# Synthesis and Opioid Activity of [D-Pro$^{10}$]Dynorphin A-(1−11) Analogues with N-Terminal Alkyl Substitution†

Heekyung Choi,‡ Thomas F. Murray,§ Gary E. DeLander,§ William K. Schmidt,∥,⊥ and Jane V. Aldrich*,‡

*College of Pharmacy, Oregon State University, Corvallis, Oregon 97331, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 North Pine Street, Baltimore, Maryland 21201-1180, and Dupont Merck Pharmaceutical Company, Experimental Station, Wilmington, Delaware 19880*

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Several N-terminal di- and monoalkylated derivatives of [D-Pro$^{10}$]dynorphin A-(1−11) were synthesized in order to explore the structure−activity relationships for antagonist vs agonist activity at κ-opioid receptors. *N,N*-Dialkylated and *N*-monoalkylated (alkyl = allyl, benzyl, and cyclopropylmethyl (CPM)) tyrosine derivatives were prepared from tyrosine *tert*-butyl ester and the corresponding alkyl halides. [D-Pro$^{10}$]Dyn A-(2−11) was prepared by solid phase synthesis using Fmoc-protected amino acids, and the tyrosine derivatives were coupled to the peptide with BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate). Both the degree of substitution and the identity of the alkyl group affected κ-receptor affinity, selectivity, and efficacy. All of the *N*-monoalkylated derivatives exhibited much higher affinity ($K_i$ < 0.05 nM) for κ receptors in the guinea pig cerebellum and greatly enhanced κ-receptor selectivity ($K_i$ ratio (κ/μ) > 200) compared to the *N,N*-dialkyl [D-Pro$^{10}$]Dyn A-(1−11) analogues, although one disubstituted analogue, *N,N*-diCPM[D-Pro$^{10}$]Dyn A-(1−11), retained high affinity ($K_i$ = 0.19 nM) for κ receptors. Thus the introduction of the second alkyl group at the N-terminus lowered κ-receptor affinity and selectivity. The *N*-allyl and *N*-CPM analogues were moderately potent agonists in the guinea pig ileum (GPI) assay, while the *N*-benzyl derivative was a weak agonist in this assay. *In vivo* in the phenylquinone abdominal stretching assay the *N*-CPM analogue exhibited potent antinociceptive activity ($ED_{50}$ = 1.1 μg/mouse), while *N*-allyl[D-Pro$^{10}$]Dyn A-(1−11) exhibited weak antinociceptive activity ($ED_{50}$ = 27 μg/mouse). For the *N,N*-dialkyl derivatives the identity of the N-terminal alkyl group affected the efficacy observed in the smooth muscle assays. The *N,N*-diCPM analogue exhibited negligible agonist activity, and *N,N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11) showed weak antagonist activity against Dyn A-(1−13)$NH_2$ in the GPI. In contrast, the *N,N*-dibenzyl compound showed appreciable opioid agonist activity in this assay. *In vivo* the *N,N*-diallyl analogue exhibited weak antinociceptive activity ($ED_{50}$ = 26 μg/mouse in the phenylquinone abdominal stretching assay). The *N*-monoalkylated peptides are among the most κ-selective opioid peptides reported to date, showing comparable or greater selectivity and higher affinity than the κ-selective nonpeptide agonists U-50,488 and U-69,593. The *N,N*-diCPM and *N,N*-diallyl peptides are lead compounds in the development of peptide-based κ-receptor antagonists.

## Introduction

Although dynorphin A (Dyn A) has been postulated to be an endogenous opioid ligand for κ-opioid receptors,[1] it has been difficult to elucidate the physiological roles of Dyn A mediated by κ receptors because of the existence of multiple opioid receptor types and the low selectivity of Dyn A for κ receptors.[2] Numerous structure−activity studies involving modification of Dyn A-(1−13) have been devoted to the development of potent and more selective derivatives in order to elicit the pharmacological mechanisms of κ receptors.[2] Experimental evidence suggests that the N-terminal tetrapeptide fragment, referred to as the "message" sequence,[3] is responsible for opioid activity and that the N-terminal tyrosine residue is important for both opioid activity and binding to opioid receptors.[4] The C-terminal extension ("address" sequence) directs the peptide to κ-opioid receptors.[3]

N-Terminal alkylation of the tyrosine residue in Dyn A has been examined in a search for selective antagonists, based on the fact that substitution of the *N*-methyl group in morphine analogues by an *N*-allyl or *N*-cyclopropylmethyl group can impart antagonist activity to the classical alkaloid opiates (e.g. naloxone and naltrexone). Previously this modification was successfully applied to [Leu$^5$]enkephalin analogues. *N,N*-Diallyl substitution of [Leu$^5$]enkephalin yields a selective δ antagonist,[5] while the introduction of a single allyl group at the N-terminus of [D-Ala$^2$,Met$^5$]enkephalin yields a mixed agonist/antagonist.[6] Introduction of *N,N*-diallyltyrosine at position 1 of [D-Pro$^{10}$]Dyn A-(1−11)[7] and Dyn A-(1−13)[8] has been reported to impart antagonist activity to these peptides at κ and μ receptors, and

† Abbreviations used for amino acids follow the rules of the IUPAC−IUB Joint Commission of Biochemical Nomenclature in *Biochem. J.* **1984**, *219*, 345−373. Additional abbreviations: Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CHO, Chinese hamster ovary; CPM, cyclopropylmethyl; DAMGO, Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH; DIC, *N,N′*-diisopropylcarbodiimide; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMA, *N,N*-dimethylacetamide; DMF, *N,N*-dimethylformamide; DPDPE, [D-Pen$^2$,D-Pen$^5$]enkephalin; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MVD, mouse vas deferens; NalBzoH, naloxone benzoylhydrazone; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid.

‡ University of Maryland.
§ Oregon State University.
∥ Dupont Merck Pharmaceutical Company.
⊥ Current address: NorthStar Research & Development Ltd., 1 Bridleshire Circle, Newark, DE 19711.

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at $\kappa$ receptors, respectively. *N,N*-Diallylation of both peptides, however, leads to decreases in binding affinity and selectivity for $\kappa$-opioid receptors and results in analogues which show only weak antagonist activity in the guinea pig ileum (GPI). *N,N*-Diallyl[D-Pro$^{10}$]Dyn A-(1−11) is reported to be devoid of agonist activity, whereas *N,N*-diallyl-Dyn A-(1−13) still possesses full agonist activity in the GPI.[7,8] Incorporation of D-Pro at position 10 in Dyn A-(1−11) is reported to enhance $\kappa$-receptor selectivity and potency compared to Dyn A-(1−11).[9] We therefore chose *N,N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11) as our lead compound for further modification.

