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

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A constrained analogue of the opioid peptide dynorphin A (Dyn A) cyclized in the "message" sequence was designed which may be compatible with the helical conformation proposed by Schwyzer (*Biochemistry* **1986**, *25*, 4281–4286) as the conformation Dyn A adopts at κ opioid receptors. On the basis of molecular modeling with AMBER, we prepared the lactam cyclo-[D-Asp^2,Dap^5]Dyn A-(1–13)NH_2 (1; Dap = α,β-diaminopropionic acid) containing a four-atom bridge between positions 2 and 5 as a possible constraint compatible with an α-helix, along with the homologues with five- (**2**) and six-atom (**3**) bridges containing Dab (α,γ-diaminobutyric acid) and Orn, respectively, in position 5. All of the cyclic peptide analogues exhibited high binding affinity for both κ and μ receptors and high potency in the guinea pig ileum (GPI) assay. As ring size increased, a trend in receptor selectivity from slightly κ selective (compound 1) to nonselective for κ vs μ (compound **2**) to slightly μ selective (compound **3**) was observed in the radioligand binding assays. The results in the GPI for antagonism of these peptides by naloxone paralleled the results of the binding assays and indicated that compound 1 preferentially interacted with κ receptors in this tissue. Novel byproducts were also obtained from the cyclization reactions with HBTU (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and characterized as [D-Asp^2,X(Tmg)^5]Dyn A-(1–13)NH_2 (where X = Dap, Dab, or Orn and Tmg = tetramethylguanidinium). All of the Tmg linear byproducts bound with high affinity to κ and μ receptors and also exhibited potent agonist activity in the GPI. Circular dichroism spectra of compound 1 and the parent peptide Dyn A-(1–13)NH_2 determined in 80% trifluoroethanol at 5 °C were consistent with some α-helical content in the peptides; comparison of the Δε at 222 nm suggested that compound 1 possessed slightly higher helical content than Dyn A-(1–13)NH_2 under these experimental conditions. The cyclic Dyn A analogues **1–3** described here represent the first Dyn A analogues constrained in the "message" sequence with demonstrated high affinity and potency at κ receptors.

Introduction

Since their discovery, considerable research has focused on understanding the physiological and pharmacological roles of opioid receptors. Establishment of clear-cut relationships between specific opioid receptors and their distinct opioid effects has been complicated, however, by the existence of multiple forms of both opioid receptors and their endogenous ligands.[1] Three opioid receptor types, the μ, δ, and κ receptors, are generally accepted, and each of these receptor types has recently been cloned.[2–5] The endogenous mammalian opioid peptides are also classified into three families, the enkephalins, the dynorphins, and the endorphins.[1] Although enkephalins have high affinity for δ receptors, dynorphins for κ receptors, and endorphins for μ receptors, each of these ligands has significant affinity for more than one receptor type.[6] A major goal in opioid research is the development of ligands highly selective for each receptor type, since these ligands can be both potential therapeutic agents and valuable pharmacological tools for understanding the biological effects produced by different receptors. During the last decade considerable effort has focused on the development of κ selective opioid agonists as potential analgesics without the significant clinical side effects associated with morphine and other μ receptor selective analgesic drugs.[7,8] We are interested in developing potent and selective ligands for κ receptors as pharmacological tools to study opioid receptor structure and function using the endogenous opioid peptide dynorphin A as the prototype.

Dynorphin A (Dyn A), a 17-amino acid peptide, is postulated to be an endogenous ligand for κ opioid

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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; CD, circular dichroism; ClZ, [(2-chlorobenzyl)oxy]carbonyl; Dab, α,γ-diaminobutyric acid; Dap, α,β-diaminopropionic acid; DAMGO, [D-Ala^2,MePhe^4,Glyol]enkephalin; DCM, dichloromethane; 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; Fmoc-OSu, 9-fluorenylmethyl succinimidyl carbonate; GPI, guinea pig ileum; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; NMR, nuclear magnetic resonance; PAL resin, peptide amide linker or 5-[4-(aminomethyl)-3,5-dimethoxyphenoxy]valeric acid resin; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; THF, tetrahydrofuran; Tmg, tetramethylguanidinium; Tos, 4-toluenesulfonyl; Z, benzyloxycarbonyl.

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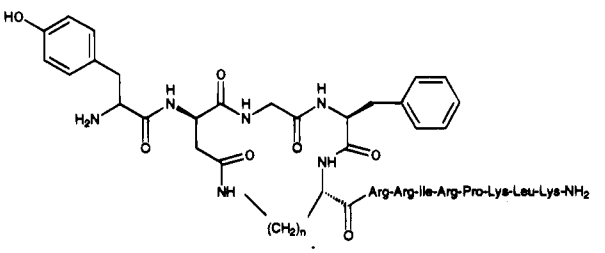
receptors.[9] Dyn A has an identical N-terminal tetrapeptide sequence (the "message" sequence)[10] to other mammalian opioid peptides and exhibits remarkably potent opioid activity.[11] Dyn A-(1−13), the peptide fragment first reported, is about 700 times more potent than [Leu]enkephalin and about 50 times more potent than β-endorphin in the guinea pig ileum (GPI) assay. Its unusually high potency is believed to result from the C-terminal sequence (residues 6−13, the "address" sequence) which directs this peptide to κ receptors.[10] Dyn A-(1−13) exhibits a similar receptor binding profile to Dyn A,[12] and therefore Dyn A-(1−13) has often served as the parent structure for developing analogues with improved κ selectivity. Several studies[10,13−16] have examined Dyn A fragments and amino acid replacement to determine the relative importance and structure−activity relationships for individual residues of Dyn A.

Dyn A is a highly flexible molecule which is capable of assuming a number of different conformations, and the biologically active conformations of this peptide are still unclear. This conformational flexibility may be one reason for the peptide's low κ receptor selectivity. Conformational constraint is one approach which can be used not only to restrict the flexibility of the peptide molecule but also to provide information on the topographical requirements of receptors.[17] In addition, potent and selective ligands may be found when appropriate conformational constraints are incorporated.

