Boc-Trp-Orn(Z)-Asp-NH_2 and Derivatives: A New Family of CCK Antagonists

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The respective roles of the benzyloxycarbonyl group (Z) and of the N-terminal tripeptide moiety in the antagonist properties of the cholecystokinin CCK_8 analogue Boc-[Nle^{28},$Orn(Z)^{31}$]CCK_{27-33} (Marseigne et al. *J. Med. Chem.* **1988**, *31*, 966.) were studied with the following derivatives: Boc-[Nle^{28},$Orn(X)^{31}$]CCK_{27-33}, Boc-[Nle^{28},$Orn(X)^{31}$]CCK_{27-32}, Boc-[$Orn(X)^{31}$]CCK_{30-33}, and Boc-[$Orn(X)^{31}$]CCK_{30-32} (X = Z, Boc, H). These derivatives, the synthesis of eight of which is reported here, were tested for their abilities to inhibit the binding of [^{3}H]pCCK_8 to guinea pig pancreatic and brain membranes and for their potencies in stimulating amylase release from guinea pig pancreatic acini. None of the Z derivatives produced amylase secretion, but they competitively antagonized the stimulation induced by CCK_8. The deletion of the N-terminal tripeptide and/or Phe-NH_2^{33} residue did not play a key role in the recognition of peripheral receptors and in the activity of these peptides, whereas replacement of the Z group by a Boc group slightly decreased the affinities of the compounds for both pancreatic and brain binding sites and their potencies as peripheral antagonists. Moreover, the tetrapeptide Boc-Trp-Orn(Boc)-Asp-Phe-NH_2 behaved as a partial agonist and analogues in which the Z or Boc groups on the ornithine residue were removed were full agonists. Interestingly, the short peptide derivative Boc-Trp-Orn(Z)-Asp-NH_2 displayed the same affinity ($K_I = 2.0 \pm 0.2 \times 10^{-7}$) and the same antagonist activity ($pA_2 = 6.63$) as its parent compound Boc-[Nle^{28},$Orn(Z)^{31}$]CCK_{27-33}. This tripeptide could be an interesting tool for studying the structural relationships between peptide and non-peptide CCK antagonists.

Introduction

The C-terminal octapeptide of cholecystokinin, CCK_{26-33} or CCK_8, is a hormonal regulator of various gut functions such as gall bladder contraction, pancreatic secretion, and gut motility.[1-3] CCK_8 is also present in high concentrations in brain, where it seems to play a neurotransmitter or neuromodulator role.[4-6] Biochemical studies have shown the existence of at least two classes of CCK receptors,[7,8] "central" receptors (CCK_B), largely distributed in the brain,[9-11] and "peripheral" receptors (CCK_A), distributed in peripheral organs but also present in some regions of the brain.[9,12] The respective roles of both types of receptors in many CCK_8-induced pharmacological responses such as satiety, analgesia, and neuroleptic effects remain to be elucidated or are still controversial.

In addition to their potential therapeutic value, receptor antagonists are important tools for elucidating the role of a natural effector in physiological as well as in pathological conditions. The CCK receptor antagonists described so far can be divided into five categories: (1) derivatives of cyclic nucleotides, such as dibutyryl cGMP;[13] (2) modified amino acids, such as proglumide,[14,15] benzotrip,[15] and its analogues;[16-19] (3) benzodiazepine derivatives[20-25] developed with the natural compound asperlicin as a model;[26] (4) substance P analogues;[27] and (5) partial sequences of CCK_8 and derivatives of its C-terminal moiety.[28-32]

Among the compounds belonging to this latter series the C-terminal fragment Z-CCK_{27-32}-NH_2 was found to competitively inhibit CCK-stimulated amylase release and the binding of labeled CCK to guinea pig pancreatic acini,[28] whereas it is a partial agonist in rat and mouse, stimulating amylase release from pancreatic acini, but in contrast to the action of CCK_8, supramaximal concentration of this compound did not cause submaximal stimulation of amylase secretion.[29] Other des-PheNH_2 CCK analogues have also been described to have the same agonist/antagonist profile.[30] On the other hand, some C-terminal tetra- and heptapeptide analogues of CCK in which the primary amide function has been deleted, such as Boc-Trp-Leu-Asp-2-phenylethyl ester[30] and Boc-Tyr(SO_3H)-Met-Gly-D-Trp-Nle-Asp-2-phenylethyl ester,[31] act as CCK receptor antagonists. Likewise, the cyclic peptide *c*-[Phe-Met-Gly-D-Trp-Met-D-Asp(OBzl)] was the most potent of a cyclic peptide antagonists family, designed to test the possible occurrence of a turn in the biologically active conformation of CCK.[32]

