Design and Synthesis of Highly Potent and Selective Cyclic Dynorphin A Analogs. 2. New Analogs†,1

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We have designed and synthesized several cyclic disulfide-containing peptide analogs of dynorphin A (Dyn A) which are conformationally constrained in the putative "address" segment of the opioid ligand. Several of these Dyn A analogs exhibit unexpected apparent selectivities for the κ and μ opioid receptors(s) of the central vs peripheral nervous systems. Thus, incorporation of conformational constraint in the putative "address" segment of Dyn A analogs has resulted in the κ/μ opioid receptor ligands [L-Pen^5,Cys^{11}]Dyn A_{1-11}-NH_2 (4), [Cys^5,Cys^{10}]Dyn A_{1-11}-NH_2 (5), [Cys^5,Cys^9]Dyn A_{1-11}-NH_2 (6), and [Cys^4,Cys^9,Arg^{10}]Dyn A_{1-11}-NH_2 (7). All of these analogs possess high κ and μ opioid receptor affinities for the central receptor (guinea pig brain), but effect only weak potency at peripheral κ and μ opioid receptors (GPI). In fact cyclic dynorphin A analog 4 shows >19 000-fold differences between central κ opioid affinity and potency in the guinea pig ileum (GPI). Additionally analog 4 is not an antagonist in the GPI, suggesting possible receptor differences between these sites. Substitution of Tyr^1 by Phe^1 in the cyclic 1–11 series gave the analog [Phe^1,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 (1) that was surprisingly potent in the guinea pig brain binding assay (IC_{50} = 15.1 nM) at the κ receptor, but was inactive in the GPI and mouse vas deferens bioassays. D-Ala^2 and Tic^4 analogs of 1 had lower affinity at brain κ receptors and had very weak potencies in the GPI and MVD bioassays. On the other hand, [Cys^6,Cys^{10}]Dyn A_{1-11}-NH_2 (8), [Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2 (9), [D-Cys^8,D-Cys^{12}]Dyn A_{1-13}-NH_2 (10), and [D-Pro^{10},Cys^5,Cys^{13}]-Dyn A_{1-13}-NH_2 (11) were surprisingly potent in the GPI bioassay, though considerable apparent selectivity for central receptors is still retained. The apparent lack of correlation between the pharmacological profiles observed in smooth muscle and in the brain binding assays, particularly with 1 and 4, may suggest the existence of different subtypes of the κ and μ opioid receptors in the brain and peripheral systems.

Since the discovery of the endogenous enkephalins,[2] there has been an enormous amount of research done in the peptidic and nonpeptidic opioid areas to understand their structure–activity relationships.[3-6] Shortly after the characterization of the endogenous enkephalins, attempts were made to determine their preferred conformations. By use of various spectroscopic methods, X-ray crystallography, and/or energy calculations, different conclusions have been reached.[3,4,7] Now it is accepted that the enkephalins are highly flexible molecules that can assume an ensemble of energetically preferred conformations.[8] There may be definite advantages for a biological system to utilize hormones or neurotransmitters of high conformational flexibility including (1) the availability of thermodynamically accessible pathways to ligand–receptor interactions via a "zipper model";[9] (2) the ability of a specific hormone or neutrotransmitter to assume different conformations which could affect different molecular pharmacological events, e.g., ligand–receptor binding, transduction, and reversal of ligand–receptor binding;[10] and (3) the availability of different conformations for a specific ligand to permit binding to multiple receptor types.[11] In regard to the latter, it has been shown that the opioid receptors are heterogeneous and consist of at least three types, namely μ, δ, κ, and possibly others.[12,13] It is thought that the complexity of the pharmacological responses (analgesia, respiratory depression, physical dependence and tolerance, gut motility, etc.) to the opioids may be due in part to their nonselective binding to the μ and perhaps other opioid receptor types.[14] Before the physiological role(s) of opioids can be understood, a prerequisite to the rational design of therapeutic opioid drugs, highly receptor-selective ligands (both agonists and antagonists) for the opioid receptor types and subtypes must be developed.[5]

Research in the development of selective and potent non-μ-opioid ligands has expanded and recently the potential of targeting the κ-opioid receptor as an effector of analgesia has been reviewed.[15] The attractiveness of utilizing ligands for the κ-opioid receptor is that the pharmacology involves low abuse potential and a milder form of dependence in comparison to the prototypic μ-opiate ligand morphine. However adverse side-effects have been implicated with synthetic κ-opioid ligands, e.g., dysphoria, psychotomimesis, and diuresis. Also it has been reported that dynorphin A, the putative endogenous κ-opioid ligand, induces a hindlimb paralysis and spinal cord injury in the rat which is non-κ opioid receptor mediated.[16] Most of the structure–function studies of the

† Dedicated to Ralph Hirschmann on the occasion of his 70th birthday for his many scientific contributions.

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κ-selective ligands have been based on the putative endogenous ligand dynorphin A (Dyn A), dynorphin B (Dyn B), and α-neoendorphin.

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH

Dyn A

Structure–activity relationships for dynorphin peptides have been reviewed,[17] and a few key conclusions should be mentioned. Sequential removal of the C-terminal amino acids of Dyn A_{1-13} established that the basic residues Arg-7, Lys-11, and Lys-13 were important for high κ-receptor selectivity and/or potency,[18] but deletion of residues 14–17 or even 12–17 did not significantly affect Dyn A potency.[18] Substitution of lipophilic residues and certain D-amino acids at position 8 of Dyn A increase κ-receptor selectivity.[19,20] These latter observations suggest a reverse turn involving this position. Similarly, substitution with D-Pro in position 10 of Dyn A_{1-11} or Dyn A_{1-13} effects greater κ selectivity and is compatible with high κ-receptor potency.[20-22] Again this suggests a reverse turn and/or the need for an N-substituted amino acid[23] in position 10 for high κ selectivity and/or potency.