The major goals of our study were to explore whether the second alkyl group in *N,N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11) is required for antagonist activity and how mono- vs disubstitution of the N-terminus affects opioid receptor selectivity and opioid efficacy. Therefore, we synthesized several *N*-monosubstituted and *N,N*-disubstituted [D-Pro$^{10}$]Dyn A-(1−11) analogues, and evaluated their affinity in radioligand binding assays and their opioid activity in the GPI assay. In a preliminary communication,[10] we described the remarkable $\kappa$-receptor selectivity of the *N*-monoalkylated derivatives. Here we describe the synthesis of these analogues and compare the effects of mono- vs disubstitution on opioid receptor affinity and efficacy. Additional pharmacological characterization of both the *N*-mono- and *N,N*-dialkylated [D-Pro$^{10}$]Dyn A-(1−11) derivatives is also reported, including determination of $\kappa$-receptor subtype affinities of selected peptides and evaluation of *in vivo* antinociceptive activities in the mouse phenylquinone abdominal stretching assay. The results of these assays are described below.

R,R′Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-D-Pro-LysOH

R = allyl, cyclopropylmethyl (CPM), or benzyl; R′ = H

R = R′ = allyl, CPM, or benzyl

*N*-alkylated [D-Pro$^{10}$]Dyn A-(1−11) derivatives

## Chemistry

The *N*-alkylated peptides were prepared by Fmoc (9-fluorenylmethoxycarbonyl) solid phase synthesis in conjunction with incorporation of an *N*-alkylated tyrosine. Several methods are available for preparation of optically pure *N*-monoalkyl-substituted amino acid derivatives, including alkylation of N-protected amino acids. This method was developed by Cheung and Benoiton[11] to make various *N*-methyl amino acid derivatives using $N^{\alpha}$-Boc (*tert*-butyloxycarbonyl)—amino acids, sodium hydride as a base, and methyl iodide. The reaction with the bulkier alkyl halides of interest for preparation of the *N*-alkyl Dyn A derivatives, however, was extremely slow and gave very poor yields. We therefore examined conditions for alkylation of the free amine with alkyl halides. The *N*-monosubstituted and a significant amount of the *N,N*-disubstituted derivatives were simultaneously produced when tyrosine *tert*-butyl ester was treated with only an equimolar amount of alkyl bromide, even in the absence of a base. Finally, the reaction conditions were optimized to obtain a better yield of *N*-monosubstituted derivatives by utilizing less reactive alkyl halides (i.e. alkyl chlorides) and an organic base. The reaction was terminated before complete consumption of the starting material in order to minimize generation of *N,N*-disubstituted derivatives, which were more difficult to separate from the desired products than the starting material. The *N,N*-dialkylated tyrosine derivatives were prepared in good yields using excess alkyl halide and an organic base.

**Table 1.** Characterization of *N*-Alkylated [D-Pro$^{10}$]Dyn A-(1−11) Derivatives by HPLC and Fast Atom Bombardment Mass Spectrometry (FAB-MS)

| peptide | HPLC $t_R$ (% purity)[a] | FAB-MS (M + 1) |
|---|---|---|
| *N,N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11) | 19.7 (100) | 1443 |
| *N,N*-dibenzyl[D-Pro$^{10}$]Dyn A-(1−11) | 25.6 (98.4) | 1543 |
| *N,N*-diCPM[D-Pro$^{10}$]Dyn A-(1−11) | 20.7 (100) | 1471 |
| *N*-allyl[D-Pro$^{10}$]Dyn A-(1−11) | 18.4 (98.5) | 1403 |
| *N*-benzyl[D-Pro$^{10}$]Dyn A-(1−11) | 20.1 (100) | 1453 |
| *N*-CPM[D-Pro$^{10}$]Dyn A-(1−11) | 18.9 (98.7) | 1417 |

[a] Conditions: Vydac 214 TP C-4 column, 0−60% solvent B (0.1% TFA in AcCN) over 40 min at 1.5 mL/min, detection at 214 nm.

[D-Pro$^{10}$]Dyn A-(2−11) was assembled on a (hydroxymethyl)phenoxyacetic acid resin using Fmoc-protected amino acids with *N,N*′-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as the coupling agents. Side chain protecting groups used were the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) group for Arg and the Boc group for Lys. The tyrosine derivatives were incorporated into the resin-bound protected Dyn A-(2−11) using (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP),[12] HOBt, and *N*-methylmorpholine in *N,N*-dimethylacetamide (DMA). The BOP reagent in the presence of HOBt has been reported to result in more rapid and efficient coupling as well as a reduction in racemization when compared with DIC plus HOBt.[13] The peptides were cleaved from the support with concentrated trifluoroacetic acid (TFA) containing 5% water as a scavenger for the released carbonium ions. After purification of the crude peptides using reverse phase HPLC, the molecular weights were confirmed by FAB-MS and the purities evaluated by analytical HPLC (Table 1). The amino acid analyses gave the expected values for each amino acid (Table 2).

## Pharmacological Results

The *N*-alkylated Dyn A analogues were examined in several assays to determine their affinity for opioid receptors and their opioid activity. All of the peptides were evaluated for $\kappa$-, $\mu$-, and $\delta$-receptor affinity in radioligand binding assays, and selected analogues were also examined in binding assays for $\kappa$-opioid receptor subtypes. In order to identify compounds to examine further for possible antagonist activity, the effect of sodium and Gpp(NH)p on the affinity of peptides for $\kappa$ receptors (sodium shift assay) was investigated. Opioid activity was examined in the GPI assay, and selected compounds were also evaluated in the mouse vas deferens (MVD) preparation. The compounds were tested *in vivo* for antinociceptive activity in the phenylquinone abdominal stretching assay.

The synthesized compounds were evaluated in radioligand binding assays in the guinea pig cerebellum, in which at least 80% of the opioid receptors are $\kappa$-binding sites,[14] using [$^3$H]bremazocine as the radioligand, and in rat brain, which contains significant populations of $\mu$ and $\delta$ receptors,[15] using [$^3$H]DAMGO ([D-Ala$^2$,-NMePhe$^4$,glyol]enkephalin) and [$^3$H]DPDPE ([D-Pen$^2$,D-Pen$^5$]enkephalin), respectively, as the radioligands. As

**Table 2.** Amino Acid Analysis of *N*-Alkylated [D-Pro10]Dyn A-(1−11) Derivatives[a]

| peptide | Gly (2) | Phe (1) | Leu (1) | Arg (3) | Ile (1) | Pro (1) | Lys (1) |
|---|---|---|---|---|---|---|---|
| *N*,*N*-diallyl[D-Pro10]Dyn A-(1−11) | 1.73 | 1.05 | 1.05 | 3.17 | 0.96 | 1.04 | 1.04 |
| *N*,*N*-dibenzyl[D-Pro10]Dyn A-(1−11) | 1.88 | 1.05 | 1.03 | 3.12 | 0.95 | 0.96 | 1.02 |
| *N*,*N*-diCPM[D-Pro10]Dyn A-(1−11) | 1.92 | 1.04 | 1.04 | 3.10 | 0.97 | 0.90 | 1.03 |
| *N*-allyl[D-Pro10]Dyn A-(1−11) | 1.92 | 1.00 | 1.08 | 3.05 | 1.02 | 0.94 | 0.98 |
| *N*-benzyl[D-Pro10]Dyn A-(1−11) | 1.87 | 1.00 | 1.00 | 3.09 | 0.91 | 1.13 | 1.00 |
| *N*-CPM[D-Pro10]Dyn A-(1−11) | 1.95 | 1.03 | 1.00 | 3.07 | 0.96 | 0.98 | 1.01 |

[a] The frequency of the amino acid is in parentheses.

