Journal of Medicinal Chemistry Article

J. Med. Chem. **2009,** *52,* 5619–5625 **5619**
DOI: 10.1021/jm900577k

Design, Synthesis, and Pharmacological Activities of Dynorphin A Analogues Cyclized by Ring-Closing Metathesis

Wei-Jie Fang,[†,§] Yanjun Cui,[‡] Thomas F. Murray,[‡] and Jane V. Aldrich*[,†]

[†]*Department of Medicinal Chemistry, The University of Kansas, Lawrence, Kansas 66045, and* [‡]*Department of Pharmacology, Creighton University School of Medicine, Omaha, Nebraska 68178.* [§]*Current address: Department of Chemical and Biological Engineering, University of Colorado, Boulder, Colorado 80309.*

Received May 5, 2009

Dynorphin A (Dyn A) is an endogenous ligand for κ opioid receptors. To restrict the conformational mobility, we synthesized several cyclic Dyn A-(1−11)NH_2 analogues on solid phase utilizing ring-closing metathesis (RCM) between the side chains of allylglycine (AllGly) residues incorporated in positions 2, 5, and/or 8. Cyclizations between the side chains of AllGly gave reasonable yields (56−74%) of all of the desired cyclic peptides. Both the cis and trans isomers were obtained for all of the cyclic peptides, with the ratio of cis to trans isomers depending on the position and stereochemistry of the AllGly. Most of the cyclic Dyn A-(1−11)NH_2 analogues examined exhibit low nanomolar binding affinity for κ opioid receptors (K_i = 0.84−11 nM). In two of the three cases, the configuration of the double bond has a significant influence on the opioid receptor affinities and agonist potency. All of the peptides inhibited adenylyl cyclase activity in a concentration-dependent manner with full or close to full agonist activity. These potent Dyn A analogues are the first ones cyclized by RCM.

Introduction

Potent clinically used narcotic analgesic agents, such as morphine and its analogues, mainly act as μ opioid receptor agonists. However, their use is associated with serious side effects, such as respiratory depression, physiological and psychological dependence, and constipation.[1] Therefore considerable effort has focused on the development of κ selective opioid agonists, especially those acting in the periphery, as potential analgesics without the side effects associated with morphine and other μ opioid receptor agonists.[2,3] Besides their roles in analgesia, κ opioid receptor agonists may also have other therapeutic applications, which include the treatment of cocaine dependence,[4] as neuroprotective and anticonvulsant agents,[5] and the treatment of HIV-1 and HIV-1 related encephalopathy.[6,7] Ligands for κ opioid receptors are very useful for studying the functions of these receptors at the molecular level, which in turn could be very important in the development of new therapeutic agents.

Dynorphin A (Dyn A,[a] Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln), a heptadecapeptide first isolated from porcine pituitary,[8] is an endogenous ligand for κ opioid receptors and is thought to be involved in a variety of physiological functions.[9] Dyn A has an identical N-terminal tetrapeptide sequence (the "message" sequence, Tyr-Gly-Gly-Phe)[10] as most other mammalian opioid peptides and a C-terminal sequence (the "address" sequence),[10] which is unique to Dyn A. Dyn A-(1−13) and Dyn A-(1−11) exhibit similar κ opioid receptor activity to Dyn A,[10] and therefore these two shorter peptides have often served as parent structures for structure−activity relationship (SAR) studies and for the development of analogues with improved κ receptor affinity, selectivity, potency, and/or altered efficacy.

Like most linear peptides Dyn A can adopt numerous conformations, and because of this the biologically active conformations are not yet clear.[11−16] This inherent conformational flexibility may be one of the reasons that Dyn A also exhibits significant affinity for μ and δ opioid receptors, resulting in low selectivity for κ opioid receptors.

Conformational constraint by cyclization is one approach that can be used to restrict the flexibility of peptides, and therefore is a valuable approach to study topographical requirements of receptors.[17−20] Cyclization of peptides can provide potent and selective ligands for receptors when appropriate conformational constraints are incorporated[18] because a well-fit preorganized conformation decreases the entropy penalty for receptor binding.[19] Furthermore, cyclic peptides are often more stable to peptidases,[21−23] and therefore they can have improved pharmacokinetic profiles and represent promising lead compounds for further development.

Conformational constraint by cyclization has been successfully employed in the development of several potent opioid

*To whom correspondence should be addressed. Phone: (785) 864-2287; Fax: (785) 864-5326; E-mail: jaldrich@ku.edu.

[a] Abbreviations: Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). Amino acids are the L-configuration except where indicated otherwise. AC, adenylyl cyclase; AllGly, allylglycine; cAMP, cyclic adenosine monophosphate; DAMGO, ([D-Ala^2,MePhe^4,-glyol]enkephalin; Dap, 2,3-diaminopropionic acid; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DPDPE, *cyclo*[D-Pen^2,D-Pen^5]enkephalin; Dyn, dynorphin; ESI-MS, electrospray ionization-mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; HOBt, hydroxybenzotriazole; HPLC, high-performance liquid chromatography; PAL-PEG-PS, peptide amide linker−poly(ethylene glycol)-polystyrene); PyBOP, benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate; RCM, ring-closing metathesis; SAR, structure−activity relationships; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TIPS, triisopropylsilane.

Published on Web 08/28/2009

peptides. Several cyclic Dyn A analogues have been synthesized and evaluated for their biological activity.[1,19,24−31] Our laboratory previously reported several cyclic Dyn A analogues where either the "message" or "address" sequence was constrained. *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2 (Dap = 2,3-diaminopropionic acid) exhibits high affinity for both κ and μ opioid receptors.[29] *cyclo*[D-Asp^5,Dap^8]Dyn A-(1−13)NH_2 shows modest affinity for κ opioid receptors compared with the linear peptide Dyn A-(1−13)NH_2, but it shows increased selectivity for κ over μ and δ opioid receptors compared to Dyn A-(1−13)NH_2.[31] Cyclic Dyn A analogues have also been prepared by other research groups utilizing either disulfide[25−28] or amide[24,30] bonds to constrain the peptides.