Thus far, the most potent and selective κ opioid receptor agonists have been non-peptides such as analogs of the prototype U-50488,[24] a *N*-methyl-*N*-((pyrrolidinyl)cyclohexyl)benzeneacetamide derivative, and U-69593[25] (*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzo[*b*]furan-4-acetamide)[26] with κ vs μ selectivity ratios of 484- and 1520-fold, respectively. The most potent and selective peptide κ opioid receptor agonist appears to be [D-Pro^{10}]Dyn A_{1-11}-OH(DPDYN).[21] DPDYN exhibits moderate κ vs μ (62) and κ vs δ selectivities with a binding affinity of 0.032 nM against the κ ligand [^{3}H]bremazocine. In tissue bioassays DPDYN displays a hamster vas deferens (HVD) (δ) vs guinea pig ileum (GPI) (μ and κ) IC_{50} ratio of 280 with an IC_{50} = 3.3 nM in the GPI.[27]

The first conformationally constrained Dyn A analog reported was the cyclic disulfide [D-Cys^2,Cys^5]Dyn A_{1-13}.[28] In the GPI and mouse vas deferens (MVD) bioassays [D-Cys^2,Cys^5]Dyn A_{1-13} exhibited high potency in the GPI bioassay, and a high MVD/GPI IC_{50} ratio was reported. The cyclic analog displayed a high μ affinity in binding studies using rat brain homogenates.[28] Recently cyclic lactams of Dyn A, [D-Orn^2,Asp^5]Dyn A_{1-8}, [Orn^5,Asp^8Dyn A_{1-13}, [Orn^5,Asp^{10}]Dyn A_{1-13}, and [Orn^5,Asp^{13}Dyn A_{1-13} were prepared.[29] The analog [D-Orn^2,Asp^5]Dyn A_{1-8} showed high potency in the GPI, but displayed a naloxone K_e value of 1.5 nM, which supported a strong interaction with the μ receptor.[12,30] The analogs [Orn^5,Asp^8]-, [Orn^5,Asp^{10}]-, and [Orn^5,Asp^{13}]Dyn A_{1-13} exhibited low potencies in both the GPI and MVD bioassays and also displayed naloxone K_e values consistent with an interaction with the μ receptor. In binding studies, the analogs [Orn^5,Asp^8]- and [Orn^5,Asp^{13}]Dyn A_{1-13} possessed high μ affinities and moderate μ vs δ selectivities.

Recently, we have designed and synthesized disulfide-containing cyclic analogs of Dyn A which incorporated conformational constraint within the "address" region of the opioid peptide.[31,32] Several of the analogs, e.g. [D-Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2 and [D-Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2, displayed high potencies (IC_{50} = 2.27 and 1.75 nM, respectively) and high κ vs μ selectivities in the GPI bioassay. Such selectivity was not observed in radioligand binding assays in guinea pig brain. κ-opioid selectivity of the agonist effect in the GPI was defined by the extent of antagonism of the analog by the μ-selective antagonists CTAP[33,34] or CTP.[35,36] In the central nervous system, the analogs possessed high κ-receptor affinity but low κ vs μ selectivity. Furthermore, [Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 and [Cys^5,Cys^{11},D-Ala^8]Dyn A_{1-11}-NH_2 displayed great differences in brain central κ-binding affinities and peripheral κ (possibly μ) bioassay potencies with the binding affinities in the brain being 3800- and 11 000-fold higher, respectively, than the potencies in the GPI. Additionally, these compounds were not antagonists in the GPI, suggesting the existence of distinct κ and μ opioid receptor types for the central and peripheral systems and prompting us to examine in more detail the effects of cyclization on κ-receptor potency and selectivity.

Results and Discussion

As mentioned previously, we recently reported the disulfide containing cyclic Dyn A analogs [Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2, [Cys^5,Cys^{11},D-Ala^8]Dyn A_{1-11}-NH_2, [Cys^8,Cys^{13}]-Dyn A_{1-13}-NH_2, [D-Cys^8,Cys^{13}]Dyn $A_{1-13}NH_2$, and [D-Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2.[31,32] In this work, we have explored further conformational constraints in the "address" region of Dyn A and their effects on κ opioid receptor selectivity and potency, and their different potencies in binding in the central nervous system (guinea pig brain, GPB) and peripheral activity (GPI). Although the Dyn A_{1-11} cyclic analogs displayed low potencies in the GPI bioassay, [Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 exhibited selective agonist activity mediated via the κ and not via the μ receptor. κ opioid selectivity of the agonist effect in the GPI was defined by the extent of antagonism by the μ-selective antagonists CTAP[33,34] (1000 nM) (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) or CTP[35,36] (1000 nM) (D-Phe-Cys-Thr-D-Trp-Lys-Thr-Pen-Thr-NH_2). Further investigation of disulfide-containing Dyn A_{1-11} undecapeptide analogs cyclized via the Cys^5 residue seemed warranted. Also the cyclic Dyn A_{1-13} analogs [D-Cys^8,Cys^{13}]-Dyn A_{1-13}-NH_2 and [D-Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2 displayed high potencies (IC_{50} = 2.27 and 1.75 nM, respectively) and high κ vs μ selectivities in the periphery (GPI), though not in the CNS (GPD). Thus, further exploration of the bioactive conformation of analogs cyclized via the Cys^8 position of Dyn A appeared promising.

All peptides were synthesized as C-terminal carboxamide analogs to impart stability to exopeptidases.[37] Previous work in our laboratories had indicated that substitution of Tyr^1 by Phe in Dyn A_{1-11}-NH_2 provided a κ ligand with high affinity (IC_{50} = 1.65 nM) and moderate κ vs μ (36) selectivity in the GPB binding assay. However, this analog, [Phe^1]Dyn A_{1-11}-NH_2 exhibited low potencies in the GPI and MVD bioassays (IC_{50} = 1600 and 6900 mM, respectively) (unpublished results). The cyclic analog [Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2[31-32] displayed a high affinity (IC_{50} = 0.285 nM) but a low κ vs μ selectivity (0.95) in the

