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Role of N- and C-Terminal Substituents on the CCK-B Agonist–Antagonist Pharmacological Profile of Boc-Trp-Phg-Asp-Nal-NH_2 Derivatives

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Abstract—Among the CCK derivatives, the tetrapeptide Boc-Trp-Phg-Asp-Nal-NH_2 (**1**) behaves as a short potent CCK-B agonist which led to the development of an efficient peptidase-resistant CCK-B antagonist by bismethylation of its terminal $CONH_2$ group. Further modifications of the N- and C-terminal moieties of **1** have been performed and are described in this paper, together with the pharmacological profile of the novel synthesized compounds. Introduction of more bulky substituents than $NalNH_2$ on the C-terminal part decreased the CCK-B receptor binding affinity. In the series of N-protected tetrapeptides X^{30}-Phg^{31}-Asp^{32}-Nal^{33}-$N(CH_3)_2$, the Boc-substituent was shown to be optimal among the N-protecting groups Boc, ^{2}Adoc, propionyl or acetyl when X = Trp. On the other hand, when X = αMeTrp, its optimal N-protecting group was ^{2}Adoc and its configuration was preferentially D. In the newly synthesized compounds, **13**: ^{2}Adoc-D-αMeTrp-Phg-Asp-NalN$(CH_3)_2$ and **16**: ^{2}Adoc-D-αMeTrp-Phg-Asp-$NalNH_2$ had the best CCK-B receptor affinities (K_i = 3.5 and 3.4 nM, respectively) and were selected for further biological evaluation. Interestingly, when tested for their capacity to influence inositol phosphate formation, induced by CCK_8 in CHO cells transfected with the rat CCK-B receptor, compound **13** behaved as a full CCK-B antagonist with an IC_{50} value of 18 ± 1 nM, being as potent as the antagonists L-365,260 and PD-134,308 (IC_{50} values respectively, 39 ± 17 and 30 ± 2 nM), whereas compound **16** was found to behave as a partial CCK-B agonist. Indeed **16** behaved as an antagonist on the firing rate of rat CA1 hippocampal neurons and acted as an agonist in the pentagastrin stimulated gastric acid secretion (EC_{50} = 12 nmol/kg) in anesthetized rats. Compound **13** in contrast, was found to inhibit the pentagastrin action at a dose (ID_{50} = 0.56 μmol/kg) similar to the potent antagonist PD-134,308 (ID_{50} = 0.4 μmol/kg). The antagonist/agonist properties of compounds **13** and **16** show that both N- and C-terminal substituents modulate the pharmacological properties in the Boc-CCK_4 derivatives presented here.

Introduction

Cholecystokinin (CCK), as well as the C-terminal octapeptide fragment CCK_8,[1] act as hormonal regulators of pancreatic secretion and induce gallbladder contraction and gut motility.[2] In the central nervous system (CNS), CCK_8 acts as a neurotransmitter and/or neuromodulator.[3] Biochemical studies have shown the existence of at least two classes of CCK receptors,[4] CCK-B receptors which are widely distributed in the brain[5] and which have been shown to be identical to gastrin receptors[6] and CCK-A receptors, which are primarily found in peripheral tissues but which also occur in certain brain regions.[7,8] The recent cloning of human CCK-A and CCK-B receptors has shown that they belong to the class of G-protein coupled receptors.[6,9–11]

Although the physiological and behavioral roles of brain CCK_8 are not yet well understood, it has been shown that brain CCK-B receptors are involved in anxiety,[12] satiety,[13] perception of pain[14,15] and in modulating memory processes.[16] In the periphery, CCK-B receptors regulate gastric acid secretion.[17] Within the past few years, numerous studies have been directed toward the development of CCK antagonists as potential therapeutic agents in many fields such as opiate analgesia,[18,19] anxiety, and neuropsychiatric disorders, particularly panic attacks.[20] In attempting to gain further insight into the respective roles of CCK receptor subtypes in the periphery and CNS, and to obtain CCK-based compounds as sources of novel therapies for the treatment of a variety of gastrointestinal and neuropsychiatric disorders, selective CCK-B antagonists have been synthesized.[21–30]

We have recently developed[31] new potent, selective and peptidase-resistant CCK-B ligands which derive from Boc-[Nle^{31}]CCK_{30-33}, by incorporation of non-natural hydrophobic amino acids. Among these compounds,

Boc-[Phg[31], Nal[33]]CCK_{30-33} (**1**) proved to be a full agonist in an electrophysiological assay on rat hippocampal CCK-B receptors. Interestingly, bismethylation of its terminal $CONH_2$ group led to Boc-[Phg[31], Nal[33]-$N(CH_3)_2$]CCK_{30-33} (**2**) which acted as a potent antagonist in the same test and which displayed a relatively good affinity for rat brain CCK-B receptors ($K_I = 52 \pm 7$ nM). It is not yet well understood how such a structural modification can change the pharmacological profile. Therefore, in order to explore this phenomenon and to obtain more potent CCK-B receptor antagonists, we have synthesized Boc[Phg[31], Nal[33]-NH_2]CCK_{30-33} analogues modified in positions 30, 33, and at their N- and C-termini. The binding affinities for CCK-A and CCK-B receptors and the pharmacological profile in various in vitro and in vivo assays of these new compounds are reported in this study.

Results and Discussion

Chemistry

Compounds **3–7** and **8–16** (Table 1) were prepared according to Scheme 1A and B, respectively. Elongation of the peptide chains was performed using DCC/HOSu, BOP, *p*-nitrophenyl ester or *N*-succinimidyl ester methods. The amino protecting group Boc was removed with trifluoroacetic acid and the carboxyl protecting benzyl and methyl esters by respectively catalytic hydrogenolysis and saponification. Compounds **8–16** were prepared by a stepwise coupling of the amino acids from the C- to N-terminus. Compounds **3–7** were prepared from the same N-terminus protected tripeptide Boc-Trp-Phg-Asp-(OBzl)OH (**18**) which was obtained by C- to N-terminus coupling steps. The different amidated amino acids **19–21** whose synthesis is reported in the Experimental Section were coupled after deprotection by TFA with compound **18**. The final tetrapeptides **3–7** were obtained, after hydrogenolysis, without any racemization as detectable by HPLC. The intermediate *N*-Boc-D,L-cyclooctylalanine derivative **22** was prepared by a simpler method, described in the Experimental Section, than that already reported.[32] Enolate alkylation of the Schiff base **22a** obtained from benzaldehyde and glycine methyl ester[33] followed by condensation with cyclooctylmethyl bromide, in the presence of LDA and HMPA and subsequent acid hydrolysis provided compound **22b**. Protection of the amino group as **22c**, followed by saponification gave the expected amino acid **22**.

Non-commercial amino acids were prepared in the laboratory: D-αMeTrp-OH was obtained as described by Blommaert et al.[25] *N*-Propionyl-Trp-OH and N-propionyl-D-αMeTrp-OH were obtained by coupling propionic acid *N*-hydroxysuccinimide ester with L-Trp-OEt and D-αMeTrp-OMe, respectively, followed by saponification in the presence of LiOH. *N*-[2]Adoc-protected tryptophan residues were prepared as described previously by Horwell et al.[22]

Structure–activity relationships

Compounds reported in Table 2 were evaluated as previously described,[34] for their affinities for CCK-A and CCK-B receptors by competition experiments with [^{3}H]pCCK_8 using guinea pig pancreatic and brain cortex membranes respectively.

