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.

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

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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$_3$H)-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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Table I. Potencies of CCK$_8$ Analogues (1-5) in Inhibiting [^{3}H]Propionyl-CCK$_8$ Specific Binding in Mouse Brain Membranes and [^{3}H]Boc-(Nle^{28},Nle^{31})-CCK$_{27-33}$ Specific Binding in Guinea Pig Pancreatic Acini[a]

| | binding K_i^b M | |
compound	mouse brain membranes	guinea pig pancreatic acini
CCK$_{26-33}$ or CCK$_8$	$(0.30 \pm 0.07) \times 10^{-9}$	$(2.5 \pm 0.6) \times 10^{-9}$
Boc(Nle^{28},Nle^{31})-CCK$_{27-33}$	$(0.13 \pm 0.03) \times 10^{-9}$	$(2.9 \pm 0.7) \times 10^{-9}$
1, Boc(Nle^{28},Phe^{31})-CCK$_{27-33}$	$(3.7 \pm 0.4) \times 10^{-9}$	$(2.2 \pm 1.7) \times 10^{-7}$
2, Boc(Nle^{28},Ala^{31})-CCK$_{27-33}$	$(1.4 \pm 0.3) \times 10^{-8}$	$(1.1 \pm 0.2) \times 10^{-6}$
3, Boc(Nle^{28},Orn^{31}(Z))-CCK$_{27-33}$	$(4.9 \pm 1.2) \times 10^{-7}$	$(5.7 \pm 2.1) \times 10^{-8}$
4, Boc(Nle^{28},Orn^{31})-CCK$_{27-33}$	$(1.1 \pm 0.2) \times 10^{-7}$	$(3.9 \pm 1.2) \times 10^{-7}$
5, Boc(Nle^{28},Glu^{31})-CCK$_{27-33}$	$>10^{-5}$	$>10^{-5}$

[a] Values represent mean ±SEM of three separate experiments performed in triplicate. [b] [^{3}H]Propionyl-CCK$_8$ was used at the concentration of 0.4 nM (K_D = 0.2 nM) with brain tissue and [^{3}H]Boc(Nle^{28},Nle^{31})-CCK$_{27-33}$ at 0.5 nM (K_D = 1 nM) with pancreatic acini.

(threonine instead of methionine at the 6-position from the C-terminus).

The methionine in position 28 (or threonine in ceruletide) has been substituted by several amino acids [valine, norleucine, leucine, α-aminobutyric acid (Abu), α-amino-γ-methoxybutyric acid (Mox or methoxine)] without significant loss of biological activity.[31-35] Nevertheless, a decrease in biological activity was observed when methionine-28 was replaced by an aromatic amino acid (tyrosine, phenylalanine, tryptophan)[31] or by a small amino acid like glycine.[31]

Though peripheral activity is completely retained after the substitution of methionine by methoxine in position 28 (gall bladder contraction), the same exchange in position 31 leads to a significant decrease in biological activity.[35]
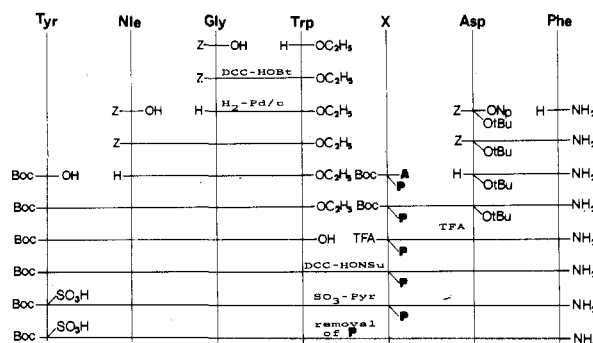

Figure 1. Scheme for the synthesis of compounds 1 (X = Phe), 2 (X = Ala), 3 (X = Orn(Z)), 4 (X = Orn), and 5 (X = Glu). A: activating group of COOH function of the amino acid X (ONp for Phe and Ala). P: protecting group of NH$_2$ or COOH side chain function of the amino acid X (Z for Orn and O-t-Bu for Glu).