We utilized ring-closing metathesis (RCM) in the design and synthesis of new cyclic Dyn A analogues. RCM has emerged as a very useful method for making cyclic organic compounds as well as cyclic peptides.[32−35] Compared with peptides cyclized by amide or disulfide bond formation, there are some advantages to using RCM. The resulting carbon−carbon bond is more stable than a disulfide or an amide bond.[34,36] Furthermore, in contrast to cyclization via amide or disulfide bond, side chain functionalities can be maintained by appropriate choice of the amino acid side chains for cyclization by RCM. In addition, cyclization by RCM can potentially stabilize different conformations of a peptide compared to a disulfide or amide as a result of the geometry of the linkage. The C−S−S−C dihedral angle for disulfide linkages is approximately ± 90°. While the amide bond in a lactam linkage can potentially be either cis (0°) or trans (180°), the conformation of this bond for secondary amides is predominantly trans, except in selected cases involving small rings that favor the cis conformation due to ring strain in the trans conformation. Also the two conformations of the amide bond cannot be isolated from one another. In contrast, in many cases both the cis and trans isomers of the double bond are obtained from RCM and can often be separated from one another by careful choice of chromatographic conditions (see below). This permits the examination of the effect of a cis double bond on the biological activity of the peptide compared to that of the trans isomer, a comparison that is not possible with cyclization via a lactam.

The application of RCM to the cyclization of opioid peptides has been very limited. To date there have been only three reports of RCM cyclic analogues of short opioid peptides, namely pentapeptide enkephalin analogues and tetrapeptides related to dermorphin.[37−39] These analogues were cyclized between D-allylglycine (D-AllGly) in position 2 and D- or L-AllGly in position 4 in the tetrapeptides or position 5 in the enkephalin derivatives. Some of these cyclic analogues showed potent activity at μ and δ opioid receptors; as expected, the enkephalin derivatives exhibited low affinity for κ opioid receptors (the κ receptor affinities of the tetrapeptides were not reported). In contrast, there have been no reports of longer opioid peptides (e.g., Dyn A analogues) or of other peptides cyclized through RCM that preferentially interact with κ opioid receptors. Here we describe our results for incorporating a cyclic constraint via RCM in the "address" (C-terminal) sequence as well as the "message" (N-terminal) sequence of Dyn A-(1−11)NH_2 and the effects of the double bond configuration on opioid receptor affinity and potency.

Results and Discussion

Analogue Design. Cyclic [2,5] and [5,8] Dyn A-(1−11)NH_2 analogues (Figure 1) were chosen to evaluate RCM for

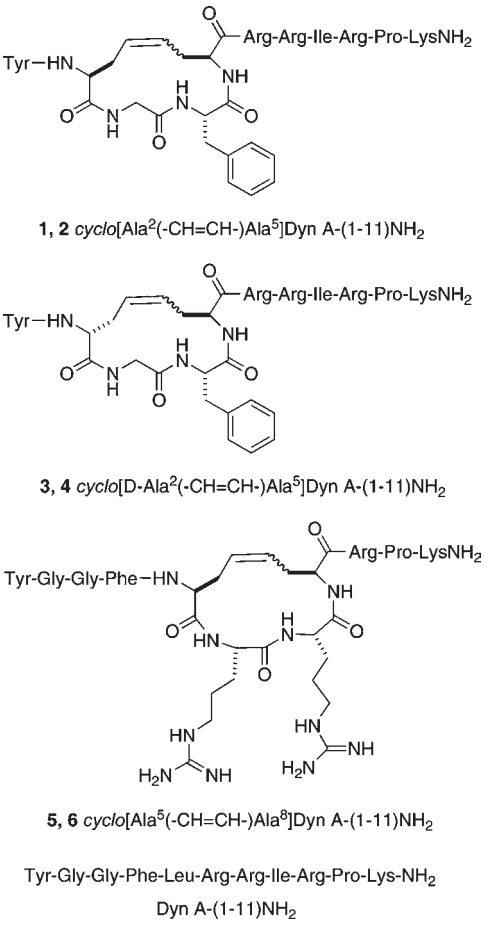


Figure 1. Structural comparison of *cyclo*[L/D-Ala^2(−CH=CH−)-Ala^5]Dyn A-(1−11)NH_2 (**1**−**4**) and *cyclo*[Ala^5(−CH=CH−)-Ala^8]Dyn A-(1−11)NH_2 (**5**, **6**) with Dyn A-(1−11)NH_2.

cyclizing Dyn A analogues and to examine the effects of these cyclizations on κ opioid receptor affinity, selectivity, efficacy, and potency. Substitution of a D-amino acid in position 2 for Gly in the linear peptides is well tolerated by κ opioid receptors; however, this modification in Dyn A analogues can greatly increase μ receptor affinity, resulting in compounds that are either nonselective or selective for μ opioid receptors.[40] Substitution of this position with an L-amino acid decreases binding affinity for all three types of opioid receptors. However, the κ opioid receptor is more tolerant of the L-configuration at this position than the other opioid receptors, and therefore the selectivity for κ opioid receptors can be increased by substitution with an L-amino acid.[40] On the basis of these observations, in the [2,5] cyclic analogues (Figure 1) both L- and D-AllGly were introduced in position 2 to evaluate their effects on the biological activity at κ opioid receptors. Leu^5 in Dyn A is not important for opioid activity,[41] and therefore this position can be used for cyclization. Similarly, Leu^5 and Ile^8 were substituted with AllGly and then cyclized by RCM to yield the [5,8] cyclic analogues **5** and **6** (Figure 1). Cyclization by RCM maintains the hydrophobic nature of these residues (Figure 1).

Synthesis. The peptides were synthesized by solid phase synthesis using Fmoc-protected (Fmoc = 9-fluorenylmethoxycarbonyl) amino acids (Scheme 1). L/D-AllGly was incorporated in appropriate positions in the linear precursor peptides, and upon completion of assembly of the peptide chains

