Full Agonists of CCK_8 Containing a Nonhydrolyzable Sulfated Tyrosine Residue

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The sulfate ester of CCK_{26-33} or CCK_8 (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) borne by the tyrosine residue is a critical determinant of the biological activity of this peptide. In order to increase the stability of this molecule, the sulfated tyrosine has been replaced by a synthetic amino acid (L,D)Phe(*p*-CH_2SO_3Na) in which the OSO_3H group was replaced by the nonhydrolyzable CH_2SO_3H group. Both isomers were separated by chromatography and the stereochemistry of the Phe(*p*-CH_2SO_3Na) residue in each peptide was established by NMR spectroscopy. The biological activities of the new derivatives Ac[X^{27},Nle^{28},Nle^{31}]CCK_{27-33} were compared with those of Boc[Nle^{28},Nle^{31}]CCK_{27-33}, an equiactive analogue of CCK_8 and Boc[D-Tyr(SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33}. Besides their highly enhanced chemical stability, Ac[L-Phe(*p*-CH_2SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33} and Ac[D-Phe(*p*-CH_2SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33} display high affinity for peripheral and central CCK receptors ($K_I \simeq 10^{-9}$ M) and proved to be full agonists in the stimulation of pancreatic secretion as well as in the in vitro CCK_8-induced contractions of the guinea pig ileum.

Sulfation is the most abundant posttranslational covalent modification of tyrosine residues found in animal proteins.[1,2] The sulfate ester group is introduced by transfer from 3′-phosphoadenosine 5′-phosphosulfate (PAPS), a universal sulfate donor, onto tyrosine residues of proteins. This transfer is catalyzed by the enzyme tyrosylprotein sulfotransferase.[3,4] Although sulfated tyrosine residues were known more than 20 years ago to occur in a few biological proteins such as fibrinopeptide B,[5] fibrinogens,[6] gastrin,[7] and cholecystokinin,[8] their presence in a large number of proteins[9-11] was not detected until recently.[1]

The biological role of protein tyrosine sulfation has been established only in the case of a few small peptides where it has been possible to compare the biological activity of the sulfated and the unsulfated forms. For example, the hormonal activity of cholecystokinin was shown to be dependent on the sulfation of tyrosine since the sulfated form was about 250 times more potent than the unsulfated one.[12] Desulfation of tyrosine also caused a considerable decrease in the biological activity of the C-terminal octapeptide of ceruletide.[13]

Moreover, in the case of ceruletide, a large decrease in biological activity was observed when the OSO_3H group was substituted by OPO_3H_2, NO_2, NH_2, and SO_2NH_2 groups whereas a small activity was retained by compounds in which the OSO_3H group was replaced by SO_3H and $NHSO_3H$ groups.[13,14] In CCK_8, substitution of tyrosine *O*-sulfate by hydroxynorleucine *O*-sulfate caused only a 4-fold decrease in potency in the amylase release test whereas replacement by serine *O*-sulfate caused a 1000-fold decrease in potency,[15-17] showing that the nature of the side chain seems to be of relatively minor importance provided that its structure allows the positioning of the sulfate ester group at a proper distance from the peptide backbone.[17] In order to study the functional importance of tyrosine sulfation, it appears to be essential to work with analogues of tyrosine which are similar enough to mimic sulfated tyrosine residues and which cannot be hydrolyzed at the level of the OSO_3H group, even in acidic conditions.

Recently, we described the synthesis of new amino acids in which the OSO_3H group of the sulfated tyrosine was replaced by a $CH_2PO_3H_2$ or CH_2SO_3H group.[18]

In this work, we report the introduction of the new amino acid Ac(L,D)Phe(*p*-CH_2SO_3Na) in the sequence of the equiactive analogue of CCK_8: Boc[Nle^{28},Nle^{31}]-CCK_{27-33}.[19] The mixture of the two isomers, Ac[L-Phe-(*p*-CH_2SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33} (compound **1**) and Ac[D-Phe(*p*-CH_2SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33} (compound **2**), obtained by the liquid-phase method, were separated by chromatography and their structures established by NMR spectroscopy, following previously reported methods.[20] The binding properties to both guinea pig brain and pancreatic membranes and the peripheral activities (amylase secretion from pancreatic acini and contractile potency on ileum) of the new CCK-related peptides were compared to those exhibited by the reference molecule Boc[Nle^{28},Nle^{31}]CCK_{27-33} (compound **3**) and its stereoisomer Boc[D-Tyr(SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33} (compound **4**), which was prepared for comparison.

