CCK-B Agonist or Antagonist Activities of Structurally Hindered and Peptidase-Resistant Boc-CCK$_4$ Derivatives

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Replacement of Met^{31} by (*N*-Me)Nle in CCK$_8$ or CCK$_4$ has been shown to improve the affinity and selectivity for CCK-B receptors. In order to obtain molecules with enhanced bioavailability, two novel series of protected tetrapeptides of the general formula Boc-Trp^{30}-X-Asp-Y^{33} have been developed. Introduction of (*N*-Me)Nle and the bulky, aromatic naphthylalaninamide (Nal-NH$_2$) in positions X and Y, respectively, does not greatly modify the affinity for guinea pig brain CCK-B receptors. In contrast, incorporation of hindering *N*-methyl amino acids such as (*N*-Me)Phe, (*N*-Me)Phg, or (*N*-Me)Chg, but not their non-methylated counterparts, in position X induced a large decrease in affinity for the CCK-B binding sites. Among the various peptides synthesized, Boc-[(*N*-Me)Nle^{31},1Nal-NH$_2^{33}$]CCK$_4$ (**2**) (K_I = 2.8 nM), Boc-[Phg^{31},1Nal-NH$_2^{33}$]CCK$_4$ (**15**) (K_I = 14 nM), and Boc-[Phg^{31},1Nal-N(CH$_3$)$_2^{33}$]CCK$_4$ (**17**) (K_I = 39 nM) displayed good affinities for brain CCK-B receptors and had good selectivity ratios. These pseudopeptides, in which the presence of unnatural and hydrophobic residues is expected to improve their penetration of the central nervous system, were shown to be very resistant to brain peptidases. Interestingly, whereas compounds **2** and **15** proved to be full agonists for rat hippocampal CCK-B receptors when measured in an electrophysiological assay, compound **17** behaved as a potent antagonist in the same test and displayed a good affinity in rat brain K_I(CCK-B) = 51 nM as compared to the Merck antagonist L365,260, K_I(CCK-B) = 12 nM. This illustrates a simple means to obtain CCK-B antagonists and suggests that the free, CONH$_2$ group plays a critical role in the recognition of the agonist state of brain CCK-B receptors.

Introduction

The peptide hormone cholecystokinin (CCK) first isolated from hog intestine[1] has been shown to stimulate many gastrointestinal functions such as gall bladder contraction and pancreatic secretion.[2] In 1975, the CCK$_8$ C-terminal fragment, ^{26}Asp-Tyr(SO$_3$H)-Met-Gly-Trp-Met-Asp-Phe-NH$_2^{33}$, was found to be present in the brain[3,4] where it seems to function as a neuromodulator and/or neurotransmitter (for review see ref 5). Several studies have demonstrated that there are at least two classes of CCK receptors,[6] the CCK-B receptor-type, which is very abundant in the central nervous system,[7,8] and the CCK-A receptor-type, which is primarily present in the periphery but which is also found in some discrete brain regions.[9,10] These receptor types can be differentiated by the relative affinities of the tetrapeptide CCK$_4$ which interacts with about 1000 times higher affinity for the CCK-B receptor[6] while the octapeptide CCK$_8$ binds with nanomolar affinities to both receptors. The biochemistry and pharmacology of brain CCK are currently being intensively studied as this neuropeptide could have potential therapeutical interest in many fields such as analgesia,[11] anxiety,[12] or neuropsychiatric disorders.[13] In particular, in humans CCK$_4$ has been shown to induce panic attacks following systemic injection.[14] However, owing to the peptidase susceptibility and the hydrophilicity of the tetrapeptide, its primary site of action, central or peripheral, remains an open question.[15] Further investigation of this phenomenon requires highly potent and selective CCK-B agonists and antagonists, capable of crossing the blood-brain barrier as well as being resistant to enzymatic degradation.

So far the best CCK-B agonists have been obtained by modifying the peptide backbone of CCK$_8$. We have previously reported that N-methylation of Nle^{31} in Boc-[Nle^{28,31}]CCK$_7$ (BDNL), an analogue equiactive with CCK$_8$,[16] did not modify the affinity for the CCK-A and CCK-B receptors. Introduction of a retro-inverso bond between residues 28 and 29 in this peptide led to BC 264[17] (Boc-Tyr(SO$_3$H)-gNle-mGly-Trp-(*N*-Me)Nle-Asp-Phe-NH$_2$) which behaves as a highly potent and selective CCK-B agonist.[18] Moreover, BC 264 was shown to be highly resistant to peptidases[17] and capable of entering the brain in its intact form after systemic administration.[19] Recently, Hruby et al.[20] described that the analogue Asp-Tyr(SO$_3$H)-(*N*-Me)Nle-Gly-Trp-(*N*-Me)Nle-Asp-Phe-NH$_2$ displays high affinities for both classes of receptor and that removal of the N-terminal part of this compound yields a pentapeptide (Gly-Trp-(*N*-Me)Nle-Asp-Phe-NH$_2$) with nanomolar affinity and high selectivity for the CCK-B receptors. Likewise, Nadzan et al.[21] incorporated, *trans*-3-*n*-propyl-L-proline, an even more constrained analogue of norleucine in Boc-CCK$_4$ and showed that this modification has a similar beneficial effect on CCK-B receptor binding as the introduction of a N-methylated norleucine. Furthermore, several studies in our laboratory have shown that incorporation of a Phe^{31} residue in BDNL induces a 55-fold selectivity for the CCK-B receptor[22] while introduction of an (Z)Orn^{31} moiety in BDNL or Boc-CCK$_4$ leads to CCK antagonists.[22,23] In addition, while it appears difficult to modify the Trp^{30} and Asp^{32} residues without a loss of biological activity,[24,25] the C-terminal amino acid Phe-NH$_2^{33}$ has been successfully substituted by bulky