Scheme 1. Synthesis of Cyclic Peptides **1** and **2**

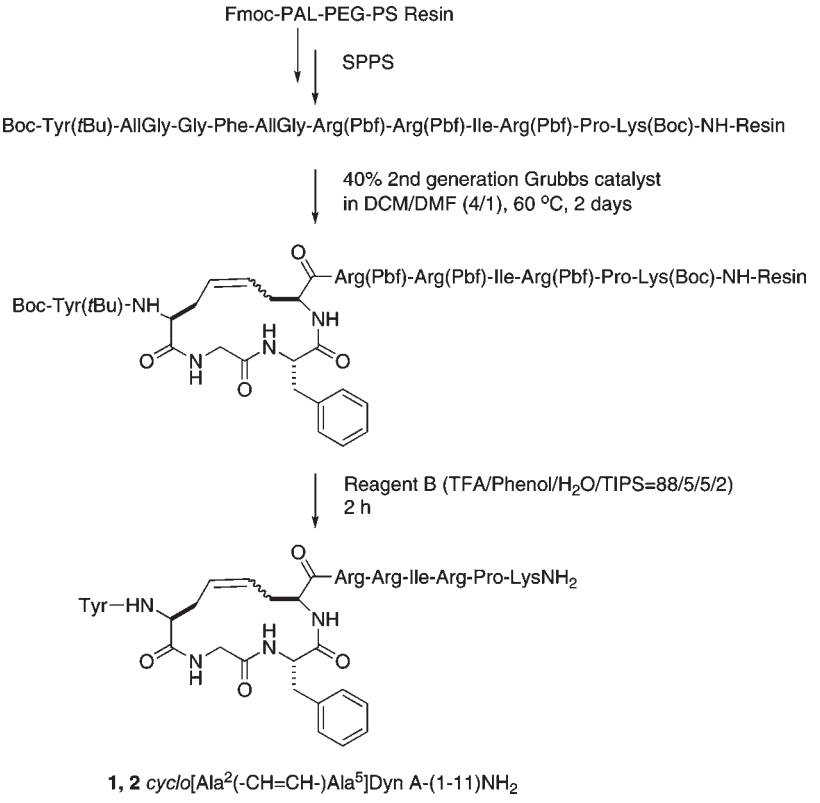


Table 1. Yields and cis/trans Ratios of Dyn A-(1−11)NH_2 Analogues Cyclized by RCM

entry	compd[a]	products (% by HPLC)[b]	cis/trans[c]
A	*cyclo*[Ala^2(−CH=CH−)Ala^5]Dyn A-(1−11)NH_2: cis, **1**; trans, **2**	56	1:1.8
B	*cyclo*[D-Ala^2(−CH=CH−)Ala^5]Dyn A-(1−11)NH_2: cis, **3**; trans, **4**	74	1:2.3
C	*cyclo*[Ala^5(−CH=CH−)Ala^8] Dyn A-(1−11)NH_2: cis, **5**; trans, **6**	63	1:1.1

[a] The alkene bridged cyclic constraints are designated as modifications to the side chains of alanine residues in the indicated positions. The structures of the peptides are shown in Figure 2. [b] The remainder was the linear precursor peptide. [c] Configuration determined by NMR, see Table 3.

second-generation Grubbs' catalyst was used to cyclize the peptides on the solid support. A mixture of dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF) (4/1, v/v) was used as the solvent for the RCM reactions. The addition of a small amount of DMF has several advantages; DMF is compatible with both the hydrophilic peptide chain and resin and allows a higher temperature to be used for the reactions. The position or stereochemistry of the AllGly residue did not have much influence on the yield of the desired cyclic peptides. Cyclizations between the side chains of AllGly generally gave reasonable yields of the desired cyclic peptides (56−74%, Table 1). Both the cis and trans isomers were obtained for all of the cyclic peptides, with the ratio of isomers, as determined by NMR, varying from approximately 1:1.1 to 1:2.3, depending on the position and stereochemistry of the AllGly (Table 1).

Because the cis and trans isomers have very similar retention times (within 0.7 min) in the standard high-performance liquid chromatography (HPLC) system (5−50% of MeCN with 0.1% trifluoroacetic acid (TFA) over 45 min at 1 mL/min, see Table 2), a very slow gradient (0.1% increase in solvent B/min) was used for purification. The two isomers were successfully separated and characterized by HPLC, electrospray ionization mass spectrometry (ESI-MS), and NMR of the purified fractions (Tables 2 and 3).

The NMR *J*-couplings and chemical shifts were used to distinguish between the cis and trans isomers.[42−44] The splitting patterns of the vinyl protons for the cis and trans isomers are very different due to the different coupling constants to the adjacent methylene protons. For the trans isomer, the coupling constants between the two vinyl protons (J_{12}) are ∼15 Hz (Table 3), while the coupling constants between the vinyl protons and their corresponding adjacent methylene protons (J_{13}, J_{14}, J_{25}, and J_{26}) are around 7−8 Hz. Because of the coupling constants, generally five peaks (approximate ratio of 1:2:2:2:1) were observed in the NMR spectra of the trans isomers (Figure 2). For the cis isomer, the coupling constants between the two vinyl protons (J_{12}) are ∼10 Hz, while the coupling constants between the vinyl protons and adjacent methylene protons are ∼10 and 2 Hz. Because of the broad line width, only three peaks were generally observed for the cis isomers in a ratio of 1:2:1 (Figure 2). The chemical shifts of the two vinyl protons in the cyclic peptides are between 5.0 and 5.5 ppm (Table 3). The two vinyl protons in the cis isomer are slightly more shielded (upfield) than in the trans isomers (Figure 2 and Table 3).

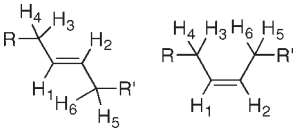
Pharmacology. The cyclic peptides were evaluated for their binding affinity at κ, μ, and δ opioid receptors using radioligand binding assays[31] (Table 4). Except for compound **1**, all of the cyclic Dyn A analogues examined exhibit low nanomolar binding affinity for κ opioid receptors (K_i = 0.84−11 nM).

Table 2. HPLC and ESI-MS Data of Purified Peptides **1**−**6**

	HPLC t_R (min)[a]		ESI-MS (m/z)	
peptides	system 1	system 2	calcd	obsd
1	13.23	24.08	$[M + 4H]^{4+} = 340.2$	$[M + 4H]^{4+} = 340.2$
			$[M + 3H]^{3+} = 453.3$	$[M + 3H]^{3+} = 453.3$
			$[M + 2H]^{2+} = 679.4$	$[M + 2H]^{2+} = 679.4$
2	13.30	25.38	$[M + 4H]^{4+} = 340.2$	$[M + 4H]^{4+} = 340.2$
			$[M + 3H]^{3+} = 453.3$	$[M + 3H]^{3+} = 453.3$
			$[M + 2H]^{2+} = 679.4$	$[M + 2H]^{2+} = 679.4$
3	12.78	24.87	$[M + 4H]^{4+} = 340.2$	$[M + 4H]^{4+} = 340.2$
			$[M + 3H]^{3+} = 453.3$	$[M + 3H]^{3+} = 453.2$
			$[M + 2H]^{2+} = 679.4$	$[M + 2H]^{2+} = 679.4$
4	13.49	26.99	$[M + 4H]^{4+} = 340.2$	$[M + 4H]^{4+} = 340.2$
			$[M + 3H]^{3+} = 453.3$	$[M + 3H]^{3+} = 453.3$
			$[M + 2H]^{2+} = 679.4$	$[M + 2H]^{2+} = 679.4$
5	9.11	16.32	$[M + 4H]^{4+} = 326.2$	$[M + 4H]^{4+} = 326.2$
			$[M + 3H]^{3+} = 434.6$	$[M + 3H]^{3+} = 434.6$
6	9.63	17.85	$[M + 4H]^{4+} = 326.2$	$[M + 4H]^{4+} = 326.2$
			$[M + 3H]^{3+} = 434.6$	$[M + 3H]^{3+} = 434.6$

[a] System 1: solvent B = MeCN; system 2: solvent B = MeOH; see the Experimental Section for details. The final purity of all of the peptides by both methods was greater than 98%.