**Table 3.** Opioid Receptor Binding Affinity of *N*-Alkylated [D-Pro10]Dyn A-(1−11) Analogues

| peptide | $K_i$ (nM)[a] | | | $K_i$ ratio[b] ($\kappa/\mu/\delta$) |
|---|---|---|---|---|
| | [3H]bremazocine ($\kappa$) | [3H]DAMGO ($\mu$) | [3H]DPDPE ($\delta$) | |
| [D-Pro10]Dyn A-(1−11) | 0.030 ± 0.001 | 0.24 ± 0.002 | 2.1 ± 0.4 | 1/8/70 |
| *N*,*N*-diallyl[D-Pro10]Dyn A-(1−11) | 3.60 ± 0.50 | 31.7 ± 3.8 | 149 ± 58 | 1/8.8/41 |
| *N*,*N*-dibenzyl[D-Pro10]Dyn A-(1−11) | 3.66 ± 0.10 | 128 ± 1 | 2770 ± 130 | 1/35/757 |
| *N*,*N*-diCPM[D-Pro10]Dyn A-(1−11) | 0.19 ± 0.002 | 3.90 ± 0.10 | 166 ± 14 | 1/21/880 |
| *N*-allyl[D-Pro10]Dyn A-(1−11) | 0.049 ± 0.001 | 10.9 ± 0.5 | 499 ± 15 | 1/222/9160 |
| *N*-benzyl[D-Pro10]Dyn A-(1−11) | 0.029 ± 0.001 | 31.1 ± 0.2 | 176 ± 17 | 1/1070/6080 |
| *N*-CPM[D-Pro10]Dyn A-(1−11) | 0.020 ± 0.001 | 9.6 ± 0.6 | 558 ± 8.9 | 1/480/27900 |

[a] Affinity for $\kappa$ receptors was determined by measuring the inhibition of [3H]bremazocine binding to guinea pig cerebellar membranes. Affinities for $\mu$ and $\delta$ receptors were measured in rat forbrain using [3H]DAMGO and [3H]DPDPE, respectively. See the Experimental Section for details of the binding assays. [b] Ratio of $K_i$ values where the lowest $K_i$ is used in the denominator.

**Table 4.** Binding Affinity of [D-Pro10]Dyn A-(1−11) Analogues at $\kappa$ Subtypes[a]

| peptide | $K_i$ (nM) | | | $K_i$ ratio ($\kappa_1/\kappa_2/\kappa_3$) |
|---|---|---|---|---|
| | [3H]U69,593 ($\kappa_1$) | [3H]bremazocine ($\kappa_2$, $IC_{50}$)[b] | [3H]NalBzoH ($\kappa_3$) | |
| Dyn A-(1−11) | 1.70 | 13.3 | 23.1 | 1/7.8/14 |
| *N*,*N*-diallyl[D-Pro10]Dyn A-(1−11) | 21.3 | 4970 | 113 | 1/233/5.3 |
| *N*,*N*-diCPM[D-Pro10]Dyn A-(1−11) | 2.61 | 388 | 44.4 | 1/150/17 |
| *N*-benzyl[D-Pro10]Dyn A-(1−11) | 3.24 | 427 | 58.1[c] | 1/132/18 |

[a] See Experimental Section for details of the assays. [b] For the $\kappa_2$ receptors, $K_i$ values are not reported because low Hill coefficients suggest that there are more than one [3H]bremazocine binding site. [c] The Hill coefficient (=2.7) for this compound is quite different from that observed for other compounds (∼1).

shown in Table 3, the parent peptide [D-Pro10]Dyn A-(1−11) showed affinity for $\kappa$ receptors similar to that reported by Gairin et al.[9] Compared to their results, however, the affinities of this peptide for $\mu$ and $\delta$ receptors were increased about 10 and 3 times, respectively, resulting in only modest selectivity for $\kappa$ receptors ($K_i$ ratio ($\kappa/\mu/\delta$) = 1/8/70). The introduction of an *N*,*N*-dialkylated tyrosine at position 1 caused a significant decrease in affinity for $\kappa$ receptors. *N*,*N*-Bis(cyclopropylmethyl) substitution affected affinity for $\kappa$ receptors substantially less than did *N*,*N*-diallyl or *N*,*N*-dibenzyl substitution. *N*-Monoalkylation of [D-Pro10]Dyn A-(1−11), however, had little influence on affinity for $\kappa$ receptors. Both N-terminal mono- and dialkylation considerably decreased affinity for $\mu$ receptors (16−530-fold) and $\delta$ receptors (70−1320-fold).

The selectivity of the *N*-alkylated peptides for $\kappa$ receptors depended on both the identity of the N-substituent and on the extent of alkylation (*N*-mono- vs *N*,*N*-dialkylation). *N*,*N*-Diallyl[D-Pro10]Dyn A-(1−11), a putative antagonist for $\kappa$ receptors, showed $\kappa$-receptor selectivity similar to that found by Gairin et al. ($K_i$ ratio ($\kappa/\mu/\delta$) = 1/6.5/17),[7] while other *N*,*N*-dialkylated analogues showed slightly improved selectivity for $\kappa$ vs $\mu$ receptors and significantly higher selectivity for $\kappa$ vs $\delta$ receptors. As described in our initial report,[10] *N*-monoalkylation of [D-Pro10]Dyn A-(1−11) resulted in a marked enhancement in $\kappa$-receptor selectivity (200−1000-fold) over $\mu$ receptors. These compounds were also extremely selective (9000−28000 times) for $\kappa$ over $\delta$ receptors. It is of interest to note that a distinct trend toward greater selectivity was associated with increasing bulk of the substituents (benzyl > CPM > allyl).