A similar result had also been observed by Pluscec et al.[22] when substituting methionine by leucine in CCK$_8$. However, both methionine residues in positions 28 and 31 can be replaced by isosteric norleucine residues leading to compounds displaying a full potency in central as well as in peripheral tests.[31,36] Thus, in contrast to methionine-28, the replacement of the sulfur atom of methionine-31 by a methylene group seems to be more favorable for receptor interaction than its replacement with oxygen.

We have investigated the role of the amino acid in position 31 in the recognition of central and peripheral receptors by replacement of Met^{31} by five different amino acids with side chains of a different chemical nature. Thus, Phe, Ala, Orn(Z), Orn, and Glu were introduced as X residues in Boc(Nle^{28},X^{31})-CCK$_{27-33}$, since the related analogue Boc(Nle^{28},Nle^{31})-CCK$_{27-33}$, synthesized in our laboratory, was shown to be equipotent to CCK$_8$.[36] 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) are reported here.

Chemistry

Compounds 1-5 were synthesized according to Figure 1. Both fragments Boc-Tyr-Nle-Gly-Trp-OH (11) and Asp(O-t-Bu)-Phe-NH$_2$ (13) were common to the five related peptides. Compound 11 was prepared stepwise, starting from the C-terminal residue, using the DCC/HOBt condensation method and deprotection by catalytic hydrogenation, the last step corresponding to a saponification of the C-terminal ethyl ester.

The intermediate 13 was prepared with the active ester (benzyloxycarbonyl)-L-aspartic acid α-$tert$-butyl-β-p-nitrophenyl ester, which was allowed to react with phe-

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Table II. Pharmacological Potencies of CCK Analogues 1–5

compound	amylase secretion by guinea pig acini			contractile activities of guinea pig ileum	
	agonist act.: EC_{50},[a] M	antagonist act.		agonist act.: EC_{50},[b] M	antagonist act.: EC_{50},[b] M
		K_A,[b] M	pA_2		
CCK_{26-33} or CCK_8	$(1.3 \pm 0.3) \times 10^{-10}$			$(0.7 \pm 0.2) \times 10^{-9}$	
$Boc(Nle^{28},Nle^{31})$-CCK_{27-33}	$(1.6 \pm 0.3) \times 10^{-10}$			$(0.4 \pm 0.2) \times 10^{-9}$	
1, $Boc(Nle^{28},Phe^{31})$-CCK_{27-33}	$(3.4 \pm 0.2) \times 10^{-9}$			$(1.1 \pm 0.4) \times 10^{-8}$	
2, $Boc(Nle^{28},Ala^{31})$-CCK_{27-33}	$(2.5 \pm 1.3) \times 10^{-8}$			$(1.1 \pm 0.3) \times 10^{-7}$	
3, $Boc(Nle^{28},Orn^{31}(Z))$-$CCK_{27-33}$	$>10^{-5}$	$(4.8 \pm 1.8) \times 10^{-7}$	6.3	$>10^{-6}$	$(1.5 \pm 0.5) \times 10^{-7}$
4, $Boc(Nle^{28},Orn^{31})$-CCK_{27-33}	$(2.2 \pm 2.3) \times 10^{-8}$			$(1.9 \pm 0.5) \times 10^{-7}$	
5, $Boc(Nle^{28},Glu^{31})$-CCK_{27-33}	$>10^{-5}$			$>10^{-6}$	

[a] Results are the mean ± SEM of three separate experiments; each value in triplicate. [b] Results are the mean ± SEM of three separate experiments.

nylalanine amide. The resulting dipeptide 12 was then partially deprotected by catalytic hydrogenation to give compound 13.

