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Synthesis and Biological Activity of CCK Heptapeptide Analogues. Effects of Conformational Constraints and Standard Modifications on Receptor Subtype Selectivity, Functional Activity in Vitro, and Appetite Suppression in Vivo†

Mark W. Holladay,* Michael J. Bennett, Michael D. Tufano, C. W. Lin, Karen E. Asin, David G. Witte, Thomas R. Miller, Bruce R. Bianchi, A. L. Nikkel, Lisa Bednarz, and Alex M. Nadzan

Neuroscience Research, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, Illinois 60064.
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A series of modifications of the CCK_7 analogue (des-NH_2)Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 was prepared and tested for binding to guinea pig CCK-A and CCK-B receptors and in CCK-A-mediated functional assays. Selected analogues also were tested for appetite suppressant activity in rats. Several conformationally restricted residues in the C-terminal tetrapeptide region, including Δ^Z-Phe^{33}, (*N*-Me)Phe^{33}, (*N*-Me)Asp^{32}, (*N*-Me)Leu^{31}, and 3PP^{31} (3PP = *trans*-3-*n*-propyl-L-proline) were found to be acceptable modifications at one or both receptor subtypes. The (*N*-Me)Asp^{32} and (*N*-Me)Leu^{31} modifications afforded potent and selective CCK-A and CCK-B ligands, respectively. SAR studies in the N-terminal acyldipeptide region examined structural requirements for the side chain at position 28, where Gly and Pro replacements were found to possess high affinity at both receptor subtypes. Other conformationally restrictive modifications were less active. All of the analogues that showed high affinity (<10 nM) for the CCK-A receptor also were full agonists in amylase release and most were full or nearly full agonists in the phosphoinositide (PI) turnover assay, the most notable exception being the Δ^Z-Phe^{33} analogue, which showed 69% of the maximal response in the PI assay. Potent activity in suppression of food intake in rats was found for selected analogues.

Cholecystokinin (CCK) is a 33-amino acid peptide that functions as a digestive hormone in the periphery and as a neurotransmitter in the central nervous system (CNS).[1,2] Two subtypes of CCK receptor have been identified.[3] CCK-A receptors, found in the periphery and in discrete regions of the CNS, mediate gall bladder contraction and pancreatic enzyme release,[4,5] and appear to be principally responsible for the satiety actions of peripherally administered CCK.[6] CCK-B receptors are widely distributed in the brain and show a pharmacological profile similar to that of gastrin receptors.[7,8] CCK-B agonists have been shown to cause panic attack in man[9] and CCK-B antagonists possess anxiolytic properties in animal models.[10]

The C-terminal octapeptide CCK_8 (CCK-27-33, H-Asp-Tyr(SO_3^-)-Met-Gly-Trp-Met-Asp-Phe-NH_2)[11] is the minimum naturally occurring fragment that retains the full potency and complete spectrum of CCK activities, and also is the predominant form found in the brain.[1,2] Numerous structure–activity studies starting with conservatively altered derivatives of CCK_8, e.g. Boc-[Nle^{28,31}]-CCK_7, have examined the effects of side chain and backbone modifications on binding to CCK-A and CCK-B receptors.[12,13] Although studies in sulfated series have yielded mostly

* Address correspondence to Mark W. Holladay, Dept. 47U, AP-10-3, Abbott Park, IL 60064.

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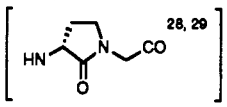
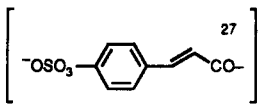
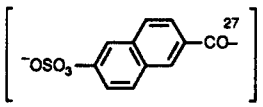
Table I. Binding Affinities and Functional Activities in Vitro for Analogues of Compound 1

(des-NH_2)Tyr(SO_3^-)^{27}-Nle^{28}-Gly^{29}-Trp^{30}-Nle^{31}-Asp^{32}-Phe^{33}-NH_2 (1)

compound	receptor binding, IC_{50} (nM)[a] pancreas	cortex	pancreas/cortex	amylase release: EC_{50} (nM)[a]	PI hydrolysis % maximal response[a,d]
1	0.77 ± 0.05 (3)	0.5 ± 0.1 (3)	1.53	0.99 ± 0.17 (3)	100 ± 6.9 (3)
(2) [NMePhe^{33}]	0.99 ± 0.28 (4)	10.0 ± 7.6 (3)	0.10	0.32 ± 0.09 (3)	94 ± 4 (3)
(3) [Δ^Z-Phe^{33}]	5.3 ± 0.7 (3)	8.7 ± 0.1 (3)	0.62	2.5 ± 0.38 (6)	69 (2)
(4) [NMeAsp^{32}]	0.50 ± 0.04 (9)	570 ± 90 (10)	0.0009	0.10 ± 0.04 (4)	100 ± 4.7 (3)
(5) [Hyp(SO_3^-)^{32}]	16.0 ● 4.4 (3)	140 ± 27 (3)	0.03	6.9 ● 0.5 (3)	82 ± 2.8 (3)
(6) [Leu^{31}]	4.7 ± 1.0 (3)	1.4 ± 0.2 (3)	3.3	3.7 ± 0.17 (3)	100 ± 4.7 (3)
(7) [NMeLeu^{31}]	140 ± 15 (3)	0.24 ± 0.09 (4)	625	15.0 ± 5.8 (4)	80 ± 2.5 (3)
(8) [Pro^{31},NMePhe^{33}]	81 ± 17 (5)	16 ± 5.2 (3)	5.1	98% (3 μM)[c]	64 ± 11 (3)
(9) [3PP^{31}, NMePhe^{33}][b]	4.2 ± 0.9 (5)	0.30 ● 0.04 (7)	14.3	1.3 ● 0.18 (3)	83 ± 3.9 (3)
(10) [D-Ala^{29}]	81 ± 10 (3)	24 ● 6.8 (3)	3.3	50 (1)	68 (1)
(11) [NH-$(CH_2)_4$-CO^{28,29}, Leu^{31}]	150 ± 57 (3)	1.1 ± 0.1 (3)	143	140 (1)	64 (1)
(12) [Gly^{28}]	5.8 ● 0.7 (3)	3.4 ± 0.9 (3)	0.6	3.7 ● 0.39 (3)	100 ± 8.1 (3)
(13) [Leu^{28}]	0.67 ± 0.22 (3)	0.59 ● 0.15 (3)	1.1	0.64 ± 0.06 (3)	120 ± 6.0 (3)
(14) [D-Nle^{28}]	150 ● 18 (3)	7.9 ● 1.1 (3)	18.5	ND	52 (1)
	130 ● 8.0 (3)	15 ± 6.2 (3)	9.0	71 (1)	86 (1)
(16) [Pro^{28}]	2.1 ± 0.7 (3)	3.1 ● 0.9 (3)	1.5	0.7 ± 0.11 (3)	100 ± 4.5 (3)
(17) [Pro^{28}, 3PP^{31}, NMePhe^{33}][b]	7.3 ± 1.5 (3)	0.9 ± 0.3 (3)	8.3	83% (5 nM)[c]	82 ● 6.2 (3)
	23 ± 6 (3)	7.7 ± 3.2 (3)	0.33	105% (20 nM)[c]	98 ± 3.1 (3)
	900 ± 70 (3)	32 ● 5.6 (3)	0.04	44% (10 μM)[c]	16 ● 6.9 (3)

[a] No. of experiments indicated in parentheses; ND, not determined. [b] 3PP = *trans*-3-*n*-propyl-L-proline. [c] EC_{50} not determined; data show % stimulation relative to CCK_8 at indicated dose. [d] Indicates % maximal response of peptide (10^{-4} M) relative to maximal response elicited by CCK_8.

nonselective analogues, certain cyclic structures[14,15] and acyclic derivatives containing N-methylation at position 31[16,17] recently have been disclosed as highly potent and selective CCK-B ligands. Analogues with high affinity and selectivity for CCK-B receptors also have been identified from structure–activity relationship (SAR) studies on nonsulfated derivatives, e.g. CCK_4 and desulfated CCK-8, that already show moderate nanomolar affinity and modest selectivity for CCK-B receptors.[18,19] Meanwhile, impressive progress has been made in the development of potent and selective antagonists at both receptor subtypes.[10,20]

In relation to our ongoing studies on CCK, and specifically as part of our program directed toward CCK analogues as anorectic agents, we undertook structure–activity studies on a series of sulfated CCK_7 analogues. Our studies followed those of Rosamond et al.,[21] who had demonstrated