Chemistry

Ac(L,D)Phe(*p*-CH_2SO_3Na) was prepared as already de-

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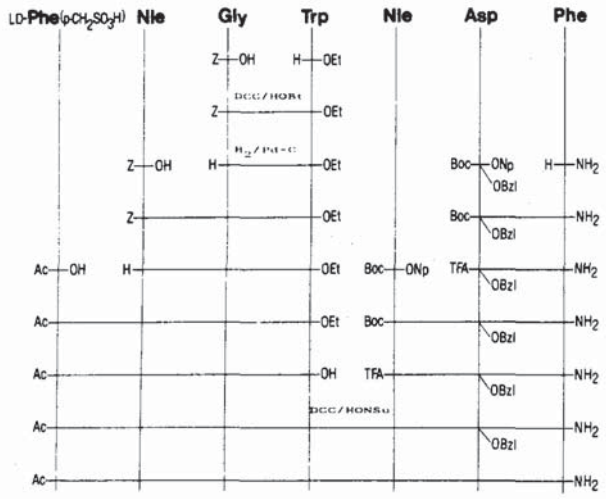

Figure 1. Scheme for the synthesis of compound **1** (Ac[L-Phe(p-CH_2SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33}) and compound **2** (Ac[D-Phe(p-CH_2SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33}), which were separated by chromatography on silica gel at the last step of the synthesis.

scribed[18] by base-catalyzed alkylation of diethyl acetamidomalonate with α-bromo-p-toluonitrile, followed by catalytic hydrogenation of the nitrile function and use of sodium nitrite in aqueous medium to obtain the hydroxymethyl derivative.

Conversion to the desired amino acid was performed with first thionyl chloride and then sodium sulfite in a sodium hydroxide solution. A final acid treatment allowed decarboxylation.

Compounds **1** and **2** were prepared in the liquid phase, according to Figure 1. Compound **4** was prepared like the equipotent analogue of CCK_8: Boc[Nle^{28},Nle^{31}]CCK_{27-33} (compound **3**).[19]

The coupling steps were performed by using the DCC/HOBt condensation method, the active p-nitrophenyl ester method, and the HONSu/DCC method.

The amine protecting groups of these fragments were removed either with trifluoroacetic acid or by catalytic hydrogenolysis. The carboxylic acid functions were deprotected by saponification, by catalytic hydrogenolysis, or with trifluoroacetic acid. The SO_3–pyridine complex in a DMF–pyridine mixture was used to introduce a sulfate ester group into the tyrosine side chain, and the final product **4** was purified by silica gel column chromatography. The two stereoisomers, compounds **1** and **2**, were separated by chromatography on silica gel, and the configuration of the α-carbon of the modified tyrosine residue was determined by ^{1}H NMR spectroscopy. The purity and the lack of racemization of each compound were checked by HPLC and ^{1}H NMR spectroscopy.

NMR Studies

The studies were performed in aqueous medium in order to control precisely the pH of the solution. Unambiguous assignment of the spectra of compounds **1**–**4** (Figure 2) was carried out by classical COSY experiments.

As compared to **3**, the only significant change in the spectrum of compound **4** is the upfield shift (0.16 ppm) of one of the two Nle resonances (Figure 2). This shielded signal can be assigned to the residue Nle 28, which is close to the site of configurational change, Tyr(SO_3^-)^{27}. On the other hand, the spectra of **3** and **1** differ only by the 0.25 ppm downfield shift of the Hα of the Ac-Phe(p-CH_2SO_3Na) residue in **1** as regards to the Hα of Boc-L-Tyr(SO_3Na) in **3**. Moreover, the position of the Hα signals