*N*,*N*-Diallyl-, *N*,*N*-diCPM-, and *N*-benzyl[D-Pro10]Dyn A-(1−11) were examined in radioligand binding assays for $\kappa$-opioid receptor subtypes in guinea pig brain membranes (Table 4). Dyn A-(1−11) showed the highest affinity for the $\kappa_1$ receptors and the lowest affinity for the $\kappa_3$ subtype. All of the synthetic derivatives preferentially interacted with $\kappa_1$ receptors; the affinities of the *N*,*N*-diCPM and *N*-benzyl derivatives were similar to that of Dyn A-(1−11) for this receptor subtype. *N*-Alkylation decreased affinity for $\kappa_2$ receptors by 30−370-fold so that these analogues had the lowest affinity for this subtype. Except for the *N*,*N*-diallyl analogue, N-terminal alkylation decreased $\kappa_3$-receptor affinity by <2.5-fold. The *N*,*N*-diallyl derivative showed much lower affinity for all three receptor subtypes than did the other analogues tested.

In the sodium shift assay (Table 5) the ability of the compounds to displace [3H]diprenorphine binding to guinea pig cerebellar membranes was determined in the absence and presence of 120 mM sodium chloride and 50 $\mu$M Gpp(NH)p. In this assay, low sensitivity to sodium ion and guanine nucleotide is expected for compounds lacking agonist activity. Except for the *N*-benzyl derivative, the rank order potency of the Dyn A derivatives in the absence of sodium and Gpp(NH)p was similar to that seen in the radioligand binding assay using [3H]bremazocine. [D-Pro10]Dyn A-(1−11) showed a large shift in the binding affinity ratio, consistent with the potent agonist activity observed in the GPI assay, but the ratio was somewhat smaller than that reported by Gairin et al. ($IC_{50}(+)/IC_{50}(-)$ = 140).[7] Gairin et al. reported that *N*,*N*-diallylation of [D-Pro10]-Dyn A-(1−11) caused complete loss of sensitivity to

**Table 5.** Effects of 120 mM NaCl + 50 $\mu$M Gpp(NH)p on Binding of [D-Pro$^{10}$]Dyn A-(1−11) Analogues at $\kappa$ Sites in the Guinea Pig Cerebellum

| peptide | $IC_{50}$ (nM)[a] (−) | $IC_{50}$ (nM)[a] (+) | ratio $IC_{50}$(+)/$IC_{50}$(−) |
|---|---|---|---|
| [D-Pro$^{10}$]Dyn A-(1−11) | 0.26 ± 0.002 | 16.5 ± 2.0 | 63.8 |
| *N*,*N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11) | 21.3 ± 2.2 | 64.6 ± 4.3 | 3.03 |
| *N*,*N*-dibenzyl[D-Pro$^{10}$]Dyn A-(1−11) | 43.2 ± 2.8 | 391 ± 47 | 9.05 |
| *N*,*N*-diCPM[D-Pro$^{10}$]Dyn A-(1−11) | 3.31 ± 0.21 | 29.1 ± 1.7 | 8.79 |
| *N*-allyl[D-Pro$^{10}$]Dyn A-(1−11) | 2.30 ± 0.25 | 229 ± 20 | 99.4 |
| *N*-benzyl[D-Pro$^{10}$]Dyn A-(1−11) | 7.32 ± 0.58 | 93.7 ± 9.4 | 12.8 |
| *N*-CPM[D-Pro$^{10}$]Dyn A-(1−11) | 0.41 ± 0.05 | 27.1 ± 2.0 | 66.9 |

[a] (−) indicates the absence of 120 mM NaCl + 50 $\mu$M Gpp(NH)p and (+) indicates the presence of the 120 mM NaCl + 50 $\mu$M Gpp(NH)p.

**Table 6.** Opioid Activity of [D-Pro$^{10}$]Dyn A-(1−11) Analogues in the Guinea Pig Ileum

| peptide | $IC_{50}$ (nM)[a] | naloxone p$A_2$[b] |
|---|---|---|
| [D-Pro$^{10}$]Dyn A-(1−11) | 0.22 (0.11−0.49) | 7.2 (6.6−7.7) |
| *N*,*N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11) | >3000[c] | |
| *N*,*N*-dibenzyl[D-Pro$^{10}$]Dyn A-(1−11) | 228 (128−562) | |
| *N*,*N*-diCPM[D-Pro$^{10}$]Dyn A-(1−11) | >10000 | |
| *N*-allyl[D-Pro$^{10}$]Dyn A-(1−11) | 18.3 (13.2−22.4) | 7.6 (7.0−8.2) |
| *N*-benzyl[D-Pro$^{10}$]Dyn A-(1−11) | 990 (657−1500) | |
| *N*-CPM[D-Pro$^{10}$]Dyn A-(1−11) | 2.16 (1.6−2.8) | 7.3 (6.8−7.7) |

[a] Ninety-five percent confidence intervals are given in parentheses. [b] The p$A_2$ value for morphine was 8.2 (7.9−8.5). [c] After treatment with *N*,*N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11), the twitch height of the ileum declined gradually with time.

sodium ion and Gpp(NH)p ($IC_{50}$(+)/$IC_{50}$(−) = 1.5),[7] but in our assay this compound displayed a slightly larger shift (ratio = 3.0). The other two *N*,*N*-dialkylated analogues gave intermediate values (8.8−9.0) for the sodium shift. Both the *N*-monoallyl and *N*-CPM derivatives showed large sodium shifts, which is consistent with their potent agonist activity seen in the GPI assay (see below). The *N*-benzyl derivative exhibited a moderate shift (12.8), which is consonant with its weak agonist activity determined in the GPI assay (see below).

Opioid activity of all of the compounds was determined in the GPI assay (Table 6), and selected analogues were examined in the mouse vas deferens. Incorporation of substituents at the N-terminus reduced agonist potency relative to the parent peptide. For *N*,*N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11), typical agonist activity was not observed following treatment with this derivative, but the twitch height of the ileum declined gradually with time. Because of this effect on the tissues, the potential antagonist activity of this compound was determined by performing individual dose rather than a cumulative dose−response curve for Dyn A-(1−13)-$NH_2$ (see the Experimental Section). At a concentration of 10 $\mu$M this compound caused a 5-fold rightward shift in the dose−response curve of Dyn A-(1−13)$NH_2$ in the GPI, which is weaker antagonist activity than reported by Gairin et al. ($K_e$ = 130 nM against [D-Pro$^{10}$]Dyn A-(1−11)).[7] The differences in results for this compound may be partially due to strain differences of guinea pigs used in our study (Hartley) vs in the experiments by Gairin et al. (tricolor). Among the *N*,*N*-dialkylated compounds only the *N*,*N*-dibenzyl derivative exhibited significant agonist activity, while the *N*,*N*-diCPM analogue showed negligible agonist activity. At doses up to 1 $\mu$M the *N*,*N*-diCPM derivative did not display any significant antagonist activity against Dyn A-(1−13)-$NH_2$ in the GPI assay. The *N*,*N*-diallyl and *N*,*N*-diCPM analogues were also examined in the MVD; no agonist activity was detected in this assay for either compound. As in the GPI, the *N*,*N*-diallyl derivative was an antagonist (p$A_2$ ∼ 6.9) in the MVD.