Table I. Potency of Dynorphin Analogs in the Guinea Pig Isolated Ileum (GPI) and Mouse Isolated Vas Deferens (MVD) Bioassays

compound	IC_{50} (nM)[a] GPI (95% CI)	MVD (95% CI)
Dyn A_{1-17}-OH	2.53 (2.176–2.882)	39.7 (31.79–47.55)
Dyn A_{1-11}-NH_2	3.81 (2.65–4.974)	28.93 (23.66–34.20)
Dyn A_{1-13}-NH_2	1.15 (0.72–1.82)	9.1 (5.78–14.4)
1, [Phe^1,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	>10000	>3000
2, [D-Ala^2,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	3972 (1927–8189)	>6000
3, [L-Tic^4,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	6180 (5523–6910)	4180 (3420–5100)
4, [L-Pen^5,Cys^{11}]Dyn A_{1-11}-NH_2	>10000	>6000
5, [Cys^5,Cys^{10}]Dyn A_{1-11}-NH_2	286 (201–408)	>3000
6, [Cys^5,Cys^9]Dyn A_{1-11}-NH_2	411 (241–703)	>3000
7, [Cys^5,Cys^9,Arg^{10}]Dyn A_{1-11}-NH_2	250 (168–372)	6262 (3009–13030)
8, [Cys^6,Cys^{10}]Dyn A_{1-11}-NH_2	6.51 (5.224–7.792)	92.6 (62.31–123.15)
9, [Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2	7.33 (3.94–13.6)	9.17 (4.30–19.54)
10, [D-Cys^8,D-Cys^{12}]Dyn A_{1-13}-NH_2	4.46 (2.54–7.70)	25.3 (17.3–37.0)
11, [D-Pro^{10},Cys^5,Cys^{13}]Dyn A_{1-13}-NH_2	24.6 (16.5–36.7)	4100 (2400–7010)

[a] For all moderately or highly potent analogs, that is compounds **2**, **3**, and **6**–**11** in the GPI and compounds **3** and **7**–**11** in the MVD, dose–response curves similar to and parallel to the dynorphins themselves were obtained with no evidence of partial agonism indicating similar efficacy.

GPB binding assay, and possessed low potencies in the GPI and MVD bioassays (IC_{50} = 1100 and 400 nM, respectively). Thus, we prepared the Phe^1-substituted analog [Phe^1,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 (**1**) to investigate the potential of incorporating κ selectivity and possibly potency into the cyclic analog. The analog [D-Ala^2,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 (**2**) was prepared to impart greater stability of the peptide in this series toward aminopeptidases.[38] Substitution at the 4 position with the highly constrained amino acid L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) resulted in [L-Tic^4,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 (**3**), which explores the possibility of biasing the conformation of the peptide toward a bioactive topology as has been shown with somatostatin-derived highly μ-selective opioid ligands.[39] Replacement of Cys with the hindered amino acid penicillamine (Pen) has been shown to confer conformational constraint in disulfide-containing cyclic peptides, resulting in highly selective and potent δ opioid ligands such as DPDPE.[40] Incorporation of L-Pen into the cyclic disulfide ring system resulted in [L-Pen^5,Cys^{11}]Dyn A_{1-11}-NH_2 (**4**). Another method to constrain a ring system would be to decrease the size of the ring. Analogs **1**–**4** contain a 23-membered ring system. Decreasing the ring system by one residue would give a 20-membered ring system such as for [Cys^5,Cys^{10}]Dyn A_{1-11}-NH_2 (**5**). This analog **5** not only should possess a more constrained ring system but would also retain the Lys^{11} residue which has been purported to be required for high κ selectivity and potency.[18] A further constraint of the ring system would be evident for the analog [Cys^5,Cys^9]Dyn A_{1-11}-NH_2 (**6**), which has a 17-membered ring. As both Dyn B and α-neoendorphin, putative ligands of the κ-opioid receptor, contain basic residues in position 10, Arg was substituted in that position in the constrained 17-membered analog [Cys^5,Cys^9,Arg^{10}]Dyn A_{1-11}-NH_2 (**7**). Constraining the ring system to a 17-membered ring while leaving the putative "message" region fully intact was achieved by cyclization at positions 6 and 10 to obtain [Cys^6,Cys^{10}]Dyn A_{1-11}-NH_2 (**8**).

The various cyclic analogs of Dyn A_{1-11} series (**1**–**8**) displayed similar pharmacological profiles as was observed for [Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2. In particular, they showed very weak potencies in the GPI and MVD bioassays (IC_{50} values of 100–10 000 nM, Table I), good κ affinities in the brain (0.05–81 nM), modest κ vs μ selectivities (0.12–14) in the GPB binding assay (Table II), and apparently high selectivity for brain (GPB) vs peripheral (GPI) receptors (46 to >19 000, Table III). The exception was analog **8** which exhibited a high potency (IC_{50} = 6 nM) in the GPI and the response was shown to be opioid receptor mediated as it was reversed by naloxone (data not shown). Furthermore, the response was mediated via the κ receptor and not the μ receptor, as the response was not antagonized by the highly μ-selective antagonist CTAP[34] but was antagonized by the highly κ-selective antagonist norBNI[41] (data not shown). In the MVD, analog **8** displayed a fair potency (IC_{50} = 100 nM). Again, the response was selectively mediated via the κ-opioid receptor since it was not antagonized by the δ-selective antagonist ICI 174,-864[42], but was antagonized by the κ-selective antagonist norbinaltorphimine (norBNI; data not shown). In the GPB binding assay analog **8** showed a high κ affinity (IC_{50} = 0.047 nM) but a poor κ vs μ selectivity (1.9) and a modest κ vs δ selectivity (10).