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Table II. Physical Properties of Sulfated Peptides

compd	amino acid analysis						MS[a]		HPLC[b]
	Nle	Gly	Trp	Asp	Phe	other	m/e	adduct	t_R (min)
1	2.1	0.9	1.0	1.0	1.0	-	1021[c]	$(M + Na)^+$	11.8
2	2.0	1.0	0.8	1.0	-	(N-Me)Phe, 0.9	989	$(M - H)^-$	12.1
							1011	$(M + Na - 2 H)^-$	
3	1.9	1.0	0.7	1.0	-	-	973	$(M - H)^-$	12.2
4	2.1	1.0	d	-	1.0	-	989	$(M - H)^-$	11.8
5	2.2	1.0	0.7	-	1.0	Hyp, 0.9	1075	$(M + Na - 2 H)^-$	9.3
6	1.1	0.9	1.0	0.9	1.0	Leu, 1.1	975	$(M - H)^-$	11.7
7	1.1	1.0	0.7	1.0	1.0	-	989	$(M - H)^-$	12.1
8	1.0	1.0	0.6	1.0	-	(N-Me)Phe, 0.9	973	$(M - H)^-$	10.0
						Pro, 1.0	995	$(M + Na - 2 H)^-$	
9	1.0	1.0	0.8	1.0	-	(N-Me)Phe, 0.9	1015	$(M - H)^-$	12.2
10	2.0	-	d	1.0	0.9	Ala, 1.1	989	$(M - H)^-$	12.1
11	-	-	0.8	1.0	1.0	Leu, 1.0	906[c]	$(M + H)^+$	10.1
12	1.0	2.2	0.9	0.9	1.0	-	919	$(M - H)^-$	9.6
							941	$(M + Na - 2 H)^-$	
13	1.0	0.9	0.7	1.1	0.9	Leu, 1.0	1021[c]	$(M + 2 Na - H)^+$	11.7
14	2.2	0.9	0.7	1.1	1.0	-	1021[c]	$(M + 2 Na - H)^+$	11.8
15	1.0	-	1.1	1.0	1.0	lactam, 1.0	947[c]	$(M + H)^+$	9.5
16	1.0	1.0	0.8	1.0	1.0	Pro, 0.9	961[c]	$(M + H)^+$	10.1
17	-	1.0	1.1	1.0	-	Pro, 1.0	1001[c]	$(M + H)^+$	10.8
						(N-Me)Phe, 0.8			
						3PP, 1.1			
18	2.1	1.0	d	1.0	1.0	-	973	$(M - H)^-$	12.1
19	2.1	1.0	d	0.9	1.0	-	997	$(M - H)^-$	12.7

[a] FAB^- unless otherwise specified. [b] Vydac C_{18}, 0.5 × 24 cm; flow rate 1 mL/min; 20% to 60% CH_3CN/0.1% aqueous TFA over 15 min. [c] FAB^+. [d] Trp present, but recovery low.

that (des-NH_2)Tyr(SO_3^-)-Met-Gly-Trp-Met-Asp-(*N*-Me)-Phe-NH_2, a relatively nonselective analogue, was potent in the suppression of food intake in rats. We chose the closely related bis-Nle analogue, (des-NH_2)Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 (1) as the starting point for our studies. Important objectives were to explore certain side chain and backbone modifications for effects on receptor affinity and subtype selectivity, and secondarily to determine whether any relationships existed between the SAR of Boc-CCK_4 analogues and their sulfated heptapeptide analogue counterparts. Also, SAR studies on the N-terminal acyldipeptide region were carried out in order to explore the possibility of eventually designing nonpeptide mimics for this portion of the molecule. These studies have resulted in the preparation of a series of analogues (Table I), including the potent and selective CCK-A agonist, A-71378 (compound 4) and the potent and selective CCK-B agonist A-72962 (compound 7). A preliminary account of these studies,[22] as well as the results of detailed pharmacological evaluations of 4 and 7[17] have been reported. Here we provide experimental details and additional in vitro and in vivo data related to these studies.

Chemistry

Compounds 1–19 (Table I) were assembled from appropriately protected forms of the constituent amino acids and 3-(4-hydroxyphenyl)propionic acid using conventional peptide synthesis techniques. Most of the syntheses were carried out in the solution phase, although standard solid-phase techniques were applied in a few cases. Fmoc-(*N*-Me)Asp(OBn)-OH was synthesized from the unmethylated precursor using the procedure developed by Freidinger[23] and either used as such or converted to the corresponding Boc derivative. Protected derivatives of the nonstandard amino acids found in analogues 3,[24] 9,[25,26] and 15[27] were prepared by published procedures. For preparation of compounds 18 and 19, commercially available *p*-hydroxycinnamic acid and 6-hydroxynaphthylene-2-carboxylic acid were converted to the corresponding *N*-hydroxysuccinimide esters prior to coupling to the C-terminal hexapeptide fragment. The assignment of *E*-geometry about the double bond of the cinnamoyl moiety of 18 rests on the coupling constant for the olefinic protons (15 Hz) in the ^{1}H-NMR of both the precursor active ester and the final product. Sulfations of assembled acylhexapeptides were accomplished using either pyridine–sulfur trioxide complex[28] or pyridinium acetyl sulfate[29] according

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to established procedures. Physical data for 1–19 are collected in Table II.

Results and Discussion

Table I shows biological data for compounds 1–19, which include binding affinities at both receptor subtypes and two types of functional data at CCK-A receptors. Functional activity at CCK-B/gastrin receptors for selected compounds in this group and a comparison of these analogues with Boc-CCK_7 and CCK_8 in standard assays for CCK activity have been reported elsewhere.[17] In this report, SAR with respect to binding to both receptor subtypes will be discussed first, followed by a discussion of the CCK-A functional data.

Compounds 2–9 incorporate modifications of the C-terminal tetrapeptide region of the molecule. Among these, 6 represents the conservative change of Nle to Leu at position 31, and is included to simplify interpretation of the results for 7 and 11, which also contain the *i*-Bu side chain at position 31. The other analogues in this group contain conformationally restricted residues in the C-terminal region, including *N*-methyl modifications at positions 31–33.

Although (*N*-Me)Phe had been shown to be a favorable modification in the related [$Met^{28,31}$] series with respect both to stability against enzymatic degradation and to potency in gall bladder contraction and appetite suppression,[21] the effect of this modification on binding to CCK receptors was not reported. Our results (cf. 2 vs 1) indicate that whereas CCK-A binding is unaffected by N-methylation at Phe^{33}, CCK-B binding affinity is diminished by a factor of 20, so that modest selectivity for CCK-A receptors results. It is noted, however, that in a related bis-sulfated octapeptide series, the corresponding (*N*-Me)Ph^{33} analogue possessed equal affinity for CCK-A vs CCK-B receptors.[30]

Compound 3 was prepared as an extension of a corresponding Boc-CCK_4 analogue, for which it was found that replacement of Phe with Δ^Z-Phe in position 33 had afforded an analogue with undiminished affinity for the CCK-B receptor.[19] Analogue 3 possesses ca. 10-fold weaker affinity than 1 for both receptor subtypes; nevertheless, the affinity for both receptor subtypes is still in the low nanomolar range, and this result is viewed as providing potentially valuable information for modeling studies.

In the course of our earlier work on analogues of CCK_4, Boc-Trp-Nle-(*N*-Me)Asp-Phe-NH_2 was synthesized and found to have weak affinity for both CCK-A (1600 nM) and CCK-B (>10 000 nM) receptors.[31] Comparison of these data with those of the parent unmethylated analogue revealed that while N-methylation on the Asp residue caused a drastic decrease in affinity for CCK-B receptors, CCK-A binding was improved by 2 to 3-fold. This observation led us to prepare the sulfated analogue 4. After detailed pharmacological evaluation,[17] 4 (A-71378) appears to be the most potent CCK-A selective agonist reported to date. It is clear that the effect of the *N*-methyl group is to disfavor interaction with the CCK-B receptor while allowing high affinity interaction with the CCK-A receptor to be preserved. Possible rationales for the observed selectivity have been discussed.[12] In further work that will be described in subsequent reports, incorporation of the (*N*-Me)Asp^{32} modification into the novel series of lysine-based tetrapeptide CCK-A agonists recently discovered at Abbott[32] affords analogues with favorable properties as CCK-A agonists.[33]

Hyp(SO_3^-) had been previously described as a replacement for Asp^{32} in a series of CCK heptapeptide analogues and noted to show selectivity for cholecystokinetic (gall bladder contraction) vs gastrin activity,[29,34] but no binding data were reported. In the present series, this modification (compound 5) resulted in a 20-fold loss of affinity for CCK-A receptors, and a nearly 300-fold loss of affinity for CCK-B receptors; thus, 5 exhibits moderate affinity and ca. 30-fold selectivity for CCK-A receptors.

Analogue 7 incorporates the (*N*-Me)Leu^{31} modification, which affords a potent and selective CCK-B ligand. Although N-methylation at position 31 has been used as a key component of other selective CCK-B ligands,[16,18,35] it is interesting to note that simple N-methylation at position 31 of [$Nle^{28,31}$]-CCK_8 affords a poorly selective analogue.[18] Thus, N-methylation in combination with some other modification, in this case a change to the *i*-Bu side chain at position 31, is necessary for high CCK-B selectivity. Compound 7 has been shown to be an agonist in the stimulation of calcium release in a human cancer cell line, an event which is mediated by CCK-B/gastrin receptors.[17]

trans-3-*n*-Propyl-L-proline (3PP), which was found to afford a potent CCK-B selective analogue when incorporated at position 31 of the Boc-tetrapeptide series,[25] was incorporated into the present series in combination with the (*N*-Me)Phe^{33} modification to provide 9, which possesses high affinity and ca. 10-fold selectivity for the CCK-B receptor. By comparison with compound 8, it is seen that the *n*-propyl group of 3PP contributes ca. 50-fold to affinity for CCK-B receptors and 20-fold to affinity for CCK-A receptors. This contrasts to the ca. 400-fold contribution to CCK-B binding and negligible contribution to CCK-A binding by the *n*-propyl group in the Boc-tetrapeptide series.[25] In DMSO solution, 8 and 9 exist as multiple conformers corresponding to restricted rotation about both tertiary amide bonds.