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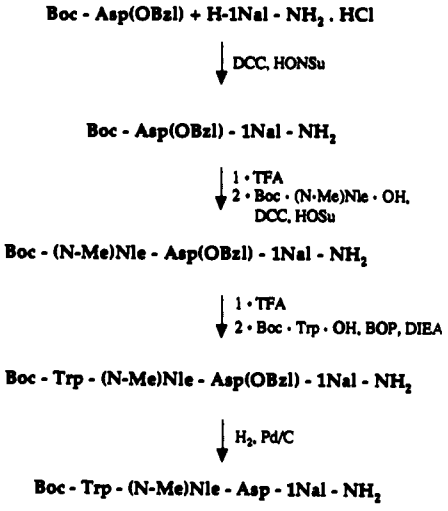
Scheme I



amino acids.[26] The removal of Phe-NH_2[33] in CCK_8 and derivatives led to an antagonist for both classes of binding sites[27,28] whereas removal of the $CONH_2$ group was reported to induce a partial agonist activity for the peripheral CCK-A receptors.[29] Furthermore, a hydrazide analogue of CCK_4 (Boc-Trp-Leu-Asp-Phe-NH-NH_2) was shown to behave as a full antagonist for CCK-A receptors but as a partial agonist for CCK-B receptors.[30] Taken together, these results show that voluminous amino acids can be introduced in positions 31 and 33 and that, depending on the nature of the side chains, an activation or blockade of CCK receptors can be obtained. In the present paper, we describe the synthesis and biological activity of Boc-CCK_4 and Boc-[(*N*-Me)Nle[31]]CCK_4 analogues modified in position 31 and 33, which were designed with the aim of obtaining small CCK-B-selective ligands which are easy to synthesize and are endowed with good stabilities and bioavailabilities.

Chemistry

The synthesis of all analogues was performed as described for the preparation of compound **2** in Scheme I. Elongation of the peptide chains was performed stepwise using DCC/HOSu, BOP, or *p*-nitrophenol ester/HOBt methods. For the difficult coupling reactions, i.e., condensation of acids with *N*-methylamino peptides, either BOP or *p*-nitrophenol ester/HOBt methods were used. The amino protecting groups Boc and Z were respectively removed with trifluoroacetic acid and by catalytic hydrogenolysis. The carboxylic acid protecting group benzylic ester was removed by catalytic hydrogenolysis.

The naphthylalanine analogues H-1Nal-NH_2, H-2Nal-NH_2, and H-1Nal-$NHCH_3$ were obtained by esterification of the carboxylic acid function of the commercial products H-3-(1-naphthyl)-Ala-OH (H-1Nal-OH) or H-3-(2-naphthyl)-Ala-OH (H-2Nal-OH) by thionyl chloride, followed by amidification with ammonia or methylamine. H-1Nal-$N(CH_3)_2$ was synthesized by coupling Z-1Nal-OH with dimethylamine followed by hydrogenolysis of the Z protecting group. Boc-(N-Me)Phg-OH was prepared by the method described by McDermott and Benoiton,[31] by reaction of Boc-Phg-OH with iodomethane in the presence of sodium hydride in THF. Boc-Chg-OH was prepared by hydrogenation of H-Phg-OH followed by protection of the amino group with di-*tert*-butyl dicarbonate. For the synthesis of Boc-(*N*-Me)Chg-OH, it was found that direct hydrogenation of Boc-(*N*-Me)Phg-OH was very slow under the conditions used (45 psi, 45 °C, PtO_2 catalyst). Therefore, Boc-(*N*-Me)Phg-OH was deprotected with trifluoroacetic acid, hydrogenated, and then protected using di-*tert*-butyl dicarbonate to give Boc-(*N*-Me)Chg-OH.

Results and Discussion

The aim of this study was to develop new small CCK_4-related ligands endowed with good affinities and selectivities for CCK-B receptors and improved bioavailabilities. The compounds synthesized were evaluated for their potency in displacing [^{3}H]pCCK_8 from guinea pig pancreatic and brain membranes, and the results are shown in Table I.

As expected, replacement of Gly in Gly-Trp-(*N*-Me)-Nle-Asp-Phe-NH_2 by a Boc group did not modify the higher preferential interaction of this peptide for CCK-B sites.[20] Compound **1** was at least 3 orders of magnitude more potent at B than at A binding sites. The methylation of the peptide bond between residues 30 and 31 in compound **12** led to a 4-fold increase in CCK-B binding affinity. In the course of structure–activity studies on BDNL analogues, it was found that Phe-NH_2[33] can be replaced by convenient bulky residues, particularly 1-Nal-NH_2, without causing alteration in biological activity and specificity.[26] Accordingly, 1-Nal-NH_2 or 2-Nal-NH_2 were introduced as C-terminal residues leading to peptides **2** and **4** which retained the binding profile of their precursor, in spite of a slight decrease in affinity for the CCK-B receptor (3.5- and 6.4-fold, respectively, as compared to compound **1**). 1-Nal-NH_2 residue was chosen to replace Phe-NH_2 in almost all the synthesized compounds, due to its hydrophobicity and its noncoded nature which was expected to improve the resistance to degrading enzymes. Indeed, the peptidase sensitivity of CCK_4 and compound **1** and **2** was investigated using crude rat brain membranes (Figure 1). It was found that whereas CCK_4 has a half-life of only 5 min, introduction of a *N*-methyl residue in position 31 (compound **1**) improves the resistance to peptidases, and additional incorporation of 1Nal in position 33 (compound **2**) almost totally protects the peptide from degradation.