Among the tested compounds, only the monosubstituted *N*-allyl and the *N*-CPM analogues exhibited potent opioid activity in the GPI. Low p$A_2$ values for antagonism of these analogues by naloxone were consistent with the $\kappa$-receptor selectivity observed in the radioligand binding assays. In spite of its high affinity for $\kappa$ receptors in the binding assays, the *N*-benzyl derivative behaved as a very weak agonist in the GPI. The intermediate value of the *N*-benzyl analogue in the sodium shift assay is in agreement with the weak agonist activity found in the GPI assay. This compound did not, however, exhibit any significant antagonist activity against Dyn A-(1−13)$NH_2$ at doses up to 1 $\mu$M. In the MVD assay, the *N*-benzyl compound exhibited negligible agonist activity (<10% inhibition at a concentration of 2 $\mu$M) and no $\mu$- or $\kappa$-receptor antagonism.

All of the analogues except the *N*,*N*-dibenzyl analogue were examined *in vivo* in the phenylquinone abdominal stretching assay following icv administration. Among the tested compounds, the *N*-CPM derivative showed the most potent antinociceptive activity with an $ED_{50}$ of 1.1 $\mu$g/mouse, while the lead compound *N*,*N*-diallyl-[D-Pro$^{10}$]Dyn A-(1−11) and the *N*-monoallyl analogue showed similar weak analgesic activity, with $ED_{50}$ values of 26 and 27 $\mu$g/mouse, respectively. Neither of these two peptides produced antagonist activity against 0.1 $\mu$g/mouse of [D-Ala$^2$]Dyn A-(1−13)$NH_2$ at a dose of 10 $\mu$g/mouse. The *N*,*N*-diCPM and the *N*-benzyl derivatives did not exhibit significant agonist or antagonist activity at doses up to 10 $\mu$g/mouse.

## Conclusions

Various *N*-alkylated tyrosine derivatives were introduced at position 1 in [D-Pro$^{10}$]Dyn A-(1−11) in order to explore the structure−activity relationships (SAR) for the N-terminus of Dyn A. Both the degree of substitution and identity of the alkyl group affected $\kappa$-receptor affinity, selectivity, and efficacy. In particular, *N*-monoalkylation led to marked increases in $\kappa$-receptor selectivity with retention of high affinity for this receptor. The *N*-allyl and *N*-CPM derivatives were potent agonists in the GPI, while the *N*-benzyl analogue exhibited weak agonist activity in this assay, in spite of high affinity for $\kappa$ receptors in the binding assay. The *N*-benzyl derivative exhibited a decreased $IC_{50}$ ratio in the sodium shift assay compared to the parent peptide or other *N*-monosubstituted derivatives, consistent with the decreased potency observed in the GPI.

The identity of the alkyl group in the *N*,*N*-disubstituted analogues affected both receptor affinity and efficacy. The disubstituted peptides had much lower affinity for $\kappa$ receptors compared to the *N*-monosubstituted derivatives, although one analogue, the *N*,*N*-diCPM peptide, retained sub-nanomolar affinity for $\kappa$ receptors. The significant decrease in affinity for $\kappa$

receptors upon disubstitution may be due to steric hindrance caused by incorporation of a second bulky alkyl group at the N-terminus. All of the *N*-alkylated analogues tested for binding affinity to $\kappa$ receptor subtypes preferentially interacted with $\kappa_1$ followed by $\kappa_3$ receptors; compared to the unsubstituted peptide these derivatives exhibited greatly reduced affinity for $\kappa_2$ receptors. In the sodium shift assay the *N*,*N*-disubstituted peptides exhibited significantly lower sodium shifts than the parent peptide, suggesting that these peptides warranted further investigation as possible antagonists. In the GPI the *N*,*N*-diallyl derivative exhibited antagonist activity, consistent with the previous report by Gairin et al.,[7] although only at high concentrations. In contrast, the *N*,*N*-dibenzyl analogue was a full agonist in the GPI. The *N*,*N*-diCPM displayed neither agonist nor antagonist activity in the smooth muscle assays at the concentrations examined.

Functional assays utilizing smooth muscle preparations and *in vivo* assays are complicated by the presence of multiple opioid receptors. With the cloning of the opioid receptors it is now possible to examine the effects of opioid ligands on second messenger systems in cells expressing a single type of receptor. Using Chinese hamster ovary (CHO) cells stably expressing rat $\kappa$ receptors, we have examined the efficacies of selected peptides as inhibitors of adenylyl cyclase production of cAMP.[16] In this assay both *N*,*N*-diCPM- and *N*,*N*-diallyl[D-Pro10]Dyn A-(1−11) exhibit greatly reduced efficacy (maximum inhibition of adenylyl cyclase <25%) and were able to reverse the inhibition of adenylyl cyclase caused by 10 nM Dyn A-(1−13)$NH_2$ in a dose-dependent manner.[16]

The *N*-substituted Dyn A analogues described here provide valuable SAR information in our search for potent and selective agonists and antagonists for $\kappa$-opioid receptors. The *N*-monoalkylated dynorphin derivatives have high affinity for $\kappa$ receptors and are among the most $\kappa$-selective opioid peptides reported to date,[17] showing comparable or greater selectivity and higher affinity than those of the $\kappa$-selective non-peptide agonists U-50,488 and U-69,593.[10] Therefore these peptides will be useful tools for future studies of the structure and function of $\kappa$-opioid receptors. The *N*,*N*-diCPM analogue represents a new lead compound which, along with the *N*,*N*-diallyl peptide, can be modified further in an attempt to identify potent and selective $\kappa$-receptor antagonists.

## Experimental Section

**Materials.** The sources of Fmoc-amino acids, reagents, and solvents for syntheses and purification were the same as previously reported.[18] Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected. Thin-layer chromatography was performed on silica gel plates (Kieselgel 60 F254, 0.20 mm thickness, E. Merck) and visualized by UV or by staining with 0.2% ninhydrin in isobutanol and heating. Medium-pressure column chromatography was performed with 230−400 mesh, 60 Å silica gel (E. Merck). Proton nuclear magnetic resonance ($^1$H NMR) spectra of *N*,*N*-dialkylated tyrosine derivatives were recorded on a Bruker 300A (300 MHz) spectrometer in the Department of Chemistry at Oregon State University. Elemental analysis was performed by M-H-W Laboratories, Phoenix, AZ.

