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

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Received June 19, 2003

cyclo[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2 (Dap, 2,3-diaminopropionic acid; Dyn A, dynorphin A), synthesized previously in our laboratory, showed sub-nanomolar affinity for κ opioid receptors and potent agonist activity in the guinea pig ileum assay (Arttamangkul et al., *J. Med. Chem.* **1995**, *38*, 2410−2417). Various modifications were made in position 3 of *cyclo*[D-Asp^2,Dap^5]-Dyn A-(1−11)NH_2 that could influence the opioid receptor affinity, selectivity, and/or efficacy of this peptide. An optimized orthogonal synthetic strategy was developed for the synthesis of these cyclic peptides in which the final peptides could be cleaved from the solid support with trifluoroacetic acid. Substitutions of Gly^3 by Ala, D-Ala, Trp, and D-Trp in *cyclo*[D-Asp^2,Dap^5]-Dyn A-(1−11)NH_2 and its linear counterpart [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 were generally well tolerated by both κ and μ opioid receptors. Despite differences in the size and stereochemistry of the substitutions, most of the peptides (except for *cyclo*[D-Asp^2,Pro^3,Dap^5]Dyn A-(1−11)NH_2 and [D-Asp^2,D-Ala^3, Dap^5]Dyn A-(1−11)NH_2) exhibited low nanomolar affinity for both κ (K_i = 0.21 to 2.2 nM) and μ (K_i = 0.22 to 7.27 nM) opioid receptors. All of the 3-substituted cyclic and linear analogues synthesized showed reduced affinity for δ opioid receptors. Incorporation of D-Ala at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 exhibited 2-fold higher κ opioid receptor affinity and 16-fold higher selectivity for κ over μ opioid receptors than the parent cyclic peptide. In contrast, substitution of Ala at position 3 resulted in an analogue with 2.4-fold lower affinity and very low preference for κ over μ opioid receptors. The Trp and D-Trp cyclic and linear analogues exhibited similar nanomolar affinities for κ opioid receptors. *cyclo*[D-Asp^2,Pro^3,Dap^5]Dyn A-(1−11)NH_2 showed the largest decreases in affinity for all three opioid receptors compared to the parent cyclic peptide. Except for *cyclo*[D-Asp^2, Pro^3,Dap^5]Dyn A-(1−11)NH_2, which was a partial agonist, all of the cyclic peptides exhibited full agonist activity in the adenylyl cyclase assay using cloned κ opioid receptors.

Introduction

Dynorphin A (Dyn A[1]), a heptadecapeptide first isolated from porcine pituitary,[2] is thought to be an endogenous ligand for κ opioid receptors[3] and is involved in a variety of biological functions.[4] Like most linear peptides, Dyn A is capable of assuming a number of different conformations,[5−10] and the biologically active conformations of Dyn A are not yet clear. This inherent conformational flexibility of Dyn A may be one of the reasons that this peptide also exhibits significant affinity for μ and δ opioid receptors, and therefore low selectivity for κ opioid receptors.[11,12]

Incorporation of a conformational constraint is a well-known approach that has been used to restrict the flexibility of peptides. This approach has been successfully used in the opioid peptide field, and a number of conformationally constrained peptides with high affinity and selectivity for μ and δ opioid receptors have been synthesized (see ref 13 for a review). However, only a limited number of cyclic analogues of Dyn A, with either a side-chain-to-side-chain lactam or disulfide bridge, have been synthesized.[14−20] Many of the Dyn A analogues cyclized either in the N- or C-terminus showed high affinity for both κ and μ opioid receptors, and therefore generally exhibited only low to moderate selectivity for κ over μ opioid receptors. To date the cyclic analogue of Dyn A with the highest selectivity (64-fold) for κ over μ opioid receptors is *cyclo*[D-Asp^3,Lys^7]Dyn A-(1−11)NH_2.[19] Although a variety of structure−activity relationship (SAR) studies have been performed on linear Dyn A analogues, such studies on cyclic Dyn A analogues have been very limited. Synthesis of high affinity conformationally constrained analogues of Dyn A will aid in studying the interactions of this peptide with opioid receptors.

In his hypothesis of membrane compartmentalization for opioid receptors, Schwyzer proposed that Dyn A-(1−13) adopts a helical conformation extending from Tyr[1] through Arg[9] when bound to κ opioid receptors.[7] On the basis of this hypothesis, we explored various *i* to *i* + *3*

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10.1021/jm030298e CCC: $27.50
Published on Web 12/17/2003

cyclic analogues of Dyn A.[18,20] The peptide cyclized between positions 2 and 5, *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2 (Dap, 2,3-diaminopropionic acid), exhibited the highest affinity for κ opioid receptors and potent agonist activity in the guinea pig ileum assay. This peptide, however, also retained high affinity for μ opioid receptors, and therefore exhibited low selectivity for κ over μ opioid receptors.[18] We chose *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 as our lead peptide to further explore the structure− and conformation−activity relationships of the residues within the cyclic region of this peptide. We were interested in exploring the SAR of residue 3 in the constrained cyclic analogues in comparison to linear analogues reported in the literature, with one goal being to obtain cyclic peptides with enhanced κ opioid receptor selectivity.

Modifications were chosen for incorporation into position 3 of the cyclic peptide that could affect the efficacy as well as the affinity and selectivity of the resulting analogues. Gly^3 in Dyn A is a noncritical residue for κ opioid receptor interaction. We anticipated that incorporation of amino acids of opposite stereochemistry at this position in *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 would result in different conformations in the cyclic portion of the peptide that in turn could result in differences in opioid receptor affinity and possibly selectivity. Substitution of this residue by either Ala or D-Ala in the linear peptide Dyn A-(1−11)NH_2 resulted in peptides with high affinity, selectivity, and agonist potency at κ opioid receptors.[21] Therefore Ala and D-Ala were chosen for incorporation in position 3 of the cyclic peptide. We were particularly interested in preparing conformationally constrained analogues of Dyn A with antagonist activity for comparison of the SAR for antagonism vs agonism. Except for a novel analogue that we recently described,[22] all of the cyclic analogues of Dyn A reported to date have been agonists. Incorporation of D-Trp in position 3 of [D-Trp^8,D-Pro^{10}]Dyn A-(1−11) was reported to impart weak antagonist activity to the peptide.[23] We recently reported that an acetylated chimeric analogue of Dyn A, Ac[Lys^2, Trp^{3,4},D-Ala^8]Dyn A-(1−11)NH_2, which has Trp at positions 3 and 4, is an antagonist at κ opioid receptors.[24] Also substitution of Gly^3 by Pro in Dyn A-(1−11)NH_2 was recently reported to result in an analogue with high affinity, high selectivity, and weak antagonist activity for κ opioid receptors.[25] Therefore we anticipated that substitution of Trp, D-Trp, or Pro at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 (Figure 1) could decrease efficacy and thus result in antagonist activity; the effects of these substitutions on opioid receptor affinity, selectivity, and efficacy were compared to analogues containing a residue at this position that was expected to result in potent agonists (i.e. Ala or D-Ala).