In our earlier studies on BDNL analogues,[22] we showed that introduction of a Phe residue in position 31 induced a 55-fold selectivity for CCK-B receptors without great change in CCK-B affinity. In the case of compound **14**, this substitution led to a 22-fold decrease in affinity and a lower selectivity for central binding sites as compared to compound **12**. However, introduction of other cyclic amino acids in this position, such as phenylglycine in **15** and cyclohexylglycine in **16**, led to compounds displaying affinities in the nanomolar range for the CCK-B receptor (14 nM and 17 nM for **15** and **16**, respectively) and are therefore only about 5 times less potent than their parent compound **12**.

Therefore, to further increase the affinity for CCK-B binding sites, the corresponding *N*-methylamino acids (*N*-Me)Phe, (*N*-Me)Phg, and (*N*-Me)Chg were introduced in position 31. Surprisingly, these modifications induced a large decrease in CCK-B binding affinity since **5** is about 740-fold less potent than **1**. Similar results were observed with compounds **7**, **8**, and **9** when compared to **2**. Moreover, all the N-methylated peptides are 10 times less potent than their unmethylated counterparts (compare **7**, **8**, and

Table I. Apparent Affinities (K_I, M) of CCK_8 and Derivatives 1–18 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 Pig

no.	compounds	K_I, (M)[a] brain (CCK-B)	K_I, (M)[a] pancreas (CCK-A)
	CCK_8	$0.3 \pm 0.01 \times 10^{-6}$	$0.7 \pm 0.04 \times 10^{-6}$
1	Boc-Trp-(*N*-Me)Nle-Asp-Phe-NH_2	$0.8 \pm 0.1 \times 10^{-6}$	$>5 \times 10^{-6}$
2	Boc-Trp-(*N*-Me)Nle-Asp-1Nal-NH_2	$2.8 \pm 0.6 \times 10^{-6}$	$1.2 \pm 0.1 \times 10^{-6}$
3	Boc-DTrp-(*N*-Me)Nle-Asp-1Nal-NH_2	$3.6 \pm 0.1 \times 10^{-6}$	$6.3 \pm 0.7 \times 10^{-6}$
4	Boc-Trp-(*N*-Me)Nle-Asp-2Nal-NH_2	$5.1 \pm 1.1 \times 10^{-6}$	$4.2 \pm 1.0 \times 10^{-6}$
5	Boc-Trp-(*N*-Me)Phe-Asp-Phe-NH_2	$5.9 \pm 0.6 \times 10^{-7}$	$3.2 \pm 0.3 \times 10^{-6}$
6	Boc-DTrp-(*N*-Me)Phe-Asp-Phe-NH_2	$3.0 \pm 0.6 \times 10^{-6}$	$8.0 \pm 0.5 \times 10^{-6}$
7	Boc-Trp-(*N*-Me)Phe-Asp-1Nal-NH_2	$7.7 \pm 1.7 \times 10^{-7}$	$3.5 \pm 0.8 \times 10^{-6}$
8	Boc-Trp-(*N*-Me)Phg-Asp-1Nal-NH_2	$1.7 \pm 0.5 \times 10^{-7}$	$8.3 \pm 0.6 \times 10^{-7}$
9	Boc-Trp-(*N*-Me)Chg-Asp-1Nal-NH_2	$1.4 \pm 0.3 \times 10^{-7}$	$2.1 \pm 0.3 \times 10^{-6}$
10	Boc-Trp-(*N*-Me)Nle-Asp-1Nal-NH(CH_3)	$6.8 \pm 0.1 \times 10^{-6}$	$1.2 \pm 0.1 \times 10^{-6}$
11	Boc-Trp-(*N*-Me)Nle-Asp-1Nal-N(CH_3)$_2$	$8.1 \pm 2.0 \times 10^{-6}$	$5.2 \pm 0.4 \times 10^{-7}$
12	Boc-Trp-Nle-Asp-Phe-NH_2	$3.2 \pm 0.3 \times 10^{-6}$	$2.9 \pm 0.1 \times 10^{-6}$
13	Boc-DTrp-Nle-Asp-Phe-NH_2	$1.6 \pm 0.1 \times 10^{-7}$	$1.1 \pm 0.1 \times 10^{-6}$
14	Boc-Trp-Phe-Asp-1Nal-NH_2	$7.1 \pm 0.8 \times 10^{-6}$	$2.4 \pm 0.2 \times 10^{-6}$
15	Boc-Trp-Phg-Asp-1Nal-NH_2	$1.4 \pm 0.1 \times 10^{-6}$	$9.9 \pm 0.6 \times 10^{-7}$
16	Boc-Trp-Chg-Asp-1Nal-NH_2	$1.7 \pm 0.3 \times 10^{-6}$	$1.4 \pm 0.3 \times 10^{-6}$
17	Boc-Trp-Phg-Asp-1Nal-N(CH_3)$_2$	$3.9 \pm 1.0 \times 10^{-6}$	$1.0 \pm 0.4 \times 10^{-6}$
18	Boc-Trp-Chg-Asp-1Nal-N(CH_3)$_2$	$7.4 \pm 0.2 \times 10^{-6}$	$5.4 \pm 1.4 \times 10^{-6}$

[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.

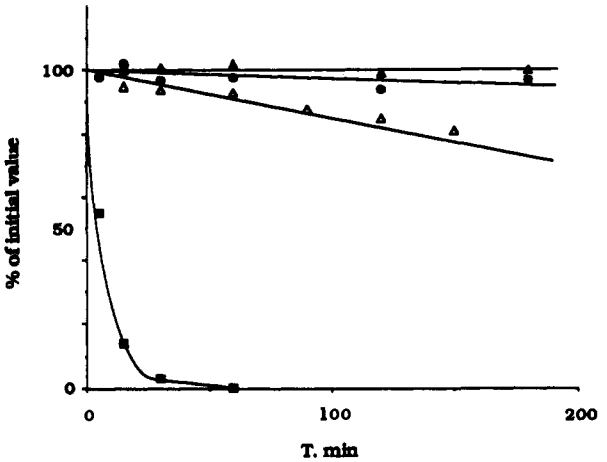


Figure 1. Time course for the hydrolysis of CCK_4 analogues by rat brain homogenate. Substrates (10^{-4} M) were incubated for varying times at 35 °C with crude membrane fractions of rat brain (3 mg protein/mL). (■) CCK_4, (▲) compound 1, (●) compound 2, (▲) compound 17. Results are the mean of two separate experiments.