When X was Phe or Ala, the corresponding tripeptides 15 and 18 were synthesized according to the active p-nitrophenyl ester method followed by a deblocking step with trifluoroacetic acid. When X was Orn(Z) or Glu(O-t-Bu), the DCC/HOBt condensation method was followed by deprotection of the Boc and O-t-Bu groups by trifluoroacetic acid to give compounds 21 and 24. Condensation of compound 11 with the different tripeptides 15, 18, 21, and 24 was obtained by the DCC/HONSu method. At this step of the synthesis, the lack of racemization of the heptapeptides was thoroughly checked by ^{1}H NMR spectroscopy.

The heptapeptides 16, 19, 22, and 25 were treated with SO_3-pyridine complex in a DMF-pyridine mixture to introduce a sulfate ester group in the tyrosine side chain.

In the case of compound 4, the last step of the synthesis was the removal of the benzyloxycarbonyl protecting group of the amine function of the ornithine residue, by catalytic hydrogenation. All the peptides were purified by silica gel column chromatography and lyophilized twice in a 0.1 M NH_4OH solution. The purity and the lack of racemization of each compound were checked by HPLC and ^{1}H NMR spectroscopy.

Biological Results

Binding Experiments. The five CCK_8 analogues reported in this paper were evaluated for their potency in displacing [^{3}H]propionyl-CCK_8 from mouse brain membranes and [^{3}H]Boc(Nle^{28},Nle^{31})-CCK_{27-33}[37] from guinea pig pancreatic acini. The apparent affinities (K_I) of the five analogues listed in Table I were lower than the one of CCK_8. Compounds 1 and 2 exhibited a significantly greater affinity for brain binding sites, whereas compound 3 displayed a higher affinity for the pancreatic binding sites. At the highest concentration used (10^{-5} M), compound 5 was unable to displace the tritiated probe while compound 4 was approximately equipotent on the two binding sites.

Amylase Release. The pancreozymin-like activities of the five CCK_8 analogues were assessed by measuring their effect on amylase secretion from guinea pig pancreatic acini, as previously described.[38] The efficiency of the five related analogues to stimulate amylase release is reported in Table II. Only three compounds (1, 2, and 4) gave dose–response curves with a similar shape to that of CCK_8. Moreover, compound 3 displayed an antagonist activity on amylase release from pancreatic acini[39] (Figure 2).
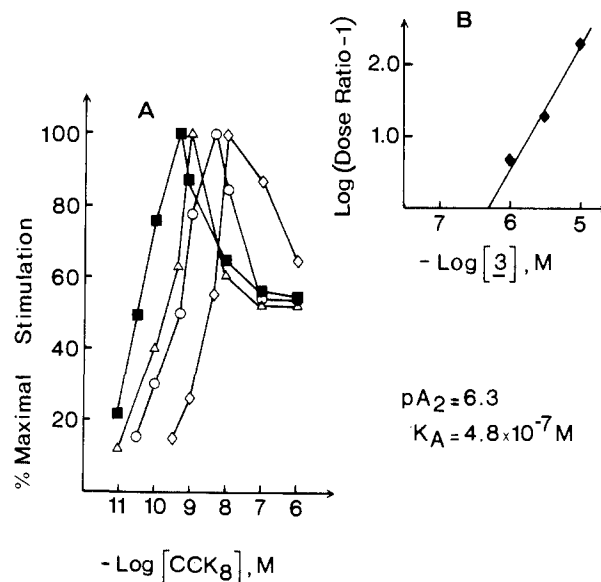

Figure 2. Antagonism by compound 3 of CCK_8-induced amylase release from guinea pig pancreatic acini. A: effect of compound 3 on stimulation of amylase release caused by different concentrations of CCK_8. Acini were incubated with increasing concentrations of CCK_8 alone (■) or in the presence of various concentrations of compound 3 [(△) 10^{-6} M, (○) 3×10^{-6} M, (◇) 10^{-5} M]. B: Schild plot of the antagonism by compound 3. Each point represents the mean value of triplicate determinations, and the experiment was made three times.