Table 3. ^{1}H-NMR Data for Vinyl Protons of Cyclic Peptides **1**−**6**



peptide	chemical shifts (δ) of vinyl protons	J (Hz)
1 (cis)	H_1 = 5.10	J_{12} = 10.4
	H_2 = 5.34	J_{21} = 12.4
2 (trans)	H_1 = 5.25	J_{12} = 14.9
	H_2 = 5.47	J_{21} = 13.9
3 (cis)	H_1 = 5.05	J_{12} = 11.4
	H_2 = 5.16	J_{21} = 12.1
4 (trans)	H_1 = 5.12	J_{12} = 14.7
	H_2 = 5.26	J_{21} = 15.4
5 (cis)	H_1 = 5.14	J_{12} = 10.5
	H_2 = 5.41	J_{21} = 12.9
6 (trans)	H_1 = 5.21	J_{12} = 14.9
	H_2 = 5.46	J_{21} = 13.9

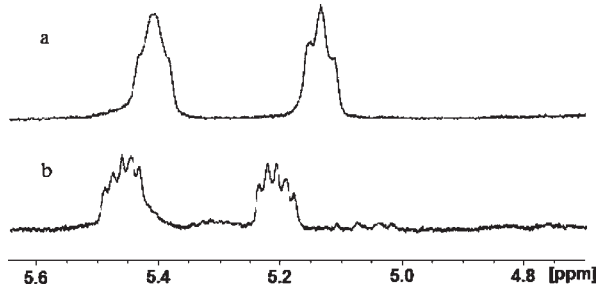


Figure 2. Chemical shifts and splitting patterns of the two vinyl protons of cyclic peptides **5** (a, cis) and **6** (b, trans).

The [2,5] cyclizations involving an L-AllGly in position 2 decreased κ opioid receptor affinity. The trans isomer of *cyclo*[Ala^2(−CH=CH−)Ala^5]Dyn A-(1−11)NH_2 (**2**) exhibits modest κ opioid receptor affinity (K_i = 9.5 nM, Table 4). However, the cis isomer **1** exhibits 10-fold lower affinity for κ receptors than the trans isomer **2**, presumably due to the differences in the peptides' conformations. Compared with Dyn A-(1−11)NH_2, both isomers **1** and **2** exhibit much lower affinity for μ and δ receptors, resulting in higher κ opioid receptor selectivity for these compounds than Dyn A-(1−11)NH_2 (Table 4). The cis isomer (**1**) has similar selectivity to the trans isomer (**2**) even though it shows about 10-fold lower affinity for κ opioid receptors. Thus while substitution of an L-amino acid in position 2 in the cyclic peptides decreases κ opioid receptor affinity compared with Dyn A-(1−11)NH_2, the selectivity toward the other opioid receptors (μ and δ) can be increased, as the latter receptors are less tolerant to the introduction of an L-amino acid in position 2 compared to κ opioid receptors.

Table 4. Opioid Receptor Binding Affinities of Dyn A Analogues Cyclized by RCM

	K_i (nM)			
compd	κ	μ	δ	K_i ratio ($\kappa/\mu/\delta$)
1	87.2 ± 6.9	763 ± 35	7670 ± 1030	1/8.8/88
2	9.46 ± 1.80	180 ± 10	1130 ± 98	1/19/119
3	0.84 ± 0.10	2.33 ± 0.20	9.30 ± 1.00	1/2.8/11
4	1.38 ± 0.31	2.33 ± 0.22	7.17 ± 0.55	1/1.7/5.2
5	10.9 ± 1.8	93.0 ± 6.0	1210 ± 90	1/8.5/111
6	2.46 ± 0.57	36.0 ± 2.1	460 ± 51	1/15/187
Dyn A-(1−11)NH_2[a]	0.57 ± 0.01	1.85 ± 0.52	6.18 ± 1.01	1/3/11

[a] From reference 45.

In contrast, the [2,5] cyclizations involving a D-AllGly in position 2 are well tolerated by κ opioid receptors. The two isomers of *cyclo*[D-Ala^2(−CH=CH−)Ala^5]Dyn A-(1−11)-NH_2 (**3** and **4**) exhibit high κ opioid receptor affinity (K_i = 0.84 and 1.38 nM for the cis and trans isomers, respectively, Table 4). However, these two compounds also exhibit high affinity for μ and δ opioid receptors, and therefore have minimal selectivity for κ over these opioid receptors. These results are similar to those found for *cyclo*[D-Asp^2, Dap^5]Dyn A-(1−13)NH_2 and *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2.[29,46]

Schiller and Hruby previously reported the synthesis of several tetrapeptide and enkephalin analogues utilizing RCM.[37−39] The two olefinic dicarba analogues of the enkephalin cyclic peptide H-Tyr-*cyclo*[D-Cys-Gly-Phe-L-Cys]-NH_2 exhibit nanomolar affinity for both μ (2.40 and 0.616 nM for the cis and trans isomers, respectively) and δ opioid receptors (6.55 nM and 1.25 nM for the cis and trans isomers, respectively).[38] As expected, these enkephalin analogues exhibit low affinity for κ opioid receptors (200 and 57.6 nM for the cis and trans isomers, respectively).[38] The addition of the C-terminal residues of Dyn A-(1−11)NH_2 substantially increases the affinity for κ opioid receptors while maintaining the affinity for μ opioid receptors and decreasing the affinity for δ opioid receptors. Interestingly, while the two enkephalin isomers reported previously have different affinities for all three opioid receptors, our two cyclic Dyn A isomers **3** and **4** exhibit very similar affinities for each of the receptors.