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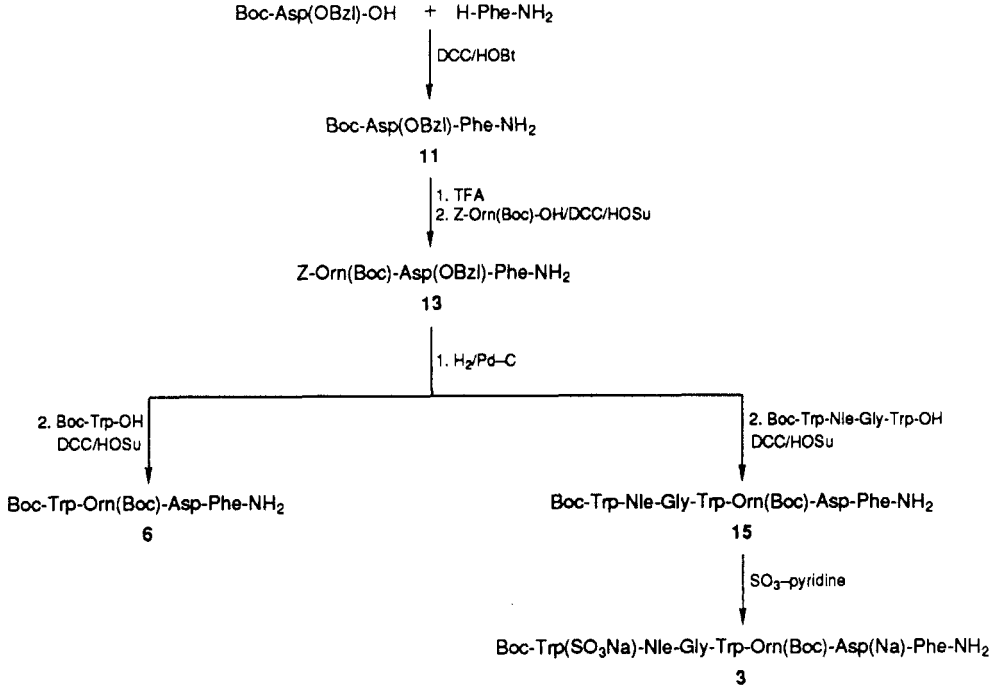
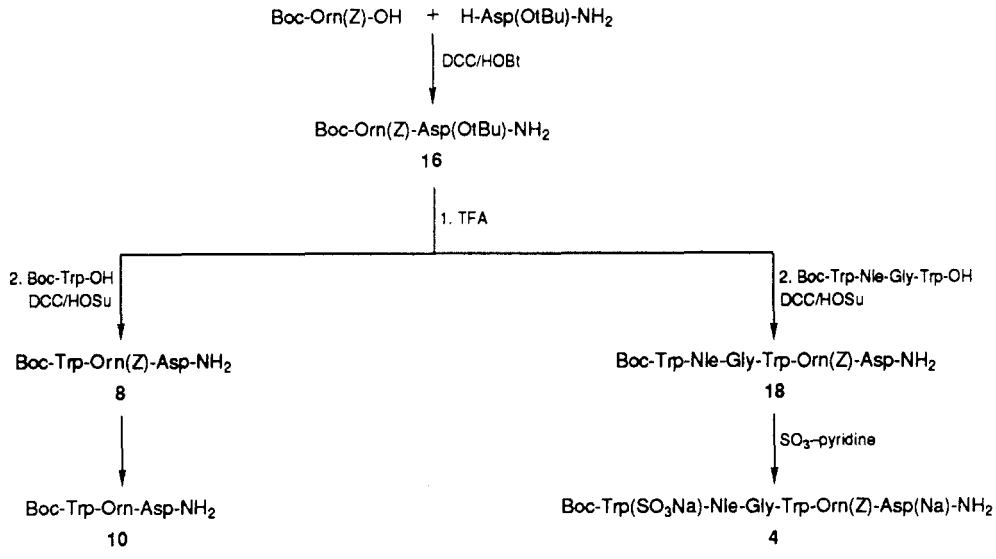
Scheme I



Scheme II



We have previously shown that the compound Boc-Tyr(SO_3H)-Nle-Gly-Trp-Orn(Z)-Asp-Phe-NH_2 (1) is an antagonist of both peripheral and central CCK receptors.[33,34] The fact that the corresponding analogue with

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Table I. Apparent Affinities (K_I) of Derivatives 1–10 on the Binding of [^{3}H]pCCK$_8$ to the Brain (K_D = 0.18 nM) and Pancreatic Membranes (K_D = 1.22 nM) of Guinea Pigs