9 with 14, 15, and 16, respectively). All these N-methylated analogues show the presence of the cis/trans isomerism around the Trp-(*N*-Me)Nle bond found in 1, excluding the possibility that the loss in affinity is due to the absence of the biologically active conformer. Furthermore, the proton of the amide bond between residues 30 and 31 does not play a critical role for CCK-B receptor recognition as shown by the high affinity of compound 1.

To further increase the lipophilicity of compounds 2 and 12, the C-terminal amide group was replaced by a mono or dimethylamide function since, in the case of BDNL, this modification was shown to produce only a small loss in CCK-B receptor affinity (1.00 nM for Boc-[Nle^{28,31},Phe-NH(CH_3)^{33}]CCK_7 versus 0.13 nM for BDNL).[26] In the present series, the reduction in affinity was greater in compounds 10 and 11 (around 25–30-fold as compared to that for compound 2), but remained in the same range in the corresponding non-methylated peptides 17 and 18 which are only 3-4 times less potent than their parent compounds for the CCK-B binding sites.

The steric constraint caused by N-methylation of aromatic or cyclic residues in position 31, particularly when associated with a large C-terminal naphthylalanine residue, therefore seems to be responsible for the large reduction in CCK-B affinity, probably by driving the bulky phenyl or cyclohexyl rings towards the excluded volume of the receptor subsite. Inversion of the configuration of Trp^{30} in the partial agonist Boc-Tyr(SO_3^-)-Met-Gly-Trp-Nle-Asp-2-phenylethyl ester[32] led to a full CCK-A antagonist while in the peptoid antagonist PD 134 308,[33] the presence of a D-α-methyltryptophan residue instead of the corresponding L amino acid led to a 100-fold higher affinity for the CCK-B receptor. In contrast, the introduction of a D-Trp in position 30 in compounds 2, 5, and 12 led to an important decrease in affinity for the CCK-B receptor (13-, 5- and 50-fold decrease for compounds 3, 6, and 13 as compared to their parent compounds).

Among the peptides synthesized, compounds 2, 15, and 17 display good affinities for the CCK-B receptors. However, these derivatives are modified in positions which are crucial for the activation of CCK binding sites. Their agonist/antagonist profile was thus investigated using a CCK-B selective electrophysiological test in which CCK_8 and CCK-B agonists have been shown to stimulate the firing rate of rat CA1 hippocampal neurones in a dose-dependent manner.[18,34] As illustrated in Figure 2, compound 2 acted as a full agonist, showing that N-methylation of Nle^{31} and substitution of Phe-NH_2[33] by 1Nal-NH_2[33] did not modify the activity of the peptides at the CCK-B receptors. Compound 15 also acted as a full CCK-B agonist, showing that Nle^{31} can be usefully replaced by Phg. In contrast, compound 17 was unable to enhance the firing rate of the hippocampal neurones but antagonized, in a dose-dependent manner, the stimulation induced by CCK_8 (Figures 3 and 4). In all cases the pharmacological potencies of the peptides were relatively well-correlated with their affinities for CCK-B receptors.

The opposing pharmacological activities of compounds 15 and 17 strongly suggest that bis-methylation of the NH_2 terminus is responsible for the antagonist properties of 17, either through a steric hindrance or by eliminating possible hydrogen bonds between the $CONH_2$ group and appropriately located donor and acceptor groups in the

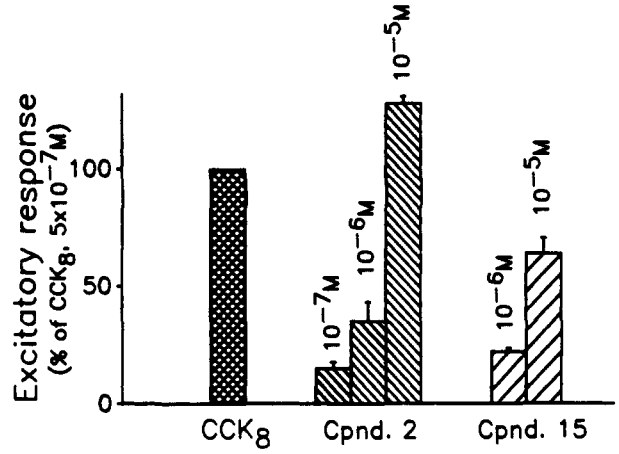


Figure 2. Agonist activity of compounds 2 and 15. Excitatory responses of hippocampal CCK-sensitive neurons to various concentrations of compound 2 (narrow-hatched bars) and compound 15 (wide-hatched bars) expressed relative to the response to 5×10^{-7} M CCK_8 (cross-hatched bars). Note the concentration-dependent excitatory effect of both compounds.

Figure 3. Responses of a hippocampal neuron to CCK_8 in the presence of increasing concentrations of compound 17. Segments of firing rate recording to a CCK-sensitive CA1-neuron. (a) CCK_8 (5×10^{-9} M) alone (control); (b) CCK_8 + compound 17 (10^{-9} M); (c) CCK_8 + compound 17 (10^{-9} M); (d) CCK_8 + compound 17 (10^{-7} M); (e) CCK_8 + compound 17 (10^{-5} M). Note the progressive decline in excitatory responses to CCK_8. Calibration bars: vertical, 20 spikes/s; horizontal, 5 min.