Analogues 10–19 represent changes in the N-terminal acyldipeptide region. The results with D-Ala^{29} (compound 10) are in accord with those reported by others,[36] and show that this modification causes a ca. 100-fold loss of affinity

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(33) Holladay, M. W.; Bennett, M. J. Unpublished data.

(34) In ref 29 the text indicates that 3-hydroxyproline was incorporated into the peptide; personal communication with one of the authors (J. Rivier) indicated that *trans*-4-hydroxy-L-proline was in fact utilized in that study. In the present work, Hyp refers to *trans*-4-hydroxy-L-proline.

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to the CCK-A receptor and a 50-fold loss of affinity to the CCK-B receptor. Compound 11 represents the deletion of the position 28 side chain as well as the replacement of the 28,29 peptide bond with ethylene. This analogue proved to be equipotent to its parent (6) in binding to the CCK-B receptor, but possessed 30-fold lower affinity than 6 for the CCK-A receptor. Simple deletion of the position 28 side chain, as in the Gly^{28}-Gly^{29} analogue 12, resulted in ca. 8-fold lower affinity relative to the parent 1 at both receptor subtypes. Mendre et al.[37] have studied a series of isosteric peptide bond replacements at the 28,29 position in a CCK heptapeptide series, including the $Nle^{28}\Psi$-$[CH_2CH_2]Gly^{29}$ analogue. This compound exhibited affinity to both receptor subtypes nearly equivalent to that of the all-amide parent. Thus, while the CCK-B receptor is highly tolerant even to multiple changes in this region, the CCK-A receptor is slightly less tolerant to either modification of the 28,29 amide bond or to deletion of the position 28 side chain, and considerably less tolerant to both changes simultaneously.

The binding results for the Leu^{28} and D-Nle^{28} analogues 13 and 14, respectively, are similar to those found for similar modifications in a caerulein-based series.[38] Thus, whereas binding affinity to both receptor subtypes is unaffected by the Leu^{28} modification, D-amino acids at this position cause at least a 10-fold decrease in affinity to the CCK-B receptor, and a >100-fold decrease at the CCK-A receptor. A similar result is found for the conformationally restricted lactam 15, which has stereochemistry corresponding to a D-amino acid at position 28. In contrast, the conformationally restricted L-Pro residue at position 28 (compound 16) is well tolerated at both receptor subtypes. Compound 17 was prepared as a multiply constrained analogue for which it was hoped that a single preferred conformation would exist in solution. Although it proved to show good activity at both receptor subtypes consistent with the activities of the individual modifications, at least three major conformations were present in all of a variety of solvent conditions as evidenced by ^1H NMR. Finally, it was hoped that an appropriately constrained (des-NH_2)$Tyr(SO_3^-)$ residue, such as that found in 18 or 19, would lead to compounds with improved potency or selectivity. However, both 18 and 19 possess considerably lower affinity than the parent to both receptor subtypes.

In Vitro Functional Data

With the exception of compounds 14 and 19, which had weak affinity for CCK-A receptors, the compounds in Table I were shown to possess maximal stimulatory activity in amylase release relative to CCK_8. EC_{50} values (or in a few cases, percent stimulation at an appropriate concentration) for these analogues are shown in Table I. In general, the potency of analogues in stimulating amylase secretion agreed with their potency in pancreatic binding assays. Table I also shows the maximal stimulation of PI hydrolysis that can be elicited by high concentrations of analogues 1–19. Most analogues with high affinity for CCK-A receptors also elicited >95% of the maximal PI response (1, 2, 4, 6, 12, 13, 16). Analogue 3, on the other hand, had affinity in the low nanomolar range, but elicited only 69% of the maximal PI response, while analogues 5, 9, and 17 also possessed high affinity but still elicited <90% of the maximal PI response. Since the probable mechanism for elicitation of enzyme secretion involves amplification of the signal from an earlier PI hydrolysis event,[4,39] the PI turnover assay would be expected to be more stringent with respect to agonist efficacy. The data in Table I are consistent with this interpretation.

Table III. Activity of Selected Analogues in Suppression of Food Intake in Rat

compd	0–60 min ED_{50} (nmol/kg)	360–420 min % suppression at 30 nmol/kg
1	3.98 ● 1.22	NS[a]
2	2.45 ± 0.73	30% ± 6%
4	0.71 ● 0.29	29% ± 3%
5	>1000	NS
9	8.77 ± 2.55	NS

[a] NS = no significant suppression.

Reduced amylase secretory activity at supramaximal concentration has been observed for many sulfated CCK analogues,[40] and was found in the present study for analogues 1, 4, 6, 12, 13, 16. All of these compounds elicited >80% of the maximal PI response in guinea pig tissue, whereas compounds 3 and 7, which stimulated 69% and 80% of the maximal PI response, respectively, did not reduce secretion at supramaximal concentration. This behavior is consistent with the results found in other series of CCK-A agonists.[32]

Appetite Suppression Studies in Vivo

Compounds 1, 2, 4, 5, and 9 were evaluated for their ability to suppress food intake in a food-deprived rat paradigm. The results for the period immediately following injection of test compounds (Table III, column 1) show that all of the above analogues except the $Hyp(SO_3^-)$ analogue 5 are potent in the suppression of food intake. The high level of activity for the CCK-A selective compound 4 is consistent with the hypothesis that appetite suppression by systemically administered CCK agents is mediated by CCK-A receptors. Compounds 1 and 2 are $[Nle^{28,31}]$ analogues of corresponding $[Met^{28,31}]$ derivatives studied by Rosamond et al.[21] In that study, N-methylation of Phe^{33} was shown to provide an analogue with increased stability to tissue homogenates and enhanced potency in gall bladder contraction, while anorectic potency after 0.5 and 3 h was similar to the unmethylated analogue. In our study, both $[(N\text{-Me})Phe^{33}]$ analogue 2 and the $[(N\text{-Me})\text{-}Asp^{32}]$ analogue 4, but not 1, 5, or 9, caused a statistically significant suppression of food intake during the afternoon feeding period. On the other hand, preliminary in vitro metabolism studies on analogues 4 and 9 indicate that 9 is considerably more stable than 4 to both kidney and liver homogenates. Thus, duration of action does not appear to correlate strictly with stability in these in vitro preparations.

Compound 5 shows poor in vivo activity despite its relatively high affinity and efficacy at CCK-A receptors.

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This result could in principle be attributable to one or a combination of several factors, including failure to achieve sufficient receptor activation as a result of the slightly lower affinity and intrinsic activity; pharmacokinetic/pharmacodynamic considerations; or differences between pancreatic receptors and those responsible for suppression of food intake. Presently, the extent to which any of these factors is operative remains to be established.

In summary, we have prepared a series of sulfated acylhexapeptide analogues of CCK that contain both standard amino acid as well as conformationally restricting residues. Analogues that are potent and selective for either the CCK-A or CCK-B receptor were found, and additional data on structure–activity relationships for the acyldipeptide region have been presented. Also, the relationship of in vivo activity to several in vitro parameters has been discussed.

Experimental Section

Methylene chloride, DMF, and pyridine were stored over 4A molecular sieves prior to use; other solvents and reagents were reagent grade and used as purchased unless otherwise indicated. Standard workup of solution-phase reactions involved dilution of the reaction mixture with EtOAc and successive washings with saturated aqueous $KHSO_4$, H_2O, saturated aqueous $NaHCO_3$, and brine, followed by drying of the organic phase over Na_2SO_4 or $MgSO_4$, filtration, and evaporation of the volatile components on a rotary evaporator. Thin-layer chromatography, flash column chromatography, NMR, MS, and elemental analyses were carried out under conditions previously specified.[26] Analytical HPLC was carried out on a Beckman instrument with effluent monitored at 254 and 280 nm and other conditions as specified subsequently. Preparative reverse-phase HPLC was carried out on either a Beckman or Gilson instrument using either a Vydac C_{18} (22 × 250 mm) or a Dynamax C_{18} (21.4 × 250 mm, plus 50-mm guard column) using mixtures of CH_3CN and 50 mM NH_4OAc, pH 4.5, as the mobile phase at a flow rate of 15 mL/min, with effluent monitored at 280 nm. Amino acid analyses (AAA) were carried out by hydrolysis (6 N HCl, 150 °C, 2 h), followed by analysis on a Beckman Model 6300 amino acid analyzer; in some cases, 5% mercaptoacetic acid was included during the hydrolysis step to improve recovery of tryptophan. Combustion analyses for selected intermediates are in agreement with theoretical values ±0.4% for the elements shown unless otherwise indicated. Final products were purified to >95% homogeneity as determined by analytical HPLC. The following procedures for synthesis of selected analogues are representative of the methods used for the synthesis of the remaining compounds.

General Synthetic Procedures. A. Mixed Anhydride Couplings. A solution of the acid component (1 mmol) and *N*-methylmorpholine (NMM) (1 mmol) in THF or other specified solvent (2–4 mL) is cooled in a dry ice/CCl_4 bath and treated with dropwise addition of isobutyl chloroformate (0.98 mmol). After 3–6 min, a chilled (−10 to 0 °C) solution of amine salt (1 mmol) in DMF (2–4 mL) is added, followed by tertiary amine base [NMM, NEt_3 or *N,N*-diisopropylethylamine (DIEA), 1–1.5 mmol]. The mixture is allowed to warm to ambient temperature and stir overnight, or until complete by TLC (if necessary, additional base is added), and then worked up as specified.