compound	no.	binding K_I,[a] M: brain (CCK_B)	pancreas (CCK_A)
Boc-Tyr(SO_3H)Nle-Gly-Trp-Orn(Z)-Asp-Phe-NH_2	1	$9.3 \pm 0.1 \times 10^{-8}$	$3.1 \pm 0.2 \times 10^{-7}$
Boc-Tyr(SO_3H)Nle-Gly-Trp-Orn-Asp-Phe-NH_2	2	$1.13 \pm 0.1 \times 10^{-7}$	$3.4 \pm 0.4 \times 10^{-7}$
Boc-Tyr(SO_3H)Nle-Gly-Trp-Orn(Boc)-Asp-Phe-NH_2	3	$2.9 \pm 0.4 \times 10^{-7}$	$6.6 \pm 0.7 \times 10^{-7}$
Boc-Tyr(SO_3H)Nle-Gly-Trp-Orn(Z)-Asp-NH_2	4	$5.4 \pm 0.6 \times 10^{-8}$	$2.3 \pm 0.5 \times 10^{-7}$
Boc-Trp-Orn(Z)-Asp-Phe-NH_2	5	$5.8 \pm 1.1 \times 10^{-7}$	$3.4 \pm 0.1 \times 10^{-7}$
Boc-Trp-Orn(Boc)-Asp-Phe-NH_2	6	$2.2 \pm 0.4 \times 10^{-6}$	$5.2 \pm 0.3 \times 10^{-6}$
Boc-Trp-Orn-Asp-Phe-NH_2	7	$1.4 \pm 0.1 \times 10^{-6}$	$1.4 \pm 0.4 \times 10^{-5}$
Boc-Trp-Orn(Z)-Asp-NH_2	8	$4.2 \pm 1.0 \times 10^{-6}$	$2.0 \pm 0.2 \times 10^{-7}$
Boc-Trp-Orn(Boc)-Asp-NH_2	9	$4.3 \pm 1.1 \times 10^{-6}$	$2.9 \pm 0.9 \times 10^{-5}$
Boc-Trp-Orn-Asp-NH_2	10	$1.0 \pm 0.2 \times 10^{-4}$	10^{-3}

[a] K_I values represent mean ± SEM of three separate experiments each performed in triplicate. The values of the Hill coefficients were close to 1 in all experiments.

Table II. Pharmacological Potencies of CCK Analogues 1–9[a]

compound	no.	amylase secretion by guinea pig acini: antagonist act.: K_b, M	pA_2	agonist act.: EC_{50}, M
Boc-Tyr(SO_3H)Nle-Gly-Trp-Orn(Z)-Asp-Phe-NH_2	1	4.9×10^{-7}	6.32	ND
Boc-Tyr(SO_3H)Nle-Gly-Trp-Orn-Asp-Phe-NH_2	2	ND		2.20×10^{-8}
Boc-Tyr(SO_3H)Nle-Gly-Trp-Orn(Boc)-Asp-Phe-NH_2	3	1.1×10^{-6}	5.94	ND
Boc-Tyr(SO_3H)Nle-Gly-Trp-Orn(Z)-Asp-NH_2	4	2.2×10^{-7}	6.64	ND
Boc-Trp-Orn(Z)-Asp-Phe-NH_2	5	5.7×10^{-7}	6.25	ND
Boc-Trp-Orn(Boc)-Asp-Phe-NH_2	6	2.6×10^{-5}	4.58	1.36×10^{-5}
Boc-Trp-Orn-Asp-Phe-NH_2	7	ND		1.38×10^{-5}
Boc-Trp-Orn(Z)-Asp-NH_2	8	2.3×10^{-7}	6.63	ND
Boc-Trp-Orn(Boc)-Asp-NH_2	9	2.4×10^{-5}	4.62	ND

[a] Values are the mean of three experiments each performed in duplicate. $r = 1.0 \pm 0.1$ for all experiments. ND means that no significant activity could be detected at 10^{-4} M.

an unprotected Orn^{31} side chain (**2**) acts as a full peripheral agonist suggested that the aromatic ring of the Z group could play a critical role in the antagonist activity of **1**. In order to test this hypothesis the Z protecting moiety was replaced by a *tert*-butyloxycarbonyl group, in **3**. The synthesis of this compound and of a series of shorter derivatives, with Orn(Z), Orn(Boc), or Orn in position 31, their binding properties to both peripheral and central receptors, and the effect on amylase release are also reported in this paper.

Results

The syntheses of CCK analogues **3**, **4**, **6**, **8**, and **10** are summarized in Schemes I and II. Elongation of the peptide chains was performed stepwise or by fragments with condensation using the DCC/HOBt or DCC/HOSu condensation methods. The amino protecting groups were removed either with trifluoroacetic acid or by catalytic hydrogenolysis. Peptides **13** and **16** were prepared as common intermediates for the synthesis of compounds **3** and **6**, and **4** and **8**, respectively (Schemes I and II). The hexa- and heptapeptides **15** and **18** were treated with an SO_3–pyridine mixture to introduce a sulfate ester group in the tyrosine side chain.

Compound **5**, Boc-Trp-Orn(Z)-Asp-Phe-NH_2, was synthesized by condensation of Boc-Trp-OH with the peptide fragment H-Orn(Z)-Asp-Phe-NH_2, prepared as previously described,[33] by using the DCC/HOSu method. Compounds **7** and **10** were obtained from peptide derivatives **5** and **8** by catalytic hydrogenation.

The synthesis of compound **9** was achieved by coupling Z-Orn(Boc)-OH to H-Asp(OBzl)-NH_2 according to the DCC/HOBt method to give derivative **19**, followed by catalytic hydrogenation and condensation of the resulting compound with Boc-Trp-OSu.

All peptides were purified by silica gel column chromatography and lyophilized. Purity and lack of racemization of final products and of all intermediates were checked by HPLC and ^{1}H NMR spectroscopy.

The synthesized compounds were evaluated for their potency in displacing [^{3}H]pCCK$_8$ from guinea pig pancreatic and brain membranes (Table I) and for their ability to induce or to inhibit amylase release from guinea pig pancreatic acini (Table II). Results were compared to those obtained with the parent compounds Boc-(Nle^{28},Orn(X)^{31})CCK$_{27-33}$ (**1**, X = Z; **2**, X = H).