The cyclizations in the "address" sequence of Dyn A involving AllGly residues substituted in positions 5 and 8 are also tolerated by κ opioid receptors. The κ opioid receptor affinity of the trans isomer of *cyclo*[Ala^5-(−CH=CH−)Ala^8]Dyn A-(1−11)NH_2 (**6**, 2.46 nM) is 5-fold lower than Dyn A-(1−11)NH_2. However, this compound exhibits significantly higher selectivity for κ over μ and δ receptors (15- and 187-fold, respectively, Table 4)

Table 5. κ Opioid Receptor Potencies and Efficacies in the AC Assay of Dyn A Analogues Cyclized by RCM

compd	EC_{50} (nM)	maximum AC % inhibition[a]
1	190 ± 19	90 ± 2
2	27 ± 1	104 ± 4
3	0.80 ± 0.34	111 ± 6
4	0.47 ± 0.11	107 ± 4
5	23 ± 11	110 ± 10
6	8.3 ± 3.7	106 ± 6
Dyn A-(1−11)NH_2	0.39 ± 0.02	100

[a] Relative to Dyn A-(1−13)NH_2 (100%).

than the linear Dyn A-(1−11)NH_2. Similar to the isomers **1** and **2** of *cyclo*[Ala^2(−CH=CH−)Ala^5]Dyn A-(1−11)NH_2, the cis isomer of *cyclo*[Ala^5(−CH=CH−)Ala^8]Dyn A-(1−11)NH_2 (**5**) shows significantly lower affinity (4.4-fold) for κ opioid receptors (K_i = 10.9 nM) than the trans isomer **6**. However, the two isomers **5** and **6** exhibit similar selectivities for κ vs μ and δ opioid receptors (Table 4).

The cyclic analogues were also examined for concentration-dependent inhibition of adenylyl cyclase (AC) (Table 5).[47] All of the compounds inhibit AC activity in a concentration-dependent manner with similar efficacy (≥90%) to the reference agonist Dyn A-(1−13)NH_2. Thus the cyclizations have little or no effect on the efficacy of these Dyn A-(1−11)NH_2 analogues. The potencies (EC_{50}) of these cyclic analogues in the AC assays (Table 5) are well correlated with their κ opioid receptor affinities. The two isomers of *cyclo*[D-Ala^2(−CH=CH−)Ala^5]Dyn A-(1−11)NH_2 (**3** and **4**) exhibit the highest potencies with EC_{50} values (0.80 and 0.47 nM, respectively, Table 5) comparable to that of the parent peptide Dyn A-(1−11)NH_2. These results are consistent with those reported for *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2.[46] For the two isomers of *cyclo*[Ala^2-(−CH=CH−)Ala^5]Dyn A-(1−11)NH_2 (**1** and **2**), where the configuration of position 2 is L instead of D, the potencies dropped significantly (487- and 69-fold for **1** and **2**, respectively) compared with Dyn A-(1−11)NH_2. Similar to the affinities for κ opioid receptors, the trans isomer **2** is about 7-fold more potent than the cis isomer **1**. The two isomers of *cyclo*[Ala^5(−CH=CH−)Ala^8]Dyn A-(1−11)NH_2 (**5** and **6**) show intermediate potency among these cyclic peptides, with the trans isomer **6** being 2.8-fold more potent than the corresponding cis isomer **5**.

Conclusions

Here we report the synthesis of the first Dyn A analogues cyclized by RCM and the first peptides that preferentially interact with κ opioid receptors cyclized by this methodology. Cyclizations in both the "message" and "address" sequences of Dyn A were explored to prepare potent κ opioid receptor agonists. While cyclization by RCM has been introduced into the "message" sequence in enkephalin analogues and tetrapeptides related to dermorphin,[37−39] this is the first application of this type of cyclization in the "address" sequence of an opioid peptide. Cyclization by RCM has some advantages over traditional approaches such as amide or disulfide bond formation in these Dyn A analogues. The carbon−carbon double bond in these peptides retains similar lipophilicity to the side chains of Leu and Ile found in positions 5 and 8 in Dyn A. The positions and stereochemistry of the residues involved in the cyclizations influenced the affinity and selectivity for κ opioid receptors. Both [2,5] cyclic analogues with the D-configuration in position 2 (compounds **3** and **4**) retain high affinity for κ, μ, and δ receptors, indicating that the conformations which are adopted by these two peptides are compatible with all three opioid receptor types. Thus, these two peptides show minimal selectivity for κ over μ receptors and low selectivity for κ over δ receptors. The [5,8] cyclic analogues (compounds **5** and **6**) show intermediate affinity for κ opioid receptors; however, their selectivity over the other opioid receptors is greater than Dyn A-(1−11)NH_2. The [2,5] cyclic analogues with the L-configuration in position 2 (**1** and **2**) show the lowest affinity for κ opioid receptors; however, these two compounds also exhibit higher selectivity for κ receptors compared with the parent peptide Dyn A-(1−11)NH_2. In the latter two cases, the configuration of the double bond has a significant influence on the opioid receptor affinity and agonist potency, likely due to the double bond configuration affecting the conformation of the cyclic portion of the peptide. In both cases, the peptides containing the trans double bond exhibits higher κ opioid receptor affinity, selectivity, and agonist potency in the AC assay than the cis isomer. Similar to Dyn A, these cyclic analogues all exhibit concentration-dependent agonist activity (≥90% efficacy) at κ opioid receptors with potencies well correlated with their affinities.

These analogues represent interesting lead compounds for further characterization of conformation−activity relationships for Dyn A at κ opioid receptors. Further studies of these and other Dyn A analogues cyclized by RCM are ongoing in our laboratory.