As part of this research we also developed a modified synthetic strategy for the preparation of these cyclic peptides. The synthesis of the original peptide *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2 required HF for the final deprotection of the side-chain protecting groups and cleavage of the peptide from the resin.[18,20] Also this peptide contains a sequence (D-Asp-Gly) which is very susceptible to aspartimide formation.[26] The new synthetic strategy developed for these peptides does not require the use of HF and does not result in significant aspartimide formation in the parent peptide.

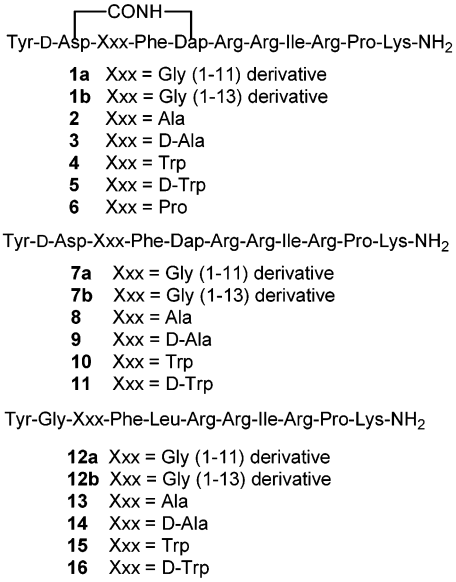


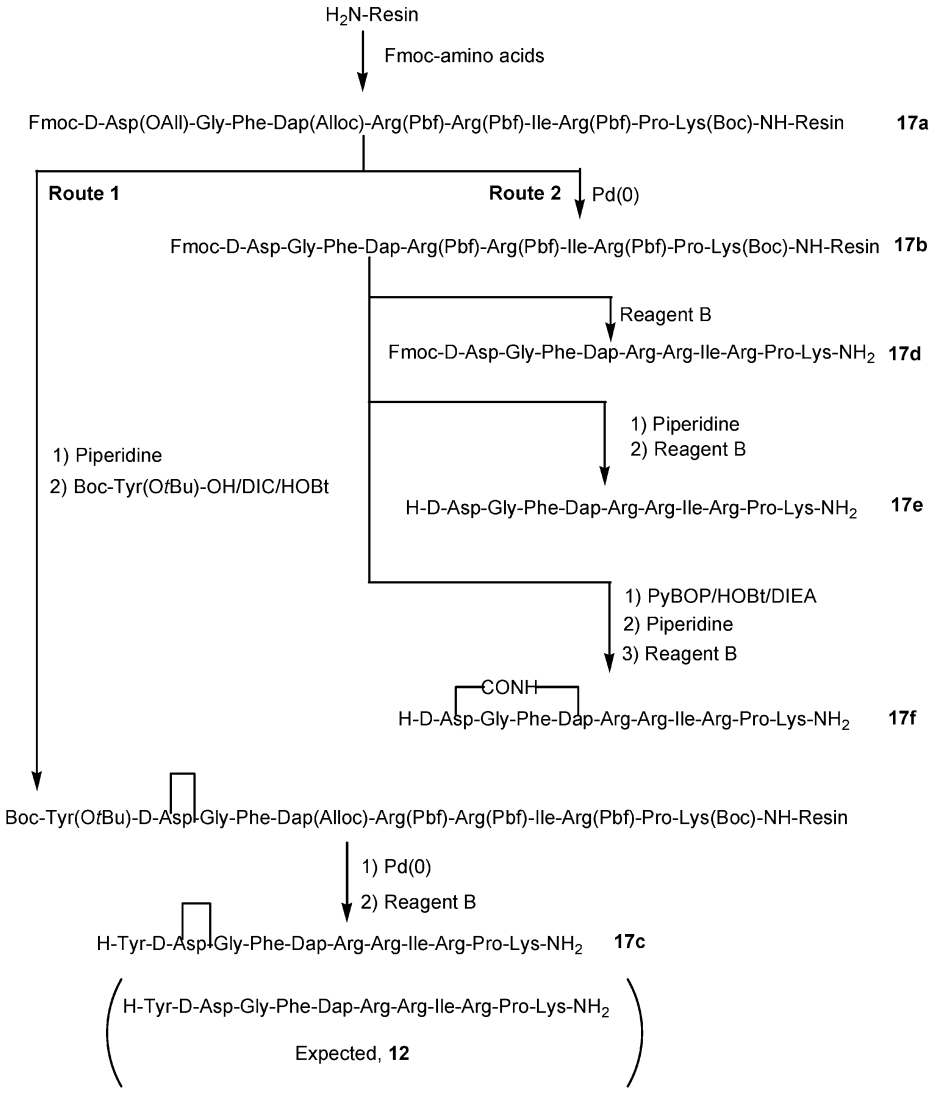
Figure 1. Structures of cyclic and linear analogues of Dyn A prepared.

Results and Discussion

Optimization of the Synthesis of the Cyclic Peptides. The synthesis of side-chain-to-side-chain cyclic peptides requires selective removal of protecting groups from the side chains of the amino acids involved in the cyclization in the presence of other side-chain protecting groups. The synthesis of *cyclo*[D-Asp^2,Dap^5]-Dyn A-(1−13)NH_2 and the other *i* to *i* + *3* cyclic analogues was originally performed on an MBHA (4-methylbenzhydrylamine) resin using Fmoc (9-fluorenylmethoxycarbonyl) protection for the N^{α}-amine, *tert*-butyl-type protecting groups for the side-chain functional groups of the amino acids involved in cyclization, and ClZ (2-chlorobenzyloxycarbonyl) and Tos (tosyl) protection of the side-chain functional groups of Lys and Arg, respectively.[18,20] After incorporating the N-terminal Tyr into the peptide chains, the *t*-Bu protecting groups were removed by trifluoroacetic acid (TFA) and the cyclization then performed on the resin. After the synthesis of the peptides, the final deprotection of the remaining side-chain protecting groups and removal of the peptide from the resin utilized HF. The use of HF for the final deprotection and cleavage has a number of disadvantages: difficulty in monitoring lactam formation, special handling of the highly corrosive gas, and side reactions due to the extremely acidic conditions. Therefore we explored modified synthetic strategies that avoided utilization of HF for the final deprotection and cleavage.

The synthetic strategies explored involved assembly of the peptide on the TFA-labile PAL−PEG−PS (peptide amide linker−poly(ethylene glycol)−polystyrene) or Tentagel S-AM resins (with (5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid as the linker) using N^{α}-Fmoc-protected amino acids with the side chains of Tyr, Lys, and Arg protected by TFA-labile protecting groups (*t*-Bu, Boc (*tert*-butyloxycarbonyl), and Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), respectively). For the amino acids involved in cyclization, D-Asp and

Scheme 1. Optimization of the Synthesis of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2.



Dap, the side-chain functional groups were protected by allyl-type protecting groups, namely the allyl ester and Alloc (allyloxycarbonyl) group, respectively. The allyl-type protecting groups (removed by Pd(0)) along with N^{α}-Fmoc protected amino acids and *t*-Bu-based side-chain protecting groups provide a true three-dimensional orthogonal protection strategy.