Previously we reported a highly κ selective and potent opioid receptor agonist, [D-Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2.[31,32] Selectivity of the agonist effect was defined by the extent of antagonism by the μ-selective antagonists CTAP[34] (1000 nM) and CTP[35] (1000 nM) or CTP[35] (1000 nM) in the GPI (κ vs μ) or by the δ-selective antagonist ICI 174,864[44] (1000 nM) in the MVD (κ vs δ). The diastereomers [D-Cys^8,Cys^{13}]-Dyn A_{1-13}-NH_2 and [Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2 also were reported previously.[31,32] The former possessed good potency in the GPI but did not show κ selectivity in the

Table II. Opioid Receptor Binding Affinities and Selectivities of Various Cyclic Disulfide-Containing Dynorphin Analogs with Guinea Pig Brain Homogenate

no.	compound	IC_{50} (nM) [^{3}H]U-69,593	[^{3}H]PL-17	[^{3}H]DPDPE	μ/κ	δ/κ
	Dyn A_{1-17}-OH	0.231 ± 0.101	5.04 ± 1.10	2.54 ± 0.23	22	11
	Dyn A_{1-11}-NH_2	0.077 ± 0.017	1.08 ± 0.07	6.99 ± 0.901	14	91
	Dyn A_{1-13}-NH_2	0.114 ± 0.036	1.29 ± 0.30	4.07 ± 1.10	11	36
1	[Phe^1,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	15.1 ± 2.3	61.2 ± 5.6	389 ± 48	4.0	26
2	[D-Ala^2,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	81.2 ± 14.5	9.69 ± 2.93	107 ± 18	0.12	1.3
3	[L-Tic^4,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	51.5 ± 2.3	106 ± 25	181 ± 26	2.0	3.5
4	[L-Pen^5,Cys^{11}]Dyn A_{1-11}-NH_2	0.522 ± 0.172	1.26 ± 0.44	86.3 ± 9.14	2.4	165
5	[Cys^5,Cys^{10}]Dyn A_{1-11}-NH_2	0.593 ± 0.254	4.26 ± 1.07	82.5 ± 5.09	7.2	139
6	[Cys^5,Cys^9]Dyn A_{1-11}-NH_2	0.868 ± 0.432	6.43 ± 2.29	31.2 ± 10.2	7.4	36
7	[Cys^5,Cys^9,Arg^{10}]Dyn A_{1-11}-NH_2	0.42 ± 0.12	5.81 ± 0.08	18.2 ± 6.1	14	43
8	[Cys^6,Cys^{10}]Dyn A_{1-11}-NH_2	0.047 ± 0.023	0.091 ± 0.025	0.475 ± 0.122	1.9	10
9	[Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2	0.035 ± 0.018	0.380 ± 0.242	1.93 ± 0.566	11	55
10	[D-Cys^8,D-Cys^{12}]Dyn A_{1-13}-NH_2	0.116 ± 0.048	0.674 ± 0.127	6.54 ± 1.78	5.8	56
11	[D-Pro^{10},Cys^5,Cys^{13}]Dyn A_{1-13}-NH_2	0.21 ± 0.10	2.48 ± 0.24	9.60 ± 2.41	12	46

Table III. CNS (guinea pig brain, GPB) vs Peripheral (Guinea Pig Ileum, GPI) Selectivities at the κ and μ Opioid Receptor(s) for Various Cyclic Dynorphin A Analogs

no.	compound	ratio of IC_{50}: GPI/GPB
	Dyn A_{1-17}-OH	11
	Dyn A_{1-11}-NH_2	49
	Dyn A_{1-13}-NH_2	10
1	[Phe^1,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	>660
2	[D-Ala^2,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	46
3	[L-Tic^4,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	120
4	[L-Pen^5,Cys^{11}]Dyn A_{1-11}-NH_2	>19000
5	[Cys^5,Cys^{10}]Dyn A_{1-11}-NH_2	550
6	[Cys^5,Cys^9]Dyn A_{1-11}-NH_2	470
7	[Cys^5,Cys^9,Arg^{10}]Dyn A_{1-11}-NH_2	600
8	[Cys^6,Cys^{10}]Dyn A_{1-11}-NH_2	140
9	[Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2	209
10	[D-Cys^8,D-Cys^{12}]Dyn A_{1-13}-NH_2	37
11	[D-Pro^{10},Cys^5,Cys^{13}]Dyn A_{1-13}-NH_2	120

GPB. The latter displayed comparable potencies in the GPI and MVD bioassays and showed κ selectivity in the GPI but not in the MVD. The three diastereoisomers of [Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2 exhibited high κ affinities (IC_{50} = 0.74–1.76 nM), but surprisingly all possessed low κ vs μ selectivities in the GPB binding assay.[31] Further investigation of this series was obviously desirable. Of the four possible diastereoisomers of [Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2, [Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2 (**9**) was not previously reported, and in this work we now do so. Analog **9** exhibited a potent response (IC_{50} = 7.3 nM) in the GPI, which was selectively mediated via the κ-opioid receptor as was evidenced by an antagonism of the response by norBNI and a lack of antagonism by CTAP (data not shown). In the MVD, analog **9** showed a response of moderate potency (IC_{50} = 9.2 nM) that was not δ receptor mediated as it was not antagonized by ICI 174,864. Typical of most of the cyclic analogs reported herein, the binding profile of analog **9** showed a high affinity (0.035 nM) for the κ receptor and modest κ vs μ selectivity (210) (Table II). The 20-membered ring system of the κ-selective and potent analog [D-Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2 was constrained further by decreasing the ring size to 17 via a cyclization at the 8 and 12 positions to give [D-Cys^8,D-Cys^{12}]Dyn A_{1-13}-NH_2 (**10**). These substitutions also allow for the presence of a Lys^{13} residue, which reportedly enhances κ selectivity and potency.[18] In the GPI, analog **10** exhibited a highly potency response (IC_{50} = 4.5 nM) which was opioid in nature as it was antagonized by naloxone and was selectively mediated by the κ receptor as evidenced by an observed antagonism by norBNI and a lack of antagonism by CTAP (data not shown). In the MVD, analog **10** showed a moderate potency (IC_{50} = 25.3 nM) and a response which was opioid in nature since it was antagonized by naloxone. The response was mediated selectively by the κ receptor, as it was antagonized by norBNI but not by CTAP or ICI 174,864. The binding profile of **10** in the GPB binding assay (Table II) showed that the analog had a high affinity (0.23 nM) for the κ receptor but a poor κ vs μ selectivity (5.8). Finally, the ring system of this series was relaxed by expansion of the cycle to a 29-membered ring achieved via the analog [D-Pro^{10},Cys^5,Cys^{13}]Dyn A_{1-13}-NH_2 (**11**). The D-Pro was incorporated to stabilize a potential reverse turn at the 10 position, which we inferred based on previous structure–activity relationships.[20-22] In the GPI bioassay, analog **11** was shown to possess a moderate potency (IC_{50} = 24.6 nM) (Table I), and a response which appeared to be selectively mediated by the κ receptor as it was not antagonized by CTAP (data not shown). Unexpectedly analog **11** displayed a very low potency (4100 nM) in the MVD bioassay (Table I) with a response that was not μ receptor mediated since it was not antagonized by CTAP (data not shown). In the GPB binding assay analog **11** showed high affinity (IC_{50} = 0.21 nM) and modest κ selectivity (12) (Table II).

