Articles

Investigation of Peripheral Cholecystokinin Receptor Heterogeneity by Cyclic and Related Linear Analogues of CCK$_{26-33}$: Synthesis and Biological Properties

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A possible heterogeneity of peripheral receptors for CCK$_{26-33}$ [Asp-Tyr(SO$_3$H)-Met-Gly-Trp-Met-Asp-Phe-NH$_2$] (CCK$_8$) was investigated by replacement of the flexible Gly^{29} residue, reported to be crucially involved in the CCK$_8$ folding, by a D-Lys residue in Boc[Nle^{28,31}]CCK$_{27-33}$, a derivative as active as CCK$_8$. The linear peptide Boc-Asp-Tyr(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$ (1) was cyclized through amide bond formation between the side chains of Asp^{26} and D-Lys^{29} to give the peptide Boc-Asp-Tyr(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$ (2). Analogues 1 and 2 were shown to stimulate secretion of amylase from rat pancreas with a potency that was respectively 40 and 80 times lower than that of CCK$_8$. In contrast, both peptides acted as weak antagonists (EC$_{50}$ ~ 10^{-5} M) of the CCK$_8$-induced contractions of guinea pig ileum. Peptides 3 and 4 obtained by removal of the phenylalanine from 1 and 2 were inactive in all bioassays despite amidification of their C-terminal Asp^{32} residue, a modification known to induce antagonist properties in CCK$_7$. Cyclization between residues 28 and 31 in Boc[Asp^{28},Lys^{31}]CCK$_{27-33}$ gave compound Boc-Tyr(SO$_3$H)-Asp-Gly-Trp-Lys-Asp-Phe-NH$_2$ (5), which was inactive in all bioassays. The pharmacological properties of these first described cyclic analogues of CCK$_8$ were in agreement with their binding affinity to brain and pancreas receptors, suggesting the existence of a heterogeneity of peripheral receptors and emphasizing the usefulness of cyclic peptides in structure–activity studies.

The sulfated peptide CCK$_{26-33}$ [Asp-Tyr(SO$_3$H)-Met-Gly-Trp-Met-Asp-Phe-NH$_2$] (CCK$_8$) behaves as a hormonal regulator of pancreatic secretion, induces contractions of the gallbladder and increases the gut motility.[1,2] CCK$_8$ has also been found in high concentrations in mammalian brains,[3] and various biochemical and pharmacological experiments have suggested a neurotransmitter role for this peptide.[4,5] Structure–activity studies as well as binding experiments done with cholecystokinin fragments or synthetic analogues of CCK$_{26-33}$ have revealed the differences between central and peripheral receptors. Unsulfated CCK$_{26-33}$ (NS CCK$_8$) and shorter fragments, such as CCK$_{30-33}$ (CCK$_4$), are between 1000 and 10000 times less potent than CCK$_8$ when interacting with receptors of pancreatic acini but only 4–10 times less active on brain receptors.[6,7] The occurrence of a multiplicity of CCK binding sites implies the concomitant existence of different biologically active conformations of the native peptide, which could be investigated by conformational analysis. As previously shown by ^{1}H NMR spectroscopy, fluorescence transfer, and energy calculations, CCK$_8$ exists, in various solvents including water, in folded conformations characterized by a N-terminal β-turn stabilized by a hydrogen bond between the CO of Asp^{26} and the NH of Gly^{29} and a C-terminal folding around the sequence Gly-Trp-Met-Asp.[8,9] These constrained structures seem to be fa-

vored by the presence of the small and flexible Gly residue in the center of the peptide. Interestingly, the replacement of Gly^{29} by D-Ala$^{10-12}$ or D-Trp^{12} led to peptides whose potency in the guinea pig ileum and gallbladder bioassays was significantly reduced but whose ability to potentiate pancreatic amylase secretion remained unchanged. In contrast, introduction of a L-Ala or an Aib residue in place of Gly^{29} in Boc[Nle^{28,31}]CCK$_{27-33}$, a derivative as active as CCK$_8$,[13] induced a loss of activity on all peripheral bioassays but did not affect the affinity for brain receptors.[10] Conformational behavior of compounds Boc-[Nle^{28,31},Ala^{29}]CCK$_{27-33}$ and Boc[Nle^{28,31},Aib^{29}]CCK$_{27-33}$ was investigated by both ^{1}H NMR spectroscopy and energy calculations, showing the presence of a β-turn involving the sequence Nle-Gly-Trp-Nle,[14] which is likely responsible for their selectivity.

These preliminary results suggested a possible modulation in the recognition of putatively different peripheral CCK receptor types by restriction in the conformational flexibility of the native peptide. Indeed, as shown for the enkephalins,[15,16] specific probes for the various opioid re-

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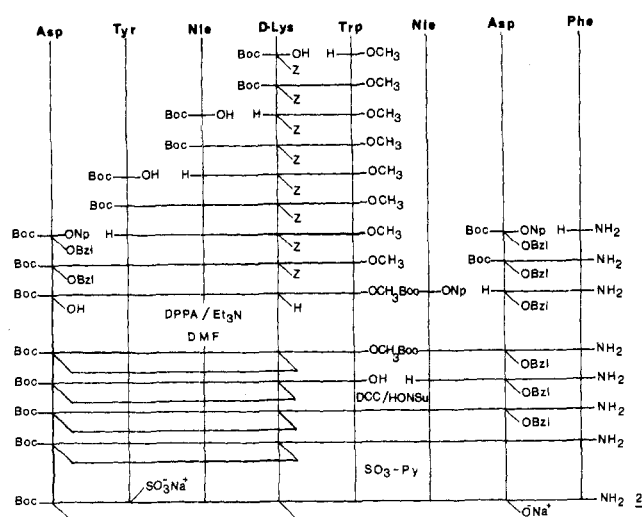
Figure 1. Scheme for the synthesis of compound Boc-Asp-Tyr(SO$_3$Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH$_2$ (2). Compound Boc-Asp-Tyr(SO$_3$Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-NH$_2$ (4) was prepared with the same strategy by coupling H-Nle-Asp-NH$_2$ (22) in the place of the tripeptide H-Nle-Asp(OBz)-Phe-NH$_2$ (18) to the cyclic moiety.