As expected, attempts to synthesize the full length linear [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 (**7a**) using N^{α}-Fmoc-protected amino acids and the allyl ester for protection of the D-Asp side chain resulted in aspartimide formation. This reaction is highly sequence dependent,[26−28] and the parent peptide contains a D-Asp-Gly sequence which is very susceptible to this side reaction.[26] This reaction can be catalyzed under either acidic conditions (such as in Boc deprotection) or basic conditions (i.e. by piperidine during Fmoc deprotection), and even by excess coupling reagent.[29] During deprotection of the Fmoc group by piperidine, this side reaction is prevalent when a small protecting group such as the allyl ester is used for the side-chain of Asp.[30,31] As expected, in our case aspartimide formation occurred during deprotection of the Fmoc group by piperidine from the protected Fmoc-[D-Asp(OAl)^2,Dap(Alloc)^5]Dyn A-(2−11)NH-resin (**17a**) to give the aspartimide **17c** after the introduction of the N-terminal Tyr (Route 1, Scheme 1 and Table 1). Removal of the allyl protecting groups first using Pd(0) (Route 2, Scheme 1) to give the linear peptides Fmoc-[D-Asp^2,Dap^5]Dyn A-(2−11)NH_2 (**17d**) and [D-Asp^2,Dap^5]Dyn A-(2−11)NH_2 (**17e**) before and after Fmoc deprotection, respectively, resulted in no detectable aspartimide formation as determined by mass spectrometry (Table 1). Cyclization of the protected Fmoc-[D-Asp^2,Dap^5]Dyn A-(2−11)NH-resin (**17b**) by PyBOP (benzotriazol-1-yloxytrispyrrolidinophosphonium hexafluorophosphate)/HOBt (1-hydroxybenzotriazole)/DIEA (*N*,*N*-diisopropylethylamine), followed by deprotection of the Fmoc group resulted in the desired cyclic peptide *cyclo*[D-Asp^2,Dap^5]Dyn A-(2−11)NH_2 (**17f**) following cleavage from the resin and final deprotection (Scheme 1). Interestingly, in the original synthetic strategy,[18] which involved cyclization after the synthesis of the full-length linear peptide, 3 to 5 days were required for completion of the cyclization reaction.[18,20] In this modified synthesis, however, the cyclization reaction was complete within 6 to 8 h (as determined using ninhydrin). Thus, in this sequence the length of the peptide chain appears to have a profound effect on the rate of cyclization. Also this modified synthesis utilizes milder acidic conditions (TFA instead of HF) for

Table 1. Structures, HPLC and MALDI-TOF Analysis of Reaction Products Obtained during the Optimization of the Synthesis of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2, **1a**

Compound	Sequence	HPLC (t_r, min)[a]	MALDI-TOF (M+H)$^+$ Obs.	MALDI-TOF (M+H)$^+$ Calcd.
17c	H-Tyr-D-Asp(-)Gly-Phe-Dap(-)Arg-Arg-Ile-Arg-Pro-Lys-NH_2	11.8	1375.9	1374.8
17d	Fmoc-D-Asp-Gly-Phe-Dap-Arg-Arg-Ile-Arg-Pro-Lys-NH_2	21.0	1451.1	1451.7
17e	D-Asp-Gly-Phe-Dap-Arg-Arg-Ile-Arg-Pro-Lys-NH_2	9.7	1229.9	1229.7
17f	D-Asp(CONH)-Gly-Phe-Dap(CONH)-Arg-Arg-Ile-Arg-Pro-Lys-NH_2	11.3	1212.8	1211.7
7a	Tyr-D-Asp-Gly-Phe-Dap-Arg-Arg-Ile-Arg-Pro-Lys-NH_2	12.6	1392.7	1392.8
1a	Tyr-D-Asp(CONH)-Gly-Phe-Dap(CONH)-Arg-Arg-Ile-Arg-Pro-Lys-NH_2	15.3	1374.7	1374.8

[a] System 1 (see Experimental Methods).

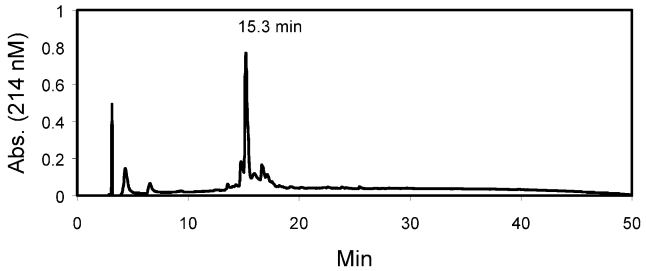


Figure 2. HPLC of crude *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)-NH_2, **1a**.

the final deprotection and cleavage of the peptide from the resin, which makes it much easier to monitor the formation of the lactam by HPLC analysis of a cleaved aliquot of the peptide.

Following cyclization and removal of the N-terminal Fmoc group, Fmoc-Tyr(O*t*Bu)-OH was attached to the peptide. After deprotection of the N-terminal Fmoc group and removal of the peptide from resin, the desired *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2, **1a**, was obtained as the major product (t_r = 15.3 min, 60% by HPLC of the crude peptide, Figure 2). On the basis of these results, the *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 analogues with substitutions at position 3 were synthesized on the PAL−PEG−PS resin using a similar strategy as shown in Scheme 2. The linear peptides were synthesized either on a Tentagel S-AM or PAL−PEG−PS resin according to standard procedures[32] using Fmoc-protected amino acids, with acid-labile groups used to protect the side chains of D-Asp and Dap. All of the peptides were cleaved from the resin using Reagent B[33] and purified by preparative reversed phase HPLC. Following synthesis and purification, the identity and purity of the final compounds was verified using mass spectrometry and analytical HPLC (see Supporting Information). The final purity of all peptides was >98%.

Pharmacology

The peptides were examined for their opioid receptor affinity as described previously[20] using Chinese hamster ovary (CHO) cells stably expressing cloned rat κ and μ and mouse δ opioid receptors (Table 2) and employing [^{3}H]diprenorphine, [^{3}H]DAMGO ([D-Ala^2,MePhe^4,glyol]-enkephalin), and [^{3}H]DPDPE (*cyclo*[D-Pen^2,D-Pen^5]-enkephalin) as radioligands for κ, μ, and δ opioid receptors, respectively. Since the receptor affinities of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2, **1b**, were originally evaluated in brain tissue membrane preparations employing [^{3}H]bremazocine, [^{3}H]DAMGO, and [^{3}H]DPDPE as radioligands for κ, μ, and δ opioid receptors, respectively,[18] this original cyclic peptide was also evaluated in the current assay system for comparison to *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2, **1a**, the parent cyclic peptide for the 3-substituted peptides. The binding affinities of **1b** determined using cloned receptors were similar to those found previously in brain tissue membranes[18] and were similar to those for Dyn A-(1−13)-NH_2. The shortened cyclic peptide **1a** exhibited affinities within 2−3-fold that of **1b**. The linear counterpart of **1b**, [D-Asp^2,Dap^5]Dyn A-(1−13)NH_2, **7b**, also exhibited sub-nanomolar affinity, but a loss in selectivity, for κ over μ opioid receptors, as observed with other linear counterparts of the Dyn A analogues synthesized in the 3-substituted series.