Conformational constraint by cyclization has been successfully employed in the development of several potent opioid peptide analogues, such as the selective μ and δ receptor agonists H-Tyr-cyclo[D-Orn-Phe-Asp]-NH_2[18] and DPDPE (cyclo[D-Pen^2,D-Pen^5]enkephalin).[19] Several cyclic Dyn A analogues have also been synthesized and evaluated for their biological activity, but studies of constrained Dyn A analogues have been far less extensive than those reported for μ and δ selective peptides. Only four Dyn A analogues have been reported with a constraint in the "message" sequence of the peptide. A disulfide bridge between D-Cys in position 2 and L-Cys in position 5 produced a peptide which is 5-fold more potent than the parent 13-mer in the GPI assay,[20] but tolerance studies suggested that it interacted mainly with δ receptors and had only minor interactions with κ receptors.[21] A cyclic lactam, cyclo[D-Orn^2,Asp^5]Dyn A-(1−8)NH_2, has also been prepared, but the high sensitivity of this peptide to antagonism by naloxone in the GPI (K_e = 1.49 nM) suggests that this peptide preferentially interacts with μ receptors in this tissue.[22] Two analogues containing *cis*- and *trans*-4-aminocyclohexanecarboxylic acid as a conformational constraint in place of Gly^2-Gly^3 were prepared in our laboratory.[23] Although exhibiting some selectivity for κ receptors, the affinity of these analogues for κ receptors was only modest (K_i = 9−14 nM). Other cyclic analogues have involved modifications in the "address" segment.[22,24−25] Several of the cyclic disulfide analogues reported exhibited unexpected selectivities for the receptors in the central vs peripheral nervous system,[24,25] which suggested possible receptor differences between these sites. Cyclic lactam analogues of Dyn A, cyclo[Orn^5,Asp^8]Dyn A-(1−13)NH_2, cyclo[Orn^5,Asp^{10}]Dyn A-(1−13)NH_2, and cyclo[Orn^5,Asp^{13}]Dyn A-(1−13)NH_2, have also been prepared.[22] 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 compounds did not interact significantly with κ receptors.

Using equilibrium thermodynamic and kinetic estimations, Schwyzer proposed a helical conformation for the N-terminus of Dyn A-(1−13) oriented perpendicular to the membrane surface when the peptide interacts with κ receptors.[26] The α-helix was proposed to extend from Tyr^1 through Arg^9 with the C-terminal residues Pro^{10}−Lys^{13} in an unordered structure. 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,[27−29] we found that a four-atom bridge containing an amide bond between residues *i* (D-configuration) and *i* + *3* (L-configuration) appeared to be ideal for spanning the distance between $C_i\alpha$ and $C_{i+3}\alpha$ in an α-helix. We were interested in incorporating this constraint into the "message" sequence and therefore prepared the cyclic analogue cyclo[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2 (1; Dap = α,β-diaminopropionic acid) containing the four-atom bridge between the α carbons of residues 2 and 5. In addition, the analogues with five- (**2**) and six-atom (**3**) bridges were synthesized by incorporating Dab (α,γ-diaminobutyric acid) and Orn, respectively, into position 5 of the peptides in order to examine the effect on opioid activity and receptor affinity of varying the bridge length.



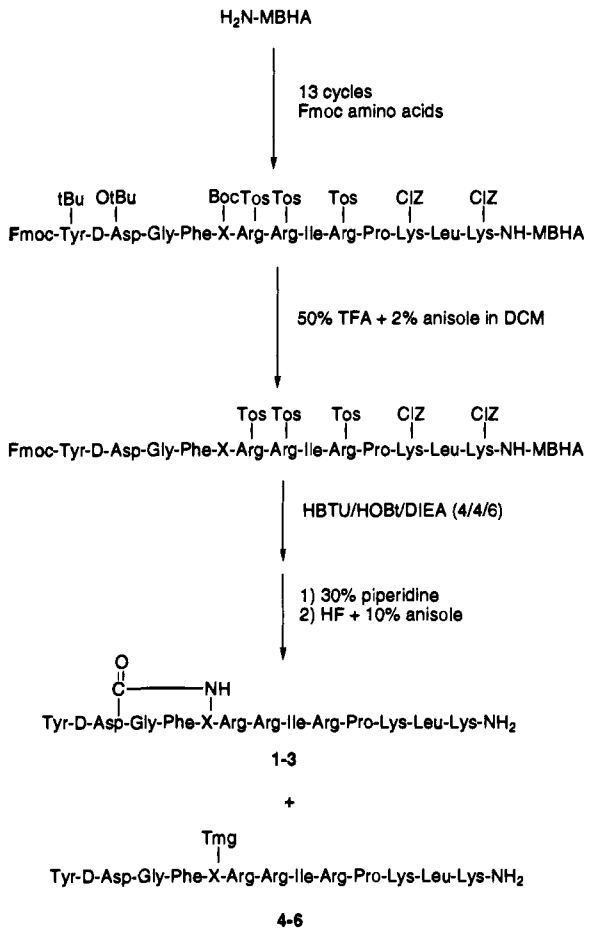
1 : n = 1 (Dap)
2 : n = 2 (Dab)
3 : n = 3 (Orn)

During the formation of these relatively small (14- to 16-membered) lactam rings using HBTU, we obtained another major peptide product in addition to the desired cyclic peptides. We evaluated the cyclic peptides, along with the unusual linear byproducts obtained from the cyclization reactions, for their affinity for κ, μ, and δ opioid receptors and for their opioid activity in the GPI assay. The results of these assays are described below.

Results and Discussion

Chemistry. All of the cyclic peptides were synthesized and cyclized on an MBHA resin. Cyclizations performed on solid phase resins utilize pseudodilution to favor intramolecular resin-bound reactions.[30] This methodology was selected in order to minimize formation of cyclodimeric or polymeric peptides which can occur when cyclizations are performed in solution, even if the solution is very dilute.[31] The Fmoc group was selected for α-amine protection of the amino acids. The side chain functional groups of the amino acids involved in the cyclization reaction, D-Asp and Dap, Dab, or Orn, were protected with *tert*-butyl and Boc groups, respectively. The side chain protecting groups for Arg and

Scheme 1



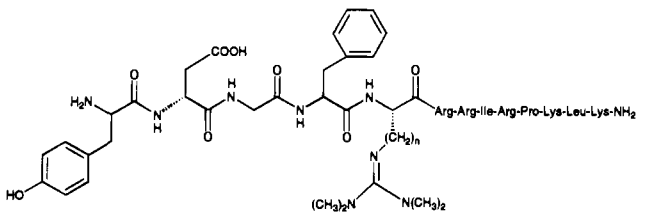
Lys, Tos and ClZ, respectively, were chosen to be stable to trifluoroacetic acid (TFA) treatment.

The synthesis of the linear peptide **7** was performed on a PAL resin (peptide amide linker resin) using standard Fmoc synthetic strategy. The side chains of Fmoc amino acids used in this case were protected with TFA-labile functional groups, namely D-Asp(OtBu), Tyr-(tBu), Lys(Boc), and Arg(Pmc).