Figure 2. Scheme for the synthesis of compound Boc-Tyr-(SO$_3$Na)-Asp-Gly-Trp-Lys-Asp(Na)-Phe-NH$_2$ (5).
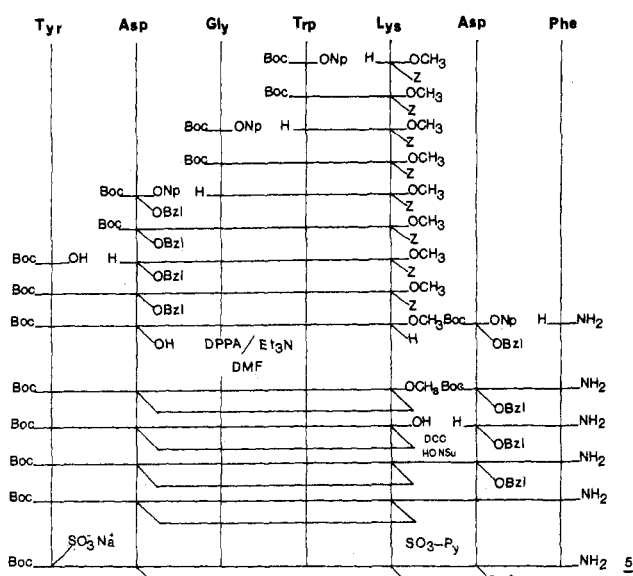
ceptors can be designed to mimic favorable conformational characteristics of the native peptide, through cyclizations between nonadjacent amino acids. Taking this into account, we report the synthesis of a series of cyclic CCK related peptides, obtained by introducing a D-Lys in place of Gly[29], followed by cyclization between the amino and carboxyl groups of the D-Lys[29] and Asp[26] side chains.[17] Moreover, as the removal of Phe[33] in CCK$_{27-33}$ (CCK$_7$) has been shown to lead to a relatively potent antagonist,[18] this modification was introduced in the above compounds.

In addition, we attempted to reproduce the folded conformation observed in Boc[Nle^{28,31},Ala^{29}]CCK$_{27-33}$ and in Boc[Nle^{28,31},Aib^{29}]CCK$_{27-33}$ through cyclization by amide bond formation between the side-chain carboxyl and amino groups of Asp and Lys residues introduced in positions 28 and 31, respectively.
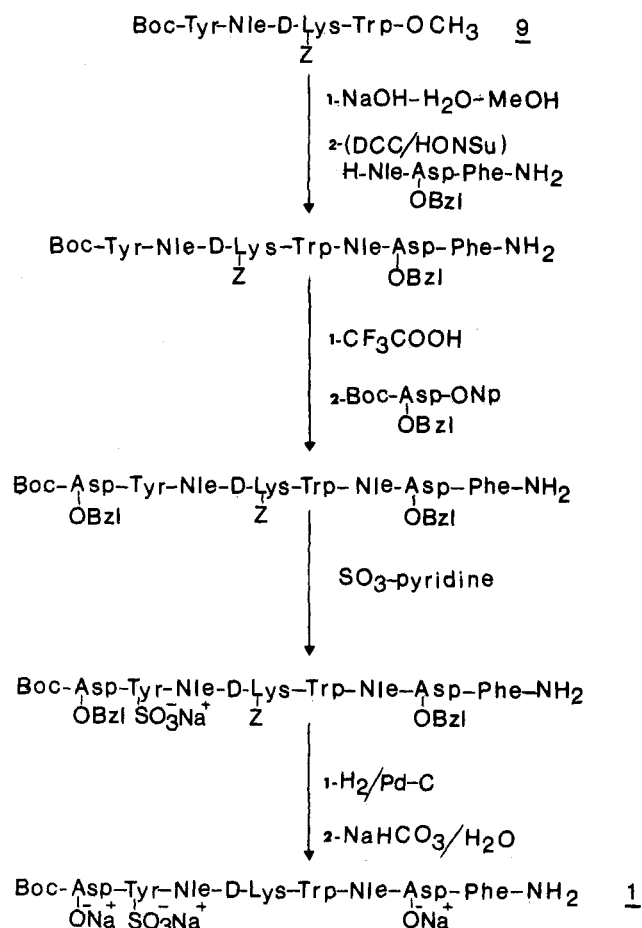
The binding properties to both mouse brain membranes and rat pancreas tissue are reported here, and the results are discussed in relation to their agonist or antagonist activity on amylase secretion by rat pancreas and contractile activities of guinea pig ileum.

Chemistry

The synthesis of both cyclic and linear CCK$_8$ related peptides was performed in liquid phase by using a fragment condensation methodology as illustrated in Figures 1–3. The final cyclic peptides were obtained by condensation of the cyclic fragment with an appropriate C-terminal di- or tripeptide. This method has been previously described for the condensation of a single N-terminal amino acid to a cyclic fragment.[19,20] The syntheses reported

Boc-Tyr-Nle-D-Lys-Trp-OCH$_3$　　**9**
　　　　　　　　|
　　　　　　　　Z

　　　　　1-NaOH-H$_2$O-MeOH

　　　　　2-(DCC/HONSu)
　　　　　H-Nle-Asp-Phe-NH$_2$
　　　　　　　　OBzl

Boc-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$
　　　　　　|　　　　　　　　OBzl
　　　　　　Z

　　　　　1-CF$_3$COOH

　　　　　2-Boc-Asp-ONp
　　　　　　　　OBzl

Boc-Asp-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$
　　OBzl　　　　　Z　　　　　　　OBzl

　　　　　SO$_3$-pyridine

Boc-Asp-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$
　　OBzl SO$_3^-$Na$^+$　Z　　　　　OBzl

　　　　　1-H$_2$/Pd-C

　　　　　2-NaHCO$_3$/H$_2$O

Boc-Asp-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$　　**1**
　　ONa$^+$ SO$_3^-$Na$^+$　　　　　ONa$^+$

Figure 3. Scheme for the synthesis of compound Boc-Asp-(Na)-Tyr(SO$_3$Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH$_2$ (1). The same strategy was used for the preparation of compound Boc-Asp(Na)-Tyr(SO$_3$Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-NH$_2$ (3) with H-Asp-Phe-NH$_2$ (22) in the second step in the place of the tripeptide H-Nle-Asp(OBz)-Phe-NH$_2$ (18).

here require only two amino-protecting groups (benzyloxycarbonyl and *tert*-butyloxycarbonyl) and two types of carboxyl protection (methyl and benzyl esters). The cy-

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Table I. Potencies of CCK Analogues 1–5 in Inhibiting [^{3}H]Boc[Nle^{28,31}]CCK$_{27-33}$ Specific Binding in Mouse Brain Membranes and in Rat Pancreas Membranes