The parent cyclic peptide *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2, **1a**, its linear counterpart [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2, **7a**, and Dyn A-(1−11)NH_2 all showed high affinity for cloned κ (K_i = 0.14 to 1.59 nM) and μ (K_i = 0.22 to 1.91 nM) opioid receptors, with slightly lower affinity for δ opioid receptors (K_i = 5.10 to 11.6 nM). These results indicate that incorporation of a lactam bridge between residues 2 and 5 of Dyn A-(1−11)NH_2 is well tolerated by all three opioid receptors. Peptide **1a** showed similar sub-nanomolar affinity for both κ and μ opioid receptors, resulting in a nonselective peptide. Compared to Dyn A-(1−11)NH_2 and **1a** the linear peptide **7a** exhibited increased affinity for μ opioid receptors and decreased affinity for κ opioid receptors, resulting in a peptide with a 7-fold preference for μ opioid receptors. This may be due to the presence of a

Scheme 2. Final Synthetic Strategy for the Synthesis of 3-Substituted *cyclo*[D-Asp^2,Dap^5]-Dyn A-(1−11)NH_2 Analogues

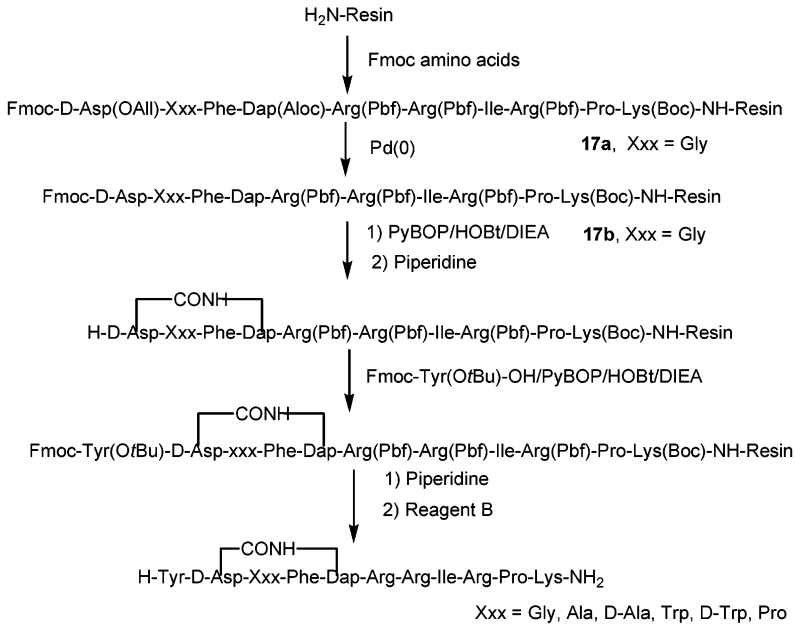


Table 2. Opioid Receptor Affinities of Cyclic and Linear Dyn A-(1−11)NH_2 Analogues

analogues of Dyn A-(1−11)NH_2[a]	K_i (nM ± SEM)			K_i ratio
	κ	μ	δ	$\kappa/\mu/\delta$
	cyclo[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 Analogues			
1a *cyclo*[D-Asp^2,Dap^5]	0.46 ± 0.14	0.52 ± 0.06	5.10 ± 0.50	1/1.1/11
2 *cyclo*[Ala^3]	1.10 ± 0.20	2.52 ± 0.13	30.8 ± 3.7	1/2.3/28
3 *cyclo*[D-Ala^3]	0.21 ± 0.05	3.89 ± 0.52	139 ± 11	1/18/662
4 *cyclo*[Trp^3]	1.33 ± 0.26	2.09 ± 0.64	7070 ± 1920	1/1.6/5320
5 *cyclo*[D-Trp^3]	2.25 ± 0.37	7.27 ± 2.01	949 ± 49	1/3.2/422
6 *cyclo*[Pro^3]	9.03 ± 2.26	125 ± 11	>10 000	1/14/>1000
	Linear [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 Analogues			
7a [D-Asp^2,Dap^5]	1.59 ± 0.44	0.22 ± 0.04	11.6 ± 2.0	7.2/1/53
8 [Ala^3]	0.86 ± 0.27	2.02 ± 0.28	55.1 ± 6.0	1/2.3/64
9 [D-Ala^3]	6.12 ± 1.37	4.21 ± 0.99	959 ± 8	1.5/1/228
10 [Trp^3]	1.34 ± 0.33	3.41 ± 0.95	927 ± 197	1/2.5/692
11 [D-Trp^3]	1.26 ± 0.29	4.57 ± 1.14	813 ± 222	1/3.6/645
	Linear Dyn A-(1−11)NH_2 Analogues			
12a Dyn A-(1−11)NH_2	0.14 ± 0.04	1.91 ± 0.42	6.18 ± 1.05	1/14/44
13 [Ala^3]	0.26 ± 0.06	16.4 ± 2.2	23.3 ± 6.3	1/63/90
14 [D-Ala^3]	0.34 ± 0.06	19.8 ± 6.3	213 ± 39	1/58/626
15 [Trp^3]	1.56 ± 0.31	42.7 ± 4.3	168 ± 12	1/27/108
16 [D-Trp^3]	5.41 ± 0.28	45.9 ± 1.4	1080 ± 210	1/8.5/199

[a] The opioid receptor affinities of the Dyn A-(1−13)NH_2 analogues were as follows: *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−13)NH_2, **1b**, K_i = 0.16 ± 0.07 nM (κ), 1.17 ± 0.32 nM (μ), 3.31 ± 0.41 nM (δ); [D-Asp^2,Dap^5]Dyn A-(1−13)NH_2, **7b**, K_i = 0.20 ± 0.06 nM (κ), 0.26 ± 0.09 nM (μ), 3.90 ± 0.41 nM (δ); Dyn A-(1−13)NH_2, **12b**, K_i = 0.39 ± 0.12 nM (κ), 1.88 ± 0.40 nM (μ), 6.07 ± 0.79 nM (δ).

D-amino acid and/or a negative charge at position 2, both of which have been shown to increase affinity for μ opioid receptors.[18,19,34,35]

In general the substitutions at position 3 were well tolerated, and most of the cyclic peptides showed high affinities for κ and μ opioid receptors (Table 2). Compared to the parent peptides (**1a**, **7a**, and **12a**), modifications at position 3 resulted in reductions in affinity for μ and δ opioid receptors for all of the cyclic and linear peptides. However, the loss in affinity was much higher in the case of δ opioid receptors. Therefore most of the cyclic peptides had very high selectivity for κ over δ opioid receptors, but the selectivity for κ over μ opioid receptors was at best modest.