The synthesis of the amino acid derivative FmocDap-(Boc), **10**, was performed via a modified Hoffmann rearrangement. Starting with ZAsn, the aliphatic amide was converted to an amine using [*I*,*I*-bis(trifluoroacetoxy)iodo]benzene[32] and the free amine then protected with Boc using *tert*-butyl pyrocarbonate[33] to yield ZDap(Boc). After removal of the benzyloxycarbonyl protecting group by catalytic transfer hydrogenation,[34] the Fmoc protecting group was introduced on the α-amine using Fmoc-OSu[23] to give compound **10**. Attempts to rearrange FmocAsn to give FmocDap directly gave lower yields and a product with lower purity.

The syntheses of **1**–**3** were performed according to Scheme 1. After incorporation of the last amino acid to the growing peptide chains, the *tert*-butyl protecting groups were removed with TFA and the cyclizations then performed on the resins using a 4-fold excess of HBTU reagent (HBTU/HOBt/DIEA, 4/4/6 equiv). The cyclization reactions all gave a negative response to ninhydrin within 2–3 h. The N-terminal Fmoc protecting groups were removed with piperidine and the peptide amides then liberated from the resins using liquid HF. Crude peptides were purified by gel filtration on Sephadex G-10 followed by reversed phase HPLC.

In all cases, two major peptide products were obtained. Analyses of the purified peptides using FAB-MS revealed that the desired cyclic analogues were obtained along with byproducts having molecular weights 116 Da higher than the corresponding cyclic peptides (Table 1). Amino acid analysis of the byproduct obtained from the cyclization reaction to yield **1** gave a peak which did not match Dap, and peptide sequencing produced a peak distinct from that of Dap or Asp-Dap in the fifth cycle. A similar result was observed in our laboratory during an attempt to form the highly strained 10-membered lactam ring in cyclo[Dab^2,D-Glu^3,Leu^5]-enkephalinamide.[35] In this case, characterization of the products using amino acid analysis, peptide sequencing, NMR, and high-resolution mass spectrometry indicated that the products were [Dab(Tmg)^2,D-Glu^3,Leu^5]-enkephalinamide and the corresponding linear dimeric peptide containing the tetramethylguanidinium (Tmg) functionality, resulting from transfer of the tetramethyluronium group from HBTU to the γ-amine of Dab. This side reaction was also observed during a backbone coupling when HBTU reagent was added to the free amine solution instead of to the carboxylate solution.[36] The analytical data for the byproduct peptides obtained following the cyclization reactions of the Dyn A analogues were consistant with this identification as the linear tetramethylguanidinium derivatives **4**–**6**. A detailed analysis indicates that the prevalence of this side reaction is dependent upon the amino acid sequence and the reagents used for the cyclization (S. Arttamangkul and J. V. Aldrich, unpublished results).



4 : n = 1 (Dap(Tmg))
5 : n = 2 (Dab(Tmg))
6 : n = 3 (Orn(Tmg))

Receptor Affinity and Opioid Activity. The peptides were evaluated for their binding affinity at κ, μ, and δ opioid receptors using competition binding assays (Table 2). κ Receptor affinity was determined in guinea pig cerebellum, a tissue which is rich in κ binding sites,[37] using [^{3}H]bremazocine as the radioligand. To measure μ and δ affinity, [^{3}H]DAMGO and [^{3}H]DPDPE were employed as selective radioligands for μ and δ receptors, respectively, in rat forebrain membrane preparations.

All of the Dyn A analogues examined exhibited high binding affinity for both κ and μ receptors (K_i < 1 nM, Table 2) and generally had moderate binding affinity for δ receptors (K_i = 4–14 nM except for [D-Asp^2]Dyn A-(1–13)NH_2, **7**). These results are similar to those observed for the parent linear peptide Dyn A-(1–13)-NH_2. At κ receptors the binding affinities of compounds **1** and **2** were similar to that of the parent peptide, while compound **3**, which contains the largest ring size in the series, exhibited a 3–4.5-fold decrease in κ receptor affinity compared to **1** and Dyn A-(1–13)NH_2. Compounds **2** and **3** had similar binding affinity at μ receptors as the parent peptide, while compound 1 had

Table 1. Analytical Data for Dyn A-(1−13)NH_2 Analogues

compd	t_R[a] (min)	FAB-MS (M + 1)	amino acid composition[b]									
			Tyr (1)	Asp (1)	Gly (1)	Phe (1)	X (1)	Arg (3)	Ile (1)	Pro (1)	Lys (2)	Leu (1)
1	17.9	1615.9	0.96	1.01	0.99	1.00	0.99	3.03	0.97	0.91	2.01	1.02
2	17.1	1630.5	1.04	1.02	1.03	1.02	1.02	3.02	0.96	0.85	2.01	1.04
3	18.2	1643.9	0.79	1.06	1.16	0.86	0.96	3.12	1.03	1.02	2.00	1.04
4	15.3	1731.9	0.90	0.93	1.00	1.01	*c*	3.10	1.01	0.90	2.13	1.08
5	15.7	1745.8	0.89	0.91	1.00	0.99	*d*	3.04	1.01	0.81	2.25	1.11
6	16.2	1759.9	0.95	0.96	1.02	1.04	*e*	3.04	0.98	0.88	2.08	1.05
7	20.6	1661.0	1.05	1.04	1.05	1.04		3.14	0.90	0.76	2.00	2.01 (2)

[a] Elution as described in the Experimental Section using the Vydac analytical column. [b] Theoretical values are shown in parentheses. [c] A peak was observed at 46.3 min which did not correspond to Dap (t_R = 47.8 min). [d] A peak was observed at 46.0 min which did not correspond to Dab (t_R = 42.5 min). [e] A peak was observed at 45.7 min which did not correspond to Orn (t_R = 40.8 min).

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

compd no.	analogues	K_i (nM)[a]			
		[^{3}H]bremazocine	[^{3}H]DAMGO	[^{3}H]DPDPE	K_i ratio ($\kappa/\mu/\delta$)
1	cyclo[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2	0.22 ± 0.002	0.49 ± 0.016	10.2 ± 0.3	1/2.2/47
2	cyclo[D-Asp^2,Dab^5]Dyn A-(1−13)NH_2	0.13 ± 0.003	0.17 ± 0.002	12.0 ± 0.4	1/1.3/92
3	cyclo[D-Asp^2,Orn^5]Dyn A-(1−13)NH_2	0.68 ± 0.033	0.19 ± 0.001	14.1 ± 0.2	3.6/1/74
4	[D-Asp^2,Dap(Tmg)^5]Dyn A-(1−13)NH_2	0.21 ± 0.003	0.16 ± 0.001	4.31 ± 0.28	1.3/1/27
5	[D-Asp^2,Dab(Tmg)^5]Dyn A-(1−13)NH_2	0.14 ± 0.005	0.32 ± 0.01	7.52 ± 0.40	1/2.3/54
6	[D-Asp^2,Orn(Tmg)^5]Dyn A-(1−13)NH_2	0.36 ± 0.011	0.28 ± 0.006	14.2 ± 0.4	1.3/1/51
7	[D-Asp^2]Dyn A-(1−13)NH_2	0.45 ± 0.07	0.013 ± 0.001	0.044 ± 0.005	35/1/3.4
	Dyn A-(1−13)NH_2	0.15 ± 0.004	0.19 ± 0.002	3.88 ± 0.091	1/1.3/26

[a] K_i ± SE.