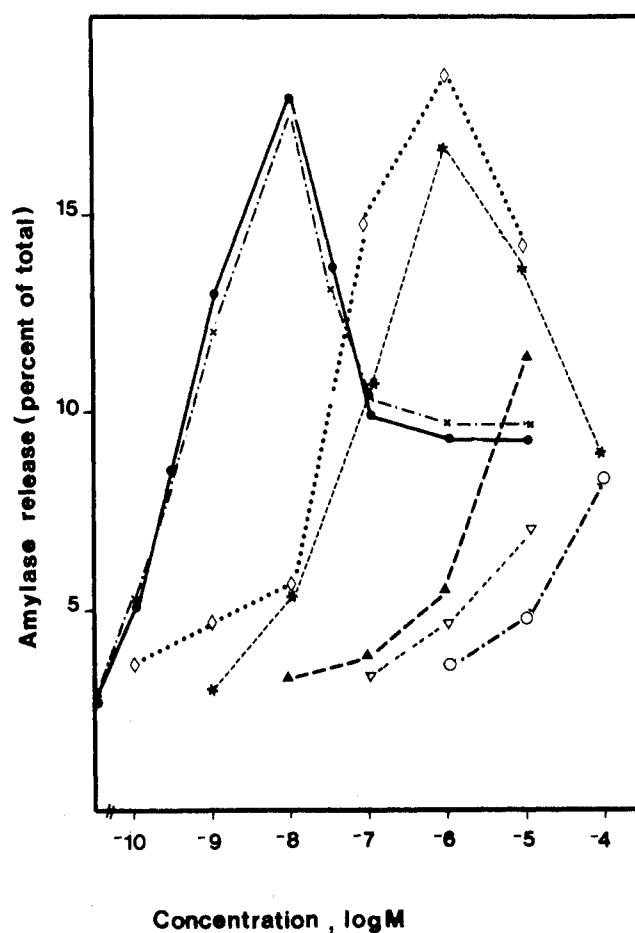|		binding: K_I,[a,c] M	
compound[b]	peptide sequence	mouse brain membranes	rat pancreas membranes
CCK$_8$	Asp-Tyr(SO$_3$H)-Met-Gly-Trp-Met-Asp-Phe-NH$_2$	$(4.9 \pm 1.2) \times 10^{-10}$	$(8.7 \pm 1.2) \times 10^{-9}$
Boc[Nle^{28,31}]CCK$_{27-33}$	Boc-Tyr(SO$_3$H)-Nle-Gly-Trp-Nle-Asp-Phe-NH$_2$	$(1.9 \pm 0.6) \times 10^{-10}$	$(4.3 \pm 1.3) \times 10^{-9}$
1	Boc-Asp-Tyr(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$	$(3.0 \pm 2.0) \times 10^{-7}$	$(3.4 \pm 1.8) \times 10^{-7}$
2	Boc-Asp-Tyr(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$	$(1.2 \pm 1.1) \times 10^{-7}$	$(1.5 \pm 0.7) \times 10^{-6}$
3	Boc-Asp-Tyr(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-NH$_2$	$>10^{-5}$	$>10^{-5}$
4	Boc-Asp-Tyr(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-NH$_2$	$>10^{-5}$	$>10^{-5}$
5	Boc-Tyr(SO$_3$H)-Asp-Gly-Trp-Lys-Asp-Phe-NH$_2$	$>10^{-5}$	$>10^{-5}$

[a] Values represent the mean ± SEM of three separate experiments performed in triplicate. [b] All the peptides were used in Na salt form. [c] [^{3}H]Boc[Nle^{28},Nle^{31}]CCK$_{27-33}$ was used at its K_D concentration, i.e., 0.19 and 4.4 nM for brain and pancreas, respectively.

clization step was performed between pH 7 and 7.5 with diphenylphosphoryl azide (DPPA)[21,22] with a diluted solution of the precursor giving a high yield (50–60%) of the cyclic peptide. After saponification, the cyclic penta-peptide was coupled to appropriate peptide fragments by the N,N'-dicyclohexylcarbodiimide–N-hydroxysuccinimide method. After removal of the Asp^{32} benzyl ester by catalytic hydrogenation, the Tyr residue was sulfated by means of a SO$_3$–pyridine complex in DMF–pyridine mixture.[23,24] As several byproducts appeared during evaporation of the solvent, the time of this step was reduced by using a lower proportion of DMF (25%) than usually employed.[25] This procedure increases significantly the yield of the sulfation reaction (about 30%). The synthesis of linear analogues was performed as shown in Figure 3. As the sulfation step, including an alkaline treatment, was carried out on peptides with the Asp side chain in the benzyl ester form, a fraction of the final product contained unprotected Asp residues related to $\alpha \rightarrow \beta$ transposition of these Asp residues.[26]

Biological Results and Discussion

Binding Experiments. The five CCK$_8$ analogues reported in this paper were firstly evaluated for their potency in displacing [^{3}H]Boc[Nle^{28,31}]CCK$_{27-33}$ from mouse brain and rat pancreatic membranes. This radioligand has been shown to display biochemical and pharmacological properties identical with those of CCK$_8$.[28] The apparent affinity of the peptides (K_I) listed in Table I shows that the introduction of a D-Lys^{29} residue in place of Gly^{29} in 1 and 2 leads to an approximate 1000- and 100-fold decrease in the affinity for brain and pancreatic binding sites, respectively. At the highest concentration used (10^{-5} M), compounds 3–5 were unable to displace the tritiated probe.

Amylase Release. The pancreozymin-like activities of the five CCK$_8$ analogues were assessed by measuring their effect on amylase secretion from rat pancreas fragments

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Concentration, logM

Figure 4. Ability of CCK analogues to stimulate amylase release from rat pancreas. In each experiment, the results are the mean from three separate experiments, each value in triplicate. EC$_{50}$ values are reported in Table II. All the peptides were used in Na salt form. (●) CCK$_8$, (X) Boc[Nle^{28,31}]CCK$_{27-33}$, (◊) Boc-Asp-Tyr(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$, (*) Boc-Asp-Tyr-(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH$_2$, (O) Boc-Asp-Tyr-(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-NH$_2$, (▲) Boc-Asp-Tyr(SO$_3$H)-Nle-D-Lys-Trp-Nle-Asp-NH$_2$, (▽) Boc-Tyr(SO$_3$H)-Asp-Gly-Trp-Lys-Asp-Phe-NH$_2$.

as previously described.[13,29] For compounds 1 and 2, the shape of the dose–response curve was similar to that obtained with both CCK$_8$ and Boc[Nle^{28,31}]CCK$_{27-33}$ (Figure 4). The efficiency of compounds 1–5 to stimulate amylase release is reported in Table II.

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Table II. Pharmacological Potencies of CCK Analogues 1–5

compound[d]	peptide sequence	amylase secretion by rat pancreas: agonist act., EC_{50},[a] M	contractile activities of guinea pig ileum	
			agonist act.: EC_{50},[b] M	antagonist act.: EC_{50},[b,c] M
CCK_8	Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	$(1.0 \pm 0.7) \times 10^{-9}$	$(2.0 \pm 0.25) \times 10^{-9}$	
$Boc[Nle^{28,31}]CCK_{27-33}$	Boc-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2	$(2.8 \pm 0.5) \times 10^{-9}$	$(3.2 \pm 0.2) \times 10^{-9}$	
1	Boc-Asp-Tyr(SO_3H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH_2	$(4.0 \pm 1.5) \times 10^{-8}$	$>10^{-5}$	$(1.5 \pm 1.0) \times 10^{-5}$
2	Bos-Asp-Tyr(SO_3H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH_2	$(8.0 \pm 3.0) \times 10^{-8}$	$>10^{-5}$	$(3.0 \pm 1.7) \times 10^{-5}$
3	Boc-Asp-Tyr(SO_3H)-Nle-D-Lys-Trp-Nle-Asp-NH_2	$>10^{-4}$	$>10^{-5}$	$>10^{-5}$
4	Boc-Asp-Tyr(SO_3H)-Nle-D-Lys-Trp-Nle-Asp-NH_2	$>10^{-6}$	$>10^{-5}$	$>10^{-5}$
5	Boc-Tyr(SO_3H)-Asp-Gly-Trp-Lys-Asp-Phe-NH_2	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$

[a] Results are the mean ± SEM of three separate experiments, each value in triplicate. [b] Results are the mean ± SEM of three separate experiments. [c] Antagonist activities of the contraction induced by 3, 10^{-9} M of CCK_8. [d] All the peptides were used in Na salt form.