If the affinities at the κ-opioid receptor in the guinea pig brain binding studies are compared to potencies in the GPI bioassay, an apparent measure of the selectivity of

the ligand for the central κ vs peripheral κ receptor system(s) can be obtained if the analog is also tested as an antagonist (Table III). As previously reported, the analogs [Cys⁵,Cys¹¹,D-Ala⁸]Dyn A_{1-11}-NH_2 and [Cys⁵,Cys¹¹]Dyn A_{1-11}-NH_2 showed an 11 000- and 3800-fold greater affinity in the guinea pig brain binding assay than in the GPI bioassay. However, the analogs [Cys⁸,Cys¹³]Dyn A_{1-13}-NH_2, [D-Cys⁸,Cys¹³]Dyn A_{1-13}-NH_2, and [D-Cys⁸,D-Cys¹³]Dyn A_{1-13}-NH_2 showed GPI/GPB IC_{50} ratios of 17, 1.3, and 16, respectively. For the analogs reported here which have high binding affinity (<1 nM) at the κ opioid receptor, i.e., analogs 4–11, 4 exhibits an GPI/GPB IC_{50} ratio >19 000 and 5–9 and 11 showed ratios greater than 100 (120–600). A GPI/GPB IC_{50} ratio of 120 for 11 was considerably better than the parent compound Dyn A_{1-13}-NH_2. The question arises whether the apparent high selectivity for κ potency in the CNS (as measured by binding in the guinea pig brain) and κ activity in peripheral tissues as measured by potency in the guinea pig ileum might be due to a difference in efficacy, so that perhaps the inactive compounds, especially 1 and 4, were antagonists. The dose–response curves indicated that the active compounds in the GPI (2, 3, 5-11) gave similar dose–response curves as Dynorphin A. We thus examined the ability of 4 (10 000 nM) to inhibit U69593 (IC_{50} = 2.2 ± 1.2 nM) in the GPI. No shift in the dose–response curve was observed (IC_{50} = 3.0 ± 0.4 nM). Thus, on the basis of this data, analog 4 appears to more strongly discriminate between the central κ-opioid receptor of the GPB and that of the periphery (GPI) than any known compound, while the analogs 1 and 5–9 appear to do the same, though somewhat less so. It is unclear whether analogs 1 and 5–9 are antagonists in GPI, so conerns about possible differences in efficacy cannot be eliminated. The results with analog 4, however, lend support to the possible existence of distinct κ and μ opioid receptor types for the central and peripheral nervous system.

Perhaps cyclization in the "address" region of Dyn A via the 5-position resulting in analogs 1–7 (17, 20, or 23-membered disulfide-containing ring systems) gives rise to a conformation(s) not unlike that possessed by [Orn⁵,Asp⁸],[Orn⁵,Asp¹⁰]- and [Orn⁵,Asp¹³]Dyn A^{1-13}, which were reported to exhibit similar pharmacological profiles, i.e., low potencies in both the GPI and MVD bioassays and high μ affinities in binding assays (κ affinities were not reported).[29] Apparently, the three-dimensional structure of analog 11 may not resemble that of [Orn⁵,Asp¹³]Dyn A_{1-13}, as 11 shows a κ-selective response of moderate potency (IC_{50} = 24.6 nM) in the GPI. Cyclization via the 6 and 10 positions to give analog 8 may afford a favorable bioactive conformation, and in addition the presence of the Lys^{11} may enhance κ-receptor selectivity and potency in the GPI system. We previously showed that [D-Cys⁸,Cys¹³]Dyn A_{1-13}-NH_2 exhibited a high potency (2.27 nM) in the GPI, but that the respose was not selective for the κ receptor in that, at least in part, it was mediated by the μ receptor.[31] Analog 9, which is a diastereomer of the mentioned analog, shows a comparable potency (7.33 nM) and a κ-selective response in the GPI in that only norBNI but not CTAP or ICI 174,864 blocks its effects. Since there has been only a stereochemical change in the two analogs, the bioactive conformation of the ligand may be influenced by the helicity of the disulfide bond.[43] The more constrained analog 10 displays a comparable potency and κ selectivity in the GPI and MVD. Thus it would appear that for κ selectivity in the peripheral bioassays a D-stereochemistry for the C-terminal Cys of the 8–13 or 8–12 cyclized ring systems is important.

While the possibility of differences in opioid κ and μ receptors between central (i.e., brain) and peripheral tissues can be inferred from the data obtained with these novel Dynorphin analogues, it must be emphasized that such conclusions are preliminary. Differences in central receptor affinity and potency such as in the GPI or MVD bioassay do not generally consider the possibility of compounds which may bind well to peripheral receptors but lack efficacy. However, the tentative conclusion of differences between receptors in central and peripheral sites appears to be supported by the observation that analog 4, one of the most potent in binding in the brain, shows poor potency in both the GPI and MVD as agonists and, further, did not show antagonist activity against a selective κ agonist. The lack of antagonist actions of these compounds suggest that they do not bind, or bind only weakly, to the κ receptor in the peripheral tissues, despite having high affinity for the κ receptor in GPB. This finding supports the concept of differences between central and peripheral opioid κ receptors. While further work will be required to determine whether other analogs support differences in the central and peripheral opioid κ receptors, the hypothesis is attractive.