C-terminus modifications. Starting from the tetrapeptide Boc-Trp[30]-Met[31]-Asp[32]-Phe[33]-NH_2, as we have reported in a preceding paper, the nature of residues 31 and 33 was optimized to provide compound **1**, Boc-Trp-Phg-Asp-Nal-NH_2, which has relatively good CCK-B agonist properties.[31] We have also reported that bismethylation of the C-terminal amide group in compound **1** led to a potent, peptidase-resistant CCK-B antagonist **2**.[31] These results suggested that the size of the C-terminal amide substituent might play a critical role in the recognition of the agonist or antago-

Table 1. Physical and analytical data of peptides **3–16** used in the biological tests

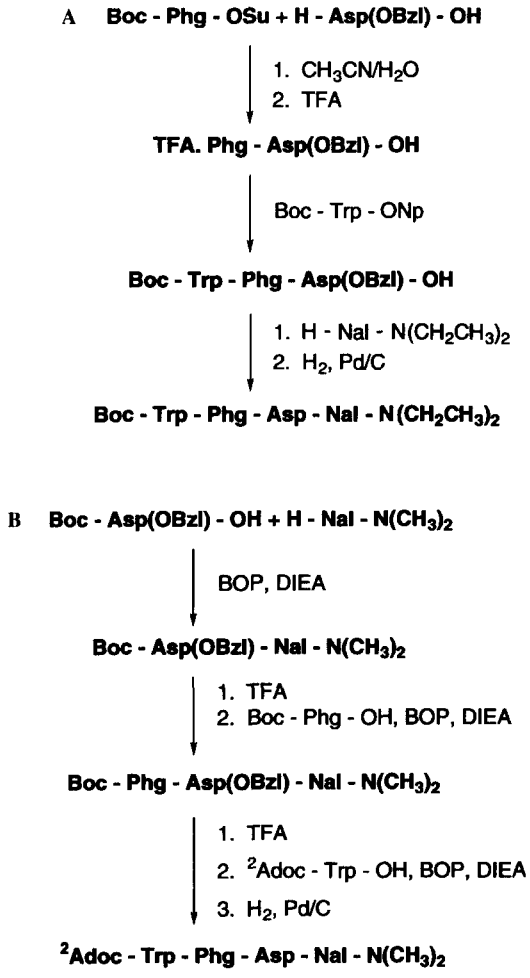
No.	Compounds	Mp (°C)	R_f (C)	Molecular formula	Anal.	HPLC R_t min (A/B)
3	Boc-Trp-Phg-Asp-Nal- 	154–156	0.52	$C_{45}H_{50}N_6O_8 \cdot H_2O$	C, H, N	12.2 (50/50)
4	Boc-Trp-Phg-Asp-Nal-$N(CH_2CH_3)_2$	137–139	0.65	$C_{45}H_{52}N_6O_8 \cdot H_2O$	C, H, N	9.3 (45/55)
5	Boc-Trp-Phg-Asp-Nal- 	198–200	0.34	$C_{45}H_{50}N_6O_9$	C, H, N	8.9 (50/50)
6	Boc-Trp-Phg-Asp-Phe-$N(CH_3)_2$	136–138	0.44	$C_{39}H_{45}N_6O_8$	C, H, N	9.2 (55/45)
7	Boc-Trp-Phg-Asp-NH-D,L-Coa-$N(CH_3)_2$	149–151	0.65	$C_{41}H_{56}N_6O_8.2H_2O$	C, H, N	7.4 (40/60)
8	[2]Adoc-Trp-Phg-Asp-Nal-$N(CH_3)_2$	161–163	0.57	$C_{49}H_{54}N_6O_8 \cdot 2H_2O$	C, H, N	9.3 (40/60)
9	CH_3CH_2CO-Trp-Phg-Asp-Nal-$N(CH_3)_2$	157–159	0.42	$C_{41}H_{44}N_6O_7$	C, H, N	6.6 (55/45)
10	CH_3CO-Trp -Phg-Asp-Nal $N(CH_3)_2$	167–169	0.26	$C_{40}H_{42}N_6O_7$	C, H, N	5.0 (55/45)
11	Boc-L-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	165–167	0.54	$C_{44}H_{50}N_6O_8 \cdot 2.5H_2O$	C, H, N	7.9 (45/55)
12	Boc-D-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	165–167	0.54	$C_{44}H_{50}N_6O_8 \cdot H_2O$	C, H, N	6.1 (45/55)
13	[2]Adoc-D-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	163–165	0.61	$C_{50}H_{56}N_6O_8$	C, H, N	8.8 (40/60)
14	[2]Adoc-L-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	163–165	0.61	$C_{50}H_{56}N_6O_8$	C, H, N	12.1 (40/60)
15	CH_3CH_2CO-D-α MeTrp-Phg-Asp-Nal-$N(CH_3)_2$	149–151	0.43	$C_{42}H_{46}N_6O_7 \cdot AcOH$	C, H, N	7.7 (55/45)
16	[2]Adoc-D-α MeTrp-Phg-Asp-Nal-NH_2	169–171	0.59	$C_{48}H_{52}N_6O_8 \cdot 2H_2O$	C, H, N	8.8 (45/55)

nist sites of brain CCK-B receptors. In order to optimize the antagonist properties of compound **2**, groups of different size, such as *N*-piperidinyl, diethyl and morpholinyl (peptides **3–5**) were introduced as C-terminal amide substituents. These modifications resulted in decreases in affinity (about 3-, 4-, and 12-fold, respectively) as compared to compound **2**, without great changes in selectivity (Table 2). This progressive loss of affinity is probably due to enhanced steric hindrance which might decrease the interactions with the antagonist state of the CCK-B receptor. Furthermore, in order to know whether similar structure–activity relationships could be developed for agonist and antagonist series, we substituted Nal[33] in compound **2** by a Phe residue, giving compound **6** since Boc[(*N*-Me)Nle[31],Phe[33]]CCK_4 was found to exhibit a four-fold higher binding affinity for the CCK-B sites than its Nal[33] analog.[31] Compound **6** showed a 10-fold decrease in CCK-B binding affinity as compared to **2**. In addition, as it has been reported that substitution of the Phe in position 33 of Ac-CCK_7, by a large cyclooctylalanine (Coa) moiety provided ligands with high binding affinities for both CCK-A and CCK-B receptors,[32] the Nal residue was changed in **2** by such a residue to provide **7**. However, this modification led to a seven- to eight-fold reduction in binding affinity and selectivity for the CCK-B receptor.

These results show that structure–activity relationships are quite different for agonist and antagonist ligands of the same receptor. This is in agreement with site-directed mutagenesis experiments which have shown that agonists and antagonists bind to different sites localized in different parts of the seven transmembrane domains.[35–37] Nevertheless, our results suggest that the CCK-B receptor antagonist site preferentially recognizes a $CO\text{-}N(CH_3)_2$ amide group and possesses a hydrophobic pocket interacting with C-terminal residues of CCK_4 ligands, preferentially aromatic and of large size, such as Nal, which appears to be the optimal group in this series.