Guinea Pig Ileum Contractions. The ability of the new compounds to stimulate the contraction of the isolated guinea pig ileum was used to further study their cholecystokinin-like activities.[30] None of the analogues displayed agonist properties, but compounds 1 and 2 antagonized the CCK_8-induced contractions of guinea pig ileum (Table II).

Preliminary results reported here show that the replacement of Gly^{29} in the $Boc[Nle^{28,31}]CCK_{26-33}$ sequence by a D-Lys residue with the side chain either free (compound 1) or cyclized through an amide bond with the carboxylic group of Asp^{26} (compound 2) induces a 100-fold decrease in affinity for both brain and pancreas receptors. However, the most interesting result concerns the differentiation in the pharmacological effects observed in the rat pancreas and guinea pig ileum. Indeed, 1 and 2 behave as agonists in the stimulation of the pancreatic secretion enzyme with potencies 40- and 80-fold, respectively, lower than that of CCK_8 but act as antagonists of the CCK_8-induced contraction of guinea pig ileum. The receptor interaction of compounds 1 and 2 seems to be similar with the Lys side chain free in 1 or cyclized with Asp^{26} in 2, suggesting that the two peptides have similar conformational properties. This could be explained by the occurrence of a salt bridge formation involving the charged side chains of Asp and Lys residues in the conditions (pH 7.4) of the bioassays. According to these features, the ammonium group of the D-Lys side chain might interact preferentially with the carboxylate group of Asp^{26} rather than with the sulfate group of the Tyr residue. Nevertheless, the conformational restriction introduced in the cyclic structure of 2 and the pseudocyclic structure of 1, which were designed to mimic the N-terminal folding of CCK_8, induce a loss of affinity for both brain and pancreatic receptors. Moreover, whereas 1 and 2 behave as agonists in the amylase assay, they act as antagonists in the ileum contraction assays. Such differentiation of the peripheral receptors has already been suggested for CCK_8 analogues in which the Gly residue was changed for either a D-Ala[10,12] or a D-Trp[12] residue, leading to compounds with the same potency as CCK_8 for pancreozymin-like activity but a large decreased activity in inducing gut contractions. However, the opposed pharmacological properties (agonist and antagonist) of compounds 1 and 2 provide further evidence for differences between pancreas and ileum receptors. The removal of the C-terminal Phe-NH_2 residue and the amidification of Asp^{32} in peptides 3 and 4 induced an important loss of affinity for all classes of CCK receptors. This result is somewhat unexpected since such a type of modification was recently described to induce in the case

of [Asp-NH_2^{32}]CCK_{27-32} antagonist properties in both peripheral[18] and central bioassays.[31] Further studies are required to explain this apparent discrepancy. As expected, the cyclic analogue 5, designed to mimic the folded conformations of $Boc[Nle^{28,31},Ala^{29}]CCK_{27-33}$ or Boc-$[Nle^{28,31},Aib^{29}]CCK_{27-33}$,[14] displayed, as do these two peptides, a complete lack of affinity for peripheral CCK receptors. However, in contrast to the linear peptides, compound 5 was also unable to interact with the brain CCK receptors. This could be due to the difference between the size of the cyclic moiety (17-membered ring) in 5 as compared with the pseudocyclic moiety (10-membered ring) corresponding to the β-turn encountered in Boc-$[Nle^{28,31},Aib^{29}]CCK_{27-33}$. Therefore, additional conformational studies concerning the size and the nature of the cyclic part of the modified peptides are required to obtain central nervous system selective peptides by this approach.

Finally, in addition to their enhanced resistance to peptidases (manuscript in preparation), the cyclic CCK related peptides reported here could be of great value to define from comparative conformational analysis the structural requirement for selective interactions with the various CCK receptors.

Experimental Section

Synthesis. All protected amino acids were from Bachem AG. Solvents are of analytical grade from Prolabo. SO_3-pyridine complex is from Janssen. Melting points were determined on a Kofler apparatus and are uncorrected. Chromatography was carried out with Merck silica gel (230–400 mesh). For thin-layer chromatography, Merck plates precoated with F 254 silica gel were used with the following solvent systems: R_f (A) $CHCl_3$–MeOH (95:5), R_f (B) $CHCl_3$–MeOH (9:1), R_f (C) $CHCl_3$–MeOH (8:2), R_f (D) $CHCl_3$–MeOH (7:3), R_f (E) EtOAc–pyridine–AcOH–H_2O (60:20:6:11), R_f (F) EtOAc–pyridine–AcOH–H_2O (80:20:6:11), R_f (G) EtOAc–pyridine–AcOH–H_2O (100:20:6:11), R_f (H) EtOAc–pyridine–AcOH–H_2O (240:20:6:11). 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 (Bruker WH 270 MHz). The purity was checked by HPLC (Waters apparatus) on a 250 × 4.6 mm Prolabo ODS2 5-μm column in a linear gradient solvent system carried out in 15 min with Et_3N–H_3PO$_4$ buffer (TEAP, 0.025 M, pH 6.5)/CH_3CN as eluent (flow rate, 1.5 mL/min) with UV (210 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 analysis was done on a LKB biochrom 4400 analyzer after hydrolysis with 6 N HCl, at 110 °C for 24 h. Mass spectra were recorded on a double-focusing VG 70-250 instrument. The FAB ionization was obtained with a FAB 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

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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; Aib, α-aminoisobutyric acid; MeOH, methanol; EtOAc, ethyl acetate; THF, tetrahydrofuran; AcOH, acetic acid; DMF, dimethylformamide; TFA, trifluoroacetic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; HOBt, 1-hydroxybenzotriazole; HONSu, *N*-hydroxysuccinimide; DCU, *N,N'*-dicyclohexylurea; t_R, retention time in HPLC; FAB, fast atom bombardment. Other abbreviations used were those recommended by the IUPAC–IUB Commission.[32]

Boc-D-Lys(Z)-Trp-OCH₃ (6). To a chilled solution of Trp–OCH₃·HCl (2.64 g, 12.1 mmol) in 120 mL of the solvent mixture THF–CHCl₃ (60:40) and Et₃N (1.69 mL, 12.1 mmol) were added Boc-D-Lys(N-εZ)-OH (4.6 g, 12.1 mmol), HOBt (1.8 g, 12.1 mmol), and DCC (2.73 g, 13 mmol). The mixture was stirred under N₂ for 1 h at 0 °C and overnight at room temperature. After filtration of DCU, the solvents were evaporated. The oily residue was dissolved in EtOAc and washed with 10% citric acid, saturated NaHCO₃, and brine, dried over Na₂SO₄, and concentrated to dryness in vacuo. The solid residue was recrystallized from EtOAc–hexane to yield 6.07 g (80%): mp 68–70 °C; R_f (B) 0.53. Anal. (C₃₁H₄₀O₇N₄) C, H, N.