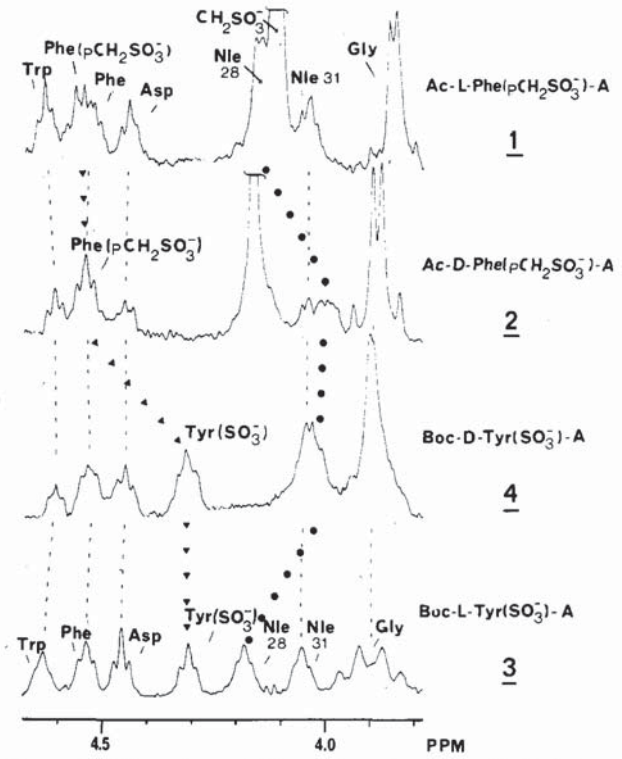

Figure 2. Hα region of 1D spectra of compounds **1**–**4**: A = Nle-Gly-Trp-Nle-Asp-Phe-NH_2, (●) full circle point the resonance of residue Nle^{28}, (▲) full triangle point the resonance of residue [Phe(p-CH_2SO_3Na) or Tyr(SO_3H)]^{27}.

Therefore it can be concluded that, in compound **1**, the Phe(p-CH_2SO_3Na) residue has the same L configuration as Tyr(SO_3Na) in **3**.

The same effects were observed with compounds **2** and **4**: downfield shift of Hα of Boc-Tyr(SO_3Na) resonance in Ac-Phe(p-CH_2SO_3Na), and unchanged chemical shift of Hα of Nle.[28] Therefore, the two peptides have a first amino acid in the D configuration.

Finally the comparison of **1** and **2** confirms the previous remarks and attributions, i.e. an upfield shift (0.16 ppm) of Hα of Nle^{28} between **1** (L-Phe(p-CH_2SO_3Na)) and **2** (D-Phe(p-CH_2SO_3Na)).

The same behavior has already been observed with the dipeptides L-Phe-L-Ala and D-Phe-L-Ala, where the methyl of Ala was more shielded in the D,L than in the L,L diastereoisomer.[20]

The small changes (including coupling constants of the glycine moiety) which occurred in the spectra of compounds **1**–**4** suggest slight modifications in the conformational states of these peptides.

The NMR attribution of the configuration of compound **1** and **2** is in accordance with HPLC studies. Compound **3** (L-Tyr(SO_3H) shows a higher retention time (t_R = 4.5 min) than that of compound **4** (D-Tyr(SO_3H)) (t_R = 3.6 min). A similar result was observed with the stereoisomers **1** (t_R = 9.0 min) and **2** (t_R = 7.8 min). Thus, one can assume that both **3** and **1** have the same L configuration and compounds **4** and **2** the same D configuration at the level of Tyr(SO_3H) or Phe(p-CH_2SO_3H).

Figure 3 shows the increased stability of compound **1**, which is in fact stable over a period of several days, whereas compound **3** is completely desulfated in 6 h in a solution of TFA (0.5 M) in DMSO.

Biological Results

Binding Experiments. The CCK-related analogues

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Table I. Potencies of CCK$_8$ Analogues (1–4) in Inhibiting [^{3}H]Propionyl-CCK$_8$ Specific Binding in Guinea Pig Brain and Pancreatic Membranes

compound	binding K_I,[a,b] M	
	brain	pancreas
CCK_{26-33} or CCK_8	$(0.28 \pm 0.01) \times 10^{-9}$	$(0.64 \pm 0.04) \times 10^{-9}$
1 (Ac(L-Phe(p-CH_2SO_3Na)^{27},Nle^{28},Nle^{31})CCK_{27-33})	$(3.20 \pm 0.60) \times 10^{-9}$	$(1.70 \pm 0.20) \times 10^{-9}$
2 (Ac(D-Phe(p-CH_2SO_3Na)^{27},Nle^{28},Nle^{31})CCK_{27-33})	$(1.60 \pm 0.20) \times 10^{-8}$	$(1.90 \pm 0.40) \times 10^{-8}$
3 (Boc(L-Tyr(SO_3Na)^{27},Nle^{28},Nle^{31})CCK_{27-33})	$(0.23 \pm 0.05) \times 10^{-9}$	$(0.93 \pm 0.08) \times 10^{-9}$
4 (Boc(D-Tyr(SO_3Na)^{27},Nle^{28},Nle^{31})CCK_{27-33})	$(3.00 \pm 0.30) \times 10^{-9}$	$(3.30 \pm 0.70) \times 10^{-8}$