The replacement of the phenyl ring of the protecting Z group by a hydrophobic *tert*-butyl group in **3** led to only a 2- and 3-fold decrease in affinities for CCK_A and CCK_B receptors, respectively, while removal of the Z group (compound **2**) did not modify the binding properties of compound **1**. In compound **4** the removal of the C-terminal phenylalanine amide present in **1** did not change significantly the binding properties of CCK_A and CCK_B receptors. The shortening of the peptide sequence by removal of the N-terminal tripeptide, Boc-Tyr(SO_3H)-Nle-Gly, led to a significant 6–10-fold decrease in the affinity of the tetrapeptides **5**–**7** for CCK_B binding sites as compared to that of their parent compounds. In contrast, the Z tetrapeptide **5** preserved the same affinity for pancreatic receptors as compound **1**, while the Boc derivative **6** and the nonsubstituted analogue **7** were, respectively, 8 and 40 times less potent than their corresponding heptapeptides **3** and **2**. As in the case of the heptapeptides, introduction of the Boc protecting group in place of the Z group induced a small reduction in affinity for both receptor types. As expected from the behavior of **4**, re-

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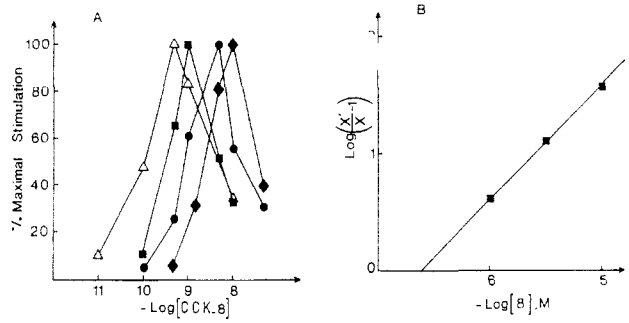

Figure 1. Effects of compound 8 on CCK_8-induced amylase release from guinea pig pancreatic acini: (A) amylase release of CCK_8 was measured alone (△) or in the presence of compound 8, (■) 10^{-6} M, (●) 3×10^{-6} M, and (◆) 10^{-5} M; (B) Schild plot of the antagonism by compound 8 [$\log x'/x - 1) = 0.96 \log B + 6.37$ ($r = 0.998$), $pA_2 = 6.63$]. Points represent the mean of three experiments each performed in duplicate.

moval of the C-terminal Phe-NH_2 group in **8** and **9** slightly modified the interaction with brain receptors when compared to that with compounds **5** and **6**. However, the importance of a phenyl ring in the peptide sequence for efficient recognition of CCK_A receptors was clearly demonstrated by the approximately 100-fold differences in binding properties of **8** and **9**. The unsubstituted tripeptide **10**, in which both aromatic rings were deleted, showed very weak affinity for both receptor types.

No agonist activity was detected for Z- or Boc-CCK derivatives in amylase bioassays at concentrations as high as 10^{-4} M, except for compound **6**, which displayed partial agonist activity. All the antagonists caused a parallel shift to the right of the dose–response curve to CCK_8. A typical experiment is shown in Figure 1. The negative log of the molar concentrations of each antagonist (-log B) was plotted against log $(x'/x - 1)$, where x'/x is the ratio between the agonist concentrations which are able to induce the same response in the presence or in the absence of the antagonists.[35] Linear-regression analysis of the data points gave the affinity constant $(1/K_b)$ of each antagonist by interception with the X axis. The results obtained are reported in Table II. The slopes of the lines were not significantly different from unity, indicating competitive inhibition. The K_b values were in good agreement with the K_I values for CCK_A receptors determined from binding studies except for compound **6**, which had a partial agonist activity, with a maximum release of amylase reaching 75% of that induced by CCK_8. Derivatives **4**, **5**, and **8** were equipotent to compound **1** ($K_b \approx 10^{-7}$ M) while the Boc-tetra- and tripeptides (**6** and **9**) were much less active ($K_b \approx 10^{-5}$ M).

Discussion

The relative affinities of the CCK_8 analogues **1**, **5** and **4**, **8** for guinea pig CCK_A and CCK_B receptors and their potencies in inhibiting amylase release from guinea pig pancreatic acini show that the removal of the N-terminal sequence Boc-Tyr(SO_3H)-Nle-Gly does not strongly modify the recognition of the CCK_A receptor type and the antagonist activity of these peptides (Table II). It can be noted that the nonpeptide benzodiazepine-derived CCK antagonists do not possess a sulfated moiety.

Moreover, elimination of the C-terminal Phe-NH_2[33] residue in **4** and **8** did not influence their antagonist properties, which remained similar to those of their parent compounds **1** and **5**. Replacing the Z group in the ornithine side chain by a *tert*-butyloxycarbonyl moiety led to a slight decrease in the affinity and antagonist potency of compound **3**, with more drastic reductions in the case of compound **9**. Nevertheless both compounds behaved as full antagonists while the tetrapeptide **6** had mixed agonist–antagonist properties. Removing the bulky and lipophilic Z or Boc groups led to weakly potent, full agonists (compounds **2** and **7**) with low CCK receptor affinities. Thus, it seems that the aromatic ring of the Z group is a major structural factor in inducing antagonist properties in the CCK analogues studied.