Table 3. Opioid Activity of Cyclic Dyn A-(1−13)NH_2 Analogues in the Guinea Pig Ileum

compd no.	analogues	IC_{50} (nM)[a]	relative potency (%)
1	cyclo[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2	0.16 (0.13−0.20)	138
2	cyclo[D-Asp^2,Dab^5]Dyn A-(1−13)NH_2	0.23 (0.18−0.31)	96
3	cyclo[D-Asp^2,Orn^5]Dyn A-(1−13)NH_2	0.40 (0.30−0.54)	55
4	[D-Asp^2,Dap(Tmg)^5]Dyn A-(1−13)NH_2	0.46 (0.36−0.58)	48
5	[D-Asp^2,Dab(Tmg)^5]Dyn A-(1−13)NH_2	0.18 (0.14−0.23)	122
6	[D-Asp^2,Orn(Tmg)^5]Dyn A-(1−13)NH_2	1.78 (0.92−3.46)	12
7	[D-Asp^2]Dyn A-(1−13)NH_2	0.46 (0.32−0.67)	32
	Dyn A-(1−13)NH_2	0.22 (0.15−0.33)	100

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

2.5-fold lower affinity for these receptors. These modest differences in affinities resulted in a trend in receptor selectivity from slightly κ selective (compound **1**) to nonselective (compound **2**) to slightly μ selective (compound **3**) as ring size increased. Since the differences in affinities were relatively small, however, none of these cyclic analogues exhibited a large preference for either κ or μ receptors. Cyclization of the peptides decreased binding affinity at δ receptors 2.5−4-fold, resulting in enhanced selectivity for κ and μ receptors over δ receptors. All of these cyclic peptides had similar affinity for δ receptors (K_i = 10−14 nM), indicating that ring size did not significantly affect affinity for these receptors.

Introduction of a D-amino acid at position 2 in linear analogues of Dyn A-(1−13)NH_2 results in compounds that are either nonselective for κ vs μ receptors (D-Asn^2)[15] or μ selective (D-Asp^2, **7**, and other D-amino acid-containing analogues[15]). Introduction of the negatively charged D-Asp residue in position 2 reduced κ binding affinity 3-fold compared to the parent peptide but enhanced affinity for μ receptors, an effect also seen for the D-Asn^2 analogue.[15] Interestingly the anionic residue increased binding affinity for δ receptors almost 90-fold, an effect not seen with the D-Asn^2 analogue. The δ receptor's preference for negative charges may explain this effect.[38]

Surprisingly, the Tmg linear byproducts exhibited high binding affinity for κ receptors, indicating that the bulky and positively charged Tmg group at position 5 did not adversely affect binding to these receptors. This functional group did have a significant adverse effect on affinity for μ and δ receptors, however, decreasing binding by 10−25-fold and 100−300-fold, respectively, compared to compound **7**. The longer length of the Tmg-containing side chain in the 5 position reduced the δ binding affinity of compounds **5** and **6** by 2- and 3-fold, respectively, compared to compound **4**. This effect of chain length was not observed for κ or μ receptors. It is interesting to note that compounds **4**−**6** exhibited binding affinities for the three opioid receptor types similar to the affinities of the corresponding cyclic analogues. This may be due to electrostatic interactions between the negative and positive charges of the D-aspartate and the Tmg functional groups, which could permit these linear peptides to adopt conformations similar to those of the cyclic peptides at opioid receptors.

To determine *in vitro* opioid activities, the peptides were tested for inhibition of electrically evoked contractions of the GPI (Table 3). All of the compounds inhibited muscle contractions in a dose-dependent manner with full agonist activity. Compounds **1**, **2**, and **5** exhibited high potency with IC_{50} values (0.16−0.23 nM) comparable to that of the parent peptide Dyn A-(1−13)-NH_2. Compounds **3**, **4**, and **7** also showed strong agonist activity with potencies 30−60% of that of the parent peptide, whereas compound **6** was a weaker agonist (IC_{50} = 1.78 nM). Interestingly, the linear peptide

[D-Asn^2]Dyn A-(1−13)NH_2, which does not have a negative charge, also exhibited decreased agonist potency (IC_{50} = 2.12 nM).[15] As observed in the binding assays, the ring size in the cyclic peptides had a slight effect on opioid activity, with opioid activity in the GPI decreasing slightly as the ring size increased. The potencies of the linear [D-Asp^2,Dap(Tmg)^5] and [D-Asp^2,Dab(Tmg)^5] byproducts were equal to or greater than that of [D-Asp^2]Dyn A-(1−13)NH_2, **7**. The length of the Tmg-containing side chain in position 5 affected the GPI activities; compound **5** was the most potent, whereas compound **4** (shorter length) and compound **6** (longer length) were 2.5- and 10-fold less potent, respectively.

Whether the opioid activity of the cyclic peptides was mediated through κ or μ receptors was examined by evaluating naloxone sensitivity. The K_e values for naloxone antagonism of compounds **1**−**3** and Dyn A-(1−13)NH_2 were 64.9, 32.3, 17.1, and 64.0 nM, respectively. These results parallelled those found in the binding assays and indicated that compound 1 preferentially interacted with κ receptors in the GPI. The naloxone K_e for compound **3** indicated some κ selectivity for this compound in the GPI, a result which differs from that reported by Schiller et al. for the μ selective compound cyclo[D-Orn^2,Asp^5]Dyn A-(1−8)NH_2 (K_e = 1.49 nM).[22] The difference between these two compounds in opioid receptor selectivity in the GPI may be due to truncation of the C-terminus in Schiller's peptide and/or reversal of the direction of the lactam bond. The 13-mer Dyn A-(1−13) has been reported to be a more κ selective ligand than the 8-mer Dyn A-(1−8),[10,12] and the direction of a lactam bond may be important in stabilizing or destabilizing helix formation.[39]

