Peripherally Acting Enkephalin Analogues. 1. Polar Pentapeptides[1]

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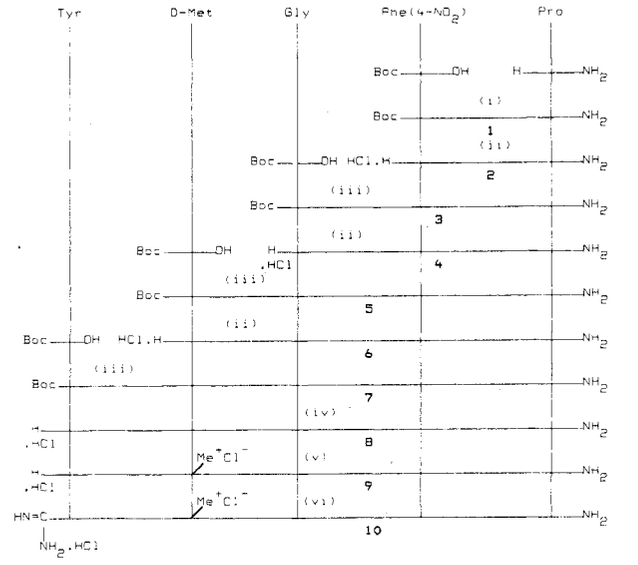
The design, synthesis, and biological activity of a series of highly polar enkephalin-related pentapeptides are reported. These analogues incorporate structural features that exclude them from the central nervous system and thereby restrict their action to peripherally located receptors. Hydrophilic analogues were obtained by introduction of polar D-amino acid residues at position 2 and, in certain cases, by conversion of the N-terminal amino group of the Tyr residue to a guanidino function. The peptides were synthesized by classical solution methods. All compounds demonstrated in vitro opioid activity in the GPI and all were shown to possess antinociceptive activity in chemically induced writhing models. The analgesic effects were shown to be predominantly peripherally mediated by antagonism of antinociception with the peripheral antagonist *N*-methylnalorphine. Comparative data obtained in writhing and hot-plate tests were also supportive of a peripheral mode of action. Compound **13a**, L-tyrosyl-D-arginylglycyl-L-4-nitrophenylalanyl-L-prolinamide (BW 443C), was identified as having a favorable pharmacological profile, indicating a high level of peripheral selectivity, and worthy of further investigation.

Preparations derived from the opium poppy (*Papaver somniferum*) have been in use for millenia for the relief of pain. The strong analgesic drugs used in the clinic today are derived from opiates.[2] The archetypal drug, morphine, is widely used despite the disadvantage of a set of severe side effects, the most serious of which are addictive liability, tolerance, and respiratory depression.[3] For many years medicinal chemists have been engaged in a search for compounds that retain the desirable analgesic property divorced from these unwanted side effects but with little success.[4] The discovery, in 1975, of the endogenous opioid peptides, the enkephalins,[5] provided a new approach to the development of novel opiate analgesic agents. Since that time a vast number of analogues of the parent enkephalin structures have been prepared and tested for analgesic and other pharmacological activities.[6] Even so, little progress has been made in attempting to obtain compounds that lack the disadvantageous side effects of the classical opiate drugs. In fact, although analgesic activity in limited clinical studies has been reported with two enkephalin derived peptides, FK-33,824[7] [Tyr-D-Ala-Gly-(*N*-Me)-Phe-Met(O)-ol] and metkephamid[8] [Tyr-D-Ala-Gly-Phe-(*N*-Me)-Met-NH_2], both compounds were also associated with a diverse set of side effects such as heaviness of limbs[8,9] not normally associated with opiates.

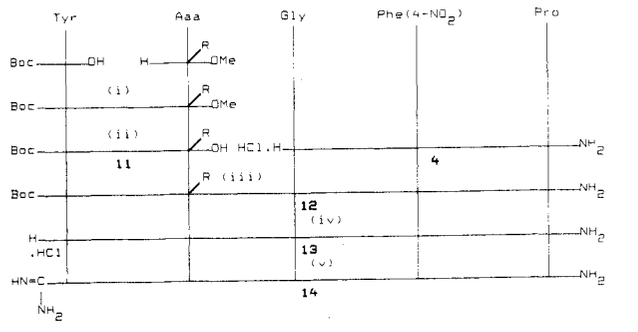
The primary site of action of the opiate drugs is generally accepted to be in the central nervous system (CNS); evidence has also been adduced to indicate that the major side effects are also centrally mediated.[3] In recent years there has been interest in the peripheral effects of opiates, and it has been suggested that at least part of the analgesic action of these agents may be mediated in the periphery.[10,11] Indeed, one of us (Smith) has demonstrated that peripherally mediated analgesia may be observed with the simple quaternized derivative *N*-methylmorphine chloride.[12] This being the case, it would be of interest to prepare opioids that exhibit greatly restricted access to the CNS. For this purpose the enkephalins are a good starting point since they represent a class of opioids more hydrophilic than the alkaloids or synthetic opiate drugs, i.e., they are intrinsically less likely to cross the blood–brain barrier. Consequently, we undertook a program of research whose objectives were to design and synthesize polar enkephalin analogues and to investigate whether they would exhibit peripherally mediated analgesia divorced from centrally mediated side effects.

Scheme I[a]



[a] Reagents: (i) DCCI, HOBt, NMM, DMF, 0 °C; (ii) 1 M HCl–HOAc; (iii) *i*-BuOCOCl, NMM, THF, DMF, −15 °C; (iv) 1 M HCl–HOAc, anisole; (v) MeI, MeOH, 7 days; (vi) ADMP, EtOH, DMF, 50 °C.

Scheme II[a]



	Aaa	R
a	D-Arg	H^+
b	D-Glu	t-Bu

[a] Reagents: (i) *i*-BuOCOCl, NMM, THF, DMF, −15 °C; (ii) NaOH, H_2O, MeOH; (iii) DCCI, HOBt, NMM, DMF, 0 °C; (iv) 1 M HCl–HOAc; (v) ADMP, EtOH, DMF, 50 °C.