The equipment used for solid phase peptide syntheses and for analytical and preparative HPLC systems was described previously.[18] The solvents used for analytical and preparative HPLC were aqueous 0.1% TFA for solvent A and 0.1% TFA in acetonitrile (AcCN) for solvent B. Fast atom bombardment mass spectra (FAB-MS) were obtained on a Kratos MS50RF (Department of Agricultural Chemistry, Oregon State University) at +8 kV in the positive or negative mode. Amino acid analysis was carried out on a Beckman System Gold HPLC amino acid analyzer system at Oregon State University as previously reported.[18]

**Synthesis of *N*,*N*-Disubstituted Tyrosine Derivatives: General Procedure.** Tyrosine *tert*-butyl ester (0.5−1.0 g, Sigma Chemical Co., St. Louis, MO) was dissolved in DMF (2 mL/mmol TyrO*t*Bu), and *N*,*N*-diisopropylethylamine (DIEA, 2.5 equiv) was added to the solution dropwise, followed by alkyl bromide (6−7 equiv). The reaction mixture was stirred either at room temperature or at 60 °C under $N_2$. The progress of the reaction was followed by TLC (EtOAc), and after completion of the reaction (generally 5−6 h), the mixture was diluted with water and extracted with $Et_2O$ (3 × 20 mL). The combined extracts were washed with saturated NaCl, dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure to yield the *N*,*N*-disubstituted tyrosine *tert*-butyl ester as an oil. The *tert*-butyl ester was then cleaved by 90% TFA/8% anisole/2% DCM at room temperature under $N_2$ for 4 h. The mixture was then concentrated *in vacuo* and dried under vacuum overnight. Crystallization and recrystallization from MeOH/$Et_2O$ gave the desired tyrosine derivatives.

***N*,*N*-Diallyltyrosine.** Tyrosine *tert*-butyl ester (0.51 g, 2.2 mmol) was reacted with DIEA (0.68 g, 5.4 mmol) and allyl bromide (1.8 g, 15.1 mmol) in DMF (4.5 mL) at 60 °C under $N_2$ for 5 h according to the general procdure. The product was isolated and the *tert*-butyl group removed as described above. Recrystallization from MeOH/$Et_2O$ provided the desired product as the free acid (361 mg, 63%): mp 196−198 °C; $[\alpha]^{25}$ = +15.4° (*c* 2.0, AcOH); HPLC $t_R$ = 8.8 min (100% purity); FAB-MS 260 (M − 1); $^1$H NMR (DMSO-$d_6$) $\delta$ 9.0 (s, 1H), 6.95 (d, *J* = 8.3 Hz, 2H), 6.64 (d, *J* = 8.3 Hz, 2H), 5.65 (m, 2H), 5.09 (m, 4H), 3.46 (t, *J* = 7.5 Hz, 1H), 3.29 (dd, *J* = 6.9 and 14.7 Hz, 2H), 3.09 (dd, *J* = 6.9 and 14.7 Hz, 2H), 2.85 (dd, *J* = 13.8 and 7.5 Hz, 1H), 2.70 (dd, *J* = 13.8 and 7.5 Hz, 1H). Anal. ($C_{15}H_{19}NO_3$) C, H, N.

***N*,*N*-Dibenzyltyrosine.** Tyrosine *tert*-butyl ester (1.0 g, 4.22 mmol) was alkylated with DIEA (1.37 g, 10.6 mmol) and benzyl bromide (4.44 g, 25.3 mmol) in 8.5 mL of DMF under $N_2$ at room temperature for 6 h and then extracted and concentrated as described above. The oily residue was purified by medium-pressure chromatography (DCM/3% EtOAc) to afford 1.67 g (94.3%) of *N*,*N*-dibenzyltyrosine *tert*-butyl ester as an oil. The residue was deprotected and then crystallized from MeOH/$Et_2O$ to yield the desired product as the free acid (0.90 g, 59%): mp 181−183 °C; $[\alpha]^{25} = -0.174°$ (*c* 0.5, MeOH); HPLC $t_R$ = 22.0 min (98% purity); FAB-MS 360 (M − 1); $^1$H NMR (MeOH-$d_4$) $\delta$ 7.31 (m, 10H), 6.91 (d, *J* = 8.3 Hz, 2H), 6.68 (d, *J* = 8.3 Hz, 2H), 4.18 (d, *J* = 13.6 Hz, 2H), 4.10 (d, *J* = 13.6 Hz, 2H), 3.81 (t, *J* = 7.3 Hz, 1H), 3.18 (dd, *J* = 8.0 and 14.1 Hz, 1H), 3.10 (dd, *J* = 8.0 and 14.1 Hz, 1H). Anal. ($C_{23}H_{23}NO_3 \cdot CF_3CO_2H$) C, H, N.

***N*,*N*-Bis(cyclopropylmethyl)tyrosine.** Tyrosine *tert*-butyl ester (0.76 g, 3.2 mmol) was treated with DIEA (1.03 g, 8.0 mmol) and cyclopropylmethyl bromide (2.6 g, 19.2 mmol) in 7 mL of DMF under $N_2$. The remainder of the procedure, including deprotection, followed the general procedure described above. Crystallization from MeOH/$Et_2O$ gave the pure product (561 mg, 61%): mp 219−220 °C; $[\alpha]^{25} = -0.482°$ (*c* 0.5, DMA); HPLC $t_R$ = 11.4 min (100% purity); FAB-MS 290 (M + 1); $^1$H NMR (MeOH-$d_4$) $\delta$ 7.14 (d, *J* = 8.5 Hz, 2H), 6.70 (d, *J* = 8.5 Hz, 2H), 4.21 (t, *J* = 7.2 Hz, 1H), 3.37 (d, *J* = 7.6 Hz, 1H), 3.31 (d, *J* = 7.5 Hz, 1H), 3.11 (m, 4H), 1.12 (m, 2H), 0.70 (m, 4H), 0.39 (m, 4H). Anal. ($C_{17}H_{23}NO_3$) C, H, N.

**Synthesis of *N*-Monosubstituted Tyrosine Derivatives: General Procedure.** Tyrosine *tert*-butyl ester (0.5−0.8 g) was dissolved in DMF (2 mL/mmol TyrO*t*Bu) under $N_2$, and the solution was cooled to 0 °C. DIEA (1 equiv) and alkyl halide (1 equiv) were added slowly. The progress of the reaction was monitored by TLC (EtOAc). The reaction mixture was diluted with water and extracted with EtOAc (3 × 20 mL). The EtOAc extract was washed with saturated NaCl, dried ($Na_2SO_4$), filtered, and concentrated under reduced pressure to dryness. Following purification of the *N*-alkylated tyrosine *tert*-butyl ester derivatives, deprotection and crystallization

were performed as described in the general procedure for *N,N*-disubstituted tyrosine derivatives above.

