by liquid HF as described above. Crude peptides were then extracted and purified by procedures analogous to those described for their corresponding cyclic peptides.

Solid Phase Syntheses of Linear Peptides 5, 10, and 15. The linear peptides **5**, **10**, and **15** were prepared on a PAL resin (peptide amide linker, Millipore, 0.31 mmol/g resin substitution) using standard Fmoc synthetic procedures.[7] Following cleavage of the peptides with Reagent K (82.5% TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol) for 2 h,[24] the peptides were purified by reversed phase HPLC on a Protein Plus C_3 column using a gradient of 0−50% solvent B over 50 min at a flow rate of 20 mL/min. The purity of the peptides (>98%) was verified by analytical HPLC using a Vydac C_4 column as described above.

Radioligand Binding and Smooth Muscle Assays. Opioid receptor binding studies were performed on membranes derived from cells expressing cloned κ, μ, and δ receptors (COS-7 cells[30] for compounds **1**−**4** or CHO cells[31] for compounds **5**−**16**). COS-7 cells were harvested 72 h following transient transfection, and CHO cells were harvested at confluency in 50 mM Tris buffer (pH 7.4 at 4 °C) and centrifuged at 45000*g* for 10 min at 4 °C. The pellet was washed twice and then resuspended in 50 mM Tris buffer to yield a protein concentration of 30−60 μg/mL. Incubations were performed in triplicate with varying concentrations of the peptides for 90 min at 22 °C using [^{3}H]diprenorphine, [^{3}H]-DAMGO, and [^{3}H]DPDPE as the radioligands for κ, μ, and δ receptors, respectively. Binding assays were carried out in the presence of peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine) and 3 mM Mg^{2+}. Nonspecific binding was determined in the presence of 10 μM unlabeled Dyn A-(1−13)NH_2, DAMGO, and DPDPE for κ, μ, and δ receptors, respectively. The reactions were terminated by rapid filtration over Whatman GF/B fiber filters using a

Brandel M24-R cell harvester. The filters had been presoaked for 2 h in 0.5% polyethylenimine to decrease adsorption of radioligand to the filter. The filter disks were then placed in minivials with 4 mL of Cytocint (ICN Radiochemicals) and allowed to elute for 6 h before counting in a Beckman LS6800 scintillation counter. The IC_{50} values derived from equilibrium competition analyses were converted to dissociation constants (K_i) using the Cheng and Prusoff equation;[32] K_D values for [^{3}H]-diprenorphine, [^{3}H]DAMGO, and [^{3}H]DPDPE were 0.45, 0.49, and 1.76 nM, respectively. The results presented are from at least two separate assays.

The GPI assays were performed as described in detail elsewhere,[8] except that peptidase inhibitors (10 μM bestatin and 0.3 μM thiorphan) were added to the tissue bath 5 min before each experiment. IC_{50} values reported were obtained from at least three replicates in tissues from different animals. The pA_2 values were also determined from at least three replicates in different animals using Schild analysis[33] with four different concetrations of naloxone (10, 100, 300, and 1000 nM).

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Supporting Information Available: Analytical data for dynorphin A-(1−13)NH_2 analogues (1 page). Ordering information is given on any current masthead page.

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