This paper describes our initial investigations, an extension of previous work from these laboratories, which

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0022-2623/88/1831-0960$01.50/0

Table I. Pentapeptides

R_1-Tyr-X-Gly-Phe(4-NO_2)-Pro-NH_2

no.	structure R_1	structure X	$[\alpha]_D$, deg (c, t °C)[a]	TLC R_f D	E	F	amino acid analysis[b]	analysis	FAB-MS[c] (M + 1)$^+$
9	H	D-Met(Me^+)	+15.8 (1.0, 21)	0.39	0.04	0.15	Tyr, 0.99; Gly, 1.02; Pro, 1.07; Phe(4-NO_2), 0.99	$C_{31}H_{42}N_7O_8SCl\cdot HCl\cdot 2.5H_2O$: C, H, N	672
10	C(NH)NH_2	D-Met(Me^+)	−8.8 (1.0, 25)	0.33	0.02	0.15	Tyr, 0.17;[d] Gly, 1.10; Pro, 1.04	$C_{32}H_{45}N_9O_8SCl_2\cdot 4H_2O$: C, H, N	715
13a	H	D-Arg	+12.7 (1.0, 24)	0.46	0.27	0.37	Tyr, 0.96; Arg, 0.98; Gly, 1.04; Phe(4-NO_2), 1.01; Pro, 1.0	$C_{31}H_{42}N_{10}O_8\cdot 2CH_3CO_2H\cdot 2H_2O$: C, H, N	683
13b	H	D-Glu	−24.8 (0.5, 24.5)	0.83	0.90	0.44	Tyr, 1.03; Glu, 1.05; Gly, 1.00; Phe(4-NO_2), 0.98; Pro, 0.98	$C_{30}H_{37}N_7O_{10}Na\cdot 2H_2O$: C, H, N	656, 678[e]
14a	C(NH)NH_2	D-Arg	−9.6 (1.0, 25)	0.68	0.04	0.27	Tyr, 0.19;[d] Arg, 1.02; Gly, 1.06; Pro, 0.92	$C_{32}H_{44}N_{12}O_8\cdot 2CH_3CO_2H\cdot 2H_2O$: C, H, N	725
14b	C(NH)NH_2	D-Glu	−67.0 (1.0, 25)	0.77	0.60	0.29	Tyr, 0.17;[d] Glu, 0.96; Gly, 1.02; Pro, 1.02	$C_{31}H_{39}N_9O_{10}\cdot 3H_2O$: C, H[f]	698
19a	H	D-Cys(O_3H)	+11.6 (1.0, 25)[g]	0.86	0.96	0.36	ND[h]	$C_{28}H_{35}N_7O_{11}S\cdot HCl\cdot 3H_2O$: C, H,[i]	678
19b	H	D-Hcy(O_3H)	−13.8 (1.0, 25)	0.88	0.93	0.40	ND[h]	$C_{29}H_{38}N_7O_{11}SCl\cdot 3H_2O$: C, H, N	692
20a	H	D-Cys(SO_3H)	ND[h]	0.89	0.97	0.41	Tyr, 0.94; Gly, 1.04; Phe(4-NO_2), 0.82; Pro 0.96	$C_{28}H_{35}N_7O_{11}S_2Na\cdot CH_3CO_2H$: C, H, S[j]	630[k]
21a	H	D-Cys[$(CH_2)_2SO_3Na$]	ND[h]	0.88	0.96	0.31	Tyr, 0.94; Gly, 0.99; Phe(4-NO_2), 0.84; Pro, 1.01	$C_{30}H_{38}N_7O_{11}S_2Na\cdot 3H_2O$: C, H, N	738, 760[e]

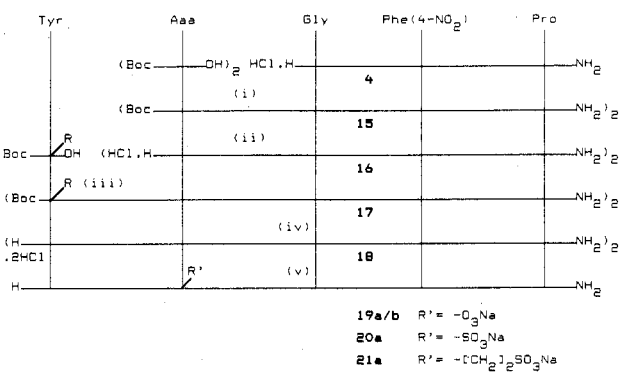
[a] Determined in MeOH. [b] Unnatural amino acids not determined unless otherwise noted. [c] (M + H^+) unless otherwise noted. [d] Tyr values consistently low when N-amidinated. [e] (M + Na^+). [f] N: calcd, 12.40; found, 13.20. [g] Determined in MeOH–H_2O (1:1). [h] Not determined. [i] N: calcd, 12.33; found, 12.77. [j] N: calcd, 16.78; found, 16.25. [k] [(M − SO_3) + 1]$^+$.

led to the synthesis of highly potent and stable pentapeptide analogues of the enkephalins.[13]

Methods

Chemistry. All of the peptides were synthesized by classical methods in solution. Three synthetic routes were used, as exemplified in Schemes I–III. The strategy of minimal side-chain protection was adopted. Thus, the phenolic group of tyrosine was unprotected, arginine was incorporated with the guanyl group in protonated form, glutamic acid was incorporated as the γ-*tert*-butyl ester, and cysteine and homocysteine[14] were incorporated as the disulfides; the *tert*-butyloxycarbonyl group was used for α-amino protection. The excess mixed anhydride (EMA)[15] method of coupling was generally used, taking advantage of the high yields and state of purity of the products so obtained. Couplings to proline and fragment condensations were mediated by DCCI–HOBt,[16] the former to avoid urethane formation and the latter to minimize racemization.[17]

Scheme III[a]



[a] Reagents: (i) DCCI, HOBt, NMM, DMF, 0 °C; (ii) 1 M HCl–HOAc; (iii) *i*-BuOCOCl, NMM, THF, DMF, −15 °C; (iv) 1 M HCl–HOAc, anisole; (v) (**19a/b**) HCO_2H, H_2O, H_2O_2, −10 °C; (**20a**) Na_2SO_3, $Na_2S_4O_6$, MeOH, H_2O; (**21a**) *n*-Bu_3P, $NaHCO_3$, *i*-PrOH, H_2O; then $Br[CH_2]_2SO_3Na$, H_2O.

(1) Abbreviations used include the following: acetic acid (AA), 1-amidino-3,5-dimethylpyrazole acetate (ADMP), diisopropylethylamine (DIPEA), diketopiperazine (DKP), dicyclohexylcarbodiimide (DCCI), dimethylformamide (DMF), 1-hydroxybenzotriazole (HOBt), high-performance liquid chromatography (HPLC), 4A molecular sieve (MS4A), *N*-methylmorpholine (NMM), phenyl-1,4-benzoquinone (PBQ), tetrahydrofuran (THF), intraperitoneal (ip), subcutaneous (sc), oral (po).