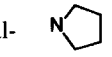
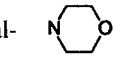
N-terminus modifications. Literature data have shown that the affinity of CCK_4 for CCK-B binding sites is improved 10-fold by protecting the N-terminal part with a bulky group such as Boc.[30,31] In order to optimize the size of this protecting group in **2**, the Boc group was replaced by alkyl carbamates of increasing or decreasing sizes. However, none of the compounds obtained (**8–10**) displayed significant changes in affinity for the CCK-B receptor. Moreover, no important change in CCK-A receptor recognition was observed (Table 2). In addition, in order to induce conformational preferences through steric hindrance and to stabilize the peptide bond against enzymatic degradation, the natural amino acid Trp was replaced by its α-methyl substituted analogue in **2**. While the peptide containing L-αMeTrp (compound **11**) had a two-fold drop in affinity and a four-fold decrease in selectivity as compared to **2**, the corresponding D-αMeTrp analogue (**12**) was found to be more potent and selective for the CCK-B receptor. This is in agreement with the fact that introduction of the non-genetically coded D-αMeTrp moiety enhanced the affinity for brain receptors 10-fold over its L-isomer in the peptoid series of CCK-B antagonists described by Horwell et al.[30] Finally, since in CCK-B antagonists the ^{2}Adoc group was shown to be one of the best N-terminal substituents, it was introduced as a replacement for Boc, providing the peptide **13** which is 7 times more active than **12** and 30 times more active than **15**, which is substituted with a N-propionyl group. Compound **13** appears as a very potent (K_i = 3.5 nM), although not highly selective (ratio CCK-A/CCK-B = 15) CCK-B ligand. Peptide **14**, containing a L-αMe-Trp[30] residue and a ^{2}Adoc N-terminal group showed only a four-fold increase in CCK-B binding potency when compared to compound **11**. Thus, the residue ^{2}Adoc-D-αMeTrp in position 30 appeared to be the most appropriate moiety in this series of tetrapeptides. This probably indicates that the size of the ^{2}Adoc moiety is optimal to fit adequately the antagonist binding site of the CCK-B receptor.

Since the N substitution of the C-terminal amide by two methyl groups changed the agonist profile of compound **1** to the antagonist compound **2**, the



Scheme 1. (A) Synthetic pathway for the preparation of compounds **3–7**. (B) Synthetic pathway for the preparation of compounds **8–16**.

Table 2. Inhibitory effects (K_I, M) of CCK_4 derivatives **1–16** on the binding of [^{3}H]pCCK_8 to guinea pig brain (CCK-B, $K_D = 0.18$ nM) and pancreatic (CCK-A) membranes ($K_D = 1.22$ nM)

No.	Compounds	K_I (M)[a]	
		CCK-B	CCK-A
1[b]	Boc-Trp-Phg-Asp-Nal-NH_2	$1.4 \pm 0.1 \times 10^{-8}$	$9.9 \pm 0.6 \times 10^{-7}$
2[b]	Boc-Trp-Phg-Asp-Nal-$N(CH_3)_2$	$3.9 \pm 1.0 \times 10^{-8}$	$1.0 \pm 0.4 \times 10^{-6}$
3	Boc-Trp-Phg-Asp-Nal- 	$1.3 \pm 0.2 \times 10^{-7}$	$1.3 \pm 0.1 \times 10^{-6}$
4	Boc-Trp-Phg-Asp-Nal-$N(CH_2CH_3)_2$	$1.7 \pm 0.3 \times 10^{-7}$	$8.0 \pm 2.7 \times 10^{-6}$
5	Boc-Trp-Phg-Asp-Nal- 	$4.9 \pm 1.0 \times 10^{-7}$	$1.8 \pm 0.4 \times 10^{-6}$
6	Boc-Trp-Phg-Asp-Phe-$N(CH_3)_2$	$3.4 \pm 1.1 \times 10^{-7}$	$7.0 \pm 2.3 \times 10^{-6}$
7[c]	Boc-Trp-Phg-Asp-NH-D,L-Coa-$N(CH_3)_2$	$2.6 \pm 0.4 \times 10^{-7}$	$1.8 \pm 0.3 \times 10^{-6}$
8	^{2}Adoc-Trp-Phg-Asp-Nal-$N(CH_3)_2$	$1.1 \pm 0.1 \times 10^{-7}$	$1.9 \pm 0.4 \times 10^{-6}$
9	CH_3CH_2CO-Trp-Phg-Asp-Nal-$N(CH_3)_2$	$7.1 \pm 1.0 \times 10^{-8}$	$5.0 \pm 0.4 \times 10^{-6}$
10	CH_3CO-Trp-Phg-Asp-Nal $N(CH_3)_2$	$8.7 \pm 1.2 \times 10^{-8}$	$2.5 \pm 0.9 \times 10^{-6}$
11	Boc-L-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	$8.1 \pm 1.4 \times 10^{-8}$	$5.0 \pm 0.5 \times 10^{-7}$
12	Boc-D-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	$2.5 \pm 0.5 \times 10^{-8}$	$2.2 \pm 0.1 \times 10^{-6}$
13	^{2}Adoc-D-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	$3.5 \pm 0.5 \times 10^{-9}$	$5.4 \pm 0.9 \times 10^{-8}$
14	^{2}Adoc-L-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	$2.1 \pm 0.3 \times 10^{-8}$	$2.9 \pm 0.6 \times 10^{-7}$
15	CH_3CH_2CO-D-αMeTrp-Phg-Asp-Nal-$N(CH_3)_2$	$1.2 \pm 0.1 \times 10^{-7}$	$5.1 \pm 0.1 \times 10^{-6}$
16	^{2}Adoc-D-αMeTrp-Phg-Asp-Nal-NH_2	$3.4 \pm 0.5 \times 10^{-9}$	$2.5 \pm 0.1 \times 10^{-7}$
	L-365,260	$2.0 \pm 0.4 \times 10^{-9}$	$8.68 \pm 0.72 \times 10^{-7}$
	PD-134,308	$4.04 \pm 0.51 \times 10^{-10}$	$1.27 \pm 0.16 \times 10^{-6}$

[a]The K_I values represent the means ± SEM of three separate experiments each performed in triplicate. The values of the Hill coefficients were close to 1 in all experiments.
[b]Compounds already described in ref 31.
[c]Coa = cyclo-octylalanine

terminal $CON(CH_3)_2$ group in **13** was changed for a $CONH_2$ in **16**, a compound endowed with a high CCK-B affinity. The pharmacological profile of these two interesting compounds was then analyzed in detail.

Pharmacological studies of compounds 13 and 16

Inositol phosphate production in CHO cells transfected with CCK-B receptor. The effects of compounds **13** and **16** on the liberation of inositol phosphate were studied in CHO cells transfected with the rat brain CCK-B receptor.[38] As shown in Figure 2B, compound **13** behaved as a full antagonist of CCK_8 induced accumulation of inositol phosphate. For **13**, as well as for the reference antagonists PD-134,308 and L-365,260, our results show that there is a good correlation between the binding affinity measured in CHO cell preparations (Fig. 1 and Table 3) and the antagonist potency of these molecules belonging to three different chemical classes: peptide for **13**, peptoid for PD-134,308, and benzodiazepine-based non-peptide structure for L-365,260 (Fig. 2B and Table 3). It is therefore tempting to conclude that these three molecules fit the same binding area within the CCK-B receptor. This hypothesis seems to agree with the recent results of site-directed mutagenesis of the receptor, showing that mutations of several amino acids produced similar affinity changes for the three classes of CCK-B antagonists.[39] Interestingly in CHO cells, compound **13** ($IC_{50} = 18 \pm 1$ nM) is more potent than PD-134,308 ($IC_{50} = 30 \pm 2$ nM) and L-365,260 ($IC_{50} = 39 \pm 17$ nM) in inhibiting inositol phosphate production.