[a] Values represent mean ± SEM of three separate experiments performed in triplicate. [b] [^{3}H]Propionyl-CCK$_8$ was used at the concentration of 0.2 nM (K_D = 0.2 nM) with brain tissue and at 0.1 nM (K_D = 1.2 nM) with pancreatic tissue.

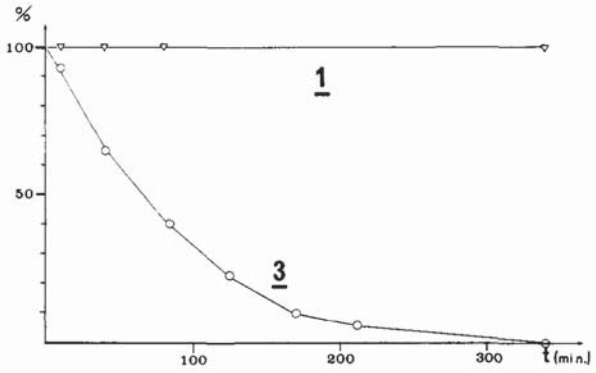

Figure 3. Stability of compounds 1 and 3 (2 mM) in a mixture of TFA (500 mM) and DMSO expressed in percentage of starting (sulfated) product: t = time in minutes; (▽) 1, Ac-Phe(p-CH_2SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2; (○) 3, Boc-Tyr-(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2.

displacing [^{3}H]propionyl-CCK$_8$ from guinea pig brain and pancreatic membranes. They all display high affinities (K_I ~ 1–30 nM) for brain and pancreatic binding sites (Table I).

Amylase Release. The pancreozimin-like activities of the CCK-related peptides were assessed by measuring their effect on amylase secretion from guinea pig pancreatic acini. For compounds 1, 2, and 4, the shape of the dose–response curves was similar to that obtained with both CCK$_8$ and Boc[Nle^{28},Nle^{31}]CCK_{27-33}. The efficiency of these CCK$_8$ analogues to stimulate amylase release is reported in Table II. Compound 1 proved to be the most active component with an EC_{50} value of 0.33 nM.

Guinea Pig Ileum Contractions. The ability of compounds 1, 2, and 4 to stimulate the contraction of the isolated guinea pig ileum was used to evaluate cholecystokinin-like activity.

All the analogues displayed agonist properties, compound 1 being the most active compound with an EC_{50} value of 3.8 nM (Table II).

Discussion

Replacement of the sulfated tyrosine residue in the sequence of CCK$_8$ by the new amino acid L-Phe(p-CH_2SO_3Na) led to a compound which displays an affinity for pancreatic binding sites (K_I = 1.7 nM) as high as that of the reference molecule Boc[Nle^{28},Nle^{31}]CCK_{27-33} (K_I = 0.93 nM). Moreover, this compound is a full agonist of CCK$_8$ in the stimulation of the pancreatic secretion enzyme (EC_{50} = 0.33 nM) as well as in the induction of guinea pig ileum contractions (EC_{50} = 3.8 nM). In addition to its potent peripheral activity, this compound displays a high affinity for brain binding sites (K_I = 3.2 nM).

Thus, compound 1 is the first described analogue of CCK$_8$ modified on the sulfate ester group which recognizes both central and peripheral CCK receptors and which

Table II. Pharmacological Potencies of CCK$_8$ Analogues 1–4

compounds	agonist act.: EC_{50}, M	
	amylase secretion by guinea pig acini[a]	contractile activities of guinea pig ileum[b]
CCK_{26-33} or CCK_8	$(0.10 \pm 0.05) \times 10^{-9}$	$(0.70 \pm 0.02) \times 10^{-9}$
1	$(0.33 \pm 0.07) \times 10^{-9}$	$(3.80 \pm 0.30) \times 10^{-9}$
2	$(1.60 \pm 0.40) \times 10^{-9}$	$(1.70 \pm 0.30) \times 10^{-8}$
3	$(0.28 \pm 0.11) \times 10^{-9}$	$(0.40 \pm 0.20) \times 10^{-9}$
4	$(4.50 \pm 0.90) \times 10^{-9}$	$(2.80 \pm 0.40) \times 10^{-8}$