The effects of incorporating Ala or D-Ala in position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 (to give **2** and **3**, respectively) and the corresponding linear [D-Asp^2,-Dap^5]Dyn A-(1−11)NH_2 analogues (compounds **8** and **9**) were examined and compared to the results for the previously reported linear Dyn A-(1-11)NH_2 analogues **13** and **14**.[21] In the assays conducted in our laboratory **13** and **14** showed similar selectivities (K_i ratios (μ/κ) for **13** = 63 and for **14** = 58) which were considerably lower than previously reported (IC_{50} ratios (μ/κ) for **13** = 190 and for **14** = 350).[21] These differences in K_i vs IC_{50} ratios may be due to differences in the assays, i.e. the source of the receptors (cloned rat opioid receptors in CHO cells in our assays vs guinea pig brain homogenates) and/or the radioligand used for κ opioid receptor binding ([^{3}H]diprenorphine in our assays vs [^{3}H]U-69,593). Although incorporation of Ala at position 3 of Dyn A-(1−11)NH_2 to give peptide **13** resulted in improved selectivity for κ opioid receptors, substitution of Ala at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2

to give peptide **2** resulted in similar decreases in affinity for both κ and μ opioid receptors compared to the parent cyclic peptide **1a**, resulting in a nonselective peptide. Incorporation of D-Ala at position 3 of *cyclo*[D-Asp^2,Dap^5]-Dyn A-(1−11)NH_2 to give **3** resulted in a 2.2-fold increase in affinity for κ opioid receptors and a 7.4-fold decrease in affinity for μ opioid receptors compared to the parent cyclic peptide **1a**. Thus the constrained Dyn A analogue **3** showed 18-fold selectivity for κ over μ opioid receptors, while the cyclic parent peptide **1a** showed similar affinity for these opioid receptors. Compared to [D-Ala^3]Dyn A-(1−11)NH_2, **14**, the cyclic peptide **3** showed similar affinity for κ opioid receptors but higher affinity for μ opioid receptors. In contrast, the corresponding linear D-Ala^3 analogue **9** showed a 29-fold decrease in affinity for κ opioid receptors while retaining affinity for μ opioid receptors comparable to the cyclic peptide **3**, resulting in a nonselective peptide. Peptide **9** also showed a decrease in affinity for all opioid receptors compared to the parent linear peptide **7a**. Compared to the linear peptides [Ala^3]Dyn A-(1−11)-NH_2, **13**, and [D-Ala^3]Dyn A-(1−11)NH_2, **14**, the corresponding linear [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 analogues **8** and **9** showed lower affinity and lack of selectivity for κ opioid receptors.

Substitution of Ala or D-Ala at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2, [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2, and Dyn A-(1−11)NH_2 resulted in compounds with reduced affinities for δ opioid receptors; the reduction in affinities for δ opioid receptors was significantly higher when D-Ala was substituted at position 3 of these peptides. This observation is consistent with the earlier report by Lung et al.,[21] where substitutions at position 3 of Dyn A-(1−11)NH_2 resulted in analogues with lower δ opioid receptor affinity. These results suggest that there is more limited space around the residue at position 3 of Dyn A analogues in the binding pocket of δ opioid receptors than in either κ or μ opioid receptors.

Incorporation of Trp and D-Trp at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 and its linear counterpart [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 resulted in peptides (compounds **4**−**5** and **10**−**11**, respectively) with similar nanomolar affinities for κ opioid receptors and similar selectivities for κ over μ opioid receptors. These results indicate that incorporation of a bulky amino acid is well tolerated at this position in these Dyn A-(1−11)NH_2 analogues. However, in contrast to the D-Ala^3 cyclic peptide **3**, both the Trp^3 and D-Trp^3 cyclic peptides **4** and **5** showed low selectivity for κ over μ opioid receptors, suggesting that the bulk of the Trp residue might be overshadowing the influence of stereochemistry. This was also true for the linear [D-Asp^2,Dap^5]Dyn A-(1−11)-NH_2 analogues **10** and **11**, but not for the linear Dyn A-(1−11)NH_2 analogues, where incorporation of Trp in position 3 to give **15** resulted in 3−4-fold higher affinity and selectivity for κ opioid receptors than exhibited by [D-Trp^3]Dyn A-(1−11)NH_2 (**16**). Although the difference is small, the latter results are also in contrast to the effects seen with incorporation of Ala and D-Ala at position 3 of Dyn A-(1−11)NH_2, where analogues **13** and **14** exhibited similar affinity and selectivity for κ opioid receptors. These results for the cyclic and linear analogues of Dyn A-(1−11)NH_2 containing Ala and Trp isomers at position 3 suggest that the affinities and selectivities of Dyn A analogues are influenced by both the stereochemistry and bulk of the residue at position 3, as well as by the conformational constraint.

Similar to the Ala^3 and D-Ala^3 peptides, the Trp^3 and D-Trp^3 cyclic and linear Dyn A analogues exhibited significant losses in affinity for δ opioid receptors. However, in contrast to the Ala and D-Ala-containing peptides, where the D-Ala^3 analogues consistently exhibited greater decreases in affinity for δ opioid receptors compared to the Ala^3 analogues, the relative δ opioid receptor affinities of Trp^3 vs D-Trp^3 analogues varied considerably depending on the other modifications in the peptides; thus, for the cyclic peptides the relative affinities were **5** > **4**, while for the linear peptides **10** ∼ **11** and **15** > **16**.

On the basis of the reported high affinity, selectivity, and weak antagonist activity of [Pro^3]Dyn A-(1−11)NH_2 for κ opioid receptors,[25] we incorporated this substitution in position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2. Compared to the parent cyclic peptide, *cyclo*[D-Asp^2,Pro^3,Dap^5]Dyn A-(1−11)NH_2, **6**, exhibited a 19-fold loss in affinity for κ opioid receptors; this peptide also showed substantially lower κ receptor selectivity (K_i ratio ($\kappa/\mu/\delta$) = 1/15/>1000) than reported for [Pro^3]Dyn A-(1−11)-NH_2 (K_i ratio ($\kappa/\mu/\delta$) = 1/2110/3260) by Goodman and co-workers.[25] The significant loss in opioid receptor affinity for **6** may be due to changes in the conformation adopted by the cyclic region of this peptide resulting from the conformational restriction around the Φ angle of the Pro residue; this could alter the spatial orientation of important functional groups (i.e. the Tyr^1 and Phe^4 residues) in the peptide.