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(17) In the case of the D-Arg^2 peptide (**7b**), 3–4% of the L-Arg^2 isomer was formed under these conditions (HPLC) but could be removed by treatment of the deprotected product with trypsin, followed by chromatographic purification (data not shown). In the other cases, the extent of racemization was not checked.

The peptides containing D-Met[2] were assembled stepwise as shown in Scheme I. The synthesis was straightforward except that the dipeptide amide **2** showed the expected propensity to cyclize and required careful manipulation. Tripeptide **4** was a key intermediate common to all of the syntheses. The deprotected D-Met[2] pentapeptide **8** was S-methylated with MeI–MeOH to yield the sulfonium derivative **9**, which was further converted to the N^{α}-amidino peptide **10** by reaction with 1-amidino-3,5-dimethylpyrazole (ADMP).[18]

For peptides containing D-Arg[2] and D-Glu[2], the sequences were assembled by 2 + 3 fragment couplings of the appropriate N-terminal tyrosine dipeptide with the common tripeptide precursor **4** as shown in Scheme II. The syntheses of the dipeptide fragments **11a,b** were generally uneventful except that the polar nature of the D-Arg compound complicated its purification (see the Experimental Section). The D-Glu[2] pentapeptide **12b** was assembled without characterization of intermediate products. The deprotected pentapeptides **13a,b** were again converted to the *N*-amidino peptides **14a,b** as described above.

The peptides containing D-Cys and D-Hcy were assembled as the disulfide-linked dimers by stepwise elongation of the common tripeptide (**4**) by using the same methodology as above, again without characterization of the intermediates (Scheme III). In the synthesis of **19b**, the phenolic group of tyrosine was protected as the *tert*-butyl ether. For the preparation of the sulfonic acid derivatives, the relevant parent D-Cys[2] (**18a**) or D-Hcy[2] (**18b**) pentapeptide dimer was oxidized with performic acid[19] (**19a,b**), subjected to sulfitolysis[20] (**20a**), or reduced with Bu_3P[21] and alkylated with 2-bromoethanesulfonic acid (**21a**).

Each of the pentapeptide derivatives required for biological testing was purified by ion-exchange chromatography and in some cases by preparative HPLC. The synthetic peptides were characterized by optical rotation, TLC, amino acid analysis, elemental analysis, and FAB-MS. The data are presented in Table I.

Pharmacology. The in vitro biological activity of the polar pentapeptides (**9**, **10**, **13a,b**, **14a,b**, **19a,b**, **20a**, and **21a**) was evaluated on the isolated guinea pig ileum by determination of the inhibition of electrically induced contractions. The opioid nature of these inhibitions was confirmed by their reversal in the presence of naloxone (1 $\mu g\ mL^{-1}$).

In vivo antinociceptive activity was determined in mice by using both chemical and thermal noxious stimuli. Chemically induced writhing assays were carried out with intraperitoneal injections of two irritants, phenyl-1,4-benzoquinone (PBQ) and acetic acid (AA). Such writhing assays have been used previously to investigate the antinociceptive effects of nonsteroidal antiinflammatory drugs[22,23] and peripherally acting opioids.[12] In the latter study, the peripheral nature of the antinociceptive effects of *N*-methylmorphine was demonstrated by antagonism of these effects by the peripherally acting opioid antagonist *N*-methylnalorphine. The centrally mediated antinociceptive effects of morphine were not antagonized by *N*-methylnalorphine. In the present study, the peripheral nature of the inhibitory effects of the pentapeptide analogues in acetic acid induced writhing was also investigated with *N*-methylnalorphine. Antagonism is expressed by means of dose ratio, which is defined as the ratio of the doses of agonist required to produce an equiactive effect in the absence and presence of the antagonist. A dose ratio of unity, therefore, signifies no antagonism, and a high ratio expresses significant antagonism and hence indicates a peripherally mediated effect.

Activity was also determined in the hot-plate assay. In contrast to the writhing assays, such heat-induced noxious assays detect only centrally acting opioids.[24] Thus a comparison of the antinociceptive potencies of opioids in writhing and hot-plate tests provides an index of peripheral or central activity. A peripheral mode of action is indicated by a high potency in the writhing tests coupled with a low potency in the hot-plate test.

Table II. log *P* Values for Selected Compounds

compd	log P^a	compd	log P^a
9	−0.89 ± 0.05	**19a**	−0.38 ± 0.02
13a	−0.79 ± 0.01	**20a**	+0.39 ± 0.01
13b	+0.02 ± 0.02	**22**	+1.23 ± 0.01
14a	−0.98 ± 0.02		

[a] Determined between 1-butanol and 50 mM phosphate buffer, pH 7.4.

Discussion

We took as our starting point the highly potent pentapeptide amide, Tyr-D-Met-Gly-Phe(4-NO_2)-Pro-NH_2, **22**, previously prepared in these laboratories.[13] Although this compound has low oral activity, it does display potent central activity after subcutaneous administration. For this work we wished to obtain opioid peptides whose activity would be restricted to the periphery. The strategy we adopted to achieve this goal was to prepare highly polar, hydrophilic analogues of **22** and thus restrict access to the CNS by reducing penetration of the blood–brain barrier. A large number of earlier structure–activity studies in the enkephalin field have shown that the glycine reside at position 2 in the parent sequences may be replaced with a variety of D-amino acid residues, of widely differing physicochemical properties, with retention of activity.[6,25] Thus our objective was to prepare analogues having a polar (usually acidic or basic) D-amino acid residue in place of the D-Met[2] residue in **22**. Initial investigations centered around the sulfonium salt **9** (Table I), which was easily obtained from **22** by methylation of the methionine thioether. The sulfonium analogue was comparatively unstable due to facile elimination, but did show a profile of biological activity, which suggested a predominantly peripheral mode of action. Encouraged by this result, we synthesized a series of analogues of **22** containing D-Arg[2], D-Glu[2], or sulfonic acid derivatives based on D-Cys[2] or D-Hcy[2] (Table I). In selected cases the N-terminal amino group of the Tyr residue was converted to a guanidino group to further increase hydrophilicity.

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Table III. In Vitro and in Vivo Biological Activities of Polar Enkephalin Pentapeptide Analogues

compd	GPI, nM	PBQ writhe: sc, ED_{50}, mg kg^{-1}	AA writhe: sc, ED_{50}, mg kg^{-1}	dose ratio[a]	hot plate: sc, ED_{50}, mg kg^{-1}
9	61.2	3.1	8.1	3.3	NE @ 50
10	525	1.1	0.5	4.2	10.9
13a	132	4.9	5.7	5.5	35.0
13b	9.0	3.0	1.4	5.5	7.6
14a	420	1.3	0.8	4.6	NE @ 25
14b	414	0.3	0.5	1.2	5.3
19a	89.4	ND	5.9	ND	19.0
19b	807	3.1	3.5	4.2	18.6
20a	7.5	0.4	0.5	3.9	9.7
21a	3.6	ND	0.1	4.0	ND
22	1.8	0.4	0.1	1.2	1.6
morphine	58.6	0.4	0.5	0.6	1.9

[a] Dose ratio in the presence/absence of *N*-methylnalorphine (11.5 mg kg^{-1}) in acetic acid (AA) writhing.