B. Carbodiimide-Mediated Couplings. A solution of the acid component (1 mmol), the amine (1 mmol) [or amine salt (1 mmol) plus tertiary amine base (1 mmol)], and 1-hydroxybenzotriazole hydrate (1 mmol) in DMF (2–4 mL) at 0 °C is treated with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) (1.1 mmol), and the solution is allowed to warm slowly to ambient temperature and stir overnight or until complete by TLC, and then worked up as specified.

C. Formation of Symmetrical Anhydrides. A solution of the acid component (2 mmol) in CH_2Cl_2 (10–20 mL) at 0 °C is treated with EDC (1 mmol). After stirring for 0.5–1 h, the solution is used directly in the coupling step.

Boc-Trp-Nle-Asp-Phe-NH_2 (20). A stirred solution of the trifluoroacetate salt of H-Nle-Asp-Phe-NH_2[28] (5.8 g, 11.4 mmol) in DMF at 0 °C was treated with DIEA (4.0 mL, 22.8 mmol) and Boc-Trp-OSuccinimidyl (4.6 g, 11.4 mmol), and the mixture was allowed to warm to ambient temperature overnight. Addition of H_2O and HOAc precipitated the crude product (8.8 g), which was recrystallized from EtOH/H_2O to afford 5.4 g (70%) of the title compound as colorless needles; mp 210–211 °C (lit.[41] 217–218 °C); MS (FAB$^+$) *m/e* 679 (M + H)$^+$; NMR (300 MHz, DMSO-d_6) δ 0.80 (m, 3 H), 1.05–1.23 (m, 4 H), 1.29 (s, 9 H), 1.35–1.61 (m, 2 H), 2.45 (1 H, obscured), 2.65 (dd, J = 7, 17 Hz, 1 H), 2.78–2.94 (m, 2 H), 2.95–3.10 (m, 2 H), 4.20 (m, 2 H), 4.32 (m, 1 H), 4.50 (m, 1 H), 6.32 (m, 1 H), 6.86 (d, J = 8 Hz, 1 H), 6.93 (t, J = 8 Hz, 1 H), 7.03 (t, J = 8 Hz, 1 H), 7.09–7.31 (m, 8 H), 7.56 (d, J = 8 Hz, 1 H), 7.81 (d, J = 8 Hz, 1 H), 7.88 (d, J = 8 Hz, 1 H), 8.24 (d, J = 8 Hz, 1 H), 10.75 (s, 1 H).

H-Trp-Nle-Asp-Phe-NH_2 Trifluoroacetate (21). Compound **20** (5.0 g, 7.4 mmol) was treated with a solution comprised of CH_2Cl_2 (100 mL), TFA (100 mL), dimethyl phosphite (4 mL), and anisole (5 mL). After standing for 1 h at ambient temperature, the mixture was concentrated and the residue treated with anhydrous Et_2O to precipitate the product, which was collected and washed with fresh Et_2O to afford the title compound (5.1 g, 100%) as a light tan solid: MS (CI/NH_3) *m/e* 579 (M + H)$^+$; NMR (300 MHz, DMSO-d_6) δ 0.81 (t, J = 6 Hz, 3 H), 1.12–1.30 (m, 4 H), 1.40–1.60 (m, 2 H), 2.45 (1 H, obscured), 2.68 (dd, J = 6, 17 Hz, 1 H), 2.81 (dd, J = 9, 14 Hz, 1 H), 2.99 (m, 2 H), 3.19 (dd, J = 5, 15 Hz, 1 H), 4.11 (m, 1 H), 4.25–4.40 (m, 2 H), 4.53 (m, 1 H), 6.99 (t, J = 7 Hz, 1 H), 7.08 (t, J = 7 Hz, 1 H), 7.10–7.25 (m, 7 H), 7.29 (s, 1 H), 7.35 (d, J = 8 Hz, 1 H), 7.69 (d, J = 8 Hz, 1 H), 7.88 (d, J = 8 Hz, 1 H), 8.36 (d, J = 8 Hz, 1 H), 8.71 (d, J = 8 Hz, 1 H), 10.96 (s, 1 H).

Boc-Nle-Gly-OBn (22). Boc-Nle-OH (3.34 g, 14.4 mmol) was coupled to TsOH·H-Gly-OBn (4.86 g, 14.4 mmol) using general procedure A with NMM as base. After 45 min, TLC analysis indicated consumption of starting materials. The mixture was diluted with EtOAc, subjected to acid–base workup, dried (Na_2SO_4), and concentrated to give an oil that was crystallized from Et_2O/hexane to afford 3.50 g (64%) as a white solid: mp 86–87 °C; MS (FAB$^+$) *m/e* 379 (M + H)$^+$; NMR (300 MHz, DMSO-d_6) δ 0.81 (t, J = 7 Hz, 3 H), 1.24 (m, 4 H), 1.38 (s, 9 H), 1.46 (m, 1 H), 1.59 (m, 1 H), 3.79–4.00 (m, 3 H), 5.11 (s, 2 H), 6.86 (d, J = 8 Hz, 1 H), 7.36 (m, 5 H), 8.28 (t, J = 6 Hz, 1 H).

(Des-NH_2)Tyr-Nle-Gly-OH (23). Compound **22** (11.38 g, 30 mmol) was treated with 1.4 N HCl/HOAc (31 mL) for 3 h, then anhydrous ether was added, and the precipitate was collected by filtration to afford the 9.22 g of the dipeptide hydrochloride, which was coupled to 3-(4-hydroxyphenyl)propionic acid (5.0 g, 30 mmol) according to general procedure A with NMM as base. The reaction mixture was diluted with EtOAc, washed successively with dilute aqueous acid and H_2O, then dried ($MgSO_4$), and concentrated. Chromatography over silica gel ($CHCl_3$/hexane/MeOH/concentrated NH_4OH, 50:50:10:0.1) afforded 8.92 g (70%) of the product as a white solid, which was dissolved in MeOH and shaken in a Parr apparatus under 4 atm H_2 in the presence of 10% Pd/C. When H_2 uptake ceased, the mixture was filtered through Celite and concentrated to afford 6.91 g (95%) of the title compound as white foam: MS (CI/NH_3) *m/e* 337 (M + H)$^+$, 354 (M + NH_4)$^+$; NMR (300 MHz, DMSO-d_6) δ 0.80 (m, 3 H), 1.09–1.29 (m, 4 H), 1.40 (m, 1 H), 1.58 (m, 1 H), 2.32 (m, 2 H), 2.65 (m, 2 H), 3.70 (m, 2 H), 4.22 (m, 1 H), 6.61 (d, J = 7.5 Hz, 2 H), 6.95 (d, J = 7.5 Hz, 2 H), 7.90 (d, J = 9 Hz, 1 H), 8.11 (t, J = 5 Hz, 1 H), 9.10 (br s, 1 H). Anal. ($C_{17}H_{24}N_2O_5$·0.25H_2O) C, H, N.

(Des-NH_2)Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 (24). Acid **23** (146 mg, 0.43 mmol) in 2 mL of THF and 0.5 mL of DMF was coupled to tetrapeptide **21** (300 mg, 0.43 mmol) according to general procedure A with NEt_3 as base. The mixture was diluted with H_2O and acidified with HOAc to afford a solid precipitate, which was collected by filtration and dried under reduced pressure to yield 342 mg of the crude product. A 100-mg portion of the crude product was purified by preparative HPLC (Dynamax column, gradient 30 to 45% CH_3CN over 40 min). Pure fractions as judged by analytical HPLC were pooled and CH_3CN was

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evaporated under reduced pressure. The precipitate was collected, washed with H_2O, and dried under reduced pressure to afford 23 mg of the title compound: mp 218–219 °C; AAA: Phe (1.0); Asp, 1.1; Gly, 1.0; Nle, 2.0; Trp, 1.1; MS (FAB$^-$) m/e 895 (M – H)$^-$; NMR (500 MHz, DMSO-d_6) δ 0.81 (m, 6 H), 1.10–1.25 (m, 8 H), 1.47 (m, 2 H), 1.60 (m, 2 H), 2.35 (m, 2 H), 2.45 (m, 1 H, partially obscured by solvent), 2.58 (m, 1 H), 2.66 (m, 2 H), 2.84 (dd, J = 5, 9 Hz, 1 H), 2.97 (dd, J = 5, 9 Hz, 1 H), 3.05 (dd, J = 3, 8 Hz, 1 H), 3.16 (dd, J = 3, 9 Hz, 1 H), 3.58 (dd, J = 2, 10 Hz, 1 H), 3.73 (dd, J = 3, 10 Hz, 1 H), 4.15 (m, 2 H), 4.34 (m, 1 H), 4.45 (m, 1 H), 4.55 (m, 1 H), 6.62 (d, J = 5 Hz, 2 H), 6.94 (m, 3 H), 7.04 (t, J = 4 Hz, 1 H), 7.10 (m, 2 H), 7.15–7.25 (m, 5 H), 7.28 (m, 2 H), 7.55 (d, J = 5 Hz, 1 H), 7.85 (d, J = 5 Hz, 1 H), 7.95 (d, J = 5 Hz, 1 H), 8.00 (d, J = 5 Hz, 1 H), 8.12 (br s, 1 H), 8.15 (d, J = 5 Hz, 1 H), 8.25 (br s, 1 H), 9.10 (br s, 1 H), 10.79 (s, 1 H).