Structure–activity relationships and conformational studies have shown that in CCK-like compounds the presence and relative position of the aromatic rings play a critical role in receptor binding and in transduction mechanism.[36-40] This is illustrated by the increased CCK_B selectivity induced by cyclization of CCK_8 analogues[41] or by the antagonist properties produced by the removal of the CCK_8 terminal amino acid Phe-NH_2.[33,28]

Moreover, the most important criteria for an efficient stimulation of CCK-receptors was shown to be not the aromatic character of the amino acid in position 33 but its size and hydrophobicity[37-42,43] and, even more critically, the presence of an amide group.[30,31] Therefore, owing to the flexibility of the ornithine side chain, the phenyl ring of the Z protecting group or the bulky *tert*-butyl moiety could fit into the hydrophobic receptor subsite corresponding to the C-terminal Phe-NH_2[33] residue, either by displacement of this amino acid from its site or directly when it is lacking. This structural change would hinder the amide group of Phe-NH_2[33] from adopting the precise orientation required for receptor activation. Another possible, although less satisfactory explanation, is that the Z group induces a conformational change which prevents the binding of the Phe-NH_2 residue to its hydrophobic pocket.

Alternatively, the Z or Boc groups of Orn could interact with an auxiliary hydrophobic site, leading to less favorable interaction of the Phe moiety with an associated decrease in affinity.

The lower affinity and antagonist potency of the tetra- and tripeptides containing a Boc group, the partial agonist profile of **6**, and the pure and full agonist properties of compounds containing an Orn residue could be explained by the relative strengths of interactions of -Orn(Z)-,

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-Orn(Boc)- or -Orn(H)- either at the Phe-NH_2 subsite or at the previously mentioned auxiliary subsite. Further studies such as those on modifications of both-Phe-NH_2 and the ϵ-amino function of Orn are now required to confirm these assumptions.

In conclusion, we have developed from the CCK-antagonist heptapeptide Boc-(Nle[28],Orn(Z)[31])CCK_{27-33} (1) a simplified derivative (Boc-Trp-Orn(Z)-Asp-NH_2 (8) with a 45-fold reduced affinity for central binding sites but the same antagonist properties at the peripheral level. One of the advantages of this new antagonist is its much better water solubility than the benzodiazepine-derived CCK antagonists. Although the most potent and selective CCK antagonists developed to date are non-peptide-like substances,[20-25] research in the peptide field could be of great interest to study the structural interrelationships among the various classes of CCK antagonists.

Experimental Section

All protected amino acids were from Bachem AG or Novabiochem. Solvents (analytical grade) were from Prolabo. Chromatography was carried out with Merck silica gel (230–400 mesh). For thin-layer chromatography (TLC), Merck plates precoated with F254 silica gel were used with the following solvent systems (by volume): A, EtOAc–pyridine–AcOH–H_2O (160/20/6/11); B, EtOAc–pyridine–AcOH–H_2O (150/20/6/11); C, EtOAc–pyridine–AcOH–H_2O (80/20/6/11); D, EtOAc–pyridine–AcOH–H_2O (65/20/6/11); E, CH_2Cl_2–MeOH (9/1); F, CH_2Cl_2–MeOH (95/5); G, CH_2Cl_2–MeOH (8/2); H, $CHCl_3$–MeOH–AcOH–H_2O (5/5/1/0.5). Plates were developed with UV, iodine vapor, ninhydrin, or Ehrlich's reagent. The structure of the compounds and of all intermediates were confirmed by ^1H 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_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 detection (210 nm). At each step of the synthesis, the lack of significant racemization of a given peptide was checked by ^1H NMR and by HPLC. FAB mass spectra were recorded on a double-focusing VG 70-250 instrument. Amino acid analyses were carried out on LKB biochrom 4400 analyzer after hydrolysis with 6 M HCl, at 110 °C for 24 h. The following abbreviations have been used: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; TFA, trifluoracetic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide. Other abbreviations used are those recommended by the IUPAC-IUB Commission (*Biochem. J.* **1984**, *219*, 345.).

Boc-Asp(OBzl)-Phe-NH_2 (11). To a solution of H-Phe-NH_2·HCl (6.4 g, 3.19 mmol) in a mixture of THF–CH_2Cl_2 (40/60) were added successively, at 0 °C, Et_3N (4.5 mL, 32 mmol), Boc-Asp(OBzl)-OH (10.31 g, 31.9 mmol), HOBt (5 g, 32.6 mmol), and DCC (6.73 g, 32.6 mmol). The reaction mixture was stirred for 30 min at 0 °C and then overnight at room temperature. After filtration of the DCU and evaporation of the solvents, the residue was dissolved in EtOAc, washed with citric acid (10%), $NaHCO_3$ (10%), and saturated NaCl solution, dried over Na_2SO_4, and concentrated in vacuo to yield, after precipitation with anhydrous ether, 13.78 g (92%) of the compound as a white solid: R_f 0.62 (E). Anal. Calcd for $C_{25}H_{31}N_3O_6$: C, 63.95; H, 6.65; N, 8.95. Found: C, 63.89; H, 6.66; N, 8.84.