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

performed on the sulfate ester group of the tyrosine of ceruletide—especially the replacement of the OSO_3H group by a $NHSO_3H$ group—led to less active analogues.[14]

In contrast, substitution of the OSO_3H group by a CH_2SO_3H group does not affect CCK$_8$ activity in vitro, suggesting that (i) the oxygen atom linked to the tyrosine ring is not involved in a crucial hydrogen bound within the receptor and (ii) the methylene group is flexible enough to position the SO_3H moiety in the appropriate receptor subsite.

The introduction of a D sulfated tyrosine residue in position 27 slightly modifies the affinity for central and peripheral receptors (13- and 35-fold decrease). Moreover, the analogue 4 is respectively 10 and 70 times less potent than Boc[Nle^{28},Nle^{31}]CCK_{27-33} on amylase release and on the CCK$_8$-induced contractions of guinea pig ileum. The same loss of affinity and peripheral activity is observed when comparing the biological properties of the two enantiomeric peptides Ac[L-Phe(p-CH_2SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33} and Ac[D-Phe(p-CH_2SO_3Na)^{27},Nle^{28},Nle^{31}]CCK_{27-33} (Tables I and II). Nevertheless, compounds 2 and 4 with D-amino acids are of interest since they could be more resistant to aminopeptidase degradation.

The use of tyrosine O-sulfate containing peptides is relatively limited as the sulfate ester bond is remarkably acid labile[5] and is therefore likely to be hydrolyzed in some of the common protein–chemical procedures.[1] The new CCK-related peptide 1 proved to be more stable in acid medium than the reference molecule Boc[Nle^{28},Nle^{31}]-CCK_{27-33} (Figure 3). Besides its enhanced chemical stability, compound 1 retains full in vitro biological activity and studies on its in vivo activity and stability are now in progress.

Extensive in vivo pharmacological studies of CCK$_8$-related peptides require compounds able to recognize specifically central receptors, protected from degrading enzymes since in vitro CCK$_8$ is relatively quickly degraded[21] and chemically resistant to desulfation. The first two requirements have already been satisfied since cyclization in the N-terminal part of CCK$_8$ yielded peptides highly

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selective for central receptors[22] and introduction of modified amino acids in the sequence led to fully peptidase-resistant compounds.[23]

As illustrated in this work, the third criteria, i.e. resistance to acid-induced desulfation, has now been achieved. The synthesis of CCK_8 analogues containing all three of these modifications is now in progress.

Experimental Section

Synthesis. All protected amino acids were from Bachem AG. Solvents were of analytical grade from Prolabo. 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 (by volume): A, $CHCl_3$–MeOH–AcOH–H_2O (70:30:6:3); B, $CHCl_3$–MeOH–AcOH–H_2O–EtOAc (35:15:3:1.5:1); C, $CHCl_3$–MeOH (90:10); D, EtOAc–pyridine–AcOH–H_2O (60:20:6:11); E, EtOAc–pyridine–AcOH–H_2O (40:20:6:11); F, EtOAc–pyridine–AcOH–H_2O (50:20:6:11).

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 ^1H NMR spectroscopy (Bruker WH 270 MHz). The purity was checked by HPLC (Waters apparatus) on a 250 × 4.6 mm Prolabo ODS2 5-μm column with Et_3N–H_3PO_4 buffer (TEAP, 0.025 M, pH 6.5)/CH_3CN 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 ^1H NMR spectroscopy and by HPLC. Amino acid analyses were carried out on a LKB biochrom 4400 analyzer after hydrolysis with 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) ionization was obtained with a FAB saddle field source (Ion Tech Ltd., Teddington, U.K.) 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; DCU, *N,N'*-dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; HONSu, *N*-hydroxysuccinimide. Other abbreviations used were those recommended by the IUPAC–IUB Commission (*Biochem. J.* **1984**, *219*, 345). All the dipeptides and tripeptides were prepared in the liquid phase, as previously described.[19,24]