Compound **16** has a more complex behavior. As shown in Figure 2A, compound **16** is able, as CCK_8, to induce inositol phosphate liberation in CHO cells with an EC_{50} value of 35 ± 14 nM. However, the effect of compound **16** is limited to 80% of that of CCK_8 and the pattern of inositol phosphate formation is biphasic, since at doses higher than 10^{-7} M, a slight decrease, of about 20%, in IP accumulation was observed. This

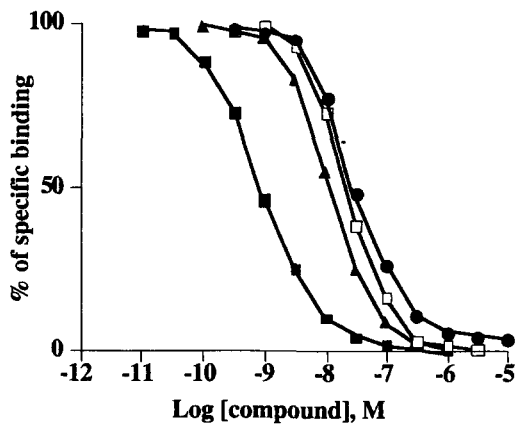


Figure 1. Displacement of the specific binding of [^{3}H]pCCK_8 on homogenate of CHO cells transfected with the rat CCK-B receptor by compounds **13** (▲), **16** (□), CCK_8 (■), and L-365,260 (●).

Table 3. Apparent affinities and pharmacological properties of various CCK ligands

No.	Compounds	K_i (nM)[a]		IP formation[b]	
		Rat	CHO	EC_{50} (nM)	IC_{50} (nM)
2	Boc-Trp-Phg-Asp-Nal-$N(CH_3)_2$	52±7	62±4		378±130
13	[2]Adoc-D-α MeTrp-Phg-Asp-Nal-$N(CH_3)_2$	7.8±0.8	5±0.7		18±1
16	[2]Adoc-D-α MeTrp-Phg-Asp-Nal-NH_2		12±1	35±14	
	PD-134,308	6±0.5	7.3±0.5		30±2
	L-365,260	11±2	15±0.5		39±17
	CCK_8		0.43±0.05	0.40	

[a]Values obtained by using [^{3}H]pCCK_8 (0.5 nM) and rat brain membranes or CHO cells membranes transfected with the rat CCK-B receptor.
[b]The experiments were assessed with CHO cells. Values correspond to induced formation (EC_{50}) of inositol phosphates (IP) or inhibition (IC_{50}) of IP formation evoked by CCK_8 (0.5 nM).

latter effect could be attributed to the affinity of **16** for the antagonist state of the CCK-B receptor, at high concentrations. In agreement with this, the compound is able to slightly antagonize the CCK_8-induced secretion of inositol phosphates at doses higher than 10^{-7} M (data not shown). Another possible explanation could be that **16** has opposing pharmacological effects (agonist or antagonist) at the level of two CCK-B binding subsites.[40,41]

Gastric acid secretion. Intravenous administration of CCK_8 or pentagastrin induced a dose-dependent stimulation of gastric acid output, with an estimated EC_{50} value of 4.1 and 12 nmol/kg, respectively, compound **16** also stimulated the gastric acid output dose-dependently with an estimated EC_{50} value of 25 nmol/kg (Fig. 3). As depicted in Figure 4A, the submaximal dose of pentagastrin-stimulated acid-output was time- and dose-dependently inhibited by the CCK-B receptor antagonists PD-134,308 and compound **13**. The inhibitory effect of **13** became significant at 0.15 μmol/kg and was maximal (100% inhibition) at 1.5 μmol/kg. A comparison of the inhibition of pentagastrin-induced acid output by PD-134,308 and **13** indicated that the peptoid antagonist PD-134,308 was slightly more active than **13** (PD-134,308, $ID_{50} = 0.4$ μmol/kg; **13**, $ID_{50} = 0.56$ μmol/kg) (Fig. 4B). Since compound **13** has a slightly better in vitro efficiency than PD-134,308 in reducing CCK_8-evoked inositol phosphates formation (Table 3), the difference observed in vivo is probably

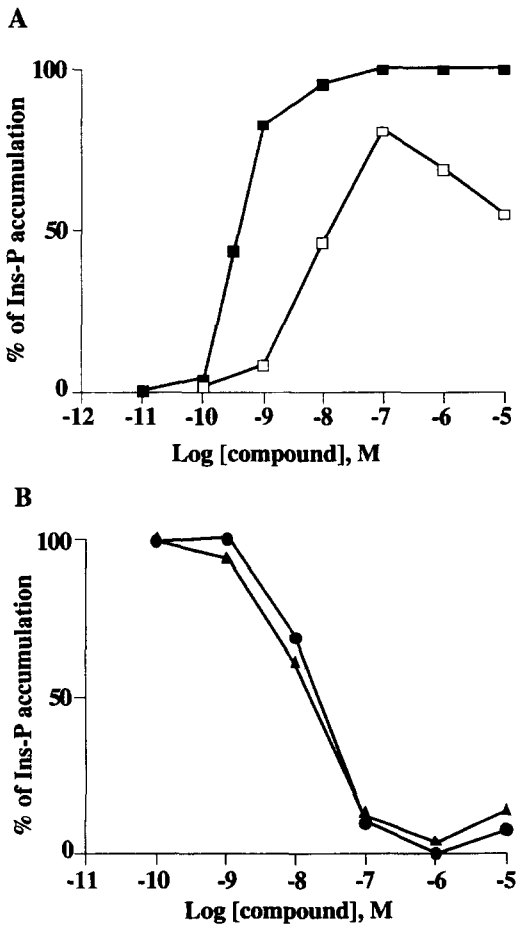


Figure 2. (A) Accumulation of inositol phosphate induced by compound **16** (□) and CCK_8 (■) in CHO cells transfected with the rat CCK-B receptor. (B) Inhibition by L-365,260 (●) and compound **13** (▲) of inositol phosphate accumulation, induced by CCK_8 (0.5 nM) in CHO cells transfected with CCK-B receptor.

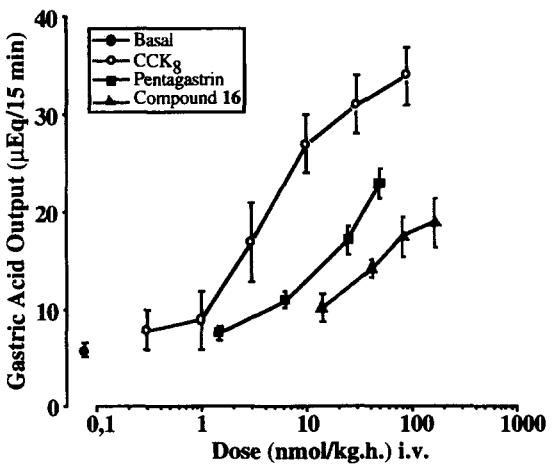


Figure 3. Dose–response curve for stimulation of gastric acid output by CCK_8, pentagastrin and compound **16** in anesthetized perfused rat stomach. Results are expressed in μEq/15 min and each point represents the mean±SEM of $n = 10$ for CCK_8, $n = 8$ for pentagastrin, and $n = 6$ for compound **16**.

due to a slightly better bioavailability of the peptoid as compared to the peptide in gastric mucosa.