TFA·Asp(OBzl)-Phe-NH_2 (12). Compound 11 (6 g, 12.8 mmol) dissolved in 30 mL of a TFA–CH_2Cl_2 (1/1) mixture was stirred at 0 °C for 1 h and at room temperature for 1 h, yielding after evaporation, precipitation, rinsing with dry ether, and filtration, a white solid: 5.45 g (88%); R_f 0.56 (D).

Z-Orn(Boc)-Asp(OBzl)-Phe-NH_2 (13). To a solution of Z-Orn(Boc)-OH (1.5 g, 2.86 mmol) in 5 mL of dry DMF were added HOSu (330 mg, 2.87 mmol) and DCC (590 mg, 2.87 mmol). The 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 12 (1.382 g, 2.86 mmol) and Et_3N (0.4 mL, 2.86 mmol) in DMF (5 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: 1.87 g (91%); R_f 0.52 (F). Anal. Calcd for $C_{38}H_{47}N_5O_9$: C, 63.58; H, 6.60; N, 9.76. Found: C, 63.61; H, 6.73; N, 9.61.

H-Orn(Boc)-Asp-Phe-NH_2 (14). Compound 13 (1 g, 1.39 mmol) was hydrogenated overnight in 40 mL of CH_2Cl_2–MeOH (1/1) mixture in the presence of 10% Pd/C catalyst (140 mg). After evaporation of the solvents, the resulting residue was dissolved in water and filtered. Evaporation of water gave a white product: 446 mg (65%); R_f 0.25 (G).

Boc-Trp-Orn(Boc)-Asp-Phe-NH_2 (6). To a solution of Boc-Trp-OH (138 mg, 0.45 mmol) in 3 mL of dry DMF were added, at −10 °C, HOSu (53 mg, 0.46 mmol) and DCC (95 mg, 0.46 mmol). The solution was stirred at −10 °C for 30 min and then at 0 °C for 1 h and overnight at room temperature. To the above mixture was added a solution of compound 14 (224 mg, 0.45 mmol) in dry DMF (8 mL) and it was allowed to stand overnight at room temperature. After filtration of DCU, evaporation, and precipitation with EtOAc–ether, the resulting residue was purified by chromatography on silica gel with EtOAc–pyridine–AcOH–H_2O (160/20/6/11) as eluent to yield 186 mg (53%) of the compound: R_f 0.37 (A); HPLC t_R = 18 min, eluent CH_3CN–TEAP (36/64); FAB-MS (MH^+) calcd 781, found 781. Anal. Calcd for $C_{39}H_{53}N_7O_{10}$: C, 60.06, H, 6.85; N, 12.57. Found: C, 60.12; H, 6.76; N, 12.42.

Boc-Tyr-Nle-Gly-Trp-Orn(Boc)-Asp-Phe-NH_2 (15). To a solution of Boc-Tyr-Nle-Gly-Trp-OH[44] (168 mg, 0.26 mmol) in dry DMF (3 mL) were added, at −10 °C, HOSu (31 mg, 0.27 mmol) and DCC (56 mg, 0.27 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 tripeptide 14 (130 mg, 0.26 mmol) in dry DMF (8 mL) was added to the above mixture. The solution was stirred overnight at room temperature and was treated as previously described for the preparation of compound 13 to give a light yellow compound: 276 mg (95%); R_f 0.72 (D). Anal. Calcd for $C_{56}H_{76}N_{10}O_{14}$: C, 60.42; H, 6.88; N, 12.58. Found: C, 60.28; H, 6.94; N, 12.39.

Boc-Tyr(SO_3H)-Nle-Gly-Trp-Orn(Boc)-Asp(Na)-Phe-NH_2 (3). A solution of compound 15 (270 mg, 0.24 mmol) in dry DMF (5 mL) and dry pyridine (5 mL) was treated with SO_3–pyridine complex (1.62 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. After filtration and evaporation, the residue was purified by column chromatography on silica gel using EtOAc–pyridine–AcOH–H_2O (65/20/6/11) as eluent to yield 98 mg (33%) of a white product: R_f 0.34 (D); HPLC t_R = 15.1 min, eluent CH_3CN–TEAP (37/63); FAB-MS (MH^+) calcd 1238, found 1238; amino acid analysis, Asp 0.98; Nle 1.02; Gly 0.97; Tyr 0.93; Phe 0.99; Orn 0.94; Trp 0.85.

Boc-Orn(Z)-Asp(OtBu)-NH_2 (16). To a solution of H-Asp(OtBu)-NH_2 (800 mg, 3.56 mmol) in 35 mL of a CH_2Cl_2–THF (1/2) mixture, at 0 °C, were successively added Boc-Orn(Z)-OH (1.304 g, 3.56 mmol), HOBt (547 mg, 3.57 mmol), and DCC (737 mg, 3.57 mmol). The reaction was stirred for 1 h at 0 °C and overnight at room temperature and was worked up as described for the preparation of compound 13 to yield the product as a white solid: 1.89 g (98%); R_f 0.45 (E). Anal. Calcd for $C_{26}H_{40}N_4O_8$: C, 58.19, H, 7.51; N, 10.44. Found: C, 58.29; H, 7.57; N, 10.39.