(Des-NH$_2$)Tyr(SO$_3^-$)-Nle-Gly-Trp-Nle-Asp-Phe-NH$_2$ (1). Dry, crude **24** (100 mg, ca. 85% purity, ca. 0.1 mmol) in 2 mL of 1:1 DMF/pyridine was treated with pyridine–sulfur trioxide (35 mg, 2.2 mmol). After 5 days, the reaction was quenched by addition of H_2O and then brought to neutral pH by addition of concentrated NH_4OH. Purification was accomplished by preparative HPLC (Dynamax column, gradient 25% to 35% CH_3CN over 30 min). Fractions judged pure by analytical HPLC were pooled and lyophilized twice to afford 20 mg (ca. 21% yield) of **1**. NMR (500 MHz, DMSO-d_6) δ 0.82 (m, 6 H), 1.10–1.30 (m, 8 H), 1.48 (m, 2 H), 1.60 (m, 2 H), 2.35–2.50 (m, 3 H), 2.61 (dd, J = 4, 10 Hz, 1 H), 2.72 (m, 2 H), 2.85 (dd, J = 5, 8 Hz, 1 H), 2.97 (dd, J = 5, 9 Hz, 1 H), 3.04 (dd, J = 2, 8 Hz, 1 H), 3.16 (dd, J = 2, 9 Hz, 1 H), 3.60 (dd, J = 3, 10 Hz, 1 H), 3.72 (dd, J = 4, 10 Hz, 1 H), 4.15 (m, 2 H), 4.35 (m, 1 H), 4.47 (m, 1 H), 4.55 (m, 1 H), 6.94 (t, J = 5 Hz, 1 H), 7.04 (m, 5 H), 7.10 (m, 2 H), 7.15–7.27 (m, 8 H), 7.30 (d, J = 5 Hz, 1 H), 7.55 (d, J = 5 Hz, 1 H), 7.81 (d, J = 5 Hz, 1 H), 7.93 (d, J = 5 Hz, 1 H), 7.97 (d, J = 5 Hz, 1 H), 8.09 (br t, 1 H), 10.76 (s, 1 H).

Boc-Asp(O*t*-Bu)-(Δ)Phe-NH$_2$ (25). Boc-Asp(Ot-Bu)-OH (0.34 g, 1.17 mmol) was converted to a mixed anhydride according to general procedure A and then treated with a solution of d,l-β-phenylserine hydrate (0.21 g, 1.17 mmol) and NMM (0.13 mL, 1.17 mmol) in 6 mL of H_2O. After warming to ambient temperature, the mixture was poured into 10% citric acid and extracted with EtOAc, and then the organic layer was dried ($MgSO_4$) and concentrated. Chromatography (silica gel, EtOAc/hexane/HOAc, 25:25:1 to 35:15:1, then EtOAc/HOAc, 50:1, and then EtOAc/acetone/HOAc, 78:20:2) gave 290 mg of a foam, which was dissolved in acetic anhydride, treated with sodium acetate trihydrate (0.26 g, 1.91 mmole), and stirred at room temperature for 6 h. The reaction mixture was concentrated to dryness and then partitioned between $CHCl_3$ and 10% citric acid. The aqueous phase was extracted twice with chloroform, and then the combined organic phases were dried ($MgSO_4$) and concentrated. Chromatography over silica gel (EtOAc/hexane, 1:6 to 1:2) gave 221 mg of the oxazalone as a foam: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.40 (s, 18 H), 2.65–3.00 (m, 2 H), 4.92 (m, 1 H), 7.36 (s, 1 H), 7.45–7.55 (m, 3 H), 7.60 (d, J = 9 Hz, 1 H), 8.20 (m, 2 H). The oxazalone (29 mg, 0.071 mmole) was dissolved in dioxane (2.5 mL) and treated with concentrated NH_4OH (2.5 mL). After 30 min, the reaction mixture was poured into 10% citric acid solution, extracted with $CHCl_3$, dried ($MgSO_4$), and concentrated. Purification by preparative TLC (silica gel, 1 mm, EtOAc) gave 15 mg of the title compound: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.39 (s, 9 H), 1.40 (s, 9 H), 2.50 (m, 1 H), 2.70 (dd, J = 6, 16 Hz, 1 H), 4.38 (m, 1 H), 7.22 (s, 1 H), 7.26–7.40 (m, 5 H), 7.57 (m, 2 H), 9.55 (s, 1 H).

Boc-Nle-Asp-(Δ)Phe-NH$_2$ (26). A solution of compound **25** (780 mg, 1.81 mmole) in 1.4 N HCl/HOAc was allowed to stand at ambient temperature for 2.5 hours, and then MeOH was added until the solution became homogeneous. The product was precipitated by addition of anhydrous Et_2O (125 mL) and collected by filtration to afford 550 mg of a white solid. A portion (250 mg, 0.80 mmol) was coupled to Boc-Nle-OH (190 mg, 0.80 mmole) according to general procedure A with NMM as base. After acid–base extractive workup, the crude product was purified by recrystallization from EtOAc/acetone to give 200 mg of the title compound: mp 180–182 °C dec, MS (FAB$^+$) m/e 491 (M + H)$^+$, 513 (M + Na)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.82 (m, 3 H), 1.15–1.65 (m, 15 H), 2.64 (dd, J = 7.5, 17 Hz, 1 H), 2.82 (dd, J = 6, 17 Hz, 1 H), 3.88 (m, 1 H), 4.57 (m, 1 H), 7.05 (d, J = 7.5 Hz, 1 H), 7.14 (br s, 1 H), 7.25–7.40 (m, 5 H), 7.55 (m, 2 H), 8.46 (d, J = 7.5 Hz, 1 H), 9.32 (s, 1 H), 12.53 (br s, 1 H). Anal. ($C_{24}H_{34}N_4O_7$) C, H, N.

(Des-NH$_2$)Tyr(SO$_3^-$)-Nle-Gly-Trp-Nle-Asp-(Δ)Phe-NH$_2$ (3). Compound **26** (184 mg, 0.375 mmol) was treated with 5 mL of 1.6 N HCl/HOAc for 1.5 h, and then the solution was frozen and lyophilized. The salt was extended to the Boc-tetrapeptide (73% yield) by using a procedure similar to that described for preparation of compound **20**. Extension to the unsulfated acylhexapeptide (mp 207.5–210 °C, 70% yield) proceeded similarly as for preparation of compound **21**. To a solution of the acylhexapeptide (128 mg, 0.14 mmol) in 1:1 DMF/pyridine (6 mL) was added pyridinium acetyl sulfate[29] (310 mg, 1.4 mmol). After 1.5 days, the mixture was concentrated under reduced pressure, and then treated with 3 mL of H_2O containing 100 mg of NaOH. The mixture was diluted with MeOH and filtered through Celite, and then purified by preparative HPLC (Dynamax and Vydac columns, gradient 25% to 50% CH_3CN over 10 min). Pure fractions were pooled and lyophilized to afford the title compound (87 mg, 64%): ^{1}H NMR (300 MHz, DMSO-d_6, selected data) δ 3.54 (dd, J = 3, 18 Hz, 1 H), 3.72 (dd, J = 5, 18 Hz, 1 H); 4.14 (m, 1 H), 4.22 (m, 1 H), 4.56 (m, 2 H) (α-protons); 6.59 (s, vinylic-H).

Fmoc-(*N*-Me)Asp(OBn)-OH (27). Following the general procedure of Freidinger,[23] a solution of Fmoc-Asp(OBn)-OH (15 g, 33.6 mmol), paraformaldehyde (7 g), and *p*-TsOH·H_2O in toluene (500 mL) was heated at reflux with azeotropic removal of water for 2 h, at which time TLC indicated consumption of starting material was complete. The solution was washed with saturated aqueous $NaHCO_3$, then dried (Na_2SO_4), and concentrated. The residue was dissolved in $CHCl_3$ (150 mL) and treated with TFA (26 mL) and triethylsilane (16 mL). Additional quantities of TFA and triethylsilane were added over several days until TLC indicated that the reaction was complete. The solution was concentrated and fresh $CHCl_3$ was added and evaporated, and then the residue was treated with Et_2O/hexane (1:9) to afford a light tan solid (9.3 g, 60%): mp 123–126 °C; $[\alpha]^{24}_D$ = –41.4° (*c* 1.16, $CHCl_3$); MS (FAB$^+$) m/e 460 (M + H)$^+$, 482 (M + Na)$^+$; ^{1}H NMR (300 MHz, $CDCl_3$) (two conformers, ca. 1:1) δ 2.35 (dd, J = 7.5, 17 Hz, 0.5 H), 2.72 (dd, J = 6, 16 Hz, 0.5 H), 2.90 (s, 1.5 H, *N*-Me), 2.95 (m, 0.5 H), 3.0 (s, 1.5 H, *N*-Me), 3.18 (dd, J = 6, 17 Hz, 0.5 H), 4.15 (t, J = 5 Hz, 0.5 H), 4.25 (t, J = 7.5 Hz, 0.5 H), 4.42 (m, 1.5 H, Fmoc CH_2), 4.50 (dd, J = 5, 10.5 Hz, 0.5 H, Fmoc CH_2), 4.62 (m, 0.5 H, α-H), 4.8 (dd, J = 6, 8 Hz, 0.5 H, α-H), 5.15–5.20 (m, 2 H), 7.20–7.45 (m, 9 H), 7.52 (m, 2 H), 7.68 (d, J = 7.5 Hz, 0.5 H), 7.75 (d, J = 7.5 Hz, 1.5 H). A second crop of 1.6 g (10%) of small, colorless needles was obtained from the mother liquors: mp 133–134 °C; $[\alpha]^{25}_D$ = –47.1° (*c* 1.1, $CHCl_3$).