In conclusion, we have prepared a series of disulfide-containing cyclic analogs of dynorphin A that have been cyclized in the address region of the peptide. Analogs 1–7, which are cyclized via the 5 position to positions 9, 10, or 11, result in low potencies in peripheral bioassays (GPI and MVD), but moderate to high affinities in the GPB binding assay. The analog [L-Pen⁵,Cys¹¹]Dyn A_{1-11}-NH_2 (4) has extraordinary selectivity for the central vs peripheral κ opioid receptor. Cyclization at the 6 and 10 positions afforded a Dyn A analog (8) which exhibited a response with good potency and κ selectivity in the GPI and a high κ affinity in the GPB binding assay. The analogs [Cys⁸,Cys¹³]Dyn A_{1-13}-NH_2 (9) and [D-Cys⁸,D-Cys¹²]Dyn A_{1-13}-NH_2 (10) were found to be the most potent and κ selective analogs of the series in the GPI and MVD bioassays. Disulfide-containing cyclic Dyn A analogs involving positions 8 and 13 or 8 and 12 appear to require D-Cys at the C-terminal for κ selectivity in the peripheral bioassays. In the GPB binding assay analogs 9 and 10 showed high κ affinities but modest κ selectivity.

Experimental Section

In Vitro Bioassays. The guinea ileum longitudinal muscle/myenteric plexus preparation was used as described previously.[44] The tissues were suspended under a final tension of 1 g in organ baths, bathed with Krebs buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.18 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.48 mM glucose), maintained at 37 °C, and aerated with 95% O_2/5% CO_2. Electrical stimuli were 0.4-ms pulses of supramaximal voltage, at a rate of 6/min. Isometric contractions were measured via strain gauge force transducers on chart recorders. The mouse vas deferens preparation was also performed as described.[45] The Krebs buffer was made as above, but without magnesium, and the tissues were suspended under a final tension of 500 mg. Pulse duration was 2 ms. Concentration–response testing in all preparations was carried out in a cumulative fashion. The compounds were tested for intrinsic agonist activity in two in vitro bioassays. Thus, concentrations showing intrinsic activity for each analog were

Table IV. Analytical Properties of Cyclic Dynorphin A Analogs

no.	compound	yields[a] (mg)	thin-layer chromatography R_f values[b] I	II	III	IV	HPLC k' values[c]	FAB-MS[d]
1	[Phe^1,Cys^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	32	0.50	0.15	0.85	0.65	2.60^2	1308 [M]$^+$
2	[D-Ala^2,Cys^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	87	0.55	0.15	0.85	0.65	4.00^2	1328 [M]$^+$
3	[L-Tic^4,Cys^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	17	0.40	0.15	0.85	0.65	3.46^2	1336 [M]$^+$
4	[L-Pen^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	43	0.50	0.15	0.90	0.65	4.25^1	1352 [M]$^+$
5	[Cys^5,Cys^{10}]Dyn A$_{1-11}$-NH$_2$	57	0.35	0.15	0.85	0.55	3.80^2	1356 [M + H]$^+$
6	[Cys^5,Cys^9]Dyn A$_{1-11}$-NH$_2$	77	0.35	0.15	0.85	0.55	4.20^2	1296 [M]$^+$
7	[Cys^5,Cys^9,Arg^{10}]Dyn A$_{1-11}$-NH$_2$	77	0.45	0.15	0.85	0.65	2.60^2	1355 [M]$^+$
8	[Cys^6,Cys^{10}]Dyn A$_{1-11}$-NH$_2$	77	0.50	0.15	0.85	0.65	2.60^2	1312 [M]$^+$
9	[Cys^8,D-Cys^{13}]Dyn A$_{1-13}$-NH$_2$	32	0.35	0.15	0.85	0.55	2.59^2	1566 [M + H]$^+$
10	[D-Cys^8,D-Cys^{12}]Dyn A$_{1-13}$-NH$_2$	47	0.30	0.15	0.85	0.55	4.48^2	1582 [M + 2 H]$^+$
11	[D-Pro^{10},Cys^5,Cys^{13}]Dyn A$_{1-13}$-NH$_2$	102	0.45	0.15	0.85	0.65	3.38^2	1566 [M + H]$^+$

[a] Yields are those following methods similar to those used for 1 (see Experimental Section). [b] Solvent systems: (I) 1-butanol/pyridine/acetic acid/water (15/10/3/8, v/v/v/v), (II) 1-butanol/acetic acid/water (4/1/5, v/v/v), (III) 2-propanol/concentrated ammonium hydroxide/water (3/10/10, v/v/v), (IV) 1-butanol/pyridine/acetic acid/water (6/6/1/5, v/v/v/v). [c] Superscripts 1 and 2 refer to HPLC solvent systems and solvent programs described in the Experimental Section. [d] Fast-atom bombardment, low-resolution full scan, glycerol or DTE/DTT matrix.

Table V. Amino Acid Analysis of Synthetic Dyn A$_{1-11}$-NH$_2$ Analogs

no.	compound	amino acids[a] Pro	Gly	Ala	Cys	Ile	Leu	Tyr	Phe	Lys	Arg
1	[Phe^1,Cys^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	0.90 (1)	2.0 (2)		2.2 (2)	0.90 (1)			1.9 (2)		2.7 (3)
2	[D-Ala^2,Cys^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	0.95 (1)	0.95 (1)	0.93 (1)	2.2 (2)	0.90 (1)		0.93 (1)	1.1 (1)		3.1 (3)
3	[L-Tic^4,Cys^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	1.1 (1)	2.0 (2)		2.1 (2)	0.96 (1)		1.0 (1)			3.1 (3)
4	(L-Pen^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	1.1 (1)	2.0 (2)		ND[b]	0.96 (1)		1.1 (1)	0.92 (1)		3.1 (3)
5	[Cys^5,Cys^{10}]Dyn A$_{1-11}$-NH$_2$		2.0 (2)		2.2 (2)	0.92 (1)		0.91 (1)	0.98 (1)	0.90 (1)	2.8 (3)
6	[Cys^5,Cy^9]Dyn A$_{1-11}$-NH$_2$	1.1 (1)	2.0 (2)		1.9 (2)	0.90 (1)		1.0 (1)	1.0 (1)	0.99 (1)	2.1 (2)
7	[Cys^5,Cys^9,Arg^{10}]Dyn A$_{1-11}$-NH$_2$		2.0 (2)		1.9 (2)	1.0 (1)		1.0 (1)	1.0 (1)	0.97 (1)	3.1 (3)
8	[Cys^6,Cys^{10}]Dyn A$_{1-11}$-NH$_2$		2.0 (2)		1.9 (2)	0.92 (1)	1.0 (1)	0.92 (1)	0.95 (1)	0.92 (1)	2.1 (2)
9	[Cys^8,D-Cys^{13}]Dyn A$_{1-13}$-NH$_2$	1.0 (1)	2.2 (2)		2.2 (2)		2.1 (2)	0.99 (1)	0.91 (1)	0.90 (1)	2.9 (3)
10	[D-Cys^8,Cys^{12}]Dyn A$_{1-13}$-NH$_2$	1.1 (1)	2.2 (2)		2.0 (2)		1.1 (1)	1.0 (1)	0.90 (1)	1.8 (2)	3.0 (3)
11	[D-Pro^{10},Cys^5,Cys^{13}]Dyn A$_{1-13}$-NH$_2$	1.1 (1)	1.8 (2)		2.0 (2)	0.95 (1)	1.1 (1)	1.0 (1)	1.0 (1)	0.99 (1)	2.9 (3)