Boc-Nle-D-Lys(Z)-Trp-OCH₃ (7). Compound 6 (7.5 g, 12.9 mmol) was dissolved in a chilled solvent mixture of CH₂Cl₂ (20 mL) and TFA–anisole (20 mL/1 mL). The resulting solution was stirred under N₂ for 45 min at 0 °C and for 45 min at room temperature. After evaporation of the solvents, the residue did not precipitate with ether; therefore the evaporation was repeated several times by the addition of CH₂Cl₂ and benzene. The resulting amorphous solid was suspended in a chilled solution of CHCl₃–THF (1:1) (100 mL), and Et₃N (1.81 mL, 12.9 mmol), Boc-Nle-OH (3 g, 12.9 mmol), HOBt (1.98 g, 12.9 mmol), and DCC (2.67 g, 12.9 mmol) were successively added. The resulting mixture was stirred under N₂ for 1 h at 0 °C and overnight at room temperature. The reaction mixture was worked up as described for the preparation of 6 to yield, after recrystallization from EtOAc–petroleum ether, 7.75 g (85%): mp 80–82 °C; R_f (A) 0.35. Anal. (C₃₇H₅₁O₈N₅) C, H, N.

Boc-Tyr-Nle-D-Lys(Z)-Trp-OCH₃ (9). A chilled solution of 7 (6.2 g, 8.93 mmol in CH₂Cl₂ (15 mL) and TFA–anisole (15 mL/0.8 mL) was stirred under N₂ for 45 min at 0 °C and for 45 min at room temperature. The solvent was removed by evaporation and the residue was purified by flash chromatography on silica gel by eluting first with CHCl₃–MeOH (95:5) and then with CHCl₃–MeOH (9:1), yielding 5.68 g (90%) of H-Nle-D-Lys(Z)-Trp-OCH₃·TFA (8), R_f (C) 0.49. To a chilled solution of 8 (3.46 g, 4.9 mmol) in CHCl₃ (30 mL) were added successively Et₃N (0.68 mL, 4.9 mmol), Boc-Tyr-OH (1.38 g, 4.9 mmol), HOBt (0.75 g, 4.9 mmol), and DCC (1.01 g, 4.9 mmol). The reaction mixture was stirred under N₂ for 1 h at 0 °C and overnight at room temperature. The *N,N'*-dicyclohexylurea was filtered off and the filtrate was concentrated in vacuo. The oily residue was purified by flash chromatography on silica gel with CHCl₃–MeOH (97:3) as eluent, yielding 3.3 g (80%): mp 152–154 °C; R_f (A) 0.34, R_f (B) 0.46. Anal. (C₄₆H₆₀O₁₀N₆) C, H, N.

Boc-Asp(OBzl)-Tyr-Nle-D-Lys(Z)-Trp-OCH₃ (11). A chilled solution of 9 (2.7 g, 3.15 mmol) in CH₂Cl₂ (5 mL) and TFA–anisole (5 mL/0.3 mL) was stirred under N₂ for 45 min at 0 °C and for 45 min at room temperature, yielding after evaporation, precipitation with dry ether, and filtration 2.3 g (84%) of H-Tyr-Nle-D-Lys(Z)-Trp-OCH₃·TFA (10), R_f (C) 0.58. To a chilled solution of 10 (1.15 g, 1.2 mmol) in DMF (8 mL) containing DIEA (0.41 mL, 2.4 mmol) were added Boc-Asp(OBzl)-ONp (0.64 g, 1.44 mmol) and HOBt (0.186 g, 1.2 mmol). Stirring was continued under N₂ for 1 h at 0 °C and 2 h at room temperature. After evaporation in vacuo, the oily residue was triturated with EtOAc and precipitated with anhydrous ether. After filtration, the residue was recrystallized from EtOAc–hexane, yielding 1.2 g (82%): mp 182–184 °C; R_f (C) 0.72. Anal. (C₅₇H₇₁O₁₃N₇) C, H, N.

Boc-Asp-Tyr-Nle-D-Lys-Trp-OCH₃ (13). Compound 11 (0.6 g, 0.56 mmol) in MeOH (20 mL) was hydrogenated in the presence

of 10% Pd/C catalyst (0.12 g) for 2 h to give after filtration and evaporation of MeOH 4.6 g (98%) of Boc-Asp-Tyr-Nle-D-Lys-Trp-OCH₃ (12), R_f (E) 0.36. A solution of 12 (0.74 g, 0.88 mmol) in DMF (145 mL) containing Et₃N (0.246 mL, 1.76 mmol) was treated at –40 °C with DPPA (0.248 mL, 1.14 mmol) in DMF (5 mL) through dropwise addition over 1 h. The reaction mixture was then stirred under N₂ for 48 h at –25 °C and for 48 h at 4 °C. During this time, the apparent pH was maintained between 7 and 7.5 through addition of Et₃N (moist pH paper, range 6–8). The DMF was evaporated in vacuo and the residual oil was purified by flash chromatography on silica gel by eluting first with CH₂Cl₂–MeOH (95:5) and then with CH₂Cl₂–MeOH (90:10) to yield 0.37 g (51%): mp 172–174 °C (recrystallization from CH₂Cl₂); R_f (B) 0.31, R_f (H) 0.34; FAB-MS (MH⁺) calcd 820, found 820.

Boc-Asp-Tyr-Nle-D-Lys-Trp-OH (14). A solution of 13 (0.33 g, 0.40 mmol) in MeOH (4.5 mL) at 0 °C was treated with 1 N NaOH (0.8 mL, 0.8 mmol). The mixture was stirred under N₂ for 2 h at 0 °C and overnight at room temperature. After evaporation of MeOH, the residue was dissolved in cold water (5 mL) and extracted with ether. The aqueous phase was acidified with cold 1 N HCl and extracted with EtOAc. The organic layer was then washed with brine and dried over Na₂SO₄ to yield after evaporation 0.24 g (74%): mp 205–207 °C; R_f (G) 0.37. Anal. (C₄₁H₅₅O₁₀N₇) C, H, N.

Boc-Asp(OBzl)-Phe-NH₂ (15). To a chilled solution of H-Phe-NH₂·TFA [obtained from Boc-Phe-NH₂ (1.07 g, 4.05 mmol)] in DMF (10 mL) and Et₃N (0.6 mL, 4.3 mmol) was added Boc-Asp(OBzl)-ONp (1.86 g, 4.05 mmol), and then the mixture was stirred at room temperature for 24 h. After evaporation in vacuo, the residue was dissolved in EtOAc and washed with 10% citric acid, 5% NaHCO₃, and brine. The solution was dried over Na₂SO₄ and evaporated to yield after recrystallization from EtOAc–ether 1.47 g (77%): mp 137–138 °C (lit.[27] mp 134–135 °C); R_f (A) 0.38. Anal. (C₂₅H₃₁O₆N₃) C, H, N.