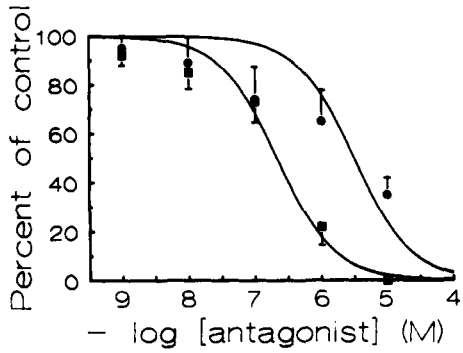


Figure 4. Antagonist activity of compound 17. Mean ± SEM (n = 2–4 neurons per point) excitatory responses of rat hippocampal neurons to CCK_8 (5×10^{-6} M) in the presence of various concentrations of compound 17 (●) or L365,260 (■). Estimated half-maximal effective concentrations were 3.2×10^{-6} M and 2.2×10^{-7} M, respectively.

receptor. This avoids, or at least strongly reduces, the possibility that the ligand will fit the agonist state of the CCK-B receptor. The presence of both a Phg and a 1Nal-$N(CH_3)_2$ residue could also contribute to the potent antagonist activity of 17. This compound was also highly potent in displacing [^{3}H]pCCK_8 from rat brain membranes (Table II) with an affinity similar to that found in guinea pig brain. In contrast, the non-peptidic antagonist L365,-260,[35] which is potent in the guinea pig, displays a 5-fold lower affinity for the rat CCK-B receptors. Therefore, 17 is only 5 times less potent than L365,260 in the rat. However, this difference in potency appears slightly larger in an electrophysiological assay since the ED_{50} were 3.2×10^{-6} M for 17 and 2.2×10^{-7} M for L365,260 (Figure 4). This feature could be due to different pharmacokinetic

Table II. Apparent Affinities (K_I, nM) of the Antagonist 17 and L 365,260 on the Binding of [^{3}H]pCCK_8 to the Brain Membranes of Guinea Pig and Rat

	K_I (nM)[a]	
compounds	guinea pig	rat
17	39 ± 10	51 ± 7
L365,260	2.2 ± 0.6	12 ± 3

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

properties between peptide and non-peptide compounds. In addition, as observed for compound 2, the modifications introduced in positions 31 and 33 in compound 17 almost fully protect this peptide from degradation (Figure 1).

In conclusion, the modifications performed in position 31 and 33 in Boc-CCK_4 provided potent and selective CCK-B agonists and antagonists. The noncoded nature of the residues incorporated significantly improves the protection of these peptides to degradation, while their high hydrophobicity is expected to improve their penetration into the brain. This feature is being studied in our laboratory by in vivo binding techniques.[36] One compound of the series proved to be a potent and specific CCK-B antagonist. The best CCK-B antagonists reported to date are the benzodiazepine derivative L365,260,[37] the peptoid PD 134,308,[33] the ureidoacetamide RP 69758,[38] and the diphenylpyrazolidinone LY 262864.[39] The peptidomimetic CCK-B antagonist 17 developed in this work displays a good affinity for CCK-B receptors, especially in the rat where it is almost as potent as L365,260 and is highly protected from enzymatic degradation. A comparison of the structure of these molecules using NMR and molecular modeling could permit the characterization of the essential structural components for the blockade of brain CCK-B binding sites. This work is now in progress in our laboratory.

Experimental Section

The following compounds were prepared as described in the literature: Boc-(*N*-Me)Nle-OH, H-Asp(OBzl)-Phe-NH_2, H-Nle-Asp(OBzl)-Phe-NH_2·TFA, and Boc-Trp-(*N*-Me)Nle-Asp(OBzl)-Phe-NH_2;[17] H-1Nal-OMe·HCl and H-1Nal-NH_2·HCl.[26] All other amino acids were from Bachem. Solvents were from SDS. 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, CH_2Cl_2–MeOH (95:5); B, CH_2Cl_2-MeOH (9:1); C, EtOAc–CH_2Cl_2–MeOH–H_2O–AcOH (22:7:3:0.6:0.3); D, CH_2Cl_2–MeOH–H_2O–AcOH (7:3:0.6:0.3). Plates were developed with UV, iodine vapor, ninhydrin, or Ehrlich's reagent. The structure and the lack of racemization of the final compounds and all intermediates were confirmed by ^{1}H NMR spectroscopy (Brucker WH, 270 MHz) in DMSO-d_6. The purity of all final compounds was checked by HPLC (Shimatzu apparatus) on a 250 × 4.6 mm Kromasil C8 5-μm column with a mixture of CH_3-CN (solvent A) and H_2O/TFA 0.05% (solvent B) as eluent (flow rate, 0.8 mL/min) with UV detection (214 nm). Molecular mass was obtained by 252 Cf-plasma desorption mass spectroscopy on a Bio-Ion 20 time of flight instrument. Melting points were taken on a Kofler apparatus and are given uncorrected. The following abbreviations have been used: DMF, dimethylformamide: DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; HOSU, *N*-hydroxysuccinimide; HOBt, 1-hydroxybenzotriazole; DIEA, *N*,*N*′-diisopropylethylamine; BOP, (benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; H-1Nal-OH, 3-(1-naphthyl)alanine; H-2Nal-OH, 3-(2-naphthyl)alanine. Other abbreviations used are those recommended by the IUPAC-IUB Commission (*Biochem J.* **1984**, *219*, 345).