Conformational Analysis. Circular dichroism (CD), a sensitive method for evaluating peptide secondary structure, was used to examine possible conformations of the cyclic peptides and Dyn A-(1−13)NH_2. In aqueous phosphate buffer (pH 7.4) at room temperature, all of the cyclic peptides and Dyn A-(1−13)NH_2 exhibited CD spectra typical of a random structure (data not shown). Trifluoroethanol (TFE) was therefore utilized to determine whether secondary structure could be induced in the constrained cyclic analogues or Dyn A-(1−13)NH_2. While lowering the temperature from 25 to 5 °C had little effect on the CD spectrum above 200 nm of Dyn A-(1−13)NH_2 in TFE, it decreased the $\Delta\epsilon$ in this region for compound **1** (data not shown), suggesting increased stability of a helical structure at the lower temperature. A comparison of the CD spectra of compound **1** and Dyn A-(1−13)NH_2 determined in 80% TFE at 5 °C is shown in Figure 1. Both peptides showed CD spectra indicative of some helical structure, with minima at 222 and 205 nm and a large positive peak near 190 nm. The $\Delta\epsilon$ at 222 nm for compound **1** was somewhat more negative than that observed for Dyn A-(1−13)NH_2 at 5 °C, suggesting a slightly higher helical content in compound **1** compared to Dyn A-(1−13)NH_2 under these experimental conditions. While the helicity indicated by the CD spectra of this cyclic peptide and Dyn A-(1−13)NH_2 in TFE appears to be modest, it suggests that it may be possible for both compound **1** and Dyn A to adopt partial helical structures at κ receptors, as proposed by Schwyzer.

NMR evidence supporting a partial helical structure for Dyn A interacting with membranes has recently been reported. On the basis of transfer nuclear Overhauser effects plus molecular modeling, Epand and coworkers[40] proposed a membrane-mediated conformation for Dyn A-(1−13) consisting of a short helical segment from Tyr^1 to Leu^5 plus a folded structure for residues Ile^8−Pro^{10}. Recently Kallick[41] examined Dyn A-(1−17) bound to dodecylphosphocholine micelles by NMR and found that the peptide adopts a helix from Gly^3 to Arg^9. We are conducting NMR studies of cyclo[D-Asp^2,Dap^5]-Dyn A-(1−13)NH_2, **1**, our constrained analogue proposed to be compatible with an α-helix, to examine this peptide's conformation in more detail.

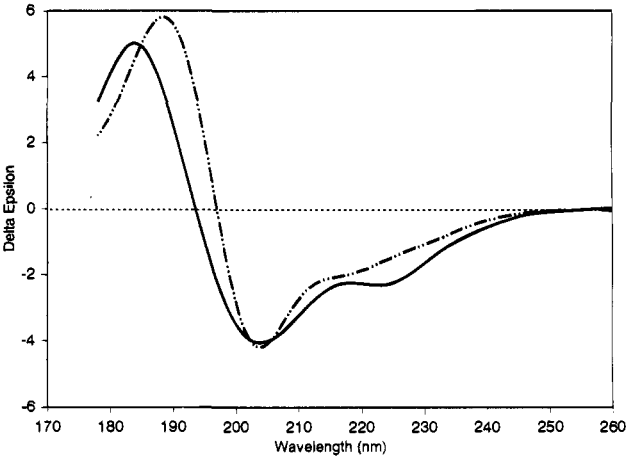


Figure 1. Circular dichroism spectra of cyclo[D-Asp^2,Dap^5]-Dyn A-(1−13)NH_2, **1** (—), and Dyn A-(1−13)NH_2 (− · · −) in 80% TFE at 5 °C.

Conclusions

Compounds **1**−**3** were synthesized in order to limit the comformational freedom of Dyn A-(1−13)NH_2. All of these analogues exhibited high opioid receptor affinities and potent opioid activity in the GPI. Cyclo[D-Asp^2,-Dap^5]Dyn A-(1−13)NH_2, **1**, the constrained analogue designed to be compatible with an α-helix, was a potent agonist, and the high naloxone K_e value for this peptide in the GPI indicated that this peptide preferentially interacted with κ receptors in this tissue. This is in contrast to the μ selectivity found for cyclo[D-Orn^2,Asp^5]-Dyn A-(1−8)NH_2,[22] the only previously reported Dyn A analogue constrained via a lactam in the "message" region. In the radioligand binding assays, compound **1** exhibited high affinity for κ receptors and a slight preference for κ over μ receptors. Increasing the ring size to give compounds **2** and **3** tended to decrease binding affinity and/or selectivity for κ receptors. The high affinity for κ receptors and the high potency in the GPI of these cyclic peptides suggest that they can adopt the appropriate bioactive conformation for κ receptors. Interestingly, the novel Tmg byproducts, compounds **4**−**6**, also exhibited high binding affinity for κ receptors and potent agonist activity in the GPI, indicating that the bulky tetramethylguanidinium group did not interfere with κ receptor interaction and suggesting that these analogues are also capable of adopting the appropriate bioactive conformation at κ opioid receptors.

Possible conformations of cyclo[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2, **1**, and Dyn A-(1−13)NH_2 were examined by circular dichroism. The CD spectra of these peptides in 80% TFE at 5 °C (Figure 1) are both indicative of some helical structure and suggest that **1** exhibits

slightly greater helical content than Dyn A-(1−13)NH_2 under these experimental conditions. The constraint in cyclic peptide **1** still retains some flexibility, however, and therefore could be compatible with other folded conformations (i.e., a β-turn); this could account for the peptide's affinity for other opioid receptors. An extended conformation has also been proposed for the N-terminus of Dyn A-(1−13), based on fluorescent energy transfer of [Trp^4]Dyn A-(1−13) in aqueous solution.[42] However, the affinity and potency of the cyclic peptides described here, particularly given the short length of the bridges in compounds **1** and **2**, would tend not to support such an extended conformation as a bioactive conformation for the N-terminus of Dyn A.

The cyclic Dyn A analogues described here represent the first Dyn A analogues constrained in the "message" sequence with demonstrated high affinity and potency at κ receptors. Thus these analogues represent interesting lead compounds for further characterization of structure− and conformation−activity relationships for Dyn A at κ opioid receptors, studies which are under way in our laboratory. Although the conformation Dyn A adopts at κ opioid receptors is still uncertain, a helical structure for part of Dyn A is a promising candidate for this peptide's bioactive conformation at these receptors.