[a] Theoretical values in parentheses. Hydrolysis in 4 N methanesulfonic acid (0.2% 3-(2-aminoethyl)indole) at 110 °C for 24 h. [b] Not determined.

tested in the presence of naloxone (1000 nM) to define opioid activity. To further define the opioid selectivity of the agonist effect, the δ selective antagonist ICI 174,864[42] (1000 nM) was utilized in the MVD, and the μ selective antagonists CTAP[34] (1000 nM) or CTP[35] (1000 nM) were employed in the GPI. NorBNI[39] (1000 nM) was used as the κ-selective antagonist. At least 2 assays run in duplicate were performed for each peptide. Analog 4 was also tested as a antagonist against graded concentrations of the selective κ agonist[15,25] U69,593 in the GPI.

Radioligand Binding Assay. Membranes were prepared from whole brains taken from adult male Hartley guinea pigs (300–500 g) obtained from Harlan Sprague–Dawley Inc. (Indianapolis, IN). Following decapitation, the brain was removed, dissected, and homogenized at 0 °C in 20 volumes of 50 mM Tris-HCl buffer (Sigma, St. Louis, MO) adjusted to pH 7.4 using a Teflon–glass homogenizer. The membrane fraction obtained by centrifugation at 48000*g* for 15 min at 4 °C was resuspended in 20 volumes of fresh Tris buffer and incubated at 25 °C for 30 min to dissociate any receptor-bound endogeneous opioid peptides. The incubated homogenate was centrifuged again as described and the final pellet resuspended in 20 volumes of fresh Tris-HCl buffer adjusted to pH 7.4 using a Teflon–glass homogenizer. The membrane fraction obtained by centrifugation at 48000*g* for 15 min at 4 °C was resuspended in 20 volumes of fresh Tris buffer and incubated at 25 °C for 30 min to dissociate any receptor-bound endogenous opioid peptides. The incubated homogenate was centrifuged again as described and the final pellet resuspended in 20 volumes of fresh Tris-HCl buffer.

Radioligand binding inhibition assay samples were prepared in an assay buffer consisting of 50 mM Tris-HCl, 1.0 mg/mL bovine serum albumin, 30 μM bestatin, 50 μg/mL bacitracin, 10 μM captopril, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 (all from Sigma, St. Louis, MO, except captopril, which was a gift of Squibb, Princeton, NJ). The radioligands used were [^{3}H]D-Pen^2,D-Pen^5]enkephalin[46] at a concentration of 1.0 nM, [^{3}H]U-69,593[25] at a concentration of 0.75 nM, and [^{3}H]PL-17[47] (all radioligands from New England Nuclear, Boston, MA). Peptide analogs were dissolved in assay buffer prior to each experiment and added to duplicate assay tubes at 10 concentrations over an 800-gold range. Control (total) binding was measured in the absence of any inhibitor while nonspecific binding was measured in the presence of 10 μM naltrexone (Endo Laboratories, New York, NY). The final volume of the assay samples was 1.0 mL of which 10% consisted of the membrane preparation in 0.1 mL of Tris-HCl buffer. Incubations were preformed at 25 °C for 3 h after which the samples were filtered through polyethylenimine (0.1% w/v, Sigma, St. Louis, MO) treated GF/B glass-fiber filter strips (Brandel, Gaithersburg, MD). The filtrates were washed three times with 4.0 mL of ice-cold 100 mM KCl before transfer to scintillation vials. The filtrate radioactivity was measured after adding 10 mL of cocktail,

consisting of 16 g of CrystalFluor (West Chem, San Diego, CA) in 1.0 L of Triton X-100 and 2.0 L of toluene, to each vial and allowing the samples to equilibrate over 8 h at 4 °C.

Binding data were analyzed by weighted nonlinear regression methods using one and two independent site models as previously described.[48] Statistical comparisons between one- and two-site fits were made using the F-ratio test using a p value of 0.05 as the cutoff for significance.[49] Data best fitted by a one-site model was reanalyzed using the logistic equation.[50] Data obtained from at least three independent measurements are presented as the arithmetic mean ± SEM.[51]