As a measure of hydrophilicity, log *P* values were determined for selected compounds (Table II). The measurements were performed in the system 1-butanol–50mM phosphate buffer, pH 7.4; the low solubility of the compounds in octanol rendered its use impractical. As can be seen from Table II, the most polar compound is the amidinated D-Arg^2 peptide **14a** and the order of decreasing hydrophilicity is **14a** > **9** > **13a** > **19a** > **13b** > **20a** ≫ **22**.

Table III summarizes the in vitro and in vivo properties of the polar enkephalin analogues. All of the analogues investigated (**9**, **10**, **13a,b**, **14a,b**, **19a,b**, **20a**, and **21a**) demonstrated in vitro opioid activity on the guinea pig ileum, the ethyl sulfonate derivative **21a** being the most potent. Similarly, the analogues all exhibited antinociceptive activity in the chemically induced writhing models, being of the same order of potency against the two irritants. In each case, N-amidination of the Tyr residue decreased potency on the guinea pig ileum but increased potency in the writhing tests (compare **10**, **14a,b** with **9**, **13a,b**, respectively). However, in general there was little correlation between the in vitro and in vivo data in these tests. This lack of apparent correlation between potencies on the guinea pig ileum and in antinociceptive assays is markedly different from the close association of these properties described for the classical alkaloid narcotics,[26] but generally poor correlations have been observed previously for enkephalin analogues.[27,28]

The introduction of sulfonium or acidic or basic amino acid residues at position 2 resulted in compounds that exhibited peripherally mediated antinociception as demonstrated by the ability of *N*-methylnalorphine to antagonize this effect. The dose ratios produced by pretreatment of the animals with *N*-methylnalorphine (11.5 mg kg^{-1} ip) were generally of similar magnitude (**9**, **10**, **13a,b**, **14a**, **19a,b**, **20a**, and **21a**: range of dose ratios = 3.3–5.5). The single exception to this general observation was the amidinated D-Glu^2 analogue (**14b**), which exhibited a dose ratio of 1.2, a value comparable to that observed for the relatively more lipophilic and centrally acting compounds **22** and morphine (Table III). This result appears particularly anomalous since **14b** is equipotent with **22** and morphine as an inhibitor of PBQ-induced writhing, but displays a ratio of potencies in the writhing and hot-plate tests akin to the general trend of the enkephalin analogues containing a basic amino acid residue and higher than that for **22** and morphine. For all of the compounds, the data from the writhing and hot-plate tests are indicative of a peripheral mode of action.

A number of the compounds were tested for oral activity in the PBQ-induced writhing assay. As expected, the observed activities were low, presumably due to poor absorption of these highly polar compounds. For example, compounds **9** and **13a** were inactive in this model at 100 mg kg^{-1} orally, and the more potent compounds such as **14b** and **21a** had ED_{50}s of 64.2 and 18.8 mg kg^{-1}, respectively.

In conclusion, this work has resulted in the development of compounds that possess the profile of pharmacological activity required for further investigation of the potential of polar opiates to mediate peripheral antinociception. From the data presented in Tables II and III, it is apparent that the D-$Met(Me^+)^2$ (**9**) and the D-Arg^2 analogues (**13a** and **14a**) have the most favorable profiles. That is, they are the most hydrophilic, have high dose ratios with respect to the peripheral antagonist, and have a good separation of potencies in the writhing versus hot-plate tests. However, the inadequate chemical stability of **9** led us to choose **13a** as the compound for further investigation. In vitro studies on the guinea pig ileum preparation with **13a** (BW 443C) and 16-methylcyprenorphine (M8008), a selective opioid antagonist with a low affinity at κ receptors,[29] has shown **13a** to be selective for μ receptors in this isolated tissue preparation. A detailed study of the pharmacology of **13a** (BW 443C) has been published elsewhere.[30–32] In addition, the compound has been shown to have antinociceptive activity in human volunteers in a model of cold-induced pain.[33] Such encouraging results have led to further investigations with structurally simpler enkephalin analogues[34] and polar analogues of other opiate drugs.[35] Further results from these studies will be reported at a future date.

Experimental Section

Melting points were determined on a Gallenkamp apparatus and are uncorrected. Optical rotations were determined with a Thorn-NPL automatic polarimeter, type 243. Amino acid analyses were performed on a Biotronik LC6001 or a Beckman 3201 amino acid analyzer with ninhydrin detection. Proton magnetic resonance spectra were recorded on Brucker WH 90, AM 200-SY, or AM 360 instruments; chemical shifts are reported in parts per million (δ) from internal tetramethylsilane. FAB-mass spectra were recorded on a VG ZAB 1F or a Kratos MS 50 TC instrument.

TLC was performed on 0.25 mm thickness silica gel plates (Merck, silica gel 60, F-254); the following solvent systems were used: (A) $CHCl_3$–MeOH, 8:1, v/v; (B) $CHCl_3$–MeOH–32% HOAc, 120:90:5, v/v; (C) $CHCl_3$–MeOH–"0.88"NH_4OH, 120:90:5, v/v; (D) $CHCl_3$–MeOH–32% HOAc, 120:90:40, v/v; (E) $CHCl_3$–MeOH–"0.88"NH_4OH, 120:90:40, v/v; (F) *n*-BuOH–HOAc–H_2O, 3:1:1, v/v; (G) CH_2Cl_2–MeOH, 95:5, v/v. Compounds were visualized by viewing under ultraviolet light and/or by spraying with

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1% ninhydrin in Me_2CO or 1% *t*-BuOCl–cyclohexane followed, after ca. 5 min, by 1% starch–1% KI in water. Ion-exchange chromatography was performed with Whatman CM52 carboxymethyl cellulose eluted with a linear gradient of aqueous NH_4OAc, pH 5.1 (0.005–0.5 M); fractions were collected with an LKB automated fraction collector. Analytical reverse-phase HPLC was carried out with a Waters ALC with Du Pont Zorbax C8 prepacked columns. Reverse-phase desalting of peptides was performed on a Waters Prep LC 500 instrument with C18 Prep Pak cartridges; samples were applied in aqueous solution, the column was washed with water, and the products were eluted with 5% HOAc–MeOH.[36]

Solutions in organic solvents were dried over anhydrous $MgSO_4$. Solvents were evaporated on a Büchi Rotavapor with a water-bath temperature < 40 °C at water-pump pressure except for DMF when high vacuum was required. Isobutyl chloroformate, anisole, NMM, and DCCI were redistilled before use; THF was distilled from CaH_2 and stored over MS4A; HPLC-grade DMF (Romil) was stored over prebaked MS4A for 24 h before use; HOBt was recrystallized from water and thoroughly dried before use.