Experimental Section

Peptide syntheses were performed by solid phase synthesis using a Milligen Biosearch 9500 automated peptide synthesizer (Novato, CA), and HF cleavages were performed on a Multiple Peptide Systems HF apparatus model 2010C (San Diego, CA). Reversed phase HPLC was done on a Beckman model 431A gradient system. The analytical HPLC columns were a DuPont Zorbax Protein Plus analytical column (C_3, 300 Å, 10 μm, 4.6 mm × 25 cm) equipped with a Protein Plus guard cartridge and a 214TP54 Vydac analytical column (C_4, 300 Å, 5 μm, 4.6 mm × 25 cm). Preparative reversed phase HPLC was performed on a Rainin gradient HPLC system using 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. Amino acid analyses were performed after hydrolysis of the peptides under standard conditions (6 N HCl plus 1.0% phenol at 110 °C for 20 h). Each amino acid was resolved using a step gradient on a Beckman Spherogel 52C ion exchange column (4.6 × 15 cm) equipped with postcolumn ninhydrin detection on a Beckman 126AA System Gold HPLC amino acid analyzer. Peptide sequencing was performed via Edman degradation on an Applied Biosystems Model 475A gas phase protein sequencer. Both amino acid analysis and peptide sequencing were performed by the Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University. Molecular weights of the peptides were determined by FAB mass spectrometry in the positive mode on a Kratos MS50RFTC spectrometer in the Department of Agricultural Chemistry, Oregon State University. NMR spectroscopy was performed on a 300 MHz Bruker NMR spectrometer in the Department of Chemistry, Oregon State University. Elemental analysis was performed by MWH Laboratories, Phoenix, AZ.

Amino Acid Synthesis. N^{α}-(Fluorenylmethoxycarbonyl)-N^{β}-(*tert*-butyloxycarbonyl)-L-α,β-diaminopropionic Acid (10). N^{α}-(Benzyloxycarbonyl)-L-asparagine (Bachem) was rearranged to N^{α}-(benzyloxycarbonyl)-L-α,β-diaminopropionic acid (56% yield) using [*I*,*I*-bis(trifluoroacetoxy)iodo]-benzene (Aldrich) as reported in the literature.[32] The crystalline ZDap obtained was then protected with Boc by a standard method[33] using *tert*-butyl pyrocarbonate (Aldrich) to give N^{α}-(benzyloxycarbonyl)-N^{β}-*tert*-butyloxycarbonyl)-L-α,β-diaminopropionic acid (75% yield). The Z protecting group was removed by catalytic transfer hydrogenation using 10% palladium on carbon (Aldrich) and ammonium formate (Aldrich),[34] and then the Fmoc protecting group was introduced at the α-amine using Fmoc-OSu (Bachem; 0.9 equiv) plus Na_2CO_3 (1 equiv) in THF/H_2O (1/1).[23] Crude **10** was purified by medium pressure liquid chromatography on silica gel (grade 60, 230−400 mesh, 60 Å, Merck 9385) using first DCM followed by DCM/MeOH/AcOH (95/5/0.1) at a flow rate of ca. 10 mL/min. Purified **10** was precipitated from MeOH with H_2O to yield a fine white powder (87% yield): mp 97−100 °C; FAB-MS *m*/*z* 425.1(M − 1); TLC R_f (DCM/MeOH/AcOH, 85/10/5) 0.77; ^{1}H-NMR (300 MHz, MeOH-d_4) δ 1.45 (s, 9H, -C(CH_3)$_3$), 3.32 (m, 2H, βCH_2), 4.25 (m, 2H, αCH, Fmoc-CHCH$_2$O), 4.34 (d, 2H, Fmoc-CHCH_2O), 7.34 (m, 4H, Ar), 7.66 (d, 2H, Ar), 7.79 (d, 2H, Ar); $[\alpha]^{25}_D$ −4.3° (c 1.0, MeOH). Anal. ($C_{23}H_{26}N_2O_6$·H_2O) C, H, N.

Cyclic Peptide Synthesis. For syntheses of the cyclic peptides, Fmoc amino acids (Bachem, Novabiochem, and Bachem Biosciences (FmocDab(Boc)) were assembled on an MBHA resin (Bachem; 0.23 mmol/g, 1% cross-linked poly(vinylstyrene)) using the HBTU (Amino Tech) coupling protocol as previously described.[35] 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 and then 30 min). Following neutralization with 10% (v/v) DIEA in DCM (3 × 30 mL) and washing with DCM (5×) and DMA/DCM (1/1, 5×), cyclizations were carried out at room temperature by the addition of HBTU/HOBt (4.0 equiv of each) in DMF containing excess DIEA (6.0 equiv). The reaction was usually complete within 2−3 h, as determined by the Kaiser test. The N-terminal Fmoc groups were then removed using 30% piperidine in DMA/toluene (1/1) and the peptide resins washed with DMA/DCM (1/1, 10×), DCM (6×), and MeOH (4×) and dried *in vacuo* over night. The peptides were cleaved from the resin by treatment with liquid HF (10 mL of HF plus 1 mL of anisole/g of resin) for 60 min at 0 °C. After evaporation of HF, the resin was extracted with Et_2O (3 × 10 mL) and then with 0.5 N AcOH (3 × 10 mL). The crude peptides were obtained by lyophilization of the aqueous extracts.

Peptides were desalted by gel filtration through a Sephadex G-10 column (2.6 × 16.3 cm) using 0.5 N AcOH. Following lyophilization, the crude peptides were purified further by reversed phase HPLC using a mobile phase gradient of 20−50% MeOH in 0.1% TFA over 30 min at a flow rate of 20 mL/min. In all cases two major peaks of peptides were obtained. The pure fractions from each peak were collected and lyophilized. Homogeneity of the peptides was established by analytical HPLC using a mobile phase gradient of 0−75% AcCN in 0.1% TFA over 50 min at a flow rate of 1.5 mL/min. All peptides were >98% pure, as judged from the HPLC elution profiles.

Linear Peptide Synthesis. The linear peptide **7** was synthesized by standard Fmoc protocol on a PAL resin (Millipore; 0.27 mmol/g) as previously described.[14] Following cleavage of the peptide with Reagent K[43] (82.5% TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol), the peptide was purified by reversed phase HPLC using a gradient of 0−50% AcCN containing 0.1% TFA in 100 min at 10 mL/min.

Circular Dichroism. Circular dichroism spectra were recorded on a J-720 Jasco spectrometer (Japan Spectroscopic Co. Ltd., Tokyo). The instrument was routinely calibrated with (+)-10-camphorsulfonic acid for proper operation.[44] The peptides were freshly prepared in aqueous buffer containing 10 mM potassium phosphate (pH 7.4) or in 80% (v/v) TFE (Aldrich) in 10 mM potassium phosphate buffer. Concentrations of the peptide solutions were determined by the guanidine hydrochloride method[45] using absorbance of the same stock solutions at 280 (ϵ = 1280 $M^{-1}cm^{-1}$) and 190 nm. Spectra were collected at 0.5 nm intervals in the range of 178−260 nm and are the average of four runs. Spectra were smoothed using a cubic spline algorithm.