TFA·Orn(Z)-Asp-NH_2 (17). Compound 16 (1.88 g, 3.5 mmol) was stirred for 30 min at 0 °C and for 4 h at room temperature in the presence of TFA (10.5 mL). Evaporation of TFA and precipitation with dry ether yielded 1.47 g (85%) of a white solid: R_f 0.15 (D).

Boc-Trp-Orn(Z)-Asp-NH_2 (8). To a solution of Boc-Trp-OH (153.8 mg, 0.505 mmol) in dry DMF (2.5 mL) were added, at −10 °C, HOSu (58.6 mg, 0.51 mmol) and DCC (105 mg, 0.51 mmol). The resulting solution was stirred for 30 min at −10 °C, for 1 h at 0 °C, and overnight at room temperature. To the above mixture was added compound 17 (250 mg, 0.505 mmol) dissolved in dry DMF (2 mL) and in the presence of Et_3N (71 μL, 0.507 mmol). The reaction was stirred for 30 min at 0 °C and overnight at room temperature and then treated as for compound 6. The residue was chromatographed on a column of silica gel using EtOAc–pyridine–AcOH–H_2O (160/20/6/11) as eluent to yield 195 mg

(58%) of the product: R_f 0.28 (A); HPLC t_R = 5.8 min, eluent CH_3CN–TEAP (43/57); FAB-MS (MH^+) calcd 668, found 668. Anal. Calcd for $C_{33}H_{42}N_6O_9$: C, 59.45; H, 6.35; N, 12.60. Found: C, 59.50; H, 6.27; N, 12.43.

Boc-Trp-Orn-Asp-NH_2 (10). Compound 8 (100 mg, 0.15 mmol) in 10 mL of MeOH was hydrogenated in the presence of 10% Pd/C catalyst (10 mg) for 3 h. After filtration of the catalyst, MeOH was evaporated. The product was lyophilized (0.1 M NH_4OH, 10 mL, overnight) to yield a white powder: 59.4 mg (72%); R_f 0.52 (H); HPLC t_R = 7.1 min, eluent CH_3CN–TEAP (25/75); FAB-MS (MH^+) calcd 551, found 551. Anal. Calcd for $C_{25}H_{39}N_7O_7$: C, 54.63; H, 7.15; N, 17.84. Found: C, 54.31; H, 7.29; N, 17.51.

Boc-Tyr-Nle-Gly-Trp-Orn(Z)-Asp-NH_2 (18). To a solution of Boc-Tyr-Nle-Gly-Trp-OH (350.7 mg, 0.55 mmol) in 3 mL of dry DMF were added, at −10 °C, HOSu (64.4 mg, 0.56 mmol) and DCC (115 mg, 0.56 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 reaction was added a solution of compound 17 (272 mg, 0.55 mmol) and Et_3N (77 μL, 0.55 mmol) in dry DMF (2 mL). The resulting mixture was stirred for 30 min at 0 °C and overnight at room temperature and then was worked up as described for the synthesis of compound 13 to give a light yellow solid: 522.6 mg (95%); R_f 0.38 (C). Anal. Calcd for $C_{50}H_{65}N_9O_{13}$: C, 60.05; H, 6.55; N, 12.60. Found: C, 59.92; H, 6.59; N, 12.47.

Boc-Tyr(SO_3H)-Nle-Gly-Trp-Orn(Z)-Asp(Na)-NH_2 (4). A solution of compound 18 (250 mg, 0.25 mmol) in dry DMF (5 mL) and dry pyridine (5 mL) was treated with an SO_3–pyridine complex (1.5 g) and stirred overnight at room temperature and under N_2. After evaporation of the solvents, the residue was treated as for compound 3 to yield, after purification with silica gel column chromatography, using EtOAc–pyridine–AcOH–H_2O (65/20/6/11) as eluent, 85.5 mg (31%) of a white product: R_f 0.25 (D); HPLC t_R = 8.6 min, eluent CH_3CN–TEAP (36/64); FAB-MS (MH^+) calcd 1125, found 1125; amino acid analysis, Asp 1.01; Nle 0.97; Gly 0.95; Tyr 0.94; Orn 0.89; Trp 0.87.

Boc-Trp-Orn(Z)-Asp-Phe-NH_2 (5). To a solution of Boc-Trp-OH (119 mg, 0.39 mmol) in 2 mL of dry DMF were added, at −10 °C, HOSu (45 mg, 0.39 mmol) and DCC (80 mg, 0.39 mmol). The mixture was stirred for 30 min at −10 °C, for 1 h at 0 °C, and overnight at room temperature. A solution of the tripeptide TFA·Orn(Z)-Asp-Phe-NH_2 (250 mg, 0.39 mmol) and Et_3N (55 μL, 0.39 mmol) in 2 mL of DMF was then added, at 0 °C, to the above mixture. The reaction was allowed to stand overnight at room temperature and was treated as previously described for the preparation of compound 6 to yield 206 mg (65%) of a white product: R_f 0.33 (A); HPLC t_R = 15 min, eluent CH_3CN–TEAP (40/60); FAB-MS (MH^+) calcd 815, found 815. Anal. Calcd for $C_{42}H_{51}N_7O_{10}$: C, 61.98; H, 6.32; N, 12.05. Found: C, 61.76; H, 6.47; N, 11.89.