***N*-(*tert*-Butyloxycarbonyl)-L-4-nitrophenylalanyl-L-prolinamide (1).** A solution of Boc-L-Phe(4-NO_2)-OH (110.7 g, 357 mmol) and HOBt (96.4 g, 714 mmol) in DMF (1 L) was cooled to −10 °C. DCCI (73.5 g, 357 mmol) was added, and the mixture was stirred at −5 °C for 30 min. H-L-Pro-NH_2·HCl (53.75 g, 357 mmol) and NMM (36.1 g, 357 mmol) were added, and the mixture was stirred at 4 °C for 18 h. The suspension was filtered, and the filtrate was concentrated in vacuo to an oil. The oil was dissolved in EtOAc (2.5 L) and filtered; the organic phase was washed successively with 500-mL portions of half-saturated aqueous NaCl (once), 5% aqueous citric acid (twice), 5% aqueous $NaHCO_3$ (three times), and half-saturated aqueous NaCl (twice). The solution was filtered through phase-separating paper and dried, and the filtrate was stored at 4 °C for 18 h. The crystalline product was collected by filtration, washed with EtOAc, and dried in vacuo, yield 69.6 g. The mother liquor was concentrated and diluted with Et_2O to give a second crop, yield 55.3 g. Total yield 124.9 g (86%): mp 186.5–188 °C; $[\alpha]^{21}_D$ −30.2° (*c* 1.0, MeOH); TLC R_f (B) 0.81, R_f (C) 0.75, R_f (F) 0.69. Anal. ($C_{19}H_{26}N_4O_6$) C, H, N.

L-4-Nitrophenylalanyl-L-prolinamide Hydrochloride (2). The following conditions must be strictly followed to avoid DKP formation. The Boc compound (1) (204 g, 502 mmol) was dissolved in a mixture of EtOH (2 L) and EtOAc (3.5 L) by warming the mixture to 55 °C, and the solution was cooled to 15 °C. Dry HCl gas was bubbled through the stirred mixture for 60 min with the temperature maintained at 15–20 °C; dry N_2 gas was then passed through the solution for 40 min. The solution was seeded and diluted with Et_2O (8 L). The solid was filtered off, washed well with Et_2O (3 L), and dried in vacuo over NaOH, yield 183 g (106%): mp 221–223 °C dec; $[\alpha]^{23}_D$ −14.2° (*c* 1.0, MeOH); TLC R_f (B) 0.24, R_f (C) 0.39, R_f (F) 0.28. Attempted recrystallization of this product or heating in vacuo to complete drying generated significant quantities of DKP; consequently, the material was used directly in the next stage. An acceptable microanalysis could not be obtained due to incomplete drying.

***N*-(*tert*-Butyloxycarbonyl)glycyl-L-4-nitrophenylalanyl-L-prolinamide (3).** A solution of (2) (150.9 g, 440 mmol) and NMM (44.5 g, 440 mmol) in a mixture of DMF (680 mL) and water (85 mL), apparent pH 7, was cooled to −25 °C. Meanwhile, a solution of Boc-Gly-OH (84.8 g, 485 mmol) and NMM (49 g, 485 mmol) in THF (850 mL) was cooled to −25 °C, a precooled (−20 °C) solution of isobutyl chloroformate (63.1 g, 462 mmol) in THF (85 mL) was added, and the mixture was stirred at −15 °C for 2.5 min to form the mixed anhydride. The above solution of the amino component (2) was added, and the mixture was stirred for 2.5 h at −15 °C; 2 M $KHCO_3$ (520 mL) was added, and the mixture was stirred at 0 °C for 30 min and then concentrated in vacuo. The residual oil was distributed between EtOAc (3 L) and half-saturated aqueous NaCl; the organic phase was washed successively with 500-mL portions of 5% aqueous citric acid (three times), 5% aqueous $NaHCO_3$ (three times) and half-saturated aqueous NaCl (twice). The solution was dried and concentrated to an oil, which solidified on trituration with a mixture of petroleum ether (bp 60–80 °C, 500 mL) and Et_2O (500 mL). The solid was collected and dried, yield 172.7 g (85%): $[\alpha]^{21}_D$ −32.1° (*c* 1.3, MeOH); TLC R_f (A) 0.48, R_f (B) 0.79, R_f (C) 0.82, R_f (F) 0.67; Anal. ($C_{21}H_{29}N_5O_7 \cdot 0.5H_2O$) C, H, N.

Glycyl-L-4-nitrophenylalanyl-L-prolinamide Hydrochloride (4). To a stirred solution of the above Boc compound (3) (198 g, 430 mmol) in glacial HOAc (1 L) was added 2 M HCl in HOAc (1 L). The mixture was stirred at ambient temperature for 45 min and then concentrated. The residual oil solidified on trituration with dry Et_2O. The solid was recrystallized from EtOH–*i*-PrOH (5:2, 1.75 L) and dried to yield 137.3 g of product; a second crop was obtained on concentration of the mother liquors, 15.8 g. Total yield 153.1 g (89%): $[\alpha]^{19}_D$ −16.2° (*c* 1.0, MeOH); TLC R_f (B) 0.25, R_f (C) 0.41, R_f (F) 0.19; Anal. ($C_{16}H_{22}N_5O_5Cl$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-D-methionylglycyl-L-4-nitrophenylalanyl-L-prolinamide (5).** Boc-D-Met-OH·DCHA (312 g, 0.726 mol) was suspended in ether (3.5 L) and stirred with a solution of $KHSO_4$ (150 g) in water (1.4 L). The phases were separated, and the aqueous layer was extracted with ether (400 mL). The combined ether phases were washed with water (3 × 500 mL), dried, and evaporated to give Boc-D-Met-OH as an oil. This material was coupled to the tripeptide 4 (263.7 g, 0.66 mol) by the mixed-anhydride method as exemplified for 3 above. After workup, a solid product was obtained by trituration with dry ether, yield 362.7 g (92.5%): $[\alpha]^{23}_D$ −3.2° (*c* 1, MeOH). Anal. ($C_{26}H_{38}N_6O_8S \cdot 0.25H_2O$) C, H, N.