Guinea Pig Ileum Contractions. The ability of compounds 1–5 to stimulate the contraction of the isolated guinea pig ileum was used to evaluate their cholecystokinin-like activities.[40] Compounds 1, 2, and 4 displayed agonist properties whereas compounds 3 and 5 were devoid of activity at a concentration as high as 10^{-6} M. Moreover, compound 3 antagonized the CCK_8-induced contractions of guinea pig ileum (Table II).

Discussion

The introduction of a phenylalanine residue in position 31 (compound 1) slightly modifies the affinity for brain binding sites (12-fold decrease when CCK_8 is taken as the reference molecule) but leads to a larger decrease (90-fold) in the affinity for pancreatic binding sites. A similar discrimination is observed when the amino acid in position 31 is an alanine residue (compound 2), i.e. a 46-fold decrease in the affinity for brain binding sites versus a 440-fold lower affinity for pancreatic binding sites. Interestingly, these substitutions lead to an increase in the

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selectivity for the central receptors since compounds 1 and 2 are respectively 60- and 80-fold more potent at the level of the brain receptors. Moreover, both compounds 1 and 2 behave as agonists in the stimulation of the pancreatic secretion enzyme (with potencies 25- and 200-fold lower, respectively, than that of CCK$_8$) as well as in the CCK$_8$-induced contractions of the guinea pig ileum (with potencies 15- and 160-fold lower, respectively, than that of CCK$_8$).

Therefore, an aromatic ring in the side chain of the amino acid in position 31 does not induce a large decrease in the affinity and the peripheral activity of the peptide. Moreover, despite the small size of the alanine side chain, compound 2 still acts as a full peripheral agonist.

However, compound 5, which possesses a glutamic acid residue in position 31, is unable to interact with the brain and the peripheral CCK receptors, even at concentrations as high as 10^{-5} M. This result suggests that the presence of a negative charge in the side chain of Glu^{31} prevents the binding of the peptide, either by a repulsion between the charged carboxyl group and the receptor subsite expected to interact with the methionine-31 of CCK$_8$ and/or through Glu^{31}-induced unfavorable folding of the N-terminal part of the peptide,[41] inhibiting the receptor-recognition process.

In contrast, the presence of a positive charge in the side chain of Orn^{31} does not induce such a decrease in the affinity for both receptors and in the biological activity. Indeed, Boc(Nle^{28},Orn^{31})-CCK$_{27-33}$ (compound 4) displays an affinity for brain and pancreatic binding sites of about 10^{-7} M and a peripheral activity not very different from that of compound 2.

Interestingly, protection of the amine function of the ornithine residue by a benzyloxycarbonyl group in compound 3 leads to a greater fall in affinity for the brain binding sites (1630-fold decrease with CCK$_8$ as reference) than for the pancreatic binding sites (23-fold decrease). Despite its good affinity for the receptors of guinea pig pancreatic acini ($K_I = 5.7 \times 10^{-8}$ M), Boc(Nle^{28},Orn^{31}-(Z))-CCK$_{27-33}$ was shown to be devoid of agonist activity both in amylase secretion and contractions of the guinea pig ileum. In fact, this compound proved to be a new antagonist of peripheral CCK receptors, able to inhibit both amylase release ($K_A = 4.8 \times 10^{-7}$ M, p$A_2 = 6.3$) and guinea pig ileum contractions (EC$_{50} = 1.5 \times 10^{-7}$ M).

Taken together, these results suggest that position 31 of cholecystokinin is one of the key positions for the differentiation between the peripheral versus central receptors and probably for the effector-induced coupling of the receptor(s) to transduction entities.[42,43] It can be noticed that the recently designed antagonists of peripheral CCK receptors such as compound CR 1409[44] and compound L-364,718[21] contain structural components belonging to the C-terminal part of CCK$_8$.