Boc-Nle-Asp(OBzl)-Phe-NH₂ (17). A solution of 15 (1 g, 2.07 mmol) in TFA (2.5 mL) and CH₂Cl₂ (2.5 mL) was stirred for 45 min at 0 °C and for 45 min at room temperature. After evaporation in vacuo, the oily residue was precipitated with dry ether to yield H-Asp(OBzl)-Phe-NH₂·TFA (16) (1.85 g, 73%), R_f (B) 0.23. To a chilled solution of 16 (1.83 g, 3.78 mmol) in DMF (20 mL) treated with DIEA (1.3 mL, 7.56 mmol) were added Boc-Nle-ONp (1.48 g, 4.2 mmol) and HOBt (578 mg, 3.78 mmol). The reaction mixture was stirred for 30 min at 0 °C and overnight at room temperature. After evaporation of the solvent, the oily residue was precipitated from EtOAc–ether to yield 1.98 g (90%): mp 134–136 °C (lit.[33] mp 135–137 °C); R_f (B) 0.61. Anal. (C₃₁H₄₂O₇N₄) C, H, N.

Boc-Asp-Tyr-Nle-D-Lys-Trp-Nle-Asp(OBzl)-Phe-NH₂ (19). Compound 17 (0.151 g, 0.254 mmol) was treated with TFA (1 mL) as described for the preparation of 16 and precipitated from ether. The resulting compound H-Nle-Asp(OBzl)-Phe-NH₂·TFA (18), after drying over KOH in vacuo, was dissolved in DMF (4 mL) containing Et₃N (35 μL, 0.254 mmol) and was treated with 14 (204 mg, 0.254 mmol), HONSu (29 mg, 0.254 mmol), and DCC (63 mg, 0.30 mmol). The mixture was stirred for 1 h at 0 °C and overnight at room temperature. The *N,N'*-dicyclohexylurea was filtered off and the solvent was evaporated in vacuo. The residue was triturated with EtOAc and precipitated through addition of dry ether to yield 300 mg (93%): mp 130–133 °C; R_f (C) 0.62. Anal. (C₆₇H₈₇O₁₄N₁₁) C, H, N.

Boc-Asp-Tyr(SO₃Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH₂ (2). Compound 19 (250 mg, 0.197 mmol) was hydrogenated in MeOH (4 mL) in the presence of 10% Pd/C catalyst (11 mg) for 4 h to give after filtration and MeOH evaporation 216 mg (93%) of Boc-Asp-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (20). A solution of 20 (200 mg, 0.169 mmol) in dry DMF (2 mL) and dry pyridine (6 mL) was treated with SO₃–pyridine complex (1.1 g) overnight under N₂ at room temperature. After evaporation in vacuo, the residue was taken up in cold saturated NaHCO₃ solution and the mixture was stirred at 0 °C for 1 h with the pH maintained at about 7. The suspended product was collected by

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centrifugation and dried in vacuo (300 mg). A second crop, less pure from the aqueous phase, was isolated by lyophilization followed by precipitation of inorganic salts in MeOH and evaporation in vacuo (100 mg). These 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 for the two separations 66 mg (30%) of **2**: R_f (E) 0.23; HPLC (t_R = 11.4 min), eluent CH$_3$CN–TEAP, gradient from 25:75 to 35:65; FAB-MS (MH$^+$) calcd 1306, found 1306. Amino acid analysis: Asp 1.91, Nle 2, Tyr 0.92, Phe 0.96, Lys 0.93.

Boc-Nle-Asp(OtBu)-NH$_2$ (21). To a chilled solution of Asp(OtBu)-NH$_2$·HCl (0.35 g, 1.55 mmol) in DMF (7 mL) containing DIEA (0.266 mL, 1.55 mmol) were added Boc-Nle-ONp (0.60 g, 1.7 mmol) and HOBt (273 mg, 1.55 mmol). The mixture was stirred for 1 h at 0 °C and 3 h at room temperature. After evaporation in vacuo, the residue was dissolved in CH$_2$Cl$_2$ and worked up as described for the isolation of **11** to yield 0.55 g (88%): mp 70–72 °C; R_f (A) 0.55. Anal. (C$_{19}$H$_{35}$O$_6$N$_3$) C, H, N.

Boc-Asp-Tyr-Nle-D-Lys-Trp-Nle-Asp-NH$_2$ (23). Compound **21** (0.5 g, 1.24 mmol) dissolved in TFA–H$_2$O (4 mL/1 mL) was stirred for 2 h at 0 °C and for 2 h at room temperature. The reaction mixture was worked up as described for the preparation of **10** to give 428 mg (96%) of H-Nle-Asp-NH$_2$·TFA (**22**), R_f (E) 0.18.

To a solution of **14** (230 mg, 0.285 mmol) and HONSu (32.5 mg, 0.285 mmol) in DMF (3 mL) at –10 °C was added DCC (58.7 mg, 0.285 mmol). This reaction mixture was stirred for 45 min at –10 °C, then for 2 h at 0 °C, and then overnight at room temperature. To the above mixture at 0 °C was added a solution of **22** (102 mg, 0.285 mmol) and Et$_3$N (40 μL, 0.285 mmol) in DMF (1 mL). The resulting mixture was stirred for 48 h at room temperature. Evaporation of DMF and trituration with EtOAc–ether yielded 250 mg (85%): mp 165–167 °C; R_f (H) 0.30. Anal. (C$_{51}$H$_{72}$O$_{13}$N$_{10}$) C, H, N.

Boc-Asp-Tyr(SO$_3$Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-NH$_2$ (4). Compound **24** (200 mg, 0.193 mmol) was sulfated with SO$_3$–pyridine complex (1.25 g), worked up, and purified by chromatography as described for the preparation of **2** to yield 58 mg (28%): R_f (F) 0.23; HPLC (t_R = 18.4 min), eluent CH$_3$CN–TEAP, gradient from 22:78 to 32:68; FAB-MS (MH$^+$) calcd 1158, found 1158. Amino acid analysis: Asp 2.1, Nle 2, Tyr 0.95, Lys 1.03.

Boc-Tyr-Nle-D-Lys(Z)-Trp-OH (24). Compound **9** (500 mg, 0.58 mmol) was saponified with 1 N NaOH (1.16 mL, 1.16 mmol) in MeOH (5 mL) overnight at room temperature. After addition of water and evaporation of MeOH in vacuo, the residue was worked up, as described for the preparation of **14**, to yield 400 mg (82%): mp 111–114 °C; R_f (D) 0.32, R_f (G) 0.62. Anal. (C$_{45}$H$_{58}$O$_{10}$N$_6$) C, H, N.