Except for the D-Ala^3 analogue, which exhibited a 4-fold decrease in κ opioid receptor affinity, modifications at position 3 of the linear peptide [D-Asp^2,Dap^5]-Dyn A-(1−11)NH_2, **7a**, resulted in peptides with similar κ opioid receptor affinity but decreased μ receptor affinity compared to the parent peptide, resulting in a net increase in selectivity for κ over μ opioid receptors. The decreases in the μ receptor affinities of the linear [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 analogues paralleled those found for the cyclic peptides, suggesting subtle differences around position 3 in Dyn A analogues in the receptor binding sites of κ and μ opioid receptors. However, the linear peptide [D-Asp^2,Dap^5]Dyn A-(1−11)-NH_2, **7a**, and its analogues with substitutions at position 3 (**8**−**11**) all exhibited higher affinity for μ opioid receptors than the corresponding Dyn A-(1−11)NH_2 analogues (**12a**, **13**−**16**), consistent with previous reports that introduction of a D-amino acid or negative charge at position 2 of Dyn A increases μ receptor affinity and selectivity.[18,19,34,35]

Similar to the cyclic peptides, losses in δ receptor affinity due to modifications at position 3 of [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 were much higher than for κ and μ opioid receptors, especially for the D-Ala^3, Trp^3 and D-Trp^3 peptides **9**, **10**, and **11**. Like the cyclic peptides the improvement in selectivity for κ opioid receptors over μ and δ opioid receptors for the [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 analogues may be due to incorporation of a bulkier amino acid at position 3; in these linear peptides the introduction of a chiral amino acid at position 3 could also affect the conformation of

Table 3. Inhibition of Forskolin-Stimulated Adenylyl Cyclase Activity by *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 Analogues in CHO Cells Expressing κ Opioid Receptors[a]

compounds	EC_{50} (nM ± SEM)
1a *cyclo*[D-Asp^2,Dap^5]	0.39 ± 0.07
2 *cyclo*[Ala^3]	0.65 ± 0.10
3 *cyclo*[D-Ala^3]	0.54 ± 0.15
4 *cyclo*[Trp^3]	4.19 ± 0.55
5 *cyclo*[D-Trp^3]	12.0 ± 2.8
6 *cyclo*[Pro^3][b]	153 ± 86

[a] All of the cyclic peptides except **6** exhibited similar maximum inhibition of adenylyl cyclase as 100 nM Dyn A-(1−13)NH_2. [b] Maximum inhibition of adenylyl cyclase at 10 μM was 37 ± 7% relative to 100 nM Dyn A-(1−13)NH_2.

the peptide backbone, which in turn could affect the spatial orientation of the important Tyr^1 and Phe^4 residues.

The functional consequences of these modifications to the cyclic peptides were investigated by measuring forskolin-stimulated adenylyl cyclase activity in CHO cells expressing κ opioid receptors as described previously.[36] Results from this assay are summarized in Table 3. Compounds **2** and **3** exhibited similar potency (EC_{50} of 0.65 nM and 0.54 nM, respectively) in this assay to the parent cyclic peptide (EC_{50} = 0.39 nM). Thus, although **3** exhibited 5-fold higher κ receptor affinity than **2**, this difference was not seen in the functional assay. At the highest concentration (10 μM) compounds **2** and **3** produced similar maximum inhibition as the reference peptide (Dyn A-(1−13)NH_2, 100% inhibition at 100 nM), indicating that these analogues are full agonists. The Trp- and D-Trp-containing cyclic peptides **4** and **5**, although exhibiting 10-fold and 29-fold decreases in potency, respectively, compared to the parent cyclic peptide, were also full agonists in the adenylyl cyclase assay. This is in contrast to the linear Trp^3 and D-Trp^3 peptides **15** and **16** which were partial agonists in this assay (53 ± 17% and 69 ± 6% maximum inhibition of adenylyl cyclase, respectively, compared to Dyn A-(1−13)NH_2; EC_{50} = 5.42 ± 2.37 nM and 47.6 ± 19.2 nM, respectively). Substitution of Pro at position 3 of the cyclic peptide had the largest effect on the functional activity of the peptide. The maximum inhibition of adenylyl cyclase produced by compound **6** was only 37% compared to the reference peptide Dyn A-(1−13)NH_2, indicating that this analogue is a partial agonist in this assay. The Pro^3 cyclic peptide **6** showed a 372-fold loss in potency compared to the parent cyclic peptide.

Conclusions

A series of side-chain-to-side-chain cyclic analogues of Dyn A were successfully prepared using a modified synthetic strategy which does not require the use of a strong acid for the final deprotection and cleavage of the peptide from the resin. The original synthetic strategy[18,20] involved the assembly of the peptides on an MBHA resin using Fmoc protection of the N$^\alpha$-amine, *tert*-butyl-type protecting groups for the side-chain functional groups of the amino acids involved in cyclization, and ClZ and Tos for protection of the other side-chain functional groups (Lys and Arg, respectively). In the modified synthetic strategy, the peptides were assembled on a PAL−PEG−PS resin using allyl-based groups for protection of the side chains involved in the cyclization, along with the N$^\alpha$-Fmoc protecting group and *tert*-butyl-type protecting groups for other side chains; this strategy offers a true three-dimensional orthogonal protection. Compared to the original synthetic strategy, which required 3 to 5 days for the cyclization reactions and utilized HF for the final cleavage,[18,20] the modified synthetic strategy required only 6 to 8 h for the cyclization and utilized TFA for the final cleavage, making monitoring of the synthesis much easier. For this peptide sequence it appears that the length of the peptide has a significant influence on the rate of cyclization, with the cyclization being much faster in the absence of the N-terminal tyrosine.

Most of the analogues synthesized with modifications in position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 showed high affinity for κ and μ opioid receptors, indicating that substitutions at this position are well tolerated by these receptors. Substitutions at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 resulted in decreases in affinity for μ opioid receptors compared to **1a**, resulting in increased selectivity for κ opioid receptors. The considerable decreases in affinity for δ opioid receptors indicate that these modifications are not tolerated by this receptor. In the case of the small amino acid Ala, the influence of the stereochemistry on the affinity and selectivity of the cyclic peptide was clearly seen. *cyclo*[D-Asp^2,D-Ala^3,Dap^5]Dyn A-(1−11)NH_2, **3**, showed higher κ opioid receptor affinity and selectivity than *cyclo*[D-Asp^2,Ala^3,Dap^5]Dyn A-(1−11)NH_2, **2**, and the parent cyclic peptide *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2, **1a**. A similar influence of stereochemistry was not observed when the isomers of the bulkier amino acid Trp were incorporated into the cyclic peptide and its linear counterpart, suggesting that the steric bulk of Trp might be overshadowing the stereochemical influence, especially in the cyclic peptides. The results obtained in this study indicate that the affinity and selectivity of the Dyn A analogue can be influenced by both the stereochemistry and size of the residue at position 3, as well as by the conformational constraint.