Experimental Section

Synthesis. All protected amino acids were from Bachem AG. Solvents were of analytical grade from Prolabo. Melting points were taken on a Kofler apparatus and are uncorrected. Chromatography was carried out with Merck silica gel (230–400 mesh). For thin-layer chromatography (TLC), Merck plates precoated with F 254 silica gel were used with the following solvent systems

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(by volume): A, CHCl$_3$-MeOH (9:1); B, EtOAc-pyridine-AcOH-H$_2$O (80:20:6:11); C, EtOAc-pyridine-AcOH-H$_2$O (60:20:6:11); D, BuOH-AcOH-H$_2$O (4:1:1); E, CHCl$_3$-MeOH (8:2); F, CHCl$_3$-MeOH-AcOH-H$_2$O-EtOAc (35:15:3:1.5:1); G, EtOAc-pyridine-AcOH-H$_2$O (45:20:6:11). Plates were developed with UV, iodine vapor, ninhydrin, or Ehrlich's reagent. The structure of the compounds and of all the intermediates were confirmed by ^{1}H NMR spectroscopy (Brucker WH, 270 MHz). The purity was checked by HPLC (Waters apparatus) on a 250 × 4.6 mm Prolabo ODS2 5-μm column with Et$_3$N-H$_3$PO$_4$ buffer (TEAP, 0.025 M, pH 6.5)/CH$_3$CN system as eluent (flow rate, 1.5 mL/min) with UV (210 nm) or fluorescence (290 nm) detection. At each step of the synthesis, the lack of significant racemization of a given peptide was checked by ^{1}H NMR spectroscopy and by HPLC. Amino acid analyses were carried out on a LKB biochrom 4400 analyzer after hydrolysis by 5.6 M HCl containing 4% (v/v) thioglycolic acid, at 110 °C for 24 h. Mass spectra were recorded on a double-focusing VG 70-250 instrument. The FAB (fast atom bombardment) ionizations were obtained with a FAB saddle field source (Ion Tech Ltd., Teddington, UK) operated with xenon at 8 kV and 1 mA. Glycerol or cesium iodide was used for calibration. Accelerating voltage was set at 6 kV and resolution was 1200. Mass spectra were obtained in different matrices and processed by means of the VG-250 software package.

The following abbreviations were used: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; THF, tetrahydrofuran; AcOH, acetic acid; DMF, dimethylformamide; CHCl$_3$, chloroform; TFA, trifluoroacetic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DIEA, *N,N*-diisopropylethylamine; HONSu, *N*-hydroxysuccinimide; DCU, *N,N'*-dicyclohexylurea. Other abbreviations used were those recommended by the IUPAC-IUB Commission (*Biochem. J.* **1984**, *219*, 345).

Compounds 6 (Z-Gly-Trp-OC$_2$H$_5$), 7 (Gly-Trp-OC$_2$H$_5$), 8 (Z-Nle-Gly-Trp-OC$_2$H$_5$), 9 (Nle-Gly-Trp-OC$_2$H$_5$), 10 (Boc-Tyr-Nle-Gly-Trp-OC$_2$H$_5$), 11 (Boc-Tyr-Nle-Gly-Trp-OH), 12 (Z-Asp(O-*t*-Bu)-Phe-NH$_2$), and 13 (Asp(O-*t*-Bu)-Phe-NH$_2$) were prepared in the liquid phase, as previously described for the synthesis of the equipotent analogue of CCK$_{26-33}$, Boc(Nle^{28},Nle^{31})-CCK$_{27-33}$,[36] with ethyl ester instead of methyl ester as a carboxyl blocking group and benzyloxycarbonyl (Z) instead of *tert*-butyloxycarbonyl (Boc) as the amino-protecting agent for compound 6.

Boc-Phe-Asp(O-*t*-Bu)-Phe-NH$_2$ (14). To a chilled solution of compound 13 (0.17 g, 0.5 mmol) in DMF (5 mL) containing DIEA (0.17 mL, 1 mmol) were added Boc-Phe-ONp (0.22 g, 0.58 mmol) and HOBt (0.08 g, 0.5 mmol). The reaction mixture was stirred for 30 min at 0 °C and overnight at room temperature. After evaporation in vacuo, the residue was triturated with 10 mL of the solvent mixture EtOAc-ether (50:50) and washed several times with ether to yield a white solid: 0.23 g (81%); R_f 0.51 (A). Anal. (C$_{31}$H$_{42}$O$_7$N$_4$) C, H, N.