***N*-Allyltyrosine.** Tyrosine *tert*-butyl ester (0.75 g, 3.2 mmol) in 7 mL of DMF was treated with DIEA (0.41 g, 3.2 mmol) and allyl chloride (0.24 g, 3.2 mmol) according to the general procedure described above. After 40 h, the crude product was isolated as described above. The residue was purified by medium-pressure chromatography (91% $CHCl_3$/8% MeOH/1% HOAc/3% 2-propanol) to separate the monosubstituted compound from the starting material and disubstituted product, yielding 358 mg (41%) of pure *N*-allyltyrosine *tert*-butyl ester. The *tert*-butyl group was cleaved as described above and the free acid crystallized from $Et_2O$ (142 mg, 20%): mp 243−244 °C; FAB-MS 222 (M + 1); HPLC $t_R$ = 3.6 min (99% purity).[19] Anal. ($C_{12}H_{15}NO_3$) C, H, N.

***N*-Benzyltyrosine.** Tyrosine *tert*-butyl ester (0.5 g, 2.1 mmol) in 4.5 mL of DMF was reacted with DIEA (0.27 g, 2.1 mmol) and benzyl chloride (0.36 g, 2.1 mmol) for 45 h according to the general procedure, the reaction mixture was diluted with water and extracted with $Et_2O$ (3 × 20 mL), and the crude product was isolated as described above. The residue was purified by medium-pressure chromatography (90% DCM/10% EtOAc/2.5% 2-propanol) to afford 390 mg (57%) of *N*-benzyltyrosine *tert*-butyl ester. After deprotection *N*-benzyltyrosine was recrystallized from $Et_2O$, yielding 298 mg (52.3%): mp 232−234 °C; FAB-MS 272 (M + 1); HPLC $t_R$ = 10.7 min (100% purity).[19] Anal. ($C_{16}H_{15}NO_3$) C, H, N.

***N*-(Cyclopropylmethyl)tyrosine.** Tyrosine *tert*-butyl ester (0.8 g, 3.37 mmol) in 9 mL of DMF was alkylated with DIEA (0.44 g, 3.37 mmol) and cyclopropylmethyl bromide (0.46 g, 3.37 mmol) according to the general procedure. The reaction was terminated after 37 h, diluted with water, and extracted with $Et_2O$ (3 × 20 mL). The crude product was isolated as described above. Three hundred milligrams of the residue (out of 824 mg) was purified by medium-pressure chromatography (91% $CHCl_3$/8% MeOH/1% HOAc/5% 2-propanol), yielding 280 mg (28%) of pure *N*-(cyclopropylmethyl)tyrosine *tert*-butyl ester. The *tert*-butyl group was cleaved by the deprotection method described above and the product recrystallized from $Et_2O$ to give 125 mg (16%) of the pure free acid: mp 227−229 °C; FAB-MS 236 (M + 1); HPLC $t_R$ = 5.0 min (100% purity).[19] Anal. ($C_{13}H_{17}NO_3$) C, H, N.

**Peptide Synthesis.** Resin-bound [D-Pro$^{10}$]Dyn A-(2−11) was assembled on a (hydroxymethyl)phenoxyacetic acid resin (PAC resin, 0.5 g, 0.21 mmol substitution) by Fmoc solid phase synthesis using DIC couplings as described previously.[18] The side chains of Arg and Lys were protected by Pmc and Boc, respectively. After removal of the final Fmoc protecting group, the *N*-alkylated tyrosine derivatives were coupled to the assembled peptide chain using BOP reagent.[12] Prior to addition to the resin, the *N*-alkylated tyrosine (2 equiv) was activated using BOP:HOBt:*N*-methylmorpholine (2:2:3 equiv) in DMA (0.2 M final concentration). The progress of the coupling reaction was monitored by ninhydrin.[20] After completion of the coupling reaction, the peptide-resin was washed with 10 × 5 mL of DCM/DMA (1:1), 7 × 5 mL of DCM, and 4 × 5 mL of MeOH and dried *in vacuo*.

The crude peptides were released from the support using 5 mL of a TFA/$H_2O$ (95%/5%) mixture under $N_2$ for 4 h. The resin was filtered and rinsed with an additional 2 mL of TFA. The filtrate was concentrated under reduced pressure and dried *in vacuo*. The crude peptide was then triturated with cold $Et_2O$ and collected by filtration.

The crude peptides were purified by preparative reverse phase HPLC employing a linear gradient of 0−50% solvent B over 50 min at a flow rate of 20 mL/min. The purity of each fraction was evaluated by analytical reverse phase HPLC, and pure fractions were combined and lyophilized. Analytical data for the purified peptides are listed in Tables 1 and 2.

**Opioid Receptor Binding Assays.** The inhibitory effects of the peptides on the binding of [$^3$H]bremazocine ($\kappa$) to guinea pig cerebellar membranes and of [$^3$H]DAMGO ($\mu$) and [$^3$H]-DPDPE ($\delta$) to rat forebrain membranes were determined as previously described.[21] Briefly, $\kappa$-receptor binding assays were carried out at 4 °C for 3 h with [$^3$H]bremazocine and guinea pig cerebellar membranes in 20 mM HEPES, pH 7.4. Each assay tube also contained 100 nM DAMGO and 100 nM bestatin, and nonspecific binding was determined in the presence of 10 $\mu$M Dyn A-(1−13)$NH_2$. The [$^3$H]DAMGO and [$^3$H]DPDPE binding assays were performed at 4 °C for 5 h with rat forebrains prepared in 50 mM Tris, pH 7.7. Each assay tube also contained bestatin, captopril, and L-leucyl-L-leucine at final concentrations of 10, 30, and 50 $\mu$M, respectively. Nonspecific binding was determined in the presence of 10 $\mu$M levorphanol for the [$^3$H]DAMGO binding assay and in the presence of 10 $\mu$M unlabeled DPDPE for the [$^3$H]DPDPE binding assay. Competition data were fitted by nonlinear regression analysis using GraphPad. The $IC_{50}$ values derived from competition analyses were converted to dissociation constants ($K_i$) using the Cheng and Prusoff equation;[22] the $K_D$ values used for the conversion of $IC_{50}$ to $K_i$ values for [$^3$H]-labeled bremazocine, DAMGO, and DPDPE were 0.0547, 0.314, and 7.63 nM, respectively.