Boc-(*N*-Me)Asp(OBn)-OH (28). Compound **27** (83 g, 180 mmol) in CH_2Cl_2 (300 mL) was treated with piperidine (100 mL), followed by isopropyl ether (1000 mL). After ca. 2 h, additional isopropyl ether was added to precipitate the product which was collected by filtration to afford 31.3 g of material. A suspension of this intermediate in CH_2Cl_2 (200 mL) was added to a stirred mixture of di-*tert*-butyl dicarbonate (42.33 g), CH_2Cl_2 (200 mL), H_2O (100 mL), and DIEA (to maintain pH 8–8.3). After stirring at 35 °C for 1 h, the acidic product was isolated by standard extractive procedures, followed by crystallization from EtOAc/heptane to afford 32.6 g (54%) of the title compound: mp 102–103 °C; $[\alpha]^{24}_D$ = –59.3° (*c* 1.2, $CHCl_3$); MS (CI/NH_3) m/e 338 (M + H)$^+$, 355 (M + NH_4)$^+$; ^{1}H NMR (300 MHz, $CDCl_3$) (two conformers, ca 1:1) δ 1.41 (s, 4.5 H) and 1.43 (s, 4.5 H) (Boc); 2.83 (m, 1 H); 2.89 (s, 1.5 H) and 2.95 (s, 1.5 H) (*N*-Me); 3.15 (dd, J = 6, 16 Hz, 1 H); 4.60 (m, 0.5 H) and 4.75 (m, 0.5 H) (α-proton); 5.15 (s, 2 H), 7.35 (m, 5 H).

H-(*N*-Me)Asp(OBn)-Phe-NH$_2$ Hydrochloride (29). Boc-(*N*-Me)Asp(OBn)-OH (12.0 g, 35.6 mmol) and H-Phe-NH_2 (6.08 g, 37.0 mmol) in DMF (75 mL) were coupled according to general procedure B. After 30 min at 0 °C and 2 h at ambient temperature, TLC indicated that the reaction was complete. The mixture was partitioned between EtOAc (100 mL) and 5% aqueous $NaHCO_3$. The separated aqueous phase was back-extracted with EtOAc (100 mL), and then the combined organic layers were washed with successive portions of 5% aqueous

$NaHCO_3$, 5% aqueous citric acid (2×), 5% aqueous $NaHCO_3$, and H_2O (2×), then dried ($MgSO_4$), and concentrated to a syrup. The crude dipeptide was dissolved in EtOAc (100 mL), heptane was added to cloudiness, and then 1.2 N HCl/EtOAc (250 mL) was added. After 2 h, the reaction was incomplete according to TLC and additional HCl (g) was introduced. After an additional 0.5 h, more heptane was added, the mixture was slowly concentrated under reduced pressure, and the precipitated solid was collected, washed with hexane, and dried at 45 °C under reduced pressure to afford 14.5 g (97%) of the title compound: MS (CI/NH_3) m/e 384 (free base $(M + H)^+$); NMR (300 MHz, DMSO-d_6) δ 2.01 (s, 3 H), 2.72 (dd, J = 11, 14 Hz, 1 H), 2.90 (d, J = 6 Hz, 2 H), 3.10 (dd, J = 4, 14 Hz, 1 H), 3.99 (t, J = 6 Hz, 1 H), 4.60 (m, 1 H), 5.10 (s, 2 H), 7.11–7.30 (m, 6 H), 7.31–7.40 (m, 5 H), 7.55 (br s, 1 H), 8.97 (d, J = 9 Hz, 1 H).

Boc-Trp-Nle-(*N*-Me)Asp(OBn)-Phe-NH_2 (30). The symmetrical anhydride of Boc-Nle-OH (2.47 g, 10.6 mmol) was formed in 10 mL of CH_2Cl_2 according to general procedure C. Subsequently, **29** (2.1 g, 5.0 mmol) was added followed by DIEA (1.78 mL, 10.2 mmol). After several days, the mixture was concentrated and subjected to standard workup, followed by crystallization from Et_2O/hexane to afford 2.5 g (84%) of the protected tripeptide as a solid. The tripeptide (2.3 g, 3.9 mmol) in CH_2Cl_2 (25 mL) was treated with trifluoroacetic acid (TFA, 25 mL), and the solution was allowed to stand at ambient temperature for 1 h and then concentrated. Addition of anhydrous Et_2O precipitated a solid, which was collected and washed with Et_2O to afford 1.7 g (72%) of the tripeptide salt. To the mixed carbonic anhydride prepared from Boc-Trp-OH (1.06 g, 3.47 mmol) according to general procedure A were added the tripeptide salt (1.63 g, 2.67 mmol) in CH_2Cl_2, followed by DIEA (0.47 mL, 2.70 mmol). Further treatment according to the general procedure afforded a crude product (2.04 g), which was crystallized from CH_2Cl_2/Et_2O to afford 1.36 g (65%) of the title compound as a colorless solid: MS (FAB^+) m/e 783 $(M + H)^+$; ^1H NMR (300 MHz, $CDCl_3$, selected data) (two conformers, ca. 1.5:1) δ 2.20 (*N*-Me, major conformer) and 2.74 (*N*-Me, minor conformer); 4.39, 4.40, 4.80, 4.98 (α-protons, major conformer); 4.80, 5.12, 5.85, 5.92 (α-protons, minor conformer).

H-Trp-Nle-(*N*-Me)Asp-Phe-NH_2 Trifluoroacetate (31). Compound **30** (600 mg, 0.77 mmol) in HOAc (100 mL) was shaken for several hours in the presence of 10% Pd/C (60 mg) under an atmosphere of H_2 (4 atm). The solution was filtered and concentrated, and then the residue was dissolved in CH_2Cl_2 (20 mL) containing anisole (0.8 mL) and dimethyl phosphite (0.8 mL) and treated with TFA (20 mL). After standing at ambient temperature for 1 h, the solution was concentrated and the residue treated with anhydrous Et_2O to precipitate a solid, which was collected and dried to afford 485 mg (90%) of the title compound as a light violet powder: MS (CI/NH_3) 593 $(M + H)^+$, 575 $(M + H - H_2O)^+$; ^1H NMR (300 MHz, DMSO-d_6, selected data) (two conformers, ca. 4:1) δ 2.21 (*N*-Me, minor conformer), 2.38 (*N*-Me, major conformer); 4.02, 4.48, 4.55, 5.14 (α-protons, major conformer).

(Des-NH_2)Tyr(SO_3^-)-Nle-Gly-Trp-Nle-(*N*-Me)Asp-Phe-NH_2 (4). Compound **31** (485 mg, 0.69 mmol) was converted to the title compound in a manner similar to that described for conversion of **21** to **1**. Purification by preparative HPLC (Dynamax C18, gradient from 25 to 40% CH_3CN over 30 min) afforded 180 mg (28%) of the title compound: ^1H NMR (500 MHz, DMSO-d_6, selected data) (two conformers, ca. 2:1) δ 2.10 (*N*-Me, minor conformer), 2.30 (*N*-Me, major conformer); 3.55–3.75, 4.11, 4.35, 4.45, 4.52, 4.62, 4.77, 5.12, 5.23 (α-protons, both conformers).

(Des-NH_2)Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Hyp(SO_3^-)-Phe-NH_2 (5). The unsulfated acylhexapeptide was assembled using a Biosearch Model 9500 automated peptide synthesizer starting with 1 g (substitution level of 0.67 mmol/g) of *p*-methylbenzhydrylamine resin (Bachem, Inc. Torrance, CA). The standard Merrified Boc/benzyl protection strategy[42] was used, except the (des-NH_2)Tyr residue was incorporated with the phenolic hydroxyl unprotected. On the basis of weight gain after incorporation of the first (Boc-Phe-) residue, the effective level of substitution was estimated as 0.47 mmol/g. Double couplings were performed in 1:1 CH_2Cl_2/DMF for 2 h each using a 3.5-fold excess of protected amino acid at a concentration of 0.3 M in the presence of 1,3-diisopropylcarbodiimide, followed by capping with acetylimidazole. Deblocking was carried out in 45% TFA, 2.5% anisole, 2% dimethyl phosphite in CH_2Cl_2 for 20 min, followed by neutralization with 10% DIEA in CH_2Cl_2. The peptide was cleaved from the resin by treatment with liquid HF (9 mL) in the presence of anisole (0.75 mL) and dimethyl phosphite (0.25 mL) at 0 °C for 1 h. After evaporation of the HF under reduced pressure, the residue was triturated with MeOH, the resin was removed by filtration, and the solvent was evaporated. The residue was subjected directly to sulfation with pyridinium acetyl sulfate as described for the preparation of **3**. When the reaction was complete as judged by TLC (ca. 3 h), the reaction was quenched by addition of H_2O, then brought to neutral pH by addition of concentrated NH_4OH. The solvent was removed by lyophilization, the residue was triturated with MeOH, and the supernatant was subjected to repeated purification by preparative HPLC (Dynamax and Vydac columns, gradient 15% to 45% CH_3CN over 40 min). Pure fractions were combined and lyophilized to afford 25 mg (5% yield) of the title compound as a fluffy solid: ^1H NMR (500 MHz, DMSO-d_6, selected data) δ 3.55–3.72 (m, 4 H) (Gly α-protons plus Hyp δ-protons), 4.15 (m, 1 H), 4.28 (m, 2 H), 4.42 (m, 1 H), 4.58 (m, 1 H), 4.76 (m, 1 H) (remaining α-protons plus C*H*OSO_3^-).