Boc-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp(OBzl)-Phe-NH$_2$ (25). Compound **17** (237 mg, 0.40 mmol) was treated with TFA (1.5 mL) as described for the preparation of **16**, precipitated from ether, and dried over KOH in vacuo. To a solution of the above resulting product and Et$_3$N (52 μL, 0.37 mmol) in DMF (3 mL) were added **24** (312 mg, 0.37 mmol), HONSu (42.5 mg, 0.37 mmol), and DCC (91.5 mg, 0.44 mmol). The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature. After the same working up as described for the isolation of **19**, the product was recrystallized from EtOAc–petroleum ether, yielding 400 mg (83%): mp 211–213 °C; R_f (D) 0.73, R_f (B) 0.42. Anal. (C$_{71}$H$_{90}$O$_{14}$N$_{10}$) C, H, N.

Boc-Asp(OBzl)-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp(OBzl)-Phe-NH$_2$ (26). Compound **25** (326 mg, 0.25 mmol) in CH$_2$Cl$_2$ (0.6 mL) was treated with TFA–anisole (0.6 mL/35 μL) as described for the preparation of **10**. The resulting powder, after drying in vacuo, was dissolved in DMF (3 mL) at 0 °C containing DIEA (86 μL, 0.5 mmol). To the above solution were added HOBt (38.2 mg, 0.25 mmol) and Boc-Asp(OBzl)-ONp (133 mg, 0.25 mmol), and the resulting mixture was stirred for 1 h at 0 °C and 3 h at room temperature. After evaporation, the oily residue was precipitated with EtOAc, filtered, and recrystallized from MeOH: yield 290 mg (77%); mp 235–237 °C; R_f (D) 0.63. Anal. (C$_{82}$H$_{101}$O$_{17}$N$_{11}$) C, H, N.

Boc-Asp(OBzl)-Tyr(SO$_3$Na)-Nle-D-Lys(Z)-Trp-Nle-Asp(OBzl)-Phe-NH$_2$ (27). Compound **26** (280 mg, 0.19 mmol) was

sulfated with SO$_3$–pyridine complex (0.9 g) as previously described for the preparation of **2** to yield after working up and chromatography on silica gel 61.6 mg (20%): eluent EtOAc–pyridine–AcOH–H$_2$O (100:20:6:11); R_f (H) 0.28. Amino acid analysis: Asp 1.85, Nle 2, Tyr 0.91, Phe 0.94, Lys 0.98.

Boc-Asp(Na)-Tyr(SO$_3$Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH$_2$ (1). Compound **27** (60 mg, 0.036 mmol) in MeOH (5 mL) was hydrogenated for 12 h in the presence of 10% Pd/C catalyst (30 mg). After filtration of the catalyst, the methanol was evaporated in vacuo. The residue was taken up in 5% NaHCO$_3$ (5 mL) and lyophilized. After precipitation of inorganic salts in MeOH followed by evaporation in vacuo, the residue was purified by chromatography on silica gel with the eluent EtOAc–pyridine–AcOH–H$_2$O (40:20:6:11) to give 30 mg (63%): R_f (F) 0.25; HPLC (t_R = 11.6 mn), eluent CH$_3$CN–TEAP, gradient from 28:72 to 38:62; FAB-MS (MH$^+$) calcd 1345, found 1345. Amino acid analysis: Asp 2.08, Nle 2, Tyr 1, Phe 1.09, Lys 1.06.

Boc-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp-NH$_2$ (28). As described for the preparation of **23**, compound **24** (375 mg, 0.44 mmol) is activated through HONSu (50.6 mg, 0.44 mmol) and DCC (90.6 mg, 0.44 mmol) and condensed with **22** (158 mg, 0.44 mmol) in DMF (1 mL) containing Et$_3$N (61 μL, 0.44 mmol). Evaporation of DMF and trituration with EtOAc–ether yielded 300 mg (64%): mp 160–162 °C; R_f (H) 0.55. Anal. (C$_{55}$H$_{75}$O$_{13}$N$_9$) C, H, N.

Boc-Asp(OBzl)-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp-NH$_2$ (29). Compound **28** (270 mg, 0.25 mmol) was treated with TFA–anisole (1 mL/0.1 mL) as previously described for the deprotection of **15** to give 240 mg (88%); R_f (H) 0.24. To a chilled solution of the above product 240 mg (0.22 mmol) and DIEA (40 μL, 0.22 mmol) in DMF (2 mL) were added Boc-Asp(OBzl)-ONp (112 mg, 0.25 mmol) and HOBt (33.6 mg, 0.22 mmol). The reaction mixture was stirred at 0 °C for 1 h and overnight at room temperature. The solvent was removed in vacuo and the oily residue was precipitated from EtOAc–ether to give after filtration 245 mg (87%): mp 153–155 °C; R_f (H) 0.58. Anal. (C$_{66}$H$_{86}$O$_{16}$N$_{10}$) C, H, N.

Boc-Asp(OBzl)-Tyr(SO$_3$Na)-Nle-D-Lys(Z)-Trp-Nle-Asp-(Na)-NH$_2$ (30). Compound **29** (235 mg, 0.18 mmol) in dry DMF (2 mL) and dry pyridine (6 mL) was sulfated with SO$_3$–pyridine complex (1.3 g) following the procedure previously described for the preparation of **2** to give, after workup and chromatography on silica gel with EtOAc–pyridine–AcOH–H$_2$O (80:20:6:11), 70 mg (28%), R_f (G) 0.25.

Boc-Asp(Na)-Tyr(SO$_3$Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-NH$_2$ (3). Compound **30** (65 mg, 0.046 mmol) in MeOH (5 mL) was hydrogenated for 2 h in the presence of 10% Pd/C catalyst (20 mg). After filtration of the catalyst, the methanol was evaporated in vacuo and the residue was taken up in 5% NaHCO$_3$ (5 mL) and lyophilized. After precipitation of inorganic salts in MeOH followed by evaporation in vacuo, the residue was purified by chromatography on silica gel with EtOAc–pyridine–AcOH–H$_2$O (40:20:6:11) to yield 40 mg (74%): R_f (F) 0.22; HPLC (t_R = 12.2 min), eluent CH$_3$CN–TEAP, gradient from 30:70 to 40:60; FAB-MS (MH$^+$) calcd 1198, found 1198. Amino acid analysis: Asp 2.09, Nle 2, Tyr 1.01, Lys 1.07.

Boc-Trp-Lys(Z)-OCH$_3$ (31). To a chilled solution of Lys(N-εZ)-OCH$_3$·HCl (6 g, 18.2 mmol) in DMF (90 mL) treated with DIEA (6.27 mL, 36.4 mmol) were added Boc-Trp-ONp (9.28 g, 21.8 mmol) and HOBt (2.78 g, 18.2 mmol). The reaction mixture was stirred under N$_2$ for 1 h at 0 °C and 2 h at room temperature. The solvent was evaporated and the residue was dissolved in EtOAc and worked up as described for the isolation of **6** to yield after crystallization in ether 9.2 g (87%): mp 99–101 °C; R_f (A) 0.43. Anal. (C$_{31}$H$_{40}$O$_7$N$_4$) C, H, N.