Phe-Asp-Phe-NH$_2$·TFA (15). Compound 14 (0.2 g, 0.34 mmol) dissolved in 1 mL of the TFA-H$_2$O (8:2) mixture was stirred at 0 °C for 1 h and at room temperature for 3 h, yielding after evaporation, precipitation, rinsing with dry ether, and filtration a while solid: 0.16 g (90%); R_f 0.15 (C).

Boc-Tyr-Nle-Gly-Trp-Phe-Asp-Phe-NH$_2$ (16). To a solution of compound 11 (0.255 g, 0.4 mmol) in dry DMF (2 mL) were added, at –10 °C, HONSu (0.046 g, 0.4 mmol) and DCC (0.083 g, 0.4 mmol). The reaction mixture was stirred for 30 min at –10 °C and then for 1 h at 0 °C and overnight at room temperature. To the above mixture was added, at 0 °C, a solution of compound 15 (0.217 g, 0.4 mmol) and Et$_3$N (56 μL, 0.4 mmol) in DMF (1 mL). The resulting mixture was stirred overnight at room temperature. After filtration of DCU and evaporation of DMF, the residue was triturated with EtOAc-ether, washed several times with ether to yield a white powder: 0.396 g (95%); R_f 0.60 (C). Anal. (C$_{55}$H$_{67}$O$_{12}$N$_9$) C, H, N.

Boc-Tyr(SO$_3$Na)-Nle-Gly-Trp-Phe-Asp(Na)-Phe-NH$_2$ (1). A solution of compound 16 (0.222 g, 0.212 mmol) in dry DMF (4 mL) and dry pyridine (4 mL) was treated with SO$_3$-pyridine complex (1.33 g) and was stirred overnight under N$_2$ at room temperature. After evaporation in vacuo, the residue was taken up in cold saturated NaHCO$_3$ solution and the mixture was stirred at 0 °C for 1 h with the pH maintained at about 7. A solid residue was collected by centrifugation and dried in vacuo (79 mg). The

suspended product was isolated by lyophilization followed by precipitation of inorganic salts in MeOH, filtration, and evaporation of MeOH in vacuo (312 mg). The two fractions were separately purified by chromatography on silica gel with EtOAc–pyridine–AcOH–H$_2$O (60:20:6:11) as eluent to yield 74 mg (31%) of compound 1; R_f 0.27 (C); HPLC t_R 8.4 min, eluent CH$_3$CN–TEAP (35:65); FAB-MS (MH$^+$) calcd 1171, found 1171. Amino acid analysis: Asp 0.94, Nle 0.99, Gly 1.02, Tyr 0.96, Phe 1.98, Trp 0.89.

Boc-Ala-Asp(O-t-Bu)-Phe-NH$_2$ (17). To a chilled solution of compound 13 (0.62 g, 1.85 mmol) in DMF (10 mL) containing DIEA (0.62 mL, 3.7 mmol) were added Boc-Ala-ONp (0.62 g, 2 mmol) and HOBt (0.28 g, 1.85 mmol). The reaction mixture was stirred for 30 min at 0 °C and overnight at room temperature and worked up as described for the preparation of compound 14 to yield a white solid: 0.90 g (96%); R_f 0.52 (A); mp 145–146 °C. Anal. (C$_{25}$H$_{38}$O$_7$N$_4$) C, H, N.

Ala-Asp-Phe-NH$_2$·TFA (18). Compound 17 (0.5 g, 1 mmol) dissolved in 3 mL of the TFA–H$_2$O (8:2) mixture was stirred at 0 °C for 1 h and at room temperature for 3 h and worked up as described for the preparation of compound 15 to yield a white solid: 0.44 g (95%); R_f 0.27 (D).