Table III. Physical and Analytical Data of Peptides 1–18 Used in the Biological Tests

	mp, °C	R_f	HPLC t_R, min (A/B)	FAB/MS (MH⁺) calcd	FAB/MS (MH⁺) found	anal. C, H, N
Boc-Trp-(*N*-Me)Nle-Asp-Phe-NH_2 (1)	132–136	0.23 (C)	9.2 (54:46)	693	693	$C_{36}H_{48}N_6O_6$
Boc-Trp-(*N*-Me)Nle-Asp-1Nal-NH_2 (2)	131–133	0.38 (C)	13.4 (54:46)	743	743	$C_{40}H_{50}N_6O_6$
Boc-D-Trp-(*N*-Me)Nle-Asp-1Nal-NH_2 (3)	136–138	0.43 (C)	12.5 (54:46)	743	743	$C_{40}H_{50}N_6O_8$
Boc-Trp-(*N*-Me)Nle-Asp-2Nal-NH_2 (4)	142–144	0.31 (C)	13.6 (54:46)	743	743	$C_{40}H_{50}N_6O_8$
Boc-Trp-(*N*-Me)Phe-Asp-Phe-NH_2 (5)	130–134	0.35 (C)	9.3 (54:46)	727	727	$C_{36}H_{48}N_6O_8$
Boc-D-Trp-(*N*-Me)Phe-Asp-Phe-NH_2 (6)	123–125	0.42 (C)	9.1 (54:46)	727	727	$C_{36}H_{48}N_6O_8$
Boc-Trp-(*N*-Me)Phe-Asp-1Nal-NH_2 (7)	199–200	0.37 (C)	8.5 (60:40)	777	777	$C_{43}H_{48}N_6O_8$
Boc-Trp-(*N*-Me)Phg-Asp-1Nal-NH_2 (8)	156–158	0.39 (C)	11.5 (54:46)	763	763	$C_{42}H_{46}N_6O_8$
Boc-Trp-(*N*-Me)Chg-Asp-1Nal-NH_2 (9)	144–146	0.28 (C)	9.5 (60:40)	769	769	$C_{42}H_{52}N_6O_8$
Boc-Trp-(*N*-Me)Nle-Asp-1Nal-$NH(CH_3)$ (10)	124–126	0.42 (C)	10.2 (60:40)	757	757	$C_{41}H_{52}N_6O_8$
Boc-Trp-(*N*-Me)Nle-Asp-1Nal-$N(CH_3)_2$ (11)	170–172	0.25 (C)	11.7 (60:40)	771	771	$C_{42}H_{54}N_6O_8$
Boc-Trp-Nle-Asp-Phe-NH_2 (12)	209–212	0.21 (C)	9.0 (51:49)	679	679	$C_{35}H_{46}N_6O_8$
Boc-D-Trp-Nle-Asp-Phe-NH_2 (13)	145–147	0.43 (C)	8.8 (51:49)	679	679	$C_{35}H_{46}N_6O_8$
Boc-Trp-Phe-Asp-1Nal-NH_2 (14)	202–205	0.34 (C)	13.9 (51:49)	763	763	$C_{42}H_{46}N_6O_8$
Boc-Trp-Phg-Asp-1Nal-NH_2 (15)	115–120	0.20 (C)	9.5 (54:46)	749	749	$C_{41}H_{44}N_6O_8$
Boc-Trp-Chg-Asp-1Nal-NH_2 (16)	225–226	0.31 (C)	7.8 (60:40)	755	755	$C_{41}H_{50}N_6O_6$
Boc-Trp-Phg-Asp-1Nal-$N(CH_3)_2$ (17)	143–145	0.43 (C)	8.9 (60:40)	777	777	$C_{43}H_{48}N_6O_8$
Boc-Trp-Chg-Asp-1Nal-$N(CH_3)_2$ (18)	130–132	0.30 (C)	11.4 (60:40)	783	783	$C_{43}H_{54}N_6O_8$

Table IV. Physical and Analytical Data of the Synthetic Peptide Derivatives

	mp, °C	R_f	anal. C, H, N
Boc-Asp(OBzl)-1Nal-NH_2 (19)	120–122	0.50 (A)	$C_{29}H_{33}N_3O_6$
Boc-(*N*-Me)Nle-Asp(OBzl)-1Nal-NH_2 (20)	99–101	0.30 (A)	$C_{36}H_{46}N_4O_7$
Boc-Asp(OBzl)-2Nal-NH_2 (21)	151–153	0.28 (A)	$C_{29}H_{33}N_3O_6$
Boc-(*N*-Me)Nle-Asp(OBzl)-2Nal-NH_2 (22)	61–63	0.50 (A)	$C_{36}H_{46}N_4O_7$
Boc-(*N*-Me)Phe-Asp(OBzl)-Phe-NH_2 (23)	65–67	0.27 (A)	$C_{35}H_{42}N_4O_7$
Boc-(*N*-Me)Phe-Asp(OBzl)-1Nal-NH_2 (24)	78–81	0.54 (A)	$C_{39}H_{44}N_4O_7$
Boc-(*N*-Me)Phg-OH (25)	101–103	0.73 (C)	$C_{14}H_{19}NO_4$
Boc-(*N*-Me)Phg-Asp(OBzl)-1Nal-NH_2 (26)	84–86	0.53 (A)	$C_{38}H_{42}N_4O_7$
Boc-(*N*-Me)Chg-OH (27)	oil	0.71 (C)	$C_{14}H_{25}NO_4$
Boc-(*N*-Me)Chg-Asp(OBzl)-1Nal-NH_2 (28)	75–77	0.48 (A)	$C_{35}H_{46}N_4O_7$
H-1Nal-$NH(CH_3)$ (29)		0.51 (B)	
Boc-Asp(OBzl)-1Nal-$NH(CH_3)$ (30)	89–91	0.53 (A)	$C_{30}H_{35}N_3O_6$
Boc-(*N*-Me)Nle-Asp(OBzl)-1Nal-$NH(CH_3)$ (31)	57–59	0.51 (A)	$C_{37}H_{46}N_4O_7$
H-1Nal-$N(CH_3)_2$ (32)		0.45 (B)	
Boc-Asp(OBzl)-1Nal-$N(CH_3)_2$ (33)	58–61	0.63 (A)	$C_{31}H_{37}N_3O_7$
Boc-(*N*-Me)Nle-Asp(OBzl)-1Nal-$N(CH_3)_2$ (34)	50–52	0.59 (A)	$C_{38}H_{50}N_4O_7$
Boc-Phe-Asp(OBzl)-1Nal-NH_2 (35)	199–201	0.28 (A)	$C_{38}H_{42}N_4O_7$
Boc-Phg-Asp(OBzl)-1Nal-NH_2 (36)	166–168	0.59 (A)	$C_{37}H_{40}N_4O_7$
Boc-Chg-OH (37)	oil	0.80 (D)	$C_{13}H_{23}NO_4$
Boc-Chg-Asp(OBzl)-1Nal-NH_2 (38)	201–203	0.50 (A)	$C_{37}H_{46}N_4O_7$
Boc-Phg-Asp(OBzl)-1Nal-$N(CH_3)_2$ (39)		0.54 (A)	$C_{39}H_{44}N_4O_7$
Boc-Chg-Asp(OBzl)-1Nal-$N(CH_3)_2$ (40)	72–74	0.52 (A)	$C_{29}H_{50}N_4O_7$