Selected analogues were evaluated for affinity for $\kappa$-receptor subtypes ($\kappa_1$, $\kappa_2$, $\kappa_3$) by Stanford Research Institute (NIDA contract no. 271-89-8159) according to standard procedures: the brains from Hartley guinea pigs were homogenized in 50 mM Tris-HCl buffer, pH 7.7 (25 mL/brain), using a Polytron, and the homogenate centrifuged at 40000*g* for 15 min, rehomogenized, and centrifuged. The final pellet was resuspended in Tris-HCl, pH 7.7, at a final concentration of 6.67 mg original wet weight of tissue/mL, except for tissues prepared for [$^3$H]-NalBzoH (naloxone benzoylhydrazone) binding which were resuspended in buffer containing 5 mM EDTA. The following radioligands (∼1 nM) were used to label the specific receptor binding sites: [$^3$H]U69,593 ($\kappa_1$), [$^3$H]bremazocine in the presence of 100 nM DAMGO, DSLET and U69,593 ($\kappa_2$), and [$^3$H]-NalBzoH in the presence of 100 nM U69,593 ($\kappa_3$). The guinea pig brain suspension (1.8 mL) was incubated in 50 mM Tris-HCl, pH 7.7, for 1 h at 25 °C with 100 $\mu$L of radioligand and 100 $\mu$L of test compound ($10^{-5}$−$10^{-11}$ M). Nonspecific binding was determined in the presence of 1 $\mu$M unlabeled U69,593 for $\kappa_1$ assays and 10 $\mu$M bremazocine and NalBzoH for the $\kappa_2$ and $\kappa_3$ assays, respectively. Each tube contained a peptidase inhibitor cocktail (6.25 $\mu$g/mL bacitracin, 10 $\mu$M bestatin, and 0.3 $\mu$M thiorphan). The suspensions were then filtered through glass fiber filters on a 48-well Brandel cell harvester and the filters washed with 3 × 3 mL of buffer. Filters were incubated overnight with 5 mL of scintillation cocktail before counting. The $K_i$ values were determined using the Cheng and Prusoff equation.[22] The $K_D$ values were obtained by computer analysis of detailed self-inhibition curves for each of the labeled ligands (L), using the curve-fitting program LIGAND.

The sensitivity of the binding of the prepared compounds to sodium and Gpp(NH)p was measured with [$^3$H]diprenorphine in guinea pig cerebellar membranes. Guinea pig cerebellar membranes were prepared as previously described,[21] except that 50 mM Tris, pH 7.4, was used as the buffer. Equilibrium binding experiments were performed at 25 °C for 60 min with peptidase inhibitors (10 $\mu$M bestatin and 1 mM Leu-Leu) using a final [$^3$H]diprenorphine concentration of 0.3 nM in either the absence or presence of 120 mM NaCl and 50 $\mu$M Gpp(NH)p, as reported by Gairin et al.[23] Final incubation volume was 1 mL, and each assay tube also contained 100 nM DAMGO. Nonspecific binding was determined in the presence of 1 $\mu$M Dyn A-(1−13)$NH_2$. The reaction was terminated by rapid filtration over glass filters (Whatman GF/B) using a Brandel M24-R cell harvester; the filters were presoaked for 2 h in 0.5% polyethyleneimine to decrease nonspecific filter binding. The filter disks were then placed in minivials with 4 mL of Cytocint (ICN Radiochemicals) and allowed to elute for 6 h before counting in a Beckman LS6800 scintillation counter.

**Smooth Muscle Assays.** Opioid activity in the GPI was determined as described previously.[21] Agonist activity was determined following addition of cumulative doses of the dynorphin analogues to the bath. Mean $IC_{50}$ values were determined from three to six replications using tissues from different guniea pigs. In order to examine antagonist activity, the tested peptides, except for *N,N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11), were added to the bath in the presence of peptidase inhibitors (100 nM bestatin (200 $\mu$L) and 3 nM thiorphan (200 $\mu$L)) 10 min prior to the determination of the $IC_{50}$ of Dyn A-(1−13)$NH_2$. For *N,N*-diallyl[D-Pro$^{10}$]Dyn A-(1−11) following de-

termination of the $IC_{50}$ of Dyn A-(1−13)$NH_2$ in the absence of the *N*,*N*-diallyl derivative, each tissue was incubated with 10 $\mu$M of the *N*,*N*-diallyl peptide for 10 min followed by treatment with a single dose of Dyn A-(1−13)$NH_2$. This method was used due to the gradual decline in twitch height with time following treatment with the *N*,*N*-diallyl derivative. Naloxone antagonism (naloxone p$A_2$) was measured by the standard procedure. Various concentrations of naloxone were incubated in the bath for 20 min before redetermination of the $IC_{50}$ of the test peptides. All Schild analyses[24] were performed using a computerized pharmacologic data analysis system.[25]

The MVD assay was performed by Stanford Research Institute as follows: the vas deferens from Swiss-Webster mice (30−35 g) were prepared as described by Hughes et al.[26] The tissues were mounted in an 8 mL, 31 °C organ bath containing a magnesium-free Krebs solution, which was bubbled with a mixture of oxygen and carbon dioxide (95:5). An initial tension of 150−200 mg was used. The field stimulation parameters were modified slightly from those described by Rónai et al.;[27] paired shocks of 100 ms delay between supramaximal rectangular pulses of 1 ms duration were delivered at a rate of 0.1 Hz. A Grass S-88 electrostimulator was used for stimulation. The concentrations were recorded using an isometric transducer (Metrigram) coupled to a Grass 7D multichannel polygraph. The agonist potency was determined as described in the GPI assay.

***In Vivo* Antinociceptive and Antagonist Determination.** Selected compounds were investigated for *in vivo* antinociceptive and possible antagonist activity by observing the phenylquinone abdominal stretching response. Male CF1 mice (Charles River Breeding Laboratories, Wilmington, MA), weighing 18−22 g at the time of testing, were housed a minimum of 6 days under carefully controlled environmental conditions (22.2 ± 1.1 °C; 50% average humidity; 12 h lighting cycle/24 h). Mice were fasted overnight (16−22 h) prior to testing (unfasted mice were used for antagonist activity determinations). For observation of stretching responses, mice were placed in individual clear plastic cages (13 cm long × 9 cm wide × 12.5 cm deep) with hinged clear plastic lids and wire screen bottoms arranged in units of 30 on a 20° incline. Randomized and coded doses of test compounds were administered by intracerebroventricular (icv) injection in a volume of 5 $\mu$L/mouse. Observation times represent the period from drug administration to the start of the observation period; the phenylquinone challenge dose (1.25 mg/kg ip phenyl-*p*-benzoquinone) was injected in a volume of 0.25 mL/20 g at 5 min prior to the specified observation time. The concentration of phenylquinone solution was 0.1 mg/mL in 5% aqueous ethanol. For scoring purposes a "stretch" was indicated by whole body stretching or full contraction of the abdomen. Mice were observed for 10 min for the presence or absence of the characteristic abdominal contraction and stretching response, beginning 5 min after the phenylquinone injection. Antinociception was indicated by a complete blockade of the stretching response. Greater than 95% of the control (vehicle-treated) mice are expected to exhibit a stretching response. Antinociceptive activity was calculated as the percentage of mice failing to respond to the phenylquinone challenge dose. $ED_{50}$ values were determined by the moving averages method[28] and statistical significance determined by $\chi^2$ analysis.

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