Boc-Trp-(*N*-Me)Leu-Asp(OBn)-Phe-NH_2 (32). Boc-(*N*-Me)Leu-OH (152 mg, 0.62 mmol) and TFA·H-Asp(OBn)-Phe-NH_2[43] (300 mg, 0.62 mmol) were coupled according to general procedure B. Standard workup after 3 days afforded a crude product that was purified by flash chromatography (EtOAc/hexane/HOAc, 75:25:2) to afford 319 mg (86%) of the protected tripeptide. Treatment of the tripeptide (283 mg, 0.47 mmol) with 4 N HCl/dioxane (ca. 2 mL) for 1 h was followed by evaporation of the volatile components and then addition and evaporation of anhydrous EtO to form a solid, which was dried under reduced pressure. The HCl salt was treated with a solution of the symmetrical anhydride prepared from Boc-Trp-OH (286 mg, 0.94 mmol) according to general procedure C, followed by addition of NMM (0.051 mL, 0.46 mmol). The mixture was stirred at 0 °C for 3 h and allowed to warm to ambient temperature and stir overnight. Standard workup followed by flash chromatography (EtOAc/hexane/HOAc, 60:40:2) afforded 220 mg (60%) of the title compound as an off-white solid: MS (FAB^+) m/e 783 $(M + H)^+$; ^1H NMR (300 MHz, DMSO-d_6, selected data) (two conformers, ca. 9:1) δ 0.48 (d, J = 4 Hz) and 0.52 (d, J = 4 Hz) (Leu δ, minor conformer); 0.81 (d, J = 3 Hz) and 0.85 (d, J = 3 Hz) (Leu δ, major conformer); 2.53 (s, *N*-Me, minor conformer), 2.83 (*N*-Me, major conformer); 4.40 (m), 4.52 (m), 4.64 (dd, J = 3.8 Hz), 5.10 (dd, J = 2, 7 Hz) (α-protons, major conformer).

(Des-NH_2)Tyr(SO_3^-)-Nle-Gly-Trp-(*N*-Me)Leu-Asp-Phe-NH_2 (7). Compound **32** (192 mg, 0.25 mmol) in CH_2Cl_2 (1.7 mL) was treated with anisole (0.075 mL), dimethyl phosphite (0.06 mL), and TFA (1.3 mL) and allowed to stand for 1 h. Anhydrous Et_2O was added, the volatile components were removed under reduced pressure, then the residue was triturated with fresh Et_2O, and the solid was collected by filtration to afford 114 mg (57%) of the trifluoroacetate salt. The salt (111 mg, 0.14 mmol) was coupled to acid **23** (44 mg, 0.14 mmol) via the mixed anhydride according to general procedure A, using NMM as the base. The reaction mixture was partitioned between EtOAc and aqueous $KHSO_4$ and then the separated organic layer was washed successively with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated to afford 139 mg of the crude protected acylhexapeptide, which was dissolved in EtOAc (5 mL) and stirred under an atmosphere of H_2 in the presence of 10% Pd/C (100 mg). Since consumption of starting material was not complete after 2 days, DMF (2 mL) was added, and the mixture was stirred for a further 4 h and then filtered using MeOH rinse.

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The filtrate was concentrated, diluted with H_2O, frozen, and lyophilized to afford 109 mg of the deprotected acylhexapeptide: MS (FAB$^+$) m/e 911 (M + H)$^+$. Sulfation of the crude product (69 mg, 0.076 mmol) according to the procedure described for preparation of 1, followed by purification by preparative HPLC (Dynamax column, gradient 10 to 40% CH_3CN over 40 min) afforded 13 mg (15% from deprotected tetrapeptide) of the title compound: ^{1}H NMR (500 MHz, DMSO-d_6, selected data) (two conformers, ca. 3:1) δ 0.43 (d, J = 4 Hz) and 0.48 (d, J = 4 Hz) (Leu δ, minor conformer); 0.83 (m) (Leu δ, major conformer plus Nle δ, both conformers), 2.56 (*N*-Me, minor conformer), 2.83 (*N*-Me, major conformer); 3.56–3.80, 4.18, 4.38, 4.52, 4.91 (α-protons, both conformers).

H-Asp(OBn)-(*N*-Me)Phe-NH$_2$ Hydrochloride (33). Boc-Asp(OBn)-OH (15.8 g, 49 mmol) and H-(*N*-Me)Phe-NH$_2$[44] (8.71 g, 49 mmol) were allowed to react for 4 days under the conditions of method A. The mixture was concentrated under reduced pressure and subjected to standard workup to afford the crude product which was flash chromatographed (Et_2O/hexane, 1:1 then 7:3). A solution of the product (12.42 g) in 1.5 N HCl/HOAc (70 mL) was allowed to stand at ambient temperature for 0.75 h, and then the hydrochloride salt was precipitated by addition of anhydrous Et_2O, collected, and dried under reduced pressure to afford 11.49 g (56%) of a colorless solid: MS (CI/NH_3) m/e 383 (M + H)$^+$: ^{1}H NMR (300 MHz, DMSO-d_6) (two conformers, ca. 1:1) δ 1.20 (dd, J = 2, 18 Hz, 0.5 H), 2.20 (dd, J = 9, 18 Hz, 0.5 H), 2.81 (m, 0.5 H); 2.88 (s, 1.5 H, *N*-Me), 2.91 (s, 1.5 H, *N*-Me), 2.98 (m, 0.5 H), 3.15–3.35 (m, 2 H), 4.50 (m, 1 H), 4.65 (dd, J = 5, 16 Hz, 0.5 H), 4.88 (dd, J = 6, 10 Hz, 0.5 H), 5.05–5.18 (m, 2 H), 7.05–7.50 (m, 11.5 H), 7.90 (0.5 H), 8.30–8.50 (br m, 3 H). Anal. ($C_{21}H_{26}ClN_3O_4$·HOAc) C, H, N.

Boc-3PP-Asp(OBn)-(*N*-Me)Phe-NH$_2$ (34). Boc-3PP-OH (1.03 g, 4.00 mmole) and HCl·Asp(OBn)-(*N*-Me)Phe-NH$_2$ (1.68 g, 4.00 mmole) were coupled using general procedure A with NMM as base. The mixture was partitioned between EtOAc and dilute aqueous HCl, and the organic phase was washed successively with H_2O, 5% $NaHCO_3$, and H_2O, then dried ($MgSO_4$), and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc/hexane, 1:1 to 7:3, then EtOAc) to give 2.01 g of the product as a foam: MS (CI/NH_3) m/e 623 (M + H)$^+$; 640 (M + NH_4)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6, selected data) (multiple conformers) δ 0.82 (m, 3 H, *n*-Pr CH_3), 1.27 (s, Boc), 1.30 (s, Boc), 1.35 (s, Boc), 2.70 (s, *N*-Me), 2.72 (s, *N*-Me), 2.89 (s, *N*-Me), 2.92 (s, *N*-Me). Anal. ($C_{34}H_{46}N_4O_7$·0.5H_2O) C, H, N.

Boc-Trp-3PP-Asp(OBn)-(*N*-Me)Phe-NH$_2$ (35). Compound 34 (2.01 g, 3.23 mmol) was treated with 10 mL of 1.4 N HCl/HOAc for 40 min, and then the product was precipitated with anhydrous Et_2O, collected by vacuum filtration, and dried in vacuo to give 1.56 g of a white solid. Anal. Calcd for ($C_{29}H_{38}N_4O_5$·HCl·H_2O) C, H, N. The hydrochloride salt (1.56 g, 2.79 mmol) was coupled to Boc-Trp-OH (0.85 gm, 2.79 mole) using general procedure B with DIEA as base. The mixture was partitioned between EtOAc and dilute aqueous HCl, and the separated organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc/hexane, 1:1 to 7:3, then EtOAc) to give 1.59 g (70%) of the title compound: MS (FAB$^+$) m/e 809 (M + H)$^+$, 831 (M + Na)$^+$. Anal. ($C_{45}H_{56}N_6O_8$·1.5H_2O) C, H, N.

(Des-NH$_2$)Tyr(SO$_3^-$)-Nle-Gly-Trp-3PP-Asp-(*N*-Me)Phe-NH$_2$ (9). Compound 35 (1.58 g, 1.96 mmol) was treated with 1.4 N HCl/HOAc (12 mL) for 1 h under N_2, then anhydrous Et_2O was added to precipitate a solid which was collected by filtration to afford 1.36 g of hydrochloride salt. The salt (1.36 g) was coupled to acid 23 (0.62 g, 1.85 mmol) according to general procedure B using DIEA as base. The reaction mixture was partitioned between EtOAc and dilute aqueous $NaHCO_3$, and then the separated organic layer was washed successively with 5% $NaHCO_3$, H_2O, 10% HCl, and H_2O, then dried ($MgSO_4$), and concentrated. Flash chromatography (EtOAc/HOAc, 50:1, then EtOAc/acetone/HOAc, 80:20:1) afforded 1.14 g of a tan foam, which was dissolved in MeOH (150 mL) and shaken in the presence of 10% Pd/C (0.21 g) under 4 atm of H_2. After confirming consumption of starting material by TLC, the solution was filtered through Celite and concentrated. A portion of the residue (0.049 g, 0.053 mmol) was sulfated as described above for preparation of 3, except that concentrated NH_4OH was used to quench the reaction. The crude product was purified by preparative HPLC (Dynamax column, gradient 10 to 40% CH_3CN over 40 min) to afford the title compound in 30% yield from 35: ^{1}H NMR (300 MHz, DMSO-d_6, selected data) (multiple conformers) δ 0.82 (m, 6 H, *n*-Pr and Nle methyls); 2.72 (s, *N*-Me), 2.95 (s, *N*-Me); 4.19 (m), 4.66 (m), 4.72–5.15 (m) (α-protons).