D-Methionylglycyl-L-4-nitrophenylalanyl-L-prolinamide Hydrochloride (6). The tetrapeptide 5 (362 g, 0.61 mol) was deprotected with 1 M HCl–HOAc (3.2 L) as described for 4 but with the addition of redistilled anisole (1.33 L) as scavenger. A solid product was obtained after exhaustive trituration with ether and was dried to yield 6, 316.7 g (97.9%): $[\alpha]^{20}_D$ −33.7° (*c* 1, MeOH). Anal. ($C_{21}H_{31}N_6O_6SCl \cdot 1.5H_2O$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-methionylglycyl-L-4-nitrophenylalanyl-L-prolinamide (7).** Boc-L-Tyr-OH (183.9 g, 0.654 mol) was coupled to tetrapeptide 6 (315.7 g, 0.595 mol) by the EMA method as described for 3. After workup, a solid product was obtained by ether trituration, yield 417.5 g (92.7%): $[\alpha]^{20}_D$ +15.1° (*c* 1, MeOH). Anal. ($C_{35}H_{47}N_7O_{10}S$) C, H, N.

L-Tyrosyl-D-methionylglycyl-L-4-nitrophenylalanyl-L-prolinamide Hydrochloride (8). The protected pentapeptide 7 (417 g, 0.555 mol) was deprotected in two batches each with 1 M HCl–HOAc (1.5 L)–anisole (610 mL). The solid (382 g) obtained after trituration with ether was treated in four portions as follows. The solid was dissolved in water (1.5 L) and extracted with EtOAc (3 × 500 mL) to remove residual anisole. The aqueous phase was concentrated to remove EtOAc, diluted with water, and freeze-dried, combined yield 349.6 g (91.5%): $[\alpha]^{24}_D$ +12.1° (*c* 1, MeOH). Anal. ($C_{30}H_{39}N_7O_8S \cdot HCl \cdot 1.5H_2O$) C, H, N.

L-Tyrosyl-D-*S*-methylmethionylglycyl-L-4-nitrophenylalanyl-L-prolinamide Chloride Hydrochloride (9). Pentapeptide 8 (11.40 g, 16.4 mmol) was dissolved in MeOH (20 mL) and MeI (4.7 g, 33 mmol) added. The solution was stored in the dark at ambient temperature for 7 days and then evaporated. The residue was dissolved in water (150 mL), washed with ether (50 mL), and purified by ion-exchange chromatography on a 9 × 36 cm CM 52 column eluted with a 20-L gradient. The eluate was monitored by HPLC, and product fractions were pooled and reverse-phase desalted. The MeOH was evaporated; water (200 mL) and 1 M HCl (20 mL) were added to the residue. The resultant solution was freeze-dried to leave the product as a white solid, yield 6.77 g (52%) (Table I).

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-arginine Hydrochloride (11).** Boc-L-Tyr-OH (51 g, 182 mmol) was coupled to H-D-Arg-OMe·2HCl (43.1 g, 165 mmol) by the excess mixed-anhydride procedure as described for compound 3. After concentration of the reaction mixture, the crude product was distributed between EtOAc (1.5 L) and water (0.5 L). The organic phase was further extracted with water (2 × 200 mL). The combined aqueous phases were adjusted to pH 7 with HOAc, saturated with NaCl, and extracted with $CHCl_3$–*n*-BuOH (5:1, 1.56 L) and then with $CHCl_3$ (2 × 1 L). The combined organic extracts were washed with saturated aqueous NaCl (2 × 400 mL),

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dried, and concentrated to an oil. Trituration with Et_2O gave the dipeptide ester as a solid, yield 77 g (103%): TLC R_f (B) 0.46, R_f (F) 0.24. Anal. ($C_{21}H_{33}N_5O_6 \cdot 1.5H_2O$) C, H, N. This material was dissolved in MeOH (720 mL)–water (180 mL), and 1 M NaOH (330 mL) was added. The solution was stirred at 23 °C for 60 min, when TLC in solvent (B) indicated complete reaction. HCl (1 M, 330 mL) was added, the MeOH was removed by evaporation, and the residual aqueous solution was freeze-dried. The resulting solid was reverse-phase desalted in four batches. The combined eluates were concentrated, and the residue was dissolved in water and lyophilized to give the product as the free base, yield 56.8 g; elemental analysis indicated that this material was a sesquihydrate. The product was suspended in water (1 L), and 1 M HCl (122.4 mL) was added; the solution was filtered, diluted to 1500 mL, and freeze-dried. The fluffy solid was collected with the aid of dry Et_2O and dried in vacuo, yield 60.3 g (77%): $[\alpha]^{23}_D$ +6.43° (*c* 1.0, MeOH); TLC R_f (B) 0.39, R_f (C) 0.42, R_f (F) 0.48. Anal. ($C_{20}H_{31}N_5O_6 \cdot HCl \cdot H_2O$) C, H, N, Cl.

L-Tyrosyl-D-arginylglycyl-L-4-nitrophenylalanyl-L-prolinamide Diacetate (13a). Boc compound 11 (47.4 g, 100 mmol) was coupled to amino compound 4 (40 g, 100 mmol) by using the DCCI–HOBt method as exemplified for 1. The reaction was allowed to proceed for 72 h at 4 °C. After filtration and concentration, the crude product was distributed between EtOAc (600 mL) and water (900 mL), the aqueous layer was further extracted with EtOAc (2 × 250 mL), and the organic extracts were discarded. The product was then extracted from the water layer with EtOAc–*n*-BuOH (5:1, 3 × 1 L), and the combined extracts were back washed with saturated NaCl (2 × 500 mL) and then washed successively with 300-mL portions of 5% $NaHCO_3$–saturated NaCl (1:1, twice), 5% Na_2CO_3–saturated NaCl (1:1, twice), 5% $NaHCO_3$–saturated NaCl (1:1, twice) and saturated NaCl (once) to remove HOBt completely. The solvents were evaporated, and the residue was reconcentrated from EtOH–water (twice) and EtOH (twice). The residue was triturated with Et_2O to give **12a** as a solid, yield 63.9 g. A further batch (6.6 g) was obtained by reworking the aqueous phases, total yield 70.5 g (86%). The product was dissolved in a mixture of HOAc (260 mL), MeOH (85 mL), and anisole (107 mL), and the stirred mixture was treated with 2 M HCl–HOAc (460 mL) for 45 min at ambient temperature. The mixture was concentrated, and the residue was triturated with dry Et_2O; the resultant solid was dissolved in water (1 L), and the solution was washed with Et_2O (3 × 250 mL) to remove residual anisole and then freeze-dried to give crude product (62.5 g). This material was purified in four batches by ion-exchange chromatography on a CM52 column (9 × 36 cm) eluted with a 20-L gradient. Fractions containing pure product were identified by analytical HPLC, pooled, and desalted. The eluate was evaporated, and the residue was dissolved in water, filtered, and freeze-dried to give the pure peptide **13a**, yield 55.3 g (66%) (Table I).