Receptor Binding Assays and Smooth Muscle Assays. Opioid receptor binding studies were performed as described in detail elsewhere.[15] Incubations of [^{3}H]bremazocine (0.7−0.8 nM) with guinea pig cerebellar membrane preparation[5] were performed for 3 h at 4 °C, and incubations of rat membrane preparations with [^{3}H]DAMGO (0.3−0.4 nM) and

[^{3}H]DPDPE (2.4 nM) were performed for 5 h at 4 °C. IC_{50} values were determined by nonlinear regression analysis to fit a logistic equation to the competition data and K_i values calculated from the IC_{50} values by the Cheng and Prusoff equation,[46] using K_D values of 0.314, 7.63, and 0.055 nM for [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]bremazocine, respectively.

The GPI assays were carried out as previously described.[15] A log dose–response curve was determined with Dyn A-(1–13)NH_2 (Peninsula Laboratories) as the standard for each ileum. K_e values for naloxone were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed concentration of naloxone (300 nM, added 20 min prior to addition of the agonist). IC_{50} and K_e values reported were obtained from four to five replicates in different animals.

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References

(1) Simon, E. J.; Hiller, J. M. Opioid Peptides and Opioid Receptors. In *Basic Neurochemistry: Molecular, Cellular, and Medical Aspect*, 5th ed.; Siegel, G. J., et al., Eds.; Raven Press, Ltd.: New York, 1994; pp 321–339.

(2) Kieffer, B. L.; Befort, K.; Gaveriaux-Ruff, C.; Hirth, C. G. The δ-Opioid Receptor: Isolation of a cDNA by Expression Cloning and Pharmacological Characterization. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 12048–12052.

(3) Evans, C. J.; Keith, D. E., Jr.; Morrison, H.; Magendzo, K.; Edwards, R. H. Cloning of a Delta Opioid Receptor by Functional Expression. *Science* **1992**, *258*, 1952–1955.

(4) Yasuda, K.; Raynor, K.; Kong, H.; Breder, C.; Takeda, J.; Reisine, T.; Bell, G. Cloning and Functional Comparison of Kappa and Delta Opioid Receptors from Mouse Brain. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6736–6740.

(5) Chen, Y.; Mestek, A.; Liu, J.; Hurley, J. A.; Yu, L. Molecular Cloning and Functional Expression of a μ-Opioid Receptor from Rat Brain. *Mol. Pharmacol.* **1993**, *44*, 8–12.

(6) Simon, E. J. Opioid Receptors and Endogenous Opioid Peptides. *Med. Res. Rev.* **1991**, *11*, 357–374.

(7) Millan, M. J. κ-Opioid Receptors and Analgesia. *Trends Pharmacol. Sci.* **1990**, *11*, 70–76.

(8) Rees, D. C. Chemical Structures and Biological Activities of Non-Peptide Selective Kappa Opioid Ligands. In *Progress in Medicinal Chemistry*; Ellis, G. P., Luscombe, D. K., Eds.; Elsevier Science Publishers B.V.: Amsterdam, The Netherlands, 1992; Vol. 29, pp 109–139.

(9) Chavkin, C.; James, I. F.; Goldstein, A. Dynorphin is a Specific Endogenous Ligand of the κ Opioid Receptor. *Science* **1982**, *215*, 413–415.

(10) Chavkin, C.; Goldstein, A. Specific Receptor for the Opioid Peptide Dynorphin: Structure-Activity Relationships. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6543–6547.

(11) Goldstein, A.; Tachibana, J. S.; Lowney, L. I.; Hunkapiller, M.; Hood, L. Dynorphin-(1–13), an Extraordinarily Potent Opioid Peptide. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6666–6670.

(12) Corbett, A. D.; Paterson, S. J.; McKnight, A. T.; Magnan, J.; Kosterlitz, H. W. Dynorphin$_{1-8}$ and Dynorphin$_{1-9}$ are Ligands for the κ-Subtype of Opiate Receptor. *Nature (London)* **1982**, *299*, 79–81.

(13) Turcotte, A.; LaLonde, J.-M.; St-Pierre, S.; LeMaire, S. Dynorphin-(1–13) I. Structure-Function Relationships of Ala-Containing Analogs. *Int. J. Pept. Protein Res.* **1984**, *23*, 361–367.

(14) Snyder, K. R.; Story, S. C.; Heidt, M. E.; Murray, T. F.; DeLander, G. E.; Aldrich, J. V. Effect of Modification of the Basic Residues of Dynorphin A-(1–13) Amide on κ Opioid Receptor Selectivity and Opioid Activity. *J. Med. Chem.* **1992**, *35*, 4330–4333.

(15) Story, S. C.; Murray, T. F.; DeLander, G. E.; Aldrich, J. V. Synthesis and Opioid Activity of 2-Substituted Dynorphin A-(1–13) Amide Analogues. *Int. J. Pept. Protein Res.* **1992**, *40*, 89–96.

(16) Kawasaki, A. M.; Knapp, R. J.; Walton, W. S.; Zalewska, T.; Yamamura, H. I.; Porreca, F.; Burks, T. M.; Hruby, V. J. Syntheses, Opioid Binding Affinities, and Potencies of Dynorphin A Analogues Substituted in Position 1, 6, 7, 8 and 10. *Int. J. Pept. Protein Res.* **1993**, *42*, 411–419.

(17) Hruby, V. J.; Al-Obeidi, F.; Kazmierski, W. Emerging Approaches in the Molecular Design of Receptor-Selective Peptide Ligands: Conformational, Topographical and Dynamic Considerations. *Biochem. J.* **1990**, *268*, 249–262.

(18) Schiller, P. W.; Nguyen, T. M.-D.; Maziak, L. A. Synthesis and Activity Profiles of Novel Cyclic Opioid Peptide Monomers and Dimers. *J. Med. Chem.* **1985**, *28*, 1766–1771.

(19) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. P. Bis-penicillamine Enkephalins Possess Highly Improved Specificity Toward δ Opioid Receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871–5874.

(20) Schiller, P. W.; Eggiman, B.; Nguyen, T. M.-D. Comparative Structure-Function Studies with Analogs of Dynorphin-(1–13) and [Leu^2]Enkephalin. *Life Sci.* **1982**, *31*, 1777–1780.

(21) Shearman, G. T.; Schulz, R.; Schiller, P. W.; Herz, A. Generalization Tests with Intraventricularly Applied Pro-enkephalin B Derived Peptides in Rats Trained to Discriminate the Opioid Kappa Receptor Agonist Ethylketocyclazocine. *Psychopharmacology* **1985**, *85*, 440–443.

(22) Schiller, P. W.; Nguyen, T. M.-D.; Lemieux, C. Synthesis and Opioid Activity Profile of Cyclic Dynorphin Analogs. *Tetrahedron* **1988**, *44*, 733–743.