Boc-Tyr-Nle-Gly-Trp-Ala-Asp-Phe-NH$_2$ (19). To a solution of compound 11 (0.224 g, 0.351 mmol) in dry DMF (2 mL) were added, at –10 °C, HONSu (0.041 g, 0.36 mmol) and DCC (0.074 g, 0.36 mmol). The reaction mixture was stirred for 30 min at –10 °C, then for 1 h at 0 °C, and overnight at room temperature. To the above mixture was added, at 0 °C, a solution of compound 18 (0.163 g, 0.351 mmol) and Et$_3$N (50 μL, 0.35 mmol) in DMF (1 mL). The resulting mixture was stirred overnight at room temperature and worked up as described for the preparation of compound 16 to yield a white powder: 0.328 g (96%); R_f 0.68 (C). Anal. (C$_{49}$H$_{63}$O$_{12}$N$_9$) C, H, N.

Boc-Tyr(SO$_3$Na)-Nle-Gly-Trp-Ala-Asp(Na)-Phe-NH$_2$ (2). Compound 19 (0.223 g, 0.223 mmol) was sulfated with SO$_3$–pyridine complex (1.33 g) in dry DMF (4 mL) and dry pyridine (4 mL) as described for the preparation of compound 1 to yield 55 mg (24%); R_f 0.28 (C); HPLC t_R 4.4 min, eluent CH$_3$CN–TEAP (35:65); FAB-MS (MH$^+$) calcd 1095, found 1095. Amino acid analysis: Asp 1.01, Nle 0.96, Gly 0.98, Tyr 0.94, Phe 1.02, Ala 0.98, Trp 0.86.

Boc-Orn(Z)-Asp(O-t-Bu)-Phe-NH$_2$ (20). To a chilled solution of compound 13 (1.67 g, 5 mmol) in 40 mL of the solvent mixture THF–CHCl$_3$ (75:25) were added Boc-Orn(Z)-OH (1.83 g, 5 mmol), HOBt (0.766 g, 5 mmol), and DCC (1.05 g, 5.1 mmol). The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature and worked up as described for the preparation of compound 14 to yield a white solid: 2.546 g (74%); R_f 0.57 (A). Anal. (C$_{35}$H$_{49}$O$_9$N$_5$) C, H, N.

Orn(Z)-Asp-Phe-NH$_2$·TFA (21). Compound 20 (0.54 g, 0.789 mmol) dissolved in 2 mL of TFA was stirred at 0 °C for 30 min and at room temperature for 2 h and worked up as described for the preparation of compound 15 to yield 0.357 g (71%); R_f 0.14 (C).

Boc-Tyr-Nle-Gly-Trp-Orn(Z)-Asp-Phe-NH$_2$ (22). To a solution of compound 11 (0.347 g, 0.545 mmol) in dry DMF (3 mL) were added at –10 °C HONSu (0.063 g, 0.55 mmol) and DCC (0.113 g, 0.55 mmol). The reaction mixture was stirred for 30 min at –10 °C, for 1 h at 0 °C, and overnight at room temperature. Then, a solution of the tripeptide 21 (0.350 g, 0.545 mmol) in 10 mL of THF and Et$_3$N (77 μL, 0.545 mmol) was added at 0 °C to the above mixture. The resulting mixture was allowed to stand overnight at room temperature and was treated as previously described for the preparation of compound 16 to yield 0.292 g (47%) of compound 22; R_f 0.34 (E). Anal. (C$_{59}$H$_{74}$O$_{14}$N$_{10}$) C, H, N.

Boc-Tyr(SO$_3$Na)-Nle-Gly-Trp-Orn(Z)-Asp(Na)-Phe-NH$_2$ (3). Compound 22 (0.282 g, 0.245 mmol) was sulfated with SO$_3$–pyridine complex (1.69 g) in dry DMF (6 mL) and dry pyridine (6 mL) as described for the preparation of 1 to yield 94 mg (31%); R_f 0.48 (C); HPLC t_R 6.5 min, eluent CH$_3$CN–TEAP (35:65); FAB-MS (MH$^+$) calcd 1272, found 1272. Amino acid analysis: Asp 0.97, Nle 1.01, Gly 0.97, Tyr 0.95, Phe 0.98, Orn 0.93, Trp 0.86.