Ac(L,D)Phe(*p*-CH_2-SO_3Na)-Nle-Gly-Trp-OC_2H_5 (5). Ac-(L,D)Phe(*p*-CH_2SO_3Na) (84.2 mg, 0.26 mmol) was dissolved in 6 mL of DMF. Nle-Gly-Trp-OC_2H_5 (105 mg, 0.26 mmol), HOBt (41 mg, 0.27 mmol), and DCC (55 mg, 0.27 mmol) were successively added at 0 °C. The mixture was stirred for 1 h at 0 °C and overnight at room temperature. After evaporation of DMF, the residue was washed with EtOAc (10 mL). The product was isolated from the solid residue by two extractions with water (2 × 25 mL) and lyophilization, to yield 147 mg (80%) of a white powder: R_f 0.24 (A), corresponding to a mixture of two stereoisomers; FAB-MS (MH^+) calcd 708, found 708.

Ac(L,D)Phe(*p*-CH_2SO_3Na)-Nle-Gly-Trp-OH (6). Compound **5** (100 mg, 0.14 mmol) was saponified with 1 M NaOH (0.3 mL) in the solvent mixture H_2O–MeOH (8 mL–1 mL) for 1 h at 0 °C and 3 h at room temperature. After evaporation of MeOH, the aqueous phase was acidified with 1 M HCl, washed with EtOAc, and lyophilized, to yield a white product: 90 mg (95%); R_f 0.08 (A) for the two stereoisomers.

Ac(L,D)Phe(*p*-CH_2SO_3Na)-Nle-Gly-Trp-Nle-Asp(OBzl)-Phe-NH_2 (7). Compound **6** (20 mg, 0.029 mmol) was dissolved in 1 mL of DMF. Nle-Asp(OBzl)-Phe-NH_2·TFA (17.5 mg, 0.029 mmol) and Et_3N (5 μL) in DMF (1 mL), HOBt (6.1 mg, 0.04 mmol), and DCC (8.2 mg, 0.04 mmol) were successively added at 0 °C. The reaction mixture was stirred for 30 min at 0 °C and overnight at room temperature.

After filtration of DCU and evaporation of DMF, the residue was triturated with EtOAc–ether and washed several times with ether to yield a white solid: 30.1 mg (91%); R_{f_1} 0.42 (B) and R_{f_2} 0.39 (B) for the two stereoisomers.

Ac(L,D)Phe(*p*-CH_2SO_3Na)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 (8). Compound **7** (22 mg, 0.019 mmol) in 5 mL of MeOH was hydrogenated in the presence of 10% Pd/C catalyst (15 mg) for 3 h. After filtration of the catalyst, MeOH was evaporated to yield a white product: 19.5 mg (97%); R_{f_1} 0.24 (B) and R_{f_2} 0.21 (B) for the two stereoisomers, which were separated by chromatography on silica gel with $CHCl_3$–MeOH–AcOH–H_2O–EtOAc (35:15:3:1.5:1) as eluent to yield 3.2 mg, R_{f_1} 0.24 (B), and 3.8 mg, R_{f_2} 0.21 (B); HPLC, t_{R_1} HPLC 9.0 min and t_{R_2} 7.8 min (eluent: CH_3CN–TEAP, 29:71); the attribution of the configuration of the α-carbon in the modified tyrosine is discussed in this paper. t_{R_1} was attributed to compound **1** and t_{R_2} to compound **2**. FAB-MS (MH^+) (compounds **1** and **2**) calcd 1054, found 1054.

Boc-D-Tyr-Nle-Gly-Trp-OC_2H_5 (9). Nle-Gly-Trp-OC_2H_5 (402.5 mg, 1 mmol) was dissolved in 30 mL of the solvent mixture THF–CH_2Cl_2 (75:25). Boc-D-Tyr-OH (281.3 mg, 1 mmol), HOBt (154 mg, 1 mmol), and DCC (227 mg, 1.1 mmol) were successively added at 0 °C. The mixture was stirred for 1 h at 0 °C and overnight at room temperature.

After filtration of DCU, the solvents were evaporated. The residue was dissolved in EtOAc and washed with citric acid (10%), $NaHCO_3$ (10%), and brine, dried over Na_2SO_4, concentrated in vacuo, and precipitated with anhydrous ether to yield a white powder: 550 mg (83%); R_f 0.30 (C). Anal. ($C_{35}H_{47}O_8N_5$) C, H, N.