Boc-Gly-Trp-Lys(Z)-OCH$_3$ (32). Compound **31** (9.1 g, 15.7 mmol) was dissolved in a chilled solvent mixture of CH$_2$Cl$_2$ (25 mL) and TFA–anisole (25 mL/1.2 mL). The resulting solution was stirred under N$_2$ for 45 min at 0 °C and for 45 min at room temperature. The evaporation was repeated several times by the addition of CH$_2$Cl$_2$ and benzene, and the resulting amorphous solid was dissolved in DMF (80 mL) at 0 °C and was successively treated with DIEA (2.6 mL, 15.7 mmol), Boc-Gly-ONp (5.1 g, 18.8 mmol), and HOBt (2.4 g, 15.7 mmol). The mixture was stirred for 1 h at 0 °C and for 2 h at room temperature. After evaporation of the solvent, the residue was worked up as described for the isolation of **6** to give after crystallization from ether 9.05 g (90%):

mp 81–83 °C; R_f (B) 0.36. Anal. ($C_{33}H_{43}O_8N_5$) C, H, N.

Boc-Asp(OBzl)-Gly-Trp-Lys(Z)-OCH₃ (33). Compound **32** (9 g, 14.1 mmol) was treated by a mixture CH₂Cl₂–TFA–anisole (20 mL/20 mL/1 mL) following the procedure described for the deprotection of **6**. The resulting amorphous solid was dissolved in chilled DMF (40 mL), and DIEA (1.65 mL, 16.9 mmol), Boc-Asp(OBzl)-ONp (4.26 g, 16.9 mmol), and HOBt (1.22 g, 14.1 mmol) were added successively. The mixture was stirred for 1 h at 0 °C and overnight at room temperature. After evaporation in vacuo, the oily residue was dissolved in EtOAc and extracted, as described for the preparation of **6**. The resulting powder was purified by flash chromatography on silica gel with CHCl₃–MeOH (95:5) as eluent, yielding 9.75 g (82%): mp 80–82 °C; R_f (B) 0.32. Anal. ($C_{44}H_{54}O_{11}N_6$) C, H, N.

Boc-Tyr-Asp(OBzl)-Gly-Trp-Lys(Z)-OCH₃ (34). After removal of the Boc protecting group on **33** (9.74 g, 11.6 mmol) with CH₂Cl₂–TFA–anisole (17 mL/17 mL/1 mL) as described for the deprotection of **6**, the residual solid was placed in chilled DMF and treated successively with Et₃N (1.62 mL, 11.6 mmol), Boc-Tyr-OH (3.25 g, 11.6 mmol), HONSu (1.33 g, 11.6 mmol), and DCC (2.38 g, 11.56 mmol). The mixture was stirred under N₂ overnight at room temperature. The solvent was removed in vacuo and the residue was worked up as described for the preparation of **6** to yield after flash chromatography on silica gel with CH₂Cl₂–MeOH (97:3) and crystallization from EtOAc–ether 9.88 g (85%): mp 103–105 °C; R_f (B) 0.41. Anal. ($C_{53}H_{63}O_{13}N_7$) C, H, N.

Boc-Tyr-Asp-Gly-Trp-Lys-OCH₃ (35). Compound **34** (950 mg, 0.94 mmol) in MeOH (40 mL) was hydrogenated in the presence of 10% Pd/C catalyst (150 mg) for 2 h to give after filtration and evaporation of the MeOH 650 mg (77%): mp 185–187 °C; R_f (E) 0.26. Anal. ($C_{38}H_{51}O_{11}N_7$) C, H, N.

Boc-Tyr-Asp-Gly-Trp-Lys-OCH₃ (36). Compound **35** (640 mg, 0.818 mmol) was cyclized with DPPA in the conditions previously described for the preparation of **13** to yield after flash chromatography on silica gel with CHCl₃–MeOH (95:5) and recrystallization from EtOAc 400 mg (64%): mp 187–189 °C; R_f (B) 0.36; FAB-MS (MH⁺) calcd 765, found 765. Anal. ($C_{38}H_{49}O_{10}N_7$) C, H, N.

Boc-Tyr-Asp-Gly-Trp-Lys-OH (37). Compound **36** (0.3 g, 0.39 mmol) was saponified following the procedure described for the preparation of **14** to give 210 mg (72%): mp 210–211 °C; R_f (F) 0.26. Anal. ($C_{37}H_{47}O_{10}N_7$) C, H, N.

Boc-Tyr-Asp-Gly-Trp-Lys-Asp(OBzl)-Phe-NH₂ (38). Compound **16** (130 mg, 0.27 mmol) was dissolved in chilled DMF (2 mL) containing Et₃N (0.038 mL, 0.27 mmol) and treated with **37** (200 mg, 0.27 mmol), HONSu (31 mg, 0.27 mmol), and DCC (66.7 mg, 0.32 mmol). The reaction mixture was stirred for 1 h at 0 °C and for 48 h at room temperature and was worked up as previously described for the isolation of **19** to give 250 mg (84%): mp 239–241 °C; R_f (F) 0.48. Anal. ($C_{57}H_{68}O_{13}N_{10}$) C, H, N.

Boc-Tyr(SO₃Na)-Asp-Gly-Trp-Lys-Asp(Na)-Phe-NH₂ (5).

Compound **38** (200 mg, 0.18 mmol) was hydrogenated in MeOH–DMF (2 mL/4 mL) for 12 h with 10% Pd/C catalyst (45 mg) to afford Boc-Tyr-Asp-Gly-Trp-Lys-Asp-Phe-NH₂ (**39**) (160 mg, 87%). The sulfation of **39** (150 mg, 0.148 mmol) with SO₃–pyridine complex (1 g) as described for the preparation of **2** and purification by chromatography on silica gel with Et-OAc–pyridine–AcOH–H₂O (55:20:6:11) as eluent gave 60 mg (42%); R_f (E) 0.20; HPLC (t_R = 11 min), eluant CH₃CN–TEAP, gradient from 25:75 to 35:65; FAB-MS (MH⁺) calcd 1136, found 1136. Amino acid analysis: Asp 1.95, Gly 1.03, Tyr 0.97, Phe 1, Lys 1.02.

Bioassays. Tissue preparation of mouse brain and rat pancreas membranes and the related binding experiments were performed as reported in detail previously.[28] Amylase secretion from rat pancreas fragments was assayed according to the method of Ceska et al.[29] using the Phadebas reagent (Pharmacia). Pancreas fragments preparation and experimental procedures were reported elsewhere.[13]

Contractile activity on guinea pig ileum was carried out according to Hutchinson and Dockray[34] in the presence of atropine (1.5 mg/L). The conditions of these test were described elsewhere.[13]

(34) Hutchinson, J. B.; Dockray, G. I. *Eur. J. Pharmacol.* **1981**, *69*, 87.