Boc-Trp-Orn-Asp-Phe-NH_2 (7). Compound 5 (75 mg, 0.92 mmol) in 10 mL of MeOH was hydrogenated in the presence of 10% Pd/C catalyst (10 mg) for 2 h. After filtration of the catalyst and evaporation, the product was lyophilized twice (0.1 M NH_4OH, 10 mL) to yield 52 mg (81%) of the compound: R_f 0.27 (C); HPLC t_R = 10.3 min, eluent CH_3CN–TEAP (30/70); FAB-MS (MH^+) calcd 699, found 699. Anal. Calcd for $C_{34}H_{48}N_8O_8$: C, 58.52; H, 7.08; N, 16.06. Found: C, 58.35; H, 7.12; N, 16.19.

Z-Orn(Boc)-Asp(OBzl)-NH_2 (19). To a solution of TFA·Asp(OBzl)-NH_2 (0.99 g, 2.96 mmol) and Et_3N (0.41 mL, 2.96 mmol) in THF (25 mL) were successively added, at 0 °C, Z-Orn(Boc)-OH (0.99 g, 2.96 mmol), HOBt (0.45 g, 2.96 mmol), and DCC (0.61 g. 2.96 mmol). The resulting mixture was stirred for 30 min at 0 °C and for 4 h at room temperature. After filtration of DCU and evaporation, the reaction was worked up as for the preparation of compound 11 to give 1.44 g (90%) of the compound: R_f 0.46 (E). Anal. Calcd for $C_{29}H_{38}N_4O_8$: C, 61.04; H, 6.71; N, 9.82. Found: C, 61.11; H, 6.59; N, 9.67.

HCl·Orn(Boc)-Asp-NH_2 (20). Compound 19 (1.25 g, 2.31 mmol) and HCl (0.2 mL, 2.31 mmol) in 100 mL of EtOH was hydrogenated for 1 h 30 min at atmospheric pressure and room temperature in the presence of 10% Pd/C as catalyst (250 mg). After filtration of the catalyst and evaporation, the residue was dissolved in water, filtered, and lyophilized to give 0.86 g (82%) of the compound as a white solid: R_f 0.16 (H).

Boc-Trp-Orn(Boc)-Asp-NH_2 (9). To a solution of Boc-Trp-OH (420 mg, 1.38 mmol) in dry DMF (7 mL) were added, at −10 °C, HOSu (159 mg, 1.38 mmol) and DCC (258 mg, 1.38 mmol). The reaction was stirred at −10 °C for 30 min and at 0 °C for 30 min and at room temperature for 2 h. A solution of compound 20 (528 mg, 1.38 mmol) and Et_3N (0.19 mL, 1.38 mmol) in dry DMF (5.5 mL) was then added at 0 °C. After stirring for 30 min at 0 °C and overnight at room temperature, the reaction was treated as for the preparation of compound 6. Purification on silica gel column chromatography using EtOAc–pyridine–AcOH–H_2O (150/20/6/11) as eluent afforded 543 mg (62%) of the product: R_f 0.34 (B); HPLC t_R = 7.4 min, eluent CH_3CN–TEAP (36/64). FAB-MS (MH^+) calcd 634, found 634. Anal. Calcd for $C_{30}H_{44}N_6O_9$: C, 56.95; H, 7.01; N, 13.28. Found: C, 56.77; H, 7.28; N, 13.14.

Bioassays. Binding experiments were performed on the brain and pancreas membranes of guinea pigs with [^{3}H]pCCK$_8$ as described previously.[45] Incubations (final volume = 1 mL) were carried out at 25 °C in 50 mM Tris-HCl buffer (pH = 7.4), 5 mM $MgCl_2$, 0.2 mg/mL bacitracin for 60 min in the presence of brain membranes (0.6 mg of protein/mL) or in 10 mM pipes-HCl buffer (pH = 6.5), 30 mM $MgCl_2$, 0.2 mg/mL bacitracin, 0.2 mg/mL soybean trypsin inhibitor, for 120 min in the presence of pancreatic membranes (0.2 mg of protein/mL). [^{3}H]pCCK$_8$ was incubated at 0.2 nM in the presence of varying concentrations of the competitor. The nonspecific binding was determined in the presence of 1 μM CCK$_8$. Incubation was terminated by filtration through Whatman GF/B glass-fiber filters precoated with buffer containing 0.1% (w/v) bovine serum albumin. The filters were rinsed with 2 × 5 mL of ice-cold buffer and dried, and the radioactivity was counted in 5 mL of Ready-solv EP scintillation cocktail (Beckman).

Pancreatic acini were prepared as previously reported.[2] 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 described.[46]

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