Boc-[(*R*)-3-amino-2-oxo-1-pyrrolidineacetyl]-Trp-Nle-Asp-Phe-NH$_2$ (36). (*R*)-3-[(*tert*-Butyloxycarbonyl)amino]-2-oxo-1-pyrrolidineacetic acid was prepared from Boc-D-Met-Gly-OH according to the method of Freidinger,[27] $[\alpha]^{23}_D$ = +28.0° (*c* 0.94, MeOH) (lit.[27] for the *S* isomer: $[\alpha]^{24}_D$ = −31.2° (*c* 1.0, MeOH)). This acid (42 mg, 0.16 mmol) was coupled to tetrapeptide 21 (124 mg, 0.18 mmol) according to general procedure A, with the mixed anhydride formed in 1:1 CH_2Cl_2:THF, and using NEt_3 as base during the coupling step. The product was isolated by concentration of the reaction mixture followed by addition of H_2O and HOAc to precipitate a solid, which was collected by filtration, washed with H_2O, and dried in vacuo to afford 111 mg (85%) of the title compound: MS (FAB$^+$) m/e 841 (M + Na)$^+$, 719 (M + H − Boc)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6, selected data) δ 1.38 (s, Boc), 3.05 (m, lactam C-5 protons); 3.67 (d, J = 17 Hz, 1 H), 3.88 (d, J = 17 Hz, 1 H), 4.09 (dd, J = 9, 18 Hz, 1 H), 4.21 (m, 1 H), 4.35 (m, 1 H), 4.51 (m, 1 H), 4.57 (m, 1 H) (α-protons).

(Des-NH$_2$)Tyr(SO$_3^-$)-[(*R*)-3-amino-2-oxo-1-pyrrolidineacetyl]-Trp-Nle-Asp-Phe-NH$_2$ (15). A solution of 36 (98 mg, 0.12 mmol) in CH_2Cl_2 (1 mL) containing anisole (0.050 mL) and dimethyl phosphite (0.040 mL) was treated with TFA (1 mL), and the solution was kept at ambient temperature for 1 h. The volatile components were evaporated, the residue was treated with anhydrous Et_2O, and the precipitate was collected by filtration to afford the TFA salt (96 mg). To a solution of the salt (95 mg, 0.11 mmol) in DMF (1 mL) at 0 °C were added NMM (0.015 mL, 0.14 mmol) and 3-(4-hydroxyphenyl)propionic acid succinimide ester (Hpp-OSu) (34 mg, 0.13 mmol). The mixture was allowed to warm to ambient temperature and stir overnight and then was treated with additional Hpp-OSu (5 mg, 0.02 mmol). After an additional 24 h, the mixture was treated with H_2O and HOAc to precipitate a gum, which solidified after addition of a small quantity of MeOH to the aqueous mixture followed by trituration. The solid was collected by filtration and dried under reduced pressure to afford 87 mg of crude acylhexapeptide. The crude acylhexapeptide (53 mg) was sulfated in a manner analogous to that described for the preparation of 1, and purified by preparative HPLC (Dynamax column, gradient 20 to 35% CH_3CN over 30 min.) to afford 29 mg (42% from 36) of the title compound: ^{1}H NMR (360 MHz, DMSO-d_6, selected data) 3.79 (m, 2 H), 4.20 (m, 1 H), 4.32 (m, 2 H), 4.50 (m, 1 H), 4.57 (m, 1 H) (α-protons); 7.82 (d, J = 7 Hz, 1 H), 8.05 (d, J = 7 Hz, 1 H), 8.12 (d, J = 7 Hz, 1 H), 8.21 (d, J = 7 Hz, 1 H), 8.31 (d, J = 7 Hz, 1 H).

In Vitro Assays. Binding, amylase release and PI turnover studies were carried out in guinea pig tissues as described previously.[8]

Feeding Assays. Adult male Sprague–Dawley rats (Sasco, Madison, WI) were housed and tested in individual hanging wire mesh cages, and they weighed between 260 and 450 g at the time of testing. Animals were allowed at least 3 days to become acclimated to the colony room after arrival from the supplier. Water was freely available, but food was restricted to a liquid diet of Ensure available for 60 min in the morning and for 30 min approximately 6 h later. Prior to testing, animals were maintained on this feeding schedule for at least 10 days, by which time intakes had stabilized. Drug solutions were prepared in a distilled water vehicle, and doses ranging from 0–30 nmol/kg were administered ip in volumes of ca. 1 mL/kg. In general, eight animals per experimental group were used. On test days, treatment groups were matched according to 60-min intakes on the previous day, and the animals were weighed and injected (ip) 7–10 min prior to the morning feeding period. Intake volumes of Ensure, which was presented in graduated feeding tubes, were measured after both the morning and afternoon feeding periods. Data for the

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morning period are expressed as mean (±SEM) dose required to suppress intakes approximately 50% compared to vehicle, as determined by least squares regression analysis.

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Analogs of the δ Opioid Receptor Selective Cyclic Peptide [2-D-Penicillamine,5-D-penicillamine]-enkephalin: 2′,6′-Dimethyltyrosine and Gly³-Phe⁴ Amide Bond Isostere Substitutions

Nizal S. Chandrakumar,*,† Awilda Stapelfeld,‡ Patrick M. Beardsley,‡ Oscar T. Lopez,‡ Bridget Drury,‡ Elizabeth Anthony,‡ Michael A. Savage,‡ L. Neil Williamson,§ and Melvin Reichman‡,§

CNS Diseases Research, G.D. Searle and Company, 4901 Searle Parkway, Skokie, Illinois 60077. *Received May 17, 1991*

In order to develop systemically-active opioid peptides, the δ-selective, opioid pentapeptide [D-Pen²,D-Pen⁵]-enkephalin (DPDPE) was modified by esterification and by substitution of 2′,6′-dimethyltyrosine for tyrosine to yield **4**. Compound **4** was on the order of 8- and 800-fold more active than DPDPE in both δ and μ opioid radioligand binding assays, respectively, in rat neural membrane suspensions. Compound **4** was considerably more potent than DPDPE at inhibiting contractions of electrically-stimulated mouse vas deferens in vitro, and this effect was very sensitive to naltrindole, a δ-selective opioid antagonist. These observations can be taken as indication that **4** exerts its effects through δ opioid receptors. This interpretation is supported by the finding that the EC_{50} value of **4** derived in the smooth muscle assay is very similar to that derived in NG108-15 neuroblastoma cells, a preparation devoid of μ receptors. Unlike DPDPE, **4** exhibited significant, naloxone-sensitive, antinociceptive activity when administered systemically, as measured by inhibition of phenylbenzquinone-induced stretching in mice (ED_{50} = 2.1 mg/kg). Compound **4** also displayed significant antinociceptive activity following systemic administration as measured by its action in mice to increase latencies for tail withdrawal from radiant heat (ED_{50} = 50 mg/kg). Compound **4** did not produce morphine-like discriminative stimulus effects in rats trained to discriminate 3.0 mg/kg morphine from vehicle at doses ranging from 30 to 120 mg/kg. This observation can be interpreted as indication that within this dosage range there is an absence of morphine-like subjective effects. Physical dependence, however, could be induced in mice at higher doses of **4** under a progressively-graded, 4-day dose regimen. Congeners of **4** with amide bond surrogates for the Gly-Phe amide bond (oxymethylene, trans-double bond, and bismethylene isosteres) in the cyclic core of DPDPE were prepared in an attempt to increase the antinociceptive activity of **4**. While some of the congeners were active in the in vitro assays, they did not display significant antinociceptive activity following systemic administration. The preparation of all the compounds was accomplished by solution-phase methods. The mechanisms which might underlie the biological and systemic activity of **4** are discussed.

Introduction

The discovery of endogenous pentapeptide brain opioids, the enkephalins,[1] led to much excitement in the arena of the medicinal chemistry of pain.[2,3] The activity was fueled by the possibility that analogs of these relatively simple linear peptides might yield safer analgesics than narcotic alkaloids typified by morphine. While many enkephalin congeners have been described, only a handful have undergone clinical trials. None of these has yet advanced into the clinician's armamentarium against pain.[4-6] An important result of this work was the recognition that a δ opioid receptor type, along with the μ and κ opioid receptor types, could mediate analgesia in mammals.[7-10] Evidence is emerging that analgesia elicited through δ opioid receptors is associated with a more benign side effect profile than analgesia elicited through other opioid receptors.[11]

† CNS Chemistry.
‡ CNS Biology.
§ Cellular Pharmacology Laboratory.

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