The cyclic constraint in the parent peptide **1a** is compatible with interaction with all three opioid receptors. The same substitutions at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 (analogues **2**−**5**) and Dyn A-(1−11)NH_2 (peptides **13**−**16**) generally resulted in high affinity (K_i < 2.5 nM) for κ opioid receptors; however, the μ opioid receptor affinities of the cyclic peptides were consistently higher than the linear peptides. Most of the modifications to position 3 did not significantly improve κ over μ opioid receptor selectivity compared to the parent cyclic peptide. Only *cyclo*[D-Asp^2,D-Ala^3,Dap^5]Dyn A-(1−11)NH_2, **3**, and *cyclo*[D-Asp^2,Pro^3,Dap^5]Dyn A-(1−11)NH_2, **6**, showed modest increases in selectivity for κ over μ opioid receptors compared to the parent cyclic peptide **1a**. Substitutions at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 (**3**−**6**), [D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 (**9**−**11**), and Dyn A-(1−11)NH_2 (**14**−**16**) all resulted in large losses in affinity for δ opioid receptors. Thus, while κ and μ opioid receptors tolerate introduction of a residue with a side chain at this position, δ opioid receptors were very sensitive to these modifications. These results suggest that there is more limited space around residue 3 of Dyn A analogues in the binding pocket of δ opioid receptors

than either κ or μ opioid receptors. The results presented here clearly indicate that incorporation of a conformational constraint and modifications within the constraint can influence significantly the interaction of Dyn A analogues with a particular receptor.

Substitutions at position 3 of *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 also affected the potency and, in the case of the Pro^3 analogue, the efficacy of the peptide at κ opioid receptors as determined in the adenylyl cyclase assay. Substitution of Ala and D-Ala at position 3 of the cyclic peptide resulted in similar potency and efficacy to the parent peptide. Substitution of the bulkier amino acids Trp and D-Trp resulted in 10−30-fold decreases in potency compared to the parent cyclic peptide **1a**, but interestingly increased the maximum response compared to the corresponding linear peptides **15** and **16**. Substitution of Pro at position 3 resulted in a compound with weak partial agonist activity at κ opioid receptors in this assay.

This series provides the first detailed SAR analysis of the constrained region of a cyclic Dyn A analogue. Cyclic peptide **3** with D-Ala^3 showed high κ receptor affinity and potency in a functional assay, making it a promising compound for use in further studies, including conformational analysis. Selected cyclic analogues including **3** are currently being examined by NMR and molecular modeling to explore the influence of the 3-substitution on the backbone conformations of the peptides and possible orientations of important side chains (i.e. Tyr^1 and Phe^4). Additional cyclic analogues with substitution in other positions have been prepared and will be reported shortly.

Experimental Methods

Materials. The PAL−PEG−PS resin, HOBt, and DIEA were purchased from Applied Biosystems (Foster City, CA). The Tentagel S-AM resin was purchased from Peptides International (Louisville, KY). PyBOP was purchased from Calbiochem-Novabiochem (San Diego, CA). Fmoc-protected amino acids were purchased from Applied Biosystems, Calbiochem-Novabiochem, Bachem (King of Prussia, PA), or Anaspec (San Jose, CA). *N,N*-Diisopropylcarbodiimide (DIC), piperidine, triisopropylsilane (TIS), and TFA were purchased from Aldrich Chemical Co. (Milwaukee, WI), and tetrakispalladium(0) ($Pd(PPh_3)_4$) was purchased from Acros Chemical Co. (Pittsburgh, PA). All HPLC-grade solvents, AcOH, MeCN, diethyl ether, *N,N*-dimethylformamide (DMF), *N,N*-dimethylacetamide (DMA), dichloromethane (DCM), MeOH, and tetrahydrofuran (THF), used for peptide synthesis or high performance liquid chromatography (HPLC) analysis were obtained from either Burdick and Jackson, Inc. (Muskegon, MI) or EM Sciences (Gibbstown, NJ). TFA for HPLC analysis was purchased from Pierce (Rockville, IL).

Common Synthetic Steps. The synthesis of the cyclic peptides was performed on the PAL−PEG−PS resin, while the synthesis of the linear peptides was performed on either the PAL−PEG−PS or Tentagel S-AM resin. The side chains of amino acids Arg, Lys, and Tyr were protected by Pbf, Boc and *tert*-butyl groups, respectively. For the Trp- or D-Trp-containing peptides, the Boc group was used for the protection of the side-chain of Trp. Typically the protected peptide-resin Fmoc-Arg-(Pbf)-Arg(Pbf)-Ile-Arg(Pbf)-Pro-Lys(Boc)-resin was first assembled on a Milligen Biosearch 9500 automated synthesizer using a 4-fold excess of Fmoc-protected amino acids (0.4 M in DMA) and coupling reagents (DIC and HOBt, 0.4 M in DCM). The deprotection of the Fmoc group was carried out with 20% piperidine in DMA (2 × 10 min). The remaining Fmoc-amino acids were coupled to the peptide manually using either DIC/HOBt or PyBOP/HOBt/DIEA as the coupling reagent as described below. All coupling reactions were usually complete within 2 h, as determined by the ninhydrin reaction.

Cleavage of the Peptides from the Resin. The protected peptide resins were treated with 10 mL of Reagent B (88% TFA, 5% phenol, 5% water, and 2% triisopropylsilane)[33] for 2 h. The solution was filtered from the resin, and the resin washed with additional TFA (1 mL). The solutions were diluted with 10% acetic acid (30 mL) and then extracted with ether (3 × 30 mL); the ether extracts were back extracted with acetic acid (2 × 10 mL). The combined aqueous layers were lyophilized to give the crude peptides.

Synthesis of Cyclic Peptides 1−6. The synthesis of the cyclic peptides was carried out on a low load (0.17 mmol/g) PAL−PEG−PS resin. The side chains of D-Asp^2 and Dap^5 that were involved in the cyclization were protected as the allyl ester and Alloc group, respectively. The protected peptide-resin Fmoc-Arg(Pbf)-Arg(Pbf)-Ile-Arg(Pbf)-Pro-Lys(Boc)-PAL−PEG−PS (Fmoc-Arg(Pbf)-Arg(Pbf)-Ile-Arg(Pbf)-Pro-Lys(Boc)-Leu-Lys(Boc)-PAL−PEG−PS for compound **1b**) was first assembled as described above. The remaining Fmoc-amino acids (5-fold excess, 0.1 M in DMF) were coupled to the peptide using PyBOP/HOBt/DIEA (1/1/2, 0.1 M in DMF) on a Symphony multiple peptide synthesizer (Rainin, Inc.). During the synthesis of the peptides on the Symphony, the deprotection of the Fmoc group was carried out with 20% piperidine in DMF (2 × 10 min).

After synthesis of the protected linear peptide up through residue 2, the allyl and Alloc groups were selectively deprotected as follows: Following swelling of the protected peptide-resins in DCM for 15 min, a solution of tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$, 0.08 M, 4 equiv) in 92.5% DCM/5% AcOH/2.5% *N*-methylmorpholine (NMM)[37] was added manually to the peptide-resin. Nitrogen was bubbled occasionally through the suspension, and DCM was added to compensate for the loss of solvent due to evaporation. The deprotection was terminated after 4 h, and the peptide-resin washed with DCM (5 × 2 min), THF (4 × 2 min), DCM (3 × 2 min), DMF (3 × 2 min), 0.5% DIEA in DMF (3 × 2 min), 0.02 M sodium diethyldithiocarbamate in DMF (3 × 15 min), and DMF (5 × 2 min).[38]

The free carboxylic acid group of D-Asp and the amino group of Dap were cyclized using a 10-fold excess of PyBOP/HOBt/DIEA (1/1/2, 0.2 M in DMF); the cyclization was complete within 8 h as determined by the ninhydrin test. The Fmoc group was then removed and Fmoc-Tyr(O*t*Bu)-OH coupled to the protected cyclic peptide. Following removal of the N-terminal Fmoc group the crude cyclic peptide was cleaved from the solid support as described above.