Boc-D-Tyr-Nle-Gly-Trp-OH (10). Compound **9** (253.7 mg, 0.38 mmol) was saponified with 1 M NaOH (0.77 mL, 0.77 mmol) in EtOH (8 mL) for 1 h at 0 °C and 4 h at room temperature. After evaporation of EtOH, the residue was dissolved in water (20 mL). The unreacted product was extracted with EtOAc. The aqueous phase was acidified with cold 1 M HCl and extracted with EtOAc, washed with brine, and dried over Na_2SO_4 to yield after evaporation and precipitation with anhydrous ether a white powder: 202.4 mg (83%); R_f 0.83 (D). Anal. ($C_{33}H_{43}O_8N_5$) C, H, N.

Boc-D-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 (11). Compound **10** (0.199 g, 0.31 mmol) was dissolved in 8 mL of dry DMF. HONSu (0.036 g, 0.31 mmol) and DCC (0.065 g, 0.31 mmol) were then added at −10 °C. 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 Nle-Asp-Phe-NH_2·TFA (0.158 g, 0.31 mmol) and Et_3N (44 μL, 0.31 mmol) in DMF (3 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 and washed several times with ether to yield a white powder: 0.192 g (61%); R_f 0.81 (E). Anal. ($C_{52}H_{69}O_{12}N_9$) C, H, N.

Boc-D-Tyr(SO_3Na)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 (4). A solution of compound **11** (0.185 g, 0.18 mmol) in dry DMF (4 mL) and dry pyridine (4 mL) was treated with SO_3–pyridine complex (1.11 g) and was stirred overnight under N_2 at room temperature. After evaporation in vacuo, the residue was taken up in a 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.

The suspended product was isolated by lyophilization followed by precipitation of inorganic salts in MeOH, filtration, and evaporation of MeOH in vacuo. The two fractions were separately purified by chromatography on silica gel with EtOAc–pyridine–AcOH–H_2O (50:20:6:11) as eluent, to yield 94 mg (48%) of compound **4**: R_f 0.40 (F); HPLC t_R 3.6 min (eluent: CH_3CN–TEAP, 35:65); FAB-MS (MH^+) calcd 1137, found 1137. Amino acid analysis: Asp 0.96, Nle 1.98, Gly 0.98, Tyr 0.94, Phe 1.02, Trp

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NMR Measurements. Samples were prepared by dissolving of peptide in D_2O (99.98% CEA). The pH was adjusted to 7.0 by addition of DCl or NaOD solutions (0.1 N in D_2O) and measured with a microelectrod Ingold 405.M3 with a Tacussel pH meter PH N 75, without correction for deuterium effect. The final concentration was 0.8 mM for 1D spectra and 2 mM for 2D experiments.

Spectra were run in the Fourier transform mode at 270 MHz on a Bruker WH 270 instrument and at 400 MHz on a Bruker AM 400 instrument equipped with an Aspect 2000 and 3000 computer, respectively, and with a Bruker temperature controller (±1 °C). Chemical shifts were given in ppm from DSS as external reference. The residual peak of HDO was suppressed by presaturation. The COSY experiments were performed by using established techniques.[25]

For stability in acidic conditions, the samples were dissolved in DMSO-d_6 (99.8% CEA), and then 20 μL of TFA was added at room temperature. The final concentrations were 1 mM in peptide and 0.5 N in acid. The proportion of sulfated and unsulfated products was determined from characteristic peaks intensity in 1D spectra (H_ϵ resonance for compound **3** and CH_2SO_3H resonance for compound **1**).

Guinea Pig Brain Membrane Preparation. Male guinea pig brain cortex were dissected on ice and homogenized (12 mL/g of tissue, wet weight) in 50 mM Tris·HCl buffer, pH 7.4, containing 5 mM $MgCl_2$. The homogenate was incubated for 30 min at 35 °C and centrifuged at 4 °C for 35 min at 100000*g*, and the resulting pellet was rehomogenized in a large excess of ice-cold buffer and centrifuged under the same conditions. The final pellet was resuspended in 50 mM Tris·HCl buffer, pH 7.4, supplemented with 0.2 mg/mL bacitracin and 5 mM $MgCl_2$ (6–7 mg of protein/mL). Protein concentration was determined by the method of Lowry et al.[26] using bovine serum albumin standards.