Experimental Section

Materials. All standard Fmoc-protected amino acids were purchased from Bachem (King of Prussia, PA), Calbiochem-Novabiochem (San Diego, CA), Applied Biosystems (Foster City, CA), or Peptides International (Louisville, KY). Fmoc-AllGly-OH and Fmoc-D-AllGly-OH were purchased from NeoMPS (San Diego, CA). Fmoc-PAL-PEG-PS (PAL-PEG-PS = peptide amide linker-poly(ethylene glycol)-polystyrene) resin was purchased from Applied Biosystems. Benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Calbiochem-Novabiochem. DCM, *N,N*-diisopropylethylamine (DIEA), DMF, acetic acid, diethyl ether, acetonitrile, methanol, and TFA were purchased from Fisher Scientific (Hampton, NH). 1-Hydroxybenzotriazole (HOBt) and triisopropylsilane (TIPS) were purchased from Acros Chemical Co. (Pittsburgh, PA). All other chemicals including phenol, piperidine, second-generation Grubbs' catalyst, and dimethyl sulfoxide-d_6 (DMSO-d_6) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Synthesis of Cyclic Dyn A Analogues. The peptides were synthesized on the Fmoc-PAL-PEG-PS resin (300 mg, 0.19−0.21 mmol/g) using a CS Bio automated peptide synthesizer, except for the couplings of Fmoc-AllGly-OH and Fmoc-D-AllGly-OH, which were performed manually. The synthesis of the peptides *cyclo*[Ala^2(−CH=CH−)Ala^5]Dyn A-(1−11)NH_2 (**1** and **2**) is shown in Scheme 1 as an example. The desired Fmoc-protected amino acids were coupled to the growing peptide chain with PyBOP, HOBt, and DIEA (4/4/10 relative to the resin substitution) in DMF (2 mL) for 2 h; for Fmoc-AllGly-OH and Fmoc-D-AllGly-OH, 2 equiv were used for the couplings (using 2, 2, and 5 equiv of PyBOP, HOBt, and DIEA, respectively, relative to the resin). The completion of the reactions was determined by the ninhydrin test.[48] Following the assembly of the linear precursor, the resin was mixed with 40 mol % second-generation Grubbs' catalyst in DCM/DMF (4/1, v/v) under reflux conditions (60 °C) for 2 d (Scheme 1). The resin was then washed with DCM (10 × 5 mL) to remove the catalyst.

Finally, the resin was washed with methanol and dried under vacuum. The crude cyclic peptides were cleaved from the resin by treating with 5 mL of reagent B (88% TFA, 5% phenol, 5% water, and 2% TIPS) for 2 h[49] and the peptides isolated as described previously.[50]

Analysis of Cyclic Dyn A Analogues. The crude peptides were analyzed by analytical reversed-phase HPLC to determine the yields and ratios of the two isomers obtained from the RCM reactions. A linear gradient of 5−50% MeCN containing 0.1% TFA over 45 min, at a flow rate of 1 mL/min, was used for the analysis.

The crude peptides were purified by preparative reversed-phase HPLC using a linear gradient of 5−23% aqueous MeCN containing 0.1% TFA over 3 h (0.1% MeCN/min) at a flow rate of 20 mL/min. The purity of the final peptides was verified using two analytical HPLC systems (Table 2). For analytical HPLC, a linear gradient of 5−50% solvent B (solvent A, aqueous 0.1% TFA and solvent B, MeCN containing 0.1% TFA (system 1) or MeOH containing 0.1% TFA (system 2) over 45 min, at a flow rate of 1 mL/min, was used. The final purity of all of the peptides by both methods was greater than 98%. Molecular weights of the peptides (Table 2) were determined by ESI-MS Waters LCT Premier time-of-flight instrument.

The configuration of the double bond in the cyclic RCM peptides was determined by NMR analysis. ^{1}H NMR spectra of these compounds (2−5 mg) were obtained at 25 °C in DMSO-d_6 on a Bruker AVANCE DRX-500 spectrometer (500.13 MHz proton frequency) equipped with a 5 mm *z*-gradient Cryoprobe. ^{1}H chemical shifts referenced to the residual DMSO signal at 2.49 ppm, and coupling constants were extracted from the 1D spectra (Table 3). The 1D ^{1}H NMR spectra of these cyclic analogues are provided in the Supporting Information.

Pharmacological Assays. Radioligand binding assays were performed as previously described[31] using cloned rat κ, rat μ, and mouse δ opioid receptors stably expressed separately on CHO cells. [^{3}H]Diprenorphine (0.4 nM), [^{3}H]DAMGO ([D-Ala^2,MePhe^4,glyol]enkephalin, 1 nM), and [^{3}H]DPDPE (*cyclo*[D-Pen^2,D-Pen^5]enkephalin, 0.15 nM) were used as radioligands in the binding assays for κ, μ, and δ opioid receptors, respectively. Incubations were carried out in triplicate with varying concentrations of peptides (0.1−10,000 nM) for 90 min at room temperature 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, or DPDPE for κ, μ and δ receptors, respectively. IC_{50} values were determined by nonlinear regression analysis to fit a logistic equation to the competition data using Prism software (GraphPad Software Co., San Diego, CA). K_i values were calculated from the IC_{50} values by the Cheng and Prusoff equation,[51] using K_D values of 0.45, 0.49, and 1.76 nM for [^{3}H]diprenorphine, [^{3}H]DAMGO, and [^{3}H]DPDPE, respectively. The results presented are the mean ± SEM from at least three separate assays.

The peptides were also evaluated for their ability to inhibit the synthesis of cyclic adenosine monophosphate (cAMP) by AC using cloned rat κ opioid receptors stably expressed on CHO cells as previously described.[47] Cells were washed twice with free F12 medium and then incubated for 4 h in 1 mL of the same media containing 12 μCi [^{3}H]adenine. The cells were then incubated at 37 °C for 40 min in the presence of 50 μM forskolin, peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine), and varying concentrations of the peptide ligand (0.1−10,000 nM in 10-fold dilutions). Incubations were terminated by the addition of 30 μL of stop solution (2% sodium dodecyl sulfate and 1.3 mM cAMP in water), followed by the addition of 100 μL of conc perchloric acid and 750 μL of water. [^{14}C]cAMP (500 cpm in 50 μL) was added to each well to correct for recovery. After transferring the contents of the wells to 1.5 mL centrifuge tubes, 12 M KOH was added to neutralize the samples. The resulting precipitates were pelleted by centrifugation at 10000*g* for 10 min. cAMP in the supernatants was isolated by sequential chromatography over Bio-Rad AG-50W-X4 cation exchange resin and neutral alumina. The concentrations of [^{3}H]cAMP and [^{14}C]cAMP in the eluants were determined simultaneously by scintillation counting. Counts were corrected for crossover and recovery. The efficacies of the analogues are expressed relative to the reference compound Dyn A-(1−13)NH_2. The results presented are the mean ± SEM from at least three separate assays.

Acknowledgment. We thank Dr. David VanderVelde from the University of Kansas NMR Laboratory for assistance with configurational assignment based on the NMR data. Funding was provided by National Institute on Drug Abuse grant R01 DA18832.