Synthesis of Linear Peptides. Synthesis of Compounds 7−11. The synthesis of the linear peptides was performed on either the Tentagel S-AM (0.25 mmol/g) or Fmoc-PAL−PEG−PS resin (0.19 mmol/g). For synthesis of compounds **7a**, **8**, and **9**, Fmoc-D-Asp(O*t*Bu) and Fmoc-Dap(Boc) were used, while Fmoc-D-Asp(Pip) (Pip, 2-phenylisopropyl) and Fmoc-Dap(Boc) were used for the synthesis of compounds **7b**, **10**, and **11**. The protected peptide-resin Fmoc-Arg(Pbf)-Arg-(Pbf)-Ile-Arg(Pbf)-Pro-Lys(Boc)-resin was first assembled as described above. The remaining Fmoc-amino acids (5-fold excess, 0.1 M in DMF) were coupled to the peptide using PyBOP/HOBt/DIEA (1/1/2, 0.1 M in DMF) on the Symphony multiple peptide synthesizer. The deprotection of the Fmoc group was carried out with 20% piperidine in DMF (2 × 10 min). Following removal of the N-terminal Fmoc group, the crude peptides were cleaved from the solid support as described above.

Synthesis of Compounds 13−16 and Dyn A-(1−11)NH_2. The synthesis of the linear peptides was performed on the Fmoc-PAL−PEG−PS resin (0.43 mmol/g) as described above. Following synthesis of the protected peptide-resin Fmoc-Arg-(Pbf)-Arg(Pbf)-Ile-Arg(Pbf)-Pro-Lys(Boc)-PAL−PEG−PS, the remaining Fmoc-amino acids (5-fold excess, Fmoc-amino acid/DIC/HOBt (1/1/1), 0.3 M in DMF) were coupled to the peptide using a manual multiple peptide synthesizer ("CHOIR") constructed in house.[39] Once the protected full-length linear

peptides were assembled, the N-terminal Fmoc group was removed; the side-chain protecting groups and the peptides were removed simultaneously from the resin as described above.

Purification and Analysis of Peptides. The crude peptides were purified by preparative reversed phase HPLC (Rainin HPXL HPLC system equipped with a Shimadzu SPD-10A detector) on a Vydac C18 column (10 μ, 300 Å, 21 × 250 mm) equipped with a Vydac guard cartridge. For purification a linear gradient of 15−50% MeCN containing 0.1% TFA over 35 min, at a flow rate of 20 mL/min, was used. The purification was monitored at 214 nm.

The purity of the final peptides was verified by analytical HPLC (Beckman HPLC system Gold, consisting of a programmable solvent module 126 and a diode array detector module 168) employing a Vydac 218-TP column (5 μ, 300 Å, 4.6 × 250 mm) equipped with a Vydac guard cartridge. A linear gradient of 5−80% solvent B over 50 min, at flow rate of 1 mL/min, was used for the analysis. The absorbance was monitored at 214 and 280 nm. Two solvent systems, system 1 (solvent A, aqueous 0.1% TFA, and solvent B, MeCN containing 0.1% TFA) and system 2 (solvent A, aqueous 0.1% TFA, and solvent B, MeOH containing 0.1% TFA), were used to assess the purity of the final peptides. Final purity of all peptides by both analytical systems was >98%. Molecular weights of the compounds were determined by either direct injection into a Thermoquest LCQ electrospray ionization mass spectrometer (ESI-MS) or on a Voyager MALDI-TOF (matrix assisted laser desorption ionization-time-of-flight) mass spectrometer (Biopolymer Laboratory, University of Maryland, Baltimore).

Radioligand Binding Assays. Radioligand binding assays were performed at room temperature by standard procedures as previously described[20] using cloned rat κ and μ and mouse δ opioid receptors stably expressed on CHO cells. [^{3}H]Diprenorphine, [^{3}H]DAMGO, and [^{3}H]DPDPE were used as radioligands in assays for κ, μ, and δ opioid receptors, respectively. Nonspecific binding was determined in the presence of 10 μM unlabeled Dyn A-(1−13)NH_2, DAMGO, and DPDPE for κ, μ, and δ opioid receptors, respectively. Binding assays were carried out in the presence of peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine) and 3 mM Mg^{2+}.

Data generated from equilibrium binding experiments was analyzed by fitting a logistic equation to data points using GraphPad Prism software as described previously.[36] K_i values were calculated from the IC_{50} values using the Cheng and Prusoff equation,[40] and 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.

Adenylyl Cyclase Assays. Adenylyl cyclase assays were performed as previously described[36] using cloned rat κ opioid receptors stably expressed on CHO cells. Adenylyl cyclase assays were carried out in the presence of a phosphodiesterase inhibitor (50 μM RO20−1724) and peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine). The cultures were incubated at 37 °C for 40 min in the presence of 50 μM forskolin and various concentrations of peptides. [^{14}C]-Cyclic AMP (5000 cpm in 50 μL) was added to each plate to correct for recovery. Concentrations of [^{3}H]- and [^{14}C]cyclic AMP were determined simultaneously using a Beckman LS 6000SC scintillation counter, and counts were corrected for crossover and recovery. The results presented are the mean ± SEM from at least two to three separate assays.

Acknowledgment. The authors would like to thank Jennif Chandler and B. Mirinda Thomas for performing the pharmacological assays and Nicholas P. Ambulos, Jr., Ph.D. and Kevin Rossamondo for performing MALDI-TOF analysis. This research was supported by grant R01 DA05195 from the National Institute on Drug Abuse.

Supporting Information Available: HPLC and mass spectral data for the Dyn A-(1−11)NH_2 analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) 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 where indicated otherwise. Additional abbreviations used are as follows: OAl, *O*-allyl; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; CHO, Chinese hamster ovary; ClZ, 2-chlorobenzyloxycarbonyl; DAMGO, [D-Ala^2,MePhe^4, glyol]enkephalin; Dap, 2,3-diaminopropionic acid; DCM, dichloromethane; DIC, *N*,*N*′-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMA, *N*,*N*-dimethylacetamide; DMF, *N*,*N*-dimethylformamide; DPDPE, *cyclo*[D-Pen^2,D-Pen^5]enkephalin; Dyn A, dynorphin A; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization−time of flight; MBHA, 4-methylbenzhydrylamine; NMM, *N*-methylmorpholine; PAL, peptide amide linker; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PEG, poly(ethylene glycol); Pip, 2-phenylisopropyl; PS, polystyrene; PyBOP, benzotriazol-1-yloxytrispyrrolidinophosphonium hexafluorophosphate; SEM, standard error of the mean; TFA, trifluoroacetic acid; Tos, tosyl.

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JM030298E