Electrophysiological profile. Compound **16** was tested in a CCK-B selective electrophysiological test in which CCK_8 and other CCK-B agonists have been shown to stimulate the firing rate of rat CA_1 hippocampal neurones in a dose-dependent manner.[42,43] As illustrated in Figure 5, compound **16** which is unable to enhance the firing rate of the hippocampal neurones, antagonizes, in a dose-dependent manner, the stimulation induced by CCK_8, as already observed for the antagonist **2**. The antagonist profile of compound **16** in this electrophysiological assay seems to be in apparent contradiction to its agonistic properties on the inositol phosphate formation measured in CHO cells transfected with the CCK-B receptor. Several explanations can be proposed to account for these observations. Firstly, for technical reasons, high concentrations of compound **16** are necessary in the electrophysiological assay and at these concentrations only the antagonistic properties of **16** could be revealed. On the other hand, as already discussed, the different pharmacological profiles of **16** could be related to the existence of CCK-B gastrin subsites with different modes of G-protein coupling, whose distribution and pharmacology might be different in the hippocampal neurons and the gastric cells of rats.[40,41] Further experiments now are under investigation in our laboratory to clarify this question.

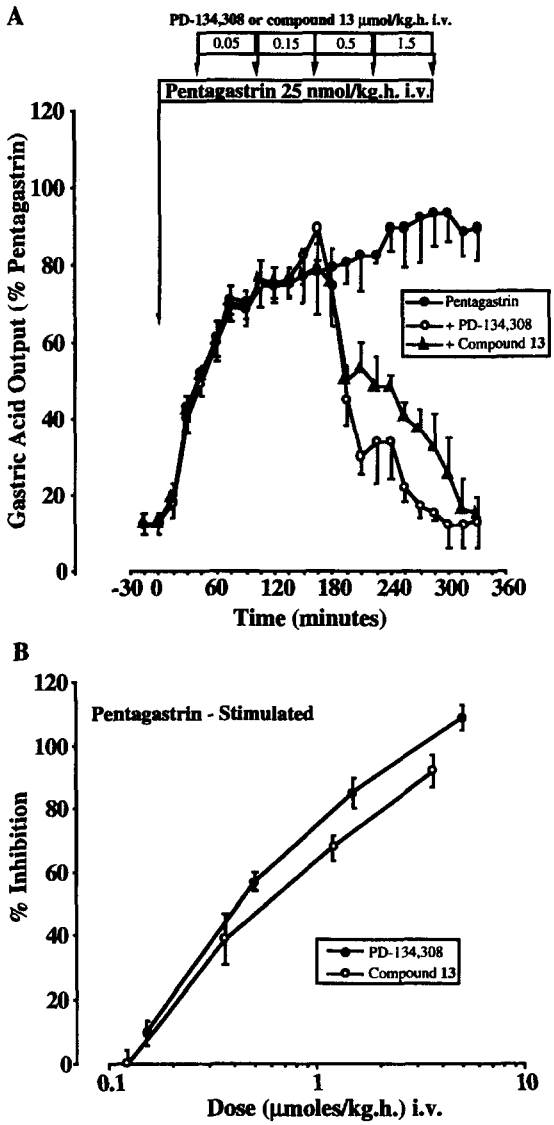


Figure 4. (A) Time–course effects of increasing doses of PD-134,308 and compound **13** on pentagastrin-stimulated gastric acid output in anesthetized perfused rat stomach. Results are expressed as percent of maximal response to pentagastrin. Each point represents the mean ± SEM of $n = 18$ for pentagastrin, $n = 7$ for PD-134,308, and $n = 10$ for compound **13**. (B) Comparative dose–response for inhibition of pentagastrin-stimulated gastric output by the CCK-B receptor antagonists, PD-134,308 and compound **13** in anesthetized perfused rat stomach. Results are expressed in percent inhibition of response to pentagastrin. Each point represents the mean ± SEM of $n = 7$ for PD-134,308 and $n = 10$ for compound **13**.

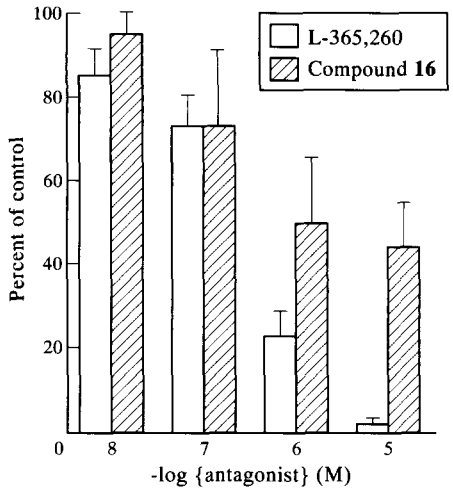


Figure 5. Antagonist activity of compound **16** as compared with that of L-365,260 on the firing rate of CCK_8 recorded on CCK-sensitive CA1-neurons. Responses of rat hippocampal neurons to CCK_8 (5×10^{-8} M) are measured in the presence of various concentrations of compound **16** or L-365,260. Mean ± SEM of $n = 2$–4 neurons per point.

In conclusion, we have obtained ligands with high binding affinity and selectivity for the CCK-B receptor. Modifications of the hydrophobic and steric character of either the C- or N-terminal amino acid substituents of CCK_4 derivatives seem to be able to change the agonist or antagonist profile of these peptides. This was shown by the fact that the agonist **1** can be chemically modified to become an antagonist, by addition of two alkyl groups on the terminal $CONH_2$ (compound **2**).[31] It was also confirmed by the fact that ^{2}Adoc-D-αMe-Trp-Phg-Asp-Nal-N$(CH_3)_2$ (**13**) behaves as an antagonist on the formation of IP induced by CCK_8 while its analogue ^{2}Adoc-D-αMe-Trp-Phg-Asp-Nal-NH_2 (**16**) behaves as an agonist in this test, and as a partial agonist/antagonist in the electrophysiological assay on rat CA1 hippocampal neurons. These compounds could therefore be used to analyze, at the molecular level, the agonist and antagonist states of the CCK-B

receptor and could be of importance to investigate the occurrence and physiological relevance of CCK-B receptor subsites.

Experimental

Amino acids and reagents for amino protections and coupling reactions were from Bachem (Switzerland). Solvents were from SDS (France). Other reagents were of high purity (Aldrich, France) and used without further purification unless otherwise noted. BOP was from Novabiochem. PD-134,308 was synthesized as previously described.[22] Chromatography was carried out with Merck Silica Gel (230–400 mesh). Thin-layer chromatography (TLC) was performed on Merck precoated Silica Gel $60F_{254}$ plates with the following solvent systems (by volume): A, CH_2Cl_2:MeOH (100:2); B, CH_2Cl_2:MeOH (9:1); C, EtOAc:$CHCl_3$:MeOH:H_2O:AcOH (22:7:3:0.6:0.3); D, EtOAc:$CHCl_3$:MeOH:H_2O:AcOH (11:7:3:0.6:0.3). Plates were developed with UV, iodine vapor, ninhydrin, or Ehrlich's reagent. Melting points were determined on a Kofler apparatus and are uncorrected. The structure of the final compounds and all intermediates was confirmed by ^{1}H NMR spectroscopy (Bruker AC, 270 MHz) in DMSO-d_6. The purity of all final compounds was checked by HPLC (Shimadzu apparatus) on a 150×4.6 mm Kromasil C 18, 5 mm column with a mixture of H_2O:TFA 0.05% (solvent A) and CH_3CN (solvent B) as eluent (flow rate 1 mL/min, UV detection 214 nm). Mass spectra were recorded on a quadrupole Nermag R10–10 C apparatus. Elemental analyses, performed by 'Service Régional de Microanalyse' (Paris, France) were within $\pm 0.4\%$ of the theoretical values unless noted otherwise.