Supporting Information Available: The ^{1}H NMR data of cyclic Dyn A analogues **1**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Aldrich, J. V.; Vigil-Cruz, S. C. Narcotic Analgesics. In *Burger's Medicinal Chemistry & Drug Discovery*; Abraham, D. J., Ed.; John Wiley & Sons, Inc.: New York, 2003; Vol. 6, pp 329−481.
(2) Millan, M. J. κ-Opioid Receptors and Analgesia. *Trends Pharmacol. Sci.* **1990**, *11*, 70–76.
(3) Riviere, P. J. M.; Junien, J. L. Opioid Receptors, Targets for New Gastrointestinal Drug Development. In *Drug Development, Molecular Targets for GI Diseases*; Gaginella, T. S.; Guglietta, A., Eds.; Humana Press: Totowa, NJ, 2000; pp 203−238.
(4) Shippenberg, T. S.; Zapata, A.; Chefer, V. I. Dynorphin and the Pathophysiology of Drug Addiction. *Pharmacol. Ther.* **2007**, *116*, 306–321.
(5) Tortella, F. C.; Decoster, M. A. Kappa Opioids: Therapeutic Considerations in Epilepsy and CNS Injury. *Clin. Neuropharmacol.* **1994**, *17*, 403–416.
(6) Chao, C. C.; Gekker, G.; Hu, S.; Sheng, W. S.; Shark, K. B.; Bu, D.-F.; Archer, S.; Bidlack, J. M.; Peterson, P. K. κ Opioid Receptors in Human Microglia Downregulate Human Immunodeficiency Virus 1 Expression. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8051–8056.
(7) Peterson, P. K.; Gekker, G.; Lokensgard, J. R.; Bidlack, J. M.; Chang, A.-C.; Fang, X.; Portoghese, P. S. κ-Opioid Receptor Agonist Suppression of HIV-1 Expression in CD4+ Lymphocytes. *Biochem. Pharmacol.* **2001**, *61*, 1145–1151.
(8) Goldstein, A.; Fischli, W.; Lowney, L. I.; Hunkapiller, M.; Hood, L. Porcine Pituitary Dynorphin: Complete Amino Acid Sequence of the Biologically Active Heptadecapeptide. *Proc. Natl. Acad. Sci. U.S.A* **1981**, *78*, 7219–7223.
(9) Caudle, R. M.; Mannes, A. J. Dynorphin: Friend or Foe? *Pain* **2000**, *87*, 235–239.
(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) Schwyzer, R. Estimated Conformation, Orientation, and Accumulation of Dynorphin A-(1−13)-Tridecapeptide on the Surface of Neutral Lipid Membranes. *Biochemistry* **1986**, *25*, 4281–4286.
(12) Schwyzer, R. Molecular Mechanism of Opioid Receptor Selection. *Biochemistry* **1986**, *25*, 6335–6342.
(13) Taylor, J. W.; Osapay, G. Determining the Functional Conformations of Biologically Active Peptides. *Acc. Chem. Res.* **1990**, *23*, 338–344.
(14) Renugopalakrishnan, V.; Paraka, R. S.; Bhargava, H. N. Conformational Features of Opioid Peptides: Ligand Receptor Interactions. In *Opioid Peptides, Biochemistry and Applied Physiology*; Szekely, J. I.; Ramabadran, K., Eds.; CRC Press: Boca Raton, FL, 1990; Vol. IV, pp 53−114.
(15) Lancaster, C. R. D.; Mishra, P. K.; Hughes, D. W.; St.-Pierre, S. A.; Bothner-By, A. A.; Epand, R. M. Mimicking the Membrane-Mediated Conformation of Dynorphin A-(1−13)-Peptide: Circular Dichroism and Nuclear Magnetic Resonance Studies in Methanolic Solution. *Biochemistry* **1991**, *30*, 4715–4726.
(16) Kallick, D. A. Conformation of Dynorphin A(1−17) Bound to Dodecylphosphocholine Micelles. *J. Am. Chem. Soc.* **1993**, *115*, 9317–9318.
(17) Deber, C. M.; Madison, V.; Blout, E. R. Why Cyclic Peptides? Complementary Approaches to Conformations. *Acc. Chem. Res.* **1976**, *9*, 106–113.