Guinea Pig Pancreatic Membrane Preparation. Male guinea pigs (250–350 g) were sacrificed by cervical dislocation, the pancreases were quickly dissected and placed into ice-cold 10 mM Pipes–HCl buffer, pH 6.5, containing 30 mM $MgCl_2$ (Pipes–$MgCl_2$ buffer). After careful removal of the fat, the pancreases of seven guinea pigs were homogenized in 25 volumes of Pipes–$MgCl_2$ buffer at 4 °C with a Brinkmann Polytron PT10, the homogenate was filtered on gauze, and the filtrate was centrifuged twice at 50000*g* for 10 min with an intermediate rehomogenization of the pellet in fresh buffer. The final pellet was resuspended in 2 vol of fresh buffer and stored frozen at −80 °C until used. This preparation usually led to 12–14 mL of a membrane suspension containing 25–30 mg of protein/mL.

Binding Assays. [^{3}H]Propionyl-CCK_8 ([^{3}H]pCCK_8, 60 Ci/mmol) was purchased from Amersham. Binding experiments with [^{3}H]pCCK_8 were performed as described previously[23] with some modifications. Briefly, incubations (final volume 1 mL) were carried out in 50 mM Tris·HCl buffer, pH 7.4, 5 mM $MgCl_2$, 0.2 mg/mL bacitracin, for 60 min at 25 °C, in the presence of brain membranes (0.6 mg of protein/tube), or in 10 mM Pipes–HCl buffer, pH 7.4, 30 mM $MgCl_2$, 0.2 mg/mL bacitracin, 0.2 mg/mL soybean trypsin inhibitor, for 120 min at 25 °C in the presence of pancreatic membranes (0.2 mg of protein/tube). For displacement experiments, the radiolabeled probes (0.2 and 0.1 nM for brain and pancreatic membranes, respectively) were incubated in the presence of varying concentrations of the competitor. Nonspecific binding was determined in the presence of 1 μM CCK_8 in all cases. The incubation was terminated by filtration through Whatman GF/B filters precoated by incubation in buffer (brain membranes, 50 mM Tris·HCl, 5 mM $MgCl_2$, pH 7.4; pancreatic membranes, 10 mM Pipes–HCl, pH 6.5) containing 0.1% bovine serum albumin; filters were rinsed twice with 5 mL of ice-cold buffer and dried, and the radioactivity was counted in 5 mL of ready-solvent EP scintillation cocktail (Beckman). The K_I values were calculated by using the Cheng–Prusoff equation.[27]

In Vitro Bioassays. Acini were prepared as previously reported.[28] Amylase release from pancreatic acini was measured after incubation for 30 min at 37 °C in the presence of CCK_8 or CCK analogues as previously reported.[22,29] Contractile activity of guinea pig ileum was measured according to Hutchinson and Dockray.[30]

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Registry No. **1**, 117942-22-0; **2**, 117942-23-1; **3**, 100654-12-4; **4**, 117942-24-2; (L)-**5**, 117942-32-2; (D)-**5**, 117942-33-3; (L)-**6**, 117942-34-4; (D)-**6**, 117942-35-5; (L)-**7**, 117942-36-6; (D)-**7**, 117942-37-7; **9**, 117942-38-8; **10**, 117942-39-9; **11**, 117942-40-2; Z-Gly-OH, 1138-80-3; Trp-OEt, 7479-05-2; Z-Nle-OH, 39608-30-5; Boc-Nle-ONp, 21947-33-1; Boc-Asp(OBzl)-ONp, 26048-69-1; Phe-NH_2, 5241-58-7; Ac-(L)-Phe(*p*-CH_2SO_3Na)-OH, 117942-25-3; Ac-(D)-Phe(*p*-CH_2SO_3H)-OH, 117942-26-4; Z-Gly-Trp-OEt, 117942-27-5; Z-Nle-Gly-Trp-OEt, 117942-28-6; Gly-Trp-OEt, 117942-29-7; Nle-Gly-Trp-OEt, 117942-30-0; Boc-Asp(OBzl)-Phe-NH_2, 60058-69-7; Boc-Nle-Asp(OBzl)-Phe-NH_2, 65864-24-6; TFA-Asp(OBzl)-Phe-NH_2, 117942-31-1; TFA-Nle-Asp(OBzl)-Phe-NH_2, 117959-03-2; Boc-(D)-Tyr-OH, 70642-86-3.

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