N^1-Amidino-L-tyrosyl-D-arginylglycyl-L-4-nitrophenylalanyl-L-prolinamide Diacetate (14a). The D-Arg^2 pentapeptide **9** (0.5 g, 0.61 mmol) was dissolved in DMF (0.5 mL) and EtOH (2 mL). 1-Amidino-3,5-dimethylpyrazole acetate (0.15 g, 0.76 mmol) and NEt_3 (0.12 mL, 0.87 mmol) were added, and the mixture was stirred at 55 °C for 6 h and then at room temperature for 16 h. The solvents were evaporated, and the residue was triturated with EtOAc to give the crude product. Purification was achieved by ion-exchange chromatography on a CM52 column eluted with a 2-L gradient. Product fractions were pooled and freeze-dried, yield 0.37 g (42%) (Table I).

L-Tyrosyl-D-3-(sulfomethyl)alanylglycyl-L-4-nitrophenylalanyl-L-prolinamide Hydrochloride (19b). (Boc-D-Hcy-OH)$_2$ (4.10 g, 8.75 mmol), HOBt (4.73 g, 35 mmol), and **4** (7.0 g, 17.5 mmol) were dissolved in DMF, and the solution was cooled to −25 °C. DCCI (3.61 g, 17.5 mmol) and NMM (1.77 g, 17.5 mmol) were added, and the mixture was stirred at 5 °C for 18 h. The DCU was filtered off, and the solvent was evaporated. The residue was distributed between EtOAc (400 mL) and water (50 mL) and filtered. The organic phase was washed successively with 40-mL portions of 5% citric acid (twice), water (once), 5% $NaHCO_3$ (twice), water (once) and dried. Evaporation gave an oil, which solidified on trituration with petroleum ether. The solid was collected and dried, yield 8.97 g (88%). TLC showed two components, the product and DCU (less polar). TLC R_f (B) 0.88, 0.92; R_f (C) 0.78, 0.81; R_f (F) 0.66, 0.92. The solid was dissolved in a mixture of anisole (154 mL) and 1 M HCl–HOAc (230 mL) and stirred at ambient temperature for 45 min. The suspension was concentrated, and the residue was triturated with dry Et_2O to give a solid, which was collected and dried, yield 8.13 g. This material was coupled to Boc-Tyr(*t*-Bu)-OH·DCHA (17.4 mmol) by the EMA method as exemplified for **3**, and the crude product was purified by preparative HPLC on a Prep 500 LC with two silica gel cartridges eluted with 10% MeOH in CH_2Cl_2. Product fractions were evaporated, and the residue was triturated with Et_2O to give a white solid: 6.92 g (55%); R_f (F) 0.81; R_f (G) 0.55. A portion (0.5 g, 0.31 mmol) of the solid was N-deprotected with HCl–HOAc as above. The product (**18b**) (0.46 g, 0.34 mmol) was stirred at −10 °C for 45 min with a mixture of formic acid (16 mL), water (0.7 mL), and 30% H_2O_2 (1.7 mL), which had been preincubated at 22 °C for 30 min. The reaction mixture was freeze-dried. The residue was relyophilized from water and finally from water containing 1 equiv of HCl to give the pure product, yield 0.33 g (67%) (Table I).

L-Tyrosyl-D-S^3-sulfocysteinylglycyl-L-4-nitrophenylalanyl-L-prolinamide Sodium Salt (20a). Pentapeptide dimer **18a** (1.27 g, 0.95 mmol) was dissolved in water (200 mL), and the solution was adjusted to pH 8.5 with aqueous ammonia. MeOH (400 mL) was added followed by Na_2SO_3 (5.13 g, 40.8 mmol) and $Na_2S_4O_6$ (2.30 g, 8.5 mmol). The solution was adjusted to pH 9.3 with aqueous ammonia and then stirred at ambient temperature for 4 days. The MeOH was evaporated, and the residue was diluted with water and desalted by reverse phase. After evaporation of the solvents, the residual oil was extracted with dilute aqueous ammonia, filtered, and freeze-dried. This process was repeated with dilute aqueous AcOH. The product was finally purified by preparative reverse-phase HPLC under isocractic conditions with 27% MeCN–0.1 M NH_4OAc, pH 4. The peptide was isolated by freeze-drying, yield 215 mg (14%) (Table I).

L-Tyrosyl-D-S^3-(2-sulfoethyl)cysteinylglycyl-L-4-nitrophenylalanyl-L-prolinamide Sodium Salt (21a). Pentapeptide dimer **18a** (100 mg, 0.073 mmol) was suspended in a mixture of propan-1-ol (50 mL) and 0.5 M $NaHCO_3$ (50 mL). A 1% solution of n-Bu_3P in propan-1-ol (2 mL, ca. 0.1 mmol) was added, and the mixture was stirred for 1 h. A second portion of the phosphine solution (2 mL) was added, and stirring was continued. After 2 h, analytical HPLC (isocratic, 35% MeCN–0.1 M NH_4OAc, pH 4) indicated complete reduction. A solution of $Br(CH_2)_2SO_3Na$ (760 mg, 1.8 mmol, 25 equiv) was added, and the mixture was stirred for 18 h. A second equal portion of the bromide was added, and stirring was continued. Analytical HPLC indicated ca. 80% reaction after 7 days. The reaction mixture was evaporated to dryness, and the residue was desalted by reverse phase. The elution solvents were evaporated, and the residue was freeze-dried (130 mg). The product was purified by preparative HPLC with 20% MeCN–0.1 M NH_4OAc, pH 4, and isolated by freeze-drying, yield 50 mg (42%) (Table I).

Pharmacological Methods. A. Isolated Guinea Pig Ileum. Segments of the terminal portion of the ileum of guinea pigs (300–350 g) were suspended in a 20-mL organ bath under 1-g tension and bathed in Krebs' bicarbonate solution gassed with 95% O_2 and 5% CO_2. Contractions were induced by coaxial stimulation of the ileum with pulses at 0.1 Hz, 0.5-ms duration, and at supramaximal voltage and recorded by means of isotonic transducers. Dose–response curves were constructed allowing 15-min washout between doses.