(18) Freidinger, R. M.; Veber, D. F.; Perlow, D. S. Bioactive Conformation of Luteinizing Hormone-Releasing Hormone: Evidence from a Conformationaily Constrained Analog. *Science* **1980**, *210*, 656–658.
(19) Hruby, V. J.; Agnes, R. S. Conformation−Activity Relationships of Opioid Peptides with Selective Activities at Opioid Receptors. *Biopolymers (Pept. Sci.)*. **1999**, *51*, 391–410.
(20) Hruby, V. J. Design in Topographical Space of Peptide and Peptidomimetic Ligands That Affect Behavior. A Chemist's Glimpse at the Mind−Body Problem. *Acc. Chem. Res.* **2001**, *34*, 389–397.
(21) Chappa, A. K. Biopharmaceutical Aspects of the Development of Peptides as CNS Drug Delivery Vectors and Therapeutic Agents: Studies with Substance P and Dynorphin A Analogs. Ph. D. Thesis. Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, KS, 2007.
(22) Sako, Y.; Goto, Y.; Murakami, H.; Suga, H. Ribosomal Synthesis of Peptidase-Resistant Peptides Closed by a Nonreducible Inter-Side-Chain Bond. *ACS Chem. Biol.* **2008**, *3*, 241–249.
(23) Miranda, L. P.; Winters, K. A.; Gegg, C. V.; Patel, A.; Aral, J.; Long, J.; Zhang, J.; Diamond, S.; Guido, M.; Stanislaus, S.; Ma, M.; Li, H.; Rose, M. J.; Poppe, L.; Veniant, M. M. Design and Synthesis of Conformationally Constrained Glucagon-Like Peptide-1 Derivatives with Increased Plasma Stability and Prolonged *in vivo* Activity. *J. Med. Chem.* **2008**, *51*, 2758–2765.
(24) Schiller, P. W.; Nguyen, T. M. D.; Lemieux, C. Synthesis and Opioid Activity Profiles of Cyclic Dynorphin Analogs. *Tetrahedron* **1988**, *44*, 733–743.
(25) 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.
(26) 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.
(27) Collins, N.; Hruby, V. J. Prediction of the Conformational Requirements for Binding to the κ-Opioid Receptor and Its Subtypes. I. Novel α-Helical Cyclic Peptides and Their Role in Receptor Selectivity. *Biopolymers* **1994**, *34*, 1231–1241.
(28) Meyer, J.-P.; Collins, N.; Lung, F.-D.; Davis, P.; Zalewska, T.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. Design, Synthesis, and Biological Properties of Highly Potent Cyclic Dynorphin A Analogs. Analogs Cyclized between Positions 5 and 11. *J. Med. Chem.* **1994**, *37*, 3910–3917.
(29) Arttamangkul, S.; Murray, T. F.; DeLander, G. E.; Aldrich, J. V. Synthesis and Opioid Activity of Conformationally Constrained Dynorphin A Analogs. 1. Conformational Constraint in the "Message" Sequence. *J. Med. Chem.* **1995**, *38*, 2410–2417.
(30) Lung, F. D. T.; Collins, N.; Stropova, D.; Davis, P.; Yamamura, H. I.; Porreca, F.; Hruby, V. J. Design, Synthesis, and Biological Activities of Cyclic Lactam Peptide Analogues of Dynorphin A(1−11)-NH_2. *J. Med. Chem.* **1996**, *39*, 1136–1141.
(31) Arttamangkul, S.; Ishmael, J. E.; Murray, T. F.; Grandy, D. K.; DeLander, G. E.; Kieffer, B. L.; Aldrich, J. V. Synthesis and Opioid Activity of Conformationally Constrained Dynorphin A Analogues. 2. Conformational Constraint in the "Address" Sequence. *J. Med. Chem.* **1997**, *40*, 1211–1218.
(32) Fu, G. C.; Grubbs, R. H. The Application of Catalytic Ring-Closing Olefin Metathesis to the Synthesis of Unsaturated Oxygen Heterocycles. *J. Am. Chem. Soc.* **1992**, *114*, 5426–5427.
(33) Miller, S. J.; Blackwell, H. E.; Grubbs, R. H. Application of Ring-Closing Metathesis to the Synthesis of Rigidified Amino Acids and Peptides. *J. Am. Chem. Soc.* **1996**, *118*, 9606–9614.
(34) Stymiest, J. L.; Mitchell, B. F.; Wong, S.; Vederas, J. C. Synthesis of Oxytocin Analogues with Replacement of Sulfur by Carbon Gives Potent Antagonists with Increased Stability. *J. Org. Chem.* **2005**, *70*, 7799–7809.
(35) Reichwein, J. F.; Versluis, C.; Liskamp, R. M. J. Synthesis of Cyclic Peptides by Ring-Closing Metathesis. *J. Org. Chem.* **2000**, *65*, 6187–6195.
(36) Nutt, R. F.; Veber, D. F.; Saperstein, R. Synthesis of Nonreducible Bicyclic Analogs of Somatostatin. *J. Am. Chem. Soc.* **1980**, *102*, 6539–6545.
(37) Berezowska, I.; Chung, N. N.; Lemieux, C.; Wilkes, B. C.; Schiller, P. W. Cyclic Dermorphin Tetrapeptide Analogues Obtained via Ring-Closing Metathesis. *Acta Biochim. Pol.* **2006**, *53*, 73–76.
(38) Berezowska, I.; Chung, N. N.; Lemieux, C.; Wilkes, B. C.; Schiller, P. W. Dicarba Analogues of the Cyclic Enkephalin Peptides H-Tyr-*c*[D-Cys-Gly-Phe-D (or L)-Cys]NH_2 Retain High Opioid Activity. *J. Med. Chem.* **2007**, *50*, 1414–1417.
(39) Mollica, A.; Guardiani, G.; Davis, P.; Ma, S. W.; Porreca, F.; Lai, J.; Mannina, L.; Sobolev, A. P.; Hruby, V. J. Synthesis of Stable and Potent δ/μ Opioid Peptides: Analogues of H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-OH by Ring-Closing Metathesis. *J. Med. Chem.* **2007**, *50*, 3138–3142.
(40) Story, S. C.; Murray, T. F.; Delander, G. E.; Aldrich, J. V. Synthesis and Opioid Activity of 2-Substituted Dynorphin A-(1−13) Amide Analogs. *Int. J. Pept. Protein Res.* **1992**, *40*, 89–96.
(41) 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.
(42) Bovey, F. A.; Mirau, P. A.; Gutowsky, H. S. *Nuclear Magnetic Resonance Spectroscopy*, 2nd ed.; Academic Press: San Diego, 1988.
(43) Braun, S.; Kalinowski, H. O.; Berger, S. *150 and More Basic NMR Experiments*, 2nd ed.; Wiley-VCH: Weinheim, 1998.
(44) Silverstein, R. M.; Webster, F. X. *Spectrometric Identification of Organic Compounds*; John Wiley & Sons, Inc.: New York, 1997.
(45) Patkar, K. A.; Yan, X.; Murray, T. F.; Aldrich, J. V. [N^{α}-BenzylTyr^1,*cyclo*(D-Asp^5,Dap^8)]dynorphin A-(1−11)NH_2 Cyclized in the "Address" Domain Is a Novel κ-Opioid Receptor Antagonist. *J. Med. Chem.* **2005**, *48*, 4500–4503.
(46) Vig, B. S.; Murray, T. F.; Aldrich, J. V. Synthesis and Opioid Activity of Side-Chain-to-Side-Chain Cyclic Dynorphin A-(1−11) Amide Analogues Cyclized between Positions 2 and 5. 1. Substitutions in Position 3. *J. Med. Chem.* **2004**, *47*, 446–455.
(47) Soderstrom, K.; Choi, H.; Berman, F. W.; Aldrich, J. V.; Murray, T. F. *N*-Alkylated Derivatives of [D-Pro^{10}]Dynorphin A-(1−11) Are High Affinity Partial Agonists at the Cloned Rat κ-Opioid Receptor. *Eur. J. Pharmacol.* **1997**, *338*, 191–197.
(48) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color Test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Anal. Biochem.* **1970**, *34*, 595–598.
(49) Sole, N. A.; Barany, G. Optimization of Solid-Phase Synthesis of [Ala^8]-Dynorphin A. *J. Org. Chem.* **1992**, *57*, 5399–5403.
(50) Bennett, M. A.; Murray, T. F.; Aldrich, J. V. Structure-Activity Relationships of Arodyn, a Novel Acetylated Kappa Opioid Receptor Antagonist. *J. Pept. Res.* **2005**, *65*, 322–332.
(51) Cheng, Y. C.; Prusoff, W. H. Relationship Between the Inhibition Constant (K_i) and the Concentration of Inhibitor Which Causes 50% Inhibition (IC_{50}) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.