[^{3}H]pCCK$_8$ (60–90 Ci/mmol) and myo-2-[^{3}H]inositol (60–90 Ci/mmol) were purchased from Amersham Life Science. Anion exchange column (Dowex AG 1-X8) was from Biorad.

Abbreviations

Boc, *tert*-butyloxycarbonyl; ^{2}Adoc, 2-adamantyloxycarbonyl; TFA, trifluoracetic acid; BOP, (benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, *N,N'*-diisopropylethylamine; THF, tetrahydrofuran; EtOAc, ethyl acetate; AcOH, acetic acid; LDA, lithium diisopropylamide; HMPA, hexamethyl phosphoramide; H-Nal-OH, 3-(1-naphthyl)alanine. Other abbreviations used are those recommended by the IUPAC-IUB Commission (*Biochem J.* **1984**, *219*, 345).

Synthesis

Compounds **3-7** were synthesized according to the general procedures detailed in this section for the preparation of compound **4** (Scheme 1A). Peptides **8-16** were prepared following the method described by Corringer et al.[31] (Scheme 1B). Particular aspects of the synthesis could be obtained from the authors. Analytical and physical data of the synthesized compounds are reported in Table 1.

Boc-Phg-Asp(OBzl)-OH (17). To a soln of Boc-Phg-OSu (1 g, 2.9 mmol) in 10 mL of CH_3CN (HPLC Grade) cooled to 0 °C was added a soln of Asp(OBzl)-OH (640 mg, 2.9 mmol) and triethylamine (557 μL, 4.0 mmol) in a mixed solvent (CH_3CN:H_2O, 25 mL:7 mL). After the reaction mixture was stirred for 1.5 h at ambient temperature, the organic solvent was removed in vacuo. The aq layer was cooled to 0 °C, acidified to pH 3 with 10% aq citric acid and extracted with EtOAc. The combined organic phases were washed with 10% aq citric acid, H_2O, brine and dried over Na_2SO_4. After evapn to dryness, the residue was purified on a silica gel column with CH_2Cl_2:MeOH:AcOH (18:1:1) as elution solvent, resulting in 1.02 g (84%) of product **17** as a yellow oil. R_f: 0.47 (B), ^{1}H NMR (DMSO): δ 1.31 (9H, s, *t*-butyl), 2.63–2.85 (2H, m, $CHCH_2$), 4.55 (1H, m, α-CH Asp), 5.14 (1H, d, $J = 10$ Hz, α-CH Phg), 7.2–7.35 (11H, m, BocNH + aro), 8.50 (1H, d, $J = 10$ Hz, CONH Asp), 12.26 (1H, br s, COOH).

Boc-Trp-Phg-Asp(OBzl)-OH (18). To a 0 °C soln of compound **17** (1.02 g 2.4 mmol) in CH_2Cl_2 (10 mL) was added TFA (1 mL). The reaction was warmed to ambient temperature and was stirred overnight. The solvent was then evapd in vacuo to yield 1.07 g (98%) of Phg-Asp(OBzl)-OH trifluoroacetate (**18a**) as a white solid which was used without further purification. At 0 °C, a stirred soln of **18a** (1.06 g, 2.3 mmol) in dry DMF (1 mL) was treated with triethylamine (807 μL, 5.75 mmol) and Boc-Trp-ONp (988 mg, 2.3 mmol), and the mixture was stirred overnight at rt. After removal of the solvent in vacuo, the residue was dissolved in EtOAc. The organic soln was washed with 10% aq citric acid, H_2O, and brine, dried with Na_2SO_4 and concd to dryness in vacuo. The residue was flash chromatographed on silica gel using a solution of CH_2Cl_2:MeOH:AcOH (36:1:1) as eluent to yield 1.04 g (70.5%) of product **18** as a white solid. R_f: 0.54 (D), mp 94–96 °C, ^{1}H NMR (DMSO): δ: 1.24 (9H, s, *t*-butyl), 2.62–3.06 (4H, m, β-CH_2 Asp + β-CH_2 Trp), 4.24 (1H, m, α-CH Trp), 4.50 (1H, m, α-CH Asp), 5.00 (2H, s, $ArCH_2O$), 5.49 (1H, s, α-CH Phg), 6.86–7.54 (16H, m, BocNH + aro), 8.32 (1H, d, $J = 10$ Hz, CONH Phg), 8.67 (1H, d, $J = 10$ Hz, CONH Asp), 10.72 (1H, s, NH indolyl), 10.26 (1H, br s, COOH).

Boc-Nal-N(CH_2CH_3)$_2$ (19). DIEA (248 μL, 1.43 mmol), BOP (232 mg, 0.545 mmol) and diethylamine (49.2 μL, 0.476 mmol) were added successively to a solution of Boc-Nal-OH (150 mg, 0.476 mmol) in DMF (0.5 mL) at 0 °C. The mixture was stirred for 1 h at 0 °C and 3 h at ambient temperature. After evapn of the solvent in vacuo, the residue was taken up in EtOAc and then washed with 10% aq citric acid, 10%

aq $NaHCO_3$, H_2O, brine and dried over Na_2SO_4. The organic soln was concd to give 158 mg (100%) of compound **19** as a yellow oil. R_f: 0.62 (B), ^1H NMR (DMSO): δ: 0.68 (3H, t, $J = 9$ Hz, CH_2CH_3), 0.77 (3H, t, $J = 9$ Hz CH_2CH_3), 1.25 (9H, s, *t*-butyl), 2.80–3.23 (6H, m, β-CH_2 Nal + 2N-CH_2), 4.64 (1H, m, α-CH Nal), 7.13 (1H, d, $J = 9$ Hz, BocNH), 7.28–7.55 (4H, m, naphthyl), 7.74 (1H, d, $J = 9$ Hz, naphthyl), 7.86 (1H, d, $J = 9$ Hz, naphthyl), 8.07 (1H, d, $J = 9$ Hz, naphthyl).

Boc-Trp-Phg-Asp-Nal-N(CH_2CH_3)$_2$ (4). Compound **19** (140 mg, 0.378 mmol) was N-deprotected with TFA (3 mL) for 0.5 h at 0 °C and 1.5 h at ambient temperature. The soln was concd and the residue treated with anhydrous Et_2O to precipitate a solid, which was collected, washed with Et_2O and dried in vacuo over KOH pellets to afford 158 mg (100%) of a yellow powder as Nal-N(CH_2CH_3)$_2$, trifluoroacetate (**4a**). To a solution of **4a** (60 mg, 0.156 mmol) in DMF (1 mL) cooled to 0 °C were added DIEA (82 μL, 0.468 mmol), BOP (76 mg, 0.172 mmol), and Boc-Trp-Phg-Asp(OBzl)-OH (100 mg, 0.156 mmol). The reaction was stirred overnight with warming to ambient temperature. The mixture was concentrated to dryness, then partitioned between EtOAc and H_2O. The organic phase was washed successively with solution of 10% aq citric acid, H_2O, 10% aq $NaHCO_3$, H_2O, and brine. After drying over Na_2SO_4, the solvent was removed in vacuo and 128 mg (92%) of (**4b**) as a yellow oil was obtained. R_f: 0.81 (B).