Peptide Synthesis and Purification. Peptide syntheses were performed by the solid-phase method[52,53] utilizing an automated synthesizer (Applied Biosystems Inc. Model 431 A) and methods similar to those used previously in our laboratory for Dynorphin synthesis.[31] Thin-layer chromatography of synthetic peptides was performed on silica gel plates (0.25 mm, Analtech, Newark, DE) with the solvent systems given in Table V. Peptides were detected with ninhydrin reagent. p-methylbenzhydrylamine resin was purchased from Advanced Chem Tech (Louisville, KY) or US Biochemical Corp. (Cleveland, OH). N^{α}-Boc-amino acids (N^{α}-Boc-O-(2,6-dichlorobenzyl)tyrosine, N^{α}-Boc-N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)lysine, N^{α}-Boc-N^{δ}-tosylarginine) were purchased from Bachem Inc., Torrance, CA, or were synthesized by standard methods. Hydrolysis of the peptides was performed in 4 N methanesulfonic acid (0.2% 3-(2-aminoethyl)indole at 110 °C for 24 h, and amino acids were analyzed with an automatic analyzer (Model 7300, Beckman Instruments). Mass spectra (fast-atom bombardment, low-resolution full scan, glycerol or dithioerythritol/dithiothreitol matrix) were performed by the Midwest Center for Mass Spectrometry, Lincolin, NE, a National Science Foundation Regional Instrumentation Facility (Grant no. CHE 8211164), or by the Center for Mass Spectrometry, University of Arizona, Tucson, AZ. HPLC was carried out by use of a ternary pump (Spectra Physics Model 8800) equipped with a UV/vis detector (Spectra Physics 8450) and integrator (Spectra Physics 4270). For analytical HPLC the solvent system used was a binary system, water containing 0.1% TFA (pH 2.0) and acetonitrile as the organic modifier, and solvent programs involved linear gradients as follows: (1) 10% to 30% acetonitrile over 20 min with flow rate of 1.5 mL/min, (2) 0% to 50% acetonitrile over 50 min with flow rate of 1.5 mL/min, (3) 15% to 30% acetonitrile ove 15 min with flow rate of 1.5 mL/min. The column used for analytical chromatography had dimensions of 4.5 × 250 mm (Vydac, 10-μm particle size, C-18). HPLC purification on a semipreparative scale (10 mg) was performed with a reverse-phase column (Vydac, 1.0 × 25 cm, C-18, 10-μm particle size) employing the same binary solvent system used for analytical HPLC. Preparative (100 mg) low-pressure (50 psi) chromatographic purification was accomplished with a reversed-phase glass column (Bio-Rex column, 2.5 × 48 cm, Vydac C-18 resin, 20–30-μm particle size). A linear gradient of 0% to 30% acetonitrile over 3 h, involving the mentioned binary solvent system, was routinely used with a flow rate of 2 mL/min. Dyn A_{1-11}-NH_2 and Dyn A_{1-13}-NH_2 were synthesized and purified using procedures as described for Dyn A_{1-11}-NH_2; Dyn A_{1-17} was a gift from Vega Biochemicals.

Protected Dyn A_{1-11}-NH_2-resin. p-Methylbenzhydrylamine resin (1.06g, 0.5 mequiv) was coupled with N^{α}-Boc-N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)lysine via its 1-hydroxybenzotriazole (HOBt) active ester.[38] N^{α}-Boc-amino acids (4 mequiv) were added to the reaction mixture as performed HOBt activated esters. DMF or N-methyl-2-pyrrolidinone was used as solvent, and the coupling reaction times were normally 30–60 min. Diisopropylethylamine was utilized as base and dichloromethane or DMF was used as solvents for washes. Side-chain protection was as follows: Lys, 2,4-dichlorobenzyloxycarbonyl; Arg, tosyl; Tyr, 2,6-dichlorobenzyl; and Cys, p-methylbenzyl. After deprotection of the last N^{α}-Boc group with 50% trifluoroacetic acid (TFA) in dichloromethane, the peptide-resin was dried in vacuo to yield the protected Dyn A_{1-11}-NH_2-resins.

Dyn A_{1-11}-NH_2. The protected peptide-resin was treated with liquid anhydrous hydrofluoric acid (HF) in the presence of anisole (10%, v/v) for 1 h at −10 to 0 °C. After removal of HF in vacuo at 0 °C, the residue was washed three times with ether and extracted with aqueous 6% acetic acid three times, followed by extraction with glacial acetic acid. The acetic acid solution was lyophilized to give a yellow solid (0.80 g) which was dissolved in 30% acetic acid and subjected to gel filtration (Sephadex G-15) with 30% acetic acid as eluant. A cream powder (0.77g) was obtained after gel filtration. This product was subjected to preparative HPLC under the mentioned conditions to yield a white powder (424 mg, 44%) after lyophilization. The structure assignment was corroborated by the results of the amino acid analysis and mass spectrometry, and the purity of the product was characterized by analytical HPLC and TLC (Tables IV and V).

[Phe^1,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 (1). The crude deprotected peptide was obtained as a white powder (530 mg) by the use of procedures as described for Dyn A_{1-11}-NH_2. After dissolving the crude product in degassed aqueous 0.1% acetic acid, the solution was diluted to a volume of 2 L with degassed deionized distilled water. The pH was adjusted to 8.5 by the addition of 3 N ammonium hydroxide, and 0.01 N $K_2Ke(CN)_6$ (20 mL) was added dropwise to a yellow endpoint. Additional 0.1 N $K_3Fe(CN)_6$ (19 mL) was added and the mixture was stirred at room temperature for 60 min, after which time the solution remained a yellow color. The pH was adjusted to 4 by the addition of 30% acetic acid and anion-exchange resin (Amberlite IRA-68, chloride form) was added to the solution. After stirring of the mixture for 60 min, the solution was colorless. The resin was filtered and washed three times with 30% acetic acid, and the solvent was evaporated at 40–45 °C to a volume of about 100 mL. Lyophilization of this solution yielded a pale-green powder, which was subjected to preparative HPLC as described to give a white powder (32 mg). The purity of the final product was verified by TLC and analytical HPLC (Table IV), and the structure assignment was corroborated by the amino acid analysis (Table V) and mass spectrum (FAB) (Table IV) results.

Synthesis of Compounds 2–11. Compounds 2–11 (See Table I for structures) were synthesized and purified by a method similar to those employed for compound 1. All were obtained as white powders of high purity (>97%) in yields ranging from 17 to 102 mg (see Table IV).

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References

(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977–983). All optically active amino acids are of the L variety unless otherwise noted. All optically active amino acids are of the L variety unless otherwise noted. Other abbreviations used are Pen, penicillamine; CTP, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; GPI, guinea pig ileum; MVD, mouse vas deferens; GPB, guinea pig brain; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

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