Synthesis. The peptides described herein were synthesized according to the general procedures detailed in this section for the preparation of compound 2. Particular aspects of the syntheses could be obtained from the authors. Analytical and physical data of the synthesized compound are reported in Tables III and IV.

Boc-Asp(OBzl)-1Nal-NH_2 (19): To a solution of H-1Nal-NH_2·HCl (3 g, 11.9 mmol) and Boc-Asp(OBzl)-OH (3.87 g, 11.9 mmol) in DMF (30 mL), cooled to 0 °C, were added successively Et_3N (1.68 mL, 11.9 mmol), HOSU (1.38 g, 11.9 mmol), and DCC (2.95 g, 14.3 mmol). The mixture was stirred for 1 h at 0 °C and overnight at room temperature. After filtration of DCU, the solvent was evaporated. The residue was dissolved in EtOAc, DCU was filtered off, and the organic solution was washed with 10% aqueous citric acid, 1 N aqueous $NaHCO_3$, water, and brine, dried over Na_2SO_4, and concentrated to yield 5.61 g (90%) of a white solid.

Boc-(*N*-Me)Nle-Asp(OBzl)-1Nal-NH_2 (20): Compound 19 (5.46 g, 10.5 mmol) was dissolved in TFA (20 mL) and stirred for 1 h at 0 °C and for 3 h at room temperature. After evaporation of the solvent, the oily residue was precipitated with dry ether to yield 4.92 g of H-Asp(OBzl)-1Nal-NH_2·TFA (88%). This compound (450 mg, 0.84 mmol) was dissolved in DMF (4 mL). The solution was cooled to 0 °C and treated successively with Et_3N (117 μL, 0.92 mmol), Boc-(*N*-Me)Nle-OH (207 mg, 0.84 mmol), HOSU (100 mg, 0.84 mmol), and DCC (190 mg, 0.92 mmol). The mixture was stirred for 1 h at 0 °C, overnight at room temperature and was worked up as described for the preparation of compound 19 to yield 373 mg (68%) of a white powder.

Boc-Trp-(*N*-Me)Nle-Asp-1Nal-NH_2 (2): Compound 20 (340 mg, 0.52 mmol) was dissolved in TFA (4 mL) and stirred for 1 h at 0 °C and for 1 h at room temperature. After evaporation of the solvent, the oily residue was precipitated with dry ether to yield 290 mg (81%) of H-(*N*-Me)Nle-Asp(OBzl)-1Nal-NH_2·TFA.

This compound (150 mg, 0.23 mmol) was dissolved in DMF (2 mL). The solution was cooled to 0 °C and treated successively with DIEA (88 μl, 0.51 mmol), Boc-Trp-OH (70 mg, 0.23 mmol), and BOP (112 mg, 0.25 mmol). The mixture was stirred for 1 h at 0 °C and overnight at room temperature. After evaporation of the solvent, the residue was dissolved in EtOAc, washed sequentially with 10% aqueous citric acid, 1 N aqueous $NaHCO_3$, water, and brine, dried over Na_2SO_4, concentrated, and purified by chromatography using CH_2Cl_2–MeOH (97:3) as eluent to yield 118 mg (61%) of Boc-Trp-(*N*-Me)Nle-Asp(OBzl)-1Nal-NH_2.

This compound (85 mg, 0.11 mmol) was hydrogenated in MeOH (6 mL) in the presence of 10% Pd/C catalyst (10 mg) for 10 h. After filtration and evaporation of the solvent, the residue was purified by chromatography using EtOAc–CH_2Cl_2–MeOH–H_2O–AcOH (20:7:3:0.6:0.3) as eluent to yield 56 mg (74%) of a white solid.

Boc-(*N*-Me)Phg-OH (25): Boc-Phg-OH (3 g, 12 mmol) and methyl iodide (6 mL, 96 mmol) were dissolved in dry THF (36 mL), and the solution was cooled to 0 °C in a flask protected

from moisture. Sodium hydride dispersion (1.08 mg, 36 mmol) was added cautiously with gentle stirring. The suspension was stirred at room temperature for 16 h. EtOAc (12 mL) was added, followed by H_2O (12 mL) dropwise. The solution was evaporated to dryness and the residue partitioned between ether and water. The ether layer was washed with 10% aqueous $NaHCO_3$, and the combined aqueous extracts were acidified to pH = 2 with 10% aqueous citric acid. The product was extracted into EtOAc and the extract was washed with water, 5% aqueous $Na_2S_2O_3$, water, and brine, dried over $HgSO_4$, and evaporated to yield 3 g (94%) of a pale yellow solid.