Compound **4b** was hydrogenated in MeOH (3 mL) at ambient temperature over 10% Pd/C (10 mg) for 10 h. After filtration and removing of the solvent in vacuo, the residue was purified by silica gel flash chromatography, eluting with EtOAc:$CHCl_3$:MeOH :H_2O:AcOH (35:7:3:0.6:0.3). The solvents were removed in vacuo, and the residue was dissolved in 0.1 M ammonia liquor and lyophilized to yield 60 mg (67%) of compound **4** as a white flocculent powder. ^1H NMR (DMSO + TFA): δ 0.40 (3H, t, $J = 8$ Hz, CH_2CH_3), 0.68 (3H, t, $J = 8$ Hz, CH_2CH_3), 1.25 (9H, s, *t*-butyl), 2.60–3.30 (10H, m, 2NCH_2CH_3 + 3β-CH_2 Trp, Asp, Nal), 4.27 (1H, m, α-CH Nal), 4.60 (1H, m, α-CH Trp), 4.87 (1H, m, α-CH Asp), 5.50 (1H, d, $J = 8$ Hz, α-CH Phg), 6.86–7.56 (15H, m, aro), 7.73 (1H, d, $J = 8$ Hz, aro), 7.86 (1H, d, $J = 8$ Hz aro), 7.86 (1H d, $J = 8$ Hz, CONH Phg), 8.76 (1H, d, $J = 8$ Hz, CONH Asp), 10.75 (1H, s, NH indolyl).

***N*-Boc-(1-naphthyl)alanine pyrrolidinyl amide (20).** Compound **20** was prepared from Boc-Nal-OH (100 mg, 0.317 mmol) and pyrrolidine (26.46 μL, 0.317 mmol) in the same manner as **19**, and obtained as a yellow oil (108 mg, 92%). R_f: 0.74 (B), ^1H NMR (DMSO): δ 1.27 (9H, s, *t*-butyl), 1.54 (4H, m, pyrrolidinyl), 2.93–3.40 6H, m, β-CH_2 Nal, + N$(CH_2)_2$ pyrrolidinyl, 4.44 (1H, m, α-CH Nal), 7.08 (1H, d, $J = 10$ Hz, BocNH), 7.30–7.55 (4H, m, naphthyl), 7.75 (1H, d, $J = 10$ Hz, naphthyl), 7.86 (1H, d, $J = 10$ Hz, naphthyl), 8.05 (1H, d, $J = 10$ Hz, naphthyl).

***N*-Boc-(1-naphthyl)alanine morpholinyl amide (21).** Compound **21** was prepared as described for **19** using Boc-Nal-OH (100 mg, 0.317 mmol) and morpholine (27.65 μL, 0.317 mmol) and obtained as an oil in 93% yield (113 mg). R_f: 0.6 (B), ^1H NMR (DMSO + TFA): δ 1.30 (9H, s, *t*-butyl), 2.83–3.30 (10H, m, β-CH_2 Nal + morpholinyl), 4.73 (1H, m, α -CH Nal), 7.37–7.55 (5H, m, BocNH + naphthyl), 7.73 (1H, d, $J = 11$ Hz, naphthyl), 7.87(1H, d, $J = 11$ Hz, naphthyl), 8.07 (1H, d, $J = 11$ Hz, naphthyl).

***N*-Boc-cyclo-octylalanine (22).** Benzylidene glycine methyl ester was prepared by treating glycine methyl-ester hydrochloride (5.0 g, 39.8 mmol) in 80 mL of CH_2Cl_2 with 1 equiv (4.05 mL) of benzaldehyde in the presence of 2 equiv (11.14 mL) of Et_3N and 3 g (21 mmol) of anhydrous Na_2SO_4, at rt for 7 h. Filtration, solvent removal (rt), water–ether partition washing (brine), drying, and removal of ether provided 7 g (99%) of **22a** as a colorless oil kept dry and cold. ^1H NMR (DMSO): δ 3.62 (3H, s, OCH_3), 4.38 (2H, s, $COCH_2N$), 7.39–7.45 (3H, m, ArH), 7.69–7.74 (2H, m, ArH), 8.33 (1H, s, ArCH=N).

A flask was charged with 30 mL of anhydrous THF under a nitrogen atmosphere. Freshly distilled diisopropylamine (2.87 μL, 20.5 mmol) was added via a syringe and the solution cooled to 0 °C, followed by the dropwise addition of *n*-butyllithium (10.2 mL of a 2.0 M soln in hexane). The soln was stirred at 0 °C for 20 min, then cooled to −78 °C and the imine (**22a**) (3.02 g, 17.06 mmol) in 30 mL of dry THF was then added via a syringe over 20 min. The mixture (red) was stirred at −78 °C for 20 min and then HMPA (3 μL, 17.06 mmol) was added. After 10 min, the cyclo-octylmethyl bromide (3.5 g, 17.06 mmol) dissolved in 30 mL of THF was then added and the reaction mixture was stirred for 6 h at −78 °C and overnight to ambient temperature. The mixture was hydrolyzed by adding 40 mL of 1 N HCl dropwise at 0 °C and stirred for 40 min at ambient temperature. Water (150 mL) was added, the aqueous phase sepd was washed with ether, pH raised to 9 with 10% aq $NaHCO_3$, and then extracted with EtOAc. Organic phase was washed with H_2O and brine, dried over Na_2SO_4 and evapd. Crude product was purified by column chromatography in EtOAc:$CHCl_3$:MeOH:H_2O:AcOH (11:7:3:0.6:0.3) and 2.45 g (72%) of cyclooctylalanine methylester (**22b**) was obtained as a yellow oil. R_f: 0.40 (D), ^1H NMR (DMSO + TFA): δ 1.44–1.84 (17H, m, CH_2CH + cyclooctyl), 2.78 (3H, s, OCH_3), 4.20 (1H, m, NCHCO), 8.50 (3H, s, NH_3^+).

To a soln of **22b** (2.45 g, 12.3 mmol) in 10 mL of dry DMF were added Et_3N (3.4 μL, 24.6 mmol) and di-*tert*-butyl dicarbonate (5.37 g, 24.6 mmol). The reaction mixture was stirred at ambient temperature for 2 h. The solvent was removed in vacuo and the residue partitioned between water and ether. The ether layer was washed successively with 10% aq citric acid, H_2O and brine. After drying with Na_2SO_4, the organic layer was concd in vacuo and the residue chromatographed

on silica gel using a soln of CH_2Cl_2:EtOAc (19/1) to yield 1.03 g (30%) of *N*-Boc-cyclo-octylalanine methylester (**22c**). R_f: 0.70 (A), ^{1}H NMR (DMSO): δ 1.10–1.50 (26H, m, CH_2CH + *t*-butyl + cyclooctyl), 3.56 (3H, s, OCH_3), 3.96 (1H, m, NCHCO), 7.20 (1H, d, $J = 8$ Hz, BocNH).