(23) Snyder, K. R.; Murray, T. F.; DeLander, G. E.; Aldrich, J. V. Synthesis and Opioid Activity of Dynorphin A-(1–13)NH_2 Analogues Containing *cis*- and *trans*-4-Aminocyclohexanecarboxylic Acid. *J. Med. Chem.* **1993**, *36*, 1100–1103.

(24) Kawasaki, A. M.; Knapp, R. J.; Kramer, T. H.; Wire, W. S.; Vasquez, O. S.; Yamamura, H. I.; Burks, T. F.; Hruby, V. J. Design and Synthesis of Highly Potent and Selective Cyclic Dynorphin A Analogs. *J. Med. Chem.* **1990**, *33*, 1874–1879.

(25) Kawasaki, A. M.; Knapp, R. J.; Kramer, T. H.; Walton, A.; Wire, W. S.; Hashimoto, S.; Yamamura, H. I.; Porreca, F.; Burks, T. F.; Hruby, V. J. Design and Synthesis of Highly Potent and Selective Cyclic Dynorphin A Analogs. 2. New Analogs. *J. Med. Chem.* **1993**, *36*, 750–757.

(26) Schwyzer, R. Estimated Conformation, Orientation and Accumulation of Dynorphin A-(1–13)-tridecapeptide on the Surface of the Neutral Lipid Membranes. *Biochemistry* **1986**, *25*, 4281–4286.

(27) Weiner, P. K.; Kollman, P. A. AMBER: Assisted Model Building with Energy Refinement. A General Program for Modeling Molecules and Their Interactions. *J. Comput. Chem.* **1981**, *2*, 287–303.

(28) Weiner, S. J.; Kollman, P. A.; Case, D. A.; Singh, U. C.; Ghio, C.; Alagona, G.; Profeta, S., Jr.; Weiner, P. K. A New Force Field for Molecular Mechanical Simulation of Nucleic Acids and Proteins. *J. Am. Chem. Soc.* **1984**, *106*, 765–784.

(29) Weiner, S. J.; Kollman, P. A. An All Atom Force Field for Simulations of Proteins and Nucleic Acids. *J. Comput. Chem.* **1986**, *7*, 230–252.

(30) Barany, G.; Merrifield, R. B. Solid-Phase Peptide Synthesis. In *The Peptides: Analysis, Synthesis, Biology; Special Methods in Peptide Synthesis, Part A*; Gross, E., Meienhofer, J., Eds.; Academic Press, Inc.: New York, 1980; Vol. 2, pp 1–284.

(31) Bodanszky, M.; Martinez, J. Side Reactions in Peptide Synthesis. In *The Peptides: Analysis, Synthesis, Biology; Special Methods in Peptide Synthesis, Part B*; Gross, E., Meienhofer, J., Eds.; Academic Press, Inc.: New York, 1984; Vol. 5, pp 111–216.

(32) Waki, M.; Kitajima, Y.; Izumiya, N. A Facile Synthesis of N^2-Protected L-2,3-Diaminopropanoic Acid. *Synthesis* **1981**, 266–268.

(33) Bodanszky, M.; Bodanszky, A. *The Practice of Peptide Synthesis*; Springer-Verlag: New York, 1984; p 20.

(34) Anwer, M. K.; Spatola, A. F. An Advantageous Method for the Rapid Removal of Hydrogenolysable Protecting Groups under Ambient Conditions; Synthesis of Leucine-enkephalin. *Synthesis* **1980**, 929–932.

(35) Story, S. C.; Aldrich, J. V. Side-product Formation during Cyclization with HBTU on a Solid Support. *Int. J. Pept. Protein Res.* **1994**, *43*, 292–296.

(36) Gausepohl, H.; Pieles, U.; Frank, R. W. Schiffs Base Analog Formation During In Situ Activation by HBTU and TBTU. In *Peptides: Chemistry and Biology Proceedings of the 12th American Peptide Symposium*; Smith, J. A., Rivier, J. E., Eds.; ESCOM: Leiden, 1991; pp 523–524.

(37) Robson, L. E.; Foote, R. W.; Maurer, R.; Kosterlitz, H. W. Opioid Binding Sites of the κ-type in Guinea-Pig Cerebellum. *Neuroscience* **1984**, *12*, 621–627.

(38) Renugopalakrishnan, V.; Rapaka, R. S.; Bhargava, N. Conformational Feature of Opioid Peptides: Ligand Receptor Interactions. In *Opioid Peptides*; Székely, J., Ramabadran, K., Eds.; CRC Press: Boston, 1990; Vol. IV, pp 53–114.

(39) Shoemaker, K. R.; Kim, P. S.; York, E. J.; Stewart, J. M.; Baldwin, R. L. Tests of the Helix Dipole Model for Stabilization of α-Helices. *Nature (London)* **1987**, *326*, 563–567.

(40) van Gorkom, L. C. M.; Lancaster, C. R. D.; St-Pierre, S.; Bothner-By, A. A.; Epand, R. M. The Membrane Mediated Conformation of Dynorphin A(1–13) as Studied by Transferred Nuclear Overhauser Effect Spectroscopy. In *Peptides: Chemistry and Biology Proceedings of the 12th American Peptide Symposium*; Smith, J. A., Rivier, J. E., Eds.; ESCOM: Leiden, 1992; pp 233–234.
(41) Kallick, D. Conformation of Dynorphin A(1–17) Bound to Dodecylphosphocholine Micelles. *J. Am. Chem. Soc.* **1993**, *115*, 9317–9318.
(42) Schiller, P. W. Fluorescence Study on the Solution Conformation of Dynorphin in Comparison to Enkephalin. *Int. J. Pept. Protein Res.* **1983**, *21*, 307–312.
(43) King, D. S.; Fields, C. G.; Fields, G. B. A Cleavage Method Which Minimizes Side Reactions Following Fmoc Solid Phase Peptide Synthsis. *Int. J. Pept. Protein Res.* **1990**, *36*, 255–266.
(44) Chen, G. C.; Yang, J. T. Two-Point Calibration of Circular Dichrometer with d-10–Camphorsulfonic Acid *Anal. Lett.* **1977**, *10*, 1195–1207.
(45) Elwell, M. L.; Schellmen, J. A. Stability of Phage T4 Lysosymes 1. Native Properties and Thermal Stability of Wild Type and Two Mutant Lysosymes. *Biochim. Biophys. Acta* **1977**, *494*, 367–383.
(46) Cheng, Y. C.; Prusoff, W. H. Relationship between the Inhibition Constant (K_i) and the Concentration of Inhibitor Which Causes 50 Percent Inhibition (IC_{50}) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

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