Boc-(*N*-Me)Chg-OH (27): Compound **25** (2.1 g, 7.92 mmol) was dissolved in TFA (10 mL) and stirred for 1 h at 0 °C and 3 h at room temperature. After evaporation of the solvent, the residue was precipitated with dry ether to yield 2.05 g (91%) of H-(*N*-Me)Phg-OH·TFA. This compound (2.0 g, 7.17 mmol) in H_2O (40 mL) and AcOH (10 mL) was treated with platinum oxide (720 mg) and placed under an atmosphere of hydrogen at 50 psi and 45 °C for 24 h. After filtration of the catalyst, the filtrate was concentrated to yield 1.55 g (75%) of H-(*N*-Me)-Chg-OH·TFA. To a solution of this compound (1.5 g, 5.26 mmol) in H_2O (5 mL) and dioxane (10 mL), cooled to 0 °C, were added Et_3N (1.84 mL, 13.15 mmol) and di-*tert*-butyl dicarbonate (1.72 g, 7.89 mmol). The mixture was stirred for 1 h at 0 °C and overnight at room temperature. After evaporation of the solvent, the residue was dissolved in 5% aqueous $NaHCO_3$, washed with ether, acidified to pH 2 with 10% aqueous citric acid, and extracted with EtOAc. The extract was washed with water, brine, dried over Na_2SO_4, and concentrated to yield 600 mg (42%) of an oil.

H-1Nal-NH(CH_3) (29): To a solution of H-1Nal-OMe·HCl (500 mg, 2.2 mmol) in ethanol (10 mL) was added a solution of 33% NH_2CH_3 in ethanol (2.2 mL). The mixture was refluxed for 24 h. After evaporation to dryness, the residue was purified by chromatography in CH_2Cl_2–MeOH (95:5) to yield 150 mg (30%) of a white solid.

H-1Nal-N(CH_3)$_2$ (32): To a solution of Z-1Nal-OH (4.55 g, 12.6 mmol) in DMF (25 mL), cooled to 0 °C, were added successively H-N(CH_3)$_2$·HCl (1.03 g, 12.6 mmol), Et_3N (1.77 mL, 12.6 mmol), HOSU (1.45 g, 12.6 mmol), and DCC (2.86 g, 12.6 mmol). The mixture was stirred for 1 h at 0 °C, overnight at room temperature and was worked up as described for the preparation of compound **19** to yield 3.65 g (72%) of Z-1Nal-N(CH_3)$_2$. This compound (4.55 g, 12.6 mmol) was hydrogenated in MeOH (20 mL) in the presence of 10% Pd/C catalyst (500 mg) overnight at room temperature. After filtration and evaporation of the solvent, the residue was purified by chromatography with CH_2Cl_2–MeOH (9:1) as eluent to yield 1.69 g (76%) of a white solid.

Boc-Chg-OH (37): H-Phg-OH (400 mg, 2.65 mmol) in 40 mL of AcOH–EtOH–H_2O (2:7:1) was treated with platinum oxide (260 mg) and placed under an atmosphere of hydrogen at 50 psi at 45 °C for 24 h, and then filtered and evaporated to yield 350 mg (84%) of H-Chg-OH. This compound (330 mg, 2.10 mmol) was treated as described for the preparation of **27** to yield 500 mg (94%) of an oil.

Binding Assays. [^{3}H]pCCK$_8$, 60 Ci/mmol, was purchased from Amersham. Membrane preparation and binding experiments with [^{3}H]pCCK$_8$ (0.2 nM) were performed as described previously.[19] Briefly, incubations (final volume 1 mL) were carried out in 50 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, and 0.2 mg/mL bacitracin for 60 min at 25 °C in the presence of brain membranes (0.6 mg protein/mL) or in 10 mM Pipes-HCl buffer, pH 7.4, 30 mM $MgCl_2$, 0.2 mg/mL bacitracin, and 0.2 mg/mL soybean trypsin inhibitor for 120 min in the presence of pancreatic membranes (0.2 mg protein/mL). Nonspecific binding was determined in the presence of 1 μM CCK$_8$. Incubations were terminated by filtration through Whatman GF/B filters precoated by incubation in buffer containing 0.1% bovine serum albumin. Filters were rinsed with 2 × 5 mL of ice cold buffer and dried and the radioactivity was counted. The K_I values were calculated using the Cheng–Prusoff equation.

Peptide Degradation. Incubation with crude rat brain membranes (3 mg protein/mL) were carried out at 35 °C in 50 mM Tris-HCl, pH 7.4, with 10^{-4} M of substrate in a volume of 500 μL. The reaction was stopped by boiling for 10 min, and after addition of acetonitrile (25% v/v) and shaking for 45 s to extract substrates, samples were centrifuged for 10 min at 13000*g* at 4 °C. The supernatants were analyzed by HPLC.

Electrophysiology. Hippocampal slices from male Sprague-Dawley rats were prepared for in vitro electrophysiological recordings as previously described.[40] 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 CA1-neurons was recorded extracellularly. A firing rate tracing was obtained by integration of the AP counts over 2–4 s. Compounds **2**, **15**, and **17** and L365,260 were dissolved in DMF and then diluted in the slice maintenance medium (composition: see ref 40) to the desired final concentration (DMF: 1% maximum). For the study of agonists, non-tachyphylactic CCK-sensitive neurons were selected by repeated bath applications of 5×10^{-7} M sulfated CCK$_8$ (CRB, Northwich, U.K.), and medium containing the compounds was then applied for 4 min. The excitatory responses were normalized relative to the last predrug response to CCK$_8$. For the study of antagonists, the compounds were applied alone for 10 min on neurons selected with a 10-fold lower concentration of CCK$_8$, and then coperfused together with CCK$_8$. Antagonist activity was measured relative to the last CCK$_8$ response as previously.

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