The methylester (**22c**) (1 g, 3.33 mmol) was dissolved in a mixed solvent (MeOH:H_2O: 10 mL:5 mL) and a soln of LiOH, H_2O (420 mg, 10 mmol) in 2 mL water was subsequently added. The reaction mixture was stirred at ambient temperature for 3 h. After removal of the solvent, 10 mL of H_2O was added, the resulting solution was acidified to pH 2 with 10% aq citric acid, and extracted with EtOAc. The combined organic layers were washed with H_2O and brine, dried over Na_2SO_4, and then concd in vacuo to give 0.95 g (99.6%) of the target amino acid as a yellow solid (**22**). R_f: 0.64 (B); mp 107–109 °C. ^{1}H NMR (DMSO): δ 1.12–1.55 (26H, m, *t*-butyl + cyclooctylmethyl), 3.86 (1H, m, NCHCO), 7.00 (1H, d, $J = 10$ Hz, BocNH), 12.27 (1H, br s, COOH).

Binding assays

[^{3}H]pCCK$_8$, (specific activity 60–90 Ci/mmol) was purchased from Amersham. Incubations (final vol 1 mL) were carried out at 25 °C in 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and 0.2 mg/mL of bacitracin for 60 min in the presence of brain membranes (0.6 mg of protein per tube) or in 10 mM Pipes–HCl buffer (pH 6.5), 30 mM $MgCl_2$, 0.2 mg/mL of bacitracin, and 0.2 mg/mL of soybean trypsin inhibitor for 120 min in the presence of pancreatic membranes (0.2 mg of protein per tube) as already described in detail.[34] [^{3}H]pCCK$_8$ was incubated with brain membranes at 0.2 nM and with pancreatic membranes at 0.1 nM, in the presence of varying concentrations of the competitor. Non-specific binding was determined in the presence of 1 μM CCK$_8$. Incubation was terminated by rapid filtration through Whatman GF/B glass-fiber filters precoated with buffer containing 0.1% bovine serum albumin. The filters were rinsed with 2 × 5 mL of ice-cold buffer and dried and the radioactivity counted. K_i values were calculated using the Cheng–Prusoff equation. Hill coefficients in all experiments were close to 1.

Establishment of a stable cell line expressing the rat CCK-B receptor

Chinese hamster ovary (CHO) cells were grown in HAM-F12 medium containing 10% fetal calf serum, 50 mg/mL gentamycin and 1 mM Na pyruvate, in 5% CO_2 at 37 °C. One day before transfection, cells were plated at a density of 3×10^5 cells/9 cm diameter tissue culture dish. Cells were transfected with 15 mg of the pcDNA3/RKB vector using the calcium phosphate method.[35] Two days after the transfection, cells were grown in the presence of 0.4 mg/mL G 418. After 3 weeks, growing clones of cells resistant to G 418 were observed. A pure cell line was obtained by cloning using the limit dilution method.[38]

Preparation of CHO membranes and ligand binding assays

Cells were plated at a density of 1×10^6 cells/15 cm diameter tissue culture dish in the presence of 0.4 mg/mL of G 418. At confluency, cells were rinsed with cold phosphate-buffered saline (PBS), scraped from the tissue culture dish and resuspended in PBS. The cells were centrifuged at 4 °C for 5 min at 2000 rpm. The pellet was homogenized at 4 °C in 50 mM Tris–HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and centrifuged at 4 °C for 35 min at 100,000*g*. The resulting pellet was rehomogenized in a large excess of ice-cold buffer and centrifuged under the same conditions. The final pellet was homogenized at 4 °C in 5 mL of Tris–HCl buffer (pH 7.4) with 5 mM $MgCl_2$. The membranes were aliquoted and frozen at −80 °C. Protein concentration was estimated using the Pierce bicinchoninic acid protein assay reagent with bovin serum albumin as a standard. The binding assays were performed in 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.2 mg/mL bacitracin as previously described.[33] Each assay contained the membrane preparation (around 40 μg of protein) and [^{3}H]pCCK$_8$ (0.4 nM) in a final vol of 1 mL. The non-specific binding was determinated in the presence of 1 μM CCK$_8$.

Inositol phosphate assays. CHO cells stably expressing wild type CCK-B rat receptor were assayed for agonists (and/or antagonists) stimulated IP hydrolysis essentially as previously described.[38,44] Cells were plated in 24 well microtiter plates. Before confluency, cells were grown in the presence of 1 μCi/mL myo-[2-^{3}H] inositol for 16 h, at 37 °C. Cells were treated with 10 mM LiCl for 30 min at 37 °C, with 0.5 nM CCK$_8$ for 5 min and various concentrations of antagonists were then added to the cells. After 45 min at 37 °C, the incubation medium was removed and the cells washed twice with 1 mL of PBS. The reaction was stopped by adding 400 mL of ice-cold 67% methanol, and 300 mL of 0.125% Triton. The cells were scrapped and the suspension subjected to chloroform extraction; 0.5 mL of the aqueous phase was added to 4.5 mL of water. The solution was then loaded onto 0.5 mL column of AG1-X8 Dowex anion exchange resin. The column was washed with 1 mL of distilled water followed by 5 mL of 5 mM sodium borate:60 mM sodium formate. Total [^{3}H]inositol phosphates were then eluted into scintillation vials with 5 mL of 1 M ammonium formate:0.1 M formic acid. Scintillation mixture was then added and the radioactivity counted. The data are expressed as the percentage of the maximal response induced by 0.5 nM of CCK$_8$.

Electrophysiology

Hippocampal slices from male Sprague-Dawley rats were prepared for in vitro electrophysiological recordings as previously described.[45] In brief, 0.5 mm thick

transverse hippocampal slices were maintained in a submersion-type recording chamber, and spontaneous action-potential (AP) discharge frequency of CA_1-neurons was recorded extracellularly. A firing rate tracing was obtained by integration of the AP counts over 2–4 s. Compounds **16** and L-365,260 were dissolved in DMF and then diluted in the slice maintenance medium (composition: see ref 38) to the desired final concentration (DMF:1% maximum). The compounds were applied alone for 10 min on neurons selected with a 10-fold lower concentration of CCK_8, and then co-perfused together with CCK_8. Antagonist activity was measured relative to the last CCK_8 response.

Gastric acid secretion test

Animals and operative technique. Experiments were performed on male Sprague–Dawley rats (Elevage Janvier, le Genest, France) weighing 300 ± 25 g fasted for 18 h with free access to water as already described in detail.[46] The technical aspects of the operation have been described by Ghosh and Schild[47] and modified by Lai.[48] Briefly, the rats were anesthetized by intramuscular injection of urethane (0.6–0.7 mL of 25% soln per 100 g body weight). A polyethylene catheter introduced in the oesophagus, passed to the level of the cardia and was connected to a peristaltic pump (Minipuls 2, Gilson Medical Electronics) to deliver a solution of 0.9% NaCl at a constant rate of 1.0 mL/min. This perfusate was collected through another catheter placed through the pylorus and secured with a ligature.

Experimental protocol. The tests started after stabilization of the gastric perfusion, which was achieved usually between 30–60 min after completion of the surgical preparation. During this period, an intravenous of physiological saline, was infused at 2.4 mL/h (perfusor Braun, Roucaire) into the dorsal vein of the penis. Stimulation by pentagastrin was obtained by continuous infusion through the same route. After 90 min infusion, the CCK-B antagonist, PD-134,308 or compound **13** was surimposed to agonist infusion, the dose being increased each hour. Gastric secretion was collected every 15 min and the H^+ amount evaluated by titrating the entire sample with 0.01 N NaOH to pH 7. The results are expressed as percentage (mean of the two experimental points preceding administration of the antagonist) of gastric acid output induced by pentagastrin. Statistical evaluations were performed using Student's *t*-test.

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