B. Writhing Assays. (a) Groups of five to six female Charles River mice of the CDl strain were injected intraperitoneally with phenyl-1,4-benzoquinone (PBQ) at 2.5 mg kg^{-1} in a dose volume of 10 mL kg^{-1}. The irritant induced a syndrome (writhing) characterized by a series of abdominal constrictions and/or hind-limb extensions, which were counted for a 2.5-min period commencing 10 min after PBQ injection. Vehicle or drugs were administered subcutaneously 30 min prior to PBQ in a dose volume of 10 mL kg^{-1}. Antinociceptive activity was assessed in terms of an ED_{50} and determined by linear regression. The ED_{50} was defined as that dose of drug that induced a 50% reduction in the number of writhes obtained compared to vehicle administration alone.

(b) Groups of five to six male Tuck mice of the TFW strain were injected intraperitoneally with acetic acid (0.6%) in a dose

volume of 25 mL kg^{-1}. Writhes were counted for a 5-min period commencing 15 min after acetic acid injection. *N*-Methylnalorphine (11.5 mg kg^{-1}) was administered intraperitoneally 20 min prior to the analogues, which were administered subcutaneously, both antagonist and test compounds being given in a dose volume of 10 mL kg^{-1}. Antinociceptive activity was determined as above. Dose ratios were determined as the shift of the parallel regression lines in the absence and presence of *N*-methylnalorphine.

C. Hot-Plate Tests. Groups of five to six male Hacking and Churchill mice of the CFLP strain were used. Each mouse was placed on to a copper surface maintained at 55 °C and observed for signs of discomfort such as licking/shaking of the paw or jumping. A cut-off time of 30-s exposure was used to prevent tissue damage. Drugs were administered subcutaneously in a dose volume of 10 mL kg^{-1}. ED_{50}s were defined as that dose of drug that increased the latency of response twofold compared to vehicle and were determined by parallel-line probit analysis.

Registry No. **1**, 73385-89-4; **2**, 88331-07-1; **3**, 88331-08-2; **4**, 88331-09-3; **5**, 94213-45-3; **6**, 113132-70-0; **7**, 73385-91-8; **8**, 88331-15-1; **9**, 88331-16-2; **9** (free base), 113132-71-1; **10**, 88331-19-5; **10** (free base), 113132-73-3; **11a**, 88331-11-7; **12a**, 88331-12-8; **13a**, 88331-14-0; **13a** (free base), 88331-13-9; **13b**, 113132-78-8; **13b** (free acid), 113132-86-8; **14a**, 88331-18-4; **14a** (free base), 88331-17-3; **14b**, 113132-79-9; **15b**, 113132-80-2; **16b**, 113132-81-3; **17b**, 113132-82-4; **18a**, 113159-95-8; **18b**, 113132-83-5; **19a**, 113132-75-5; **19a** (free base), 113132-87-9; **19b**, 113132-84-6; **19b** (free base), 113132-88-0; **20a**, 113132-76-6; **20a** (free acid), 113132-89-1; **21a**, 113132-77-7; **21a** (free acid), 113132-90-4; **22**, 70668-70-1; BOC-Phe(4-NO_2)-OH, 33305-77-0; H-Pro-NH_2·HCl, 42429-27-6; BOC-Gly-OH, 4530-20-5; BOC-D-Met-OH·DCHA, 61315-59-1; BOC-D-Met-OH, 5241-66-7; BOC-Tyr-OH, 3978-80-1; H-D-Arg-OMe·2HCl, 78851-84-0; BOC-Tyr-D-Arg-OMe, 88331-10-6; (BOC-D-Hcy-OH)$_2$, 113132-85-7; BOC-Tyr(Bu-*t*)-OH·DCHA, 30845-23-9; $BrCH_2CH_2SO_3Na$, 4263-52-9.

Synthesis and Biological Activity of CCK_{26-33}-Related Analogues Modified in Position 31

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Received July 1, 1987

The role of the amino acid in position 31 of cholecystokinin CCK_{26-33} in the recognition of central and peripheral receptors was investigated by replacement of methionine-31 by amino acids with side chains of various chemical nature. Thus, phenylalanine, alanine, glutamic acid, and ornithine and its analogue with the ϵ-amino group protected by a benzyloxycarbonyl group were introduced as X residues in Boc(Nle^{28},X^{31})-CCK_{27-33} since the related analogue Boc(Nle^{28},Nle^{31})-CCK_{27-33} was shown to be equipotent to CCK_{26-33}. The binding properties to both mouse brain membranes and guinea pig pancreatic acini and the peripheral activities (amylase secretion and contractile potency on guinea pig ileum) were determined. Whereas the introduction of phenylalanine, alanine, or ornithine residues in position 31 led to compounds that still displayed peripheral agonist properties, the presence of a negative charge in the side chain of the amino acid in position 31 prevented the binding of the peptide to both pancreatic and brain binding sites. Introduction of Phe^{31} and Ala^{31} residues increased the specificity of the peptides for the central receptors. Interestingly, when the amine function in the side chain of the ornithine-31 was protected by a benzyloxycarbonyl group, an unusual high affinity for pancreatic binding sites was observed and the related analogue proved to be a new peripheral CCK antagonist.

The sulfated C-terminal octapeptide of cholecystokinin Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (CCK_{26-33} or CCK_8), which possesses all the biological activities of the full hormone CCK_{33}, has been found discretely distributed in various areas of the central nervous system[1-4] where it appears to play either a classical neurotransmitter[5,6] or neuromodulator[7,8] role, especially at the level of the mesolimbic dopaminergic pathway.[9-11] At the present time, it is not clear if the various physiological actions of CCK_8 in the central nervous system are related to the occurrence of different classes of binding sites[12,13] or to an appropriate topological distribution of a single receptor.[14]

Furthermore, a number of behavioral effects have been obtained after systemic administration of low doses of CCK_8 in animals.[15,16] This raises the question of a possible involvement, in these pharmacological responses, of peripheral receptors[17,18] located for instance on the vagus nerve.[19] The peripheral receptors for CCK_8 seem to be structurally distinct from the brain receptors,[20] offering therefore the possibility for their respective roles to be studied with use of selective agonists and antagonists.[21] However, until now, few structure–activity studies on CCK_8 have been reported.[22-30] The most extensive studies used the C-terminal octapeptide fragment of ceruletide,[31,32] which differs from CCK_8 only in one amino acid residue

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0022-2623/88/1831-0966$01.50/0