Boc-Tyr(SO$_3$Na)-Nle-Gly-Trp-Orn-Asp(Na)-Phe-NH$_2$ (4). Compound 3 (14 mg, 0.012 mmol) in 2 mL of MeOH was hydrogenated in the presence of 10% Pd/C catalyst (5 mg) for 7 h. After filtration of the catalyst, MeOH was evaporated. The product was lyophilized (0.1 M NH$_4$OH, 10 mL, overnight) to yield a white powder: 10.2 mg (77%); R_f 0.25 (C); HPLC t_R 6.7 min, eluent CH$_3$CN–TEAP (30:70); FAB-MS (MH$^+$) calcd 1138, found 1138. Amino acid analysis: Asp 0.99, Nle 0.96, Gly 0.97, Tyr 0.94, Phe 0.98, Orn 0.91, Trp 0.87.

Boc-Glu(O-t-Bu)-Asp(O-t-Bu)-Phe-NH$_2$ (23). To a chilled solution of compound 13 (1.67 g, 5 mmol) in 50 mL of the solvent mixture THF–CHCl$_3$ (70:30) were added Boc-Glu(O-t-Bu)-OH (1.52 g, 5 mmol), HOBt (0.781 g, 5.1 mmol), and DCC (1.05 g, 5.1 mmol). The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature and worked up as previously described for the preparation of compound 14 to yield a white solid: 2.96 g (95%); R_f 0.44 (A). Anal. (C$_{31}$H$_{48}$O$_9$N$_4$) C, H, N.

Glu-Asp-Phe-NH$_2$·TFA (24). Compound 23 (1 g, 1.61 mmol) dissolved in 8 mL of the TFA–H$_2$O (8:2) mixture was stirred at 0 °C for 30 min and for 3 h at room temperature and worked up as described for the preparation of compound 15 to yield 0.735 g (89%); R_f 0.17 (D).

Boc-Tyr-Nle-Gly-Trp-Glu-Asp-Phe-NH$_2$ (25). To a solution of compound 11 (0.244 g, 0.383 mmol) in dry DMF (2 mL) were added at –10 °C HONSu (0.045 g, 0.39 mmol) and DCC (0.081 g, 0.39 mmol). The reaction mixture was stirred for 30 min at –10 °C, for 1 h at 0 °C, and overnight at room temperature. Then a solution of the tripeptide 24 (0.2 g, 0.382 mmol) in 2 mL of DMF and Et$_3$N (54 μL) was added at 0 °C to the above mixture. The resulting mixture was allowed to stand overnight at room temperature and was treated as previously described for the preparation of compound 16 to yield a white solid: 0.275 g (70%); R_f 0.40 (F); R_f 0.58 (G). Anal. (C$_{51}$H$_{65}$O$_{14}$N$_9$) C, H, N.

Boc-Tyr(SO$_3$Na)-Nle-Gly-Trp-Glu(Na)-Asp(Na)-Phe-NH$_2$ (5). Compound 25 (0.260 g, 0.252 mmol) was sulfated with SO$_3$–pyridine complex (1.56 g) in dry DMF (5 mL) and dry pyridine (5 mL) as described for the preparation of compound 1 (eluent EtOAc–pyridine–AcOH–H$_2$O, 45:20:6:11) to yield 66 mg (24%); R_f 0.22 (G); HPLC t_R 3.0 min, eluent CH$_3$CN–TEAP (28:72); FAB-MS (MH$^+$) calcd 1175, found 1175. Amino acid analysis: Asp 1, Nle 0.98, Gly 1.01, Tyr 0.96, Phe 0.98, Glu 0.97, Trp 0.87.